Models for Risk Assessment of Reactive Chemicals in Aquatic Toxicology

Modellen voor de risico-evaluatie van reactieve stoffen in de aquatische toxicologie
(met een samenvatting in het Nederlands)

Proefschrift

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Qui tractaverunt scientias aut empirici aut dogmatici fuerent. Empirici, formicae more congerunt tantum et utuntur: Rationales aranearum more, telas ex se conficiunt: Apies vero ratio media est, quae materiamex floribus horti et agri elicit; sed tamen eam propria facultate vertit et digerit.

Francis Bacon (1620), Novum Organicum, Lib. 1, XCV

Experimental scientists are like the ant: they collect and use; the theoretical scientists resemble spiders; who make cowebs out of their own substance. But the bee takes the middle course, it gathers its material from the flowers of the garden and of the field but transforms and digests it by a power of its own.

(engl. transl. given in: Yates, F.E. (1978), *Am J Physiol* 3:R159-R160.)

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INTRODUCTION

CHAPTER 1

INTRODUCTION

With industrialization, a tremendous number of chemical substances has entered our daily life (*1*). The increasing awareness of potential chemical hazards calls for a thorough risk assessment of these chemicals, both for humans health and the environment. In spite of that, it must be concluded today, that available information for most chemicals is not sufficient to perform a comprehensive risk assessment (*2*). Therefore, agencies like the environmental protection agency (EPA) in the US or the European chemical bureau (ECB) are developing methods to predict the risk of a substance from its chemical structure (*3-7*). Such models will help to fill data gaps in risk assessment. In this thesis, some toxicological models for reactive chemicals in aquatic organisms will be presented. This introduction will discuss structural properties of reactive chemicals, the use of these chemicals, and give some examples of exposure situations and toxicological effects. Additionally, a short overview of available toxicological models will be given. The introduction is concluded with an outline of the thesis.

STRUCTURE OF REACTIVE CHEMICALS

A large number of synthetic and natural substances fall in the class of organic chemicals. A close look at these organics reveals that most of them contain carbon (C) and a few other elements, namely hydrogen (H), oxygen (O), nitrogen (N), sulfur (S), phosphorus (P) and the halogens fluorine (F), chlorine (Cl), bromine (Br) and iodine (I) (*8*). Certain combinations of atoms within a molecule can be identified as functional groups. As the name already indicates, these groups are linked to an observable function of the molecule, generally a chemical reaction. Certain functional groups are known to react with each other. Reactive organic chemicals can thus be defined as organic chemicals with reactive functional groups. It should be noted that reactivity is a relative term as it always refers to a reaction with something else. Some organic chemicals are called electrophiles because they contain functional groups which tend to acquire electrons during a chemical reaction. Electrophiles react preferably with nucleophilic functional groups. This classification is important, because many biological substances such as proteins, enzymes or the DNA contain nucleophilic functional groups. They can be altered by chemical bonding of electrophilic xenobiotics and thereby lose their biological function (*9-11*). It is not surprising, that electrophilic groups are often present in carcinogenic, irritating or allergenic chemicals. Several authors have presented overviews of electrophilic groups which are connected to such toxic effects (*12-16*). In table 1, the structure of four electrophilic organic chemicals is shown.

Table 1: Some structures of electrophilic organic chemicals.

(1) Ethyl acrylate
(2) Epichlorohydrin
(3) Acrylamide
(4) Trimethylammonium-ethylmetacrylate

These chemicals are all used in large amounts for very diverse purposes. In this introduction, they will serve as examples to illustrate the risk, which is involved in the use of electrophilic organic chemicals. In this chapter, 'reactive oraganic chemicals' will be used as a synonym for 'electrophilic chemicals'.

USE OF REACTIVE CHEMICALS

Reactive (electrophilic) chemicals are often used as intermediates for products of the chemical industry. They are used in large amounts and many of them are included in the high production volume chemical (HPVC) list of the EU (*5, 15*) which means that their annual production exceeds 1'000 tons. To get a better idea in which products these chemicals eventually end up we will have a more detailed look at the four chemicals from table 1. Ethyl acrylate (1), a reactive α,β-unsaturated ester is used for the manufacturing of poly-

mers which are used in latex paints, binders, polishes and adhesives (*17*). Epichlorohydrine (2) has two functional groups, an epoxide and a carbon atom with a good leaving group (Cl). This compound is used to make epoxy resins, synthetic glycerin- and glycidyl ethers. It is also used as insecticide and rodenticide and in the production of paper, textiles and pharmaceuticals (*18*). The next compound, acrylamide (3) has a double bond similar to ethyl acrylate, but a more water soluble amino group (NH_2). Like compound (1), it is used to make polymers. They are used as flocculants for wastewater treatment, in mining industry, for soil stabilization, as papermaking aids and thickeners. Polymers of acrylamide are furthermore used to promote adhesion and dye acceptance and as additives for textiles, paints and cement. The last example in table 1 is trimethylammoniumethyl methacrylate (4), a cationic chemical. Like acrylamide, it is used as flocculating aid but also as ion-exchange resin, anti-static finishing, in hydrophilic glass fibers and as superabsorbents (*19*). It can be seen, that chemicals with quite similar structures (1, 3 and 4 all χονταιν α,β-unsaturated carboxyl groups) can have very different applications. Furthermore, each chemical is used for the production of multiple products. It is therefore difficult to link a functional group to a certain use or to a certain production process.

RISK ASSESSMENT OF REACTIVE CHEMICALS

Risk assessment of chemicals generally contains two parts: the exposure assessment and the effect assessment (*20*). In the following two sections, both parts shall be discussed using the four chemicals from table 1.

POSSIBLE EXPOSURE SITUATIONS

The use of large amounts of chemicals requires frequent transport. This increases the risk for accidents. For both ethyl acrylate (1) and epichlorohydrin (2), accidents have been reported during transportation by freight trains. In the summer of 1994, a tank car derailment took place in the central station of Lausanne, Switzerland. The tank car which contained epichlorohydrin did not start leaking. Otherwise, the central city of Lausanne would have been exposed to an extremely toxic, mutagenic and explosive gas-cloud. A smaller, but nevertheless potentially dangerous accident occurred in 1998 on the busy train tracks of the Gotthard tunnel (Göschenen, Switzerland). A leaking tab on a tank car caused the spill of approximately 100 l of ethyl acrylate on the tracks (*21*). Because fire and widespread contamination could be prevented, this accident did not cause any casualties. From these

two examples, we can already draw some conclusions. Three properties of the involved reactive chemicals, namely the volatility, explosivity, and toxicity can turn an accident into a difficult to predict hazard.

Accidents not only happen during transport but also during normal use as will be shown with the following examples. Acrylamide (3) was used as component of a novel water-tight sealing layer in the construction of a railway tunnel in southern Sweden in 1997. After successful small scale tests, a large segment of the tunnel was treated with the sealing. Soon thereafter, the construction company realized that large amounts of acrylamide did not polymerize, but remained in the chemically reactive monomer form, contaminating the air in the tunnel and the groundwater. Cattle from nearby farms were intoxicated by drinking surface water and had to be killed (*22*). The whole area around the tunnel had to be monitored, and a long term clean-up program was set up. Accidental release of the last compound on the list (4) caused a massive fish dying in Basel, Switzerland in 1998 (*23*). The chemical was used in a paper manufacturing plant as anti-static coating. After a tank had been cleaned a valve was left open accidentally and the next morning, one ton of the cationic methacrylate leaked in a nearby river. The resulting high concentrations killed all fish in the river and in a fish hatchery, located downstreams. Ironically this hatchery was almost ready to release a large number of salmons, which should compensate the large fish kill caused by the 'Schweizerhalle'-accident some years ago.

From these illustrative examples, it can be concluded that the large-volume transport and the specific use of reactive chemicals can cause accidental releases in workplaces, densely populated areas but also into the aquatic environment. Aquatic risk assessment of reactive chemical is therefore closely linked to occupational and calamity risk assessment. For reactive organic chemicals exposure scenarios are obviously not the same as for the 'classical environmental pollutants' like DDT,PCB's or PAH's, for which the chemical structure and the exposed ecosystems are relatively well known by now. The enormous variability in the use of reactive chemicals makes it virtually impossible to determine which ecosystem will be exposed to which chemical.

TOXICOLOGICAL EFFECTS

General remarks

To better understand what happens with organisms that are exposed to reactive chemicals, let us first consider some general properties of a stressed system by using a simple

example: The runner. When running, the body of a runner is exposed to a stress depending on the runners speed. At a low pace, the increased oxygen demand, caused by contracting muscles is compensated by an increasing heart-rate, cardiac output and breathing rate. This results in an increased oxygen uptake and the body will reach a new steady state where the stress of running is (almost) completely compensated by physiological adaptation. If the runner runs harder, a point will be reached where this adaptation can no longer compensate the increasing oxygen demand of the muscles. An anaerobic (without oxygen) energy production in the muscles takes over. This anaerobic process, however, is not sustainable anymore, as it causes a fast acidification of the muscle. The runner has changed from a steady state into a decompensated system, which depletes its own reserves very fast. Eventually, out of breath and with painful legs, the runner has to stop because the decompensation has reached a critical level. (It should be stressed here, that running can be an excellent way of dealing with stress.) An aquatic organism, that is exposed to a certain concentration of a chemical is in a comparable situation. Physiological and biochemical adaptations can protect the organism from harmful effects at low exposures. There is a critical exposure concentration, however, where adaptation can no longer compensate the stress and the organism will enter a decompensated state. Under continuos exposure above the critical concentration, the organism will eventually die.

The glutathione system

Among the many adaptation mechanisms in an organism, the glutathione (GSH) system is one of the most versatile and widespread 'defense' systems against electrophilic chemicals on a cellular level. This tripeptide which contains a nucleophilic SH-group (cysteine) is present in almost all species. GSH fulfills endogenous functions as well as functions related to xenobiotic metabolism. A diverse set of enzymes has been characterized that use GSH as a (co-) substrate. GSH can act as scavenger of reactive electrophiles because it is present in high concentrations in the cytosol. It thereby protects more vital SH groups of proteins and enzymes from reacting with xenobiotic electrophiles (*24*). This detoxifying function adds up to the endogenous functions where GSH is the co-substrate of GSH-Peroxidase, the enzyme that oxidizes H_2O_2 and other cytotoxic radicals produced as by-product of the oxidative phosphorylation in mitochondria. It has been demonstrated that depletion of the GSH (cellular and in mitochondria) by conjugation with electrophiles leads to oxidative damage of a cell (*25, 26*). GSH can thus be seen as a central pillar of cell-defense against various chemical stresses. In this thesis, the depletion of GSH in exposed organisms will often be used to characterize toxicological effects in stressed systems.

Table 2: Observed toxicity levels and toxic effects of compounds from table 1. For trimethylammonium ethylmethacrylate, no toxicity data was found.

Chemical name	oral LD_{50} rat[a] ($mgkg^{-1}$ body w.)	4 day LC_{50}[b] (mgL^{-1})	toxic effects/ mode of action
Ethyl acrylate	760-1020	2.5	local irritation[c]
Epichlorohydrin	40	12	carcinogenicity[d]
Acrylamide	570	120	neurotoxicity[e]

[a] From Hazardous Substance Data Base (HSDB), US-National Library of Medicine.
[b] Collected from ECOTOX database, US-EPA. Original data from (*28, 29, 43*).
[c] From (*17*)
[d] From (*18*)
[e] From (*44*)

Examples of toxicological effect

In this paragraph, we will give a short overview of the toxic effects of three chemicals from table 1 (1,2 and 3). For the fourth chemical, several databases were searched (US-EPA: AQUIRE and ISIS, US-NLM: Toxline, HSDB) but no information could be found about toxic effects or effect levels. Some effect levels and toxicological effects of the three chemicals, for which data was present, are summarized in table 2.

For ethyl acrylate, the chemical reactivity of the double carbon bond is generally recognized as the reason for its toxicity. This double bond can react by a so called Michael addition, preferably with a SH- group, as shown in figure 1. Such thiol-groups are nucleophilic functional groups, found in proteins and in active sites of enzymes. Ethyl acrylate was shown not to react with DNA molecules in vitro (*27*) and was not positive in bacterial mutagenesis assays (*17*). In chronic oral dosing experiments with rats, however, ethyl acrylate caused neoplasma formation in the forestomach. This only occurred at high doses, which also caused severe local irritation and cytotoxicity. Therefore, and because of the negative in vitro assays, ethyl acrylate was not judged to be a genotoxic carcinogen (*17*). The acute toxicity of ethyl acrylate towards fish is relatively high. The 4-day LC_{50} (aqueous concentration were 50% of the fish died within 4 days of exposure) for the fathead minnow (p. promelas) is 2.5 mg/L (*28*).

Epichlorohydrin can also react with biological thiol groups (Freidig, unpublished results) but due to different functional groups also with DNA. In vitro data shows that epichlorohydrin can bind to DNA and it was found carcinogenic in rats (*18*). Compared to ethyl acrylate, epichlorohydrin has a higher oral toxicity in rats but a lower acute toxicity towards fish (4-day LC_{50}=12 mg/L). For neither of the two compounds, long-term exposure data in aquatic species is available.

Acrylamide has a structure, similar to ethyl acrylate. The acute rodent toxicity of these

Figure 1: Michael addition of glutathione (left) to the double bond of ethyl acrylate. This chemical reaction is important for the toxicological effects of ethyl acrylate.

two chemicals is quite similar, but acrylamide is much less toxic in fish (*29*). Just like ethyl acrylate, acrylamide is not a genotoxic carcinogen. Furthermore, it affects the nervous system, an effect which is neither reported for ethyl acrylate nor for epichlorohydrin.

From the above presented data we can conclude that (i) reactive chemicals express different toxicity in different species, (ii) chemicals which share a functional group can cause very different toxic effects, and (iii) for some chemicals toxicological data is simply not available.

MODELS FOR EFFECT ASSESSMENT OF REACTIVE CHEMICALS

As mentioned before, risk assessment consists of both, an exposure and an effect assessment. This thesis will focus on the effect assessment and will try to shed some light on the complex relation between structure and toxic effects of reactive organic chemicals, in particular for aquatic species. Below, we will give a short summary of the most important strategies presently used to describe and predict aquatic toxicity. Some of these strategies were applied in this thesis. To put the different model approaches in a context, let's first look at the cascade of events that takes place between chemical exposure and toxicological response. Three intermediate steps can be identified (figure 2): (i) the disposition (uptake, distribution and metabolism) which governs the concentration of the chemical at the target site, (ii) the interaction with the target site and (iii) the decompensation of the organism which eventually results in a toxic response (e.g. lethality).

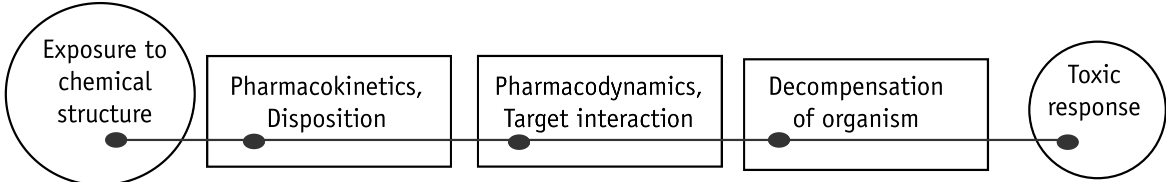


Figure 2: Chain of events, leading from exposure to a toxic response or a toxic endpoint (e.g. irritation or lethality). Knowledge about the three intermediate steps is essential to establish a structure-effect relationship.

A first model strategy is to classify reactive chemicals according to their functional groups. Such classification schemes were proposed by several authors (*12, 13, 15*) and also adapted for computers using so called expert systems (*16*) and advanced statistics (*5*). A classification approach can indicate, whether a chemical causes a certain toxic effect. Quantitative predictions (e.g. effect concentrations) can be made by quantitative structure-activity relationships (QSAR's). To establish a QSAR, the structure of a chemical is translated to one or several numerical values, the so called descriptors, which can be correlated with an observed toxic effect. Descriptors can be very simple (e.g. number of chlorine atoms), or very complex (e.g. Free energy of formation of a transition state). They can be measured (e.g. chemical reaction rate with SH groups), derived from empirical observations (e.g. Hammett constant σ), or calculated using quantum-chemical approaches (e.g. electron distribution within a molecule). A number of models have been proposed to describe fish toxicity of groups of reactive chemicals sharing the same functional group (*7, 30-35*). In figures 3, one of the first published QSAR for fish toxicity of reactive chemicals is shown (*36*). The chemi-

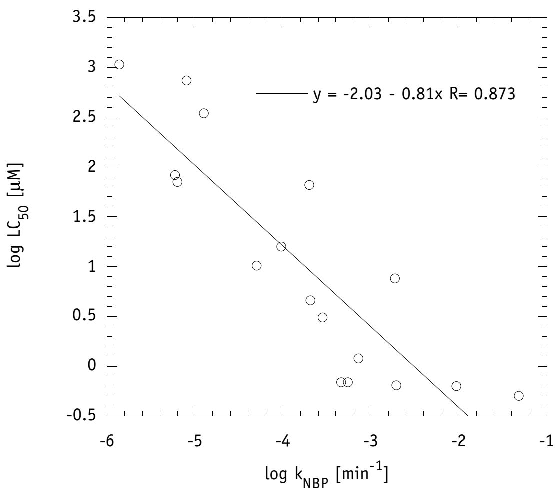
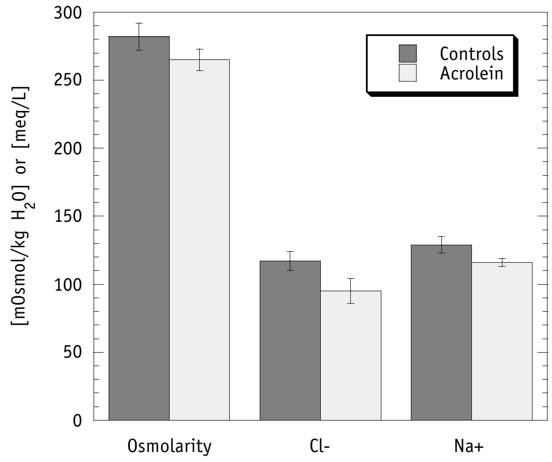


Figure 3: Quantitative structure activity relationship (QSAR) by Hermens et al. (*36*) for reactive organic halides. Acute toxicity towards guppy correlates with the chemical reactivity of the compounds. Nitrobenzylpyridine, a nucleophilic chemical was used to determine the reaction rate, k_{NBP} of the electrophilic organics.

4.a)



4.b)

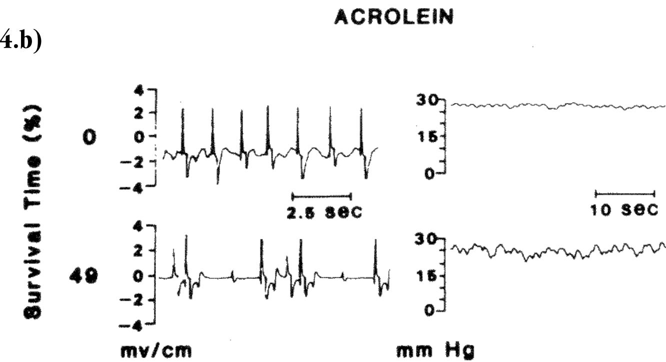


Figure 4 a and b: Some fish acute toxic syndromes (FATS) reported by McKim et al (*37*). These clinical signs were recorded in rainbow trout exposed to a lethal concentration of acrolein (α,β-unsaturated aldehyde). The concentration of ions in the plasma, given as total osmolarity, chloride and sodium ion concentration, is reduced in exposed fish (figure 4 a). This can be explained by damaged gills, where ions leak into the external water. In figure 4 b, it can be seen on the electrocardiogram that the heart of an exposed trout is severly stressed. The heartbeat becomes arrhythmic (graphics to the left) and consequently, the blood pressure is unstable. These are typical signs of a decompensated system.

cals, used to establish this QSAR shared a labile halogen-carbon bond. Measured chemical reactivities of electrophilic halogenated compounds were thereby correlated with 14-day LC_{50} values for guppy (p. reticulata). The measured reaction rate, k_{NBP} of the electrophilic compounds with the model-nucleophile nitrobenzyl-pyridine, was used as descriptor for the reactive structures.

The two approaches discussed above are clearly structure-driven. In contrast , a more physiological approach for effect assessment was proposed by McKim and coworkers by introducing 'fish acute toxic syndromes (FATS)' (*37-39*). The physiological response (clinical signs) resulting from exposure to the chemical, instead of the chemical structure was used as a classification criteria. These clinical signs were recorded in a phase of decompensation, to get an idea about the mode of action of a chemical. Some of the FATS-data of a rainbow trout exposed to acrolein, are presented in figure 4 (from McKim et al. (*37*)). Acro-

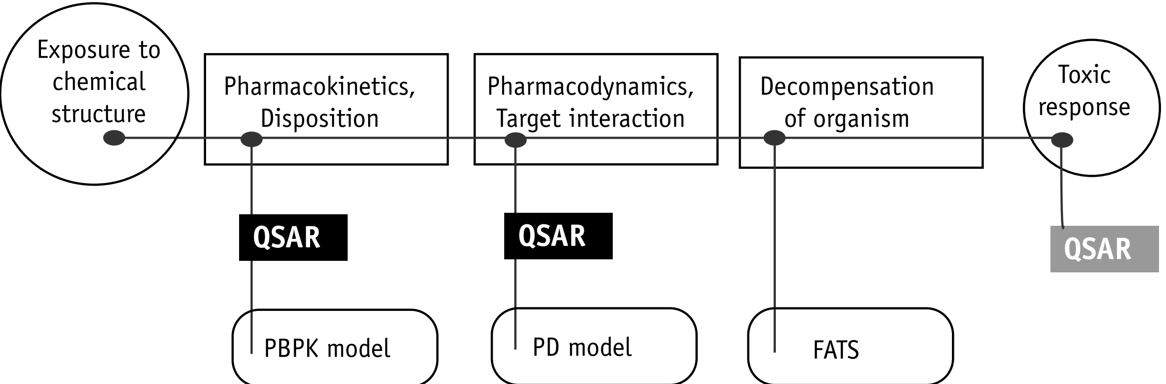


Figure 5: Different models can be used to describe the intermediate steps that lead to a toxic effect. QSAR models (as well as classification models) are very versatile. It should be noted, however, that QSAR's are more difficult to interpret, when several intermediate steps are bypassed (as e.g. by modeling acute toxicity with structural descriptors only).

lein contains an unsaturated C=C bond, similar to compounds (1), (3) and (4) and reacts therefore easily with thiol groups. The clinical changes recorded prior to the lethal intoxication of the fish, were changes in blood ion composition (figure 4 a), arrhythmic heartbeat (figure 4 b), and a very high cough rate (data not shown) and have been summarized as 'respiratory irritant syndrome'. Because measurement of a FATS requires specialized experimental facilities, this approach can not be used to screen a large number of chemicals on a routine base. The FATS, however, give valuable information about the cause of death and the ultimate target site of a chemical, information which is not provided by other toxicity data such as an LC_{50} value.

Physiologically based pharmacokinetic and pharmacodynamic models (PBPK-PD) are situated between a structural and a clinical approach. A PBPK-PD model can link the mechanistic information derived from the chemical structures with physiological information about an organism. Uptake and disposition of a chemical in the animal, but also interaction of the chemical with a target site and the toxicological response in the target organ can be modeled. This promising technique, however, has yet only been applied once for one reactive chemical in fish. Abbas and coworkers showed that the disposition and the enzyme inhibitory action of paraoxon in the rainbow trout (Oncorhynchus mykiss) could be described with a PBPK-PD model (*40*).

The models mentioned above can be located along the chain of events, as shown in figure 5. Each approach covers certain aspects of the chain of events leading to a toxic re-

sponse and is therefore limited in its possible answers. Regarding the risk assessment of chemicals, a model should focus on predicting the toxicity of untested chemicals. There are, however, more reasons why modeling efforts are worthwhile to undertake. As mentioned by Yates (*41*) and by Andersen et al. (*42*), models can also be used to:

- Organize existing information
- Expose contradictions in existing data and beliefs
- Explore implications of beliefs about toxic mechanisms
- Expose serious data gaps
- Predict toxicity under new conditions
- Identify essential structures and rate limiting steps
- Suggest and prioritize new research

This short overview of existing models is far from complete, but it gives a fair idea about the most important directions which are used currently to develop models for risk assessment purposes.

OBJECTIVES OF THIS THESIS

Because more toxicological information about existing and new reactive chemicals is needed urgently, models should be established that can predict effects from chemical structures or that can extrapolate toxic effects under different exposure conditions or for different species. The difficulty with reactive chemicals is that toxic responses are often species specific and that small changes in functional groups can change their mode of action. Quite a number of QSAR's for aquatic animals and reactive compounds have been established. Most of them relate chemical structure directly with a toxicological endpoint. Therefore, their application is very limited regarding extrapolation between species or between functional groups. There is a need for more understanding of intermediate processes such as kinetics, dynamics and compensatory mechanisms that govern the resulting toxic effect.

Major objectives of this thesis were defined as follows:

- Get more insight in the chain of events that cause the toxic effect of reactive chemicals.
- Develop approaches that can be used to model existing toxicity data from a more physiological point of view.
- Develop predictive models for electrophilic organic chemicals, especially for aquatic animals.

OUTLINE

In this thesis, a number of approaches were tested and used to establish models. Thereby, two classes of reactive organic chemicals were used, α,β-unsaturated carboxylates (which share the functional groups of compound 1 and 4 in table 1) and organophosphorus pesticides (OP-esters). Glutathione (GSH) depletion was chosen as an intermediate toxic effect that could be linked on one hand to the chemical reactivity of the chemical and on the other hand to observable toxic endpoints in organisms.

In ***chapter 2***, experimental data about the effects of α,β-unsaturated carboxylic esters was collected in purely chemical systems and modeled with QSAR's using both empirical and quantum-chemical descriptors.

In ***chapter 3***, the relation between structure and acute toxicity of α,β-unsaturated carboxylic esters in fathead minnow was investigated. Narcosis and GSH-depletion were tested as two alternative modes of action which both could cause the observed effects.

Some theoretical considerations about the homogeneity of commonly used QSAR test-sets were presented in ***chapter 4.***

In ***chapter 5***, a non-aquatic system, namely isolated rat liver cells (hepatocytes) was used to test whether the effect of α,β-unsaturated carboxylic esters in a mixture could be predicted from the effects of individual compounds.

The disposition of ethyl acrylate in the rainbow trout and its effect on GSH in the gills was investigated in ***chapter 6.*** A preliminary PBPK-PD model for the rainbow trout was established to organize and evaluate data from in vivo and in vitro experiments.

In ***chapter 7***, a general model to describe toxic effects of reactive chemicals in aquatic organisms is presented. The model was based on a simplified pharmacodynamic approach and validated with toxicity data from animals exposed to OP-esters. Parallels were found to Haber's Law, an empirical relation between exposure time and effective dose.

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QUANTITATIVE STRUCTURE-PROPERTY RELATIONSHIPS FOR THE CHEMICAL REACTIVITY OF ACRYLATES AND METHACRYLATES

CHAPTER 2

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ABSTRACT

Reactivity towards three different nucleophiles was measured for a training set of 6 acrylates and 7 methacrylates. The reactions studied were, neutral and base-catalyzed hydrolysis and Michael addition of reduced glutathione (GSH). A linear free energy relationship (LFER) was established for the base-catalyzed hydrolysis rate constants of methacrylates, with the Taft parameter σ^* as single descriptor. GSH reactivity could be modeled with a partial least square regression (PLS) using four quantum chemical ground state parameters, describing the difference in frontier orbital interaction and coulombic forces within the training set. Literature data for GSH reactivity was used to test the applicability of the PLS model. Differences in acute fish toxicity for structurally similar acrylates and methacrylates could be explained by their different potency as Michael-type acceptors.

INTRODUCTION

Acrylic and methacrylic acid esters are chemicals that are produced in large quantities. Their toxicity for humans and rodents as well as for aquatic organisms has been documented (*1*). In ecotoxicological research, acrylates and methacrylates are either classified together in one group as 'unspecific reactive chemicals' (*2*) or as two groups: the acrylates as electrophiles and the methacrylates as ester narcotics (*3*). Structure activity relationships have been established for acute fish toxicity of acrylates based on empirical (*4*), as well as quantum-chemical parameters (*5*). An important link between chemical structure and toxicity for these electrophilic compounds is their chemical reactivity (*6*) which influences both their toxicokinetic and -dynamic behaviour. Modeling of reaction rates can therefore help to explain differences in toxicity. Data about chemical reactivity can furthermore be used to predict the fate of compounds in the environment.

Chemical reactivity has been modeled successfully for a number of chemical classes, using quantum chemical parameters (*7*). Quantitative structure property relationships (QSPRs) for organic electrophiles have been proposed among others for small chlorinated alkenes by Verhaar et al. (*8*), for organophosphorus esters by Hermens et al.(*9*) and by Schüürmann (*10*) and for epoxides by Eriksson et al. and Purdy (*11,12*). For the reactivity of chlorinated alkenes, activation energies were calculated. For the other compounds semi-empirical molecular orbital (MO) parameters or empirical substituent constants proved to be successful.

In this work, a training set of 6 acrylates and 7 methacrylates was created to gain more insight in the reactivity of acrylic and methacrylic acid esters. Quantum chemical descriptors of electronic structure that were hypothesized to bear a relationship to the test compounds' reactivity or empirical descriptors were used to establish QSPRs with experimental reac-

Figure 1: Chemical structure of acrylic and methacrylic acid esters. R_1: H for acrylates, C for methacrylates, R_2: alcohol moiety.

tion rates. Because acrylates and methacrylates are both electrophilic chemicals, their reactivity was tested against three nucleophiles of different strength: water (H_2O), the hydroxyl anion (OH) and reduced glutathione (GSH). From a toxicological point of view, reaction rates of electrophilic compounds with biological nucleophiles are of special interest. In combination with effect data, they provide information about target sites within the cell and possibly about their mode of action (*13-15*). Glutathione is a nucleophile which is often used as a model for cellular thiol groups. It has an important function in the phase 2 metabolism of xenobiotic compounds where it acts as scavengerof free electrophiles and as co-substrate of glutathione transferases. In the literature, GSH-reaction rates towards different electrophiles have been reported along with a number of QSARs (*16-20*). Michael addition, a nucleophilic addition on C_β (Figure 1) is suggested as the predominant reaction mechanism of negatively charged thiols with α,β unsaturated carboxyl groups such as acrylates and methacrylates (*5,21*).

