Molecular Mechanisms in Ecotoxicology: An Interplay between Environmental Chemistry and Biology

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Abstract. A close collaboration between environmental chemistry and biological sciences is required for a complete understanding of ecotoxicological effects. Bioavailability and uptake of pollutants cannot be regarded as isolated chemical or biological questions. Knowledge of the effective concentrations in the organism or at the target site(s) is essential to link the fate and effects of a chemical and is a prerequisite for quantitative investigation of the modes of toxic action. These modes of action need to be unraveled using whole-organism or in vitro systems in order to be able to develop specific biomarkers and biosensors that can be applied as early warning systems. Our mode-of-action-based approaches, in which chemical and biological analytical tools are combined, should improve the understanding of ecotoxicological effects and should be implemented in the future in risk assessment.

1. Introduction

Ecotoxicology is the science of the impact of toxic substances on living organisms, encompassing all levels of biological organization from single organisms to ecosystems [1]. Ecotoxicology integrates environmental chemistry, biochemistry, toxicology, and ecology in a multidisciplinary manner. The unifying theme of ecotoxicological research is to provide general concepts to evaluate the potential harmfulness of pollutants. This research is the basis for the development of tools that can be used in environmental regulation.

Despite major advances in the last decade, descriptive studies still make up the majority of ecotoxicological research concerned with the effects of chemicals. This has led to an accumulation of valuable empirical data on the effects of specific pollutants on selected species that are currently used for regulatory purposes. Much less emphasis has so far been placed on the development of general concepts that allow assessments of effects on the basis of explanatory principles.

In this more conceptually oriented approach to ecotoxicology, essentially two trends have evolved in the last decade. One trend is directed towards the understanding of underlying molecular mechanisms and modes of toxic action on different levels of biological organization, while the other trend is aimed at understanding the complex interactions and feedback mechanisms in ecosystems disturbed by pollutants. Taken together, these two approaches complement each other and will finally enhance the understanding of the effects of pollutants on living systems. In this paper, we focus on the mode-of-action-based molecular approach.

2. Ecotoxicology at EAWAG

Fig. 1 shows an overview of the processes considered in the molecular approach to ecotoxicology. Emphasis is placed on the importance of understanding the interplay between environmental chemistry and toxicology, thereby linking the concepts of bioavailability, effective concentration in the organisms or at the target sites, and the mechanism of ecotoxicity.

Evaluation of the adverse effects of pollutants in aquatic ecosystems requires discrimination between the total concentration of a chemical, the bioavailable fraction, and the final concentration at the target site(s) (Fig. 1, left). Environmental chemistry plays a major role in assessing the influence of environmental processes on the fate of a substance in the environment [2]. The fate of a chemical is affected by its physicochemical properties, the characteristics of the environment, and by biological processes. As a result, only a fraction of the total input into the ecosystem will be available for uptake by organisms.

![Fig. 1. The effective concentration of a pollutant in an organism (e.g., fish, daphnia, algae) or at the target site inside the organism is the link between the environmental fate of a pollutant and its toxic effect.](image-url)
Properties of a compound that affect its fate include its speciation and the hydrophobicity of the different chemical species. Speciation is important for metals (see Sect. 5), organometallic compounds [3][4] and hydrophobic ionizable organic compounds (HIOC) (see Sect. 4). In case of organotin compounds, charged species usually show a lower but still significant bioaccumulation as compared to the corresponding neutral species [5]. The freely dissolved fraction of a compound is primarily available for uptake by organisms. Sorption to minerals and organic matter reduces the bioavailable concentration. Even the presence of dissolved organic carbon reduces the bioavailability of organic compounds [5].

The uptake and effect of chemicals is not solely determined by the bioavailable concentration of a chemical, but is also influenced by biological factors, particularly differences in lipid content and sensitivity of organisms. Differences in sensitivity among species or populations of the same species are related to differences in morphology, developmental stage, sex, genotype, metabolic activity, and individual history. Moreover, organisms can evolve protective mechanisms in response to continuous exposure to elevated concentrations of a chemical. This results in an increased tolerance to the chemical, which further obscures concentration-effect relationships. The link between total, bioavailable, and effective concentrations is further illustrated in the following section using the example of uptake and effects of metals in algae.

