Critical Review

Modes of Action in Ecotoxicology: Their Role in Body Burdens, Species Sensitivity, QSARs, and Mixture Effects

BEATE I. ESCHER*,# AND JOOP L. M. HERMENS§

Swiss Federal Institute for Environmental Science and Technology (EAWAG) and Swiss Federal Institute of Technology (ETH), CH-8600 Dübendorf, Switzerland, and Institute for Risk Assessment Sciences, Toxicology Division, Utrecht University, 3508 TD Utrecht, The Netherlands

In contrast to the general research attitude in the basic sciences, environmental sciences are often goal-driven and should provide the scientific basis for risk assessment procedures, cleanup, and precautionary measures and finally provide a decision support for policy and management. Hence, the prominent role of mechanistic studies in ecotoxicology is not only to understand the impact of pollutants on living organisms but also to deduce general principles for the categorization and assessment of effects. The goal of this review is, therefore, not to provide an exhaustive coverage of modes of toxic action and their underlying biochemical mechanisms but rather to discuss critically the application of this knowledge in ecotoxicological risk assessment. Knowing the mechanism or, at least, the mode of toxic action is indispensable for developing descriptive and predictive models in ecotoxicology. This review seeks to show the crucial role of target sites, interactions with the target site(s), and mechanisms for an adequate and efficient ecotoxicological risk assessment. Emphasis in the discussion is on target effect concentrations (or target occupancy), species selectivity and species sensitivity, time perspective of effect studies, Quantitative Structure−Activity Relationships (QSAR), and mixture toxicity. A particular focus of this review is on multiple mechanisms. Although the illustrative examples were mainly taken from studies in aquatic ecotoxicology, the proposed conceptual approach is also in principle applicable and even particularly useful for soil and sediment systems. Recommendations for further research and developments include the use of internal effect concentrations and target site concentrations in site-specific risk assessment and as a mixture toxicity parameter as well as general considerations for the derivation of mechanistically meaningful QSAR and other predictive models.

Introduction

Environmental Risk Assessment: A Complex Effort. The objective of environmental risk assessment is to protect the environment from adverse effects. Society is faced with the enormous task to assess numerous chemicals and complex chemical mixtures while protecting many different species in, and the diversity of, ecosystems.

Because the large number of existing chemicals (*1*) does not allow an in depth risk assessment at the level of disturbance of an ecosystem, the generic risk assessment scheme has developed into a system, in which a predicted no-effect concentration (PNEC) is derived from a limited set of acute and semichronic data for only a few representative species (*2, 3*). Methods have been developed to extrapolate the available experimental data for a few species to many species (*4*−*6*). These methods attempt to account for uncertainty stemming from factors that can influence the sensitivity of an organism, including the life-history strategy (*7*) and the test conditions, which are usually better controlled in laboratory tests than under field conditions (*8*). Factors that must not be neglected during these extrapolations are differences in bioavailability and speciation of chemicals between laboratory tests and real ecosystems.

Another factor, which is subject to extrapolation, is time. Since for most chemicals only data from acute tests are available, acute-to-chronic ratios (ACRs) are applied in environmental risk assessment to estimate a chronic or semichronic no-observed effect concentration (NOEC) for sublethal effects based on acute effect data, mostly with survival (LC_{50}) as endpoint. Species sensitivities and ACRs are often analyzed chemical by chemical for a class of structurally similar compounds and much less attention is paid to toxicological and organism-specific criteria to classify chemicals. Knowledge of the abundance of target sites as well as on metabolic activity and defense mechanisms is of particular relevance for understanding species selectivity and sensitivity. Despite the biological variability, it is hypothesized that behavior at the target site is relatively constant across different biological systems.

It is not only the many species, which complicates the risk assessment process, but also the sheer number of chemicals, which hampers the progress and requires methods that enable the prediction of fate and effect parameters based on a chemical's structure or its physicochemical parameters via Quantitative Structure−Activity Relationships (QSARs). This explains the interest of regulatory agencies in QSAR (*9*−*12, 1*). For practical applications of QSARs, several computer programs have been developed that supply predictions of fate and effect parameters (*13*−*15*). Several reviews discuss these toxicology prediction systems in more detail (*16, 17, 12*).

Most QSARs that are used in risk assessment are for effects at the whole organism level (*2*). A clear insight into what the actual target site is, the concentration at this target site, the mode of action, and the interaction at the target are desirable

* Corresponding author phone: 0041-1-823 5068; fax: 0041-1-823 5471; e-mail: escher@eawag.ch.
# Swiss Federal Institute for Environmental Science and Technology (EAWAG) and Swiss Federal Institute of Technology (ETH).
§ Utrecht University.

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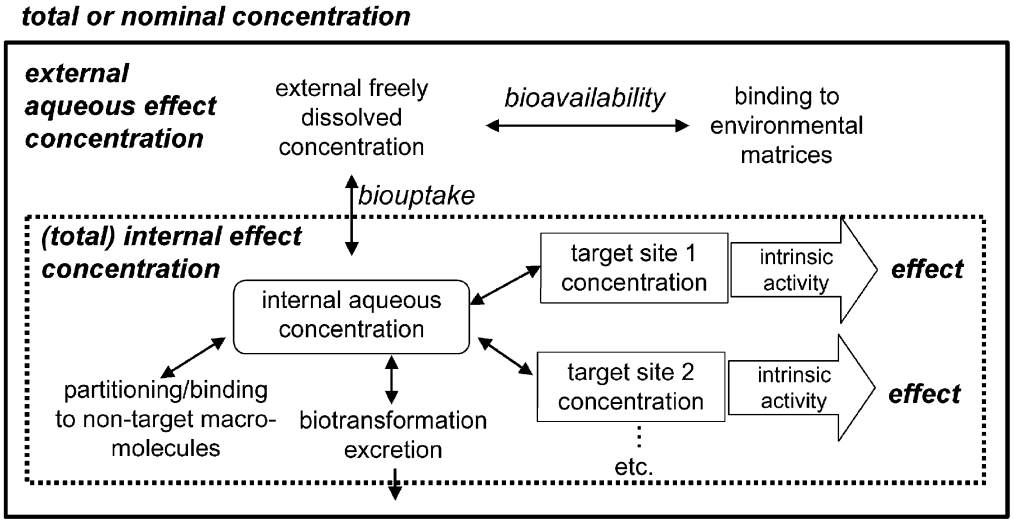


FIGURE 1. Relationship between total, external, and internal effect concentrations and distribution to different target sites.

in developing the models and in guiding the selection of relevant physicochemical parameters (*18*−*21*).

Field oriented risk assessment of actual polluted sites does not only deal with individual compounds but also has to evaluate the potential effects of complex mixtures present in the environment. Much experimental work has been focused on evaluating the combined effects of mixtures of pollutants with the objective of deriving general principles that can then be applied in the risk assessment process (*22*). In addition, group or sum parameters are often applied to measure "total concentrations" of particular classes of chemicals (*23*). Information on the mode of action is crucial not only in understanding joint toxic effects and potential interactions between chemicals in mixtures but also for developing both sound mixture toxicity parameters and other (bio- and *in vitro*-) assays for the evaluation of complex mixtures in the field. Site-specific risk assessment is often based on external effect concentrations (for example in water, soil, or sediment), and these heavily depend on the biological species, the specific test and exposure conditions, and on differences in a chemical's bioavailability and speciation. Internal effect concentrations more directly reflect the intrinsic activity of a chemical (*24*).

The Scope of This Review. Mechanistic aspects are of great importance in many topics addressed in ecotoxicology. In this review paper, we try to give an overview on the past developments in this area as well as on potential applications in the future. Emphasis is on chemical aspects and examples are mainly for organic chemicals, but the fundamental statements are meant to be generally applicable. In a first step, we attempt to categorize molecular mechanisms of toxicants that are relevant in ecotoxicology. In our opinion, crucial aspects in understanding and categorizing mechanisms are the target site and the type of interaction of a chemical with the target. Based on this framework, the core of the review is divided in three main parts: baseline toxicity, specific modes of toxic action, and multiple modes of action. Baseline toxicity (also termed narcosis) is the reference case because it is the minimal toxicity of any given chemical. Specific modes of action encompass reactive and receptor-mediated mechanisms. The often overlooked issue that a chemical may have multiple modes of action is specifically addressed to show approaches how to deal with this complexity.

These three parts rise in complexity and build upon each other but each part is structured in the same way by consecutively addressing the topics outlined in the Introduction: internal effect concentrations, species sensitivity, time dependence, QSARs, and mixture effects. The "Conclusion and Recommendation" section breaks up the rigid structure by linking the interconnected topics and emphasizing potential future applications of mechanistic information in hazard and risk assessment.

Modes of Toxic Action: Target Sites and Mechanisms

There is a long tradition in toxicological research to investigate the mechanistic principles underlying a toxic effect. A *mode of action* is defined as a common set of physiological and behavioral signs that characterize a type of adverse biological response, while *toxic mechanism(s)* refer(s) to the crucial biochemical process(es) and/or xenobiotic-biological interaction(s) underlying a given mode of action (*25*). There are further and sometimes contradictory definitions of these terms, and it is virtually impossible to draw a clear borderline. In this review, we employ the term "mechanism" whenever there is a more detailed description of molecular events available.

Basal cellular structures and functions are highly conserved biological entities. Therefore, a large number of toxic effects that target these basal functions are universal in all organisms and target tissues. On the other hand, there are toxic mechanisms that are specific for particular groups of organisms, e.g., disturbance of photosynthesis. Categorization of effects according to their associated target site is therefore a first step for setting up predictive models across different organisms. Xenobiotics may interact specifically with certain receptors but they may also be nonspecifically toxic. For instance, persistent hydrophobic compounds tend to accumulate in the membranes of cells, leading to nonspecific disturbance of the membrane integrity and functioning. Reactive and multifunctional compounds usually cannot be assigned to a single mode of action, but they often exhibit multiple toxic mechanisms. This is a difficulty for the effect assessment that hitherto has rarely been specifically addressed in the literature.