MATERIAL AND METHODS

Material

The following chemicals were used as received: o-phthalaldehyde from Acros ('s-Hertogenbosch, The Netherlands); ethyl acrylate, 2-hydroxyethyl acrylate, diethyl fumarate, isobutyl acrylate, lauryl acrylate, isobutyl methacrylate, methyl methacrylate allyl methacrylate, hydroxypropyl acrylate (mixture of isomers), hexyl acrylate, benzyl methacrylate, tetrahydrofurfuryl methacrylate and reduced glutathione from Fluka, Sigma-Aldrich (Zwijndrecht, The Netherlands); isopropyl methacrylate from Pfaltz & Bauer (Waterbury, CT, USA). Deionized water was treated with a Millipore filter-system before use. Methanol, acetone, sodium-citrate, citric acid, KH_2PO_4, Na_2HPO_4 and sodium-tetraborate decahydrate and sodium-EDTA were of analytical grade.

Assay to measure the neutral and base-catalyzed hydrolysis

Stock solutions of acrylates and methacrylates were prepared in acetone or in methanol. Aqueous buffer solutions were prepared with a pH 7.0 (1.0 mM H_2PO_4/HPO_4) and a pH 10 (1 mM sodium-tetraborate decahydrate). Mixtures of two or three compounds were used in the determination of the hydrolysis rates. The choice for the composition of the mixture was based on the retention time on the HPLC-column of the individual compounds, in order to assure proper identification and quantification. To achieve final concentrations of

100 µM of the electrophile (max. 1.5% organic solvent), 50 µl stock solution of the mixture were added to 10 ml aqueous buffer solution. The reaction vials were kept at 20°C. Samples were taken in duplicate immediately after mixing and after 24 and 48 hours (after 1 and 2 hours for diethyl fumarate at pH 10). The disappearance of the parent compound was used to calculate the hydrolysis rates.

HPLC-analysis for hydrolysis rate measurements

Reversed phase HPLC separations of the mixtures were performed on an Inertsil C-18 bonded silica column, 100 mm in length, 3 mm i.d (Chrompack, Bergen op Zoom, The Netherlands). Isocratic elution at 0.4 ml/min with a 20% to 40% water in methanol mobile phase was used, depending on the hydrophobicity of the most hydrophobic compound in the mixture. Analyte detection was with a UV detector using a wavelength of 215 nm. Concentrations were quantified using standard solutions of the compounds in methanol.

Assay to measure the reactivity with reduced glutathione

Stock solutions of reduced glutathione (GSH) (1mM) were made every second day. GSH was dissolved in water containing 50 µM sodium-EDTA to prevent oxidation. Because the deprotonated form of glutathione, GS^- is a much stronger nucleophile, the reaction was carried out at a pH of 8.8. Therefore, 1 ml of GSH stock solution was diluted with 9 ml of a 1mM sodium-tetraborate buffer solution resulting in a final GSH concentration of 100 µM. To start the reaction, 0.1ml of a methanol stock solution of an acrylate or methacrylate was added. The reaction temperature was 20°C. Electrophile concentrations ranged from 0.4 to 1.6 mM. Immediately after adding the electrophile, as well as after a given reaction time (1 hour for acrylates and 24 hours for methacrylates) 0.1ml sample was taken and diluted with 0.9 ml of a buffer with pH 3.9 (2.5 mM citric acid/ 2.5 mM sodium-citrate) to stop the reaction with GS^-. The GSH concentration of the samples was subsequently analyzed on the HPLC as described below. All reaction rates were measured in duplicate. No decrease of reduced GSH within 24 hours was found in control incubations, spiked with methanol only.

HPLC-analysis for glutathione depletion

Reduced glutathione was separated from the reactive test chemical and the GSH-conjugate on a C-18 column, as described above, using isocratic elution at 0.4 ml/min with 10% methanol in an aqueous phosphate buffer (5 mM, pH 3.0) as mobile phase. An RDR-1 reagent delivery unit (Timberline, Boulder, CO, USA) was used for the post column derivatization of reduced glutathione with o-phthalaldehyde. Reaction conditions according to Cohn and Lyle (*22*) and by Fujita et al. (*23*) were used with the following modifica-

tions: the aqueous derivatization solution contained 7.5 mM o-phthalaldehyde, 5%(v/v) methanol and was buffered at pH 8.0 with 35 mM phosphate buffer. The solution was delivered at 0.3 ml/min in a thermostated reaction coil (1m, 60°C). The fluorescence of the formed isoindole was monitored at λ_{ex}=350 nm and λ_{em}=420 nm. GSH concentrations were quantified with standard solutions of GSH in citric acid/sodium-citrate buffer.

Calculation of kinetic parameters

To determine the observed hydrolysis rate $k_{H,obs}$, a linear regression was calculated from the measured electrophile concentrations ($C_{El,t}$) and the reaction time (t) according to equation 1. The slope of the resulting equation ($-k_{H,obs}$) was tested for significant deviation (p=0.05) from zero with a *t*-test.

$$\ln C_{El,t} = \ln C_{El,0} - k_{H,obs} t \qquad \text{(EQ 1)}$$

In preliminary experiments at pH 4 (data not shown) we found that the acid catalyzed hydrolysis of the acrylates as well as of the methacrylates is too slow to be of any importance at room temperature at a pH of 7 and above, which is in agreement with literature data for other carboxylic esters (*24,25*). Therefore, only the base-catalyzed and the neutral hydrolysis were considered; the observed hydrolysis rate can then be expressed according to equation 2. Reaction rates were determined at pH 7.0 and 10.0, which resulted in two observed hydrolysis rates: $k_{H,obs}(7)$ and $k_{H.obs}(10)$. These two rates were used with equation 2.1 and 2.2 to calculate k_N and k_B, the neutral and base-catalyzed reaction rate constant, respectively (*25*). For six substances $k_{H,obs}(7)$ did not differ significantly from 0. Consequently, only the base-catalyzed hydrolysis rate was calculated using equation 2.2 and setting k_N to zero. Relative errors were calculated from the standard deviations of the slope of the regressions of equation 1 and are given as (%SE) in Table 1.

$$k_{H,obs}(7) = k_N + k_B[10^{-7}] \qquad \text{(EQ 2.1)}$$

$$k_{H,obs}(10) = k_N + k_B[10^{-4}] \qquad \text{(EQ 2.2)}$$

To determine the reactivity of the acrylates and methacrylates towards GSH, $k_{GSH,obs}$ was calculated according to equation 3, assuming a pseudo first order kinetic (electrophile in excess) for the decrease of GSH concentration, $C_{GSH,t}$. For isopropyl methacrylate, $k_{GSH,obs}$ was not significantly different from zero.

$$k_{GSH,obs} = \frac{\ln C_{GSH,0} - \ln C_{GSH,t}}{t} \qquad \text{(EQ 3)}$$

The reaction rate constant with GSH, k_{GSH} was calculated as follows (equation 4):

$$k_{GSH} = \frac{k_{GSH,obs}}{C_{El,0}} \quad \text{(EQ 4)}$$

Relative deviations from the average of two measurements are given as (%SE) in Table 3.

Quantum chemical calculations

Quantum chemical descriptors were calculated for both the training set and the test set acrylates and methacrylates. Individual structures were built manually, using the SPARTAN builder subprogram, and preoptimized using a modified MM2 force field (*26*). Global minimum conformations were located manually, using this force field. Optimized structures were subsequently submitted to a full AM1 minimization using either SPARTAN or AMSOL (*27*). Results from both programs should be the same, within machine precision. Properties were derived from the fully minimized eigenvector matrix. Additionally, AM1 optimized structures were submitted to a single point energy ab initio calculation at the Hartree-Fock/3-21G(*) level, and the same properties as for the semi empirical results were extracted from the ab initio results. Parameters used as descriptors were electrostatic potential fitted charges on selected atoms ($q(C_i)$) and ε_{LUMO}, the energy of the lowest unoccupied molecular orbital.

QSPR models and statistical analysis

Table 1: Calculated rate constants for neutral and base-catalyzed hydrolysis and estimated half-lives in aqueous solutions at pH 8.8. Rate constants were calculated according to equation 2 from measured hydrolysis rates.

Chemicals	CAS nr.	k_N (s^{-1})	(% SE)	k_B ($s^{-1}M^{-1}$)	(% SE)	Half-life at pH 8.8(days)
2-Hydroxyethyl acrylate	818611	n.d.[a]		1.1E-01	14%	12
Hydroxypropyl acrylate	999611	n.d.		3.2E-02	48%	40
Diethyl fumarate	623916	5.3E-07	14%	2.9E+00	7%	0.4
Ethyl acrylate	140855	n.d.		5.0E-02	3%	25
Hexyl acrylate	2499958	n.d.		8.7E-02	1%	15
Isobutyl acrylate	106638	1.6E-07	101%	2.0E-02	9%	28
2-Ethoxyethyl methacrylate	2370630	n.d.		3.8E-02	0.1%	33
Allyl methacrylate	96059	7.9E-07	62%	5.9E-02	9%	7
Benzyl methacrylate	2495376	n.d.		1.1E-01	9%	12
Isobutyl methacrylate	97869	4.3E-07	9%	7.1E-03	15%	17
Isopropyl methacrylate	4655349	9.2E-07	16%	7.7E-03	75%	8
Methyl methacrylate	80626	9.0E-07	30%	2.6E-02	28%	8
Tetrahydrofurfuryl methacrylate	2455245	3.9E-07	4%	4.4E-02	1%	12

[a] n.d.: No significant decrease measured.

All QSPR modeling and statistical analysis were performed with the chemometrics package SCAN (Minitab, State College, PA, USA). A partial least square (PLS) model was favored over a multiple regression (MLR) model because descriptor variables were correlated. The cross validated r^2 (Q^2) was calculated with a leave one out procedure.

RESULTS AND DISCUSSION

QSPR for hydrolysis

The measured neutral (k_N) and base-catalyzed (k_B) hydrolysis rate constants of the training set are presented in Table 1 along with their predicted hydrolysis half-lives in the GSH-assay reaction buffer at a pH of 8.8. For all but one acrylate (isobutyl acrylate), the neutral hydrolysis rate was below the detection limit of our assay. Methacrylates generally have a higher neutral hydrolysis rate but the differences between them is small. We found this data set too small to establish a QSPR for the reactivity of the training set compounds towards H_2O.

The variability in the base-catalyzed hydrolysis was more pronounced and reaction rates were measured for all compounds in the training set. Based on the work of Taft (*28*), we tried to establish a linear free energy relationship (LFER). The acrylate data set was too small for this purpose. For six of the seven methacrylates, literature values of the Taft parameters σ* and E(s) for the leaving alcohols were found (*29-31*) and are given in Table 2. With these parameters, a significant correlation could be established:

Table 2: Base-catalyzed reaction rates relative to methyl methacrylate along with Taft parameters for the alcohol moieties of the methacrylates in the training set. For tetrahydrofurfuryl no constants were found in the literature.

Chemicals	σ*[a]	E(s)	log (k_B/k_B-methyl)
Isobutyl methacrylate	-0.19	-0.93	-0.565
Benzyl methacrylate	0.75	-1.62	0.616
Allyl methacrylate	0.23	-1.60	0.350
Methyl methacrylate	0.00	0.00	0.000
Isopropyl methacrylate	-0.19	-1.71	-0.534
2-Ethoxyethyl methacrylate	0.27	-2.21	0.161

[a] Values for σ* and E(s) for the alcohol moiety from (29) and (30).

$$\log \frac{k_B}{k_B^{methyl}} = 1.31(\pm 0.28)\sigma* + 0.08(\pm 0.13)E(s) - 0.08(\pm 0.19)$$
$$r^2 = 0.884, \quad F = 11.4, \quad n = 6 \qquad \text{(EQ 5)}$$

Equation 5 shows, that the electronic effects of the substituents, expressed as σ*, are comparable with the effects found for other esters like e.g. phenyl acetic acid ester (*24*). Steric properties, expressed by E(s) seem to have less influence on k_B. An equation with only σ* indeed has the same statistical quality (equation 6), showing that the influence of E(s) on the base-catalyzed hydrolysis of methacrylates is negligible.

$$\log \frac{k_B}{k_B^{methyl}} = 1.25(\pm 0.25)\sigma* - 0.18(\pm 0.09)$$
$$r^2 = 0.871, \quad F = 26.9, \quad n = 6 \qquad \text{(EQ 6)}$$

The estimated hydrolysis rate at pH 8.8 (Table 1) shows, that for all but one compound, hydrolysis does not interfere with the GSH reactivity assay. Half-lives between 4 and 30 days were predicted. Only diethyl fumarate, a diester, had a much higher base-catalyzed hydrolysis rate and consequently a low half life at pH 8.8. It was therefore excluded from the GSH reactivity assay.

QSPR for reactivity with reduced glutathione

A clear separation in reaction rates towards GSH could be seen between the readily reacting acrylates and the slowly reacting methacrylates (Table 3). Our aim was to derive one QSPR that could describe the reactivity of both groups. Calculated partial charges on the attacked electrophilic carbon as well as Hammett constants have been used successfully by VanderAar et al. (*17*) to describe the chemical reactivity of a series of 2-substituted 4-nitrobenzenes with GSH. Frontier orbital energies (ε_{LUMO}) have been used recently by Soffers et al. (*16*) to describe the reaction rate of fluorinated nitrobenzenes with glutathione. The rather small variability in reaction rate within the methacrylates, respectively acrylates, in our training set indicates that the electronic effect of the alcohol moiety is less important than the effect of the substitution pattern on the α -carbon. The unsaturated β-carbon atom in the acid group is the most probable site of attack in the Michael addition. Therefore, we used local descriptors of this part of the molecule to establish a QSPR for the reactivity with GS^-. Based on frontier orbital theory (*21,32*), an equation containing two terms can be used to explain differences in reactivity of electrophiles towards a nucleophile: 1) a coulombic attraction/repulsion term and 2) the overlap of the frontier orbitals, the highest occupied, HOMO of the nucleophile and the lowest unoccupied, LUMO of the electrophile. This framework led us to choose the following quantum chemical descriptors for a QSPR for the reac-

Table 3: Measured reaction rate constants with GSH at a pH of 8.8 at 20 °C as well as quantum chemical descriptors of the training set that were used in the PLS model.

Training set	k_{GSH} ($M^{-1}min^{-1}$)	(% SE)	Charge density (au) C_β	C_α	C_1	e_{LUMO} (eV)	log k_{GSH} meas.	pred.
Ethyl acrylate	39.7	4.7%	-0.21	-0.51	1.06	0.0981	1.60	1.37
2-Hydroxyethyl acrylate	50.9	4.4%	-0.21	-0.50	1.04	0.0935	1.71	1.51
Hydroxypropyl acrylate	42.1	13.6%	-0.19	-0.53	1.08	0.0880	1.47	2.01
Isobutyl acrylate	29.3	11.4%	-0.19	-0.49	1.04	0.0975	1.62	1.35
Hexyl acrylate	20.3	8.5%	-0.22	-0.48	1.03	0.0988	1.31	1.16
Isobutyl methacrylate	0.19	18.9%	-0.40	-0.09	0.91	0.1042	-0.73	-0.52
Isopropyl methacrylate[a]	n.d.[b]		-0.45	-0.03	0.88	0.1056	-1.00	-0.91
Benzyl methacrylate	0.33	14.7%	-0.40	-0.09	0.88	0.1040	-0.49	-0.61
Methyl methacrylate	0.20	17.8%	-0.37	-0.15	0.97	0.1045	-0.70	-0.17
Allyl methacrylate	0.51	42.9%	-0.44	-0.07	0.94	0.1030	-0.29	-0.49
Tetrahydrofurfuryl methacrylate	0.30	8.7%	-0.43	-0.05	0.84	0.1014	-0.52	-0.76
2-Ethoxyethyl methacrylate	0.25	15.8%	-0.43	-0.07	0.92	0.1036	-0.60	-0.57

[a] For the PLS-model, the log k_{GSH} of isobutyl methacrylate was set to -1.00.
[b] n.d.: No significant decrease measured.

tion rate with GS^- (log k_{GSH}). For the first term we used the charge densities ($q(C_i)$) on the three carbon atoms in the acid part of the esters (C_β, C_α and C_1 in Figure 1). For the second term, we used the energy of ε_{LUMO} of the electrophilic acrylates and methacrylates as single descriptor for orbital overlap in terms of energy. According to Fleming (*21*), a lower ε_{LUMO} of the electrophiles correlates with a smaller difference between the energy of the two interacting molecular orbitals which in turn yields more energy for bond formation.

These four parameters, $q(C_\beta)$, $q(C_\alpha)$, $q(C_1)$ and ε_{LUMO} were calculated within two quantum chemical formalisms; semi empirical (AM1) and ab initio (3-21G(*)). Correlation matrices for resulting parameters, calculated with the two different approaches, as well as towards log k_{GSH}, are given in Tables 4.a and 4.b. Generally, the ab initio results correlated better with log k_{GSH}. The two algorithms yielded very similar energies for the ε_{LUMO}s of the training set. For the charge densities, however, the ab initio algorithm revealed a pronounced difference between acrylates and methacrylates whereas the semi empirical algorithm (data not shown) did not. Therefore, and because we expected the ab initio calculations to be more precise than semi empirical ones, ab initio results were used in the QSPR. High reactivity of the acrylates correlated with less negative charge density on C_β, more negative charge density on C_α and a high positive charge density on C_1. These correlations indicate the importance of the coulombic interaction of the thiol anion with the carbon in β-position. As predicted by the theory, a lower ε_{LUMO} correlates with higher reactivity towards GS^-. The

Table 4. Correlation matrices between the quantum chemical descriptors calculated with two different formalisms, ab initio 3-21G(*) and semi-empirical AM1) and with the reaction rate with GSH, log k_{GSH}.

	$q(C_\beta)$	$q(C_\alpha)$	$q(C_1)$	e_{LUMO}
Ab initio 3-21G(*)				
$q(C_\alpha)$	-0.995			
$q(C_1)$	0.930	-0.943		
e_{LUMO}	-0.835	0.847	-0.791	
log k_{GSH}	0.968	-0.98	0.902	-0.848
Semi-empirical AM1				
$q(C_\alpha)$	-0.944			
$q(C_1)$	0.500	-0.517		
e_{LUMO}	-0.667	0.669	-0.418	
log k_{GSH}	0.853	-0.939	0.384	-0.682

full descriptor set, calculated with the ab initio algorithm is given in Table 3. All four descriptor variables correlate with each other, so the use of a multiple linear regression model (MLR) might give misleading results (*33*). Hence, we used a PLS model to derive a relation between these four descriptors and the reaction rate. In a PLS model, the descriptor variables are transformed to orthogonal (non-correlating) latent variables (*34*). For isopropyl methacrylate, with a reaction rate below the detection limit of our assay, we used a k_{GSH} of 0.10 [$M^{-1}min^{-1}$] for the PLS modeling.

PLS model with 1 latent variable (EQ 7)

X-variables: $q(C_\beta)$, $q(C_\alpha)$, $q(C_1)$ and ε_{LUMO}

Y-variable: log k_{GSH}

Descriptor	Regression coeff.	Relative importances
$q(C_\beta)$:	2.65	0.264
$q(C_\alpha)$:	-1.37	-0.267
$q(C_1)$:	3.39	0.246
ε_{LUMO}:	-49.33	-0.231

r^2: 0.932 Q^2: 0.872 n=12

The resulting predictions for log k_{GSH} are given in Table 3. The selected PLS model (equation 7) contains only one latent variable and all four descriptor importances are almost equal. The proposed site of attack of a thiol anion is the β-carbon but, as pointed out by Fleming (*21*), the molecular orbitals in an allyl system are strongly influenced by electron

withdrawing or donating substituents on C_1, so it is not surprising that all three carbon atoms in the allyl system are included in the PLS model.

Validation of the QSPR with a test set of literature data

Although GSH-reactivity of acrylic compounds has been measured by several researchers (*19,20,35*), we only found one data set containing different acrylates and methacrylates. We used this data, reported by McCarthy et al. (*20*), to test the validity of the QSPR presented above (equation 7). Two compounds of this test set, ethyl acrylate and methyl methacrylate were also present in the training set. The quantum chemical descriptors for all six test set chemicals were calculated as described above using the ab initio formalism and are presented in Table 5. The reaction rates of this literature test set were given at 37°C in an aqueous buffer solution of pH 7., whereas we used a temperature of 20 °C and a pH of 8.8. The two chemicals, which are both in the training and the test set, allowed us to compare the influence of the different reaction conditions. Their reaction rates for both conditions, given in Table 3 and 5, show that the effect of a lower pH (lower concentration of GS^-) is partially compensated by a higher temperature. The reported reaction rates of the test set were therefore used without correction, although it is clear that model predictions for this test set could not be as accurate as for the training set. Table 5 shows the predicted reaction rates of the test set calculated with the PLS-model (equation 7). It can be concluded, that the model is able to predict the large difference in GSH-reactivity between methacrylates and acrylates for the four compounds not included in the training set.

Quantum chemical ground state parameters, like the ones used in equation 7 generally give information about the first step of a reaction. For the Michael addition, this step would be the approach of a thiol anion towards the electrophilic carbon, C_β. Due to the good correlation of our PLS model, we think that this is the rate limiting step. This conclusion is strength-

Table 5: Quantum chemical descriptors that were used to predict the reactivity of the test set towards GSH.

Test set	CAS nr.	Charge density (au) C_β	C_α	C_1	e_{LUMO} (eV)	log k_{GSH} ($M^{-1}min^{-1}$) literature[a]	predicted
Methyl acrylate	96333	-0.15	-0.57	1.14	0.1040	1.72	1.56
Ethyl acrylate	140885	-0.21	-0.51	1.06	0.0981	1.42	1.37
Butyl acrylate	141322	-0.21	-0.51	1.15	0.1053	1.59	1.31
Methyl methacrylate	80626	-0.37	-0.15	0.97	0.1045	-0.48	-0.17
Ethyl methacrylate	97632	-0.36	-0.14	0.92	0.1066	-0.85	-0.46
Butyl methacrylate	97881	-0.36	-0.16	0.98	0.1073	0.00	-0.23

[a] Values taken from McCarthy et al. (*20*).

ened by the findings of Osman (*36*) who evaluated the relative reactivity of acrylic and methacrylic acid towards the fluoride anion with ab initio calculations. He found that differences in charge distribution of the ground state structures can explain the difference in reactivity.

GSH-reactivity and toxicity

Because chemical reactivity is often mentioned as a cause of toxicity (*1-6*) we compared the measured reaction rates with reported LC_{50} values for four day acute fish toxicity. For this comparison we used two acrylates and two methacrylates which were very similar regarding hydrophobicity and alcoholic moiety. Log K_{OW} and acute LC_{50} values were taken from Karabunarliev et al. (*5*). Predicted baseline toxicity and toxic ratios (TR) were calculated according to Russom et al. (*3*). This data for the four compounds is given in Table 6. Based on an expert classification system to predict modes of action from chemical structure (*3*), acrylates and methacrylates are supposed to act by different modes of action. Our experimental results show that the low Michael-type acceptor potency of the methacrylates corresponds with their low TR values which in turn suggest that baseline toxicity (also referred to as narcosis 1) is the predominant mode of action. For the acrylates, which are good Michael-type acceptors, the high TR reveals that they are much more toxic than baseline toxicity would imply. We conclude that reaction rates with glutathione can be used to discriminate between different modes of action.

The comparison was made with four compounds which have simple aliphatic alcohol groups. It should be noted, however, that the alcohol moiety of the ester may be of toxicological importance. This is shown for allyl methacrylate, for which the relevant data is included in Table 6. This compound has a low k_{GSH} value, but is much more toxic than predicted by the baseline QSAR (*3,5*). The hydrolysis product of allyl methacrylate is allyl alcohol, which is known to be a potent hepatotoxine in mammals and fish (*37,38*).

CONCLUSIONS

Two approaches have been used to describe the reactivity of acrylates and methacrylates towards different nucleophiles. For the base-catalyzed hydrolysis of the methacrylates, a linear free energy relationship could be established based on Taft's substituent constants for the leaving alcohols. Two problems were identified for this approach: First, a substituent constant based QSPR is only valid within structure analogues, so that an equation for methacrylates is not a priori valid for acrylates. Secondly, although many substituent val-

Table 6: Comparison of acute fish toxicity data for two methacrylates (methyl, isopropyl) and two acrylates (ethyl, isobutyl) with similar hydrophobicity and similar aliphatic alcohol moieties. Allyl methacrylate is included to show the possible impact of a toxic alcohol moiety.

Chemicals	Log K_{OW} [a]	LC_{50} [a] (µM)	Baseline LC_{50} [b] (µM)	TR [c]	log k_{GSH} ($M^{-1}min^{-1}$)
Methyl methacrylate	1.38	2588	2841	1.1	0.20
Isopropyl methacrylate	2.25	296	436	1.5	n.d. [d]
Ethyl acrylate	1.32	25	3234	129	39.7
Isobutyl acrylate	2.22	16	465	28	29.3
Allyl methacrylate	1.57	8	1885	240	0.51

[a] Log K_{OW} and experimental 4-day LC_{50} data for fathead minnow taken from Karabunarliev et al.(*5*).
[b] Predicted 4-day LC_{50}, calculated with a QSAR for baseline toxicity for fathead minnow (*3*).
[c] Toxic ratio: Ratio between predicted and observed LC_{50}.
[d] n.d.: No significant reaction could be measured.

ues have been reported in the literature, these compilations are not complete which hampers the general use of the fragment based approach.

For the reactivity with glutathione, quantum chemical descriptors were calculated from the 3-D structure of the compounds. For a reaction like Michael addition to unsaturated carboxylates, substituent values of the alcohol moiety are less useful, because the site of addition is far away from the substituent and there is no large delocalised Π-system, like e.g. in benzene rings, that could communicate electronic effects. The presented data shows that ab initio calculations of the electronic structure in the ground state can produce descriptors, which are able to explain the observed reaction rates. QSPRs based on these parameters can then be used to predict the reactivity of closely related chemicals. Recent investigations in toxicity of ethyl acrylate and other acrylic acid esters in rodents (*39,40*) and in fish show that the reactions described above are relevant for the toxicity. Measuring and understanding the reactivity of organic electrophilic compounds will help to identify their predominant modes of toxic action.

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COMPARING THE POTENCY OF CHEMICALS WITH MULTIPLE MODES OF ACTION IN AQUATIC TOXICOLOGY: ACUTE TOXICITY DUE TO NARCOSIS VERSUS REACTIVE TOXICITY OF ACRYLIC COMPOUNDS

CHAPTER **3**

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ABSTRACT

A series of acrylates and methacrylates were used to illustrate a strategy to compare the importance of two modes of action (MOA) and thereby identify the predominant cause of acute fish toxicity. Acrylic compounds are known to be Michael acceptors and may therefore react with glutathione (GSH) causing GSH-depletion in vivo (reactive mechanism). On the other hand, acrylates may also act by a non-specific mechanism (narcosis). The following two, physiologically meaningful parameters were calculated in order to estimate the contribution of these two mechanisms to the overall acute toxicity: (i) a lipid normalized body burden for narcosis and (ii) the potential degree of GSH depletion by chemical reactivity. The degree of GSH depletion was found to be related to the product of the reactivity towards GSH and the exposure concentration. This model was validated with four model compounds and an in vivo study. For both MOA, toxic ratios were calculated and compared for all chemicals in the series. The approach enables the comparison of the contribution to toxicity of chemicals with more than one mode of action.

INTRODUCTION

Identification of the toxic mode of action (MOA) of chemicals is essential for a correct risk assessment. The chemical structure is thereby used as a starting point in many expert systems and computer based risk assessment programs. Compounds with reactive chemical structures are of special interest because they are often found to be very toxic *(1-3)*. For a number of electrophilic and proelectrophilic structures Lipnick *(3)* proposed molecular mechanisms with biological targets to explain their excess toxicity. A more general classification, suited for computer based evaluation of large databases was given by Verhaar et al.*(4, 5)*. In this system, the presence or absence of certain reactive substructures is used to classify chemicals in four distinct classes. These classes reflect two different MOA, namely narcosis and polar narcosis and two groups including electrophilic chemicals and chemicals with a receptor mediated mechanism such as pesticides. Often, toxic modes of action are only well defined for a few compounds and subsequently, additional chemicals are associated to these MOA based on chemical similarity. If physiological or mechanistic information is available for the toxic action, a more elaborate classification may be possible. Wenzel et al. *(6)* proposed the use of a battery of specific in-vitro tests to identify the correct MOA of a compound. Russom et al. *(7)* presented a computer assisted classification system that is based on work of McKim, Bradbury and coworkers *(8, 9)* Fish acute toxicity syndromes (FATS) of model compounds in rainbow trouts form the bases of that classification scheme. This approach is able to separate eight different MOA: Narcosis 1 and narcosis 2, ester narcosis, inhibition of oxidative phosphorylation, respiratory inhibition, AChE inhibition, central nervous system seizures and an electrophilic reactivity mechanism. Chemicals are assigned to a specific MOA based on observed FATS, behavioural syndromes, joint toxic action, LC_{50} ratios, comparisons to baseline toxicity or on structural similarities. From their work, as well as from the in vitro work of Wenzel, it can be seen that sometimes more than one MOA is plausible for a structure and a classification may become ambiguous *(7)*. A classification system should therefore include a way to weight and compare the potency of a chemical for different MOA.