Once inside the organism, the pollutant may initiate a variety of effects, ranging from cellular impairment to lethality. Any observable effect ultimately has a molecular cause (Fig. 1, right). This paradigm is supposed to be valid not only for effects on individual organisms, but also for the reproductive capability of populations and the functioning of ecosystems. To gain insight into the toxic effects of environmental chemicals, we look at the mode(s) of toxic action of a given chemical or mixture of chemicals and correlate the responses to effective concentrations at the target site(s). Particularly for compounds that are present in several chemical forms and/or act concomitantly according to different modes of action, the understanding of the overall toxic effect requires methods for distinguishing and quantifying the different modes of action. This approach is illustrated in Sect. 4.

Understanding the mode of action of pollutants is relevant in the development of sensitive ecotoxicological endpoints. In vitro assays can, to a certain extent, replace toxicity tests on whole organisms and can be used for the assessment of the toxic potential of chemicals and environmental samples. Since they are simplified model systems, in vitro systems offer the opportunity to focus in detail on specific modes of actions [3]. From an ethical point of view, they are less problematic than animal testing. However, a major problem of in vitro systems is the difficulty to extrapolate the results to whole organisms. Another disadvantage is shared with animal testing on single species in that they typically cannot account for species-specific differences in sensitivity. Therefore, species-specific systems have to be developed. In Sect. 5, different applications of a fish-specific in vitro system are shown, employing a permanent fish hepatoma cell line (PLHC-1).

Further processes whose importance cannot be unraveled by looking only at lethality or other observable effect endpoints include defense and repair mechanisms. Organisms have developed protective mechanisms (such as mobilization of various cellular constituents and enzymes that in a highly coordinated way minimize disturbances of cellular homeostasis) that allow them, within certain limits, to resist adverse conditions, including the negative effects of anthropogenic chemicals. If the disturbances become too large or are chronic, however, organisms react with stress responses, which are accompanied by an increased production of enzymes that dampen the deleterious effects of the stressors, or by repairing damaged cellular components. When protective mechanisms are overridden, toxic effects can follow, resulting in deleterious effects on individual organisms and eventually on populations. Examples of these effects are detailed in Sect. 6.

In the final section of this paper, the focus is on how the results of fundamental research on the impact of pollutants from a molecular to organism level can be used to develop tools and methods for environmental regulation and risk management. The majority of the assays presented here can be used as biomarkers and biosensors. A biomarker is defined as a measurable response at any level of biological organization that can be related to an impact of contaminants [6]. Biomarkers serve as screening tools for environmental contamination of mixtures of unknown composition and are used as an early warning system of exposure and effects. Just as biomarkers are an important tool for assessing biochemical effects, biosensors are used to examine the stress response of an organism. In the biosensor approach, the expression of affected genes can be linked to artificially introduced marker genes whose products can easily be measured (e.g., luciferase, β-galactosidase, green-fluorescence protein, or arylsulfa-
tase) [7]. Biosensors work in both prokaryotic and eukaryotic organisms and may be used for on-line monitoring of the environment.

3. Uptake and Effects of Metals in Algae

Most studies on the ecotoxicology of metals in the aquatic environment try to relate biological responses to nominal concentrations [8]. In aquatic systems, metals occur in various chemical forms, the formation of which is influenced by the locally prevailing physicochemical conditions, e.g., acidity, salinity, inorganic and organic ligands, and the presence of particles [9]. Chemical speciation is an important determinant of metal uptake and toxicity although its characterization is not trivial considering the diversity of chemical constituents of aquatic ecosystems. Moreover, in many cases, the concentrations of the free and other bioavailable forms cannot be directly measured but have to be calculated. Studies of metal-algae interactions in chemically defined culture media, in combination with thermodynamic calculations of the equilibrium speciation, are an approach to gain insights into the influence of the speciation of a metal on its biological availability [10].