As a starting point, we believe that internal concentrations in an organism provide a better basis for assessing the intrinsic toxicity of a given compound than external concentrations (for reviews see refs *26*−*29*). Aqueous effect concentrations are related to the concentration at the site of toxic action through several, partially independent processes: on one hand bioavailability and on the other hand uptake, metabolism, and excretion (toxicokinetic phase), as is depicted in Figure 1. The concentration at the site of toxic action or target site and the strength of the interactions determine the

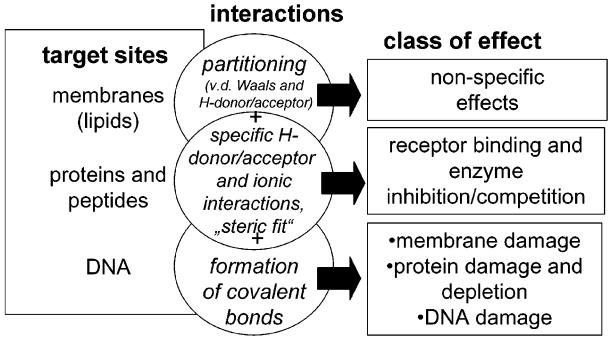


FIGURE 2. Rationale behind the classification of chemicals according to mechanism: target sites and type of interaction.

toxic effect (toxicodynamics). Target site concentrations are therefore more suitable in comparisons of the toxicity of chemicals as well as comparisons of species sensitivity.

The three main target domains in biological organisms are membranes, proteins, and genetic material. The toxic effect is directly related to the type and degree of interaction of the chemical with the target (Figure 2). Partitioning processes lead to mostly nonspecific effects, e.g., partitioning into membranes leads to baseline toxicity or narcosis. Sterically favorable van der Waals and hydrogen bond donor/acceptor interactions are the "adhesive" for the binding to specialized receptors and specific enzyme inhibition, which is the molecular basis of receptor-mediated toxicity.

Formation of covalent bonds of reactive chemicals with the target site leads often to irreversible processes. Here, we consider electrophiles as reactive chemicals that can form covalent bonds with nucleophilic entities in biological molecules (peptides and proteins, DNA), and, in particular, we will address alkylating agents. The reaction of allyl chloride with glutathione is an example of such an alkylation reaction. Examples of electrophiles are aldehydes, epoxides, and unsaturated aliphatic chlorinated hydrocarbons. Overviews of structures with electrophilic properties are given in review articles (*30*−*32*). Depending on the reactivity of the electrophile, the actual target site, the dose level, and duration of exposure, the effects of exposure to such reactive chemicals may vary from protein damage to DNA damage, causing ultimately carcinogenicity and mutagenicity. Indirectly, such effects can also be caused through the production of reactive intermediates or reactive oxygen species, which we do not address in detail. Note that the interactions depicted in Figure 2 do not necessarily lead to a biological effect because defense mechanisms and or metabolic deactivation may prevent the occurrence of visible effects. However, these interactions are the first and indispensable steps of a cascade of events that finally lead to observable toxic effects.

Refinement of the concept of interaction with target sites for specific targets, e.g., differentiation of biological membranes from generic membranes over energy-transducing membranes to photosynthetic membranes, allows a classification of compounds into 10 groups of modes of action. Each mode of action can be explained by one or more mechanisms (Table 1).

This general scheme can be stepwise further refined to very specific receptor-mediated mechanisms, e.g., the inhibition of specific enzymes such as acetylcholine esterase (AChE) or the interaction with specific receptors such as the estrogen receptor. Each of these mechanisms can be separately investigated with a selective in vitro test system. Examples of such test systems are also listed in Table 1. In the proposed classification scheme no explicit distinction is made between acute and chronic effects because, in principle, there is no difference in the fundamental interactions with biomolecules between acute and chronic effects, even though the resulting secondary effects may well be dramatically different between acute and chronic exposure. Note that indirect effects like effects on the energy status, redox status, or defense mechanisms also play an important role but are not considered explicitly here.

The approach to classification given in Table 1 is an extension of earlier proposed classification schemes. It builds up on an approach that is based on the assessment of behavioral (*33*) and physiological responses of fish (*34*−*37*). Responsiveness to stimulants, locomotive activities, body color, etc. as behavioral responses and ventilatory pattern, cough rate, heart rate, etc. as physiological responses were attributed to eight modes of toxic action using discriminant function analysis. These proposed modes of action include three types of baseline toxicity (nonpolar and polar narcosis and ester narcosis), uncoupling, respiratory inhibition, electrophile/proelectrophile reactivity, acetylcholine esterase (AChE) inhibition, and several mechanisms of central nervous system seizure.

Russom et al. developed an expert system to predict these eight modes of toxic action from chemical structures on the basis of the acute toxicity syndromes in combination with joint toxicity studies and QSARs (*38*). Out of a database of 225 industrial organic chemicals with known behavioral syndromes, 71% were classified as baseline toxicants, 3% as uncouplers, 4% as central nervous system seizure agents, 2% as respiratory inhibitors, and 20% as reactive chemicals (*38*). Mode-of-action classification did not agree with chemical class as defined by structural similarity.

Nendza, Wenzel, and co-workers introduced a similar classification scheme (*39, 40, 21*). They proposed a test battery of in-vivo and in vitro tests, which are selective for each one out of nine modes of action, including the above-mentioned, but without ester narcosis and additionally with the class of photosynthesis inhibition and mutagenicity and/or endocrine disruption. Within a set of representative model compounds, each compound yielded a characteristic toxicity profile. An unknown compound can consequently be classified by comparison to the reference toxicity profiles. These authors also emphasized that a chemical could fall into multiple categories. Nevertheless it was possible to set up predictive models based upon this classification and to correctly classify almost 90% of a test set of 115 chemicals to the appropriate mode of action class using stepwise discriminant analysis with 10 physicochemical descriptors (*21*). Each class of mode of action could be defined by a characteristic set of descriptors.

The following two sections demonstrate for baseline toxicity and specific/reactive mechanisms how the approach introduced here can be further developed and discusses future research that needs to be conducted in light of the conclusions reached in this review.

Baseline Toxicity

Internal Effect Concentration and Target Site Concentration. The idea of narcosis or baseline toxicity originates from the pioneering work of Meyer and Overton (*41, 42*) and was taken up in ecotoxicology by Könemann (*43*) and Veith et al. (*44*). Baseline toxicity is believed to be a result of nonspecific disturbance of membrane integrity and functioning as a result of partitioning of pollutants into biological membranes (*45*). Baseline toxicity constitutes the minimal toxicity of every chemical. Concentrations or volumes of different baseline toxicants are virtually constant in biological membranes for a defined endpoint effect such as lethality (*46, 45*). In other words, the intrinsic potency for baseline toxicity is equivalent for every chemical. Effects are reversible and occur if a certain threshold concentration level is exceeded. Exposure to sublethal concentrations of narcotics does not increase the sensitivity of test animals to consecutive exposure to lethal

TABLE 1. Classification According to Targets, Interaction with Targets and Mechanisms Relevant in Ecotoxicology

site of action	subclass	domain[a]	interaction[b]	molecular mechanism(s)	mode of action	selective test system (examples)
biological membrane	general	m	ns	nonspecific disturbance of membrane structure and functioning	baseline toxicity	neutral red assay (*40*) Kinspec (*59*)
	general	m	s	formation of reactive intermediates (e.g., ROS[c]) causing peroxidation of membrane lipids and proteins	degradation of membrane lipids and membrane proteins	reaction of reaction product (malondialdehyd) with thio-barbituric acid (*211*)
	energy-transducing	m	ns, s	ionophoric shuttle mechanisms	uncoupling	O_2-consumption in mitochondria (*40*) Kinspec (*109*)
		m	s	blocking of quinone and other binding sites etc.	inhibition of the electron transport chain	O_2-consumption in submitochondrial particles (*40*) Kinspec (*109*)
		m	s	blocking of proton channels and other transport channels	inhibition of ATP synthesis/ depletion of ATP	ATP assay, e.g. with luciferin/ luciferase (*212*)
	photosynthetic	m	s	blocking of photo-synthetic electron transport	photosynthesis inhibition	O_2-production in chloroplasts (*40*) chlorophyll fluorescence (*213*)
proteins, peptides	general	m, c	ns, s	electrophilic reactivity, alkylation and oxidation of proteins and glutathione (GSH)	damage and depletion of biomolecules	GSH depletion (*173*)
	specific enzymes and receptors	m, c	s	noncovalent or covalent binding to enzymes and receptors	inhibition or competition, e.g.,: 1. acetylcholine esterase 2. estrogen receptor 3. A_h receptor, etc.	receptor binding studies or enzyme activity measurements; e.g.,: 1. enzyme activity (*40*) 2. yeast estrogen screen (*214*) 3. CYP1A induction (*215*)
	specific enzymes and receptors	c	s	noncovalent or covalent binding to enzymes of the nucleic acid metabolism, replication or repair	indirect mutagenicity (DNA repair, recom-bination, regulation)	induction of repair mechanisms, e.g., SOS chromotest, or bacterial revertants, e.g., Ames test (for review see ref *216*)
DNA, RNA	general	c	ns, s	base modification and damage: electrophilic (alkylation) and oxidative damage, bulky adducts	direct mutagenicity (frameshift, cross-links, strand breaks, deletion, etc.)	measurement of DNA adducts (*173*), induction of repair mechanisms (for review see ref *216*)

[a] Cellular environment: m = membrane, c = cytosol and other aqueous compartments in the cell. [b] Selectivity: ns = nonselective, s = selective. [c] ROS = reactive oxygen species.

concentrations, when effects are expressed in terms of internal concentrations (*47*).