In the present work, we use a series of Michael acceptors, mainly acrylates and methacrylates, to illustrate a strategy to compare the importance of two MOA and thereby identify the predominant cause of acute fish toxicity for this chemical class. The two modes in question are narcosis or baseline toxicity on one hand and an electrophilic reactivity mechanism on the other. This second MOA is based on FATS data for acrolein, a very potent Michael acceptor *(10)*, as well as on the PBPK model studies of ethyl acrylate in rats by Ghanayem

(11, 12) and Frederick *(13)*. One of the major differences between narcosis and toxicity of reactive chemicals is the fact that the interaction of reactive (alkylating) chemicals with the target is irreversible, while narcosis is due to a reversible interaction. In an irreversible interaction, it is not just the concentration at the target site (relevant for narcosis), but the amount of target that is occupied or depleted, which is the relevant parameter. In order to emphasize these differences, Legierse et al. *(14)* and Verhaar et al. *(5)* have introduced the term Critical Target Occupation (CTO) for toxicity due to an irreversible interaction as an alternative approach for the Critical Body Residue (CBR) approach that is used for narcosis. Using a CTO model, they analyzed the time dependance of LC_{50} data for reactive compounds. We assumed that GSH-depletion could serve as an excellent monitor of the interaction between Michael acceptors and critical target sites in the cell.

For both modes of action a physiologically meaningful effect parameter is defined. This parameter is used in a simple model to predict the potency of both mechanisms for every chemical in the test series. This approach should be seen as an intermediate stage between simple correlations of physico-chemical properties with toxicity on one side and elaborated PBPK models on the other. The models used here include specific, physiological information of the target site but exclude kinetic processes and organ specificity. A comparison of the model properties is given in table 1.

Table 1: Comparison of two MOAs which are expected to be of importance for the acute toxicity of acrylic and methacrylic acid esters in fish along with a model for their respective potency.

Mode of action	Narcosis	Electrophilic reactivity by Michael acceptors
Target site	Cell membrane	GSH-pool
Toxicodynamics	Bioaccumulation in membrane	Covalent chemical reaction
Physico-chemical descriptor	K_{OW} [a]	k_{GSH} [b]
Physiological parameter	Body residue (BR)	Depl. rate const. for GSH (DR_{GSH})
Model used for prediction [c]	$BR=0.05K_{OW}LC_{50}$	$DR_{GSH}=k_{GSH}LC_{50}$
Critical value causing lethaliy	CBR: 2 $mmolkg^{-1}$ [d]	CDR_{GSH}: 1.8 d^{-1} [e]
Toxic ratio (TR)	$TR_{Narcosis}=BR/CBR$	$TR_{Reactivity}= DR_{GSH}/CDR_{GSH}$

[a] Octanol/water partition coefficient.
[b] Pseudo 2nd order reaction rate with glutathione.
[c] LC_{50}: Reported fish LC_{50}.
[d] Critical body residue taken from McCarty et al. *(15)*.
[e] Critical depletion rate constant, this study.

THEORY

Narcosis

For narcosis, such a model is already available *(15)* in form of the critical body residue (CBR) model . According to this model, the effects of a chemical can be related to a constant, structure independent critical or lethal body burden or critical body residue (CBR). The physiological meaning of this model is, that a constant concentration of xenobiotics in cell membranes will disable the membrane function *(16)*, causing narcotic syndromes and eventually death.

For lipid based-whole body burden the critical body residue for acute toxicity due to narcosis for fathead minnow is reported to be 2-5 mM *(15, 17)*:

The critical body residue can be estimated from a measured LC_{50} and the bioconcentration factor (BCF) as follows:

$$CBR = LC_{50}BCF \qquad \text{(EQ 1)}$$

For neutral organic chemicals, BCF can be estimated from the octanol-water partition coefficient (K_{OW}):

$$BCF = 0.05K_{OW} \qquad \text{(EQ 2)}$$

$$CBR = 0.05LC_{50}K_{OW} \qquad \text{(EQ 3)}$$

Electrophilic reactivity by Michael acceptors

For Michael acceptors, we propose a MOA which is based on the effects of covalent reaction with a biological target and its subsequent depletion. GSH is the main non-protein thiol in most animal cells and has a number of vital functions such as conjugation and transport of harmful endogenous and exogenous electrophiles, protecting membranes by scavenging of endogenous radicals and helping to maintain the redox-state of the cell *(18, 19)*. The thiol of the cysteine moiety reacts in a Michael addition with a,b-unsaturated compounds like acrylates and methacrylates to form a stable S-conjugate *(20, 21)*. Depletion of the GSH-pool will impair the self-protection of the affected cells. This has been shown clearly for ethyl acrylate in a study with rodents by Frederick et al. *(13)*. The GSH-depletion, which was observed in the stomach after oral dosing of ethyl acrylate, was correlated with tissue necrosis and neoplasm formation. A physiologically based pharmacokinetic model (PBPK), which included GSH-concentrations, showed that the area under the curve for GSH depletion exceeding 50% was a good dose surrogate for the toxicity of ethyl acrylate. The relation

between severe GSH-depletion and subsequent toxicity has also been shown among others by Comporti et al. *(22)*. These findings suggest that chemical reactivity leading to GSH-depletion might be the cause of the high acute toxicity of acrylates and methacrylates observed by Russom et al. *(23)* in fish toxicity test. PBPK models for the description of GSH depletion in rodents are given among others by Frederick et al. *(13)* and by D'Souza et al. *(24)*. In order to find a suitable descriptor for this MOA, we tried to translate these complex PBPK models to a simple model for the relation between aqueous exposure concentration and GSH depletion in a nonspecific fish tissue.

The model for the depletion of GSH is based on the following assumptions:

(1) The internal concentration, C_{int} of the Michael acceptor is constant. Lien et al. *(25)* have shown that tetrachorethane (log Kow=2.6) reaches a steady state concentration in the tissue of fathead minnow within 20 minutes. In their PBPK model, the time to reach steady state depends mainly on the hydrophobicity of the compound. In our test set, all but one compound (hexyl acrylate) are equal or less hydrophobic than tetrachloroethane. Therefore, we assume a constant internal concentration in the tissue of fathead minnow during the 4 day LC_{50} tests.

(2) The internal concentration, C_{int} is equal to the external exposure concentration C_{AQ}. Glutathione, the proposed initial target of Michael acceptors, is an aqueous soluble tripeptide. In modeling the covalent reaction of GSH with a Michael acceptor we use aqueous concentrations and not tissue concentrations of the chemical, because we assume the reaction to take place in the cytosol. Nichols et al. *(26)* suggest that, at steady state, the cytosolic concentration is equal to the external exposure concentration. McKim et al. *(27)* showed that phenol, a readily metabolized compound in rainbow trout, reached steady state concentrations in blood plasma equal to the external exposure concentrations within four hours. This assumption however, may not hold for tissues with a very high clearance of the chemical (e.g. liver or kidneys).

(3) Steady state glutathione in the cell can be modeled by a zero order synthesis and a first order endogenous consumption. Such a model was proposed by D'Souza et al. *(24)* to describe the effect of ethylene dichloride on the GSH pool in rodent tissue.

(4) The reaction of the Michael acceptor with GSH is dominated by chemical reactivity. Enzymatic conjugation of GSH with ethyl acrylate was found to be negligible compared to non-enzymatic reaction rates *(13)*.

This model describes the interaction of a reactive chemical with a biological target (GSH) with a high endogenous turnover. In case of exposure, the target concentration will be a

balance between syntheses, consumption and conjugation and such a balance can be written as a differential equation:

$$\frac{dC_{GSH}}{dt} = S^0 - k_E C_{GSH} - k_{GSH} C_{\text{int}} C_{GSH} \quad \text{(EQ 4)}$$

where: C_{GSH}: GSH concentration in cytosol.

k_{GSH}: 2nd order reaction rate constant of Michael acceptor with GSH.

C_{int}: Concentration of Michael acceptor in the cytosol.

S^0: Zero order GSH-synthesis rate.

k_E: 1st order endogenous consumption rate constant.

Equation 4 can be rearranged to:

$$\frac{dC_{GSH}}{dt} = -C_{GSH}\left(k_E + k_{GSH} C_{\text{int}}\right) + S^0 \quad \text{(EQ 5)}$$

The exact solution of this differential equation is *(28)*:

$$C_{GSH}(t) = \frac{S^0}{\left(k_E + k_{GSH} C_{\text{int}}\right)} + \left(C_{GSH}(0) - \frac{S^0}{\left(k_E + k_{GSH} C_{\text{int}}\right)}\right) e^{-\left(k_E + k_{GSH} C_{\text{int}}\right)t} \quad \text{(EQ 6)}$$

Under a constant external exposure, the GSH concentration will reach a new steady state, $C_{GSH}(\infty)$:

$$C_{GSH}(\infty) = \frac{S^0}{\left(k_E + k_{GSH} C_{\text{int}}\right)} \quad \text{(EQ 7)}$$

As mentioned by Comporti *(22)* and Frederick *(13)*, the cell will start to accumulate damage if the glutathione concentration falls below a critical level. Given enough time, this damage will sum up to a concentration which is lethal for the cell. From the point of view of GSH, it doesn't matter which chemical is responsible for the depletion. We can assume a constant, critical equilibrium concentration for GSH below which lethal damage can be expected within the given time frame of four days testing. Our model is focused on the acute exposure where only cytotoxic effects will be of importance. Carcinogenic effects will not manifest in the time span of a four-day toxicity test. The model shows that, for a given lethal steady state GSH-level, the product of k_{GSH} and C_{int} has to be constant (equation 7). Under the given assumptions (see above) C_{int} will be equal to the external aqueous concentration. A critical depletion rate constant (CDR) can be defined according to equation 8.

$$CDR_{GSH} = k_{GSH} LC_{50} \quad \text{(EQ 8)}$$

We tested the hypothesis of a constant GSH-depletion rate constant, k_{GSH}*LC_{50} by comparing data of four Michael acceptors (acrylamide, acrylonitrile, ethyl acrylate and acrolein) with a large range in reactivity. Because we do not have data on endogenous synthesis

or consumption of GSH in fish tissue, we can not a priori predict the lethal GSH-level from the model. An acute exposure experiment was therefore conducted to get insight in the degree and the time dependance of GSH depletion at a near lethal exposure concentration. In order to study individual organs, we used small rainbow trouts instead of fathead minnow.

EXPERIMENTAL SECTION

Chemicals

Ethyl acrylate, acrolein, acrylonitrile, acrylamide, diethyl fumarate and Na_3tetraborate-4-hydrate (Fluka, Bornem, the Netherlands) and reduced glutathione (Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) were all used as received. All solutions were prepared with water purified by a Millipore Milli-Q system.

Animals

Four month old rainbow trout from our own hatchery with a mean weight of 2.0±0.9 g were used. The animals were held in copper free tab-water at 11°C, with a light-dark cycle of 12 hours.

METHODS

Reaction rates with GSH

For acrylamide, acrolein and acrylonitrile, second order reaction rates with glutathione were measured by following the decrease of GSH with the electrophile in excess at 20 °C and at pH of 8.8 (tetraborate buffer, 0.4 mM). Solutions containing acrylamide, acrylonitrile or diethyl fumarate were sampled every 10 minutes during 50 minutes and solutions with acrolein every 30 seconds during 3 minutes. The samples were immediately diluted 1:10 with HPLC-eluens (pH 3.0) to stop the reaction and they were subsequently analyzed for decrease of GSH concentration on a HPLC (Separations, H.I.Ambacht, the Netherlands) equipped with a C-18 column (200x3mm, 5µM particle size, Chrompack, Bergen op Zoom, the Netherlands) and a UV-detector. The column was eluted isocratically with 10% methanol/90% phosphate buffer (5 mM, pH 3.0) at 0.4 ml/min. GSH was quantified with standards using UV absorption at 205 nm. Reaction rates were measured in threefold. Reaction rates for five acrylates and seven methacrylates with GSH, measured under the same conditions in our laboratory were taken from Freidig et al (*30*).

Table 2: Michael-acceptors that were used to establish the critical GSH-depletion rate constant, CDR_{GSH}.

SUBSTANCES	log k_{GSH} [$M^{-1}min^{-1}$]	log K_{OW} [a]	-log (LC_{50}) [b] [M]	BR [c] [mM]	CDR_{GSH} [d] [d^{-1}]
Acrylamide	-0.33	-0.67	2.81	0.016	1.038
Acrylonitrile	0.87	0.23	3.57	0.023	2.837
Acrolein	3.92	0.10	6.74	0.000	2.131
Ethyl acrylate	1.60 [e]	1.32	4.60	0.026	1.427

[a] StarList measured octanol/water partitioning coefficients, taken from MedChem *(49)*.
[b] Four day LC_{50} data for fathead minnow from *(23, 31, 32)*.
[c] Body residue at an external aqueous concentration equal to the LC_{50}, calculated with equation 3.
[d] Critical depletion rate constant, calculated with equation 8.
[e] 2nd order rate constant, taken from *(29)*.

In vivo exposure to ethyl acrylate

20 four month old rainbow trouts were exposed to a near-lethal concentration of ethyl acrylate in a 8 l aquarium under steady state conditions. Samples of three fish and water samples in duplo were taken after 1, 2, 4, 6 and 24 hours. From a control aquarium three fish were sampled at the beginning of the experiment and three after 24 hours. Ethyl acrylate concentrations of the water samples were measured on a HPLC system (Varian, Houten, the Netherlands) with a C-18 column (100x3 mm) which was eluted under isocratic conditions (40% methanol/60% water, 0.4 ml/min). UV absorption at 215 nm was used to quantify ethyl acrylate using standard solutions in destilled water. To measure the GSH concentration in different tissues, fish were killed with a blow to the head. Then, the liver, the gills and a part of the dorsal muscle were removed. The tissues (30 -150 mg) were homogenized with a Potter-Homogenizer in a 10% Trichloroacetic acid (TCA) solution on ice and centrifuged for 2 min at 13,000 g. The supernatant was diluted 1:10 with HPLC-eluens. To reduce matrix interferences, reduced GSH was separated on a HPLC equipped with a C-18 RP-column (200x3 mm) and analyzed using post-column derivatisation with o-phthalaldehyde and fluorimetric detection (λ_{ex}:340 nm, λ_{em}:420 nm) as described by Freidig et al. *(29)*. Calibration solutions of GSH in water with 1% TCA were used to quantify the GSH from the tissue samples. Limits of detection for GSH were calculated as the blank value plus three times the standard deviation of the blank.

Toxicity data

Four day LC_{50} data for fathead minnow were collected using the AQUIRE database *(23, 30-32)*, except for isobutyl methacrylate, where a 2-day LC_{50} value for golden orfe, reported by Greim et al *(33)* was used.

RESULTS AND DISCUSSION

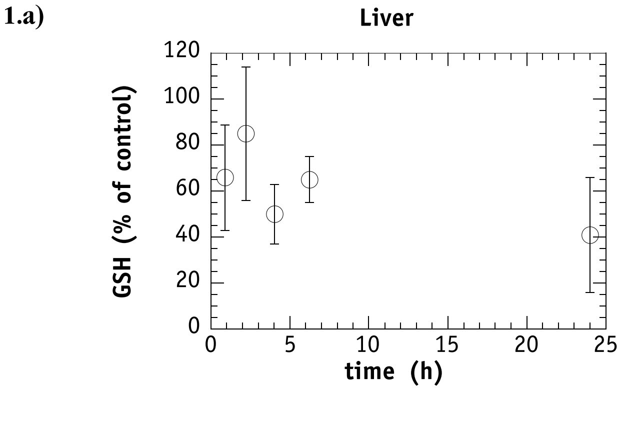
Critical depletion rate constant (CDR) for four Michael acceptors

In table 2, measured second order reaction rates with GSH are given for acrylamide, acrolein and acrylonitril along with a literature value for ethyl acrylate. The table includes acute fish toxicity data for fathead minnow, log K_{ow} values, body residues (BR) and critical depletion rate constants (CDR_{GSH}) which were calculated using equation 3 and 8, respectively. Although the 2nd order reaction rate constants of the four compounds with GSH span 4 orders of magnitude, their CDR_{GSH} is almost equal. The four model Michael acceptors have an average CDR_{GSH} of 1.8 ±0.8 [d^{-1}]. Regarding acute fish toxicity, they seem to act by the same mechanism of action which can be described as critical GSH depletion. Although GSH-depletion may be the predominant MOA for more classes of electrophiles, the validity of the derived CDR_{GSH} is probably limited to Michael acceptors due to the different assumptions in the model.

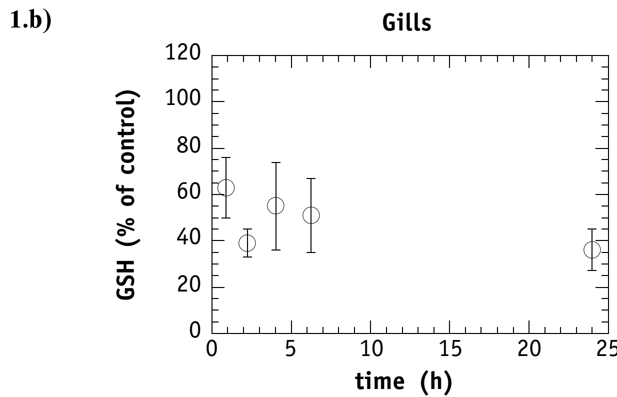
In vivo measurement of GSH depletion caused by ethyl acrylate

If the causal effect of the toxicity of ethyl acrylate is linked to the depletion of glutathione, as we propose by the CDR-model, then a significant decrease of GSH would be expected in fish exposed to a near lethal level of this chemical. To test this assumption, rainbow trouts were exposed to half the reported 4-day LC_{50} (4.6 mgL^{-1}) for this species *(34)*. During the exposure of one day, the aqueous concentration of ethyl acrylate in the static exposure system dropped from 2.3 to 1.7 mgL^{-1}. Mortalities of exposed fish were recorded fter 6 hours (1 of 11) and 24 hours (4 of 7). Tissue samples were only taken from fish that ere alive after the exposure period. In Figure 1.a-c, the time dependent concentration of SH in three tissues is shown. After 24 hours, all three tissues showed a depletion of GSH-l evel to approximately 40% compared to non-exposed individuals. Average GSH concentrations of the 6 control fish were 1.28±0.41, 2.25±0.33 and 0.45±0.08 $\mu molg^{-1}$ tissue for gill, iver and muscle, respectively with detection limits of 0.02, 0.10 and 0.06 $\mu molg^{-1}$. A comparable GSH concentration in gills of 1.6 $\mu molg^{-1}$ (measured as non-protein thiol) has been eported by Nimmo et al. *(35)*. Gill and liver tissue was significantly depleted after 1 hour lready, whereas muscle tissue showed depletion after 24 hours only. Although we used mall rainbow trouts they were still 10 times bigger than the fathead minnows for which he PBPK-model of Lien et al. *(25)* was developed. Their results, showing a fast steady state n fish tissue for low hydrophobic chemicals, should therefore be used with care in extrapolation to the rainbow trouts used in this experiment. PBPK models of adult rainbow trouts

1.a)



1.b)



1.c)

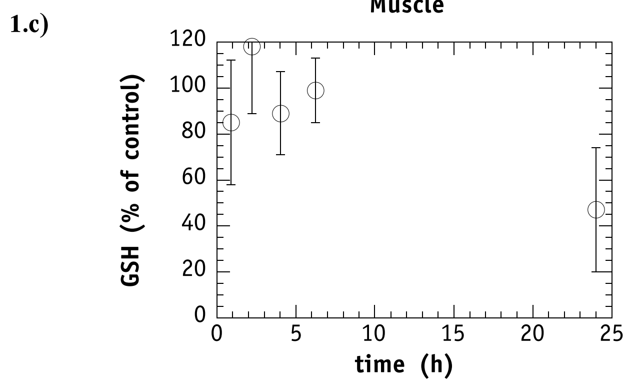


Figure 1. a-c: Time dependent tissue concentrations of glutathione in rainbow trouts exposed to a near lethal concentration of ethyl acrylate.

weight: 1 kg), on the other hand, show that low hydrophobic chemicals do still equilibrate in fast perfused tissue within hours. This can explain, that the GSH depletion in muscle occurs at a later stage than the depletion in richly perfused tissues.

Based on the above presented in vivo experiment, we concluded that ethyl acrylate, at a near lethal concentration, induces severe GSH depletion throughout the body of the fish. The near lethal steady state of GSH was found to be around 40 % of control levels. This level will be close to the hypothesized critical GSH level $C_{GSH}(\infty)$, defined in equation 7 and 8. It

is informative to compare these depletion levels with findings of in vivo experiments with rodents. Casini et al. *(36)* reported a threshold for GSH depletion in liver of 10-20% of control level, below which liver necrosis occurred in mice treated with either bromobenzene or diethyl maleate. Frederick et al. *(13)* used depletion of GSH below 50 % of the control level as a surrogate for the effective dose in forestomach necrosis in rats orally treated with ethyl acrylate.

Based on our experiment, we can not a piori assign a target organ for critical GSH depletion in fish. FATS, which were recorded during lethal acrolein exposure by McKim et al. *(10)* show signs of respiratory stress and loss of ion balance in the plasma. These clinical signs may be indicative for gill damage *(37)*. The early depletion of GSH in gills found in our experiment also suggests, that the gills are the primary target of Michael acceptors.

Comparing the potency due to narcosis and electrophilic reactivity by Michael addition

Calculating the contribution of two modes of action to the acute toxicity

We used a set of acrylic and methacrylic acid esters and diethyl fumarate (table 3) to model and compare their potency of acting by two different MOAs. Methacrylates were shown to be approximately 100 times less reactive with GSH than acrylates and diethyl fumarate *(20, 29, 38)*. For both MOA, a critical level is defined. For narcosis, a critical body residue (CBR) of 2 mmol/kg is used *(15)*. For GSH-depletion by Michael acceptors, a critical depletion rate constant (CDR_{GSH}) of 1.8 d^{-1} is observed (see above). For all chemicals in the set, we predicted the body residue and the depletion rate constant for an aqueous exposure concentration equal to the reported LC_{50} values for fathead minnow. Body residues were calculated using equation 3, whereas depletion rate constants were calculated with equation 8. To compare a chemicals potency in both MOA, we used toxic ratios (TR), as introduced among others by Lipnick et al. *(2, 39)* and Verhaar et al. *(4)* (table 3). TRs were calculated as the ratio of predicted BR to critical BR for the narcotic MOA ($TR_{Narcosis}$) and as the ratio of predicted DR_{GSH} to the critical DR_{GSH} (CDR_{GSH}) for electrophilic reactivity MOA ($TR_{Reactivity}$). These ratios are shown in figure 2 for all tested compounds. Compounds with a TR close to one for a given MOA can be expected to act by that specific MOA, while a TR of less than 0.1 indicates that this MOA is not responsible for the observed lethality.

Strong Michael acceptors: Acrylates and diethyl fumarate

The four model compounds, two hydrophilic acrylates, isobutyl acrylate and diethyl fumarate have a $TR_{Reactivity}$ which is close to one. It is therefore highly probable that they all

Table 3: Data for the set of strong and weak Michael-acceptors that were used to calculate toxic ratios (TR) for two modes of action.

SUBSTANCES	log k_{GSH} [a] $[M^{-1}min^{-1}]$	log K_{OW} [b]	-log (LC_{50}) [c] [M]	BR [d] [mM]	DR_{GSH} [e] $[d^{-1}]$	$TR_{Narcosis}$	$TR_{Reactivty}$
2-Hydroxyethyl acrylate	1.71	-0.21 (m)	4.38	0.001	3.031	0.001	1.630
Hydroxy propyl acrylate	1.47	0.35 (m)	4.59	0.003	1.084	0.001	0.583
Diethyl fumarate	2.05	1.84 (c)	4.58	0.090	4.229	0.045	2.273
Isobutyl acrylate	1.62	2.22 (m)	4.79	0.136	0.992	0.068	0.534
Hexyl acrylate	1.31	3.44 (c)	5.15	0.983	0.209	0.491	0.112
Tetrahydrofurfuryl methacrylate	-0.52	1.30 (c)	3.69	0.203	0.088	0.102	0.048
Methyl-methacrylate	-0.70	1.38 (m)	2.59	3.103	0.743	1.551	0.399
2-Ethoxy ethyl methacrylate	-0.60	1.45 (c)	3.76	0.247	0.063	0.123	0.034
Allyl methacrylate	-0.29	1.68 (c)	5.11	0.019	0.006	0.009	0.003
Isopropyl methacrylate	-1.00	2.25 (m)	3.53	2.636	0.043	1.318	0.023
Isobutyl methacrylate	-0.73	2.66 (m)	3.64	5.236	0.061	2.618	0.033
Benzyl methacrylate	-0.49	2.87 (c)	4.58	0.982	0.012	0.491	0.007

[a] 2nd order rate constant, taken from *(29)*.
[b] StarList measured (m) and calculated (c) octanol/water partitioning coefficients, taken from MedChem *(49)*.
[c] Four day LC_{50} data for fathead minnow, taken from *(23)*, except for isobuthyl methacrylate *(33)*.
[d] Body residue at an external aqueous concentration equal to the LC_{50}, calculated with equation 3.
[e] Depletion rate constant, calculated with equation 8.

share the acute toxic effect of GSH-depletion. Their $TR_{Narcosis}$ does not exceed 0.1. For hexyl acrylate, however, the $TR_{Narcosis}$ is higher (0.5) than $TR_{Reactivity}$ (0.1) which indicates that narcosis is the predominant MOA for this compound. By comparing TRs one can easily understand why, within homologues series of chemicals, the predominant MOA can change with increasing hydrophobicity.

Weak Michael acceptors: Methacrylates

For three tested methacrylates with alkyl alcohol moieties (methyl, isopropyl and isobutyl) and benzyl methacrylate, the $TR_{Narcosis}$ is very close to 1. This suggests that the acute toxicity of these chemicals is caused by narcosis. For methyl methacrylate, however, the $TR_{Reactivity}$ is also quite close to one (0.4), and it can be expected that both MOA contribute to acute toxic effects of this compound. For the three other methacrylates, both TRs do not exceed 0.1. Consequently, neither of the two MOA can be held responsible for the lethal effects of these compounds. For allyl methacrylate, the compound with the lowest TR, we suggest the following alternative MOA. The hydrolysis product of this ester, allyl alcohol, is known to be a hepatotoxine due to its rapid oxidation to acrolein *(40, 41)*. Specific hepato-

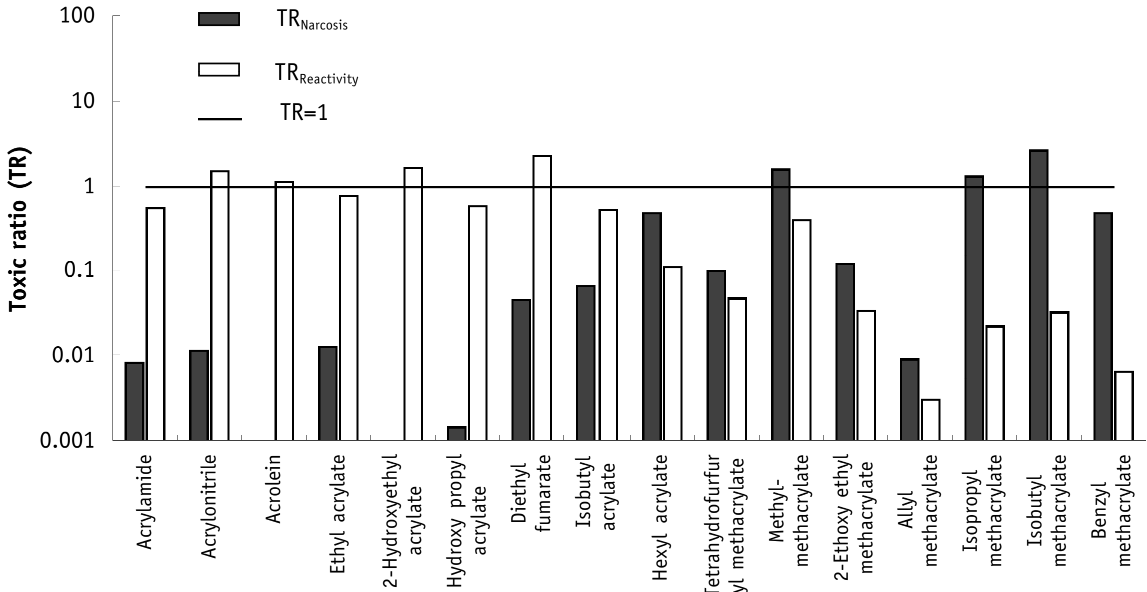


Figure 2: Predicted toxic ratios for the two modes of action at the observed LC_{50}. A mode of action may be causing a lethal effect if its toxic ratio is close to one.

toxicity in rainbow trout was also reported for allyl formate, another allyl ester *(42)*. We suggest that the high toxicity of allyl methacrylate is caused by the hydrolysis of its alcohol moiety which is subsequently metabolized to become the hepatotoxin acrolein. It remains unclear, whether this pathway can also be responsible for the toxicity of the other two methacrylates (2-ethoxy-ethyl- and tetrahydrofurfuryl) which have TR values below 0.1.

Applicability for classification of chemicals and QSAR development

Most chemicals in the test set (9 out of twelve) could be classified to act by either of the two different MOA. Three chemicals did not fit in the classification scheme because their acute toxicity was much higher than predicted by both models (TR<0.1) and consequently neither of the two MOA could be assigned.

The importance of narcosis for hydrophobic electrophiles has been noted earlier e.g. by Roberts *(43)* and Deneer et al. *(44)*. Due to the non-specificity of this MOA, all organic chemicals can be expected to act at least by narcosis unless another MOA is predominant. Often, data sets that are used to model the toxicity of electrophilic chemicals cover a large hydrophobicity range *(44-47)*. The present findings suggest that such data sets could be improved by identifying and excluding chemicals that act by narcosis. In our own data set five out of 12 chemicals were found to act predominantly by narcosis.

