Peer Reviewed: Challenges in Ecotoxicology

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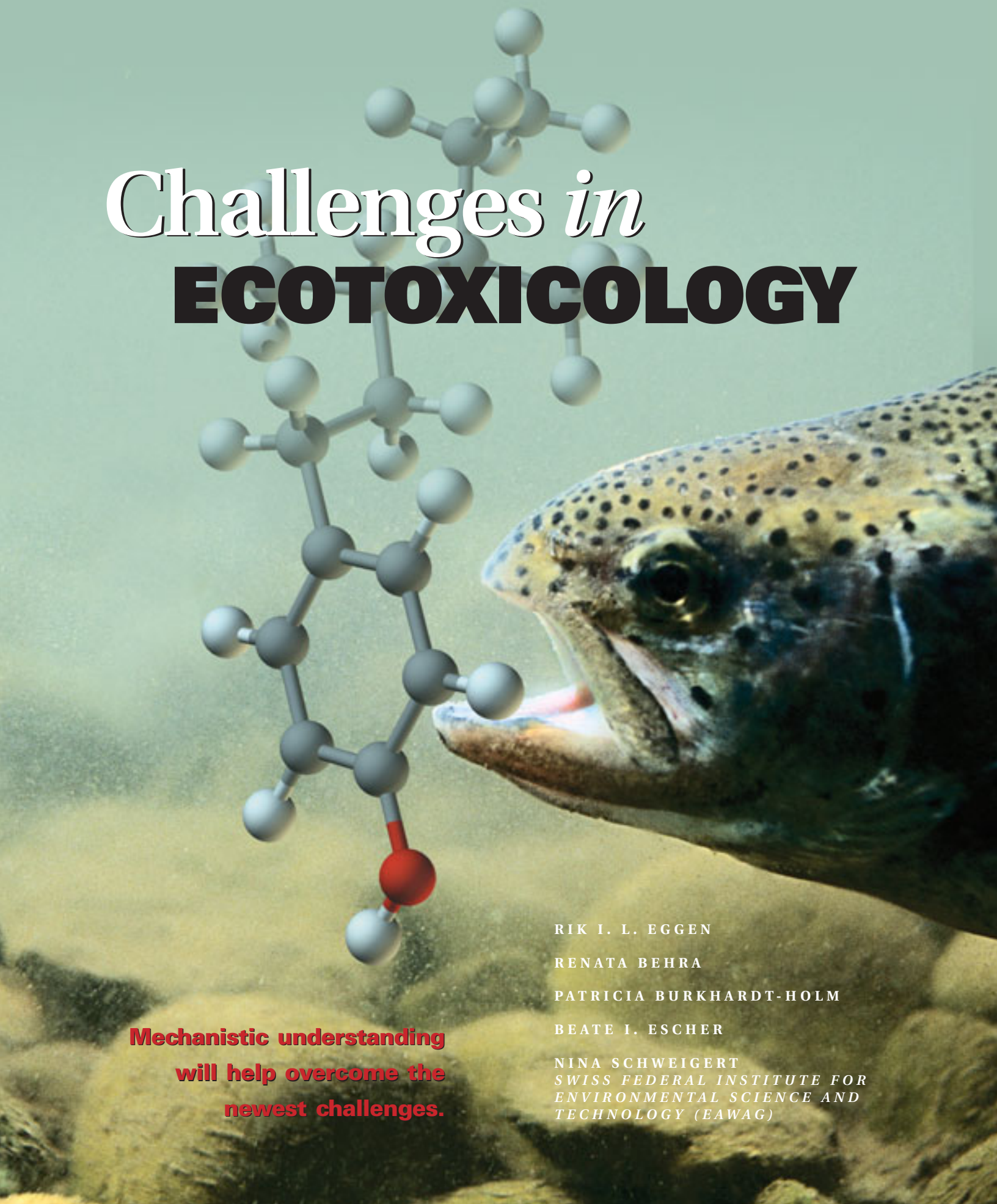
Challenges *in* ECOTOXICOLOGY



Mechanistic understanding will help overcome the newest challenges.