Target site concentrations are difficult to obtain directly. As a surrogate, total concentrations in an organism that elicit a critical effect, termed critical body residues (CBR) or internal effect concentrations (IEC), have been used. McCarty defined CBR as the molar concentration per body weight of the organism that elicits a defined toxic endpoint (*48*). If this endpoint is lethality, one often refers to the lethal body residue (LBR), internal lethal concentration (ILC), or lethal body burden (LBB). In the following, we use the term IEC and its variations because it is less ambiguous than any other terminology since it is clearly defined as a concentration and refers to a specific endpoint, e.g. ILC_{50} refers to lethality for 50% of the test animals.

The rationale behind the use of ILC was the observation that the QSARs of bioconcentration factors (BCF) and of lethality (LC_{50}) for baseline toxicants in aquatic organisms were inversely related to each other, resulting in a more or less constant internal effect concentration, e.g. ILC_{50}, for compounds with an octanol−water partition constant log $K_{ow} > 2$.

$$ILC_{50} = BCF * LC_{50} = \text{constant} \quad (1)$$

The ILC_{50} of baseline toxicants were predicted to be on the order of 2.5 $mmol/kg_{body\ weight}$ for acute toxicity (*48*), and measurements later confirmed the prediction giving an average range of 2−8 $mmol/kg_{body\ weight}$ (*49*−*54*). For juvenile fish, whose average lipid content is about 5%, the $ILC_{50,lip}$ normalized to the overall fish lipid is then around 50 $mmol/kg_{fish\ lipid}$. This value is consistent with earlier studies with erythrocyte ghost membranes, where a constant concentration in the lipid phase of 30−60 $mmol/kg_{lipid}$ in membranes was measured for the endpoint of 25% reduction in osmotic hemolysis (*55, 56*). For compounds of low hydrophobicity (log $K_{ow} < 2$), the partitioning into the lipid phases is not dominating the overall partitioning, and therefore the contribution of the toxicant concentration in the aqueous compartments of the organism has to be considered in calculating the overall IEC (*50*).

Compounds that may undergo strong H-donor/H-acceptor interactions (often simply referred to as polar molecules) exhibit distinctly lower IEC values than apolar molecules in various organisms. ILC_{50} found for polar narcotics range from 0.6 to 2 $mmol/kg_{body\ weight}$ (*57, 27, 58*). However, the effective membrane concentrations were indistinguishable between the nonpolar and polar compounds in an in vitro test system that contains only energy-transducing membranes, which represent a target lipid membrane with intercalated proteins (*59*). This observation shows that there is no real difference between the $IEC_{membrane\ lipid}$ (based on membrane lipid concentration) of nonpolar and polar chemicals and that the observed difference in whole-body concentrations can be related to differences in the distribution between target and nontarget compartments.

Van Wezel et al. and Vaes et al. proposed a simple three-compartment equilibrium partitioning model to relate the ILC_{50} of fish to the concentration in the target lipids (*52, 60*). The model compartments consist of 95% aqueous phase and 5% lipoid phase. Partitioning to protein and other hydrophobic macromolecules is neglected. 75% of the lipids were assumed to be neutral storage lipids and 25% were polar lipids, i.e., mainly the lipid bilayers of membranes. When reevaluating the simple partitioning model with liposome−water partition coefficients K_{mw} as descriptors for membrane−water partitioning and hexane−water partition coefficients K_{hw} as descriptors for storage lipid−water partitioning (Figure 3) and when extending it to other aquatic organisms, using

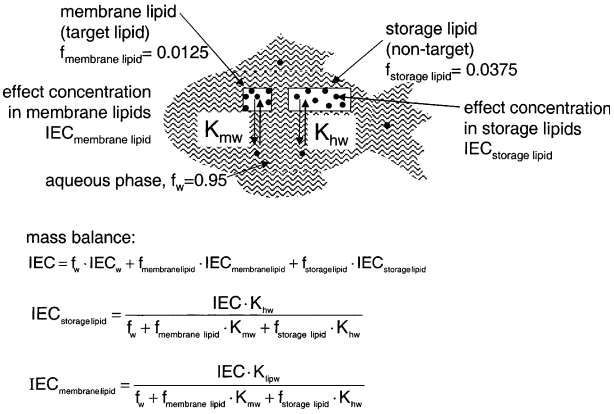


FIGURE 3. Three-phase equilibrium partitioning model for fish used to derive effective membrane concentrations from measured internal effect concentrations (IEC). Partitioning to storage lipids is described by the hexane−water partitioning coefficient K_{hw}, partitioning to membrane lipids by the membrane−water partitioning coefficient K_{mw}. The fraction of chemical in a given phase is denoted by f; f_w refers to the fraction in the aqueous phase, $f_{membrane\ lipid}$ refers to the fraction in the membrane lipids, and $f_{storage\ lipid}$ refers to the fraction in the storage lipids.

the appropriate lipid contents of the respective organisms (*61*), it can be clearly seen that the lethal membrane concentration is within the same order of magnitude for all baseline toxicants (nonpolar and polar chemicals) in algae, daphnids, and fish (Figure 4A), while the concentration in the storage lipids varies over several orders of magnitude (Figure 4B).

Body residues have also been measured for sublethal endpoints and different life stages of animals and were found useful for defining sublethal hazards (*62, 63*).

Species Sensitivity. Barron et al. (*29*) evaluated a large compilation of IEC and ILC data of various species from a database compiled by Jarvinen and Ankley from a wide variety of different literature sources (*64*). They observed that, even for baseline toxicants, IECs affecting survival varied by 5 orders of magnitude, concluding that species sensitivity, life stage, biotransformation, and physicochemical parameters such as pH and salinity might influence the IECs. Despite this apparent variability, some general conclusions related to species sensitivity can be drawn.

Differences in sensitivity for baseline toxicants are generally smaller than for specifically acting compounds, which is not surprising because of the nonspecific character of baseline toxicity. Every organism may suffer from the baseline effect and physiological differences in target sites (the membrane) are probably minor, which is corroborated by the modeling depicted in Figure 4.

The variability of the IECs for a given endpoint within one study with one species at an acute exposure time (24−96 h) varies typically only within 1 order of magnitude and is reduced by 50% if the IECs are not expressed per wet weight of the organism but in units of mol per mass or volume of lipid in the organism (*52*). Organisms with a higher storage lipid content survive higher total internal effect concentrations (*65*). Hence variation in tolerance of a subpopulation of fish is accounted for partially by the variation in lipid content (*65*), but there remain susceptibility differences in different fish species and within a population (*51, 52, 54*).

In particular, in nonequilibrium situations, differences in aqueous effect concentrations may occur, because smaller organisms will reach equilibrium faster that an organism with a smaller surface-volume ratio. In addition, uptake routes may also be variable and uptake via food ingestion should also not be neglected. For example, cutaneous uptake is

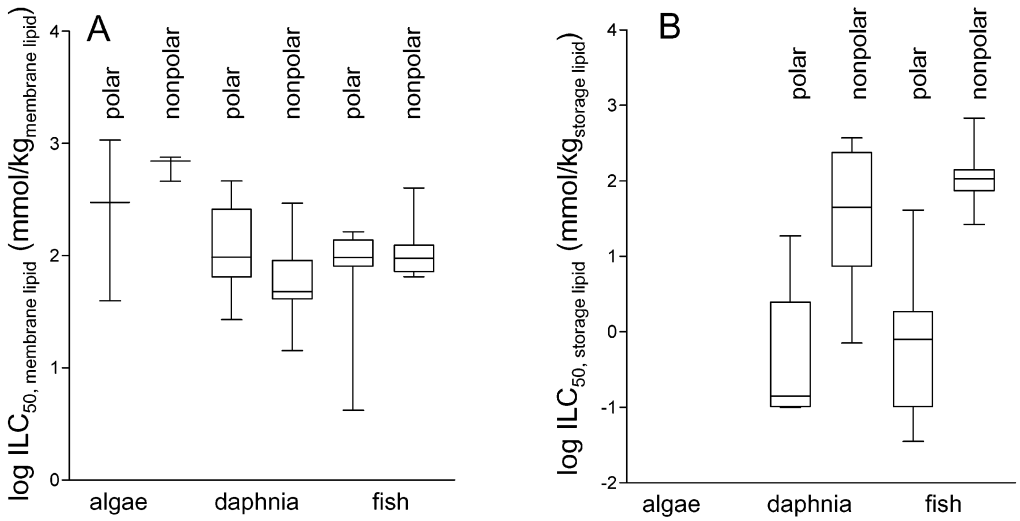


FIGURE 4. Comparison of internal lethal effect concentration ILC_{50} (A) in the membrane and (B) in the storage lipids for algae, daphnids, and fish. The graph shows boxes that extend from the 25th to the 75th percentile, with a line marking the median. The whiskers extend above/below to the highest/lowest value. Note that no storage lipids were assumed for algae. Raw data and calculations are given in ref *61*.

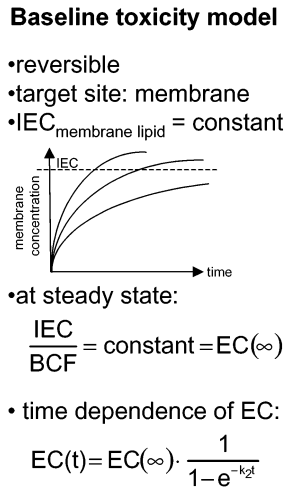


FIGURE 5. Baseline toxicity model; EC = effect concentration (referring to external aqueous phase), IEC = internal effect concentration, BCF = bioconcentration factor, k_2 = elimination rate (*119, 68*). Time dependence of toxicity is due to uptake kinetics. Membrane concentration rise with time due to bioconcentration until it reaches the IEC, where lethality is observed.

important for small fish while uptake via the gills dominates for larger fish (*66, 67*).