In our opinion, the strength of the above presented approach is that it allows to compare different modes of action of one chemical in a quantitative manner. The approach is not limited to two MOA but other mechanistic models or QSARs can be added. Although the present approach does not explicitly treats synergistic effects it can offer some insight. A chemical like methyl methacrylate e. g. can be suspected to exert combined effects between the two MOA, because both TR are close to 1 (figure 2). However, results of the proposed approach should be used carefully when going from acute to chronic situations. For some MOA, it is known that LC_{50} values are decreasing with time. This time dependence of toxic effect levels was discussed by Lipnick *(48)* and recently by Legierse et al. *(14)* for organophosphorous esters and by Verhaar et al. *(5)* for electrophiles. For such a MOA, a TR of less than 1 in an acute experiment is likely to increase during a chronic exposure regime. This might lead to a change of the predominant MOA and therefore to a change in expected toxic effects.

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NARCOSIS AND CHEMICAL REACTIVITY IN ACUTE FISH TOXICITY QSARs

CHAPTER 4

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ABSTRACT

Quantitative structure activity relationships (QSAR) that describe the acute fish toxicity have been published for many different groups of reactive organic chemicals. The structural similarity of chemicals within such groups, suggests that they share a common mode of action (MOA) which is based on their common chemical reactivity. Often, however, a descriptor for this reactivity alone can not explain the observed toxicity satisfactory but addition of a hydrophobicity parameter, like log K_{OW}, is found to improve the relationship. In the present paper, an alternative strategy was proposed and tested with three different literature data sets. Instead of searching for better descriptors to establish a QSAR for the whole data set, the assumption that all compounds within the set act by the same MOA was critically reviewed. We tested the hypothesis that some of the compounds within the data sets acted by narcosis (general anesthesia), a second plausible mode of action in acute fish toxicity. Narcosis potency at observed lethal exposure levels was modeled with a baseline toxicity QSAR. The literature data sets were split in a narcosis and a reactive subset and for each of them a separate, one-parameter QSAR was established. For a set of OP-esters, nine out of 20 compounds were identified as possible narcotic compounds and their toxicity could be described with a narcosis QSAR. For the 11 compounds remaining in the reactive subset, a good correlation between acute toxicity and measured, in-vitro AChE inhibition rate was found (r^2=0.68) which would have been overlook if the whole data set was used. The use of two separate QSARs instead of one mixed QSAR was also tested for literature data sets of nitrobenzenes and α,β-unsaturated carboxylates. It was shown that for the description of toxicity data of all three groups of reactive compounds, a model which uses two separate modes of action was superior to a mixed model which uses a reactivity and a hydrophobicity parameter in a multiple linear regression.

INTRODUCTION

The prediction of acute toxicity in aquatic species has received considerable attention during the past two decades. With a growing number of tested chemicals the possibilities for quantitative structure activity relationships (QSAR) have increased. Effect concentrations of inert organic chemicals are generally well predicted by narcosis or so called baseline toxicity QSARs. These QSARs predict the toxicity from the hydrophobicity of the compound, often using log K_{OW} as predictive descriptor. For more reactive chemicals however, these baseline QSARs often underestimate the acute toxicity by up to four orders of magnitude. *(1-6)*. To identify these chemicals, classification schemes have been established for acute fish toxicity *(5, 7)*. The quantitative prediction of their toxicity however, seems more difficult. There are no general applicable QSARs for reactive chemicals but numerous QSARs have been published for group of structurally related compounds *(8-19)*. In most of these QSARs, a suitable descriptor for reactivity was selected based on a mechanistic hypothesis about the type of chemical reaction involved, the target site structure or the involvement of reactive metabolites. Often, however, the reactivity descriptor alone could not explain the observed toxicity satisfactory. The addition of a hydrophobicity parameter, like log K_{OW}, was often found necessary to improve the relationship. The question arises whether this hydrophobicity parameter is needed to account for hydrophobic interactions or uptake of the chemicals, as suggested by Hansch *(20)*, or whether the importance of such a parameter indicates that there might be a second, independent mode of action (MOA) present. The majority of literature data on acute fish toxicity does not provide symptomatic descriptions of the effects but reports LC_{50} values only. Therefore, additional information must be generated to identify the mode of action of these chemicals and to answer the above risen question.

Recently, we showed that within a set of reactive acrylic and methacrylic acid esters some compounds cause acute toxicity to fish due to narcosis despite their chemical reactivity *(21)*. By modeling two separate toxic mechanisms, narcosis and GSH-depletion we were able to compare for each chemical the potency to act by either MOA. Because narcosis is a common mode of action in aquatic toxicology *(5, 22)*, we expected that narcosis acting compounds may also be present in QSAR data sets for other groups of reactive chemicals. The target site of narcosis is the membrane and the narcosis potency of a compound is directly related to its membrane/water partitioning coefficient *(23)*. If a certain percentage of compounds in a data set are acting by narcosis, including a hydrophobicity term will increase the quality of a mixed QSAR but will at the same time obscure the relationship with the

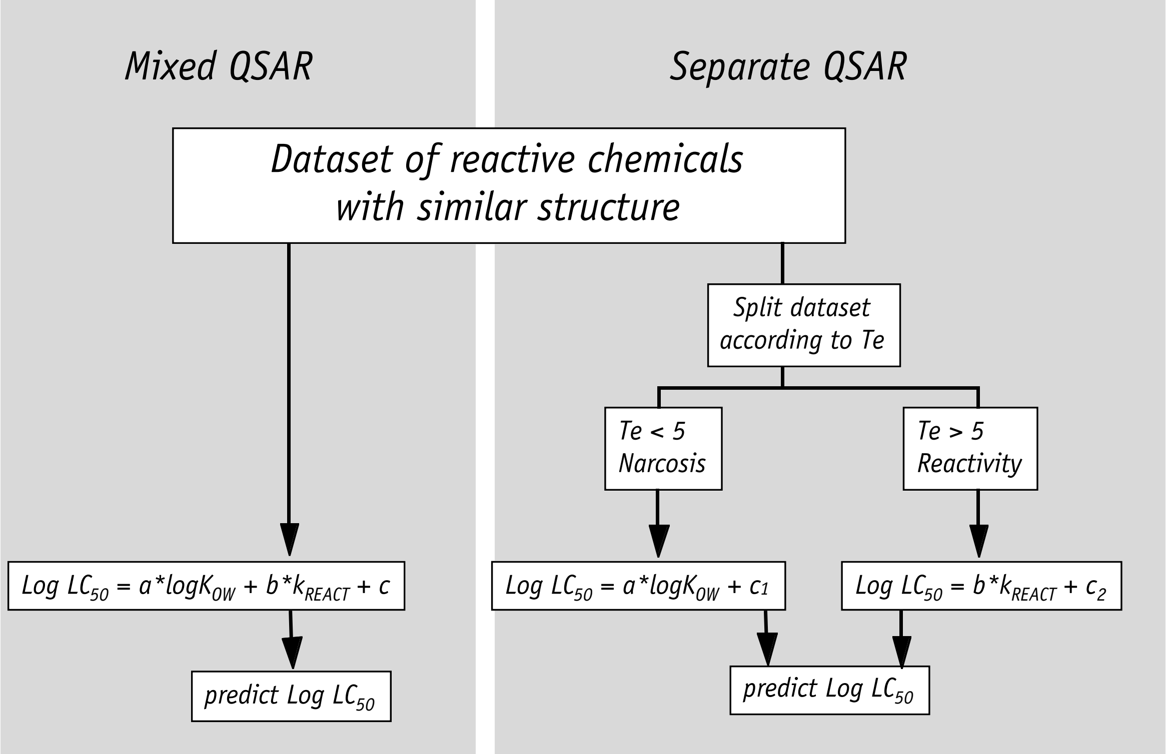

Figure 1: Comparison of the two models that were used to describe acute fish toxicity data of reactive organic chemicals. For each data set, predicted LC_{50} values were compared with observed data to estimate the power of prediction of each model.

reactivity descriptor. As an alternative to the mixed QSAR approach we suggest to split the data set in two subsets on the basis of the difference between observed and predicted LC_{50} values (Te ratio), and use separate QSARs for each MOA, as shown in figure 1.

The aim of the present study was to test whether an independent MOA approach using two separate QSARs could describe the observed toxicity as well as a mixed QSAR using multiple linear regression (MLR) with two parameters. First, a reasonable criteria had to be established to classify reactive chemicals either as acting by narcosis or by a reactive MOA. The excess toxicity ratio (Te), as defined by Lipnick *(24)* was used to establish this criteria for effect-based separation. Then, three literature data-sets for acute toxicity QSARs of reactive organic chemicals were split according to this criteria. Eventually, the predictive power of separate and mixed QSARs were compared to see if the alternative hypothesis of two MOAs was sustained by the model.

METHODS

Definition of a separation criteria

To test the hypothesis that narcosis chemicals are responsible for the frequent appearance of Log K_{OW} in QSARs for reactive chemicals we had to find a way to identify possible narcosis chemicals within a test set. The ratio of excess toxicity, Te *(3, 24)* was applied to split data sets in possible narcosis chemicals on one hand and chemicals acting through their reactivity on the other. Two data sets of narcosis chemicals with reported Te values were used to define the variation in Te for narcosis compounds. Based on this variation it was possible to set a rational limit to separate narcosis and non-narcosis compounds. In the first set given by Verhaar et al *(5)*, 50 compounds had been assigned by the authors to act by narcosis (denoted as class 1) on the bases of structural requirements. Te values were calculated using 14 day LC_{50} data of guppy (*Poecilia reticulata*) and a baseline QSAR using Log K_{OW} based on data from Köneman *(25)*. The second set, from Russom and coworkers *(7)* contained 258 compounds that were classified as narcosis 1 substances by different criteria such as behavioral effects, fish acute toxic syndromes (FATS), time dependence of effect concentration but also structural rules based on expert knowledge. Excess toxicity for this set had been calculated using 4-day fathead minnow (*Pimephales promelas*) LC_{50} data and a baseline QSAR from Veith et al *(26)*. From this data set we excluded five compounds classified with the highest uncertainty (Level D). Cumulative frequency distribution were calculated from the original data and used to estimate a 10 percentile limit for Te. This limit was used as a probabilistic criteria to split the original data sets in two separate subsets for further analysis. By setting the limit to 10% we tried to minimize the number of chemicals probably acting by narcosis but still end up with a reasonable number of compounds in the data sets representing the reactive MOA.

Data sets, models and statistics

Three literature data sets *(16, 18, 21)* for acute fish toxicity of reactive chemicals were chosen to compare the separate and the mixed QSAR approach. For all data sets, descriptors for reactivity as well as log K_{OW} were given in the original publication together with toxicity data for either P. reticulata (14 d LC_{50}) or for P. promelas (4 d LC_{50}) (table 1). The descriptors for reactivity were: 2nd order reaction rate with glutathione (k_{GSH}) for acrylic compounds *(21)*, 2nd order inhibition rate of oxon-analogues with eel acetyl cholinesterases for the set of organophosphorous esters *(18)* and $\Sigma\sigma$- for nitrobenzene data *(16)*. Each data-set was split, as defined above according to the 10-percentile Te value, in a reactive (non-narcosis) and in

Table 1: Three literature data sets that were used to test the importance of narcosis for the acute fish toxicity of reactive chemicals. LC_{50} values, reactivity parameters and log K_{OW} were teken from the original publications *(16, 18, 21)*. The potency of acting by narcosis was calculated for each chemical and is given as Te. For compound with a Te of less than 5, narcosis is considered to be the predominant mode of action.

OP-esters	log LC_{50} [µM]	log ki-oxon [$M^{-1}min^{-1}$]	log K_{OW}	Te
Iodofenphos	0.32	4.32	5.51	0.57
Fenthion-S2145	1.4	5.17	3.74	1.6
Bromophos	0.09	4.01	5.21	1.8
Pyrimiphos-methyl	0.79	4.35	4.32	2.1
Fenthion	0.89	4.81	4.17	2.2
Ronnel	0	3.58	5.07	2.9
Thiometon	1.53	3.93	3.2	3.6
SV5	1.68	1.43	3	3.8
Etrimphos	1.09	6.11	3.67	3.9
Cyanophos	1.75	3.78	2.71	**5.8**
Fenitrothion	1	4.65	3.47	**7.1**
Dicapthon	0.43	4.95	3.72	**16.0**
Methylisocyanothion	0.23	4.16	3.58	**33.5**
Methylparathion	0.61	5.28	3.04	**41.2**
Chlorthion	-0.19	5.26	3.63	**79.8**
Malathion	0.36	6.37	2.94	**89.6**
Phenthoate	-0.99	6.61	3.96	**260**
Phosmet	-0.12	6.39	2.81	**351**
Azinphos-methyl	-0.74	7.08	2.76	**1617**
Methidathion	-0.96	6.70	2.5	**4519**

Nitrobenzenes	log LC_{50} [µM]	σ^-	log K_{OW}	Te
4-Nitrotoluene	2.43	-0.15	2.34	2.5
3-Nitrotoluene	2.34	-0.07	2.4	2.8
2-Nitrotoluene	2.38	-0.15	2.3	3.1
Nitrobenzene	2.70	0	1.89	3.4
3,4-Dimethyl-nitrobenzene	1.79	-0.22	2.91	3.5
2-Chloro-nitrobenzene	2.28	0.27	2.26	4.2
4-Chloro-2-nitrotoluene	1.56	0.22	3.05	4.5
3,5-Dichloro-nitrobenzene	1.47	0.74	3.13	4.8
2-Chloro-6-nitrotoluene	1.48	0.22	3.09	5.0
3-Chloro-nitrobenzene	1.99	0.37	2.49	**5.2**
2,3-Dimethyl-nitrobenzene	1.61	-0.22	2.83	**6.3**
2,4-Dichloro-nitrobenzene	1.54	0.54	2.9	**6.4**
2,3-Dichloro-nitrobenzene	1.34	0.64	3.01	**8.2**
2,5-Dichloro-nitrobenzene	1.41	0.64	2.9	**8.6**
2,6-Dinitrotoulene	1.99	0.56	2.02	**13.3**
3-Nitroaniline	2.57	-0.16	1.26	**16.0**
4-Chloro-nitrobenzene	1.58	0.27	2.35	**17.6**

Table 1, continued

2,4-Dinitrotoluene	1.84	0.56	2.04	**18.0**
4-Nitroaniline	2.59	-0.15	1.16	**18.7**
2-Nitroaniline	1.85	-0.15	1.67	**36.9**
2,3 Dinitrotoulene	1.00	1.06	1.99	**137.6**
1,3-Dinitrobenzene	1.36	0.71	1.52	**154.0**
3,4-Dinitrotoluene	0.92	1.09	1.99	**165.5**
1,2-Dinitrobenzene	0.85	1.24	1.55	**469.4**
1,3,5-Trinitrobenzene	0.71	1.42	1.18	**1359.6**
1,4-Dinitrobenzene	0.37	1.24	1.45	**1731.8**
α,β-Unsaturated carboxylates	log LC_{50} [μM]	log k_{GSH} [$M^{-1}min^{-1}$]	log K_{OW}	Te
Isobutyl methacrylate	2.36	-0.73	2.66	0.4
Methyl-methacrylate	3.41	-0.70	1.38	0.6
Isopropyl methacrylate	2.47	-1.00	2.25	0.8
Hexyl acrylate	0.85	1.31	3.44	2.0
Benzyl methacrylate	1.42	-0.49	2.87	2.0
2-Ethoxy ethyl methacrylate	2.24	-0.60	1.45	**8**
Tetrahydrofurfuryl methacrylate	2.31	-0.52	1.3	**10**
Isobutyl acrylate	1.21	1.62	2.22	**15**
Diethyl fumarate	1.42	2.05	1.84	**22**
Ethyl acrylate	1.40	1.60	1.32	**77**
Acrylonitrile	2.43	0.87	0.23	**88**
Acrylamide	3.19	-0.33	-0.67	**122**
Hydroxy propyl acrylate	1.41	1.47	0.35	**694**
2-Hydroxyethyl acrylate	1.62	1.71	-0.21	**1569**
Acrolein	-0.74	3.92	0.10	**176111**

a narcosis sub-set. For each sub-set, a separate, one-parameter linear regression was calculated. For the narcosis sub-set, log K_{OW} was used as sole descriptor and for the reactive sub-set the reactivity descriptor, given in the original publication was used. Additionally, a mixed MOA QSAR was established using a multiple linear regression (MLR) with two parameters for the three complete data sets as shown in figure 1.

To compare the two models (two equations for separate data sets versus one equation for the whole set), we used the Akaike Information Criteria (AIC) *(27)* as given in equation 1. This criteria is used to compare no-nested predictive models with different numbers of parameter. The model with the smaller AIC has superior predictive power. However, the AIC can not provide a significance level.

$$AIC = N_{obs} \ln(RSS) + 2N_{par} \qquad \text{(EQ 1)}$$

N_{obs}: numbers of compounds in the complete data set.

RSS: residual sum of squares for predicted toxicity values.

N_{par}: number of parameters to fit:

separate: 2 one-parameter models = 2+2 = 4.

mixed: two parameter MLR model = 3.

RESULTS AND DISCUSSION

Te as criteria for separation of data sets

Narcosis QSARs are well established to predict the acute toxicity. Although the exact mechanism of narcosis still remains unclear, the target site is generally assumed to be somewhere within the membrane. Log K_{OW}, which is used in many "Narcosis QSARs" however does not exactly reflect the membrane-water partitioning behavior of a chemical. Especially polar chemicals with hydrogen donor or acceptor groups partition significantly stronger into membranes than predicted by Log K_{OW} *(28)*. For some substituted benzenes and phenols, membrane-water partition coefficients were up to 6 times higher than K_{OW} *(29, 30)*. This indicates, that narcosis QSARs based on log K_{OW} can contain a substantial uncertainty in their predicted effect concentration.

We examined two large data sets of narcosis compounds for the variation between observed and predicted LC_{50} values (Te ratio). Using this variation a probabilistic limit of 10%

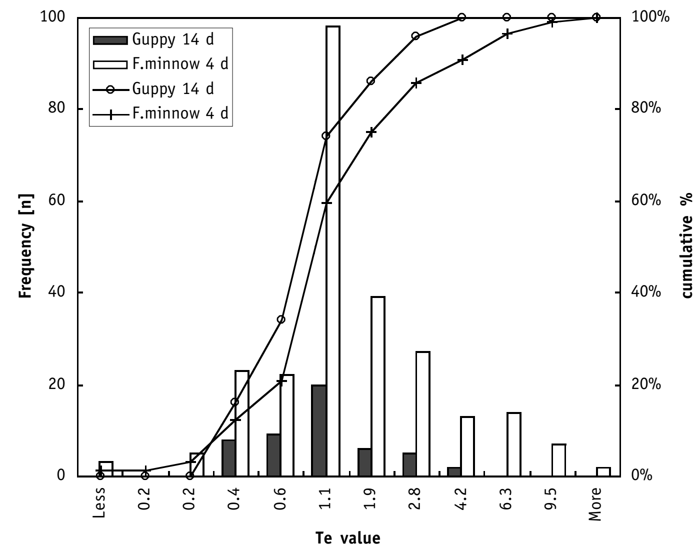

Figure 2: Histogram and cumulative percentage for the distribution of Te values for narcosis chemicals. Four day LC_{50} data for fathead minnow were taken from Russom et al. *(7)* and 14 day LC_{50} values for guppy were from Verhaar et al. *(5)*. A probabilistic limit of 90% was set to define a Te value that was used to separated narcosis from non-narcosis chemicals. Chemicals with a Te of more than five are considered not to act by narcosis.

was set to exclude chemicals acting by narcosis from data sets that were used for the development of QSARs for reactive chemicals. The distribution of the Te values of the two narcosis data sets are presented in figure 2. The frequency distribution of the data from Verhaar et al. *(5)* shows that 90 % of the narcosis compounds fall below a Te value of 2.3. The larger data set of Russom et al. *(7)* shows a larger deviation and here the 90 % limit is found at a Te value of 5.0. We choose to take the value from the larger data-set to establish the following rule:

A compound with a Te value of less than five will be considered as acting by narcosis and will not be used in the development of a QSAR for acute toxicity of reactive chemicals.

Of course, this does by no means guarantee that this chemical actually acts by narcosis but it will give a reasonable certainty that there will be very few narcosis chemicals within the sub-set for reactive chemicals. The rule was followed to devise the different literature data sets in reactive and narcosis sub-sets.

QSAR descriptors and QSAR models for acute toxicity

When the above derived rule was applied to devise the three data sets, a considerable number of chemicals (40%) fell into the sub-set of acting by narcosis. Splitting up the data set had a profound effect on the correlation of descriptors with acute toxicity, as can be seen in figure 3a-d for OP-esters. Although the complete set of OP-esters has a low correlation with ki and no correlation with K_{OW}, each separate subset shows a good correlation with its

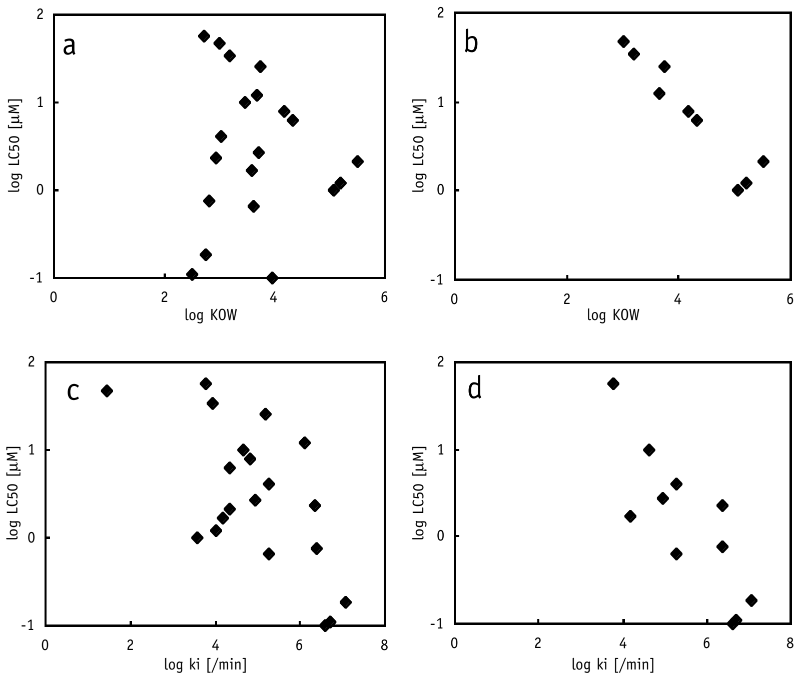

Figure 3 a-d: Correlations of the toxicity of organophosphorous esters with two descriptors, log K_{ow} and log k_i. For the whole literature set, neither log KOW (3.a) nor log ki (3.c) are well correlated with toxicity. If the whole data set is split in a narcotic and a reactive sub set however, these subsets correlate well with their expected descriptor. In figure 3.b, only the narcotic subset is plotted, and in figure 3.d, only compounds from the reactive subset are shown.

Table 2: Correlation matrix for descriptors with log LC_{50} for complete and separate data sets.

OP-esters	**log K_{OW}**	**log k_i-oxon**	**n**
narcosis subset	0.91	0.02	9
reactive subset	0.00	0.68	11
complete set	0.00	0.41	20
Nitrobenzenes	**log K_{OW}**	**σ^-**	
narcosis subset	0.97	0.34	9
reactive subset	0.00	0.74	17
complete set	0.00	0.71	26
α,β-unsaturated carboxylates	**log K_{OW}**	**log k_{GSH}**	
narcosis subset	0.93	0.57	5
reactive subset	0.02	0.82	10
complete set	0.01	0.70	15

Table 3: QSAR models for mixed and separate approach. Predictive power is measured by the Akaike informtion criteria (AIC). The better model has a lower AIC.

	QSAR used to predict LC_{50}	r^2 for pred. vs observed LC_{50}	AIC
OP-esters			
mixed	Log LC_{50}=–0.28*log K_{OW}–0.45*k_i+3.66	0.49	43.8
separate, narcosis	Log LC_{50}=–0.66*log K_{OW}+3.66		
reactive	Log LC_{50}=–0.62*k_i+3.59	0.80	26.7
Nitrobenzenes			
mixed	Log LC_{50}=–1.07*log K_{OW}–0.18*s^-+2.53	0.75	29.1
separate, narcosis	Log LC_{50}=–1.03*log K_{OW}+4.72		
reactive	Log LC_{50}=–1.00*σ^-+2.07	0.83	20.3
α,β-unsaturated carboxylates			
mixed	Log LC_{50}=–0.30*log K_{OW}–0.67*k_{GSH}+2.67	0.82	20.6
separate, narcosis	Log LC_{50}=–1.25*log K_{OW}+5.25		
reactive	Log LC_{50}=–0.68*k_{GSH}+2.45	0.86	18.5

appropriate descriptor (table 2). As stated earlier by DeBruijn et al. *(18)*, a one-parameter QSAR for the whole data set does not seem feasible. If, K_{OW} is used together with ki in a two parameter MLR for the whole data set, a better correlation is obtained than with ki alone (table 3). This contradiction can be understood if the OP-ester data set is considered as two separate subsets, acting by different toxic mechanisms. Although the complete data set does not correlate well with either of the descriptors, the good correlation of the two subsets with one descriptor improves the MLR correlation. It can be concluded, that the AChE-inhibition rate (k_i) of the oxon-analogue is a good descriptor of toxicity for the reactive

subset, which is in agreement with findings on OP-ester toxicity in other species *(31, 32)*. The same contradiction can be observed in the other two data sets. Although K_{OW} does not correlate with toxicity of the whole data set (table 2), its addition to a two-parameter QSAR increases the r^2 compared to the reactivity descriptor alone. For nitrobenzenes from 0.71 to 0.75 and for acrylic compounds from 0.70 to 0.82 (table 2 and 3).

In table 3, the predictive capacity of the two QSAR approaches is compared for the three data sets. The AIC, which corrects for the additional fit parameter used in the separate QSAR-model, shows that the independent MOA approach has a higher predictive power.

The conclusion of the QSAR models is also supported by experimental evidence. Several reports agree with the classification of some of the chemicals as narcosis acting. Behavioral symptoms of guppies exposed to Bromophos and Fenthion were characterized as narcosis-like by DeBruijn et al. *(33)*. Urrestarazu Ramos at al. *(34)* used Nitrobenzene, 3-Nitroaniline and 2-Nitrotoluene as model polar narcotics, based on critical body burdens measured in 14 day toxicity tests with guppies. Russom et al. *(35)* recorded behavioral indices during LC_{50} test with fathead minnow and assigned narcosis as MOA for 5 methacrylates (allyl-, benzyl-, 2-ethoxyethyl-, tetrahydofurfuryl- and isopropyl-methacrylate). These findings during toxicity tests, together with the improved quality of QSARs that consider narcosis as an independent MOA strongly indicate that narcosis should always be considered as an alternative MOA for acute fish toxicity.

The splitting of a data set based on effect related criteria seems a promising strategy for QSAR development. However it requires a priori assumptions about at least one of the suspected modes of action.

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GSH DEPLETION IN RAT HEPATOCYTES: A MIXTURE STUDY WITH α,β-UNSATURATED ESTERS

CHAPTER 5

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ABSTRACT

GSH depletion is often reported as an early cytotoxic effect, caused by many reactive organic chemicals. In the present study, GSH depletion in primary rat hepatocytes was used as an in vitro effect-equivalent to measure the toxic potency of α,β-unsaturated esters (acrylates and methacrylates). When these compounds were administered as a mixture, GSH depletion was found to be dose additive. The results of the mixture study show that GSH depletion may be a useful effect-equivalent for the risk assessment of mixtures of α,β-unsaturated esters. To get more insight in the underlying mechanisms of GSH depletion, the metabolism of two esters was investigated in greater detail. One of them, allyl methacrylate was found to be metabolized to acrolein. This metabolic pathway can explain the high potency of allyl methacrylate to deplete GSH despite its low intrinsic chemical reactivity.

INTRODUCTION

Risk assessment of mixtures is recognized as an important issue in environmental and human toxicology (*1, 2*) because most exposures are complex in nature. From air or water samples, typically hundreds of xenobiotic chemicals are isolated (*1, 3*). For many compounds in such complex mixtures, the chemical structure remains unknown. On the other hand, many commercially available substances are in fact complex mixtures (e.g. petroleum products or polymer products like paints or adhesives). They consist of mixtures of structurally related chemicals with different physico-chemical and toxicological properties. Often, only a tentative characterization is available for such mixtures, like the range of boiling point or the average chain length. To provide a scientific framework for effect assessment of mixtures, sumparameters for toxic effects of similar acting compounds have been proposed. A well known example of this strategy is the use of toxic equivalent factors (TEF) for the assessment of the effect of dioxin-like compounds (*4, 5*). Other effect equivalents are the inhibition percentage of acetylcholinesterase, which is used for pesticide exposure assessment (*6*) or the critical body burden which is used in aquatic toxicology as a measure for narcosis potency (*7, 8*). Effect equivalents are generally based on internal target concentrations. To link a sumparameter with an external exposure concentration, a physiologically based pharmacokinetic (PBPK) model can be used, which can account for differences in kinetics of each compound in a mixture. This combination was used by Verhaar et al. (*2, 9*) for the risk assessment of jet fuel in a workplace exposure scenario.

Recently, Frederick et al. (*10*) established a PBPK model that uses time integrated glutathione (GSH) depletion as a effect equivalent for toxic effects of ethyl acrylate, an α,β-unsaturated ester. Because many other reactive chemicals are known to cause GSH depletion, and because GSH depletion is well established as a cytotoxic effect it may be worthwhile to test whether GSH depletion can be used as an effect equivalent for mixtures of reactive chemicals. α,β-unsaturated esters are used in various combinations in polymer chemistry (*11*), and a mixture toxicity model for these compounds may improve their risk assessment. Many of these chemicals are Michael acceptors and react easily with biological thiols to form a covalent bond (*12-16*). Metabolism of α,β-unsaturated esters was shown to occur by hydrolysis and by conjugation with GSH (*10, 11, 17-20*). Toxicity tests in rodents and fish showed that toxic effects can be organ specific (*10, 15, 19-24*) and that differences in chemical reactivity alone seems not enough to explain differences in potency (*25, 26*).

