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Review

(Eco)toxicological tests for assessing impacts of chemical stress to aquatic ecosystems: Facts, challenges, and future

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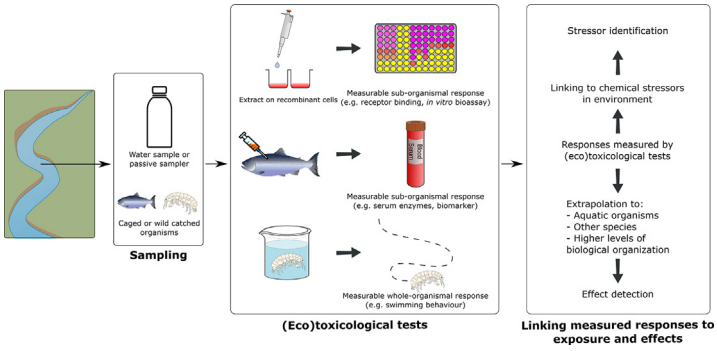
HIGHLIGHTS

- We reviewed ecotoxicity tests' availability and suitability for aquatic monitoring.
- Over 1200 ecotoxicity tests were identified from suborganismal- to ecosystem-level.
- *In vitro* bioassays and biomarkers could aid in chemical stressor identification.
- Sublethal and population/community responses are valuable for effect detection.
- Combining ecotoxicological tests with models is key for a comprehensive assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

Monitoring of chemicals in the aquatic environment by chemical analysis alone cannot completely assess and predict the effects of chemicals on aquatic species and ecosystems. This is primarily because of the increasing number of (unknown) chemical stressors and mixture effects present in the environment. In addition, the ability of ecological indices to identify underlying stressors causing negative ecological effects is limited. Therefore, additional complementary methods are needed that can address the biological effects in a direct manner and provide a link to chemical exposure, *i.e.* (eco)toxicological tests. (Eco)toxicological tests are defined as test systems that expose biological components (cells, individuals, populations, communities) to (environmental mixtures of) chemicals to register biological effects. These tests measure responses at the sub-organismal (biomarkers and *in vitro* bioassays), whole-organismal, population, or community level. We performed a literature search to obtain a state-of-the-art overview of ecotoxicological tests available for assessing impacts of chemicals to aquatic biota and to reveal datagaps. In total, we included 509 biomarkers, 207 *in vitro* bioassays, 422 tests measuring biological effects at the whole-organismal level, and 78 tests at the population- community- and ecosystem-level. Tests at the whole-organismal level and biomarkers were most abundant for invertebrates and fish, whilst *in vitro* bioassays are mostly based on mammalian cell lines. Tests at the community- and ecosystem-level were almost missing for organisms other than microorganisms and algae. In addition, we provide an overview of the various extrapolation challenges faced in using data from these tests and suggest some forward looking perspectives.

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Although extrapolating the measured responses to relevant protection goals remains challenging, the combination of ecotoxicological experiments and models is key for a more comprehensive assessment of the effects of chemical stressors to aquatic ecosystems.

Contents

1. Introduction

One of the key environmental problems for freshwater systems is the increasing worldwide pressure of anthropogenic chemical stressors (Reid et al., 2019). Although most of these chemicals are present at low concentrations, they raise ecotoxicological concerns by occurring in complex mixtures together with transformation products and unknown chemicals that may interact with each other. To further complicate matters, detecting chemicals in the environment by chemical analysis does not necessarily mean that they are bioavailable, nor that they will cause detectable or harmful effects on biological systems. Hence, for assessing the risk that chemical mixtures might pose harm to aquatic ecosystems, chemical monitoring will increasingly be less informative and provides a weak link to ecological effects (Brack et al., 2019).

In fact, chemical monitoring is often not done alone but in combination with ecological monitoring. Ecological indices are the most common method to assess ecological status worldwide and involve sampling of organisms in the monitored system to assess structural or functional endpoints (for reviews on this subject see Birk et al. (2012); Pander and Geist (2013); Siddig et al. (2016); Verdonschot and van der Lee (2020)). Indices summarize species diversity into a single value and additionally serve in describing the overall ecological status (Siddig et al., 2016). Hence, the major benefit of ecological monitoring is the high ecological relevance since it provides comprehensive information on the ecosystem and integrates the overall effect of chemical stressors including mixtures effects and bioavailability. However, a drawback of ecological indices is their limited ability to identify underlying stressors causing negative ecological effects. Additionally, measured community responses reflect alterations of the ecosystem that already took place, and are less useful as a preventive tool.

Clearly, there are some concerns with regard to chemical- and ecological-based monitoring and there is increasing consensus that complementary methods are needed (Altenburger et al., 2019; Brack et al., 2019; Lam, 2009; Wernersson et al., 2015). These concerns can be addressed by (eco)toxicological testing methods, which might provide a bridge between chemical monitoring and ecological indices. Within the present review, (eco)toxicological tests are defined as test systems that expose biological components to an environmental

medium and subsequently evaluate the biological effects of chemical stressors across different levels of biological organization, from molecular up to communities and ecosystems. These tests range from measuring sub-organismal responses in *in vitro* models to *in vivo* ecosystem-level effects, include effect-based methods, (standard) toxicity tests, bioassays, biomarkers, as well as micro-, mesocosm experiments, and can be performed in laboratories or in the field (*in situ*). The integration of ecotoxicity tests measuring biological effects into monitoring practices could overcome the limitations of ecological indices and of chemical-based monitoring through three routes: i) by providing a more comprehensive and realistic assessment of exposure and responses of aquatic organisms to chemical stressors (Altenburger et al., 2019; Brack et al., 2019; Lam, 2009; Wernersson et al., 2015), ii) by helping to unravel underlying mechanisms resulting in adverse effects on aquatic ecosystems (Leusch et al., 2014b; van der Oost et al., 2017), and finally, iii) by functioning as early-warning signal that allow taking preventive measures (Martinez-Haro et al., 2015). This makes ecotoxicological testing methods adequate tools for both prospective and retrospective risk assessment.

Worldwide, threshold levels of effects with names like 'regulatory acceptable concentrations', 'predicted no effect concentrations', etc. are derived using the results of ecotoxicological tests. In the lower tiers of, for instance, the risk assessment framework of pesticides, the results of standard toxicity tests performed with standard test species are used to derive protective threshold levels of effects (Brock et al., 2006). In the higher tiers, however, non-standard tests using non-standard test species and non-standard effect endpoints are also allowed to refine the risks identified by the lower tier, and can address any level of biological organization which is relevant for the risk assessment at stake. For instance, in Europe it is common that the results of microcosm and mesocosm experiments are used in the higher tiers of the risk assessment of pesticides (EFSA, 2013).

Broadly speaking, this review can be divided into two parts. The first part will focus on the effects of chemical stressors on aquatic systems, ranging from sub-organismal responses up to ecosystem responses, and the availability and role of (eco)toxicological tests in measuring and quantifying these effects (Sections 2–5). The aim is, on the one hand, to introduce and provide a categorization of available methods, as many methods have been developed in the last decade. On the other hand, we will reveal gaps in the available methods, *e.g.* in terms of missing or underrepresented species groups, modes of action, biological endpoints and levels of biological organization by the literature research performed. Researchers, risk assessors and other professions involved in water quality assessment and protection can use this part of the review to select the appropriate methods for their research and management questions.

The second part of the this review focusses on the revealed gaps and various extrapolation challenges faced in using these tests and data, including their diagnostic potential, cross-species extrapolation and extrapolation to higher levels of biological organization (Section 6). We conclude with future perspectives and research needs for using (eco) toxicological tests to assess the risks of chemical stressors to aquatic systems (Section 7). This part of the review serves as a starting point to outline the shortest path towards predictive ecotoxicology in the 21st century.

2. Principles of ecotoxicological tests assessing responses to chemical stress in aquatic ecosystems

Once anthropogenic chemicals enter surface waters, these chemicals may cause toxic effects on aquatic organisms. (Eco)toxicological tests can be used to measure and quantify biological responses to these chemicals for different levels of biological organization. Therefore, in this review (eco)toxicity tests will be categorized into three broad levels (Fig. 1A); tests measuring responses at the sub-organismal (Section 3), whole-organismal (Section 4) and population/community level (Section 5). However, it is important to note that the choices in sampling strategy impacts the results obtained with ecotoxicity tests (see SI 'Environmental sampling strategies').

At the sub-organismal level tests are classified as biomarkers or *in vitro* bioassays. While biomarkers refer to sub-organismal responses measured *in vivo* in field or lab exposed organisms (Fig. 1, part B4), *in vitro* bioassays use isolated cell lines (*e.g.* mammalian, fish, yeast and bacteria) exposed to extracted and enriched surface water to detect mechanistic responses (Fig. 1, part B2). Crucial difference between both is that chemicals within organisms (*in vivo*) go through complex toxicokinetic processes (absorption, distribution, metabolism and excretion), which are generally not reproduced *in vitro*.

Here we define biomarkers as a tool to quantitatively measure a sub-organismal change, including molecular, biochemical, cellular, physiological and (histo-) pathological changes, within organisms in response to external chemical stress (Smit et al., 2009). Hence, measured responses include DNA damage, effects on enzyme system functioning and effects on cell signalling. Using this definition, mortality of individuals is not considered a biomarker, but instead distinguished as whole-organismal responses. Additionally, we regard measuring analytically toxicant concentrations in collected organisms (bioaccumulation markers) as a form of chemical-based monitoring and since this review focusses on biological effects, this group of biomarkers will not be considered further.

In vitro bioassays use cell cultures or subcellular systems isolated from organisms or modified bacteria. Often *in vitro* bioassays are developed by using genetically modified cells, integrated with a specific receptor (*e.g.* human or mammalian receptor), and followed by a reporter gene that encodes an easily measured fluorescent protein or an enzyme (*e.g.*, β-galactosidase or luciferase) (Fig. 1, part B2). Ideally, the measured response (*e.g.* fluorescence intensity or enzyme activation) should correlate with the amount of receptor binding by the chemical (Escher and Leusch, 2012). In this way, *in vitro* bioassays measure responses to chemicals on a molecular level, *e.g.* receptor binding and (in)activation, or on cellular levels such as specific enzymatic activity. Other types of *in vitro* bioassays can have different read-outs.

In contrast to sub-organismal methods, ecotoxicity tests assessing responses at the whole-organismal and population/community level include both lethal and sublethal effects, measured by traditional (*e.g.* survival, reproduction) and non-traditional endpoints (*e.g.* behaviour) (Fig. 1, part B3). Tests assessing whole-organismal responses often only include single species (Connon et al., 2012), while tests that consider the simultaneous exposure of multiple species, *e.g.* by using microcosms and mesocosms, can assess responses at the population/community level. A great advantage of multi-species tests is that interactions among species, such as competition and predation, and food chains resulting in bioaccumulation, are included in the experimental set-up. These interactions are lacking in single-species tests.