RIK I. L. EGGEN

RENATA BEHRA

PATRICIA BURKHARDT-HOLM

BEATE I. ESCHER

NINA SCHWEIGERT

SWISS FEDERAL INSTITUTE FOR ENVIRONMENTAL SCIENCE AND TECHNOLOGY (EAWAG)

Modern environmental management practice has significantly reduced the risk of acute and high-volume pollution in ecosystems in industrialized countries (*1*). Concentrations of many well-known pollutants have decreased because new, less toxic, and less persistent substances with low bioaccumulative potential are being used; their use has been optimized; recycling processes have been introduced; and wastewater treatment technology has improved. Nevertheless, ecosystems are still threatened by low concentrations of a steadily increasing number of pollutants that cause subtle and chronic effects. Ecotoxicology, however, has not yet adapted accordingly and is still based on testing methods and concepts that deal with and avoid acute environmental chemical pollution.

Pollutants rarely occur alone; they are usually mixtures or combined with nonchemical stressors. The potential threat to ecosystems by multiple stressors—ranging from radiation to alterations in temperature and CO_2 concentrations—is increasingly recognized (*2*). As a consequence, we are faced with changing and increasingly complex challenges in ecotoxicology (see "Overview and examples of current challenges in ecotoxicology" below). Dealing with these challenges will require new concepts, tools, and approaches.

In this article, we will illustrate how molecular approaches could change ecotoxicology by providing a better understanding of underlying processes. This knowl-

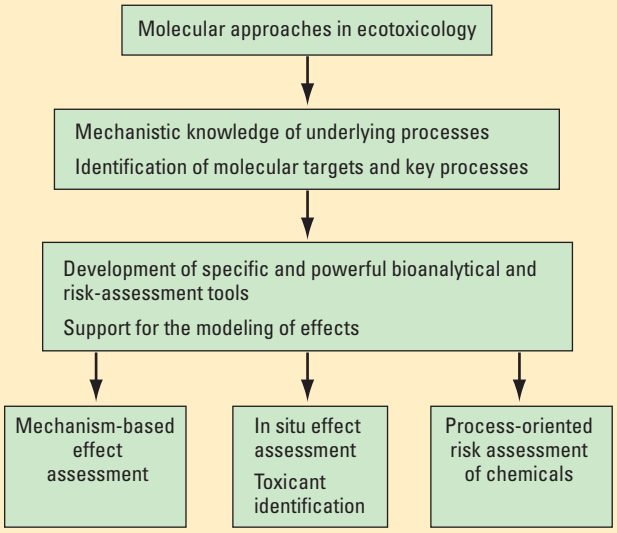
Examples of current challenges in ecotoxicology

	Challenges in ecotoxicology	Examples
1	Low concentrations of pollutants and long exposure times (chronic effects)	Endocrine disruption DNA damage/mutagenesis Deficiencies in the immune system Neurological effects
2	Multiple effects by single pollutants	Multiple target sites and multiple modes of toxic action Time- and tissue-dependent effects
3	Complex mixtures of pollutants	Wastewater treatment plant effluents Field runoff Pollutants and their degradation products Complexes of chemical compounds
4	Multiple stressors	UV and pollutants Temperature and pollutants Pathogens and pollutants
5	Ecosystem complexity	Variations in species sensitivities Effect of propagation from organisms to populations and ecosystems Identification of the stressor–effect relationship

FIGURE 1

Understanding ecotoxicology on a molecular level

Mechanistic knowledge is the basis for the development of bioanalytical and risk assessment tools, modeling of effects, mechanism-based effect assessment, identification of the causative toxic agent, assessment of effects in wildlife (in situ), and risk assessment of chemicals.



edge is essential for establishing mechanism-based assessments of the risks and effects of pollutants, developing powerful bioanalytical tools, and identifying causative agents and their effects on wildlife (Figure 1). Challenges in ecotoxicology will be presented in more detail, starting with single pollutants. Multiple effects by single compounds, chemical mixtures, and multiple stressors are addressed as well as ecosystem complexity. Finally, we will provide examples of how molecular approaches can be used to confront these challenges.