In the present study, we used primary rat hepatocytes to measure GSH depletion caused by 11 individual α,β-unsaturated esters and two mixtures. The objective of this study is to

test, if effect data from individual chemicals could be used to predict the effects of mixtures and if GSH depletion might serve as an effect equivalent for these compounds in further studies. Hepatocytes were chosen as a model system, because the liver is the primary producer of GSH and because hepatocytes contain many enzymes that are related to GSH metabolism (*27*). It should be noted however, that because of their high metabolic activity and their intrinsic high GSH concentration, hepatocytes may be more resistant to GSH depletion than cells from target organs, like e.g. gills gastro-intestinal tract or respiratory tract. The effect of two mixtures was tested for dose additivity (*28*) and for response additivity (*29*).

The concept of an effect equivalent on the basis of GSH depletion might be applicable across different mechanisms. Based on the experimental EC_{50} data in hepatocytes, we wanted to get some more insight in the underlying mechanistic aspects of GSH depletion. In particular, the low EC_{50} of allyl methacrylate, which is in contrast to the low chemical reactivity of this compound suggested that different modes of action were present within our test set. To get an idea about alternative mechanisms of GSH depletion, we compared allyl methacrylate with the strongest Michael acceptor in the set, diethyl fumarate using a number of biochemical parameters. Dose response curves for cellular GSH levels were compared with the concentration of a lipid peroxidation marker (malondialdehyde) and with the production of acrolein and acetaldehyde, both being probable metabolites of allyl methacrylate and diethyl fumarate, respectively.

MATERIALS AND METHODS

Chemicals

The following chemicals were used: o-phthalaldehyde (OPA), purchased from Arcos ('s Hertogenbosch, The Netherlands), reduced glutathione (GSH), pentafluorobenzyl-hydroxylamine (PFB), acetaldehyde, acrolein, 1,1,3,3-tetraethoxypropane, $NaWO_4$, 3,4-dichlorotoluene, ethyl acrylate, 2-hydroxyethyl acrylate, isobutyl acrylate, dietyl fumarate, allyl methacrylate, benzyl methacrylate, 2-ethoxyethyl methacrylate, tetrahydrofurfuryl methacrylate, isobutyl methacrylate and methyl methacrylate from Fluka Sigma-Aldrich (Zwijndrecht, The Netherlands) and isopropyl methacrylate from Pfalz&Bauer (Waterbury, CT).

Animals

Male Wistar(U:Wu) rats were fed *ad libitum* with a grain-based diet and had free access to drinking water.

Cell culture and exposure

Hepatocytes were isolated by whole liver perfusion using the two step collagenase technique as described by Seglen (*30*). The cells were incubated at 37°C in air-tight 50 ml tissue culture flasks (Greiner, Alphen a/d Rijn, The Netherlands) at a density of $8*10^5$ cells/ml. Initial culture medium consisted of Williams' E medium, supplemented with 0.1 M HEPES, 26 mM $NaHCO_3$, 2 mM L-glutamine, 1 μM insuline, 10 μM hydrocortisone, 70 μM gentamycine and 3 % newborn calf serum (NCS). After 3 hours a cell monolayer was formed and initial culture medium was removed and replaced by culture medium without NCS. Non-attached and dead cells were thereby washed off. Exposure of hepatocytes started 24 hours after isolation. The old medium was removed and replaced by culture medium without NCS in which the tested chemical had been dissolved. Geometric dilution series with a factor of two and five concentration steps were used. The hepatocytes were harvested after 4 hours of exposure. Culture medium was removed and an aliquot was used to determine LDH activity. For some chemicals, samples of culture medium were frozen and stored at -20°C for subsequent analysis of aldehyde production. The cell monolayer was suspended with a TritonX-100 solution (0.5%) and homogenized by vortexing the cell-suspension for 10 min. Aliquots of the homogenate were used to determine LDH activity, reduced GSH concentration and protein content.

Protein content

Protein content of the homogenate was measured according to Bradford (*31*).

Cell viability

The viability of the cells was assessed by lactate dehydrogenase (LDH) leakage. The percentage of LDH leakage was determined by comparing LDH activity in the culture medium with total LDH activity in the culture flask (medium + cell homogenate). LDH activity was measured according to Bergmeyer et al.(*32*).

GSH measurement

Cell homogenates were precipitated with 5% trichloroacetic acid, centrifuged at 10000 g and the supernatant was kept frozen at - 20°C until analysis (max. 48 hours). The supernatant was diluted 1:10 with destilled water and reduced glutathione was measured by RP-HPLC

with post-column OPA derivatisation as described by Fujita et al. (*33*) and modified by Freidig et al. (*13, 25*). Cellular GSH concentrations were calculated as nmol/mg protein.

Analysis of aldehydes in culture medium

Three aldehyde products from metabolism of unsaturated esters as well as from endogenous metabolism of the hepatocytes were analyzed in the culture medium of exposed cells. Extraction of the medium, derivatisation with PFB and analysis of the aldehydes on a gas chromatograph with electron capture detector was performed according to DeZwart et al. (*34*), with the following minor adjustments: PFB-derivates were extracted from aqueous solutions with cyclohexane using 3,4-trichlorotoluene as internal standard. Standard solutions of acrolein and acetaldehyde were prepared in water. Standard aqueous solutions of malondialdehyde were prepared from 1,1,3,3-tetraethoxypropane according to DeZwart (*34*). Aqueous standards were derivatized along with the samples and tentative identification and quantification of the three aldehydes in the culture medium were achieved using retention times and responses of the pure compounds.

Calculations

Two dose response curves for GSH depletion and LDH leakage (each concentration in duplicate) were measured for each tested ester using hepatocytes from different isolations. For GSH depletion, EC_{50} values and slopes were fitted from each dose-response curve using the sigmoidal dose-response algorithm of Prism software (GraphPad Software, San Diego, CA) given in equation 1 with (C) being the nominal exposure concentration. Because many compounds caused only a partial LDH leakage at the highest tested concentration, LOECs ($p<0.05$, one tailed) were used to assess changes in cell viability.

$$GSH(\% \quad of \quad control) = \frac{100}{1+10^{-slope(\log(EC_{50})-\log(C))}} \qquad \text{(EQ 1)}$$

Mixture toxicity

Two different models, dose addition and independent response addition were used to test for a possible additivity of GSH depletion. Two equitoxic mixtures of six esters were prepared based on their individual EC_{50} for GSH depletion. Concentrations of the compounds were transformed to toxic units (TU) according to equation 2 and each of the six compounds was added at equal TU(i) (equitoxic mixtures) to the mixture. The potency of a mixture is thereby given by the ΣTU, defined in equation 3 (*28, 35*).

$$TU(i) = \frac{conc(i)}{EC_{50}(i)} \quad \text{(EQ 2)}$$

$$\sum TU = \sum_{i=1}^{n} TU(i) \quad \text{(EQ 3)}$$

Each mixture was tested in a geometric dilution series starting with a ΣTU of 6. Dose response curves were recorded for GSH depletion and LDH leakage as described for the individual compounds. If a mixture is dose additive, a ΣTU of 1 is expected to cause 50 % GSH depletion. Furthermore, a model for response additivity was applied to the experimental data, to compare the dose addition model with. The probabilistic addition model describing independent joint action (*29, 36*) was chosen to describe a mixture situation where each compound would deplete GSH by an independent pathway. Percentages of GSH depletion, caused by individual compounds below their EC_{50} were estimated from fitted sigmoidal dose response curves and used as effect probabilities, P_i. To calculate the effect probability of the mixture,P_{mix} the P_i's of each compound in the mixture were added up according to equation 4.

$$P_{mix} = 1 - \left[(1 - P_1)(1 - P_2)...(1 - P_n)\right] \quad \text{(EQ 4)}$$

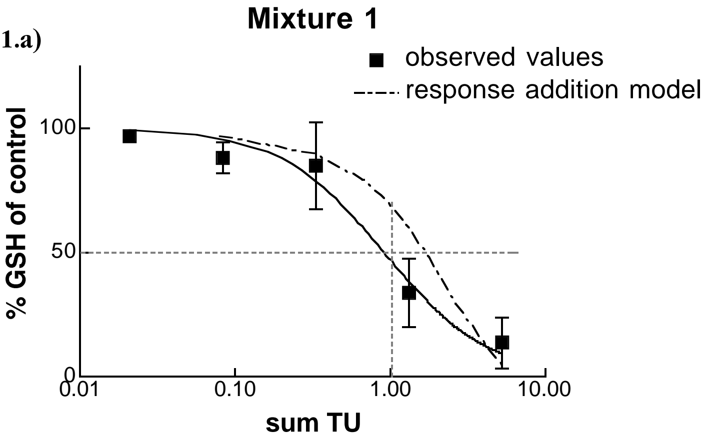
RESULTS

Single chemical tests

A series of α,β-unsaturated esters, which were known to react chemically with GSH (*13*) were tested for their potency of inducing GSH depletion in-vitro. EC_{50} values for GSH depletion of single chemicals after 4 hours are presented in table 1. They span a range from 0.14 mM for allyl methacrylate to 7.42 mM for methyl methacrylate. Slope factors for the dose response curves (table 1) were found to vary between 0.8 and 4.0. Cell viability, as determined by LDH leakage, was not affected by a concentration causing 50 % depletion of GSH, except for two compounds (allyl- and isobutyl methacrylate). However, at higher exposure concentrations more esters were found to decrease the cell viability during the 4 hour assay.

Mixture tests

Two mixtures, each containing six esters, were tested under identical conditions as the single substances. Their compositions is given in table 1. Dose - effect relations for both mixtures are presented in figure 1 a and b. The ΣTU was used as dose -equivalent on the x-



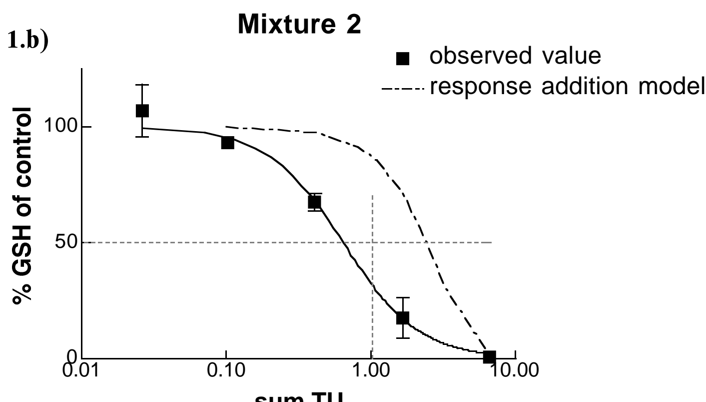


Figure 1 a-b: Dose response curves for two mixtures of α,β-unsaturated esters. The composition of the mixtures are given in table 1. Concentration of the mixtures were expressed as sum of toxic units (STU). For dose addition, a 50 % depletion is expected at STU of 1. An alternative mixture model (response addition) was less accurate and underestimated the observed effect of the mixtures.

Table 1: α,β-unsaturated esters tested for induction of GSH depletion in hepatocytes. EC_{50} and slope factors were fitted using a sigmoidal dose-response curve (equation 1).

substance	k_{GSH} [$M^{-1}min^{-1}$]	**GSH depl** EC_{50} [mM]	(SE) [mM]	slope	(SE)	**LDH-leakage** LOEC [mM]	Included in mixture:
Allyl methacrylate	0.51	0.14	0.02	1.5	0.30	0.14	1
2-Hydroxyethyl acrylate	50.00	0.28	0.18	2.2	0.80	1.2	
Diethyl fumarate	112.00	1.44	0.65	2.0	0.60	>7.3	2
Isobutyl acrylate	42.00	1.70	0.38	3.4	2.60	6.5	2
Ethyl acrylate	40.00	1.85	*1.21*	2.9	0.70	15.0	2
Benzyl methacrylate	0.33	2.36	0.49	0.8	0.60	>2.1	1
2-Ethoxyethyl methacrylate	0.25	2.73	*1.11*	2.0	0.30	>15.9	1,2
Tetrahydrofurfuryl methacrylate	0.30	3.05	0.24	1.7	0.36	>17.3	1,2
Isobutyl methacrylate	0.19	5.00	1.93	1.3	1.90	4.0	1
Isopropyl methacrylate	0.00	5.35	*1.08*	3.4	1.10	14.5	
Methyl methacrylate	0.20	7.42	*1.21*	4.0	1.80	>15.5	1,2

Italic SE-values were determined from one dose-resp. curve

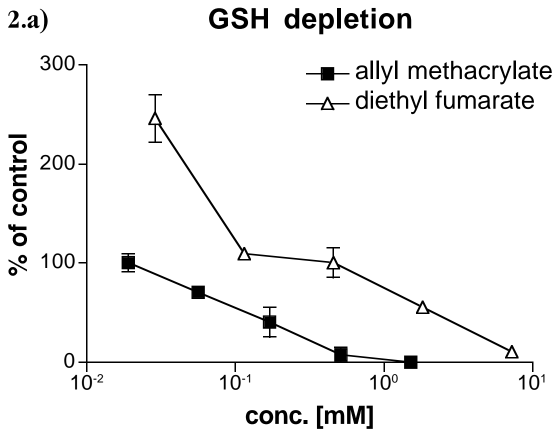
axis. Mixture 1 was found to be dose additive for GSH depletion (EC_{50} = 1.00 ΣTU, 95% confidence interval = 0.49-2.01 ΣTU), whereas mixture 2 was more potent than predicted by dose additivity (EC_{50} = 0.58 ΣTU, 95% confidence interval = 0.39-0.86 ΣTU). The model for response addition underestimated the potency for both mixtures, as can be seen in figure 1. Cell viability was only affected at the highest tested concentration for both mixtures. LOECs for LDH-leakage were 5.3 ΣTU for mix 1 and 6.6 ΣTU for mix 2, respectively.

Comparison of two esters

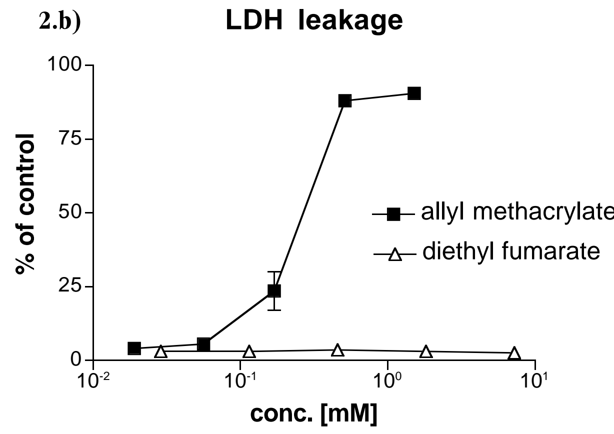
Allyl methacrylate (AMA) and diethyl fumarate (DF) differ in chemical reactivity toward GSH, DF being 200 times more reactive than AMA (table 1). However, AMA was found to be 10 times more potent than DF depleting GSH in vitro. We chose to investigate the effect of these two compounds on primary rat hepatocytes in greater detail, to see whether different modes of action could be identified that cause GSH depletion. Figure 2 a and b show the dose dependent GSH depletion and cell viability of hepatocytes exposed to both substances. For AMA, loss of cell integrity measured by LDH leakage was parallel with a decrease in cellular GSH. Loss of GSH might therefore be caused by an increasing damage of cell membranes. The lowest exposure concentration of DF caused a GSH induction with a factor 2.5 above levels of unexposed hepatocytes (32 ± 7 nmol/mg protein). Increasing DF concentrations depleted GSH but there was no effect on cell viability.

Three aldehydes were determined in the culture media of exposed cells. Malondialdehyde (MAD) was used as a marker of lipid peroxidation in AMA and DF exposed cells, whereas acetaldehyde and acrolein were used as an indicator for the metabolism pathways of DF and AMA, respectively. Figure 2 c shows that in AMA exposed cultures, MDA concentration increases together with LDH leakage. For DF exposed cells no increase of MDA-production was detected for the tested concentrations. Acrolein, which was suspected to be formed by alcohol dehydrogenase after hydrolysis of AMA, was detected above background levels at the two highest exposure concentrations (Figure 2 d). Because cell integrity was partially lost at these two concentrations, acrolein could have been formed by cytosolic enzymes that leaked into the medium. In the medium of DF exposed cells, acetaldehyde concentrations up to 50 μM were detected (figure 2 e). This oxidative metabolic product of DF was most probably formed in the cells because hepatocytes exposed to DF showed no sign of membrane damage or leakage.

2.a)



2.b)



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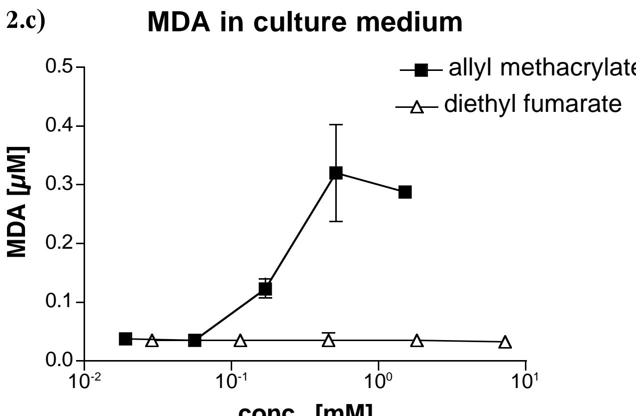
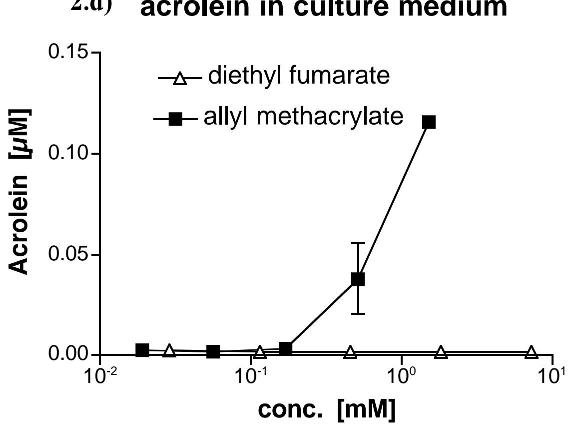


2.d)



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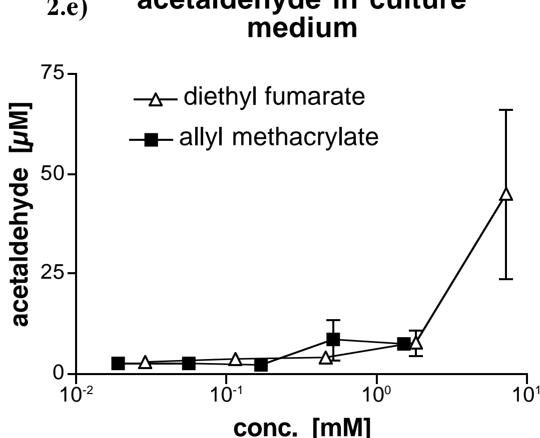

Figure 2 a-e: Dose-effect curves for allyl methacrylate and diethyl fumarate. Both compounds induce GSH depletion (2 a), but vary in LDH leakage (2 b), lipid peroxidation as measured by malondialdehyde production (2 c) and formation of the oxidized hydrolysis product (2 d and e).

DISCUSSION

GSH depletion by a,b-unsaturated esters

Chemicals with α,β unsaturated carbonyl groups are known to cause GSH depletion in rat livers (*12*). Accordingly, all esters tested in the present investigation depleted GSH in primary rat hepatocytes. Their potency to deplete GSH was only weakly correlated with their chemical reactivity with GSH (table 1). Two compounds (allyl methacrylate and 2-hydroxyethyl acrylate) were substantially more potent than the other compounds. EC_{50} for the other nine esters differ only by a factor of 5 although their chemical reactivity, given in table 1, differs by more than 2 orders of magnitude. It can be concluded that chemical reactivity with GSH is not the predominant mechanism that leads to GSH depletion in isolated hepatocytes. Moreover, because most esters share a relatively high effect concentration (1-10 mM) and because they are easily hydrolyzed by hepatic carboxylases (*10*), the metabolites can be expected to play an important role. There are a number of processes apart from pure chemical reactivity, that can govern the extent of depletion of GSH in the hepatocytes.

First, fast enzyme-catalyzed conjugation of the less reactive methacrylates by glutathione transferases (GST) might be responsible for the small difference in observed EC_{50}. Enzymatic conjugation by GST was described for ethyl acrylate in rat liver (*16*). However, no information is available about the selectivity of GST towards acrylates and methacrylates. Second, fast enzymatic hydrolysis of the esters can decrease the extent of GSH-depletion. In vivo inhibition of hydrolysis with tri-orthotolyl phosphate caused an increase of thioether production in rats exposed to methyl acrylate and methyl methacrylate (*37*) and increased the extent of depletion of non-protein thiols in various tissues of rats exposed to ethyl- and methyl acrylate (*38*). Third, metabolites resulting from hydrolysis that form or accumulate in cells can have an impact on the GSH level. This seems to be the case for allyl methacrylate. Allyl alcohol, a possible product of AMA metabolism was found to produce GSH depletion in vitro (*39-41*) and in vivo in rats (*42, 43*). This was explained by oxidation of allyl alcohol to acrolein by alcohol dehydrogenase. Acrolein, the reactive metabolite, was shown to be approximately 100 times more reactive with GSH than any of the esters tested in this work (*25*). In the present investigation, cells exposed to allyl methacrylate produced MDA, an indicator of lipid peroxidation. MDA was also found in allyl alcohol exposed cells (*44-47*). Furthermore, acrolein was detected in the culture medium. Neither acrolein nor MDA were found in DF exposed cells. We therefore suggest, that the low EC_{50} for GSH depletion of allyl methacrylate is caused by its metabolite acrolein which is formed through hydrolysis and subsequent oxidation. This is in agreement with findings about the toxicity

of other esters of allyl alcohol (*48, 49*). The induction of GSH, observed at the lowest exposure concentration of DF (figure 2 a) may be caused by the "electrophilic counterattack" as described by Talalay and co-workers (*50-53*). They reported that many chemicals with unsaturated carboxyl and carbonyl groups were able to co-induce a battery of phase 2 enzymes in hepatoma cells at very low concentrations.

Formation of acid equivalents by ester-hydrolysis in the cell could be another mechanism by which GSH is depleted. Lowering of intracellular pH from 7.35 to approx. 7.05 was found to cause GSH depletion in hepatocarcinoma cells (*54*). Acrylic acid itself was furthermore found to induce membrane permeability transitions in isolated mitochondria (*55*). This, in turn could lead to an energy deprivation and subsequently to a loss of reduced glutathione.

GSH depletion of mixtures

In the mixture experiment it was clearly shown, that GSH depletion of α,β-unsaturated esters is an additive effect in hepatocytes. Mixtures of compounds, diluted to 10-16% of their individual effect concentration induced a comparable GSH depletion as one compound at 100% of its EC_{50}. Two additivity models, dose addition and response addition were used to predict the effect of mixtures of α,β-unsaturated esters. Both mixtures were well predicted with dose addition. Response addition, which should be able to predict the joint effect of compounds with independent modes of action (*29*) underestimated the potency of both mixtures. It may be, however that the effects at low concentrations were underestimated with the current test protocol using a dilution series of a factor two.

Use of GSH depletion as effect equivalent in risk assessment

The advantage of an effect equivalent above a target concentration is, that it allows to aggregate the effect of several compounds. This study clearly shows, that GSH depletion in hepatocytes can be used as additive effect-equivalent for the toxic effects of acrylic and methacrylic acid esters in primary rat hepatocytes. The use of GSH depletion data from individual esters made it possible to predict the potency of a complex mixture. For a translation to in-vivo effects, information about the toxicokinetics of each compound in the mixture is needed to define their target tissue concentrations. Verhaar et al. (*9*) showed, how an existing PBPK model can be adapted to predict target tissue concentrations of compounds of jet fuel mixture with varying physico-chemical properties. Using such a distribution model together with a toxicodynamic model for GSH depletion (*10, 56*), it may be possible to predict the GSH depletion due to a complex mixture of e.g. acrylic monomeres.

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A PRELIMINARY PHYSIOLOGICALLY BASED PHARMACOKINETIC AND PHARMACODYNAMIC MODEL FOR ETHYL ACRYLATE IN THE RAINBOW TROUT

CHAPTER 6

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ABSTRACT

A preliminary PBPK-PD model was developed to describe the disposition of ethyl acrylate in rainbow trout. Furthermore, glutathione depletion in the gills due to conjugation with ethyl acrylate was modeled. For the pharmacokinetic model, three metabolic processes were characterized in gills, liver and in muscle tissue. Headspace solid phase microextraction was used to measure elimination rates of ethyl acrylate from tissue homogenates. In vivo data from exposure to different Michael acceptors were used to establish a model for endogenous GSH turnover in the gills. The preliminary model indicated, that for ethyl acrylate, no presystemic elimination occurred in the gills under steady state exposure conditions. Fat tissue was found to govern both, whole body kinetics and body burden. The predicted glutathione depletion in the gills was in close agreement with experimentally observed values after 24 hours.

INTRODUCTION

Acrylates and methacrylates are industrial chemicals with high production volumes. They are used in various combinations for the production of polymers, which are processed in e.g. coatings, paint or adhesives. For some of these chemicals, acute toxicity towards aquatic species was reported by Russom et al. (*1*) and by Greim et al. (*2*). Unspecific reactivity with biological target sites was thereby suspected as predominant mode of action, because these chemicals are Michael acceptors which have a high reactivity with thiol groups. Recently, we showed that a simple quantitative structure activity relationship (QSAR) which uses the reaction rate of Michael acceptors with glutathione can be used to predict the acute toxicity towards the test fish fathead minnow (*3*). Such a QSAR may assist in understanding experimental data and formulation of hypotheses, but it remains a more or less empirical based relationship or correlation. A step further in understanding experimental effect and exposure data may come from a better insight into the kinetic aspects of a certain chemical. Physiologically based pharmacokinetic (PBPK) modeling represents an excellent tool to analyse and predict concentrations in tissues and at the target site. Combined with dynamic aspects via a pharmacodynamic (PD) model, concentration-effect relationships can be modeled. We believe that the use of a PBPK-PD model will eventually give insights into rate limiting steps in the whole chain from external dose to effect. Such a model is furthermore valuable in the development of sound QSAR's, because the choice of chemical parameters and of a mathematical form of the QSAR should be coherent with the identified crucial processes in the above mentioned chain of events.

PBPK models have been developed for various species, like humans, rodents and fish. The fish models were developed to describe the toxicokinetics of inert organic chemicals (*4-7*), but have also been adapted for chemicals which undergo metabolism like paraoxon (*8*) or ethyl-hexyl phthalate (*9*).

For highly reactive compounds, like acrylic esters, it seems that the organs at the site of absorption are the most vulnerable ones. In rodents, ethyl acrylate (EA) was shown to cause cytotoxicity in the fore stomach (*10*) or the nasal cavity (*11*) following oral or inhalation exposure, respectively. Furthermore, high presystemic clearance was found in these two organs due to high carboxylesterase activity in the respiratory and in the gut epithelia (*12, 13*). Thus, both the pharmacokinetics and the -dynamics of EA occur at the site of absorption. PBPK models are generally validated with measured tissue concentration data. This, however is not always possible for fast metabolizing compounds like EA. Frederick and coworkers (*13*) e.g. could not detect EA in rat tissue after oral gavage.

Up to now, there is no PBPK model for acrylates or methacrylates in fish. The primary uptake for moderate hydrophobic organic chemicals in the fish is known to proceed through the gills (*5, 14, 15*) and therefore, cytotoxic effects as well as presystemic clearance could be expected in this organ. For acrolein, a structurally related Michael acceptor, respiratory-cardiovascular responses in trout were found, which were specific for gill damage (*16*). Petersen et al. (*17*) showed the acrylamide produced gill and liver lesions in trouts, exposed for 15 days.

In the present work, we have attempted to build a PBPK-PD model for EA, with the emphasis on the following three important aspects:

- Relation between external concentration and concentration in tissue.
- Influence of biotransformation on the target dose.
- Extent of GSH depletion at the target site as hypothesized critical effect parameter.

Because we were not able to cover all aspects, we had to make a few assumptions and therefore the model should be regarded as preliminary. As mentioned by Andersen et al. (*18*), PBPK modeling can be a valuable tool to structure existing beliefs, to identify data gaps and to direct new experimental research. We expect the PBPK-PD model to be useful as a first approximation which may guide further and more detailed studies.

MATERIAL AND METHODS

Chemicals

Ethyl acrylate, methyl methacrylate, isobutyl acrylate, isobutyl methacrylate, 2-hydroxyethyl acrylate, allyl methacrylate (Fluka, Bornem, the Netherlands) o-phthalaldehyde (OPA) (Arcos, 's Hertogenbosch, The Netherlands) and reduced glutathione (GSH) (Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) were all used as received. All solutions were prepared with water purified by a Millipore Milli-Q system.

Animals

42 four month old rainbow trout (Oncorhynchus mykiss) from our own hatchery with a mean weight of 5g were used in the exposure experiments. The animals were held in copper free tab-water at 11°C, with a light-dark cycle of 12 hours.

In vivo GSH depletion by six a,b-unsaturated esters

Rainbow trouts were exposed to the different esters in covered, 8 l aquarium under steady state conditions. The test compounds were dissolved in oxygenated, copper free

tab-water. For each compound, three fish were exposed to a concentration close to its reported 4-day LC_{50} value during 6 hours. Aqueous concentrations of the esters were measured at the beginning and after the exposure period with a HPLC-UV system as reported earlier (*19*) and an average concentration was calculated. Measured concentrations at the end of the exposure period varied between 110% (allyl methacrylate) and 53 % (2-hydroxyethyl acrylate) of initial concentrations. Non-exposed fish were kept under the same conditions to serve as controls. After exposure, the fish were killed by a sharp blow to the head and the liver, the gills and a part of the dorsal muscle were removed. The tissue samples were directly homogenized and analyzed for reduced glutathione as described by Freidig et al. (*3*) using HPLC with OPA post-column derivatization.

Partition coefficients

Tissue-water and blood-water partition coefficients for EA in rainbow trout were estimated by QSAR equations (*20*). These QSAR's have been developed using chlorinated ethanes of different hydrophobicity. For EA, an octanol-water partition coefficient of 21 was used in the calculations (*21*).