At EAWAG, evaluation of the effects of chemical speciation on growth, metal uptake, and accumulation in algae is carried out at metal concentrations relevant for freshwater phytoplankton. Consideration of the kinetic properties of a copper uptake system indicates that Scenedesmus subspicatus has a high ability to accumulate copper, reflecting its adaptation to the bioavailable copper concentration [11]. According to the free-ion activity model for metal-organism interactions [12], copper and zinc uptake in S. subspicatus is related to the free and not to the total concentration in the culture medium [13]. This was indicated by experiments carried out in the presence of two different concentrations of the synthetic ligand EDTA. Because EDTA acts as a metal buffer, manipulations of the total metal concentration allow regulation of the free metal concentration. As shown in Fig. 2 for copper, when plotted against the free Cu²⁺ concentration in the media containing 10⁻⁴ and 10⁻³ M EDTA, the copper con-

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contents in the cells are comparable. Under environmental conditions, the influence of metal speciation on biological availability may, however, be more complicated. A comparison of the cellular copper contents of *S. subspicatus* grown in a synthetic culture medium or in lake water indicated that although grown at the same free Cu\(^{2+}\) concentration, the metal content was higher in cells grown in lake water. This observation was substantiated by the isolation of algae from the field which showed a higher copper content than algae grown in a synthetic culture medium with similar free Cu\(^{2+}\) concentrations. Possible explanations include the release of ligands by the algae, which may bind and therefore reduce the free ion concentration in the medium. It is also possible that other copper species such as lipophilic metal–organic complexes are available for uptake. To what extent some of the copper–organic complexes are bioavailable is not yet fully understood.

The role of biological variables in the control of metal availability is evidenced by comparative studies in which the growth rate of various species, including field isolates, was related to the free copper concentration. With regard to the range of free metal concentration in which growth was optimal, each species had a distinct tolerance range for metals [13]. Tolerance to metals may result from intracellular immobilization through metal-binding proteins and peptides, reduced metal uptake and enhanced exclusion, extracellular binding through organic chelators released by algae, or metal transformations [14]. Our current research is directed towards an understanding of the tolerance mechanisms.

Intracellular regulation of metals was indicated by the fact that *S. subspicatus* retained an optimal growth rate over a broad range of intracellular metal contents. Interspecific difference in sensitivity to metals was also indicated by experiments in which natural algal communities were subjected to a long-term exposure to copper in river water [15]. At high copper concentrations, the community was reduced to a few species. This reduced community showed a remarkably high tolerance to copper, but also a co-tolerance to other metals such as zinc, nickel, and silver. These results again raise the question of the strategies employed by organisms for controlling the cellular speciation of a metal. We are currently examining this question. Preliminary results are indicative of a reduction of bioavailable metal by extracellular complexation, and of cellular immobilization. In similar cases where metals are accumulated in organisms, the most effective approach for predicting toxicity may be to relate it to the concentration of toxicant taken up. Studies on the molecular mechanisms of protection and on the toxic action of metals may allow tests of this hypothesis.

**4. Inhibition of Energy Metabolism**

A variety of environmental pollutants interfere with energy metabolism through different modes of action, particularly, inhibition, uncoupling, and narcosis. In fact, the targets of certain pesticides are energy-transducing membranes, which are a common feature in unicellular organisms, mitochondria, and chloroplasts. Here, energy in the form of redox equivalents in oxidative phosphorylation or light in photosynthesis is transformed into an electrochemical proton gradient. This proton gradient drives ATPase, an enzyme that synthesizes ATP from ADP and inorganic phosphate. Pesticides and a variety of other environmental pollutants can directly inhibit membrane energization by blocking the electron-transfer chain. Hydrophobic ionizable organic compounds such as weak organic acids (e.g., the pheno/estric pesticides diosgenes and penta-chlorophenol) can in addition destroy the electrochemical proton gradient thereby short-circuiting the energy cycle. Finally, any hydrophobic compound can disturb membrane energization by nonspecific membrane perturbation, the so-called narcotic effect or baseline toxicity.

In our work, we apply time-resolved spectroscopy to distinguish and quantify these different modes of membrane toxicities, using the simple cyclic photosynthetic system of the purple bacterium *Rhodobacter sphaeroides*. Biomembrane vesicles isolated from this bacterium contain the complete functioning photosynthetic system. The decay kinetics of the membrane potential that is induced by a short flash of light are used to evaluate the uncoupling activity and the narcotic effect, whereas the redox kinetics of several components of the electron-transfer chain are used as indicators of specific inhibition [16].