Challenge 1: Low-concentration and long-term exposure

Long-term, chronic exposure to low concentrations of pollutants may impair the fitness and viability of organisms, populations, or ecosystems. Recent discussions about the nature of the dose–response curve have stressed the importance of mitigating low-concentration effects (*3*). Research on endocrine disruption, which influences developmental processes, sexual differentiation, and reproduction, has impressively shown that routinely tested endpoints for growth and survival are not sufficient to detect these chronic effects. With the currently used ecotoxicological effect assessment tools, we have to wait a long time for visible and significant reactions in ecosystems to occur. The discrepancy, shown in Figure 2, between ecotoxicological effect assessment in practice (mostly relatively high concentrations and short exposure times) and effects under realistic scenarios (mostly low concentration and low exposure times) is not only restricted to endocrine disruption but is of a more general nature. For example, other, much less studied toxic mechanisms underlying possible chronic effects include influences on the neurological system, which affect developmental and differentiation processes or behavior; effects on the immune system, which alter susceptibilities for infectious diseases; and oxidative and photo-oxidative stress and increased radical production, which cause important biomolecules such as chromosomes to change or degrade.

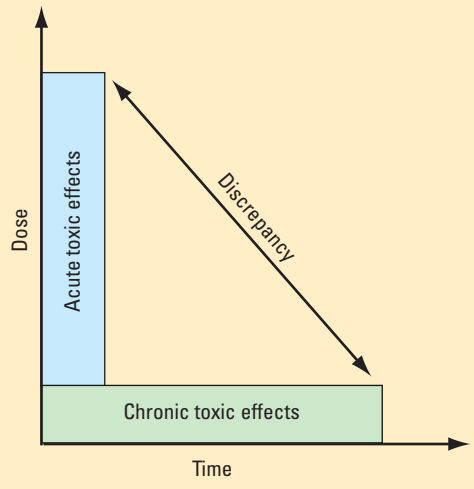
We do not fully understand the effects of low concentrations of pollutants at the molecular level, the link between chronic effects on organisms and effects on populations, and the ecotoxicological processes that lead to chronic effects. Furthermore, we do not know if we can extrapolate meaningful results from effects caused by high doses and short exposure times to effects caused by low doses and long exposure times. Both exposure scenarios may result in different effects with possibly different consequences (*4*).

We also do not yet have enough validated, effect-specific, sensitive, and fast bioanalytical tools that would allow us to assess the potential for chronic effects within practically acceptable time frames. A possible solution to this challenge would be to focus on initial triggers of toxicity instead of trying to observe subtle secondary effects at the population and ecosystem levels. Initial events like the onset of defense mechanisms or deviations from cell homeostasis can be meaningful indicators, if not of the actual effects then at least of the hazard potential of a given pollutant.

FIGURE 2

Relationship between dose and exposure time

Current ecotoxicological effect assessment tools require waiting for long time periods until visible and significant reactions in ecosystems occur. High dose and short exposure times are the most common ecotoxicological tests in practice, whereas low dose and long exposure times would be the more realistic exposure scenario.



Challenge 2: Multiple effects induced by single compounds

The physicochemical characteristics of a pollutant determine the type and degree of interaction with biological target molecules (*5*). Multiple types of interactions or interactions with different biological targets cause multiple biological effects.

For example, chemically reactive pollutants, such as electrophiles, react with different nucleophilic biological molecules. Depending on its electrophilicity, an electrophilic pollutant may prefer reactions with "soft" nucleophiles, such as thiol groups in proteins and peptides, or with "harder" nucleophiles, such as nucleotides in DNA. A reaction with peptides and proteins interferes with the cellular-reducing capacity through conjugation with glutathione or will interfere with enzyme activities, while DNA damage leads to mutations. The two types of effects are also relevant on different time scales. Although direct enzyme inhibitions may lead to acute effects, DNA damage is mainly relevant under chronic exposure conditions because of the increasing probability of failures in the efficiency of the DNA repair system over time.

Another example involves hydrophobic organic pollutants. Such pollutants accumulate primarily in membranes and will influence their permeability, the proton gradient, and/or the activity of enzymes, such as the ATP-generating ATPase, embedded in these membranes. Heavy metals, such as copper or iron, can also disturb the structure and function of membranes, block active enzyme centers, or form complexes with organic pollutants. These complexes may exhibit different effects than their constituents (*6*).

Cells react to pollutants and their effects by specific, fine-tuned defense and repair systems aimed at protecting a balanced metabolic network and cellular functioning. These reactions may occur by the activation of already available enzymes to remove or convert pollutants, or when stress-specific genes are activated and stress gene-encoded protein levels rise. Molecular regulation of the biological response is determined by the physiological status of the cells, the physiological status and function of the various exposed organs, and the developmental phase of the organisms. Thus, depending on a pollutant's chemical characteristics and the site and time of exposure, a single pollutant can activate a different array of genes in various organs and life stages.