3. Sub-organismal responses to chemical stress and available methods

Sub-organismal responses encompass biochemical and/or physiological changes. These responses precede effects on whole organisms and are generally more sensitive, making them suitable as an early warning. To explore the effects of chemicals on the sub-organismal level and present an overview of available methods to assess these effects for aquatic organisms, we searched for review papers that included (eco)toxicological tests, and subsequently compiled the available biomarkers and *in vitro* bioassays, their measured responses and test species or biosystem onto one large table (Supporting Information 2, Table S1). While not being an exhaustive review (approach and searching strategy described in the Supporting Information 1), 716 ecotoxicological methods that measure sub-organismal responses for different groups of organisms and species have been included. Of this 509 are considered biomarkers and 207 *in vitro* bioassays.

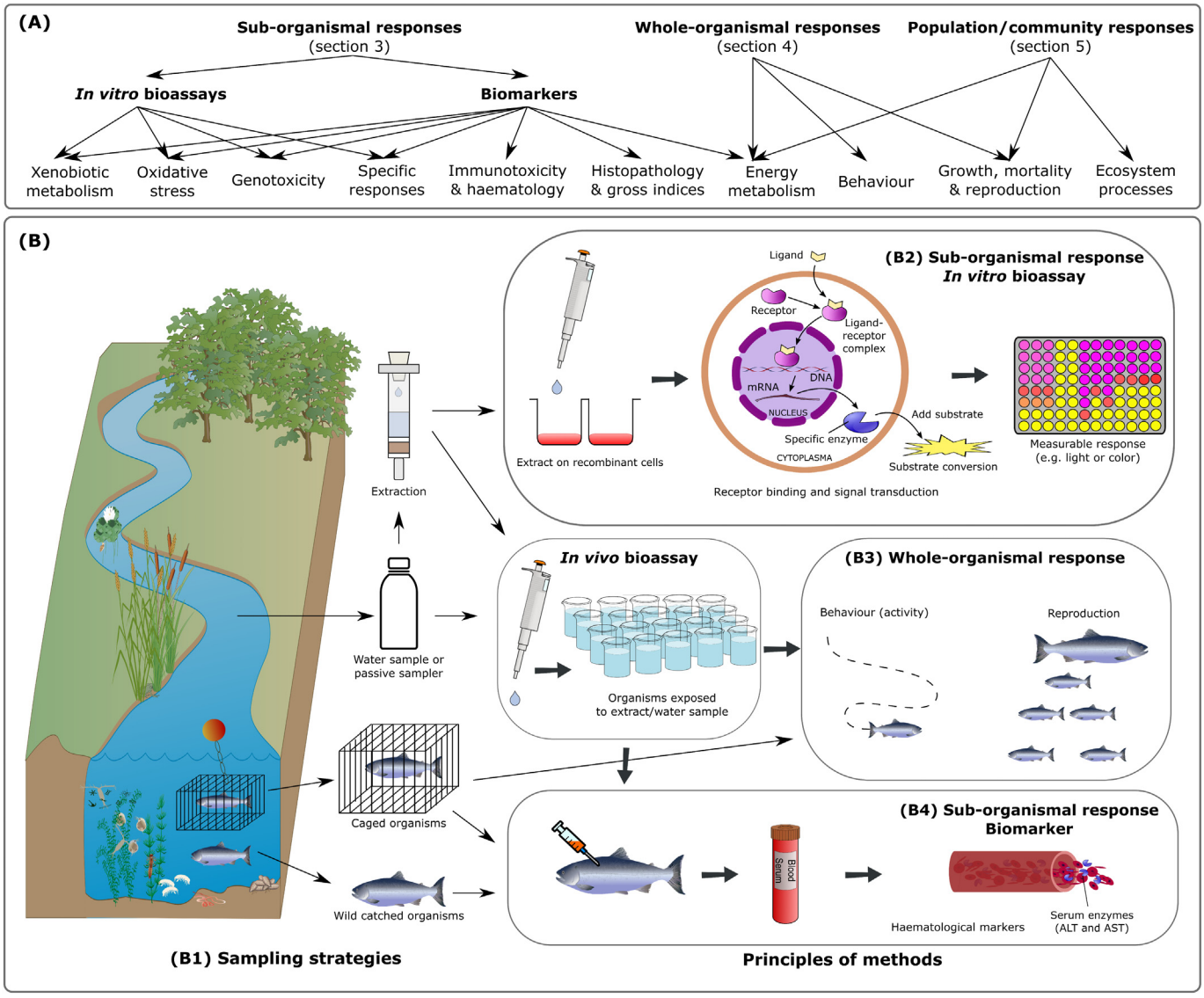

Fig. 1. (Eco)toxicological tests categorization and principles for assessing responses to chemical stress in aquatic ecosystems. Tests can be categorized according to level of biological organization measuring: sub-organismal, whole-organismal and population/community responses (A). Aquatic sampling strategies and principles of (eco)toxicological tests (B). Environmental sampling can be done by water sampling, using grab or passive sampling whereafter the sample can be concentrated by solid phase extraction. Alternatively, organisms can be caught or caged and exposed in the field (B1). Once organisms or water have been sampled, (eco)toxicological tests can be performed measuring responses at the sub-organismal level by *in vitro* bioassays (B2) and biomarkers (B4) or at the whole-organismal level (B3). B1: symbols for diagrams courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science, ian.umces.edu/symbols. ALT, alanine transaminase; AST, aspartate transaminase.

For descriptive purposes, we grouped the sub-organismal responses according to their mode of action (MOA) or to higher levels of biological responses. MOA is the process initiated by chemical-target molecule interaction and cellular response and progress up to physiological and/or morphological changes of organisms (Escher and Leusch, 2012). We will discuss for each sub-organismal response group (i) what the sub-organismal responses to pollutants are, followed by (ii) available biomarkers, what they measure and for which groups of organisms and close with (iii) *in vitro* bioassays used for water monitoring practices and since most receptor-based *in vitro* bioassays make use of human receptors, whether the used receptors are conserved in aquatic organisms (Table S3).

3.1. Xenobiotic metabolism

After xenobiotic chemicals have been taken up by the organism, transported internally, and finally, absorbed by the cells, these chemicals can be metabolized in two phases (called biotranformation): I) modification, II) conjugation (Fig. 2A). In phase I, the structure of a chemical can be modified resulting in a less active and more water soluble (easier to excrete) metabolite. This is facilitated by various enzymes, of which the cytochrome P450 monooxygenase is the most important. In phase II endogenous ligands (such as glutathione (GSH) and glucuronyl molecules) can be attached to the metabolite (conjugation). The addition of these molecules increases the water solubility of the metabolite, and thereby its excretion. Important phase II enzymes are glutathione S-transferase (GST), that catalyse the conjunction of the chemical with GSH, and UDP-glucuronyl transferases that catalyse the conjunction with glucuronic acid. Finally (sometimes referred to as phase III), the metabolite can be (actively) transported and excreted out of the cell. In vertebrates and invertebrates, the active transport of chemicals across the membrane is done in particular by ATP-binding cassette (ABC) transporters (Lee et al., 2018). Although the xenobiotic metabolism generally detoxifies chemicals, it can happen that the metabolite produced during the process is even more toxic than the precursor compounds (called bioactivation) (Van der Oost et al., 2003).

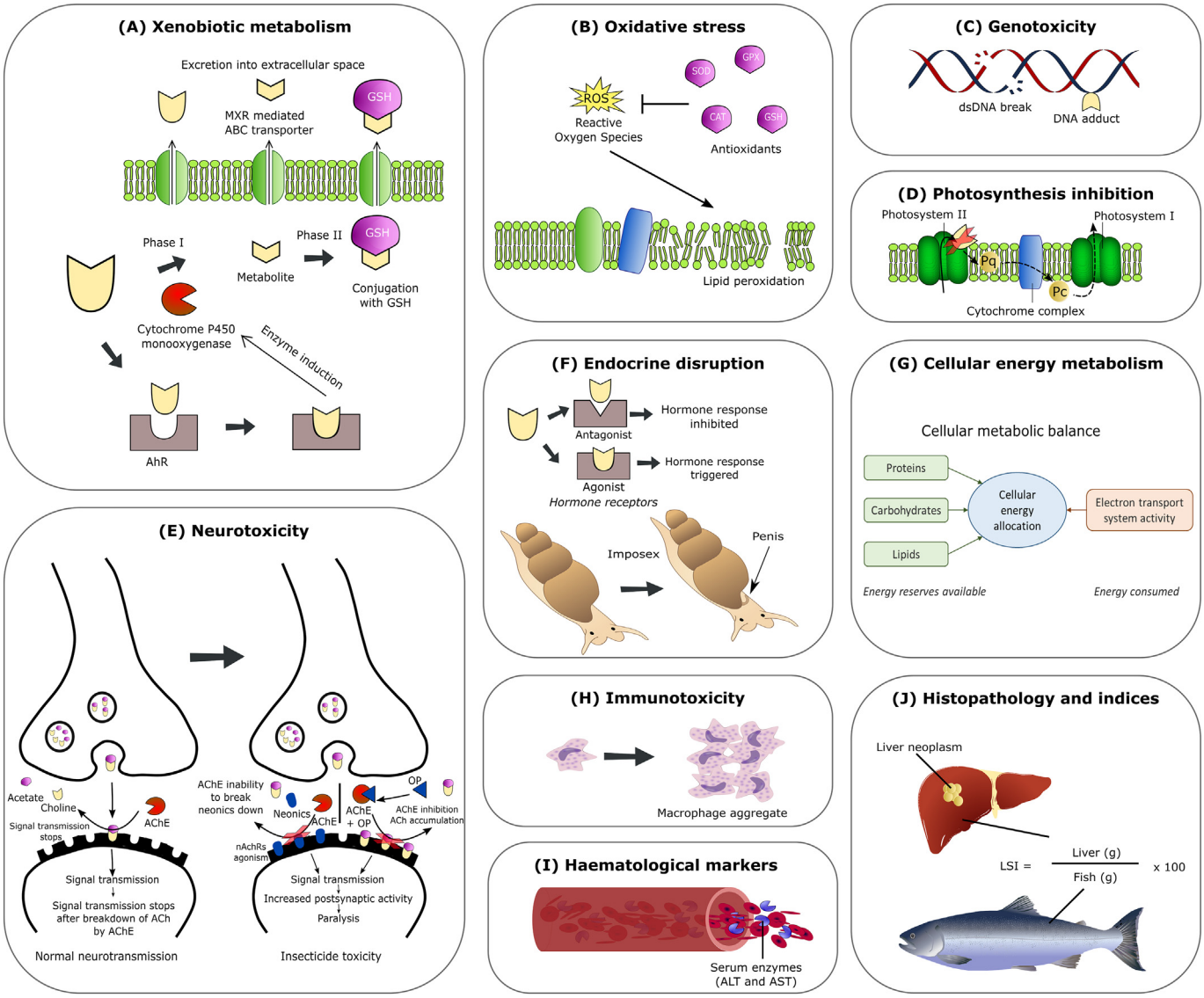

Fig. 2. Sub-organismal responses to chemical stress. Biotransformation pathways of xenobiotic chemicals in a cell (A). Increased formation of reactive oxygen species (ROS) because of the inhibition of protective antioxidant enzyme production by chemicals. This can lead in oxidative damage lipid peroxidation (B). Chemicals can cause genotoxicity by double strands DNA break or binding to a segment DNA (called DNA adduct) (C). Herbicides can block the electron flow in the Photosystem II complex and thereby inhibit photosynthesis (D). Under normal neurotransmission, acetylcholine (ACh) is released from the cholinergic neuron into the synaptic cleft and binds to the acetylcholine receptors at the postsynaptic cell, causing signal transmission. Acetylcholinesterase (AChE) breaks down ACh which stops signal transmission. Organophosphate insecticides (OP) or Neonics, interfere with this signal transmission resulting in blockage of receptors and overstimulation (E). Chemicals can interfere with human hormone receptors, present in *in vitro* assays, and act as antagonists or agonists. Chemical stressors can cause feminization of male fish and the development of male sex organs, such as a penis, in female snails (F). Effects of exposure to chemicals on cellular energy metabolism can be assessed by using cellular energy allocation (G). Macrophage aggregates, focal accumulations of pigmented macrophages formed in response to tissue damage, is a biomarker for immunotoxicity (H). Haematological parameters include increases in serum enzymes such as alanine transaminase (ALT) and aspartate transaminase (AST) that can be measured in fish and are indicators of cellular damage (I). Histopathology can be used to investigate tumour formation (neoplasms) in fish tissues such liver neoplasms. Additionally, gross indices can be used to reflect fish condition such as the liver somatic index (LSI) (J). Note, size differences of the frames in the figure do not have a meaning.