Besides their use for assessing toxic effects and modes of action, the membrane vesicles of *R. sphaeroides* may also serve as a model membrane for uptake studies of pollutants. This allows one to correlate uptake and speciation at the target site with the actual toxic effect. It is generally assumed that charged organic molecules cannot penetrate biological membranes and are consequently not biologically active. This assumption is based on the partitioning of charged organic molecules into the organic bulk solvent octanol, which is more than three orders of magnitude smaller than that of the corresponding neutral compound [17]. Sorption studies of substituted phenols and substituted anilines to the membrane vesicles of *R. sphaeroides* or to pure phospholipid model membrane vesicles have shown, however, that a significant amount of charged organic molecules is incorporated into the membrane [18]. Only the uptake of neutral molecules into membranes can be satisfactorily modeled by octanol-water partitioning, whereas the uptake of charged molecules is underesti-
mated by several orders of magnitude. In the membrane-water systems, the distribution ratio of the neutral species is at most only one order of magnitude higher than that of the corresponding anionic or cationic species.

By comparing the effective concentrations of substituted phenols in the membrane with the inhibitory effect on the electron transport, we have shown that the dissociated phenolate species is a more potent inhibitor of electron transport than the corresponding neutral phenol species \([16]\).

The effective concentration and speciation in the membrane play an even more important role for the uncoupling effect of substituted phenols \([19]\). The mechanism underlying the uncoupling is a protonophoric shuttle: a proton is taken up by a phenolate from the aqueous side with the higher proton concentration. The neutral phenol thus produced diffuses across the membrane, discharges the proton in the aqueous phase of the opposite side, and the residual phenolate diffuses back across the membrane to close the cycle. Experimental findings show that both species are required to cause the uncoupling effect. If the hydroxy function is blocked by methylation, or if only one species is present at physiological \(pH\), then only non specific narcotic effects are observed. The results obtained so far suggest that substituted phenols with acidity constants in the range of 4–8 act according to the uncoupling mechanism and exert their maximum activity at a \(pH\) at which there is an approximately equal ratio of neutral and charged species in the membrane (Fig. 3). Although there are differences in the intrinsic activity of the various substituted phenols, due to differences in the substitution pattern on the phenol ring, the major determinant of the overall effect is the membrane burden of the chemical together with its speciation.

It is well accepted that the narcotic effect of environmental pollutants is directly related to the membrane burden of the chemical \([20]\). The results obtained from the uncoupling study of substituted phenols show that this concept of membrane burdens can be extended to specifically acting compounds, if speciation is taken into consideration. This is a promising starting point for developing predictive methods for toxic effects of specifically acting compounds and for assessing the toxicity of mixtures.

5. Fish-specific in vitro Systems: Interactions with Enzymes and Proteins

As most environmental chemicals act species-specifically, toxicity towards fish can only be assessed in fish-specific systems. With the use of a permanent fish hepatoma cell line such as PLHC-1 we want to develop novel in vitro assays for the assessment of the environmental toxicity to fish and to reduce and replace animal testing, and ii) to demonstrate and validate the usefulness of such concepts in basic and applied ecotoxicological research and practice. Our studies show that fish cell-culture systems are suited for assessing the cytotoxicity of chemicals, for studying interactions of pollutants with cells, and for deriving structure-activity relationships. The in vitro cytotoxicity on fish cells of more than 50 environmental chemicals with different modes of toxic action, including organotins, chloro- and nitrophenols, sulfonic acids, and alkylphenols have been shown to correlate with the in vivo acute toxicity to fish \([21]\).

One well-established and important biomarker in the exposure assessment of aquatic systems is the induction of cytochrome P450-dependent monoxygenases (CYP) \([6]\). These enzymes are important in the metabolism and detoxification of pollutants, but may also cause toxicity via bioactivation of xenobiotics or by alteration of steroid-hormone balance. At least 50 different genes are known in mammals, and they are also present in lower animals and plants \([22]\,\[23]\). In vertebrates including fish, they are localized mainly in the endoplasmatic reticulum of the liver and several other organs. The monoxygenase system consists of two enzymes, CYP and NADPH-cytochrome P450 reductase, which are interrelated via the transfer of electrons resulting in the oxidation of a variety of substrates. Two distinctly different reactions are elicited in the fish cell line after exposure to environmental chemicals: induction and inhibition of CYP. In our studies, we have addressed both aspects by focusing on the isofrom CYP1A, which is important in ecotoxicology. We use an enzyme-linked immunosorbent assay (ELISA) technique that has been established in the permanent fish cell line PLHC-1 for the quantification of CYP1A protein with a fish-specific