These multiple and subtle effects cannot be identified if reduced growth, death, or reproduction are used as integrative toxicological endpoints. They are missed if the exposure occurs at the wrong time window or organism age. They can also be missed or misunderstood if single biomarker or gene responses are measured in a selected cell type or tissue, particularly if the biological context and relevance of these responses are unknown.

To examine the principles of multiple effects by single compounds, we propose to use relatively simple biological systems, such as subcellular systems or unicellular organisms, in combination with biomolecular tools such as the ones described in the proceeding sections. Using this approach, we have indeed been able to examine multiple effects under well-defined experimental conditions in low-complexity systems (*7*–*9*). On the basis of that acquired know-how, we could subsequently study biological systems with increasing complexity.

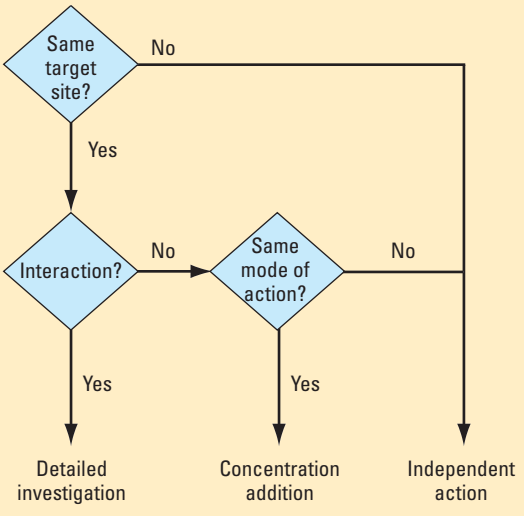
Challenge 3: Complex mixtures

Organisms are continuously exposed to chemical mixtures made up of various pollutants, different chemical forms (chemical species), metabolites, or pollutants in complexes. The general paradigm for assessing mixture effects stems initially from pharmacological research but has found wide application in ecotoxicology (*10*). This paradigm is depicted in Figure 3. If chemicals in a mixture have different molecular target sites, do not interact, and have different mechanisms, they act independently. If chemicals do not interact, but act at the same target site and have similar mechanisms, the effects are typically concentration-additive. If chemicals in a mixture interact on their way to their target site, their mixture effect cannot easily be predicted. These situations require case-by-case investigations to identify possible antagonistic or synergistic effects. The applicability of these concepts in ecotoxicology has been convincingly demonstrated (*11*, *12*) and illustrates that the correct assignment of the relevant mechanisms is a prerequisite for the effect assessment of mixtures. The previously proposed mechanism-based approach is also suitable for classification according to toxic mechanisms.

FIGURE 3

Addressing mixture toxicity in ecotoxicology

Answering the questions in the diamonds guides researchers to the conceptual model and/or research activities required to address or analyze the respective mixture toxicity issue.