Biomarkers evaluating exposure to xenobiotics are often based on the enzymatic activity of enzymes known to be involved in the first and second phase of the metabolization (Ferrat et al., 2003) (Fig. 3). For example, several enzymatic biomarkers exist to measure the induction of cytochrome P450 (*e.g.* ethoxyresorufin O-deethylase (EROD), but see Table S1 for more examples), or GST and GSH for phase II (Ferrat et al., 2003; Hyne and Maher, 2003; Kroon et al., 2017). In fact, we found 118 biomarkers of xenobiotic metabolism of which 81 belonged to either of the two-phases (Table S1). Although Phase I and Phase II in the metabolization process are ubiquitous among vertebrates, invertebrates and plants, and we found biomarkers for species within these groups (Fig. 3), species can differ in *e.g.* cytochrome P450s and GST classes/families involved in the metabolization process (Frova, 2006). An additional complicating factor is that Phase I enzymes are far less active and/or inducible in invertebrates and plants (Snyder, 2000), making them less valuable as biomarker compared to fish species. For vertebrates and invertebrates it is know that ABC transporters play an important role and hence expression and activity of these transporters have been used as biomarkers (Fig. 3).

Half of the *in vitro* bioassays found (15 out of 30) and used in water quality monitoring for xenobiotic metabolism assess aryl-hydrocarbon receptor (AhR) binding directly, based on human nuclear xenobiotic receptor binding, or indirectly by EROD induction (Fig. 3, Table S1). The remaining *in vitro* bioassays are based on the constitutive androstane receptor (CAR), peroxisome proliferator activated receptor (PPAR) or pregnane X receptor (PXR), all of them human nuclear receptors. In humans, chemicals that bind to xenobiotic receptors trigger metabolic pathways. The binding of a nuclear receptor to its binding site is not a

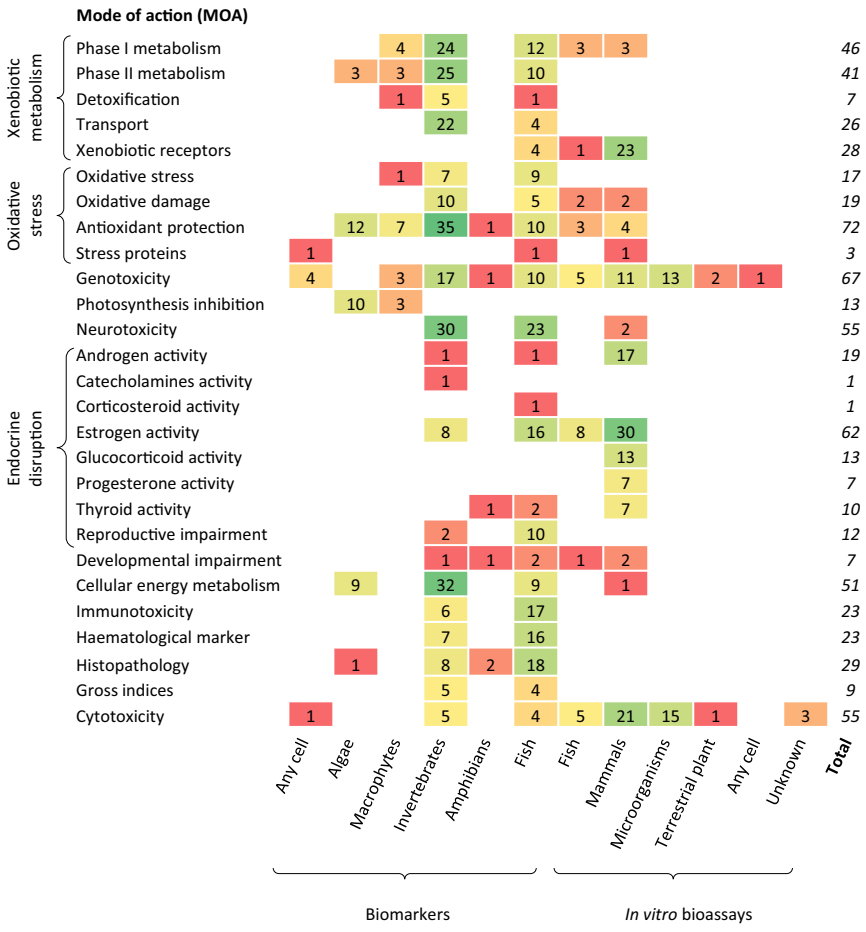

Fig. 3. Available ecotoxicological tests (biomarkers and *in vitro* bioassays) for measuring sub-organismal responses to chemical stressors for different groups of organisms. In total we included 509 biomarkers and 207 *in vitro* bioassays from our non-exhaustive literature search (Table S1, for methods, see Supporting information 1). Different colours indicate the number of *in vitro* bioassays or biomarkers found for the different sub-organismal responses and for each organism group (red = 1–2, orange = 3–4, yellow = 5–9 and green > 10).

toxic process in itself but indicates the presence of chemicals. After to xenobiotic receptors, this activates expression of genes in Phase I, Phase II and Phase III of biotransformation pathways (Bainy, 2007). Some of those human receptors are conserved among vertebrates and can be found in fish as well (Table S3).

3.2. Oxidative stress

Exposure to chemicals can also lead to the formation of reactive oxygen species (ROS) within organisms, potentially resulting in oxidative stress, a process ubiquitous among vertebrates, invertebrates and primary producers. Actually, the production of ROS is a natural phenomenon and remains generally reduced under normal growth conditions. However, when the system is not able to detoxify the level of ROS in cells by compensatory antioxidant enzymes, oxidative stress occurs. Mechanisms through which chemicals affect ROS is either by inhibiting protective antioxidant enzyme production or by increasing ROS formation. Disrupting this balance between antioxidant/pro-oxidant systems might lead to oxidative damage by enzyme inactivation, DNA damage (genotoxicity), lipid peroxidation and ultimately cell death (Fig. 2B).

Aquatic organisms have a range of antioxidant enzymes and since they can be quantitatively altered by exposure to chemicals (Colin et al., 2016; Ferrat et al., 2003; Hook et al., 2014; Van der Oost et al., 2003), this has frequently led to their use as biomarker for primary producers, invertebrate and vertebrates (Fig. 3, Table S1). Also oxidative damage can be assessed in aquatic organisms and in particular lipid peroxidation has been routinely measured in field studies (Colin et al., 2016; Ferrat et al., 2003; Hook et al., 2014). For a more in depth description on oxidative stress and biomarkers in aquatic animals see Lushchak (2011) and for primary producers see Brain and Cedergreen (2008).

A number of *in vitro* bioassays are available that target oxidative stress (11 out of 207 see also Fig. 3, Table S1), for example, the activation of the Nrf2 pathway. In mammals, the Nrf2 pathway is the primary cellular defence mechanism against oxidative stress responsible for the induction of detoxification and antioxidant genes (Escher and Leusch, 2012; Nguyen et al., 2009). The Nrf2 pathway has been identified in fish too (Table S3).

3.3. Genotoxicity

Many chemicals released in the environment have been found to be genotoxic for aquatic organisms. Chemicals can directly or indirectly damage DNA (Fig. 2C). However, organisms exhibit repair mechanisms by which damaged DNA can be repaired. Failure to repair might trigger cell death *via* apoptosis or lead to irreversible mutations with errors during replication, transcription and/or in protein synthesis.

Genotoxicity is commonly measured by using biomarkers, with the most adopted methods being the comet assay and the micronuclei (MN) and nuclear abnormalities in blood cells (ENAs) tests (Colin et al., 2016). The main difference lies in DNA damage detected by the comet assay can still be repaired by DNA-repair mechanisms, while chromosome breakages identified by MN/ENAs tests are hardly repairable (Colin et al., 2016). Both methods have been used as biomarker for vertebrates and invertebrates, and the comet assay for primary

producers as well. We found 35 biomarkers that address genotoxicity (Table S1, Fig. 3).

In vitro bioassays for genotoxicity can be performed with any cell type (Fig. 3), including established cell lines and primary cultures (Brack et al., 2016). The comet and micronuclei assay can also be performed *in vitro*. Other genotoxicity assays that have been used frequently, but are not specific for water samples, include the Ames fluctuation test and the SOS Chromotest. The Ames fluctuation test detects point mutation in *Salmonella typhimurium* while the SOS Chromotest shows primary DNA damage on *Escherichia coli*. The SOS response can likewise be detected by the umuC test. The main difference between the umuC test and SOS Chromotest is that UmuC uses a *Salmonella typhimurium* strain. Additionally, the p53 CALUX® reporter gene assay can be used to detect genotoxic carcinogens by monitoring the modulation of the p53 pathway. The p53 family of transcription factors plays an important role for regulation of DNA repair, with p53 being activated in response to DNA damage and initiating a series of DNA repair mechanisms.

3.4. Photosynthesis inhibition

When chemicals bind to a receptor, it can trigger or inhibit a response. Agonists trigger a response, whereas antagonists block receptors and inhibit their responses. Many herbicides can act as antagonist of photosystem 2 by blocking the electron flow in the Photosystem II (PSII) complex and thereby inhibit photosynthesis (Fig. 2D). Herbicides can also indirectly affect photosystem II efficiency by inhibiting biosynthesis of carotene, fatty acids, microtubule formation or through the formation of reactive oxygen species (DeLorenzo et al., 2001).

Photosystem II performance parameters have been regularly used as biomarker including maximum PSII photochemical efficiency (Fv/Fm), effective PSII quantum yield (ΦPSII, or Φm or ΔF/Fm′), and electron transport rate (ETR) (Almeida et al., 2019; Petsas and Vagi, 2017) (Table S1). Since all the endpoints are measured in macrophytes or algae (intact organisms), we grouped them under biomarkers and did not find any *in vitro* bioassay to measure phytotoxicity (Fig. 3).