![Fig. 3. Uncoupling activity of some substituted phenols (□, dimoseb, \(pK_a = 4.62\); ○, 3,4-dinitrophenol, \(pK_a = 5.48\); ×, 2,3,4,5-tetrachlorophenol, \(pK_a = 6.35\)) as a function of \(pH\) (left) and as a function of the chemical speciation (right), expressed as ratio of neutral phenol [HA] to charged phenolate [A−] in the biological membrane (m). The relative activity corresponds to the effect that is elicited by a certain total concentration of phenols in the membrane (0.0015 mol·kg−1) (adapted from \([19]\), see for details).](image-url)
monochlorinated biphenyls (PCBs), polychlorinated dibenzodioxins and furans, and polycyclic aromatic hydrocarbons (PAHs) are potent inducers of CYPIA in fish [23], in fish cell lines [24], and in other aquatic animals. The induction of CYPIA can be regarded as a process resulting in an altered metabolism and in disturbances of hormone balance, but also as an adaptive process to chronic exposure to such pollutants. The induction of CYPIA can be used as a biomarker for the ecotoxicological assessment of environmental samples. This is illustrated in Fig. 4, where a landfill leachate contaminated by PAHs and additional compounds is analyzed for its CYPIA induction potential. Comparison with known model compounds such as chrysene allows the development of toxic equivalency factors, and thus the employment in ecotoxicological risk assessment.

On the other side, we focus on chemicals such as organotin compounds and heavy metals that cause an inhibition and destruction of CYPIA [25], and on mixtures of both inducing and inhibiting compounds [26][27]. Tributyltin (TBT) and triphenyltin (TPT) chloride, both of which are based on the experimental pH, strongly inhibit the hepatic CYP system both in vivo [28] [29] and in vitro [25]. The CYP protein, its activity, and the reductases were affected. Of the P450 system, the isof orm CYPIA was selectively affected in various fish species by TBT and TPT in vivo [28][29]. At high concentrations, however, additional CYP forms were affected as well. The mechanisms responsible for the loss and inactivation of cytochrome P450 forms in vivo are based on the direct destruction of P450 and formation of P420 with subsequent rapid degradation and breakdown of the apoprotein by proteases [29].

The in vitro studies have shown that in fish microsomes [25] and in a fish cell line [26] TBT and TPT strongly interact with micromolar monooxygenase systems, resulting in the inhibition of CYPIA activity. inhibition of NAD(P)H cytochrome c reductase activity, and thus the loss of an enzyme system responsible for the detoxification of environmental pollutants and the metabolism of endogenous substances. It was shown that the activity of organotins was due to the parent compound and not to metabolites. CYPIA activity is inhibited by a noncompetitive mechanism, which means that the inhibitor does not compete with the substrate on the binding site of the enzyme. These findings gave evidence that organotins do not interfere with the binding of an inducer with the arylhydrocarbon receptor (Ah receptor) of the cell, and do not interfere with CYPIA protein synthesis. However, they act at the level of the CYP protein (destruction of apoprotein) and inhibit the catalytic activity. Heavy metals including CdII, CoII, CuII, NiII, PbIV, and ZnII have been shown to act similarly [27]. Organotins seem to inhibit the catalytic activity by binding to amino acids such as cysteine and histidine at the active site, or on other sites of the enzyme [26][29]. The lipophilic TBT and TPT most likely penetrate hydrophobic membranes, in which cytochromes P450 are embedded, thereby gaining access to these enzymes. The action is directed to CYP, as cytochrome b5 is unaffected and the reductases are affected differently by TBT and TPT pointing to a specific mode of action [28-30].

Presently, we are adapting the permanent fish hepatoma cell line PLHC-1 to be able to assess chemicals with estrogenic activity. Environmental chemicals that negatively affect the endocrine system of organisms are of particular importance in ecotoxicology, as they limit the fitness of populations to a significant degree by inhibiting reproductive success [31]. An increasing number of widely used industrial and agricultural chemicals mimic the effects of natural estrogens [32]. These estrogenic chemicals often exert their action by binding to the estrogen receptor or by regulating the activity of estrogen-responsive genes. In recent years, various systems have been developed for detecting the estrogenic potential of such compounds. However, established permanent fish cell culture systems are missing. Before using fish cell cultures for estrogenicity assays, these cells had to be adapted to hormone-free medium [33]. We are presently focusing on the induction of vitellogenin and are searching for other markers for estrogenicity. Vitellogenin is one of the most important biomarkers of estrogenicity, as it is the precursor of the yolk protein in the eggs of fish, amphibians, reptiles, and birds, and is specifically synthesized in females under the control of estradiol [34]. Hence, estrogenic chemicals that act via binding to the estrogen receptor may be detected with this biomarker [34].