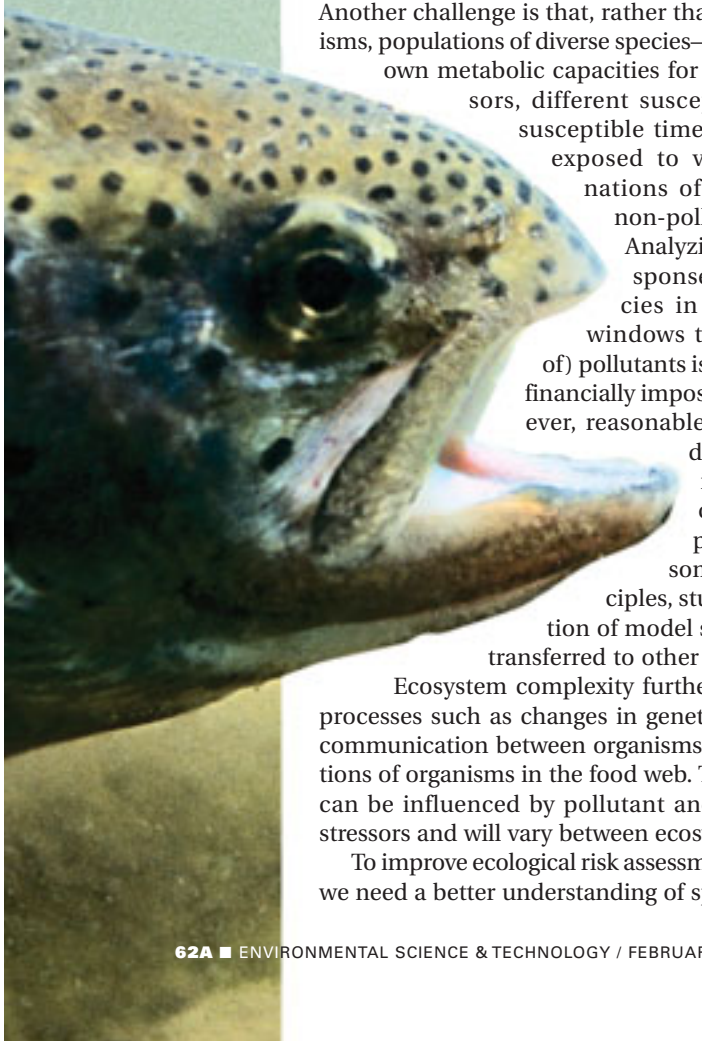
Challenge 4: Multiple stressor effects

Organisms are simultaneously or sequentially exposed to multiple stressors—for example, pollutants and nonpollutant stressors such as rising temperatures, acid rain, altered CO_2 concentrations, or increasing radiation (*1*). Changes caused by the combination of nutrients, such as phosphate or nitrate, with pollutants can also be considered as multiple stressor effects. Organisms exposed to ultraviolet radiation encounter direct effects, such as the formation of thymidine dimers in DNA, or they suffer oxidative and photooxidative stress, which leads to increased concentrations of reactive oxygen species that damage biomolecules, injure cells, or even cause cell death (*13*). Higher temperatures influence the biological metabolism, including enzyme-dependent defense activities against pollutant effects. Temperature also influences interactions between organisms and pathogens in humans (*14*) and in aquatic organisms (*15*). Nonchemical stressors can also directly affect chemical pollutants. For example, ultraviolet radiation induces degradation processes, which result in mixtures of chemical pollutants and their metabolites. Acidification caused by acid rain, for example, changes the chemical speciation and thus the bioavailable concentrations of pollutants (*16*).

We believe the best way to address this issue is to use a systematic approach: First, we must understand the mechanisms for the effects of each stressor alone, and then we can increase the complexity by combining stressors.

Challenge 5: Ecosystem complexity

Another challenge is that, rather than single organisms, populations of diverse species—each having its own metabolic capacities for handling stressors, different susceptibilities, and susceptible time windows—are exposed to various combinations of pollutant and non-pollutant stressors. Analyzing the toxic responses of each species in different time windows to all (mixtures of) pollutants is practically and financially impossible. It is, however, reasonable to expect that different organisms will have common endpoints and that some general principles, studied in a selection of model systems, can be transferred to other organisms.



Ecosystem complexity further encompasses processes such as changes in genetic diversity, the communication between organisms, or the interactions of organisms in the food web. These processes can be influenced by pollutant and nonpollutant stressors and will vary between ecosystems.

To improve ecological risk assessment procedures, we need a better understanding of species sensitivities and population responses, as well as their effects on communities and ecosystems (*4*). Insight into such complex interactions in ecosystems will require systematically examining processes to understand the underlying mechanisms of the various levels of biological organization—within organisms (molecule to organism), between organisms (differences in fitness, reproduction rates, and susceptibilities of the various species), and within ecosystems (food web interactions).

Implementing molecular approaches

Using the example of endocrine-disrupting chemicals (EDCs), we will illustrate how to approach the challenges just described. We will focus on estrogenic compounds and their effects on fish in aquatic ecosystems—an area in which much work has been recently reported (*17*, *18*)—to elucidate the potential of molecular approaches for risk assessment and the development of bioanalytical tools. We will also demonstrate the need for a mechanistic understanding of ecotoxicological processes.

Estrogenic compounds are a structurally diverse group consisting of natural and synthetic steroids, phytoestrogens, pesticides, and bulk industrial chemicals (*19*). Most of the currently known estrogenic compounds enter the aquatic ecosystems via wastewater treatment plants. After dilution in the receiving waters, these compounds occur at nanogram-per-liter concentrations for steroids and up to microgram-per-liter concentrations for industrial chemicals.