Time Dependence of Toxicity. Since baseline toxicity is a reversible mechanism, in which the response is directly related to the concentration in the membrane, the influence of time on the effect concentration is determined by the time it takes for the organism to reach equilibrium with the surrounding aqueous phase, i.e., by the bioaccumulation kinetics (Figure 5). Because the time to reach equilibrium increases with the hydrophobicity of a chemical, the relatively short exposure of 4 days in most acute tests may not be sufficient to reach a time independent EC_{50} value and longer test durations have been recommended (*68*).

A detailed discussion of bioaccumulation kinetics is beyond the focus of this review and is reviewed in detail elsewhere (*69*−*71, 28*). Traditionally, empirically based kinetic models have been used to relate toxic effects to absorbed chemicals. An alternative approach involves the use of physiologically based toxicokinetic models, which account for the organism's anatomy, physiology, and biochemistry

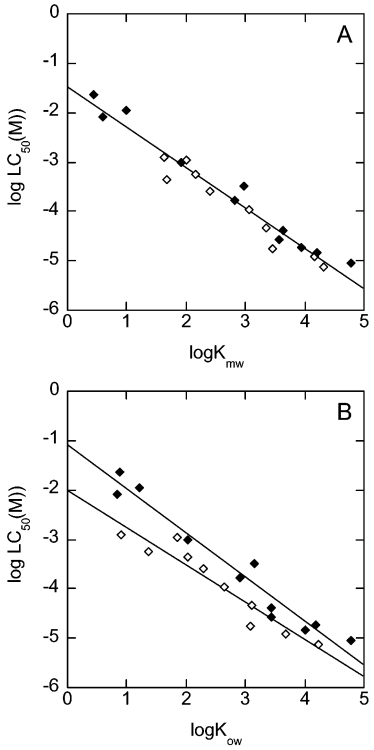


FIGURE 6. LC_{50} data for guppy of baseline toxicants (*219*) plotted versus (A) logK_{mw} (*220*−*222*); (B) logK_{ow} (*223*−*225, 222*); (◆) nonpolar chemicals, (◇) polar chemicals.

(*72, 73*). Such models not only account for the time-dependence of toxicity but also provide information regarding distribution of chemicals within an organism as well as species sensitivity, size and age of the organism, and exposure conditions.

QSARs. Since the effect concentrations in the membrane are virtually constant for baseline toxicants, a QSAR with a membrane vesicle−water partition coefficient, K_{mw}, as single descriptor can be used to describe the external effect concentration LC_{50}/EC_{50} (Figure 6A and eq 2) (*60, 74, 59*).

$$\log EC = a \cdot \log K_{mw} + c \qquad (2)$$

TABLE 2. QSARs of Baseline Toxicity Based on the Membrane−Water Partition Coefficient K_{mw} as Descriptor

	based on ref	QSAR for general baseline toxicity[a]
Poecilia reticulata	(*43*)	$\log LC_{50}\ (M) = -0.83 \log K_{mw} - 1.46$
Vibrio fischeri	(*217*)	$\log LC_{50}\ (M) = -0.79 \log K_{mw} - 1.54$
Chlorella vulgaris	(*218*)	$\log LC_{50}\ (M) = -0.91 \log K_{mw} - 0.63$
Daphnia magna	(*217*)	$\log LC_{50}\ (M) = -0.77 \log K_{mw} - 1.89$
Tetrahymena pyriformis	(*74*)	$\log LC_{50}\ (M) = -0.68 \log K_{mw} - 1.42$

[a] Transformation of QSAR for nonpolar narcosis based on K_{ow} as descriptor from literature into QSAR for general baseline toxicity (encompassing nonpolar and polar compounds) by replacing K_{ow} by K_{mw} using eq 3 (*91*).

Historically a distinction has been made between nonpolar and polar compounds because if K_{ow} was used as descriptor, two separate QSARs were obtained for the two groups of chemicals (Figure 6B) (*75*−*77*).

This difference is a consequence of the difference in molecular interactions of nonpolar and polar chemicals with water, octanol, and membrane vesicles. Following the work from Kamlet and Abraham (*78*), Gunatilleka and Poole applied linear free energy relationships using solute characteristics such as volume, excess molar refraction, dipolarity/dipolarizability, and the solute's effective hydrogen-bond acidity and basicity to explain partitioning processes as well as toxicity data (*79*, *80*). In fact, there was a significant influence of the dipolarity/dipolarizability-factor for octanol−water partitioning but no contribution to membrane−water partitioning and toxicity, confirming that octanol is not an optimal surrogate for biological membranes (*79*, *80*, *59*).

With some restrictions, for practical applications, K_{ow}-based QSARs for baseline toxicity, which are available for many endpoints and species are useful in establishing quality criteria for aqueous systems as well as for sediments (*81*−*83*). However, in future work, conventional physicochemical descriptors should be replaced with toxicologically relevant parameters.

QSARs from the past, that would bring together nonpolar and polar narcosis type chemicals, included additional parameters for describing the difference in polarizability/dipolarity and for hydrogen bond donor/acceptor interactions between octanol as surrogate and the properties of the real target site, the lipid bilayer membrane (*79*). A few examples are mentioned in the following. Karabunarliev et al. (*84*) applied the "maximal acceptor superdelocalizability" as parameter in modeling acute toxicity of substituted benzenes. In a series of articles, Mekenyan and Veith (*85*−*87*) have applied the "acceptor superdelocalizability" and the energy of the lowest unoccupied molecular orbital (E_{LUMO}) as parameters in QSARs for what they call soft electrophiles, which happened to include many of the polar baseline toxicants. Cronin and Schultz (*88*) and Bearden and Schultz (*89*) used E_{LUMO} as parameter in modeling acute toxicity data of polar narcosis chemicals. Urrestarazu Ramos et al. (*58*) included parameters for hydrogen bonding acceptor and -donor properties in QSARs for polar narcosis type compounds in three aquatic species. Dearden et al. (*90*) applied polarizability and free hydrogen acceptor factors in an analysis of toxicity data for nonpolar and polar narcosis.

It is also possible to merge K_{ow}-based QSARs from nonpolar and polar compounds into a common K_{mw}-based QSAR by applying the correlations between K_{ow} and K_{mw} that were derived separately for nonpolar and polar compounds (eqs 3 and 4) (*91*).

nonpolar compounds:

$$\log K_{mw} = 1.05 \log K_{ow} - 0.32 \quad (3)$$

polar compounds:

$$\log K_{mw} = 0.90 \log K_{ow} + 0.521 \quad (4)$$

A selection of resulting general baseline toxicity QSARs is listed in Table 2 and details of the derivation are given in ref *61*. Though it is possible to reuse these methods, as pointed out above, the use of toxicologically relevant parameters should have an increasing priority in ecotoxicology.

Mixtures. In numerous studies, it was confirmed that the effects of mixtures of baseline toxicants can be explained with concentration addition, i.e., a component of a mixture can be replaced by an equipotent concentration of another compound without affecting the overall effect. In 1981, Könemann performed a classical study on the acute toxicity of mixtures of 3−50 nonspecifically acting chemicals toward guppy fish (*Poecilia reticulata*) (*92*). These early experimental results suggested concentration addition. In a number of similar studies on the acute and sublethal effects of mixtures of large numbers of compounds, concentration addition was generally confirmed (*93*−*97*). A study on the immobilization of *Daphnia magna*, showed that very small fractions of a toxic concentration of the single compound (down to 0.0025 of the inhibitory concentration IC_{50}) still contributed to the overall toxicity (*98*).

For baseline toxicants, IEC can in principle be summed up to derive a risk estimate for mixtures. Van Wezel and co-workers proposed an empirical approach for the risk assessment of chemical mixtures of baseline toxicants (*53*). In this study, a fish with an unknown burden of environmental pollutants was exposed to an increasing concentration of 1,2- or 1,4-dichlorobenzene until a defined endpoint, e.g., lethality, was observed. From the additional internal concentration of the dichlorobenzene and the ILC_{50} of the given endpoint, the initial pollutant burden from exposure in the field could be back calculated. This method is very simple and appealing, although limited to compounds that are pure baseline toxicants or where baseline toxicity dominates the mixture effect.

Hermens and Leeuwangh put forward a hypothesis, which is very relevant with regard to the balance of baseline toxicity and specific mechanisms and the above-mentioned practical approach (*99*). For mixtures of large numbers of chemicals with diverse specific modes of action, where the individual concentrations are well below the threshold of effect, the underlying baseline toxicity may add up to a significant effect. However, the experimental data of this study was not fully supportive of this hypothesis. In a field survey on fish communities, Dyer et al. showed by analyzing fish tissue residues that concentration addition of the specific effects of the components overpredicted the mixture effect, while adding up the baseline toxicity was a good indictor of the overall effect as long as the single concentration levels were below some threshold level for the specific effect (*100*). More experience regarding the balance of cumulative baseline and mixture effects of specifically acting compounds are needed before any general conclusions for risk assessment can be drawn.

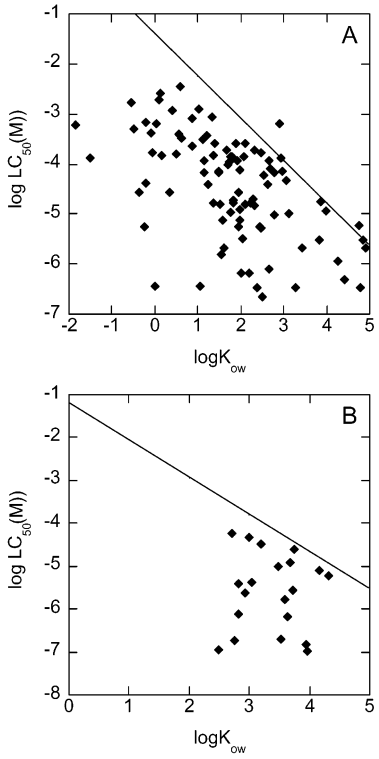


FIGURE 7. LC_{50} data of (A) reactive chemicals and (B) of specifically acting compounds plotted against K_{ow}. (A) Experimental toxicity data of reactive chemicals toward fathead minnow (data taken from the EPA ERL-D fathead minnow database (*38*)). The line corresponds to the QSAR for baseline toxicity (LC_{50}) toward fathead minnow (*Pimephales promelas*) described by ref *218* from data by ref *44*. (B) Experimental toxicity data for specifically acting compounds toward guppy (*Poecilia reticulata*) (*32*), QSAR for baseline toxicity from ref *43*.