A priori parameters for glutathione turnover in the gills

Zero-order synthesis rate, S^0 and first-order degradation rate, K_{DEG} of GSH in the gills were estimated by fitting the results of the in vivo exposure experiments to a steady-state model (*3, 8*). The model assumes, that after 6 hours, the GSH concentration in the gills is controlled by a zero order synthesis rate, a first order endogenous degradation rate and a first order conjugation rate, K_{GSH} with the Michael acceptor as given in equation 1.

$$C_{GSH}^{gill} = \frac{S_0^G}{K_{DEG}^G + K_{GSH}} \qquad \text{(EQ 1)}$$

Linearization of equation 1 shows, that the inverse of the steady state GSH concentration is linearly related to the pseudo first-order conjugation rate (equation 2).

$$\frac{1}{C_{GSH}^{gill}} = \frac{1}{S_0^G} K_{GSH} + \frac{K_{DEG}^G}{S_0^G} \qquad \text{(EQ 2)}$$

To use the GSH depletion data from the in vivo experiments in this model, pseudo first-order conjugation rates for each chemical with GSH were estimated. Because there was no

information available for enzyme catalyzed conjugation for most of these compounds, we used the product of aqueous exposure concentration and the chemical 2nd-order reaction rate with GSH at pH 8.8, $k^{8.8}_{GSH}$ ($M^{-1}h^{-1}$) (*19*) as given in equation 3.

$$K_{GSH} = C_{AQ} k_{GSH}^{8.8} \qquad \text{(EQ 3)}$$

In vitro metabolism of ethyl acrylate

Headspace sampling followed by gas-chromatography is a well established analytical method to follow in-vitro metabolism of volatile compounds (*22-24*). Instead of conventional gas-phase sampling we used headspace solid phase microextraction (*25-27*) to determine the aqueous concentration of EA in tissue homogenates. Fish tissue S-9 homogenates were prepared according to Barron et al. (*28*) from gill, liver and muscle samples of rainbow trout. Reduced glutathione in the homogenates was analyzed as described above. Per tissue, three different solutions were prepared to estimate the three different rate constants: (1): tissue homogenate alone, (2): tissue homogenate, where 1 mM GSH was added and (3): 1 mM GSH in PBS buffer at pH 7.4. The reaction was started by adding ethyl acrylate to all solutions, resulting in a final concentration of 100 µM. The reaction was carried out at 18°C and samples of 500µl were taken after 0, 15, 30 and 60 minutes. Each sample was mixed with 100 µl H_2SO_4 (0.5 M) in an air-tight 2ml autosampler vial to stop all reactions by precipitating the proteins and acidifying the solution. Immediately after taking the last sample the vials were transferred to a thermostated autosampler (30°) and allowed to equilibrate for 10 minutes. EA was analyzed by headspace SPME using a 85 µM polyacrylate fiber (Supelco, Zwijndrecht, the Netherlands) on a gas chromatograph with flame ionization detector (Varian, Houten, the Netherlands). The analyte was absorbed for 2 minutes on the fiber and desorbed for 1 minute, splitless at 225°C in the injector. A 30 m DB5.625 column 0.32mm inner diameter and 0.25µM film thickness was operated at 40 °C to separate EA. Aqueous standard solutions of EA in PBS buffer with H_2SO_4 were analyzed the same way and used to quantify the amount in the samples.

Three different loss processes of EA were expected in tissue homogenates: (i) chemical conjugation with GSH (*19*), (ii) enzymatic conjugation with GSH by glutathione transferase (GST) (*29*) and (iii) hydrolysis by carboxyl esterases (*12, 30*). To quantify the three different processes, a pseudo-first order model was used to describe the observed elimination rates of EA in the tissue homogenates. The model gives a correct approximation for enzymatic kinetics if both EA and GSH concentrations are below their K_M values. Equation 4 describes the processes in solution 1 (only tissue homogenate).

$$k_{OBS}^{1} = k_{HYDR} + k_{GSH}^{7.4} GSH_{HOM} + k_{GST} GSH_{HOM} \quad \text{(EQ 4)}$$

where k_{HYDR} (min^{-1}): pseudo-1st order hydrolysis rate constant by carboxylesterases.
$k^{7.4}_{GSH}$ ($M^{-1}min^{-1}$): 2nd order chemical reaction rate constant for GSH with EA at pH 7.4
GSH_{HOM} (M): GSH concentration in tissue homogenate
k_{GST} ($M^{-1}min^{-1}$): pseudo 2nd order reaction rate constant for enzymatic glutathione conjugation by GST

Equation 5 and 6 represent processes in solution 2 and 3:

$$k_{OBS}^{2} = k_{HYDR} + k_{GSH}^{7.4} GSH_{HOM} + k_{GST} GSH_{HOM} + k_{GSH}^{7.4} GSH_{ADD} + k_{GST} GSH_{ADD} \quad \text{(EQ 5)}$$

$$k_{OBS}^{3} = k_{GSH}^{7.4} GSH_{ADD} \quad \text{(EQ 6)}$$

where GSH_{ADD}: added GSH (1mM)

The 2nd order reaction rate between EA and GSH at pH 7.4 is obtained from equation 6. The pseudo 2nd order reaction rate which describes the enzyme catalyzed reaction between EA and GSH is calculated according to equation 7 and the pseudo 1st order reaction rate for the hydrolysis by carboxylases (equivalent to V_{MAX}/K_M) is given by equation 8.

$$k_{GST} = \frac{(k_{OBS}^{2} - k_{OBS}^{1} - k_{OBS}^{3})}{GSH_{ADD}} \quad \text{(EQ 7)}$$

$$k_{HYRD} = k_{OBS}^{1} - GSH_{HOM}(k_{GSH}^{7.4} + k_{GST}) \quad \text{(EQ 8)}$$

Observed loss rates, k_{OBS} (h^{-1}) were calculated by linear regression from semi-logarithmic plots of EA concentration versus sampling time. Standard errors for the reaction rate constants were calculated using the errors of the regression slopes and standard error propagation formula (*31*).

PBPK-PD model

The PBPK model for EA in rainbow trout presented here (figure 1 a) was based on a PBPK model by Nichols et al. (*4*). Metabolism of EA was modeled in three tissue compartments, in gill, muscle and liver. Liver was chosen because of its expected high metabolic activity and muscle because of its large fraction of total body volume. Based on our own measurements, a gill compartment with 2.5% of the total body weight, was added to the

original model structure. In the PD-model (figure 1 b) GSH concentration in the gills was modeled, using a zero order synthesis rate and 1st order endogenous elimination rate and second order conjugation rate with EA as suggested by Frederick et al. (*13*) and D'Suoza et al. (*32*). Our model, however, was simplified by assuming a constant synthesis rate for GSH. For the muscle and liver compartment, first order elimination terms were added to account for hydrolysis and conjugation with GSH. GSH concentration was not modeled for these two compartments but kept at control levels because parameters for synthesis and endogenous elimination of GSH could not be estimated from the in-vivo experiments. No metabolism was modeled in the kidney, fat and in the richly perfused tissue group, because

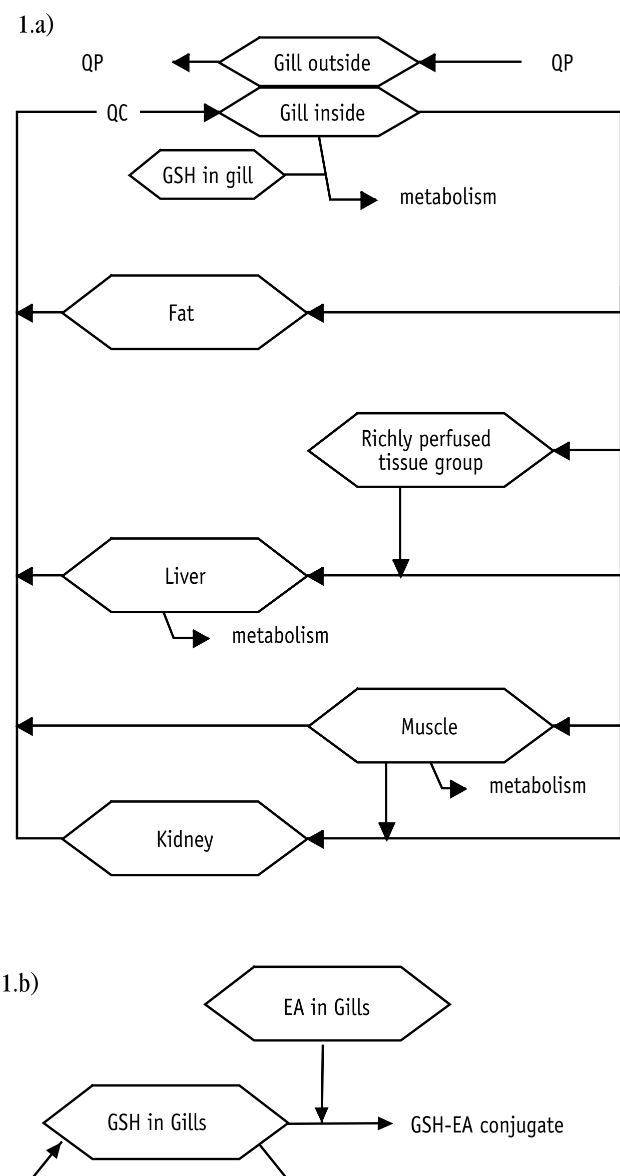


Figure 1 a and b: PBPK-PD model structure for ethyl acrylate in rainbow trout. The PBPK model was adapted from Nichols et al. (*4*). A PD model was added in the gills compartment (figure 1 b) to describe observed GSH depletion due to chemical reaction with EA.

data from the rodent model suggested that elimination of EA in these organs was low (*13*). Ventilation volume and cardiac output were scaled to a body weight of 5 g as suggested by Nichols et al. (*4*) and Erickson et al. (*33*). The model equations were written in ACSL and are given in the appendix. Model parameters and their values are given in table 1. The sensitivity of the PBPK-PD model was tested by increasing input parameters by 10 % and recording the resulting difference in predicted GSH depletion (*8*). Numerical computations were performed with ACSL for Windows, version 11.4.1, MGA Software Inc. Concord, MA). On request, electronic copies of the model code are available from the authors.

RESULTS

In vitro metabolic rate constants of ethyl acrylate

Incubation of fish tissue S-9 homogenate with 100 μM EA resulted in first order loss curves for all incubations. In figure 2.a and b, data for gill and liver tissue homogenate are shown. Resulting rate constants from S-9 incubations for k_{HYDR}, k_{GSH} and k_{GST} were scaled to represent activities in tissue and are given in table 2. Highest activities were found in the liver where enzymatic conjugation with GSH was found to be the predominant metabolic pathway for EA. No carboxylase activity was found in the gills whereas in the muscle no GST activity could be detected. The presence of enzymatic activity found in tissue homogenates are in agreement with literature data. Carboxylesterase activity was reported for several tissues of rainbow trout using different substrates (*28, 34*), and GST activity towards chloro-dinitro-benzene was reported in liver and gill of the rainbow trout (*35, 36*). The only discrepancy was found for carboxylase activity in the gill which was reported by Barron et al (*28*) for ethyl -hexylphthalate but which was not detected in the present investigation for EA.

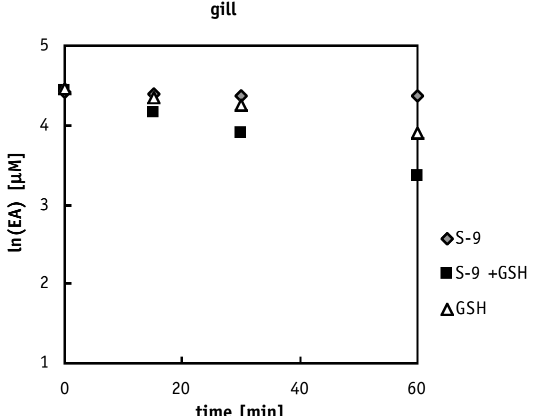
For liver and muscle tissue, over-all first order reaction rates were calculated to describe the elimination of EA in the PBPK model. GSH concentrations of control fish were used (table 2). Resulting half live times were 20 sec. in liver and 17 minutes in muscle tissue. For the gills, where GSH concentration was modeled separately, a 2nd order reaction rate for EA, $K^{G}_{GSH+GST}$ that accounted for chemical and enzymatic conjugation with GSH of 93.7 ($M^{-1}min^{-1}$) was calculated, adding $k^{7.4}_{GSH}$ and k^{G}_{GST} (taken from table 2). The chemical reaction rate between EA and GSH at pH 7.4, $k^{7.4}_{GSH}$ was determined to be 9.3 ($M^{-1}min^{-1}$). This means that, contrary to the rat model (*13*), the enzyme catalyzed reaction is more important than the chemical reactivity.

Table 1: Abbreviations and units of the parameters used in the PBPK-PD model for ethyl acrylate. The model structure is given in the appendix. Tissue volumes and perfusion rates were estimated, using data from Nichols et al. (*4*).

Parameter		
BW	Body weight (kg)	0.005
V_F	Fat tissue (ml)	0.5
V_G	Gill tissue (ml)	0.13
V_L	Liver tissue (ml)	0.07
V_M	Muscle tissue (ml)	3.7
V_K	Kidney (ml)	0.04
V_R	Richly perfused tissue group (ml)	0.3
QP	Effective ventilation volume (Lh^{-1})	0.2
QC	Cardiac output (ml h^{-1})	21
	Blood flow to tissue Q_i	
Q_G	Gills (ml h^{-1})	21
Q_F	Fat (ml h^{-1})	1.8
Q_L	Liver (ml h^{-1})	0.6
Q_M	Muscle (ml h^{-1})	12.6
Q_K	Kidney (ml h^{-1})	1.2
Q_R	Richly perfused tissues (ml h^{-1})	4.8
P_{BW}	Calculated blood/water partition coefficient	2.3
K_{OW}	Octanol/water partition coefficient of EA	21
	Calculated tissue blood partition coefficient, P_{iB}	
P_{LB}	Liver	0.7
P_{KB}	Kidney	1.7
P_{FB}	Fat	13.8
P_{MB}	Muscle	1.2
P_{RB}	Richly perfused tissues	0.7
P_{GB}	Gills	0.7
A_i	Amount of EA in compartment i (µmol)	
C_i	Concentration of EA in tissue i (µM)	
S^G_0	zero-order synthesis rate of GSH in gills (µM h^{-1})	63
K^G_{DEG}	1st-order endogenous degradation rate of GSH in gills (h^{-1})	0.068
$K^G_{GSH+GST}$	2nd-order rate const. of EA with GSH in gills ($M^{-1}min^{-1}$)	93.7

GSH turnover model for gills

A 6 hour exposure to α,β unsaturated esters close to a lethal concentration resulted in a marked depletion of GSH throughout the body of the fish (table 3). Depletion of GSH in the gills varied between 0% and 50%. The data from fish exposed to the four hydrophobic esters tested in this report, together with data for EA (*3*) and 6 control fish were correlated with estimated conjugation rates, K_{GSH} according to equation 2, to see whether a simple model was appropriate to describe GSH metabolism in the gills. 2-Hydroxyethyl acrylate,



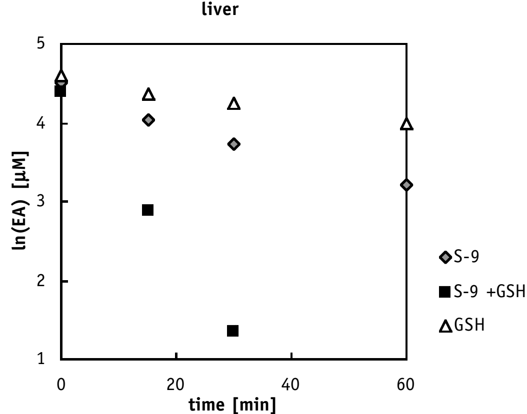


Figure 2: Semi-logarithmic plot for the elimination of ethyl acrylate (EA) in rainbow trout S-9 tissue homogenate. EA concentrations were measured by headspace SPME and gas chromatography.

Table 2: First order rate constants for elimination reactions of ethyl acrylate in rainbow trout tissue. Rate constants for tissue were scaled up from in vitro experiments and are given as (h^{-1}) to allow comparison between the different metabolic processes. Tissue GSH concentrations were taken from non-exposed control fish.

		First order rate constants (h^{-1})					
Tissue	C^i_{GSH} (mM)	k^i_H	±SD	K^i_{GSH} ($k^{7.4}_{GSH}$*C^i_{GSH})	±SD	K^i_{GST} (k^i_{GST}*C^i_{GSH})	±SD
Gill	0.94	n.s.		0.53	11%	4.82	14%
Liver	2.36	8.66	17%	1.32	11%	114.92	5%
Muscle	0.39	2.13	26%	0.22	62%	n.s	

with a log K_{OW} of -0.2, falls outside established models for gill uptake of organic compounds (*14, 33*). Because uptake behavior of this compound may deviate significantly from the other, more hydrophobic chemicals, 2-hydroxyethyl acrylate was excluded from the data set. The resulting relationship (figure 3) was significant ($p<0.01$, $n=24$) but showed a large variation between individual fish ($r^2=0.48$). The estimated model parameters (±SD) were 63.8±14 ($\mu M kg^{-1} h^{-1}$) for zero order synthesis of GSH and 0.068±0.016 (h^{-1}) for 1st order endogenous consumption of GSH. Steady state GSH concentration in gills was estimated to be 943±81 µM. Marked differences in GSH depletion were observed between tissues (table 3). In the liver, four chemicals (isobutyl acrylate, isobutyl methacrylate, methyl methacrylate and al-

Figure 3: Correlation to estimate S^G_0 and K^G_{DEG} for the GSH-turnover model in the gills according to equation 2 (n=24, r^2=0.48, F=20.7, p<0.0001). Experimental data of GSH depletion in gills from exposure to five different Michael acceptors was used.

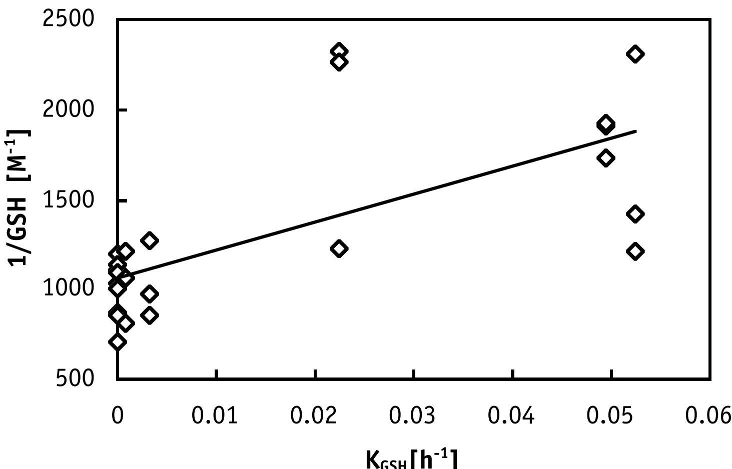


Table 3: Observed effects of Michael acceptors on GSH concentration in gills, liver and muscle of exposed fish. The estimated reaction rate is based on the chemical reaction rate at a pH of 8.8.

Chemical	Measured exposure conc. (µM)	Reported 4-day LC_{50} (µM)	GSH conc. (% of control) after 6 h exposure Gills	Liver	Muscle	Estimated reaction rate K_{GSH} (h^{-1})
Ethyl acrylate	22	25	68%	63%	90%	0.052
Isobutyl acrylate	28	16	46%	22%	72%	0.049
Methyl methacrylate	1875	2587	60%	19%	79%	0.023
Isobutyl methacrylate	288	230	85%	21%	69%	0.003
Allyl methacrylate	22	8	105%	41%	74%	0.0007
2-Hydroxyethyl acrylate[a]	127	41	68%	66%	89%	

[a] Because of its low K_{OW}, 2-hydroxyethyl acrylate did probably not reach equilibrium in the fish tissue after 6 hours and was therefore not included in the GSH turnover model.

lyl methacrylate) produced a much stronger depetion of GSH than in the gills. Allyl methacrylate, did even affect the liver at an exposure concentration were no effect in the gills could be observed. This compound, however, is known to be transformed by hepatic enzymes to the very reactive metabolite acrolein (this thesis, chapter 5).

PBPK-PD model

For a 5 g rainbow trout, a scaled effective ventilation volume of 0.20 (Lh^{-1}) (*4*) and a cardiac output of 0.021 (Lh^{-1}) (*33*) was calculated. Estimated blood-water and tissue-blood partitioning coefficients are given in table 1. Because no QSAR was available for gill and

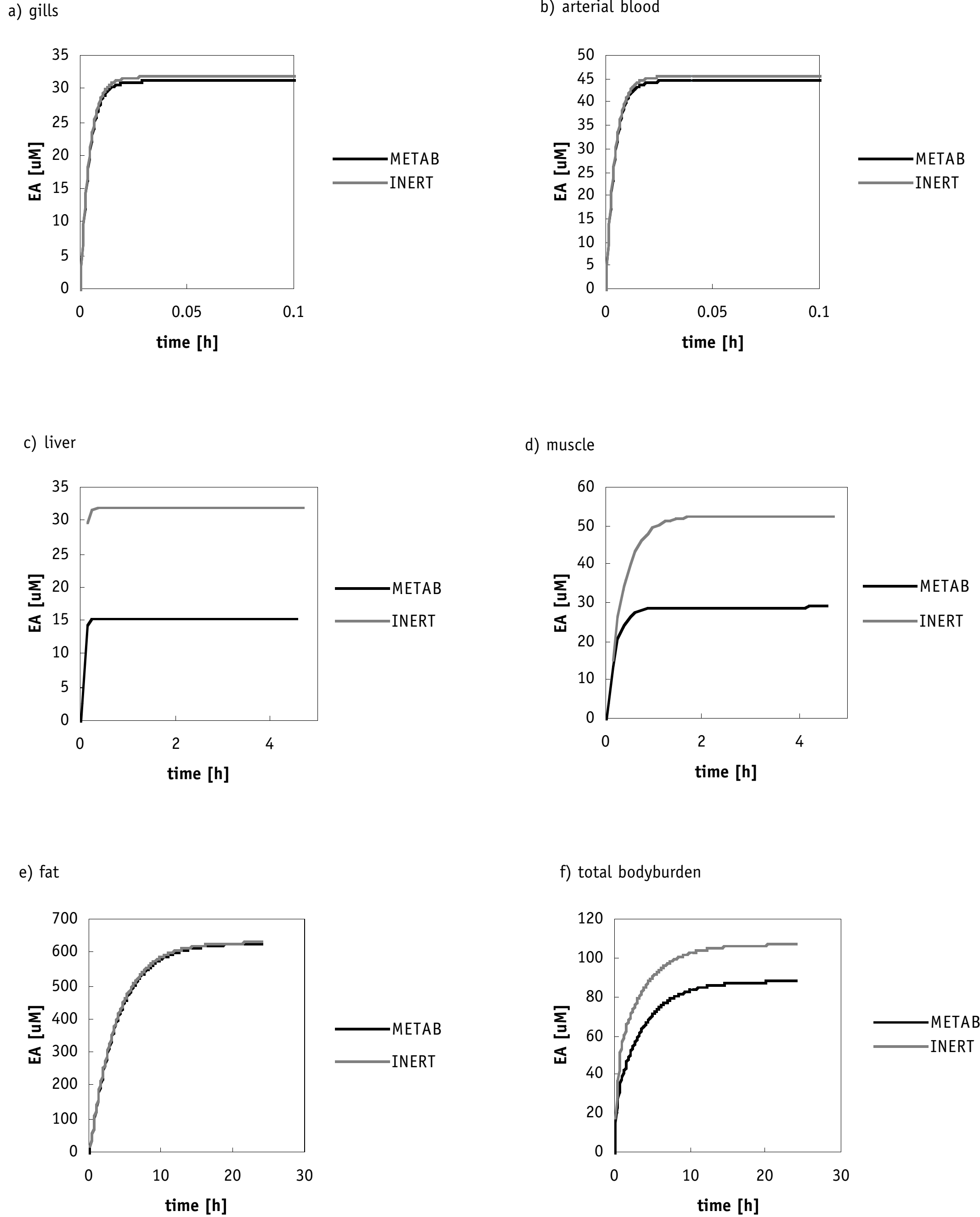


Figure 4.a-f: Forecasted concentration profile of EA during 24 hours using two models: In the first model, INERT no biotransformation of EA was added to see how an inert chemical with similar partition coefficients as EA would distribute in the fish. In the second model, METAB elimination of EA was modeled in the gills, the liver and the muscle compartment as described in the appendix. For the fastest compartments, gills (a) and arterial blood (b), only the first 6 minutes are shown, after which a steady-state is reached. For liver (c) and muscle (d) an equilibrium is reached within an hour. The model predicts that metabolism in these two tissues leads to approximately 50 % reduction of EA concentration. About 10 hours are needed for the fat (e) and the total body concentration (f) to reach a steady state.

richly perfused tissue, we used the liver/blood partition coefficient instead. The estimated tissue/blood partition coefficients for EA in rainbow trout, were between 2 and 5 times higher than measured partition coefficients of EA for rat tissue (*13*). The predicted partition coefficient for fat tissue however, was 20 times higher than the one reported for the rat.

Uptake and distribution of EA from a constant external aqueous concentration was modeled for a 24 hours exposure period. To test the influence of metabolism, a simulation run with metabolism in gill, liver and muscle was compared to a simulation run, where all metabolic constants were set to zero. This would reflect the pharmacokinetics of an inert compound with similar hydrophobicity. Resulting time-dependent tissue concentrations are given in figure 4 a-f. It can be seen that gill and arterial concentrations do not significantly differ for the two situations. Muscle and liver concentrations are reduced by metabolism to approximately 50% compared to an inert compound. Concentration in fat tissue remains unaffected. With the given tissue concentrations a bioconcentration factor of 4.4 was calculated for EA on a wet weight bases. This is almost equal to the calculated BCF of 5.4 of the hypothetical, inert compound. A mass balance over 24 hours showed, that of the 9.7 µmol which were absorbed, 95 % had been metabolized, 30% in the liver and 60% in the muscle. Only 5% of the total dose remained unchanged in the body after 24 hours. It could be concluded from the pharmacokinetic model that the metabolism in the gills did not influence the uptake of EA in the whole fish and therefore no significant first pass effect was present.

A pharmacodynamic model, which used in vitro conjugation rates with GSH (table 2) and a GSH turnover model was used to predict GSH concentrations in gills during 24 hours. In figure 5 the model predictions for GSH in the gills are compared to observed GSH levels

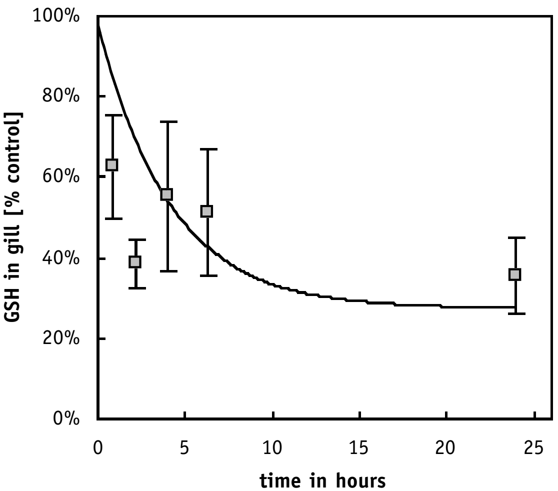


Figure 5: GSH depletion in gills of rainbow trout by ethyl acrylate. The PBPK-PD model includes metabolism of EA by hydrolysis and GSH-conjugation in liver, muscle and gill. Observed GSH depletion after 24 hours is close to the forcasted value (solid line).

Table 4: Sensitivity of gill GSH depletion (% of control) towards 10 % change in the model parameters.

Parameter	% change in gill GSH depletion due to 10 % change in parameter
S^{G}_{0}	0.1
K^{G}_{DEG}	7.1
QP	<0.01
QC	<0.01
$k^{7.4}_{GSH}$	-0.9
k^{G}_{GST}	-7.8
K^{L}_{GST}	<0.01
K^{L}_{H}, K^{M}_{H}	<0.01
K_{OW}	-4.5

of fish exposed to 20 μM EA (*3*). The model predicts a GSH depletion after 24 hours of 72 %, comparable to an observed depletion of 64 %. The model estimates that after 6 hours of exposure, GSH levels is depleted by 60 %. The very fast kinetics of EA in the gills is thus not paralleled by fast changes of GSH levels. The sensitivity analysis (table 4) showed that the reaction rates with GSH, the endogenous degradation rate and the K_{OW} (used to calculate tissue blood partition coefficients) were the parameters with the strongest influence on the outcome of the forecast. Of these parameters, $K_{DEG}G$ and $k_{GST}G$ are animal specific parameters and could therefore be responsible for the large observed variability in GSH concentration of gill in exposed fish.

DISCUSSION

In vitro biotransformation of EA

Variation in GSH and tissue homogenate concentration in the incubation mixtures were used to separate the three major metabolism pathways as described above. It should be mentioned however, that the approach used here assumes first order kinetics for all processes involved. This is only true if EA and GSH concentrations are below the K_M value of the involved enzymes. Because no information on K_M's of EA in rainbow trout was available, literature data of related K_M's was used to get an indication whether these conditions were met. Studies on enzymatic transformation of EA in rats (*13*) reported that K_M's for EA are in the millimolar range for both carboxylases and GST. This is much higher than the 100 μM used in our in vitro experiments. If data for GST of different fish species are comparable,

then the concentration of GSH used here (1mM) was also below reported K_M's for fish-GST (*37*). The model might however, underestimate the importance of enzymatic conjugation due to the presence of high affinity GST. The advantage of this 'sum of 1st order' approach was that neither the use of enzymatic inhibitors nor dialysis of the homogenate were necessary. Although these two methods are frequently used to isolate metabolic pathways they contain some inherent drawbacks. Often, high concentrations of inhibitor are needed to achieve complete inhibition (*34*) and enzymatic activity can be lost during the dialysis process (*29*).

The applied test system, using headspace SPME for estimating biotransformation rates is a promising and very simple technique. For volatiles like EA, headspace SPME injections were found to have a very low background signal compared to classical solvent injections.