Results from in vitro and in vivo studies indicate that the steroid estrogenic compounds are most probably the primary cause for the estrogenic effects observed in wild fish. However, additional effects from estrogenic industrial chemicals cannot be excluded.

Estrogenic effects described in fish include increased plasma vitellogenin (VTG) levels; inhibited testicular growth and ovotestis, which probably occur as a consequence of male fish being exposed during a critical period of gonadogenesis (*20*); and reduced reproduction and fertility (*21*). Researchers hypothesize that fish populations became even smaller as a consequence of endocrine disruption (*22*). Despite significant work on estrogenic effects in the environment, major knowledge gaps do exist.

These gaps have raised numerous questions: How can we detect the hazard of estrogenic compounds occurring at low concentrations? Where in the organism does endocrine disruption take place? When does endocrine disruption occur—both from a seasonal point of view and from a developmental biology point of view? Which multiple effects occur in the various tissues, and what are the underlying mechanisms? What are the principal endocrine-active compounds, and what are the effects of mixtures of EDCs? Do nonchemical stressors have additional effects on endocrine disruption? What are the population-level consequences of effects that have been mainly observed in individual fish?

Initially, pollutant interactions occur at the cellular level. Genes are activated in a fine-tuned and coordinated way to maintain cellular homeostasis.

General stress and stress-specific responses may occur simultaneously. To assess the hazard potential of estrogenic compounds, either in pure form or in an environmental sample, researchers must focus on early biological effects. Hence, they have to analyze primary molecular events or immediate subsequent biological reactions.

Detailed examination of the molecular, biochemical, and cellular reactions are necessary to identify the relevant and stress-specific molecular processes, key regulatory components, and target genes. These early reactions at the molecular level can be used to develop specific bioanalytical tools and to assess the hazard potential of chemical or environmental samples. Taking other classes of compounds and biological effects into the broader perspective requires a battery of mechanism-based assays that cover the various biological effects and can be used for ecotoxicological risk assessment and environmental screening purposes (*23*).

Already, single estrogenic compounds can have multiple effects on cells, depending on the organ and organism. Steroid homeostasis, for example, encompasses steroid synthesis, transport, and clearing. These processes are influenced by the physiological status of the cells and the tissue, time of exposure, and cell- and tissue-specific signaling pathways, which can subsequently lead to different local expression levels of the estrogen target genes. Multiple effects in various tissues are thus to be expected.

A good example is the enzyme cytochrome P450 aromatase (CYP19), which converts androgens to estrogens and hence plays a dominant role in hormonal balance. Recently, two aromatase encoding genes (*cyp19a* and *cyp19b*) have been characterized in fish and their tissue distribution and expression profiles following exposure to endocrine-active compounds (*24–26*). The *cyp19a* gene is expressed primarily in gonads and *cyp19b* primarily in the brain.

Both genes are regulated differently during development and are induced upon exposure to EDCs, albeit with varying kinetics and responses (*27*). These results clearly illustrate the multiple effects of a single compound on only two genes coding for functionally identical proteins in two tissues. These findings also show how important it is to understand the relevance of gene expression and protein synthesis in a biological context. To use the expression of the aromatase gene or the enzymatic aromatase activity for bioanalytical purposes, we must know when during development, at what exposure time, where in the tissue, and in which tissue to conduct the measurement.

In situ detection methods, genomics, and proteomics make it possible to assess multiple effects of pollutants in different tissues of one organism. Genomics and proteomics are based on the ever-increasing number of known genome sequences and allow the simultaneous analysis of the expression of all genes from a genome (genomics) or the analysis of all proteins encoded by the genome (proteomics) (*28*). Therefore, ecotoxicologists can obtain a much more holistic and integrative view of chemical effects. Genomics and proteomics, combined with a functional approach for understanding the physiological role and relevance of the genes and proteins, represent great strides in understanding multiple effects.

However, novel technologies can also have problems and pitfalls (*29*). It will take time to realize the full potential of genomics in ecotoxicology. Many questions must be answered: Which species will be model organisms? What are the concentrations of pollutants used? When are we going to analyze gene expression profiles, and where in the organism are they found? What are the differences in global gene expression between individuals of the same species? A specific challenge will be to link gene expression profiles with whole-organism effects.