Reactive and Specific Modes of Toxic Action

From various studies it is evident that reactive compounds (e.g., electrophiles) (Figure 7A) and specifically acting compounds (Figure 7B) are usually more toxic than baseline toxicity models predict (*18, 31, 101, 32, 38, 102*). Furthermore, it can be observed that LC_{50} -values for more hydrophobic electrophiles tend to deviate less from baseline models than hydrophilic electrophiles (*103*−*106*) (Figure 7A).

Target Concentration and Target Occupation. While for baseline toxicants, the whole-body internal effect concentrations are a reasonable first approximation of the amount of chemicals present at the target site (the membrane), reactive and specifically acting compounds may act through binding to receptors or enzymes. Hence, the toxic effect is determined not only by the concentration of the toxicant at the receptor in the membrane or in the cytosol but also by the intrinsic activity. The intrinsic potency of specifically acting compounds may additionally be dependent on their affinity to, or type of interaction with, a receptor (e.g., aryl hydrocarbon-receptor for dioxin-like compounds) or the internal speciation (e.g., uncoupling of weak organic acids). Therefore, IECs of specifically acting compounds are not only lower than those of baseline toxicants but also cover a wider concentration range for each mode of action. A compilation of data from literature is presented in several reviews (*27, 107, 28, 29*) and can be found in databases (*64, 108*).

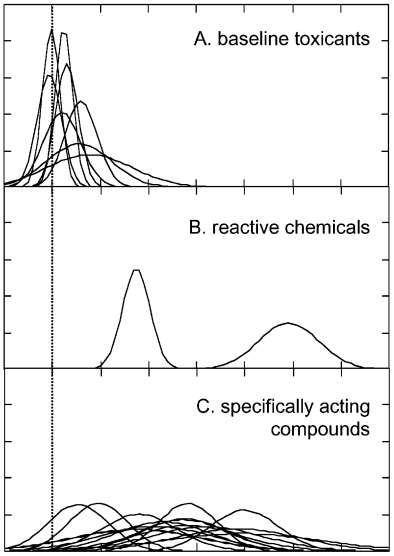
Within a category of modes of action, the assessment of internal and target site concentrations, i.e., concentrations in the membrane for membrane bound receptors or cytosolic concentrations for soluble enzymes as targets, is crucial for understanding the potency differences. For example, Escher et al. developed an in vitro test system for the assessment of baseline toxicity and uncoupling in isolated energy-transducing membranes (*109, 59*). This test system is basically a model for the pertinent internal concentration because it contains only the target site (energy-transducing membrane) in an aqueous suspension. The concentration in the membrane can be estimated with membrane−water partition coefficients (*110*). Comparison of the IEC based on membrane concentrations with the critical membrane concentration of baseline toxicants in this test system (*59*) allowed a clear assignment of a diverse set of substituted phenols to the appropriate mode-of-action category (*61*). While chlorophenols are often classified as polar narcotics because their LC_{50}-values lie within the range predicted by typical QSAR equations for polar narcosis based on log K_{ow}, the comparisons based on the membrane concentrations gave clear evidence that trichloro- and higher substituted phenols act as uncouplers. Whereas $IEC_{membrane\ lipid}$ are constant for all baseline toxicants, there is a specific $IEC_{membrane\ lipid}$ for each uncoupler, which represents its intrinsic activity as an uncoupler at the internal pH. ILC_{50}-values based on membrane-lipid concentrations were modeled for various organisms with the three-compartment model described in the section on baseline toxicity and resulting ILC_{50}-values were similar in the in vitro test and in aquatic organisms (*61*). In addition, the modeling showed that the dependence of the LC_{50}-values of substituted phenols from the external pH is in fact an artifact due to the pH-dependence of bioaccumulation. Internal membrane concentrations were usually constant for a given compound and independent of pH (*61*). Note, however, that it may well be possible for compounds exhibiting multiple specific mechanisms that dominance of a given mechanism is different in different biological species and may depend on the toxicokinetics in the respective biological species, as was shown by comparing the effect of substituted phenols in various biological tests (*111*).

For reactive chemicals and chemicals that bind irreversibly to a receptor, the extent of reaction with some target molecule or receptor at the target site, i.e., the target occupation, is a potential measure of the effect. For example, Freidig et al. have used the depletion rate constant of glutathione as parameter for describing the activity of a series of acrylic acid esters (*112*). Glutathione reacts with these compounds in a Michael addition. The depletion of glutathione was directly correlated with an adverse effect. A critical depletion rate constant of 1.5 d^{-1} was defined as the critical effect parameter related to 50% lethality.

Parameters such as critical membrane concentrations and other critical effect parameters can be used to classify chemicals and to judge the relative intrinsic potency as compared to other compounds and to other mechanisms.

Species Sensitivity Distributions. Vaal et al. carried out a systematic study evaluating the differences in sensitivities of species in relation to the mechanism of action (*113, 114*). The following mechanisms were included in the analysis: baseline toxicity, reactive toxicity, and a few specific mechanisms (inhibition of AChE, neurotoxicity). The sensitivity distributions were expressed as toxic ratio (TR), the ratio of the EC for baseline toxicity to the experimental EC and are plotted in Figure 8.

It is obvious from these plots that TRs of baseline toxicants are close to 1 and interspecies differences are small. In contrast, TRs are several orders of magnitude higher and vary strongly for reactive and specifically acting chemicals and differences in sensitivities are much larger (Figure 8). This is not surprising because the modes of action of specifically acting compounds are complex and involve many more intermediate steps. In addition, the intrinsic activity of chemicals not only depends on the structure of the chemical but also may be different in different species and sometimes



$$\log TR = \log\left[\frac{EC_{50}\,(baseline)}{EC_{50}\,(experimental)}\right]$$

FIGURE 8. Species sensitivity distribution (SSD) of toxicities of various chemicals according to classes of mode of action. Each of the bell-shaped curves corresponds to a normal distribution with a median corresponding to the 50th percentile of the experimental SSD and a standard deviation derived from the experimental $SR_{95:5}$-ratio, which is defined as the ratio of 95th to 5th percentile. The experimental data was collected by Vaal et al. (*113, 114*), and the plots were adapted from a figure in ref *113*. A. SSD of the baseline toxicants acetone, *o*-cresol, ethyl acetate, heptanol, phenol, propanol, pyridine, and trichloroethylene. For the derivation of the toxic ratio (TR), K_{ow} values given in ref *113* were converted to K_{mw}-values through eqs 3 and 4. EC-values for baseline toxicity were estimated with the QSAR for *Poecilia reticulata* given in Table 2. B. SSD of two reactive chemicals, salicylaldehyde and propenal. TRs were taken directly from ref *113*, i.e., derived from K_{ow}-based QSARs. C. SSD of specifically acting compounds, including AChE inhibitors and neurotoxic agents: methomyl, carbaryl, parathion, dibrom, fenthion, malathion, dichlorvos, diazinon, aldrin, dieldrin, endrin, heptachlor, lindane, and toxaphene.

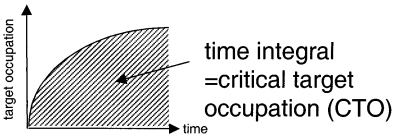
the target for a particular mode of action is specific for certain organisms (e.g., photosynthesis in plants, endocrine system only in higher organisms). The large variation in effect concentrations for organophosphates is certainly due to differences between organisms in their capacity to biotransform the parent compound into inactive or active metabolites and the sensitivity of acetylcholine esterase. In fact, species sensitivity distributions for AChE inhibitors and photosynthetically active compounds often show several maxima corresponding to sensitive and nonsensitive subgroups of organisms (*115, 116*).

Time Dependence of Toxicity. For the reversible mechanism of baseline toxicity, the internal effect concentration is virtually constant, and the time dependence of EC-values is caused by the bioconcentration kinetics (see Figure 5). If the mode of action is related to an irreversible chemical reaction, the influence of time on effect concentrations is much more pronounced and can follow the so-called Haber's law: the product of concentration and time is constant for a given response (*117, 118*). This rule was established initially for gaseous reactive compounds.

The validity of this distinction of irreversible and reversible processes and its influence of LC_{50}-time relations has been shown in an analysis of experimental data for a few reactive and receptor-mediated active compounds (*119, 68*). Verhaar, Legierse, and co-workers proposed a critical target occupation (CTO) model, in which it is assumed that a given effect

Critical target occupation model

- irreversible
- target site: receptor, enzyme, etc. in cytosol or membrane
- CTO = constant



- time dependence of EC:

$$EC(t) = EC(\infty) + \frac{\dfrac{CTO}{BCF}}{t - \dfrac{1 - e^{-k_2 t}}{k_2}}$$

FIGURE 9. Critical target occupation model (*119*, *68*). EC = effect concentration (referring to external aqueous phase), BCF = bioconcentration factor, k_2 = elimination rate, CTO = measure of the critical target occupation (fit parameter of the model representing the time integral of binding to the target).

endpoint is elicited at a constant target occupation or constant depletion rate (*119, 68*). If the interaction of the toxicant with the target is instantaneous and completely irreversible, then the integral of the target occupation over time is a means to quantify the effect (Figure 9). Consequently, the corresponding (I)EC-values show a strong time-dependence, while the time dependence of baseline toxicity is only determined by the bioconcentration kinetics and a constant (I)EC. In cases, where the reaction with the target receptor is not completely irreversible or some replenishment mechanism is active, the observed effect will occur somewhere between the model of constant internal concentration (Figure 5) and the CTO-model (Figure 9). The results of the two models are illustrated in Figure 10A for the example of a time-series of LC_{50}-values of the reactive chemical benzyl alcohol toward guppy (*68*).