3.5. Neurotoxicity

Insecticides are examples of chemicals that are interfering with signal transduction and can cause neurotoxicity, *e.g.*, disruption of nervous system functioning or structure by chemicals. A well-studied mechanism is the binding of organophosphate and carbamate insecticides to acetylcholinesterase (AChE), resulting in AChE inhibition in both vertebrate and invertebrate organisms (Fig. 2E). This inhibition prevents the degradation of the neurotransmitter acetylcholine, which can results in overstimulation of the central and peripheral nervous system with negative effects on locomotion activity and ultimately death (Fulton and Key, 2001). Pyrethroid and neonicotinoid classes of insecticides can cause paralysis and death by a different main MOA, *i.e.* sodium channel inactivation and nicotinic acetylcholine receptor agonism, respectively (Casida and Durkin, 2013). Next to pesticides, other chemicals such as pharmaceuticals can cause neurotoxicity by many different mechanisms, *e.g.* cholinesterase (ChE) inhibition (Wu and Li, 2015), altered brain neurotransmitter pathways (Bidel et al., 2016) and sensory deprivation (Brodin et al., 2014; Legradi et al., 2018).

Inhibition of acetylcholinesterase is the most well-known biomarker for neurotoxicity as this is the main MOA for organophosphates and carbamates and has been measured in fish, insects, crustaceans and molluscs (Domingues et al., 2010). Other cholinesterases have also been used as biomarkers for environmental monitoring (Fig. 3, Table S1). More information about other available neurotoxicity biomarkers such as GABA transaminase and the implementation of neurotoxicity assessment in different *in vivo* organisms and *in vitro* models can be found in the reviews by Basu (2015) and Legradi et al. (2018).

Acetylcholinesterase inhibition can also be measured *in vitro* in the commercially available isolated acetylcholinesterase inhibition assay (Fig. 3, Table S1), and this is currently still the only *in vitro* bioassay for neurotoxicity used in monitoring practices that we found.

3.6. Endocrine disruption

The endocrine system has a critical function in regulating internal homeostasis of organisms. Chemicals can interfere with the endocrine system by modulating or disrupting hormone biosynthesis, metabolism or action and consequently lead to deviations in homeostasis, development, reproduction, and behaviour of organisms (Segner et al., 2003). Endocrine systems differ between organisms. The main sex steroids in fish are estradiol and testosterone, while in invertebrates the primary coordinators are peptide hormones and ecdysteroids and the role of estradiol and testosterone is not yet clear (Lafont and Mathieu, 2007; Rotchell and Ostrander, 2003). Since endocrine systems differ between animal groups, chemicals interfering with those systems and exhibiting endocrine disrupting activity consist of a diverse group of chemicals prevalent in the aquatic environment. While for example insect growth regulators are ecdysteroid agonists and interfere with insect endocrine systems (Soin and Smagghe, 2007), organotins interferes with gastropod endocrine system with imposex as a consequence (Oberdörster and McClellan-Green, 2002) and synthetic oestrogens can cause feminisation of male fish (Corcoran et al., 2010). However, the level of understanding of endocrine disruption and the mechanisms of action of chemicals in invertebrates (see Rotchell and Ostrander (2003) for a review) are far less developed compared to vertebrates (see Kloas et al. (2009)), being hampered by the lack of detailed knowledge on invertebrate endocrinology.

It is, therefore, not surprising that more biomarkers for endocrine disruption have been developed for fish compared to invertebrates, as is demonstrated by the results of our literature search, in which we found 12 biomarkers for invertebrates and 30 for fish (Fig. 3). Biomarkers to study endocrine disruption in fish include changes in hormone and protein (*e.g.* spiggin, vitellogenin) levels and abnormal gonad development (see Table S1 for a total overview). In oviparous male fish the induction of vitellogenin, the precursor protein of yolk, is a well-known effect of endocrine disrupting compounds in surface waters (Sumpter and Jobling, 1995) and has been extensively used as biomarker (Porte et al., 2006). Although the role of hormone signalling is not fully understood in invertebrates, some endocrine disrupting biomarkers in invertebrates have actually been studied fairly well. One of the clearest examples of endocrine disruption is the development of imposex in gastropods (Fig. 2F) after exposure to tributyltin (Oberdörster and McClellan-Green, 2002), but also vitellogenin induction has been observed in aquatic invertebrates and can be used as biomarker (for reviews see Matozzo et al. (2008); Porte et al. (2006); Tran et al. (2019)).

For humans it is known that chemicals can interfere with hormone receptors and can act as agonist or antagonists (Fig. 2F). There are several commercially available *in vitro* bioassays developed for the detection of specific agonistic and antagonistic endocrine effects on the different human nuclear receptors estrogen (ER), androgen (AR), glucocorticoid (GR), progesterone (PR) and thyroid (TR) (Fig. 3, Table S1 and for reviews see Wagner et al. (2017) and Leusch et al. (2017)). Additionally, *in vitro* bioassays are available on the retinoic acid receptor (RAR) and retinoic X receptor (RXR) which are important in development. All these receptors are conserved in some fish species (see Table S3), but invertebrate endocrinology knowledge is still limited and the presence and physiological function of these receptors is not fully understood (Köhler et al., 2007; Rotchell and Ostrander, 2003).

3.7. Cellular energy metabolism

Chemical stress can affect cellular energy metabolism either directly or indirectly. By causing mitochondrial dysfunction, chemicals can

directly alter energy metabolism (Calow and Forbes, 1998). Indirectly, chemicals can alter energy metabolism *via* detoxification or general stress (Ericson et al., 2010). In both ways, the elevated energy demand resulting from chemical exposure can be rapidly provided by energy reserves (reserve in the form of lipids, proteins, and/or carbohydrates) and energy allocation (Goodchild et al., 2019).

The biomarker "cellular energy allocation" can be used for measuring cellular energy metabolism (Goodchild et al., 2019) which is often measured in invertebrates exposed to chemical and environmental stressors (Table S1). But also in fish an increased metabolic rate can be found at polluted sites, as recently demonstrated by van der Oost et al. (2020). Cellular energy allocation is the difference between energy reserves available and energy consumption and is an estimate of cellular metabolic balance (Fig. 2G). This can be determined by measuring lipids, proteins, carbohydrates and the electron transport system activity using standard spectrophotometric assays (De Coen and Janssen, 2003; Smolders et al., 2004). In addition, the scope for growth concept can be considered a biomarker for energy metabolism, but will be discussed under whole-organismal responses (Section 4.4).

The only *in vitro* bioassays grouped into this category is the MitoScan™ mitochondrial assay (Fig. 3, Table S1). Although this assay does not measure cellular energy metabolism but the inhibitory activity of chemicals on enzymes of oxidative phosphorylation instead, it fits best in this group since oxidative phosphorylation is integrated into cellular metabolism and provides energy by creating ATP (Wilson, 2017).

3.8. Immunotoxicity

Exposure to chemicals can lead to compromised immune function in aquatic invertebrates (see Galloway and Depledge (2001) for a review) and fish (see Segner et al. (2012) for a review). The immune function in aquatic organisms exposed to chemicals can be impaired through a variety of mechanisms, including by suppressing immune functions such as induction of apoptosis and causing interference of signalling pathways in immune cells (Segner et al., 2012).

In fish, the immune system consists of 2 different components; innate and adaptive. The innate (non-specific) immune system is the primary defence mechanism and is evolutionary conserved with key features shared among mammals, invertebrates and plants (Buchmann, 2014). Aquatic invertebrate species rely mainly on this system for immunological defences (Galloway and Depledge, 2001). The adaptive immune system in vertebrates is triggered by the innate immune system and is an antigen-specific response. Especially for the innate immune system, a wide set of biomarkers are available to assess immunotoxicity in fish and invertebrates (Fig. 3, Table S1), although it remains difficult to attribute specific chemicals to changes in immune function (Weeks et al., 2018). Next to leukocytes and haemocytes, commonly used biomarkers are production of antimicrobial peptides, lysozyme production, macrophage aggregate (Fig. 2H), cytokines expression and phagocytic activity (Torrealba et al., 2019). For bivalves, especially phagocytosis of haemocytes, the mechanisms of cellular defence, is often used as parameters to evaluate exogenous toxicity (Zhang et al., 2019). For a review on biomarkers of immunotoxicity for invertebrate see Galloway and Depledge (2001) and for fish see Segner et al. (2012).

Within our compiled table of ecotoxicological tests for sub-organismal responses, no *in vitro* bioassays are present that target immunotoxicity (Table S1).

3.9. Haematological markers

Transportation of molecules between different compartments of the body is done by blood and lymph in vertebrates. In contrast, invertebrates have an open circulatory system and the cells analogous to blood cells are called haemocytes or coelomocytes. Chemical stressors can affect the viability of red blood cells, which can result in hypoxia (Escher and Leusch, 2012), or affect the hemoglobin synthesis (Van der Oost et al., 2003). In invertebrates, chemicals are capable of some morphological damage of haemocytes (Calisi et al., 2008). Next to this, chemicals might cause the leakage of specific enzymes (such as serum transaminases; Fig. 2I) from cells into the blood. Increased serum activity in the blood are sensitive indicators of cellular damage since the levels of these enzymes within the healthy cell exceed those in the extracellular fluids by more than three orders of magnitude (Moss et al., 1986).

Examples of haematological biomarkers includes measurements of transaminases in fish and invertebrates *e.g.* alanine transferases and aspartate transaminase and parameters, like hematocrit, hemoglobin, and protein (Fig. 3, Table S1). Additionally other parameters can be measured in blood such as white blood cells and endocrine parameters covered in the immunotoxicity and endocrine disruption sections respectively. However, in our literature search, we did not find *in vitro* bioassays for detection of haematoxicity in surface waters.

3.10. Histopathology and gross indices

Finally, chemical exposure can also result in histopathological changes such as neoplasms in tissues and morphological changes including malformation. These are responses following chemical and cellular interaction (Van der Oost et al., 2003), and are responses measured at higher levels of biological organization as opposed to many of the previous mentioned sub-organismal responses.

Some biomarkers of morphological changes and indices can be grouped to a specific sub-organismal response, such as imposex to endocrine disruption, as mentioned earlier. Other histopathological and morphological biomarkers, not directly related to a certain MOA, are for example liver neoplasms and the liver somatic index (LSI) of fish (Fig. 3, Table S1). The liver somatic index (LSI, Fig. 2J) is the ratio between the weight of the liver and the total body weight of the fish and is used to identify liver disease (Slooff et al., 1983). Commonly used morphological biomarkers are diatom malformation and larval morphological deformities in chironomidae. In response to chemical stressors, diatoms can display deformities (teratologies) in their valves (see Lavoie et al. (2017) for a review) and for *Chironomus* sp. morphological alterations most frequently show on their mouthparts (mentum) or wings (Montaño-Campaz et al., 2019). For these high level sub-organismal responses no *in vitro* bioassays are available.