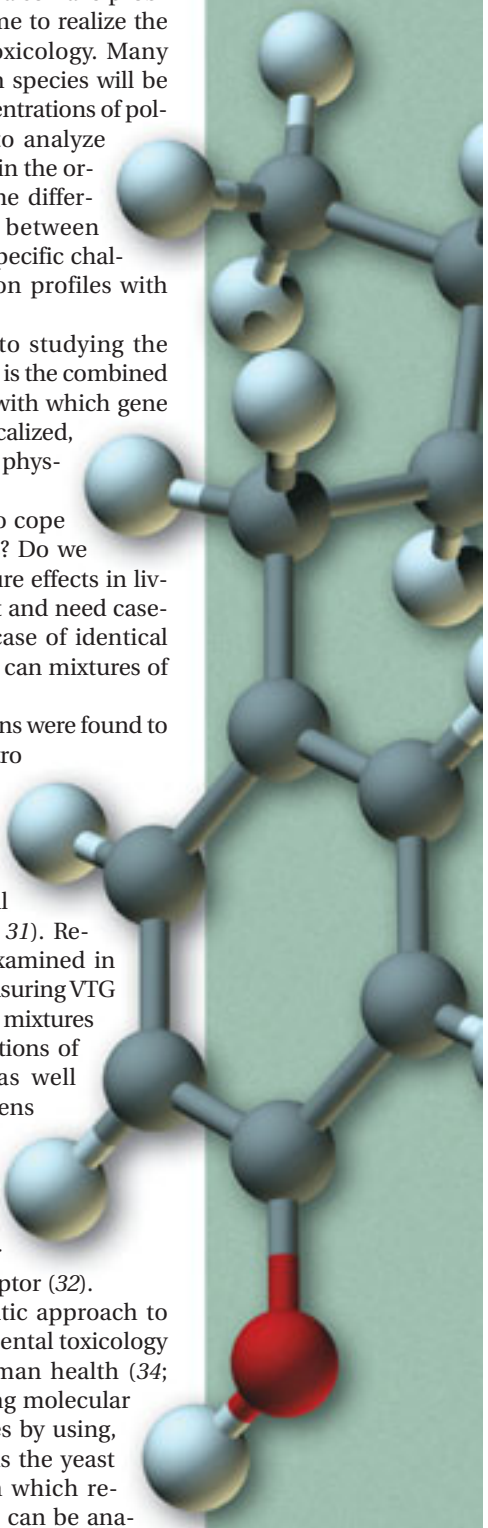
The most promising approach to studying the function of genes in whole organisms is the combined use of genetic and molecular tools with which gene expression levels can be modified, localized, and quantified, and with which their physiological role can be studied.

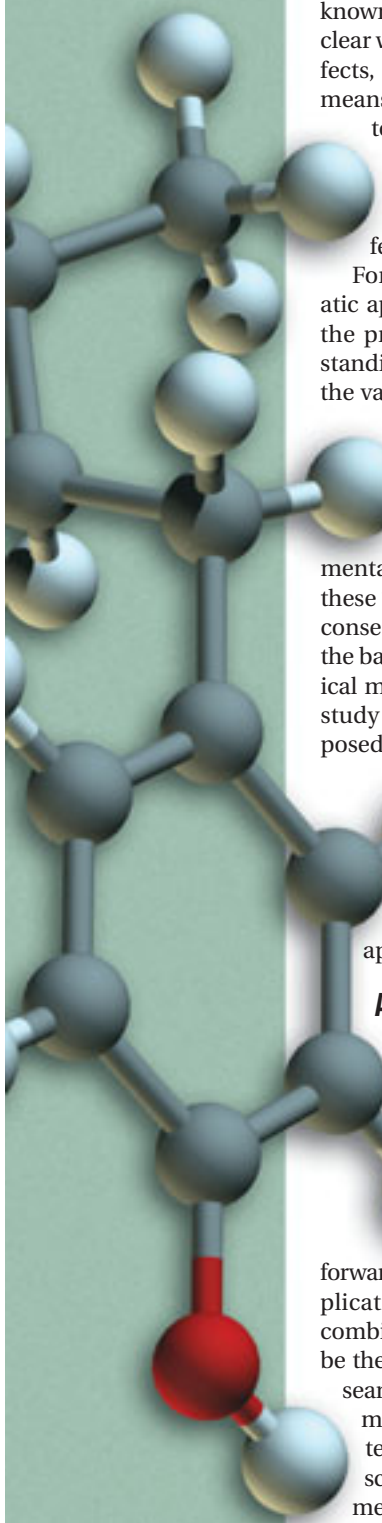
Do molecular approaches help to cope with the problem of mixture effects? Do we need mechanistic knowledge? Mixture effects in living organisms are difficult to predict and need case-by-case investigation. Only in the case of identical target sites and similar mechanisms can mixtures of chemicals act additively.