6. Oxidative Stress

Oxidative stress has been recognized to be associated with the toxicity of numerous chemicals and with the pathogenesis of many diseases, e.g., carcinogenesis, Alzheimer's disease, or atherosclerosis. It has been defined as a situation in which an overabundance of oxidants or free radicals damage or destroy a cell. In aerobic organisms, a major threat is caused by an increase in the production of oxidants and a decrease in the action of antioxidants. The former is the result of increased respiration or increased exposure to oxidants. The latter is the result of decreased action of antioxidants. The former is the result of increased respiration or increased exposure to oxidants. The latter is the result of decreased action of antioxidants.

![Fig. 4. Assessment of the CYPIA induction potential of different landfill leachates (a,b,c are different samples, ref refers to uncontaminated reference sample) that are contaminated by PAHs and other compounds. PLHC-1 cells were exposed to extracts of the leachates in DMSO in minimal essential medium (containing ±1% DMSO) for 24 h (n = 3 ± SEM). CYPIA enzyme activity is inhibited by a non-competitive mechanism, which means that the inhibitor does not compete with the substrate on the binding site of the enzyme. These findings gave evidence that organotins do not interfere with the binding of an inducer with the arylhydrocarbon receptor (Ah receptor) of the cell, and do not interfere with CYPIA protein synthesis. However, they act at the level of the CYP protein (destruction of apoprotein) and inhibit the catalytic activity. Heavy metals including CdII, CoII, CuII, NiII, PbIV, and ZnII have been shown to act similarly. Organotins seem to inhibit the catalytic activity by binding to amino acids such as cysteine and histidine at the active site, or on other sites of the enzyme. The action is directed to CYP, as cytochrome b5 is unaffected and the reductases are affected differently by TBT and TPT pointing to a specific mode of action.](image-url)
by the partial reduction of oxygen, leading to the production of highly toxic reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide, or hydroxyl radicals [35]. These are produced during normal enzymatic or metabolic processes, and they can also be produced upon transfer of electrons from organic radicals to oxygen. Transition metals play a key role in ROS formation since they may catalyze the formation of organic radicals, but also catalyze the Fenton reaction in which the most reactive hydroxyl radical is produced from hydrogen peroxide [36][37]. This metal-ion-catalyzed reaction is most damaging when it occurs site-specifically with metals in proteins, DNA, or lipids. As a consequence, in cells that are exposed to single environmental pollutants like nitroaromatics, azo compounds, halogenated compounds, pyridinyl compounds, quinones, metalloids and metals [38], or to mixtures of pollutants and transition metals [39], an increased production of ROS is observed and an increased oxidative threat is created. Fortunately, cells maintain a variety of defenses to protect against these ROS [40]. Essentially, three general antioxidant defense systems exist, water-soluble reductants (e.g., glutathione, ascorbate), lipophilic reductants (e.g., α-tocopherol, or β-carotene), and enzymes (e.g., catalases, peroxidases or superoxide dismutases). In addition, cells have the capability to remove or repair damaged molecules, e.g. by DNA repair systems, nucleases, or proteases.

The major goal of our research is to understand the protective systems of organisms using the oxidative stress response as a model system by answering the following questions: i) by what chemicals and at what concentration is the oxidative stress response activated, ii) which individual components are involved in the oxidative stress response and what is their function, iii) how is the stress response regulated, iv) what are the limits of the adaptive response, and v) which of the components could be used as sensitive and specific monitors for the detection of oxidative stress or oxidative-stress-inducing compounds?

Oxidative stress can be assessed through the detection of ROS themselves. As a result of the high reactivities and as a consequence of the short half-lives of radicals, however, determination of organic radicals and reactive oxygen has been and still is a major challenge for analytical chemists. Detection is difficult, particularly in biological systems, since, once produced, the radicals may react instantaneously. However, such measurements are required to unequivocally link biological effects with a specific toxic molecule. Analytical methods to quantify radical production include infrared phosphorescence, pulse radiolysis, ESR spin trapping, or the use of quenchers [41].