4. Whole-organismal responses to chemical stress and available methods

Sub-organismal changes caused by chemicals can translate into whole-organismal responses, which is traditionally in ecotoxicology the most commonly assessed level. Traditional approaches are typically based on measures of individual growth, reproduction, immobilization and survival of a simplified food chain, usually including algae (producers), daphnids (primary consumers) and fish (secondary consumers) (Calow and Forbes, 2003). However, research in ecotoxicology has progressed substantially from simplified, single species laboratory tests on standardized endpoints, to a wide-range of potentially more sensitive and accurate non-standard endpoints including behaviour and energy metabolism (Ågerstrand et al., 2020; Calow and Forbes, 2003).

From our literature search, we included 422 ecotoxicological tests measuring a variety of biological effects at the whole-organismal level (Supporting Information 2, Table S2). In this section we will discuss first (i) what the biological responses to pollutants are at the whole-organismal level, followed by (ii) a description of the available methods found through our literature search to measure these responses and a descripion of the ecotoxicological space these methods currently cover, simultaneously revealing possible gaps. Even though the different effects on whole-organismal level are considered under different headings, these effects are in fact inter-related (Fig. 4).

4.1. Behaviour

Responses of organisms to changes in the environment often initially manifest as changes in the behaviour (Tuomainen and Candolin, 2011), which in turn might trigger additional responses (Fig. 4). The integration of many physiological systems, including sensory, hormonal, neurological, and metabolic systems, contribute to behaviour. Consequently, chemical stress can induce a variety of behavioural changes by interfering with one or more of these systems. In both fish and invertebrates, chemicals may affect sensory systems such as mechanoreceptors, essential for movement. Especially for insecticides it is known that they can affect transmission of mechanoreceptors leading to paralysis and immobilization (Fig. 2E). Interference with light receptors could lead to the deterioration of visual sensors, and thereby influence the behaviour of organisms that rely on vision (Candolin, 2009). Chemicals affecting chemoreceptors might disrupt the transfer of olfactory cues necessary for locating food, navigation, predator detection, social recognition and communication (Legradi et al., 2018). Next to the sensory systems, also chemically-induced hormonal and metabolic changes might impact behaviour. For instance, chemicals causing endocrine disruption can have adverse effects on social behaviour (*e.g.* aggression (Colman et al., 2009)), reproductive behaviour (*e.g.* courtship and parental care (Saaristo et al., 2010)) and cognitive performances (Jacquin et al., 2020). With respect to metabolic changes, chemicals that cause metabolic dysfunction have likely implications for numerous types of behaviours, since energy availability and requirement influences optimal foraging strategies. Thus, chemical stressors can by disrupting aspects of an organisms' behaviour, affect its physical fitness.

Measuring behavioural changes as response to chemical stressors has gained more and more attention in the past years (Ågerstrand et al., 2020; Amiard-Triquet, 2009; Peterson et al., 2017). The most frequently measured behaviour for invertebrates have been immobilization (see also Section 4.5) and feeding behaviour (Fig. 5). For fish, activity levels are often used as behavioural response in tests (Fig. 5), which can nowadays be analysed by high-throughput platforms using video cameras (*e.g.* Zebrabox™, Daniovision™). However, tests measuring reproductive behaviour appear to be scarce (Fig. 5).

4.2. Individual growth and development

Growth is an important fitness component of individuals, and is known to influence the survival and reproductive success and thereby the persistence of a population in the field. Chemicals can affect an organisms' growth and development, and ultimately body size, through the following mechanisms (Moore and Folt, 1993): (1) reduced food intake, leading to a reduction in growth and smaller body size,

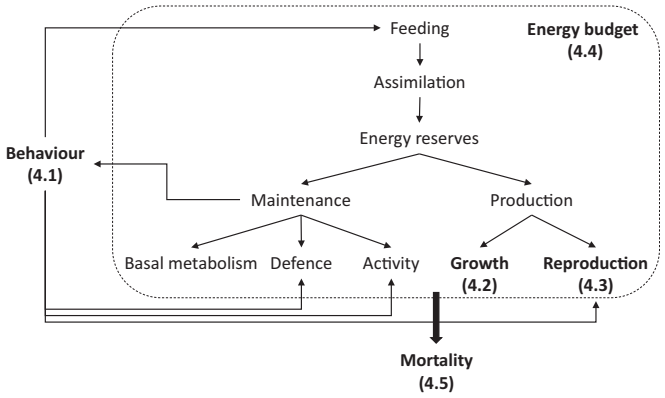

Fig. 4. Schematic visualization of the relationships between responses at the whole-organism level. The different whole-organism responses that are covered in this review are indicated in bold, and followed by the corresponding paragraph number between brackets.

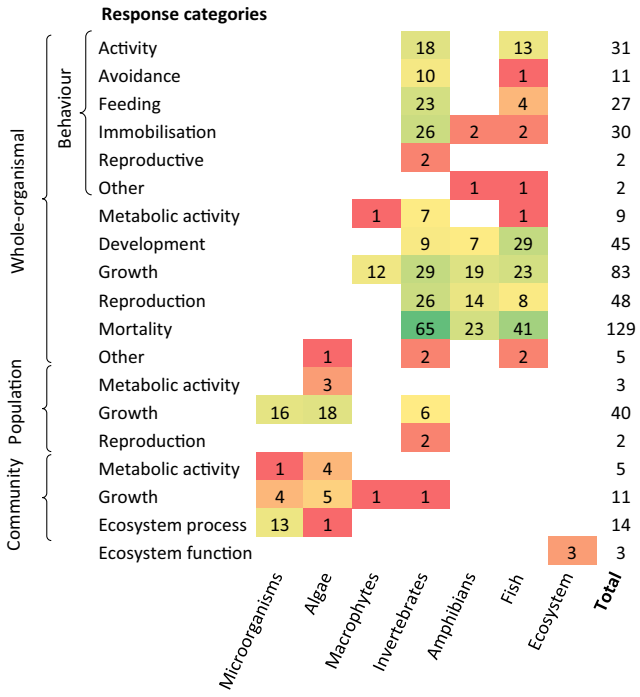

Fig. 5. Available ecotoxicological tests measuring whole-organismal, population, community and ecosystem responses to pollutants for different groups of organisms. In total we included 500 ecotoxicological tests from our literature search for these levels of biological organization (Table S2 and for searching methods see Supporting information 1). Different colours show to the number of tests found per response categories for each organism group (red = 1–2, orange = 3–5, yellow = 6–13 and green > 14).

(2) suppressed growth rates, leading to smaller body size at maturity (Moore and Folt, 1993) and (3) increased maintenance costs, thereby decreasing growth (see Section 4.4). Ultimately, inhibition in development and growth can result in a smaller mean body size, and, for species with a body size-dependent fecundity, in a lower reproduction rate, leading to population level effects (Galic et al., 2017). All in all, chemical stressors can impair growth and development by a variety of mechanisms, making them important but non-specific responses.

Individual growth has been widely used for assessing chemical stress on aquatic organisms. Measuring growth typically involves changes in size during a certain period of time. However, this measure of growth can be misleading when tracking the growth of an individual through time or comparing individuals of different sizes, since absolute gain in weight is dependent on initial size (Smith et al., 2012). Relative growth rate, expressed as growth during a certain period of time relative to the size of the individual, is a more appropriate measure of growth (Smith et al., 2012). However, while some studies indeed use relative growth rate during toxicity testing, this becomes difficult when testing multiple individuals in the same container. Hence, in such cases total length or weight at the end of the exposure period (divided by number of individuals) is often used as a measure of growth. Individual growth is a standard endpoint for macrophytes, invertebrates and vertebrates and this endpoint is covered well by the available tests. Whereas for microorganisms and algae, often population growth (see Section 5.1) rather than individual growth is measured (Fig. 5, Table S2), which is actually more related to reproduction than to individual growth.

4.3. Reproduction

Chemical stress can affect the reproductive output of organisms by influencing reproductive behaviour (Section 4.1), including mating

success and care of juveniles, by disrupting endocrine systems resulting in for example direct impairment of ovulation, germ cell maturation, intersex and imposex (Section 3.6), and by impacting genotoxic compounds on germ cells (Amiard-Triquet (2009)). Moreover, reproductive gametes (and zygotes) are often more sensitive to chemical stressors compared to adults, and exposure to chemicals may result in impaired fertility, especially in organisms that depend on external fertilisation (Hudspith et al., 2017). Next to these specific effects of chemicals, energy allocation also strongly impacts reproduction (Fig. 4, Section 4.4).

Reproductive output can be expressed in various ways at the organismal level. Examples include eggs or number of neonates per female. For macrophytes and algae, individual reproduction is often not measured, but instead growth in the form of biomass is used as endpoint. However, it is possible to assess impacts on macrophyte reproduction by measuring sexual reproductive outputs such as percentage of flowering shoots or number of seeds produced (Sesin et al., 2021). Available ecotoxicological tests with reproduction as endpoint usually use organisms with a short lifecycle such as *Daphnia* (Table S2), while many other species, including fish and amphibian species, reproduce only once a year, making reproductive tests challenging and more scarce (Fig. 5).

4.4. Energy budget

Organisms can assimilate energy through feeding, and storing it in energy reserves. Assimilated energy can be allocated towards maintenance and production. Maintenance costs are essential for individuals to survive and are distributed between basal metabolism, defence and activity (Fig. 4). Energy allocated to production favours fitness-related functions like growth and reproduction (Mouneyrac et al., 2011). However, as mentioned in Section 3.7, chemical stressors can impact energy budget directly by causing mitochondrial dysfunction and impairing an organism's ability to assimilate energy, or indirectly, *via* general stress, detoxification and repair pathways (Goodchild et al., 2019). At the whole-organism level, compensatory mechanisms may involve changes in behaviour such as escape, avoidance and feeding behaviour (see Section 4.1). Hence, exposure of organisms to chemical stressors is regarded as energetically costly (metabolic cost hypothesis see Calow (1991)), and energy may be diverted from fitness-related functions to maintenance and repair (Sokolova et al., 2012). For in-depth reviews on the effects of chemical stress on metabolic costs and energy budgets see (Calow and Forbes, 1998; Goodchild et al., 2019; Kooijman et al., 2009; Mouneyrac et al., 2011; Sokolova et al., 2012).

A common method to measure metabolic activity at the whole-organism level in ecotoxicological tests is by measuring oxygen consumption (Table S2). Oxygen is needed in the metabolic pathway oxidative phosphorylation to produce ATP, and, since ATP provides energy for almost all the main processes of organisms, oxygen consumption can be considered as an estimate of the rate of metabolism (Clarke, 2019). Besides that, the scope for growth concept is an example of the successful application of the metabolic cost hypothesis and oxygen consumption is one of the measured parameters (Mouneyrac et al., 2011). Scope for growth is defined as the difference between energy intake of an organism from its food and the total metabolic losses (production of both somatic tissue and gametes, respiratory energy expenditure and energy lost through excretion) (Widdows et al., 1995). Since all organisms need energy, ecotoxicological tests that involve the measurement of energy budgets or their components (*i.e.*, ingestion, egestion, excretion, respiration, production) are available for a wide range of organisms (Fig. 5, Table S2).