Legierse et al. further extended the CTO-model for organophosphates, which are acetyl choline esterase (AChE) inhibitors after metabolic activation from the thio- to the oxo-ester (*119*). In the model, the active oxo-analogue is formed in a pseudo-first-order reaction, and it binds to AChE with a second-order rate constant. Both back reactions are assumed to be negligible. The endpoint effect is observed when a critical amount of AChE is inhibited, which is proportional to the time-integral of the whole-body or aqueous internal concentration. The time-dependence up to 14 days of exposure of the LC_{50} of five organophosphate pesticides was in excellent agreement with the CTO model, both, in the guppy *Poecilia reticulata* (see the example of malathion depicted in Figure 10B) and in the pond snail *Lymnaea stagnalis* (*119*).

In addition, physiologically based toxicokinetic/toxicodynamic models (PB-TK/TD) are a means to fully account for the complexity of pathways and mechanisms of receptor-mediated toxicants, as is illustrated, e.g., by the PB-TK/TD model for paraoxon in rainbow trout developed by Abas and Hayton (*120*).

The time-dependence of toxicity is also related to acute-to-chronic ratios (ACR). The upper 90th percentile of ACR in a large compilation of literature data was around 70 (*121*), but the ACR vary strongly depending on the mode of toxic action. The ACR are typically lower for pure baseline toxicants and can be much higher if reactive metabolites, which are more likely to be formed during chronic exposure, cause the effect (*38*) or if the mode of action in the chronic test is

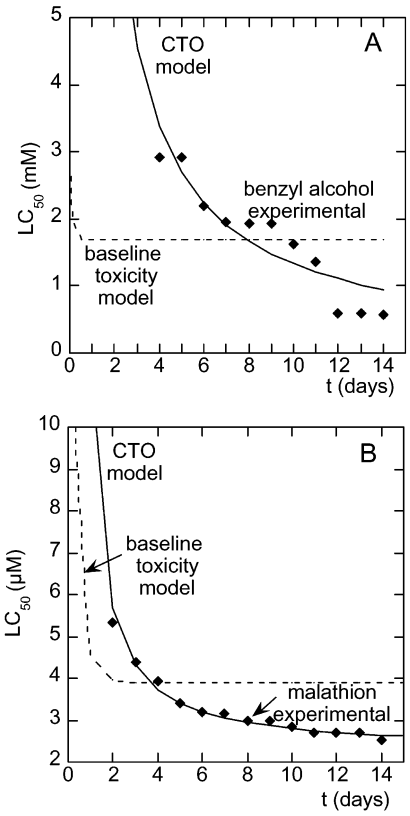


FIGURE 10. Comparison between baseline toxicity model and critical target occupation model for (A) a reactive chemical (benzyl alcohol) and (B) a specifically acting compound (malathion). For model equations see Figures 5 and 9, data and model parameters from ref *119* and *68*.

different from the acute test (*122*). Therefore inclusion of mechanistic information in the derivation of ACR will improve precision of prediction, as has also been shown by Roex et al. (*123*).

QSARs for Reactive Chemicals. It is obvious that any hydrophobicity descriptor like K_{ow} is not sufficient for describing effect data in QSAR models for reactive toxicants and many attempts have been made to describe effect concentrations (EC) via equations of the form

$$\log \mathrm{EC} = a \cdot \log K_{ow} + b \cdot R + c \qquad (5)$$

in which K_{ow} models the uptake into the organism and R represents a parameter reflecting the differences in reactivity in a certain reaction with a biologically relevant target.

Early QSAR studies with reactive chemicals have used experimentally measured reactivities (*124, 104, 105*). Quantum chemical calculations provide the opportunity to calculate, from the chemical structure of a compound as the sole input, parameters that define the (relative) reactivity of the compound (*125*). The application of quantum chemical parameters in QSARs for ecotoxicity started in the early 1990s (see for example refs *126* and *127*).

An overview of quantum chemical parameters was given recently (*128*). Quantum chemical descriptors may have shortcomings because often solvation effects are neglected in the calculations and the absolute values may not always be meaningful, but they are very useful as a relative scale within structurally related series (*125*). Some approaches explicitly include solvation effects in calculating physicochemical properties such as pK_a values (see ref *129*).

Several reviews discuss the application of quantum chemical descriptors in QSAR studies (*87, 125, 20, 128*). QSAR models with quantum chemical descriptors have been derived for fish acute toxicity data of, for example, epoxides (*127, 130*) and reactive carbonyl-containing aliphatic chemicals (*131*). Karabunarliev et al. (*84*) developed QSAR models for acute toxicity data to fathead minnow of electrophiles based on molecular mechanisms. They developed separate models for electrophiles reacting via nucleophilic substitution reactions, Michael type additions, and Schiff-base formation, and descriptors were selected based on these reaction mechanisms. These two steps, the recognition of the reaction mechanism and the selection of a specific descriptor for this mechanism, are relevant for getting meaningful and successful models. Of course, this is easier in studies of a single process (for example a certain biotransformation reaction) than in integral effect studies, in which several processes are involved (*128*).

QSARs for Receptor Mediated Toxicity. Receptor mediated processes are complex, because receptor interactions are often very specific and the strength of such interaction highly depends on the three-dimensional structure of molecules. Modeling these interactions usually demands sophisticated approaches. Approaches such as COMFA (*132*) and GRID (*133*) implicitly represent the lowest-energy, gas-phase, shape of the pharmacophore (e.g., steric and electrostatic energies derived from the interaction with "probe" atoms). If the 3-D structure of the receptor is known, the interactions of ligands with the receptor can be modeled using a "docking" procedure, based on the energy minimization of steric (van der Waals) and electrostatic interactions (*134*). The conformation of the ligand may, however, change as a consequence of binding to a receptor and this has led to the development of the COmmon REactivity PAttern (COREPA) approach by Mekenyan et al. (*135*). The approach has been used to model binding to estrogen and androgen receptors (*136*−*138*).

Modeling the activity of receptor-mediated processes is a relevant issue for the development of predictive models for estrogenic effects of environmental pollutants. It is a specialized field and we have chosen not to discuss this topic in detail. Several reviews are available which discuss QSARs for endocrine disruption (see, e.g., ref *139*).

Modeling of integral (overall) effects of chemicals with specific (receptor mediated) effects is even more complex than modeling receptor interactions. The final effect is preceded by several steps, including uptake, distribution within the organism, biotransformation to active or inactive metabolites, excretion, and interaction with the actual target. Organophosphates are a good example to illustrate this complexity. In these complex situations, or also if information on mechanisms is simply unknown, multivariate techniques such as principal component analyses (PCA) and partial least-squares analyses (PLS) can be a good starting point for analyzing experimental data (*140*−*142*). Fish LC_{50} data, but also data on biotransformation and enzyme inhibition for a series of organophosphates have been analyzed via QSAR using linear regression techniques and PLS analysis with quantum chemical parameters (*126, 143, 87, 144*). Although modeling the individual processes was in some cases successful, the QSARs for the in vivo LC_{50} data in general were of lower statistical quality than models for baseline toxicants and reactive chemicals.

Mixtures. How chemicals behave in mixtures is strongly influenced by their mode of toxic action. If two or more chemicals have different target sites, their effect can usually be treated independently, even if an integral response of the organism (e.g., lethality) is investigated. Mixtures of chemicals with a common target site and the same mode of action act according to concentration or dose additivity. If the mixture components interact with each other, they might cause antagonistic or synergistic effects. The term "synergism" is

used with respect to concentration addition, i.e., stronger effect than expected from concentration addition, and the term "antagonism" refers to an activity, which is lower than independent action predicts. This classification of mixture effects stems originally from Plackett and Hewlett (*145*) and is based on pharmacological studies but has found wide acceptance in the ecotoxicological community and has been applied also for integrative endpoints such as lethality (*146, 92, 147*). Many alternative terminologies have been used as well. The concepts of mixture toxicity and related mathematical formulations have been extensively reviewed in refs *146, 148, 147,* and *149−152*. In this review, we are focusing on the role of the mechanism of toxicity for mixture effects. An extensive compilation of mixture toxicity studies is presented elsewhere (*153*).

While the mixture effects of baseline toxicants have been thoroughly investigated, there is very little accomplished in this area for specifically acting compounds. Examples of specifically acting compounds, which share a common mode of toxic action and do not interact, therefore acting according to concentration additivity, include triazines (*154*), dioxin-like compounds (*155, 156*), and polycyclic aromatic hydrocarbons (*157*). If concentration-effect curves of additive compounds are parallel and have the same maximum, the relative potency of a given compound can be expressed in toxic equivalent concentrations. The toxic equivalent concentration of a compound is defined by its concentration times its relative potency, i.e., the ratio of the EC_x-values of the reference compound of high potency to the EC_x-value of the compound in question. This concept of toxic equivalency has been initially developed for aryl hydrocarbon receptor binding of polychlorinated dibenzodioxins, dibenzofurans, and planar polychlorinated biphenyls (*156*). This concept is appealing due to its simplicity and attempts have been made to extend this concept to integral effects (*156*) and other modes of action, e.g., endocrine disruption (*158*), but great caution should be applied when extending a concept that is strictly valid only for receptor binding assays to integral effects and to other modes of toxic action.