Alternatively, biological measurements are used to indirectly assess oxidative stress. This approach takes advantage of the fact that damaged biomolecules are more stable and more easily detectable than the radicals that caused them. It should however be kept in mind that as a result of defense and repair mechanisms, the magnitude of oxidative stress is underestimated. This points to the importance of knowledge about the components, genes, and enzymatic activities that are involved in these defense and repair mechanisms.

Upon oxidative stress in prokaryotes, the expression of ca. 60 genes is induced; some of the regulatory genes have been identified [42][43]. The current knowledge of the oxidative stress response in the bacterium Escherichia coli has been successfully applied in a biosensor approach in which DNA damage could be specifically measured using luciferase as the marker gene. In the eukaryotic organism Saccharomyces cerevisiae, the number of reacting genes has been determined to be at least 16 [44]. The regulation of the response is, however, complex as a result of compartmentalization and the higher structural organization of eukaryotic organisms. In our research, we focus on the antioxidant response in the green alga Chlamydomonas reinhardtii [45]. Recently, we have isolated a glutathione-peroxidase-homologous gene (gpxh) whose expression is strongly induced upon exposure to pollutants that cause an oxidative stress, as shown by Northern blot (Fig. 5) and RNA synthesis assays [46]. We are currently investigating the specificity, the sensitivity, and the limits of the induction of the gpxh gene by studying its expression after exposure to various stressors.

The regulatory sequences of the gpxh gene have been coupled with a arylsulfatase gene, a marker gene that can be easily measured in C. reinhardtii. Arylsulfatase activity will be measured under various growth conditions and in the presence of various pollutants. These experiments will give insights about the physiological function of gpxh and about its potential use as biosensor for oxidative stress.

7. Concluding Remarks and Outlook

Prediction of the toxicity of chemicals and assessment of their impact in the environment are major goals of ecotoxicological research. The number of modifying factors that determine ecotoxicological effects is almost countless and requires a systematic organization and stepwise deduction of principles for a holistic understanding. The fate and effect of a pollutant are influenced both by the biophysico-chemical properties of the chemical and the properties of the abiotic and biotic environment. Consequently, chemical, physical, and biological analytical tools need to be developed and combined.

Since ecotoxicological evaluations are based on concentration-effect relationships, the significance of measures of con-

![Fig. 5. Northern blot analysis of total RNA (10 μg) from Chlamydomonas reinhardtii cells that were exposed to 2 mM H2O2 for 0, 30, and 180 min. Using the gpxh gene as a probe, its mRNA levels were determined.](image-url)
concentration and of the choice of the effects to be investigated is evident. Many factors influence the availability of chemicals to organisms. One way to deal with these complexities is to relate biological responses to the concentration of a chemical taken up by organisms. It can be anticipated that observed differences in the interspecific sensitivity to chemicals will be less pronounced when effective concentrations are expressed as body residue or tissue-specific residues instead of in terms of the exposure concentration. For specifically acting compounds, the dynamics of the chemicals in different tissue and compartments should be considered in addition to whole-body concentration (physiologically based toxicokinetic modeling). The in vitro systems presented here are useful for reducing the complexity of this approach without losing relevance. More research work is still required to apply the information obtained from in vitro systems to model processes in organisms.

The classical approach to quantifying the risk associated with a pollutant is to estimate the exposure concentration and to compare it with the predicted no-effect level extrapolated from acute and chronic toxicity studies. This empirical approach has many limitations, particularly for chemicals that exhibit specific modes of action [47]. For this group of chemicals, lethality and other physiological endpoints should be supplemented by mode-of-action endpoints. The biomarkers and biosensors discussed in this article focus on such types of endpoints.

Besides their use in risk assessment, mechanism-based biomarkers and biosensors can furthermore be applied to set up quantitative structure-activity relationships and other predictive tools. After validation, these biomarkers and biosensors can also be applied as early warning systems. Environmental samples contain a mostly unknown mixture of various chemicals. The use of mode-of-action-based biochemical tools in combination with chemical analytical tools gives the opportunity to assess the integral of the effects of mixtures and to perform fast, sensitive, and specific screening of environmental samples.

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