Pharmacokinetic model

The pharmacokinetic model for EA forecasted that an equilibrium concentration was reached quickly (less than 0.1 min) in gills and arterial blood (figure 4 d-e). If all elimination constants were set to zero, only a very small increase for these concentrations could be detected. Presystemic branchial elimination, which was found to be important for the kinetics of ethyl-hexylphthalate (*28*) did not seem to be important for EA under the given exposure scenario. In less than one hour, an equilibrium EA concentration was reached for all but the fat compartment. The mass balance showed, that the size of an organ can be as important for the disposition of EA as the specific metabolic activity. Muscle tissue was predicted to eliminate twice the amount of liver tissue although, enzyme activity in the liver is approximately 60 times higher. Additionally, the model suggests, that even for a moderately hydrophobic compound like EA, the fat compartment governs both whole-body kinetics (figure 4 e . and f) as well as the whole-body burden. After 24 hours, 70% of the body burden was located in the fat tissue and 25 % in the muscle. These findings, however should be taken with care because EA metabolism in rainbow trout fat tissue had not been determined.

Pharmacodynamic model: GSH turnover

Parameters for GSH turnover in gills of rainbow trout have not been measured earlier, so we only could compare our data with data from rat models. In rat hepatic tissue, a synthesis rate, S_0 of 2500 μM/h was reported by D'Suoza et al. (*32*) and of 630 μM/h by Frederick et al. (*13*). The synthesis rate was much lower in other tissues; 12.5 μM/h in the lung and 4.2 μM/h in muscle . These synthesis rates are closer to the value of 63 μM/h that was esti-

mated from in vivo depletion data for gills in the present report (figure 3). An endogenous elimination rate for GSH, K_{DEG} of 0.068 /h was calculated for the gills. Again, this value lies between reported degradation rates for rat liver (0.14 /h) and rat muscle (0.006 /h) (*13*).

The model prediction for depletion of GSH in the gills after 24 hours were in close agreement with the experimental data. Predicted depletion during the first 6 hours could not be compared with experimental data because the variance of experimentally observed GSH concentrations in exposed fish was very large (figure 3). The pharmacodynamic model for GSH turnover and conjugation gives a reasonable forecast of the observed GSH depletion. However more data for exposure periods longer than 6 hours are needed to validate the model and to provide a more definitive link between GSH depletion and lethality.

The preliminary PBPK-PD model was able to provide insight in the governing processes of pharmacokinetics and -dynamics of ethyl acrylate. Major data gaps are the partition coefficients and the metabolism rate of EA in the fat tissue. Important uncertainties remain for the gill/water partition coefficient and the parameters of the endogenous turnover of GSH in the gills.

Comparisons with the rat model

The preliminary model for EA in the rainbow trout shows, that not first pass effect occurs, because transport across the gills outruns gill metabolism. The opposite was found for oral and inhalation exposure of EA in rats (*12, 13*). This leads to a marked difference in distribution between the two species. In the rat, EA is only present at the site of absorption, while a systemic distribution is predicted for the fish. Despite these differences in distribution, local toxic effects seem to dominate in both species. Severe GSH depletion occurs in gills, as well as in the stomach epithelia. For EA, histological lesions have not yet been investigated, but from trouts exposed to acrylamide (a comparable Michael acceptor) it is known that gill lesions occur at sub-lethal levels of exposure (*17*).

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APPENDIX

Mass balance differential equations

Amount in gills:

$$\frac{d}{dt}A_G = MIN(QP, QC * P_{BW}) * (C_{AQ} - \frac{C_V}{P_{BW}}) + C_V * QC - C_A * QC - (K^G_{GSH+GST}) * C_G * C^G_{GSH} * V_G$$

Concentration in arterial blood

$$C_A = \frac{A_G}{V_G * P_{GB}}$$

Amount in liver:

$$\frac{d}{dt}A_L = (Q_L + Q_R) * \left\{ \frac{Q_L * C_A + Q_R * C^R_V}{Q_L + Q_R} - C^L_V \right\} - \left\{ k^L_H + K^L_{GSH} + K^L_{GST} \right\} * C_L * V_L$$

Amount in muscle:

$$\frac{d}{dt}A_M = (Q_M) * \left\{ C_A - C^M_V \right\} - \left\{ k^M_H + K^M_{GSH} \right\} * C_M * V_M$$

Amount in other tissues (i: K, F and R):

$$\frac{d}{dt}A_i = (Q_i) * \left\{ C_A - C^i_V \right\}$$

Concentration in efferent blood from tissue:

$$C^i_V = \frac{C_i}{P_{iB}}$$

Amount in venous blood:

$$\frac{d}{dt}A_V = Q_F * C^F_V + 0.4Q_M * C^M_V + (0.6Q_M + Q_K) * C^K_V + (Q_L + Q_R) * C^L_V - QC * C_V$$

Amount glutathione in gills:

$$\frac{d}{dt}A^G_{GSH} = S^G_0 * V_G - K^G_{DEG} * C^G_{GSH} * V_G - (k^{7.4}_{GSH} + k^G_{GST}) * C_G * C^G_{GSH} * V_G$$

AN ELEMENTARY PHARMACODYNAMIC MODEL (EPD) FOR THE ANALYSIS OF TIME DEPENDENT AQUATIC TOXICITY DATA OF REACTIVE CHEMICALS: HABERS LAW REVISITED

CHAPTER 7

Andreas P. Freidig
Joop L. M. Hermens

ABSTRACT

Few existing models in aquatic toxicity are able to explain the time dependence of toxic effects of reactive chemicals. None of these models can give a satisfactory explanation for the often observed threshold concentration, below which no toxic effects is observed. A new model is proposed that can describe the dynamics of a toxicologically relevant target site and the interaction between this target site and a reactive chemical. Under certain conditions, this elementary pharmacodynamic (EPD) model can be reduced to an equivalent of Haber's law, which states, that exposure concentration multiplied by exposure time is constant (Cxt=constant). The EPD-model can furthermore explain the appearance of a threshold concentration. To test the model, literature data of time dependent acetylcholinesterase inhibition and acute toxicity caused by a series of organophosphorus esters were reanalyzed. Data from four species and seven chemicals was used. The EPD-model fits of the observed effect data very well and yields physiologically meaningful parameters. The model was also successfully applied to toxicity data of reactive chemicals. It is concluded, that the EPD model provideds a useful framework for the comparison of species, target sites and chemical potencies.

INTRODUCTION

Today, organic chemicals are used in large volumes in several industrial processes. Part of these chemicals are reactive organic substances. Often, they are so called electrophiles, which means that their structure prefers certain types of chemical reactions with other molecules (*1*). Reactive organics can have a very high acute toxicity. This high toxic potential together with the fact that they are used and transported in large quantities requires a thorough risk assessment. It is therefore necessary to characterize both occupational as well as environmental hazards and exposure . Because experimental information about toxic effects is often incomplete it is usually not possible to characterize the hazard for all exposure scenarios. In response to these data gaps, risk assessment procedures strongly depend on empirical safety factors to extrapolate results from laboratory studies to real exposure scenarios. Faced with the large number of chemicals submitted for registration each year, regulatory offices have a growing acceptance for models which try to fill such data gaps. During the last years, a number of models have been presented in the field of aquatic toxicology, which address different aspects in the process of risk assessment of reactive organic chemicals (*2-11*). Only few of these models models can describe the relation between toxicological endpoint and exposure time. In this paper, we present a new approach that can address the time dependence of toxic effects in aquatic animals and that can give a meaningful interpretation of observed threshold concentations. This elementary pharmacodynamic (EPD) approach can furthermore give a mechanistic interpretation of the empirical formula *Cxt=constant*, more commonly known as Haber's law in inhalation toxicology (*12*).

A short scope of the EPD model for aquatic toxicity of reactive compounds

The toxicity of reactive chemicals is thought to be caused by the interaction of the chemical with essential biological target molecules in sensitive organs. A PBPK-PD model would be the first choice to model all processes involved in the toxicity of a reactive compound. Yet, the data which are necessary to establish such a model are often not available from literature. Here, we propose the use of a simplified PD model which only describes the interaction between chemical and target. Doing so, we ignore the kinetics of uptake, elimination and distribution of the chemical. This seems to be a serious limitation of the model. However, this is not necessarily the case for small aquatic test organisms which are exposed to constant aqueous concentrations. Fast kinetics and a low first-pass elimination are more likely the rule than the exception in such animals. The fast kinetics of low to medium hydrophobic compounds was shown by Lien et al. (*7*) for fathead minnows, a commonly used test species. In this thesis (Chapter 6) we showed with a PBPK model for rainbow trout, that

the concentration of a compound in the gills and in the arterial blood is at equilibrium with the external exposure concentration after a very short exposure time. Metabolism in the gills was furthermore found not to influence the systemic concentration of the compound. Together, these findings indicate that kinetics probably have a minor influence on the concentration at the target.

The formalism of the proposed elementary pharmacodynamic model (EPD) is given below. It is in essence a 1st order linear inhomogenous differential equation. Under certain conditions, the model merges with the so called Haber's law, used mainly in inhalation toxicology, which states that Cxt will be constant.

MATERIAL AND METHODS

The model is based on three assumptions:

Assumption 1:

The chemical reacts with the biological target according to a second order rate law.

Assumption 2:

The concentration of the biological target results from a balance between zero order synthesis and 1st order elimination.

With these two assumptions, the following differential equation for the target concentration can be established:

$$\frac{dT}{dt} = S - k_E * T - k_R * C_R * T \qquad \text{(EQ 1)}$$

with:

T: concentration of the biological target

C_R: concentration of the reactive chemical at the target site

k_R: 2nd order reaction rate constant between chemical and target

S: endogenous synthesis rate of target

k_E: endogenous (1_{st} order) elimination rate constant of target

As the pharmacokinetics of organic chemicals are very fast in small aquatic animals ((*7*) and Chapter 6), a third assumption can be introduced in the model:

Assumption 3

The concentration of the reactive chemical, C_R at the target site is constant and can be approximated by a measured aqueous exposure concentration C_{EXT}.

Based on these assumptions, the differential equation 1 becomes a first order linear inhomogenous differential equation, which can be solved as follows (*13*):

$$T(t) = T^{\infty} + \left(T^0 - T^{\infty}\right) * e^{-\left(k_E + k_R * C_{EXT}\right)*t} = T^0 e^{-\left(k_E + k_R * C_{EXT}\right)*t} + T^{\infty}\left(1 - e^{-\left(k_E + k_R * C_{EXT}\right)*t}\right) \quad \text{(EQ 2)}$$

with t: exposure time and

$$T^{\infty} = \frac{S}{\left(k_E + k_R * C_{EXT}\right)} \quad \text{(EQ 3)}$$

Equation 2 can be used to model the depletion of target concentration (which could also be given e.g. as activity of an enzyme) during chemical exposure. If the concentration (or activity), T^0 for non-exposed animals is known, equation 3 can be expressed as follows:

If T^0 is known, $S = T^0 k_E$ and T^{∞} can be written as

$$T^{\infty} = \frac{T^0}{1 + \frac{k_R * C_{EXT}}{k_E}} \quad \text{(EQ 4)}$$

The synthesis rate, S is thereby eliminated from equation 2.

Dependence on exposure time and aqueous concentration

The model, as given in equation 5 can be used to describe time dependent decrease of a target under different exposure concentrations. C_{EXT} and t are thereby the independent variables, $T(t, C_{EXT})$ is the dependent variable. k_E and k_R are parameters which can be fitted to experimental observations.

$$T(t) = T^0 \frac{1}{1 + \frac{k_R * C_{EXT}}{k_E}} + \left(T^0 - T^0 \frac{1}{1 + \frac{k_R * C_{EXT}}{k_E}}\right) * e^{-(k_E + k_R C_{EXT})t} \quad \text{(EQ 5)}$$

In figure 1, an example for a hypothetical compound and target is given for a high and a low aqueous exposure concentration to visualize equation 5

LC_{50} - time relation

So far, we only tried to model the target concentration. However, it is important to link the target model with observable toxic effects. Therefore we make a fourth assumption:

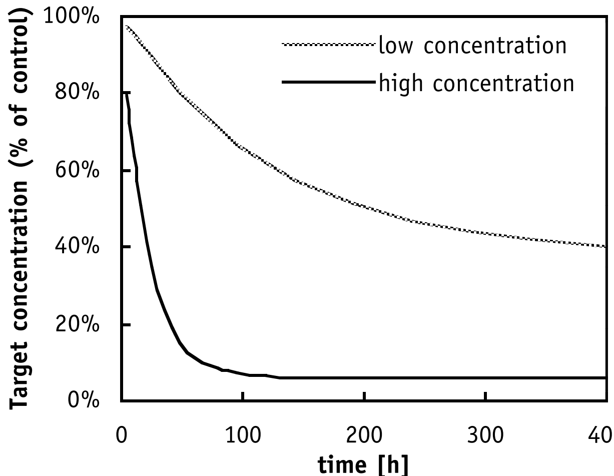


Figure 1: A hypothetical example of a target, described by the elementary pharmacodynamic model (EPD). Target concentrations which are caused by exposure to two different concentrations of a reactive chemical were predicted by equation 5. Both exposures will lead to a new steady state target concentration, T^{∞}.

Assumption 4

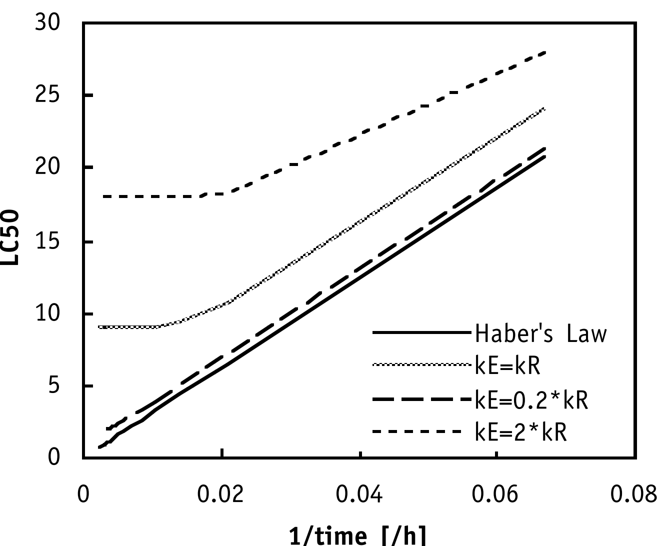
A toxic effect will appear if an essential target is depleted below a critical level, T^{CRIT}. This level is time independent.

If the target model (equation 5) can be established, the species specific parameter k_E and the compound specific parameter k_R can be estimated. The model can then be applied to explain the time dependence of the effect concentration. Observed LC_{50} values and exposure times are entered in equation 5, together with k_E and k_R. The resulting target concentration, T(t) can be interpreted as the critical level, T^{CRIT} that leads to 50 % mortality. Using equation 5 and a given critical depletion level, predictions for $LC_{50}(t)$ are possible. Due to the nature of the equation, no analytical solution for $LC_{50}(t)$ can be given and a numerical solution must be fitted. Another interpretation of equation 5 yields the threshold value for toxicity, $LC_{50}(\infty)$ (also called incipient LC_{50} (*14*)) below which the critical target concentration will not be reached, even after an infinite exposure period. $LC_{50}(\infty)$ can be calculated from equation 4 if T^{CRIT} is set equal to T^{∞} and the equation is solved for LC_{50} (equation 6).

$$Threshold \quad LC_{50} = LC_{50}^{\infty} = \frac{k_E}{k_R}\left(\frac{T^0}{T^{CRIT}} - 1\right) \qquad \text{(EQ 6)}$$

In general, effect data about a possible target are not available and only mortality data are reported. Although equation 5 can not be applied in these cases, it is possible to use the basic approach, given in equation 2 to explain time trends of such data and to extract meaningful parameters from such data sets. For a hypothetical example, the influence of the target dynamic (expressed by the elimination rate constant, k_E) on the form of the time-LC_{50} relationship is shown in figure 2. Predicted LC_{50} values are thereby plotted against the in-

Figure 2: Relationship between LC_{50} and time as predicted by the EPD model. A fixed critical target concentration of 10 % and a k_R value of 0.007 ($h^{-1}\mu M^{-1}$) was used for the model calculations. The resynthesis rate k_E was varied to investigate the effect of the dynamics of the target site. For a low k_E (slow target site resynthesis) the EPD model falls togeter with Haber's law, which states the product of effect concentration and time (Cxt) is constant.



verse of time. A T^{CRIT} of 10 % and a k_R of 0.007 ($h^{-1}\mu M^{-1}$) were used in this example. For high target turnover rates (high k_E), a threshold concentration is predicted, which is constant above a certain exposure time (low 1/t). If the turnover of the target is slow (low k_E) the EPD-model can be approximated by a straight line. This situation can be described with equation 5 if the terms T^{∞} and k_E are omitted. T(t) can then be approximated by an exponential decay curve as shown in equation 7.

$$T(t) = T^0 e^{-(k_R C_{EXT})t} \qquad \text{(EQ 7)}$$

Consequently, C_{EXT} can be expressed as given in equation 8:

$$C_{EXT} = \frac{1}{t} * \frac{\ln\left(\frac{T^0}{T^{CRIT}}\right)}{k_R} \qquad \text{(EQ 8)}$$

or more trivial:

$$LC_{50} = \frac{a}{t} \qquad \text{(EQ 9)}$$

Generally it can be concluded, that the EPD model predicts a constant LC_{50} value if exposure times are sufficient. A threshold value for toxicity is thus an intrinsic property of the EPD model.

The linear relation, as given in equation 9, between exposure concentration and exposure time has already been described by Haber and Flury (*15*). It is known since then as Haber's law. In a recent review paper on the history of Haber's law (*12*) it can be seen that a deviation from linearity for long exposure times was already noted by Flury. He intro-

duced a ‘detoxification factor’ to account for this behavior.

To test the EPD model, time dependent fish toxicity data was collected from the literature. Coefficients of determination (R^2) were calculated according to Miller at al. (*16*). Numerical solutions of equation 5 were calculated with the spreadsheet program Excel (Microsoft, Redmond, WS).

RESULTS

Modeling target interaction

To test the EPD model we analyzed literature data about enzyme inhibition caused by organophosphorous (OP) esters. Data about four different aquatic species and two different organophosphothionates were used (*17, 18*). Equation 5 was fitted to the reported data, using the exposure time and the exposure concentration as independent variables and the acetylcholinesterase (AChE) activity as a surrogate for the target concentration. Both, the original data and the fitted model are shown in figure 3 a-d. Two model parameters, k_E and k_R were fitted for each dataset and are given in table 1 together with the resulting coefficient of determination (R^2). The model provided an excellent fit for the pond snail and the mosquitofish data, a good fit for the water flea and a reasonable fit for the guppy, where the 1st timepoint of the low exposure group was excluded from the fitting process. Once, the enzyme inhibition models were established, reported LC_{50} values were used to calculate the

Table 1: Fitted parameters for the EPD-models which describe the enzyme inhibition data from figure 3 a-d. Equation 5 was applied to the data using time and concentration as independent variables and resulting enzyme inhibition as dependent variable. For the mosquitofish, cholinesterase activity was used instead of acetylcholinesterase.

	normal AChE act. (nmolmin^{-1}mg prot.$^{-1}$)	k_E (h^{-1})	k_R ($h^{-1}\mu M^{-1}$)	R^2	LC_{50}[a] (µM)	critical AChE act. (% contr.)
Chlorthion						
Pond snail	226	0.00055	0.0074	0.94	4.3	1.6%
water flea	10	0.0040	16.99	0.87	0.021	1.1%
guppy	576	0.0057	0.025	0.63	4.03	5.4%
Chlorpyrifos						
mosquitofish (muscle)	208	0.058	0.713	0.99	0.43	16.0%
mosquitofish (brain)	331	0.040	0.568	0.99	0.43	14.1%

[a] 4 d LC_{50} for guppy taken from Pickering et al. (*24*), water flea 2 d LC_{50} and pond snail 14 d LC_{50} data taken form Legierse (*17*) and 4 d LC_{50} for mosquitofish from Boone et al. (*18*).

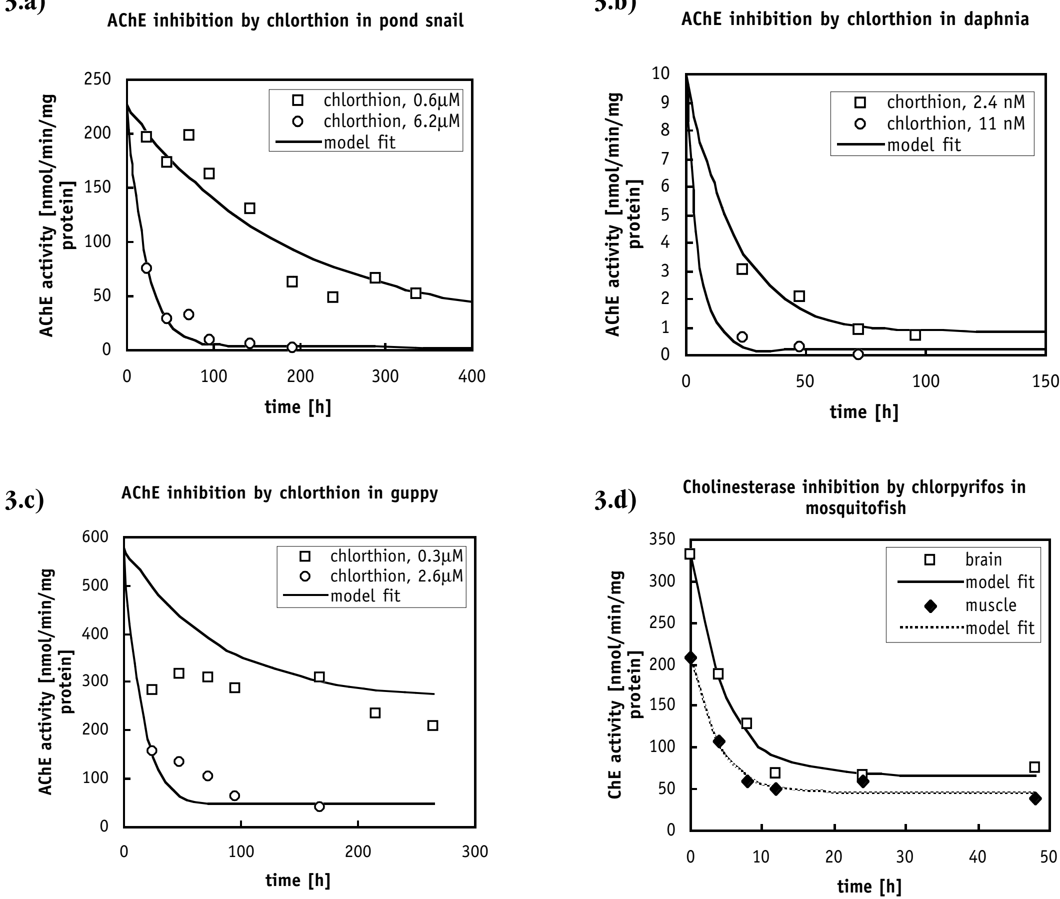


Figure 3 a-d: Experimental data of acetylcholinesterase (AChE) inhibition in aquatic organisms (*17, 18*) was fitted using the EPD model. Data from high and low exposure concentrations were fitted simultanously by a weighted least square fit (3 a-c). Parameters of all fits are reported in table 1.

critical target concentration, T^{CRIT} according to equation 5 (table 1).

Modeling of LC_{50} data

As already discussed above, it is also possible to link observed, time-dependent toxicity with the target model using T^{CRIT}. This could be done for the pond snail, where a set of time dependent toxicity data was published for chlorthion (*6*). In figure 4, the predictions of the EPD model using the parameters from table 1 are given. Predicted values are in reasonable agreement with the observed values (R^2=0.67), but the model overestimates the toxicity of chlorthion for short term exposures (predicted LC_{50} are lower than observed ones).

As discussed above, an approximation of the EPD can be used to analyze time dependent toxicity data sets. For five OP-esters LC_{50} data from one to 14 days were reported by Legierse et al. (*6*) for the guppy (Poecilia reticulata). Plots of LC_{50} data vs. the inverse of time

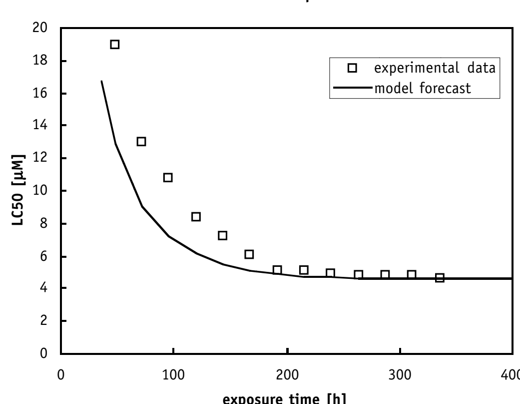


Figure 4: Using the AhE inhibition model (table 1) for the pond snail, the time dependence of the LC_{50} was predicted by the EPD model. A critcal AChE activity of 1.6% of normal activity was used to link target activity and lethality in the model.

(equation 9) are shown for three compounds in figure 5 a-c. The slope of the linear regression is given for all five datasets in table 2. Additionally, using the critical AChE inhibition, T^{CRIT} for guppy, given in table 1, k_R could be estimated for the five OP-esters according to equation 7. It can be seen that toxicity data of all five compounds are well described by a linear approximation (table 2). The estimated reaction rates with AChE vary by a factor of 5 between the six different OP-esters (table 1 and 2).

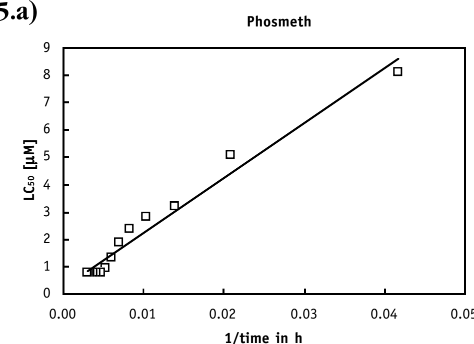
Additional to the OP-ester data, we tested the applicability of the EPD model on data sets for bluegill sunfish given by Bailey et al. (*19*). Reported time dependent fish LC_{50} data for four different compounds, copper sulfate, acrylonitrile, 2-ethoxyethyl acetate and chlorobenzene were transformed according to equation 9 and are presented in figure 6 a-d. Only 2-ethoxyethyl acetate can be described by Haber's law approximation. For acrylonitrile and copper sulfate, the long exposure data deviates from a straight line. Using the EPD model, this can be interpreted as a case where the threshold concentration is reached quickly within the experimental time frame. For chlorobenzene, a threshold value is reached after the first exposure time already (1 hour). The mechanism of chlorobenzene toxicity in fish is known to be narcosis (*11, 20*). The target site for this mode of action is located in the cell membrane and the interaction seems to be completely reversible. Therefore, the EPD model should not be applied to interpret fish toxicity data of narcosis chemicals.

DISCUSSION

Application of EPD model

The fitted parameters, k_E, k_R and T^{CRIT} could be used to compare species and compounds. The parameter k_R is found useful to establish the potency of different chemicals and to

5.a)



5.b)

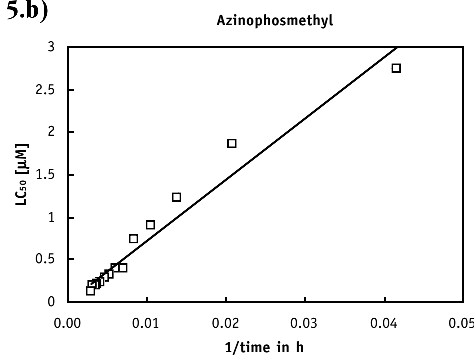
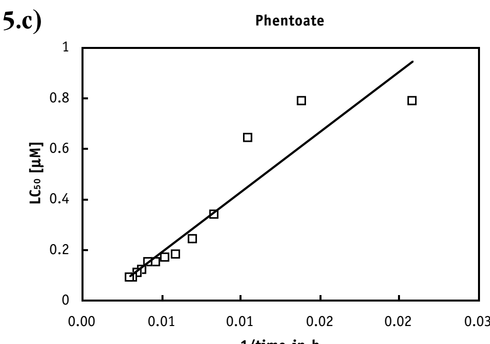


Figure 5 a-c: Plot of time/toxicity data for OP-esters in guppy (P. reticulata). The data are linearized according to Haber's law, using the inverse of the exposure time as the x-axis. According to the EPD model, a linear relation can be expected if the target site has a slow turnover. Using a linear regression, a k_R can be estimated for each chemical if the critical target concentration, T^{CRIT} is known. Fitted parameters for the OP-ester data presented above, are given in table 2.

5.c)



compare the interaction of a chemical with a target in different species. For targets with a slow turnover, k_R can be approximated using a plot of LC_{50} versus the inverse of time. Such a relation has already been reported for toxicity experiments with war gases by Haber and Flury (*12, 15*).

From the first examples (figure 3 a-d and table 1) it can be concluded that the overall 2nd order reaction rate between AChE and chlorthion (k_R) differs about three orders of magnitude between the water flea the pond snail and the guppy. The estimated reaction rate of chlorthion in the water flea (table 1) was close to the in vitro inhibition rate, k_i of chloroxon with eel AChE (k_i=11 $h^{-1}\mu M^{-1}$), as reported by DeBruijn et al. (*21*). This indicates, that chlorthion may be transformed very efficiently to chloroxon in the water flea, which is in agreement with the high sensitivity of waterflea towards this compound (table 1). For the other species, k_R was much lower than the k_i for the oxon-analog. This can be caused by a slow activation of the thionate to the active oxon and consequently, a low chloroxon concentration.

From comparison of the species specific T^{CRIT} in table 1, it can be concluded that AChE is more vital in fish than in invertebrates: the two fish species tolerate less enzyme inhibition (T^{CRIT}=5-15% of control) than the invertebrates (T^{CRIT}=1-2%).