Mixtures of four xenoestrogens, including DDT and 4-nonylphenol, showed concentration-additive effects in the recombinant yeast estrogen screen assay (*159*). Xenoestrogens are by a factor of 10^4-10^5 less potent than the natural hormone 17-β estradiol, and therefore there was doubt expressed that xenoestrogens would affect the estrogen receptor at all if estradiol was present. However, equipotent mixtures of estradiol with DDT or with bisphenol A did show additive effects (*160*). Note that the yeast estrogen screen assay just represents binding to the estrogen receptor. For more integral endpoints, such as the vitellogenin induction in rainbow trouts, toxicokinetic interactions may have to be considered. While 4-nonlyphenol and 17-β estradiol are concentration additive, methoxychlor and 17-β estradiol acted less than additive in this assay, presumably due to the need for activation of methoxychlor through metabolism in the liver (*161*).

Since specifically acting compounds usually contain specific or reactive functional groups, they are also prone to interactive effects in mixtures. For example, in mixtures of substituted phenols, which act as uncouplers of oxidative and photophosphorylation, the phenoxide species of a stronger phenolic acid may interact with the neutral species of a weaker acid or a H-donor molecule thus forming a mixed dimer, which is a more efficient uncoupler species (*162*). As a result, such mixtures exhibit a synergistic effect. However, if the formation of the mixed dimer is not particularly favorable or the mixed dimer is not an intrinsically better uncoupler than the dimers of the single components, the overall mixture effect can still be described by concentration addition (*162*).

A prominent and environmentally relevant example of dependent action is the synergistic effect of atrazine in combination with organophosphate insecticides. The organophosphates have to be activated from the thio-ester to the oxo-ester before becoming inhibitors of the acetylcholine-esterase. Stronger effects than concentration addition were not only reported for acute toxicity (*163, 164*) but also when investigating directly the activity of the AChE (*165*). Nevertheless, the role of synergistic and antagonistic effects in mixture studies should not be overemphasized. For a variety of pesticides the joint effect in aquatic animals could be predicted satisfactorily by concentration addition in 90% of over 200 mixtures (*166*). The good agreement was interpreted as a result of the small difference between the prediction of concentration addition and independent action, which are often within one order of magnitude.

Very few studies have been conducted with mixtures of reactive chemicals. This is an important research gap because further complexity is added to the problem due to the possibility of chemical−chemical interactions and other indirect effects. One recent study has dealt with the effect of phototoxic polycyclic aromatic hydrocarbons (PAH) that induce oxidative stress. Binary mixtures of phototoxic PAHs acted according to concentration addition (*167*). The same holds true for glutathione depletion by acrylates, which was dose-additive (*168*). Chen and Yeh showed that reactive electrophilic chemicals acting via the same mode of action are likely to show antagonism (*169*). In addition some severely synergistic combinations of reactive chemicals with dissimilar mode of action could be identified, e.g. malonitrile in combination with formaldehyde (*169*). These observations open up a route to more systematic investigations of the mixture effects of reactive compounds.

Earlier studies comparing concentration addition and independent action suffered very often from the fact that the effects were assessed only at one effect level and cumulative low dose effects were not considered. Grimme et al. performed a series of studies where the full concentration effect curves of a large number of single compounds and mixtures of compounds, with similar and dissimilar mode of action, at fixed concentration ratios corresponding to ratios of effective concentrations at different effect levels, were analyzed (*22, 170, 154, 171*). The toxicity of a mixture of 14 strictly dissimilarly acting compounds toward the luminescent marine bacteria *Vibrio fischeri* showed very good agreement with the prediction according to independent action (*154*). Analogously, the toxic effect of 16 triazines (inhibitors of photosystem II) in a 24-h synchronized algal growth test on *Scenedesmus vacuolatus* and 16 substituted phenols (uncouplers of oxidative phosphorylation) and other uncouplers was consistent with prediction for concentration addition (*170*). The main conclusion from these experiments is that concentrations of toxicants below a measurable effect can contribute to the overall toxicity. The effect elicited by the EC_{01} is indistinguishable from the control but the effect from the 14 to 16 compounds, each present at its EC_{01}, is measurable and corresponds to the effect predicted by the respective mixture model (*170*). Similar results were obtained for estrogenic compounds with the yeast estrogen screen (*172*). This conclusion confirms that concentration addition has to be considered in the risk assessment of chemicals, also at very low effect levels. Still missing, however, are systematic studies comparing concentration-additive effects of the underlying baseline toxicity with the independent action of groups of compounds with dissimilar mode of action.

Multiple Mechanisms

Within the proposed framework of relating effects to target sites and the interaction with the target, it is possible to

rationalize the role of multiple mechanisms fairly easily. For example, an electrophilic compound will be able to react with any biological nucleophile. The extent of an effect to the organism will on one hand be related to the abundance at the target site. If an electrophile is also very hydrophobic, it will primarily accumulate in membranes and exert its reactivity on the lipid molecules and will not react with a soluble enzyme or other molecules that are floating in the cytosol. On the other hand, any electrophile will have a preference for a specific nucleophile. For example, a soft electrophile, like acrylate, is more prone to react with thiol groups in proteins than a hard electrophile like an epoxide, which is prone to attack the DNA bases (*173*).

Chemicals that have a high affinity to ligands, which are common in biological molecules, are likely to exhibit multiple mechanisms. For example, triorganotin compounds may interfere with energy-transduction through several different mechanisms, including inhibition of the electron-transfer chain, inhibition of the adenosine triphosphate (ATP) synthase, and destruction of the electrochemical proton gradient through chloride/hydroxide antiport or through hydroxide uniport. Tributyltin partitions well into biological membranes and consequently appears to exert its dominant effect on the electrochemical proton gradient, while triphenyltin, which is too bulky for easy membrane permeation, but has a higher affinity to oxy-ligands, appears to be a direct inhibitor of the ATP synthase (*174*). In addition to these multiple mechanisms on energy transduction, triorganotin compounds interfere with many other receptor-mediated processed, leading also to chronic effects such as neurotoxicity, carcinogenicity, immunotoxicity, teratogenicity, and mutagenicity (*175*).

Multiple effects can also stem from a single first interaction with the toxicant as depicted in Figure 2 if there is a cascade of secondary effects or if effects with a common mechanistic basis result in differential organ toxicity. These topics are not addressed here in detail but still need consideration.

Concomitant Baseline Toxicity and Specific Mechanisms. Any specific effect will compete with baseline toxicity. The more hydrophobic a specifically acting molecule is, the more likely it is that its toxicity can be described through baseline toxicity (see also Figure 7). Benzylhalides may act as reactive electrophiles or as narcotics depending on their substitution pattern (*102*). The mode of action of chlorophenols and chlorocatechols changes with an increasing number of chloro substituents, i.e., with increasing hydrophobicity and acidity, from baseline toxicity to uncoupling (*176*, *177*).

Another example are acrylic and methacrylic acid esters, which are soft electrophiles and react with glutathione, which is an important metabolic conjugant and redox buffer in the cell. The stronger electrophilic acrylates will mainly act because of their reactivity (Figure 11, group 1), the more hydrophobic methacrylates may act mainly via narcosis (Figure 11, group 2), and some of the (meth)acrylic acid esters will act via both mechanisms.

Time Scales of Multiple Mechanisms. Multiple mechanisms may also occur at different time scales, which is particularly relevant for the comparison of acute and chronic effects. For example, chlorocatechols in combination with copper may produce reactive oxygen species (*178*), which may be of relevance for chronic effects, but the acute toxicity is caused by membrane toxicity, either baseline toxicity or uncoupling (*177*).

Information on the toxicodynamic phase, i.e., the interaction with the target site, the mechanism of toxicity, and the intrinsic activity (e.g., the reactivity in case of reactive chemicals), needs to be complemented by information of the preceding toxicokinetic phase, when predictive models for effects on whole organisms are to be developed and time-dependence of effects are to be investigated. The toxicokinetic

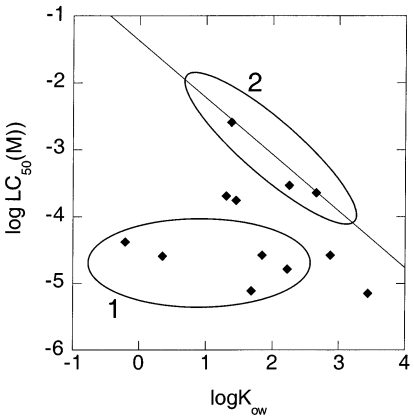


FIGURE 11. LC_{50} toward fathead minnow (*Pimephales promelas*) for reactive acrylates plotted against K_{ow}; ◆ data from ref *112*; line corresponds to QSAR equation for baseline toxicity described in ref *218* with data from ref *44*. Group 1 refers to chemicals, which act according to their reactive mode of action and group 2 includes all pure baseline toxicants. The remaining molecules exhibit features of both modes of action.

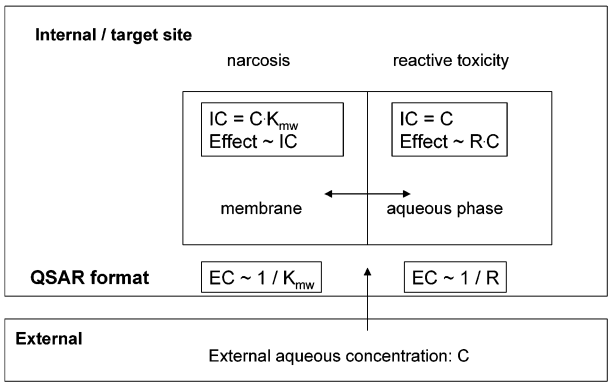


FIGURE 12. Two modes of action of reactive chemicals in relation to their target site: baseline toxicity and toxicity related to the reactivity. IC = internal concentration; C = external aqueous concentration; R = a reactivity parameter, e.g., a rate constant; K_{mw} = membrane−water partition coefficient.

phase encompasses the processes of uptake, distribution within an organism, and its compartments, biotransformation, and excretion. It is beyond the scope to discuss the toxicokinetics in detail in this review, but their role for the overall effect should not be underestimated. In particular, the role of the metabolites for the toxic effect has rarely been investigated. In many cases, metabolites act according to a different mechanism. In principle, the toxic effects of metabolites have to be assessed iteratively, and parent compound and metabolites have to be looked at as mixtures (*179*).