4.5. Mortality

Ultimately, when compensatory mechanisms are overwhelmed, stress levels become too high, or all previously described sub- and whole-organismal responses become too severe, which can lead to the death of an organism. Although all organisms can die, mortality as response to chemical stressors is most frequently measured for invertebrates, amphibians and fish (Fig. 5). In general, mortality is the most frequently measured endpoint for these groups of organisms (Fig. 5). Although mortality of microorganisms and algae is often expressed by population growth, methods exist to assess bacteria and microalgae cell death, such as the cell digestion assay, staining methods and instrumental analysis (Wang et al., 2020). However, these methods were not identified by our literature search.

Alternatively, immobilization is often taken as a proxy for ecological death (*i.e.* being ecologically inappreciable) (see also Section 4.1), especially for when invertebrates and neurotoxic compounds are concerned. The immobility of an organism make them an easy prey, might cause starvation and impedes reproduction (Sánchez-Bayo and Goka, 2006).

5. Population, community and ecosystem responses to chemical stress and available methods

Lastly, responses to chemical stressors can propagate up to the population-, community- and ecosystem-level. We will discuss in this section (i) how chemical stressors might affect these levels of biological organization, followed by (ii) whether and how these responses can be measured by the available ecotoxicological methods. A total of 78 ecotoxicological tests measuring responses at those levels were found by our literature search.

5.1. Population responses

Dynamics of population abundances are primarily driven by the demographic processes of birth and death (Smith et al., 2012). Hence, chemical stressors can affect population growth rate directly by increasing mortality rate and/or decreasing birth rate. In consequence, increasing concentrations of chemical stressors generally result in decreasing population growth rates (Walker et al., 2016).

For assessing population growth rates, it is necessary to follow the population of an organism over enough generations (Rohr et al., 2016; Stark and Banks, 2003). For microorganisms and algae, population-level tests can be completed within hours to days, whilst for invertebrates these can last from a couple of weeks to several months or years. For vertebrates, population-level tests can even last several years. As a consequence of this variation in test duration, we found many tests conducted on microorganisms and algae (organisms with a short life span), whilst for vertebrates, population-level test were scarce (Fig. 5, Table S2).

5.2. Community and ecosystem responses

Communities are a set of populations of different organisms that interact through competitive, trophic and other relationships. Ecosystems are communities considered in their physical-chemical environment. While attributes of communities are structural measures such as trophic organization and species diversity, ecosystems include functional measures of primary production and element cycling rates (Suter II, 2016). The most frequent structural changes of a community in response to chemicals are that some species decrease in abundance, some species increase in abundance and some species remain stable. This range of structural changes depends on differences in species sensitivity, and on indirect effects of trophic and competitive relationships (Fleeger et al., 2003).

Except for microbial and algal communities, ecotoxicological tests to assess community- and ecosystem-level effects of chemical stress are scarce. Particularly, the pollution-induced community tolerance (PICT) concept (Blanck et al., 1988) has been applied to biofilms (*e.g.* Blanck (2002); Guasch et al. (2012); Rotter et al. (2015); Tlili et al. (2020)), testing responses of microbial and algal communities by measuring metabolic activity and shifts in community composition (Fig. 5,

Table S2). This method rests on the assumption that under chemical stress exposure sensitive individuals and species will be replaced by more tolerant ones and thereby resulting in increased community tolerance (Schmitt-Jansen et al., 2008). Therefore, a previously exposed community should have higher tolerance to chemical stressors than an unexposed reference community (Tlili et al., 2016).

Another ecotoxicological method to assess responses the chemicals at the community and ecosystem level are by using microcosms and mesocosms, together termed model ecosystems. Model ecosystems are artificial, small-scale, sometimes replicated ecosystems that contain multiple species and usually multiple media (Suter II, 2016). Microcosm are smaller in size and can therefore more easily be maintained in the laboratory (Clements and Newman, 2003) and range from microbial communities in small beakers to aquaria containing multiple trophic levels. Mesocosms are larger in size and are usually (partially enclosed) outdoor experimental setups with some exchange with the natural environment (Clements and Newman, 2003). Endpoints measured on community- and ecosystem-level in model ecosystems include structural metrics (*i.e.* species abundance (population growth), biomass and diversity) and functional indicators (*i.e.* ecosystem processes and energetics) (Fig. 5, Table S2). Although, invertebrate species are often included in cosm experiments, ecotoxicological tests at the community level for amphibians and fish were not found by our literature search (Fig. 5).

Lastly, as already briefly discussed in the introduction, ecological indicators, indices and metrics are regularly used to assess impacts of chemical stressors on the community level during monitoring practices in the field (Maloney, 2019; Martinez-Haro et al., 2015). However, these methods are beyond the scope of this review and therefore not included in Fig. 5.

6. Challenges for ecotoxicological tests in assessing impacts of chemical stress to aquatic ecosystems

As illustrated by the previous sections, responses to chemical stressors in aquatic ecosystems can be measured at all levels of biological organization,. In ecotoxicology, measured or expected effects are frequently linked to environmental protection goals, which often concern populations, communities and ecosystems. Therefore, it would be ideal to have a method that is i) able to measure responses to chemical stressors at an early stage (*i.e.* at a low level of biological organization), ii) able to indicate specific chemicals or groups of chemicals that cause the response, and finally, iii) tightly and consistently linked to population and community level effects. These goals were already formulated in the 1970s, and initiated the start of biomarker research with the aim to link a biochemical response in fish to the presence of chemicals with a specific MOA, and at the same time linking those biochemical responses to whole organismal level and potentially populations, communities and ecosystems (McCarty and Munkittrick, 1996). However, it soon became evident that this was not easily achieved (McCarty and Munkittrick, 1996).

Key for extrapolation between different levels of biological organization is mechanistic understanding of the relationships between the underlying complex systems (Celander et al., 2011). This can be relating quantitative changes at the sub-organismal level (such as proteins) to cellular-, organismal- or population-level outcomes, or by using responses measured in a limited range of organisms, or even *in vitro*, to predict the impact on a community or ecosystem. Now, 50 years after the first aquatic biomarkers studies, the number of methods has increased and mechanistic understanding has progressed substantially. Although some biomarkers have been around for decades, often they are not generally being applied in regulatory risk assessments. It still remains challenging to identify linkages and extrapolate measured responses in ecotoxicological tests to ecosystems. These challenges have been ranked as being of the greatest importance for the field of environmental toxicology and chemistry during a European horizon-scanning exercise (Van den Brink et al., 2018). In the following sections we will address challenges of identifying linkages to specific chemical stressors and on extrapolating measured responses to higher levels of biological organization in more detail (Fig. 6).

6.1. Linking sub-organismal responses to specific chemical stressors and MOA

Biomarkers and *in vitro* bioassays can be used to assess exposure to chemical stressors. Some biomarkers can be considered as 'specific', meaning that it is known that they respond primarily to a specific group of substances, often with a shared MOA. Examples of such biomarkers are induction of vitellogenin for environmental estrogens or metallothionein for metals. However, with research progressing, it has become evident that even those biomarkers are not entirely specific and respond to other (nonchemical) stressors too. Similarly, it is increasingly recognised that chemicals grouped within a specific MOA can also induce other effects. For example, it has been shown that chemicals that induce oxidative stress also induce metallothionein (Bauman et al., 1991). Thus, although biomarkers can be used to indicate exposure to a particular chemical stressor or MOA, based on the resulting effect, additional research is needed (such as EDA, see Section 7) to confirm the causes or substances causing the observed biomarker responses.

Other than biomarkers, most of the *in vitro* bioassays are developed to measure specific responses such as receptor binding or enzyme inhibition and provide information about the MOA of the chemicals present in the water sample. Although the specific substance causing the response is still unknown by using MOA-based systems, knowledge of MOA can support the identification of the responsible chemicals (Brack et al., 2016). However, *in vitro* bioassays can be sensitive to a broad range of chemicals and in these cases, detected chemicals might only explain a small fraction of the responses (Neale and Escher, 2020).

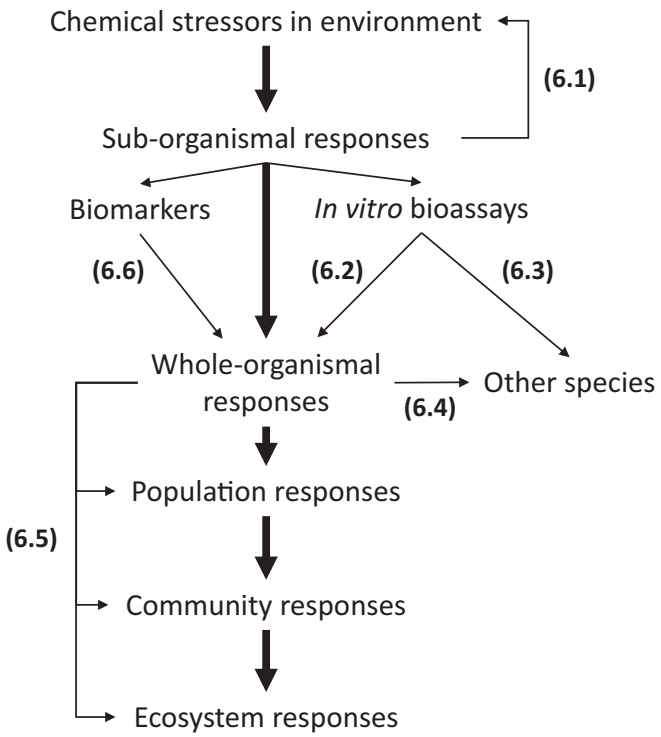

Fig. 6. Schematic visualization of the propagation of responses to chemical stressors from lower to higher levels of biological organization (bold arrows) and the challenges of identifying linkages and extrapolating measured responses by ecotoxicological tests to other species and higher levels of biological organization as discussed in Section 6 (indicated by the corresponding paragraph number between brackets).

Specifically, less than 1% of the measured responses could be explained with extensive chemical analyses in the p53 response (Neale et al., 2015), peroxisome proliferator-activated receptor (PPAR) (König et al., 2017), pregnane X receptor (PXR) (Neale et al., 2017a), oxidative stress response (Escher et al., 2013; Neale et al., 2015; Neale et al., 2017b) and androgen receptor (AR) (Neale et al., 2017b). These examples indicate that there are unknowns present, many or a few, with low or high activity, supporting the need for a combination of approaches (such as targeted, non-targeted analysis and (eco)toxicity tests). Importantly to note, however, is that in other cases the measured *in vitro* bioassay responses could be explained for a good part by a few chemicals, as illustrated in Neale et al. (2015) were up to 80% of ER activation was explained by five chemical and up to 71% of AhR activation by three chemicals.

Furthermore, only a few *in vitro* bioassays are available for neurotoxicity, immunotoxicity, inhibition of mitoses or energy metabolism (Fig. 3, Table S1) or for contaminants of emerging concern with alternative MOA (Altenburger et al., 2019; Dingemans et al., 2019). Consequently, relevant chemicals could escape attention. This poses a challenge for constructing a comprehensive *in vitro* bioassay panel, capturing all known MOAs of chemical groups and identifying MOA of chemicals of emerging concern. More *in vitro* bioassays for environmental monitoring based on mechanistic assays developed for toxicity testing, prioritized based on knowledge of the most relevant MOAs, can become available by addressing methodological specifications that currently preclude implementation (Schriks et al., 2015).