Estrogens and several xenoestrogens were found to act as concentration additives in in vitro test systems like the yeast estrogen screen, which is basically a measure of the binding to the human estrogen receptor. In this test, the encoding gene is introduced to a yeast cell and coupled to a marker gene (*30*, *31*). Recently, mixture effects have been examined in vivo in juvenile rainbow trout by measuring VTG levels in the blood of fish exposed to mixtures of estrogenic compounds. Combinations of estradiol and nonylphenol (*32*), as well as combinations of steroidal estrogens (*33*), showed the expected additivity. Combinations of estradiol and methoxychlor exhibited antagonistic effects, presumably because of a metabolic conversion of metoxychlor to an antagonist of the estrogen receptor (*32*).

Therefore, we suggest a systematic approach to mixture toxicity studies in environmental toxicology that is similar to proposals for human health (*34*; Figure 3). We also suggest integrating molecular approaches in mixture effect studies by using, for example, reporter gene assays as the yeast estrogen screen, mutant strains in which responses upon exposure to mixtures can be analyzed more specifically, or genomics and proteomics techniques for a more integrated picture of the responses.

Can molecular approaches also help to address toxicological processes that occur in complex ecosystems? In our opinion, yes. Endocrine-disrupting effects have mainly been analyzed on the level of





individual organisms—effects such as increased VTG levels in males or increased intersex ratios. Effects that might have impacts on population level (fecundity, hatching success, male reproductive health) are known (*21, 35, 36*). However, it is almost always unclear whether such influences result from primary effects, like VTG induction, or are generated by other means. Extrapolating effects from the molecular level to the cellular level or from individuals to groups of many individuals and species is a major objective in ecotoxicology that has yet to be achieved. This issue was the topic of a recent feature in *ES&T* by Calow and Forbes (*4*).

For this problem, we again recommend a systematic approach aimed at understanding each step in the propagation of effects, identifying and understanding the responsible toxicological mechanism at the various levels of biological organization, and the interactions between these levels. For example, by studying mutant organisms and their phenotypes, clues about the importance of a particular pathway are uncovered. The gene expressions during specific developmental phases can be deregulated, and the effects of these "disruptions" on developmental processes and consequences for reproduction can be examined. On the basis of detailed mechanistic understanding, critical molecular targets can be identified and used to study these targets in vivo in organisms being exposed in the environment (*37, 38*). By using genetic approaches, we can examine the genetic composition of populations and changes following environmental stress (*39*). Hence, even on the highest level of ecosystem complexity, mechanistic knowledge and molecular approaches will be beneficial.

Approach for the future

Modern molecular and genetic tools should be applied to mechanistic studies in ecotoxicology. The challenges in ecotoxicology also call for novel bioanalytical test systems for routine screening. For their development and validation, we need to use various tools in the exploratory phase and reduce them to simple, straightforward test systems for risk and hazard assessment applications. We expect that a sound scientific base combined with molecular biological approaches will be the key to success. At the same time, however, researchers need to know how subtle changes at the molecular level affect whole organisms and ecosystems. By using the strategies and approaches described in this article, we are convinced that we can meet these challenges in ecotoxicology.

Rik I. L. Eggen is a molecular biologist and ecotoxicologist, Renata Behra is a population ecotoxicologist, Beate I. Escher is an environmental chemist and ecotoxicologist, and Nina Schweigert is an ecotoxicologist at EAWAG. Patricia Burkhardt-Holm is currently an ecologist at the University of Basel in Switzerland. Address correspondence to Eggen at eggen@eawag.ch.

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