QSARs. Chemicals having multiple modes of action can obscure QSAR analyses. A good example is the study of fish toxicity with acrylates mentioned above. Some of the acrylates will mainly act because of their reactivity (Figure 11, group 1), some of them may act mainly via narcosis (Figure 11, group 2), and some of them will act via both mechanisms. Freidig et al. (*112*) suggested that the target site for reactive acrylates is in the cytosol (see Figure 12). Because the concentration in the cytosol will be similar to the external aqueous concentration at equilibrium, the effect concentration will probably only be influenced by differences in reactivity (R). In a QSAR of the type given in eq 5, the EC value will be proportional to $1/R$ in which R represents the reactivity of a chemical in a certain reaction with the target. Chemicals within the second group will predominantly act

via narcosis and the EC will be proportional to $1/K_{mw}$ (Figure 12). Based on these arguments, Freidig and Hermens (*180*) have suggested to separate a series of chemicals into groups, in which chemicals act by one single mechanism, and then to establish a QSAR model for each group separately. Analogous comparisons were made for organophosphates, which inhibit acetylcholine esterase, and for nitrobenzenes that are reactive chemicals (*180*).

Establishing QSARs for chemicals having multiple modes of action may in principle be possible if models can be derived for both modes of action separately and, at the same time, if the joint effect of the two modes of action is included in the model formulation. In QSARs for reactive compounds, the coefficient for the influence of log K_{ow} (the parameter a in eq 5) is often in the order of 0.3−0.4. This low coefficient is very likely a mean value of the coefficient for narcosis (around 0.9) and for purely reactive toxicity (around 0) and reflects the fact that two modes of action are combined with one equation.

These examples show the importance of information about the target site and distribution of a chemical between nontarget and target compartments. This distribution between the cell membrane and the cytosol, which of course is more in favor of the membrane compartment for hydrophobic chemicals, also explains the observations that LC_{50}-values for more hydrophobic electrophiles usually deviate much less from baseline models than the hydrophilic electrophiles do (*103*−*106*) (Figure 7A). The concentrations in the target compartment is just getting too low for the reactive compound to express its effects in the cytosol and narcosis starts to be the predominant mode of action.

Mekenyan et al. (*87*) also addressed the change in the influence of K_{ow} by calculating QSARs for sets of chemicals with a similar electrophilicity (an isoelectrophilic window). They observed a decrease in the influence of K_{ow} for the more electrophilic chemicals and explain these observations by an influence of interaction of the chemical with nontarget nucleophiles in the transport of the chemical to the target site. The lack of a positive hydrophobicity term in QSAR was recently discussed by Hansch et al., and several explanations were suggested (*181*).

Conclusion and Recommendations

Internal Effect Concentrations in Hazard and Risk Assessment. The advantages of mechanistic information in ecotoxicological studies as well as for hazard and risk assessment are obvious. If the target site(s) and the affinity to the target sites are known, the inherent hazard potential of a given compound can be better estimated and further research work can be directed to the appropriate questions. Replacing exposure-based (external aqueous) effect concentrations by internal effect concentrations is a first step toward a measure for inherent toxicity as was pointed out by McCarty (*24*) and taken up in a recent debate in SETAC Globe initiated by Gobas (*182, 183*). Further refinement from total internal concentration to target site concentration will greatly extend the applicability of this concept. Consideration of the internal concentrations also allows a better comparison of variable exposure conditions, e.g. in sediments, soils, and other complex matrices (*53, 184*). Remaining research gaps include the necessity for developing strategies on how to deal with multiple mechanisms and with metabolites.

By defining the intrinsic potency as the ratio of internal effect concentration IEC to an internal reference concentration, e.g. the $IEC_{baseline}$ of baseline toxicants, an intensive measure of toxicity can be introduced. This measure of intrinsic potency is independent of concentration or dose and is therefore better applicable in a categorization of persistence-bioaccumulation-toxicity (PBT) (*12*) than any presently used classification and labeling criteria for toxicity (*185*) that are usually derived as a cutoff aqueous effect concentration and therefore implicitly include both bioaccumulation and toxic effect. Persistent and bioaccumulative compounds are penalized twice with the presently used system because they have low aqueous effect concentrations even if they only act as baseline toxicants. Compounds of low hydrophobicity may not be classified as hazardous if the aqueous effect concentration is too high to fall below the cutoff value, even if their intrinsic potency is high. In contrast, in the proposed classification based upon IEC, baseline toxicants are only penalized for their bioaccumulation (if applicable) and specifically acting compounds are unambiguously identified. Intrinsic potency offers many advantages as toxicity-indicator, and further research should be directed to evaluation and validation of this measure.

Internal effect concentrations values are useful for the risk assessment of chemicals. Not only can they be applied in the classical risk assessment of single compounds as proposed by McCarty et al. (*27*) and Connell and co-workers (*186*) but also they pose particular advantages for assessing the risk of pulsed exposure (*187*), of mixtures of chemicals (*53, 83*), and effects in sediments (*188, 184, 82*).

Pulse exposure, which involves one or more isolated exposure periods, or fluctuating exposure, is observable in the event of accidents or during normal application of pesticides. Hickie et al. developed a simple one-compartment, first-order toxicokinetic model, which allows prediction of internal concentration in fish during pulsed exposure (*187*). If the internal concentration accumulated rises above the IEC value, the given endpoint effect is likely to be observable.

Mixture toxicity assessment and site-specific risk assessment can also be greatly facilitated by working with internal concentrations. Summation of external effect concentrations is of limited use, while the summation of IEC, in the case of baseline toxicity, offers a sensible mixture toxicity parameter. Procedures for estimating total internal concentrations have been proposed by several groups (*189*−*192*). Bioaccumulation and biomagnification models can be applied to calculate effective concentrations in the compartment of interest based on the IEC (*184, 193*). In such a way, the risk assessment becomes more general and less dependent on specific environmental conditions. The same holds true for differences in species. Nevertheless the concept of adding up internal concentrations is strictly true only for baseline toxicants. It needs further development for specifically acting compounds. In particular, the question needs to be addressed in which cases the underlying cumulative baseline toxicity dominates the effect in a mixture and in which cases the concentration or response addition of the fraction of specifically acting compounds are dominating the overall toxicity in an complex environmental sample. Development of mixture toxicity parameters or other (in vitro) assays for specifically acting chemicals should be based on insights into both the target site and the critical effect. In addition, the problems of bioavailability in in vitro assays (*194, 195*) and dosing (*196*), which are often overlooked, should be addressed in the development of such assays.

Predictive Models. We believe that a mechanistic perspective improves any attempt to set up predictive models. Only a profound understanding of the underlying mechanism and appropriate assignment of chemicals to a mode of action, or even to a mechanism, makes it possible to choose the right descriptors for QSAR and to define the chemical domain appropriately (*197, 19, 198, 199, 38, 200, 201*). The choice of chemical parameters and the mathematical form of a QSAR should represent, and be coherent with, the crucial molecular processes leading from external concentration to effects.

The decision on the chemical domain of applicability of a QSAR depends strongly on the mode of action concerned. Chemical similarity does not necessarily imply toxicological

similarity. Also, the development of test (training) sets and a thorough validation of models are crucial aspects (*140*−*142*). QSARs for groups of chemicals, which exhibit various or multiple modes of action, will always be less robust than for a single mechanism. Making the uncertainty in predictive models more explicit will save us from surprises and will make the decision process more transparent.

In addition, species sensitivity distributions and acute-to-chronic ratios heavily rely upon the correct assignment of mode of toxic action. ACRs are usually smaller if both acute and chronic effects have the same mode of action than if they differ in the mechanistic basis. Information on the distribution of sensitivities of species is nowadays often used in extrapolation methods (*4*−*6*) such as in the calculation of the HC_5 (hazardous concentration for 5% of the species). A log-normal distribution of species sensitivities is then often assumed, although there have been studies that show that this assumption may not always be valid (*202*). Species sensitivity distributions are influenced by factors related to the uptake, distribution, biotransformation (toxicokinetics), and interaction of a chemical in the organism (toxicodynamics). Therefore, information on target sites, biotransformation, and mode or mechanism of action are of particular relevance for understanding and predicting the toxicological aspects of species selectivity and sensitivity.

A step beyond QSARs in setting up predictive models would be to combine the toxicodynamic information with toxicokinetic modeling. Physiologically based toxicokinetic (PB-TK) modeling represents an excellent tool to analyze and predict concentrations in target tissue and target sites. If combined with dynamic aspects via physiologically based toxicodynamic (PB-TD) modeling, a more or less complete picture is obtained. We believe that the use of PB-TK and TD modeling will finally give insights into rate-limiting steps in, and theoretically based mathematical model of, the whole chain of events from external dose to observable effect. Such a mathematical model will then feed back into the development of QSARs but will also help to understand better the influence of biotransformation on target and whole-body residues. Such models were already shown to be useful for human health risk assessment of single compounds and mixtures (*203*−*207*) and have potential for application in environmental risk assessment (*208*). Toxicokinetic models for fish have already been introduced in aquatic sciences (*72, 209, 210*). Scaling these models to other species, maybe even to invertebrates, would be a first step in generalizing these models for application in predictive models. Then information on the physiology of the organism could be combined with toxicodynamic information of the chemical from in vitro test systems to perform a full risk assessment of a new chemical.

In conclusion, considering modes of action in ecotoxicology not only will improve our understanding on the effects of pollutants on ecosystems but also will be useful in setting up models and avoiding pitfalls in applied environmental risk assessment of chemicals and of polluted sites.

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