Ultimately, as shown in Section 3, for certain MOAs both biomarkers and *in vitro* bioassays exist that measure the same responses. For example CYP1A induction can be measured in fish (biomarker) and cells exposed *in vitro*. However, the most important difference between measuring CYP1A induction in a fish species and CYP1A induction in cells exposed *in vitro* are the toxicokinetic aspects (see Section 6.2). As this complicates extrapolation of *in vitro* bioassay responses to a potential risk to aquatic organisms, the direct use of biomarkers in these organisms can be a more suitable option. When the goal is to detect activity of chemicals in surface waters, this uncertainty may be acceptable and *in vitro* bioassays are a fitting alternative.

6.2. Translating in vitro responses to in vivo effects

Although *in vitro* bioassays responses do not only show the bioavailability of chemicals but also interaction at subcellular level, the question remains whether the measured responses can be translated into effects on aquatic organisms. One goal of the use of *in vitro* bioassays is to replace, reduce and refine (3R) *in vivo* tests, especially vertebrate tests (Rehberger et al., 2018). Since in ecotoxicology fish are the most common vertebrates used for toxicity testing, efforts to implement the 3R principle are primarily directed towards replacing *in vivo* fish tests (Rehberger et al., 2018).

This resulted in the development of *in vitro* cytotoxicity assays with fish cells as a potential alternative to the *in vivo* lethality test with fish. Expected was that when a certain chemical concentration causes cell death *in vitro*, the same concentration will cause cell death *in vivo*, eventually leading to systemic failure and death of the organisms. However, several studies showed that the absolute sensitivity of fish-cell based *in vitro* bioassays appeared to be lower than fish lethality of *in vivo* bioassays (Kilemade and Quinn, 2003; Rehberger et al., 2018; Segner, 2004), in this case demonstrating that the results cannot be directly translated to *in vivo* effects (Rehberger et al., 2018).

The challenge here entails translating the *in vitro* results to corresponding *in vivo* exposures. One important and obvious difference between *in vitro* and *in vivo* bioassays is the toxicokinetics (Yoon et al., 2012). In *in vitro* bioassays the processes of absorption, distribution, metabolism and/or biotransformation, and excretion (ADME) of the chemical, in addition to cell-to-cell interactions, are absent. To be able to translate *in vitro* concentrations to equivalent doses in organisms, appropriate (quantitative) *in vitro* to *in vivo* extrapolation (IVIVE) models are needed (Villeneuve et al., 2019). Such models have been developed in human toxicology, but are still lacking for aquatic species (*e.g.*, fish) (Brinkmann et al., 2016). Mixtures create an additional challenge for conducting IVIVE analyses due to different characteristics of individual chemicals and potential interactions.

Another proposed method for interpreting *in vitro* responses is by deriving effect-based trigger values (EBT) for reporter gene assays. For derivation of EBTs for surface waters, safe concentrations *in vivo* are translated to equivalent *in vitro* concentration. This is typically based on a reference compound for that specific bioassay (Leusch et al., 2014a) or, alternatively, on different potencies of the bioactive chemicals and by using a read-across approach also accounting for mixture effects (Escher et al., 2018) or bioavailability (Brand et al., 2013). Another approach, by van der Oost et al. (2017), integrates all available *in vivo* effect concentrations and SSD models to derive an EBT for aquatic systems. In a recent study, a framework is developed to determine the protective power of derived EBT values and the chance that potentially harmful substances might not be detected (Been et al., 2021). Due to the inherent assumptions and limitations of underlying datasets, current EBTs should merely be considered as indications for a level where a risk can no longer be excluded and additional water quality research is warranted. Research is ongoing to further refine EBT and support their applicability in practice.

6.3. Linking mammalian-based in vitro bioassays to aquatic organisms

While some fish-based *in vitro* bioassays are available, most *in vitro* bioassays are developed for human toxicology and are therefore strongly mammalian-biased (Fig. 3, Table S1). In order to use mammalian-based *in vitro* bioassays to assess potential effects of chemical stressors to aquatic organisms, the predictivity of mammalian receptors for aquatic organisms needs to be evaluated.

Some human receptors used for *in vitro* bioassays are conserved among vertebrates and can be found in fish, as we showed in Section 3. However, research on cross-species extrapolation from human to fish is largely focused on pharmaceuticals, since pharmaceuticals are actually produced to interact with specific human targets (*e.g.* enzymes, receptors). The read-across hypothesis predicts that if these human molecular targets are evolutionary conserved and functional among aquatic organisms (especially in fish), the presence of pharmaceuticals in the environment could potentially lead to toxicological effects (Brown et al., 2014; Gunnarsson et al., 2008; Margiotta-Casaluci et al., 2014; Rand-Weaver et al., 2013). Thus, the principle idea behind read-across approaches to predict the sensitivity towards chemical stressors between taxonomic groups is based on the conservation of a specific target that is responsible for initiating the biological response (McArdle et al., 2020). Therefore, identifying the presence (or absence) of this target within different organisms, and understanding this conservation with respect to function among vertebrates, is critical to identify (eco)toxicity tests that are predictive for effects on aquatic ecosystems.

The presence of a specific target across species can be addressed by the Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) tool developed by the USEPA (LaLone et al., 2016). By using the SeqAPASS tool, protein sequence similarities can be evaluated and used to predict the sensitivity towards chemical stressors across species. Expected is that with increasing protein sequence similarities between a known sensitive species and another species, the likelihood that the chemical might interact with that protein in the other species increases as well (LaLone et al., 2018).

The presence of a target, however, does not mean that the physiological function is the same. The ligand binding region must be highly conserved between species for functional cross-activity (Gäde and Marco, 2006). Due to differences in amino acid sequences of the receptor between humans and fish, cross-activity may not or only partially occur. Also the relative effect potencies of chemicals can be different if the

target is similar. For example, for AhR phase I effects, differences in relative effect potencies of substances are observed for different species (Hahn, 2002). Next to that, if the role of a receptor is not understood in the normal physiology of an organism, it remains unclear whether organismal effects observed after exposure to chemical stressors are due to specific effects caused at a this particular target (Rand-Weaver et al., 2013).

Thus, while above described extrapolation approaches are already challenging for fish, for invertebrates it becomes even more difficult to extrapolate from *in vitro* assays with human receptors, as the knowledge on the presence and understanding of receptors and their function is largely unknown. For example, for molluscs it is known that they have true estrogen receptors, but they seem unresponsive to estrogens in at least two cases, indicating that estrogens may act through a non-estrogen receptor mediated pathway in molluscs (Köhler et al., 2007). For most invertebrate species, endocrine pathways have not been well studied and also can be quite diverse in some of these species (Thornton, 2003). Since receptor pathways have diversified so thoroughly and the role of these nuclear receptors is unknown for most invertebrate species, it is in most cases not appropriate to use indicators of endocrine disruption based on their function in mammals for invertebrates (Thornton, 2003). Hence, at present we still lack *in vitro* bioassays for invertebrates and since the background knowledge of many invertebrate species is limited, extrapolating from the mammalian-based *in vitro* bioassays to potential effects on invertebrates has to be considered unreliable.

6.4. Interspecies extrapolation of whole-organism responses

The next challenge in assessing impacts of chemical stress to aquatic ecosystems is that the number of species used in ecotoxicological tests, and whose outcomes form the basis of environmental quality thresholds, constitute a miniscule fraction of the extremely large number of species that are actually exposed in aquatic ecosystems all over the world (Wilson, 1999). Traditionally, the uncertainty and variability associated with testing only a couple of species in the laboratory to determine the sensitivity of all species, is captured by the use of assessment factors. However, this assessment factor approach is applied to all species assemblages, and therefore lacks realism and specificity in capturing the real variability of species sensitivity over space and time. A way to overcome this is by extrapolating the sensitivity of known species to the sensitivity of unknown species. Here, we will briefly discuss two approaches that can be applied to extrapolate chemical sensitivity across species: a descriptive and a mechanistic approach.

A potential descriptive approach used to perform cross-species extrapolation of chemical sensitivity, is to use the species sensitivity distribution (SSD) concept. SSD are used in ecotoxicology to map the variation in species sensitivity to chemicals, and usually result in a potentially affected fraction (PAF) of species potentially affected by the concentration of the chemical under study (Posthuma et al., 2001). Cross-species extrapolation in this approach usually occurs two-fold: i) by transforming the experimentally tested set of species into a distribution, resulting in a presumably representative threshold value for all potentially exposed species (usually at a PAF-value of 0.05, which constitutes the hazardous concentration for 5% of the organisms (HC5), thereby protecting 95% of the species), and ii) by adding an additional assessment factor on the PAF value, to accord for any other potential extrapolation necessary (*e.g.* to species which are not easy to rear in the laboratory). However, the SSD does not provide a mechanistic explanation to why a certain species is sensitive or tolerant and is mostly descriptive. As pointed out by Segner et al. (2014), it is important to move away from those descriptive approaches towards an integration of physiological and ecological traits of species in mechanistic approaches. This, because only with mechanistic understanding we will be able to predict sensitivity with little uncertainty from the limited amount of available data.

Therefore, the next approach we want to address, concerns a mechanistic approach. In case of the whole-organismal level, ecological or phenotypical attributes of organisms can be used to differentiate organisms according to their sensitivity towards chemicals (*i.e.*, size, mode of respiration, presence of enzyme and receptor systems, Van den Brink et al., 2011). These are referred to as trait-based approaches, and have been introduced into the field of ecological risk assessment to link chemical exposure and effect mechanistically, and thereby enable extrapolation of species sensitivities over chemicals with the same MOA (Van den Berg et al., 2019). By linking traits like size and mode of respiration to sensitivity, this approach aims to predict the sensitivity of untested species from the traits it possesses (Van den Berg et al., 2019). Combining this mechanistic approach with the mechanistic sub-organismal level approaches discussed in Section 6.3 is likely to result in the strongest cross-species extrapolation models, as also has been suggested by van den Berg et al. (2020).

6.5. Extrapolating whole-organism responses to higher levels of biological organization

One of the main challenges in ecological risk assessment is to provide useful predictions of effects at population and/or community level, that is, the levels which are usually found the most relevant for protection goals in aquatic ecosystems (see also Forbes et al. (2006); Rohr et al. (2016); Van den Brink et al. (2018)). Since population dynamics are based on the rates of birth, death and migration, whole-organismal endpoints like mortality and reproduction have a direct link to population effects. However, adaptive and compensating mechanisms can modulate effect propagation across taxa, hampering extrapolation from whole-organismal responses to population and ecosystem-level effects.

What complicates extrapolation from whole-organismal to population level even further are intra- and interspecific interactions, which play an important role in ecosystems, and these interactions cannot be predicted by measuring responses to chemical stressors at the whole-organismal level. Thus, to extrapolate effects of chemical stressors from whole-organismal responses such as growth, reproduction or mortality to demographic changes of populations, methods are needed that integrate toxicological with ecological information (Segner, 2011). These methods are being developed, for instance, in the shape of population-level effect models taking into account species life history (Diepens et al., 2016; Dohmen et al., 2016), as well as methods considering indirect effects and interactions within communities (De Laender et al., 2015; Rohr et al., 2016; Schmitt-Jansen et al., 2008).

For sublethal responses, an additional step is required to extrapolate to higher levels of biological organization. This will be discussed in more detail in the next Section 6.6.

6.6. Extrapolating sublethal responses to higher levels of biological organization

Sublethal responses will only affect the population and/or community when the measured response are 1) affecting organisms' normal functioning, and 2) can mechanistically be linked to the individual demographic processes of birth and death rates (Baird et al., 2007; Forbes et al., 2006; Lam, 2009). With respect to the first argument, organisms exposed to chemical stressors can undergo physiological changes to adjust to the new (stressed) situation and maintain normal function (called accommodation or adaptation) (Nichols et al., 2011). These compensatory mechanisms can mask the impacts of chemical stressors. This highlights the important distinction between a measured sublethal response and actual biological consequences, complicating the issue when these responses indicate actual adverse effects of chemicals or are just indicators of exposure. However, compensatory mechanisms have energetic costs resulting in changed energy allocation (see Section 4.4), and can therefore still result in ecological effects when exposure is long-term. Such mechanisms can be captured by modelling

approaches, two of which will be addressed in the next paragraphs: Dynamic Energy Budget (DEB) models, and Adverse Outcome Pathways (AOPs).

DEB models are one of the most frequently used bioenergetic models to characterize sublethal effects of chemicals, and provide quantitative measures of energy costs related to chemical exposure (Kooijman et al., 2009). An advantage of using energy parameters is that they are suitable to provide an integrated measure of mixture toxicity. When exposed to chemical stressors, the energy costs of an organism increases and can be related to the duration and intensity of the combination of chemical stressors presence, instead of only focusing on only one specific chemical stressor (Segner et al., 2014). Additionally, energetic models have been used to extrapolate bioenergetics of individuals to population growth (Baas et al., 2018; Jager et al., 2014; Martin et al., 2013). One of the main groups of DEB models in ecotoxicology is DEBtox, a toxicokinetic-toxicodynamic (TKTD) model that contains a DEB model to describe the toxicodynamics (Jager et al., 2006). For a more elaborate discussion on these models, we refer to the extensive body of literature that exists on this topic (*e.g.* Ashauer et al. (2011), Jager and Selck (2011) and Jager (2020)).

Another way to extrapolate sublethal responses to higher levels of biological organizations is by using the adverse outcome pathway (AOP) framework. To model AOPs, processes at many sub-organismal levels of organization need to be characterized (molecular interactions and responses at the cellular, organ and organism level), thereby providing mechanistic information that can be used for interspecies extrapolation (LaLone et al., 2014), and for the development and refinement of toxicological assays (Knapen et al., 2015). However, in most cases, well-developed, quantitative AOPs that can aid in the extrapolation of sublethal responses do not exist (yet), and need further development (Goodchild et al., 2019; Maloney, 2019; Rohr et al., 2016). More often, putative or potential AOP are available that need more information on quantitative relationships between key events before they can support risk assessment, in the form of quantitative extrapolations (Villeneuve et al., 2019).

Some recent advances and promising developments in combining AOPs and DEB approaches exist. Goodchild et al. (2019), for example, link the AOP framework to models used in ecotoxicology for higher levels of biological organization. Recently, they developed a conceptual model (the bioenergetics-AOP framework) linking bioenergetic models (such as DEB) to the AOP framework, and thereby translating sublethal responses to whole-organismal and population-level effects (Goodchild et al., 2019).

7. Future for assessing responses to chemical stress in aquatic ecosystems

Our literature search revealed that many different tests exist in the ecotoxicological universe, measuring biological responses from the sub-organismal level up to the ecosystem level. However, regardless of this extensive availability, we still revealed some gaps in the available methods in terms of underrepresented species groups, biological endpoints and levels of biological organization. This leads us to discuss where in our opinion future research should focus on, how the different ecotoxicological tests complement each other and how ecotoxicological tests effectively could be used to assess impacts of chemical stressor to aquatic ecosystems in a comprehensive way in the future. We will not go into detail about the general application of these tests in a legislative context, since the EU-funded project SOLUTIONS recently produced a series of policy briefs translating their major finding into a legislative context and providing recommendations on the application of these tests (Brack, 2019; Brack et al., 2019).

7.1. Stressor identification

Existing ecotoxicological tests can be a valuable addition to complement existing monitoring efforts by providing help with stressor identification. Especially *in vitro* bioassays and biomarkers can aid in measuring responses of the complete pollution universe present in surface waters instead of specific individual compounds, thereby providing more insight into the chemical stressors (MOA) and mechanisms underpinning observed biological responses, although it is not always possible to discern which compound(s) are causing the measured sub-organismal responses, as discussed in Section 6.1. Some issues of repeatability, reliability, sensitivity, specificity and robustness still need to be overcome. As an example, the phagocytosis assay is one of the more popular tests to determine immunotoxicity but has shown not to be very discriminative between immunotoxicants and chemical stressors with other MOAs (Rehberger et al., 2021). An advantage, however, of these methods above a mere chemical analysis, is that they provide additional information. Biomarker and *in vitro* bioassay responses to water samples are caused by all (chemical) stressors present and bioavailable in the surface water sample, thereby overcoming limitations posed by analysing specific target compounds by chemical analysis (Brack et al., 2018). By using biomarkers and *in vitro* bioassays, an ecotoxicity profile of surface water samples can be generated and cumulative ecotoxicological risks can be calculated for monitored aquatic systems (De Baat et al., 2019).

Identification of chemicals, after indication of potential risks by ecotoxicological tests, could be pursued by effect-directed analysis (EDA) or related methods (Altenburger et al., 2019; Brack et al., 2019). Basically, in EDA, environmental sample extracts are reduced by fractionation to less complex mixtures. Subsequently, these subsamples are tested by bioassays so that the chemicals of subsamples for which toxic responses are measured by the bioassays can be isolated and identified by chemical analysis (Brack et al., 2016). While there are several limitations to the EDA approach (*e.g.* it does not allow for quantifying mixture effects, but see Brack et al. (2016) and Hecker and Hollert (2009) for in-depth reviews), we believe that the combination of chemical monitoring, (eco)toxicological tests and EDA can aid in the understanding of effects that are driven by the interaction of different compounds (Altenburger et al., 2019; Brack et al., 2019; Faust et al., 2019).

7.2. Effect detection

As mentioned in the introduction, the quality of ecological status is most frequently assessed based on ecological indices (Birk et al., 2012). However, using ecological data to establish the link to chemical stressors is still elusive due to the presence of multiple stressors and the lack of diagnostic power. Therefore we recommend the complementary use of ecotoxicological tests and ecological indicators, since they might aid in discriminating the impact of chemical stressors from other environmental factors affecting communities and ecosystem such as habitat loss and can serve as an early warning.

However, not all ecotoxicological tests are in their current state suitable in detecting effects relevant for aquatic ecosystems, and require further development. To take an example, estrogen disruption is relatively well studied for vertebrates with a number of fish biomarkers available (Fig. 3) and some mammal-based *in vitro* bioassays may be suitable alternatives due to the conservation of receptors (Table S3). However, no *in vitro* bioassays for invertebrates are available and since the level of understanding of invertebrate endocrinology is far less developed, extrapolating from mammalian-based *in vitro* bioassays to potential effects on invertebrates might be unreliable or impossible. Not only for endocrine systems, but for most biochemical pathways relevant for invertebrates, including invertebrate neurological and immune systems, *in vitro* bioassays are currently unavailable (Villeneuve et al., 2019). Hence, to be able to use *in vitro* bioassays as an alternative for invertebrate *in vivo* methods, research should focus on establishing a better understanding of basic processes and relevant pathways in invertebrates, and on developing *in vitro* bioassays and biomarkers for those pathways and processes.

Recent developments in omics technology could aid in this by providing a suite of new biomarkers including metabolomics, transcriptomics, and proteomics. Changes in DNA, RNA, protein products, and cellular metabolites can help unravel the mechanisms underlying observed responses and might help in cross-species extrapolation. Additionally, one can assess hundreds to thousands of molecular responses simultaneously within an organism in a systematic way, which will contribute to a more holistic understanding of effects of chemical stressors on organisms. As a result, omic technologies have also been suggested to annotate AOPs (Lee et al., 2015), since well-developed AOPs are still scarce. Hence, that omic technologies can play a role in assessing sublethal effect of chemicals is unquestionable, but the technology is only in its infancy. Improvements are needed to produce quantitative and reproducible omics data (Simons, 2018) and, just as we discussed earlier with respect to ecotoxicological tests, the challenge of discriminating between normal adaptive responses and changes associated to chemical stressors needs to be overcome.

Finally, for extrapolating measured responses to relevant effects we recommend the mutual development of new ecotoxicological tests and effect models like 1) toxicokinetic-toxicodynamic (TKTD) models to describe the fate, exposure and effects of the chemical at the individual level (*e.g.* EFSA (2018)), 2) individual based models to extrapolate these effects to the population level, by addressing, among others, intraspecific competition, individual dispersal and spatio-temporal variability of exposure (*e.g.* Focks et al. (2014)) and 3) food-web level models to address interspecies competition and food-web effects (*e.g.* Zhang et al. (2018)). Ecotoxicological tests should be available to parameterise these models as well as to test (validate) them. For the latter, modified exposure tests may be used to calibrate and/or validate TKTD models (Focks et al., 2018), while population, community and ecosystem level experiments, *e.g.* using microcosm and mesocosms, can be used to calibrate and/or validate models describing higher levels of biological organization. At the lower levels of biological organization the AOP concept can be used to mechanistically link sub-individual level observations made in experiments with each other, which can be laid down in TKTD models. Ecological population and food-web theory can be used to mechanistically extrapolate effects observed on individuals to the higher levels of biological organization. It is, therefore, of importance that more tests are developed that focus on these higher levels of biological organization as these tests are almost missing for organisms other than microorganisms and algae (Table S2).

Concluding, there is a clear need to better assess and understand how chemical stressors and mixtures can effect aquatic ecosystems and how this differs between species and can propagate to higher levels of biological organization. Important advances have been achieved in terms of the development of ecotoxicological tests, but also many challenges for future research remain before they can be generally applied in regulatory risk assessments. These challenges include the interpretation of the measured sub-organismal responses, *e.g.* can they indeed function as early warning indicators or are they false positives, challenges of extrapolation, across species and/or levels of biological organization, and more specific challenges associated with their practical use like using harmonized EBTs and the right selection of tests for each specific situation. Ultimately, when those challenges can be overcome, the combination of ecotoxicological experiments, models and tools that allow for high-throughput will lead to a more comprehensive assessment of the effects of chemical stressors to aquatic ecosystems.

CRediT authorship contribution statement

LS and PVdB designed the study. LS and FP performed the literature search. LS made the figures and wrote the manuscript, and LS, FP, SvdB, MD and PVdB contributed to improved versions of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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