The parameter which describes the dynamics of the target site, K_E could be used to

Table 2: Fitted parameters obtaind from the fish toxicity data of OP-esters in guppy, shown in figure 5 a-c (data for methidathion and malathion not shown). Linearization of the time/toxicity data reveals a good agreement of the experimental data with Haber's law. Using a critical activiy, T^{CRIT} for AChE in fathead minnow of 5.4% (table 1), k_R could be calculated from the slope of the regression.

	slope (µMh)	k_R ($h^{-1}µM^{-1}$)	R^2	LC_{50} (4d)[a] (µM)
Methidathion[b]	40	0.073	0.89	0.48
Azinophosmethyl	72	0.041	0.96	0.9
Phosmeth	201	0.015	0.97	2.84
Malathion	219	0.013	0.98	3.92
Phenthoate	48	0.061	0.88	0.64
Chlorthion		0.025		4.03

[a] LC_{50} data taken from deBruijn et al. (*21*).
[b] First timepoint (1h) was excluded from analysis.

compare species differences in sensitivity and target site differences. From the k_E for AChE in table 1, half-lives of resynthesis of enzyme activity were found to range between 0.5 and 60 days for different species. These values could be compared to in vivo experiments from literature. Abbas et al. (*2*) reported resynthesis half-lives of AChE in rainbow trout of 7 days in liver and heart and of about 400 days in the brain after exposure with paraoxon. The characterization of AChE derived from the EPD model seems comparable to in vivo experiments.

Evaluation of EPD-model

Concluding, the EPD model was found to be a powerful approach to describe time dependent AChE inhibition and acute toxicity due to OP-esters. The EPD model was furthermore able to explain the time dependence of LC_{50} data of other reactive chemicals which were expected to have a different target site (figure 6). For compounds like acrylonitrile, we showed earlier that the reaction rate constant towards glutathione, k_{GSH} can be used as a surrogate for k_R ((*22*) and Chapter 3 and 4). In a PBPK-PD model (Chapter 6 of this thesis), GSH depletion in the gills of rainbow trout could be explained successfully with a PD-model for GSH, similar to the EPD model proposed above. Eventually, the EPD model was found to provide an explicit description of the threshold concentration in toxicity experiments.

Apart of all these advantages, the EPD model has certain drawbacks. It yet lacks a probabilistic link between target concentration and toxic effect. Such a link was incorporated in the DEB models of Kooijman et al. (*5, 23*). The EPD could be improved by such an

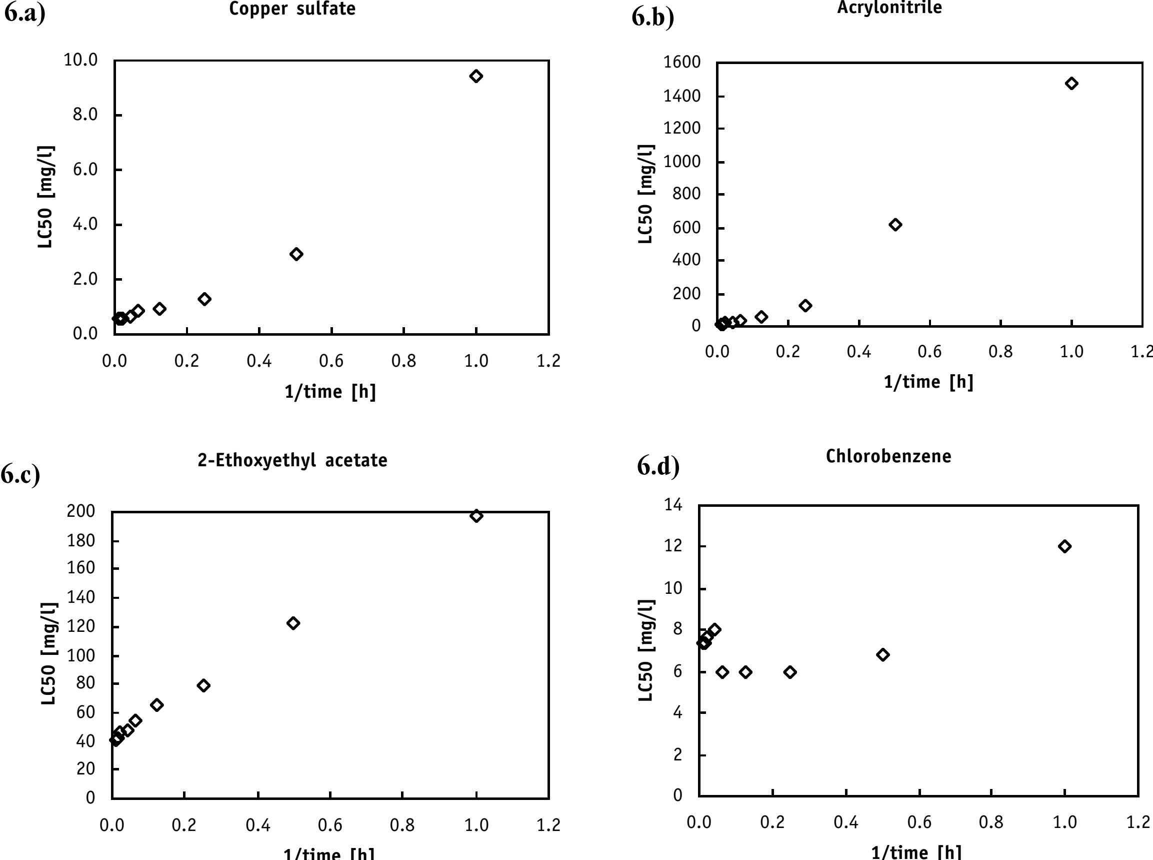


Figure 6 a-d: Experimental data of time/toxicity relationship of different chemicals for the blugill sunfish (L. macrochirus) (*19*). The data are plotted using the inverse of exposure time as x-axis. For target sites with slow dynamics, a straight line could be expected. Deviation from the straingt line towards a threshold toxicity value can be expected for target sites with a fast turnover. Fish toxicity of chlorobenzene is known to be caused by narcosis (figure 6.d). The toxicity this compounds shows almost no time dependence. The EPD model can not be used to explain the toxicity of narcosis compounds because of their reversible interaction with the target site.

extension. Another drawback is formed by the assumptions which were necessary to establish the model. They exclude *a priori* some chemicals, species and target sites.

CONCLUSIONS

This paper provided a short overview of existing models, which describe the aquatic toxicity of reactive organic chemicals. From this overview it can be concluded, that neither of the modeling approaches can cover all aspects of a risk assessment of these chemicals. Instead, each model covers a specific area and can consequently answer only specific questions. The simultaneous use of different models is certainly the most promising way to use

models for risk assessment purposes.

The new approach, forwarded here as EPD model, has its strength and limitations, just as the other existing models discussed above. The aim of the EPD model was to improve the understanding of time dependent toxicity data of reactive organic chemicals. Its strength is the use of physiologically relevant parameters in a simple pharmacodynamic model. The EPD model was found to be able to describe a target site and the interaction of the reactive chemical with this site. It suggests that steady state kinetics are a good first approximation when analyzing aquatic toxicity data. The model can provide a basic framework to analyze toxicity data of reactive chemicals. It can thereby fill a gap between the complex PBPK-PD models and the simple classification schemes.

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CHAPTER 8

SUMMARY AND GENERAL DISCUSSION

"All generalizations are dangerous,
-even this one"
Alexandre Dumas

THESIS SUMMARY

A quantitative structure property relationship (QSPR) for α,β-unsaturated carboxylates (mainly acrylates and methacrylates) was established in ***chapter* 2**. Chemical reaction rate constants were measured for 12 different chemicals with three different nucleophiles, namely H_2O, OH^- and glutathione (GSH). Relatively small differences were found in hydrolysis rates (reaction with H_2O and OH^-). At an elevated pH (8.8) the hydrolysis half-life of the compound ranged between 7 and 40 days, with exception of diethyl fumarate (0.4 day). A separation in two groups was observed for the reaction with GSH (Michael addition), where acrylates reacted approximately 100 times as fast as methacrylates. This difference was consistent with differences found in electronic structure, which was determined by quantum-chemical calculations. Because no single parameter could describe the electrophilic character of the unsaturated carboxylates satisfactory, four descriptors were pooled, using a multivariate correlation (partial least squares regression, PLS). The resulting QSPR for Michael addition was able to predict the reactivity of structurally related, unsaturated carboxylates.

Acute fish toxicity of a set of acrylates and methacrylates was evaluated in ***chapter* 3**. Published four-day LC_{50} data for fathead minnow were compared to the chemical reactivity of the compounds towards GSH, because Michael addition was expected to be the mechanism that causes harmful binding to essential biological thiol-sites in the fish (e. g. proteins and enzymes). A simple equation was used to model the interaction of electrophilic chemicals with GSH. The degree of GSH depletion, which was used to estimate the toxic effect, was found to be related to the product of aqueous exposure concentration and chemical reaction rate of the reactive compound. Although, all acrylates and methacrylates potentially could react with GSH, narcosis was judged to be an alternative mode of toxic action responsible for the observed acute toxicity. Potencies for GSH depletion and narcosis were compared on the bases of critical body residues and critical depletion rates. Five out of 12 compounds were thereby identified as narcosis chemicals on the bases of their high calculated lethal body burden. It was concluded that, although the tested chemicals all contained a similar functional group, their mode of action regarding acute fish toxicity was not the same. Therefore, a correlation between chemical reaction rate and LC_{50} for the whole test set of chemicals would not be meaningful.

The results from chapter 3 indicated, that narcosis was an interfering mode of action in QSAR's for fish toxicity of reactive chemicals. To evaluate this hypothesis, data of reactive chemicals from three different classes (unsaturated carboxylates, organophosphorus esters

and nitrobenzenes) were taken from the literature and subjected to an analysis for multiple modes of action (***chapter 4***). The Toxic ratio, being the ratio between the observed LC_{50} and the LC_{50}, predicted for the same compound by a narcosis QSAR was used to estimate the probability of a compound to act by narcosis. In total, 40 % of the 61 compounds tested were identified as "probably acting by narcosis". For these compounds, a narcosis QSAR using the octanol/water partitioning coefficient (K_{OW}) as sole descriptor was found to describe the toxicity. QSAR's using reactivity descriptors, which in earlier work had been found insufficient to describe the toxicity of these classes of compounds improved considerably, if the "narcotic chemicals" were excluded from the data sets. It was concluded, that narcosis should always be considered as a possible alternative cause of death in acute fish toxicity test, even if the chemicals seem to have a very specific mode of action. Additionally, it was shown, that QSAR's should only be established for sets of chemicals with an identical mode of action. Modes of action clearly should not be confused with functional groups.

The toxic effect of acrylates and methacrylates on a cellular level were investigated in ***chapter 5***. Cellular glutathione (GSH) concentrations were recorded in isolated cells of rat livers. These cells have a continuous high expression of GSH and a broad range of metabolism. Potentially toxic metabolites of the acrylates and methacrylates were therefore likely to be produced in these cells. Furthermore, the additivity of the toxic effect of these chemicals was investigated in this in-vitro test. For each chemical, an EC_{50} for GSH depletion was determined and used as an effect equivalent to compare their potencies. By testing two mixtures, each containing six individual chemicals, it could be shown that the depletion of GSH was dose-additive. This means that in a mixture of acrylates and methacrylates each individual chemical will contribute to the total toxic effect of the mixture. As expected, the compounds were metabolized by the hepatocytes. For one of them, allyl methacrylate, the very toxic metabolic product acrolein could be identified in the cell-culture medium. The production of this metabolite is most probably responsible for the high toxicity of this specific compound towards the liver cells as well as towards fish (chapter 3).

A preliminary physiologically based pharmacokinetic and -dynamic model (PBPK-PD) for ethyl acrylate (EA) was presented in ***chapter 6***. It was based on an existing PBPK model for inert compounds in fish, which had been established by the US-EPA in Duluth, MN (*1, 2*). The model was adapted to be used with EA by adding elimination processes in several tissue compartments. Elimination rates of EA, which had been measured in-vitro, were extrapolated to whole organs. The turnover of GSH in the gills was modeled separately and was used to describe the toxic effect of EA on biological targets. Once the model was estab-

lished, several aspects of an aqueous exposure scenario were investigated. The uptake of EA in different organs of the fish was predicted to occur very rapidly (steady state concentrations reached in minutes to a few hours) with exception of the fat tissue. The metabolic elimination of EA in the gills was not sufficient to cause a notable first pass effect. Consequently, the EA concentration in the gill tissue was predicted to be almost instantaneously at equilibrium with the aqueous exposure concentration. The EA concentration in the gills was subsequently used in the biological effect sub-model to describe the depletion of GSH. For a simulated exposure scenario close to a lethal aqueous concentration, the GSH concentration in the gills decreased by 60 % during the first 6 hours. This forecast was in agreement with experimental observations. In contrast to an existing rat model for EA, the trout model did not predict a first pass elimination of EA and therefore a systemic distribution can be expected in the fish. In both models, however, a local depletion of the GSH level at the site of adsorption was evident.

In ***chapter 7***, several findings from the previous chapters were combined to postulate an elementary approach to model toxic effects of reactive chemicals in aquatic organisms. The most important simplification of this approach was, to disregard the pharmacokinetics of moderately hydrophobic reactive chemical in aquatic organisms. This resulted in a elementary pharmacodynamic model (EPD), which describes a target and the interaction of a reactive chemical with this target. This approach can be used to describe time and concentration dependent toxicological effects. Models, based on this approach were found to give excellent description of experimental data on acetylcholine-esterase inhibition due to OP-esters in several aquatic animals. The approach was also able to predict time dependent effect concentrations (e. g. LC_{50}). Under certain conditions, the EPD model can be reduced to an equivalent of Haber's Law, which states that the product of concentration and exposure time will be constant. In addition to this, the EPD model can give a rational interpretation of threshold concentration, which are often observed in toxicity experiments.

GENERAL DISCUSSION

Risk assessment of α,β-unsaturated carboxylates

A group of α,β-unsaturated carboxylates was used in this thesis as model compounds to develop and test different approaches for the risk assessment of reactive organic chemicals. Mechanistic understanding of the toxic effects was thereby an important guideline for the development. Within the group of chemicals, three different modes of action (MOA) were

identified that could cause acute toxicity in fish.

Narcosis or general anesthesia was identified as predominant MOA for some compounds by comparing predicted body burdens with literature values for lethal narcosis body burdens (chapter 3 and 4). The outcome of the PBPK model pointed out that despite the rapid metabolism of the compounds, a systemic distribution will take place in fish exposed to constant aqueous concentrations (chapter 6). This supports the validity of the predicted body burdens, which were calculated using a log K_{OW} based bioconcentration factor.

GSH depletion in gills was identified to be the predominant MOA for most acrylates and for the less hydrophobic methacrylates. Histological gill damage as well as loss of plasma ions had been reported in fish exposed to different Michael acceptors (*3, 4*). In this thesis, exposure of rainbow trout to near lethal levels of α,β-unsaturated carboxylates was found to cause significant depletion of GSH in their gills after 6 hours. For Michael acceptors, a constant first order reaction rate can be defined as effect equivalent, which is the product of k_{GSH} and the aqueous exposure concentration. For 4-day lethality of fathead minnows, this critical reaction rate was found to be 1.8 (d^{-1}).

Hepatotoxicity is suggested as predominant MOA of allyl methacrylate. Hepatotoxic effects of the structural related allyl formate in fish were already known from literature (*5*). In in-vitro assays with rat hepatocytes, the toxicity of allyl methacrylate was demonstrated by LDH leakage and GSH depletion at much lower concentrations than for the other tested methacrylates (chapter 5). Additionally, the very reactive metabolite acrolein was shown to be produced in the in vitro system. Enzymatic hydrolysis and subsequent oxidation of allyl methacrylate are required to form acrolein. Both enzymes of this pathway, carboxylases and aldehyde dehydrogenases are present in rainbow trout liver (*6, 7*). Therefore production of acrolein can be expected to occur in the fish as well as in the rat upon allyl methacrylate exposure.

Mixture toxicity of α,β-unsaturated carboxylates was addressed in chapter 5, using primary rat hepatocytes as a model system. GSH depletion was thereby suggested as a useful effect equivalent to measure joint effects of exposure to Michael acceptors. A similar approach may be possible for gill tissue of fish. However, the effect equivalents (EC_{50} for GSH depletion) for hepatocytes can not be applied directly for fish gills as can be seen for ethyl acrylate: a concentration of 2 mM was required to deplete GSH by 50 % in 4 hours (chapter 5) in liver cells, whereas in gills of trout, a hundred times lower exposure concentration (20 μM) was already enough to reduce the GSH concentration by 60 % (chapter 3).

The strategy for effect assessment, as outlined above, uses a comparison of multiple

Table 1: Physico chemical properties of methyl vinyl ketone, a potent Michael acceptor.

name	CAS nr.	MW	log K_{OW} [a]	k_{GSH} ($M^{-1}min^{-1}$) [b]
(3-butene-2-one) methyl vinyl ketone	78944	70.09	0.117	7620 ± 360

[a] estimated using MedChem (*44*).
[b] Freidig, unpublished results.

modes of action which are possible for a compound. Once the most probable mode is identified, a QSAR (if available) can be used to predict the toxicity of this compound. This strategy will be illustrated with ***Methyl vinyl ketone***, a Michael acceptor for which only few toxicological information is available. In table 1, some basic information about the chemical is provided. The high chemical reaction rate with GSH suggests that methyl vinyl ketone acts by the second MOA, namely GSH depletion in the gills. Using the critical reaction rate of 1.8 (d^{-1}) and the model from chapter 3, a 4-day LC_{50} of 0.2 μM can be estimated for the fathead minnow. At this concentration the estimated lipid based whole body burden of the chemical will be about 0.01 μM. This is far below a concentration known to cause narcosis (2-5 mM) and therefore narcosis can be excluded as a predominant MOA. Because methyl vinyl ketone reacts with GSH much faster than ethyl acrylate, a first pass elimination in the gills, which would reduce the systemic distribution can not be ruled out. Nevertheless, lethal defects in the gills of exposed fish can be expected at exposure concentrations around 0.2 μM.

Predictive models for aquatic toxicity of reactive chemicals

Models, presented in this thesis

One of the objectives of this thesis was to develop predictive models for the toxic effects of reactive chemicals in aquatic animals (chapter 1). The models that were developed followed two different approaches. On one hand a statistical approach was chosen, establishing QSAR's presented in chapter 2 and 4. On the other hand, a physiologically oriented approach was used for the PBPK-PD model (chapter 6) and for the EPD model (chapter 7). The EPD model is the most versatile and most interesting model that was developed and therefore, a short scope of this model seems appropriate in this discussion. Before the main features of the EPD model will be discussed, however, it may be useful to critically review the main model strategies that have been used and published for predictive purposes by other authors.

A critical review of existing models

A first, straight forward approach uses the chemical structure to identify reactive chemicals with a high toxic potential. Lipnick (*8-10*) proposed a number of electrophilic functional groups, which were supposed to react directly or after bioactivation with biological nucelophilic targets. If these groups are present in the structure, a chemical is likely to have a high toxicity. Similar expert knowledge models have also been presented by Hermens (*11*) and Verhaar et al. (*12*). Russom et al. (*13*) described a computer assisted model for classification, developed by the EPA in Duluth, MN which essentially follows the same methodology. To estimate the hazard potential of these reactive chemicals, their toxicity can be compared with so called narcosis chemicals of equal hydrophobicity and a ratio of excess toxicity can be calculated (*10, 14*). For acute fish toxicity data, it was shown that reactive chemicals can be as much as 10'000 times more toxic than comparable narcosis chemicals (*12*). Classification models based on expert knowledge rules have distinct advantages for risk assessment procedures. They are easy to apply and to expand when new toxic groups and mechanisms are identified. Furthermore, computers can be used to screen large chemical data sets to identify potential hazardous substances (*15-17*). A drawback of classification models however, is the large uncertainty about the actual toxicity of a compound which forces the use of (probably unnecessarily) large safety factors.

The use of quantitative structure activity relationships (QSAR) for the prediction of acute fish toxicity of reactive organic compounds was introduced by Hermens and coworkers (*11, 18-22*). QSAR's for different substance classes were established using chemical properties which were related to the expected mode of action, such as chemical reaction rates, Hammett constants or pKa values. In the last decade, computational chemistry opened a new field for QSAR research in toxicology (*23*) and many QSAR's with quantum chemical descriptors have now been published for aquatic species (*24-29*). The majority of QSARs are established for structurally related groups of substances. They can be seen as sub-units of the classification models. Only few attempts have been undertaken to establish general models that would be applicable for all reactive chemicals (*30*). In our opinion, however, such huge-models are of little predictive value for the risk assessment of new chemicals and

Figure 1: (Opposite page) Comparison of different modeling strategies that are used to understand and predict the aquatic toxicity of reactive compounds. A graphical representation for each model is given in the second column. Examples and references for the models are given in the text.

Model	Scheme	**Advantages**	**Disadvantages**
Classification schemes	Reactive / Non-Reactive	-useful in prioritizing -uses expert knowledge -no experimental data needed -easy to apply and extend	-large safety factors needed -no variation in exposure scenario possible
QSAR	Log LC_{50} vs. Log $k_{Reactivity}$	-gives quantitative predictions -uses mechanistic kowledge	-predictions only within groups -difficult to establish and apply -no variation in exposure scenario possible
Continous time models	LC_{50} vs. time; NOEC	-different exposure times possible -use of standard test data -easy to apply	-model is compound and species specific -no physiological parameters -uses no mechanistic information
PBPK-PD model	Gills, Liver, Fat, Muscle; C(at target) vs. time; C(of target) vs. time	-different exposure scenarios are possible -uses physiological and mechanistic information -extrapolation to different species and compounds	-lots of experimental data needed -difficult to establish and apply
Elementary PD-model (EPD) (Haber's Law)	C(at target) vs. time; C(of target) vs. time; LC_{50} vs. $\frac{1}{time}$	-different exposure scenarios are possible -uses physiological and mechanistic information -extrapolation to different species and compounds	-many assumptions needed, which increase uncertainty

they will not improve the mechanistic understanding of toxicity. It can be concluded that QSAR models have become a valuable addition to expert systems. For reactive chemicals, however, the predictive capacity of QSAR's remains limited to small, structurally related groups.

Only few theoretical models have been proposed in aquatic toxicology to describe the relation between toxicological endpoints and exposure time. A model which explicitly incorporates time in the interpretation of acute toxicity data was proposed by Legierse et al. (*31*) and Verhaar et al. (*32*). The integral of the concentration in the body of the organism (area under the curve (AUC)) was thereby used to describe time dependent LC_{50} values. The advantage of the model is that an analytical function for $LC_{50}(t)$ can be fitted to experimental data. The fit-parameters of the model are the threshold effect concentration for infinite exposure time (LC_{50}^{∞}) and the critical area under the curve (CAUC). A drawback of the model is, that neither CAUC nor LC_{50}^{∞} can be easily linked to properties of the organism or of the chemical. Kooijman et al. (*33-35*) presented a model for toxic effects in aquatic animals, based on a dynamic energy budget (DEB) approach. Their approach has a very wide applicability as it can model lethality as well as effects on growth and reproduction in relation to exposure time. The lethality model uses a hazard rate, a no-effect-concentration and an elimination rate to fit experimental data. Uptake -and hazard rate can be linked to a mechanistic interpretation of toxic effects and pharmacokinetics of a compound. The no-effect-concentration, however, remains a pure fitting parameter that cannot be related to physiological processes. Both models can be used to extrapolate from short to long exposure times.

Based on the pioneering work by Nichols and coworkers on PBPK modeling for fish (*1, 2, 36-39*), two PBPK-PD models have been established for reactive organic chemicals in rainbow trout (*40*), (this thesis, chapter 6). The models use physiological data (e.g. organ size or blood perfusion rate) to describe the distribution of a chemical in the animal. A relevant target site (e.g. an enzyme or a oligopeptide) is added to model the harmful interaction between chemical and target. One advantage of the PBPK-PD model is that it uses target site concentrations instead of external exposure concentrations. The use of a target site model gives furthermore the possibility to explicitly include the recovering capacity of an organism, a process which is reduced to an empirical no observed effect level (NOEL) in other models.

A short overview of the different approaches, discussed above and of the EPD-approach, which will be presented below, is given in figure 1.

Scope of the EPD model

Mechanistic models form an essential link to understand the relation bewteen chemical structure and toxic effects of reactive chemicals. The EPD model combines a simple pharmacodynamic model with a simple chemical reaction equation. The concept of the EPD model includes the concentration of the target as an explicit variable. Both, the concentration of ***the chemical at the target*** as well as the concentration ***of the target itself*** are of importance. The target is thereby hypothesized to fulfill a vital function in the organism. If the target concentration falls below a critical level, the viability of the organism will be affected. The basic assumptions of that concepts have already been elaborated in chapter 7, so only the resulting differential equation (1) and a graphical representation of the model (figure 2) will be presented here:

Differential equation:

$$\frac{dT}{dt} = S - k_E * T - k_R * C_{\text{target}} * T \qquad \text{(EQ 1)}$$

T: concentration of the biological target

C_{target}: concentration of the chemical at the target

k_R: 2nd order reaction rate constant between chemical and target

S: endogenous synthesis rate of target

k_E: endogenous (1st order) elimination rate (turnover) constant of target

A closer look at figure 2 reveals two possible approximations of equation 1 which yield simple time-effect and concentration-effect relations.

If the critical target concentration is reached within the left shaded area of the curve, the target depletion can be approximated by an ***exponential decay*** curve. This is possible for targets with a slow turnover (recovery) or for chemicals with a high reactivity. If this approximation is solved for the effect concentration (e. g. LC_{50}) it turns out to be an equivalent to the so called Haber's Law (*41*), given in equation 2.

$$LC_{50} * t = \text{constant} \qquad \text{(EQ 2)}$$

A practical example for an exponential-decay approximation of the EPD model is given in chapter 7 of this thesis for the acute toxicity of OP-esters in fish. The slow turnover of the target enzyme acetyl cholinesterases makes such an approximation possible.

The second approximation is possible if the critical target concentration is reached in the right shaded area of the curve (figure 2), close to a ***steady state*** of the target. This implies either a high turnover of the target or a low reactivity of the chemical The steady-state approximation may be useful to describe toxic effects of chemicals on targets with a high

Figure 2: Theoretical mode of the time dependence of a toxicological relevant target (e. g. a protein or enzyme) during exposure to a reactive chemical. The solid line represents a complex model (equation 3) while the two dotted lines show approximations that are possible under certain conditions.

turnover. Depletion of the target will not immediately lead to an observable toxic effect, but the critical target concentration acts more as a threshold, below which damage will accumulate and eventually manifest in a toxic effect. In chapter 3, the depletion of GSH caused by different unsaturated carboxylates could be explained with this approximation. The steady state approximation can be summarized in equation 3.

$$LC_{50} * k_R = \text{constant} \qquad \text{(EQ 3)}$$

The exponential-decay and the steady-state concept were developed to describe toxic effects of reactive chemicals based on a physiological target model. Time-concentration relations but also comparisons between different chemicals are possible with these two approximations. We hope that the EPD model and the two resulting approximations can provide a framework to improve mechanistic approaches for structure toxicity relationships of reactive chemicals.

Computational systems in (aquatic) toxicology and risk assessment.

One of the most important changes during the last 20 years in predictive toxicology was the introduction of computers. In toxicology, computational systems are mainly used for two purposes, for management of existing toxicological data and for generating predictions for new chemicals (*42*). Computer aided data management has tremendously simplified the search and retrieval of toxicological information. Databases like ECOTOX (US EPA, Duluth, MN) ECDIN (ECB, Ispra, Italy) or TOXLINE (NLM, Bethesta, MD) are maintained by regulatory agencies and are (with some limitations) open to the public, providing easy

access to relevant literature references, publications and experimental data. Development of new and better user interfaces and the inclusion of more data can be expected to continue in the next years. For computer based predictive systems, the development seems to proceed considerably slower. Three models have been developed in the last years to predict fish toxicity data (*13, 30, 43*). Yet, no comprehensive external review of these models is available which would allow to judge their performance. A number of critical reviews, however, have addressed scope and limitations of available predictive programs for general toxicity (*15, 16, 42*). Although most of these programs were evaluated on the basis of their capacity to predict mutagenicity, the conclusions of these reviews will also hold for programs that predict aquatic toxicity endpoints because the programs share similar model approaches and algorithms. One of the main problems for the application of computer programs to predict toxic effect, seems to be the gap between developers and users, as stated by Wang et al. (*42*): "Developers, testing their own development report impressive accuracy. The 'real world' is less felicitous." Furthermore, available programs are limited by available data and knowledge so that one should be careful not to place unrealistic expectations in their predictive capacities (*16*). But even if the programs still are in their infancy, they open very interesting perspectives and might in the future lead to a more complete and faster risk assessment of the huge number of existing chemicals. A clear account of what is needed to improve predictive programs was given by Richard (*16*): "Nothing replaces the need, ultimately, for better characterization of biological mechanisms of toxicity and the underlying chemical interactions, and for the use of good judgment in the overall evaluation process of a chemical."

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Nederlandse samenvatting

Achtergrond

Meer dan 100.000 verschillende chemicaliën worden tegenwoordig geproduceerd en jaarlijks komen er honderden nieuwe bij. Bij de introductie van nieuwe chemische stoffen moet de overheid het belang van de producent tegen de belangen van het publiek afwegen. Met name de veiligheid van een nieuwe stof voor mens, dier en milieu speelt daarbij een grote rol. Omdat het onmogelijk is voor alle stoffen voor alle denkbare blootstellingssituaties een risico-evaluatie te maken, wordt vaak gebruik gemaakt van voorspellende methoden. In dit proefschrift worden voor een bepaalde groep chemicaliën, reactieve organische verbindingen, een aantal methoden voor de voorspelling van toxiciteit getoetst en verder ontwikkeld.

Doel van het onderzoek

Om voorspellende methoden een bredere basis te geven moeten ze gebaseerd worden op een betere kennis van toxicokinetische en toxicodynamische mechanismen binnen de organismen die onderzocht worden. Dit proefschrift heeft daarom een drietal doelen:

- Meer inzicht verkrijgen in de werkingsmechanismen die ten grondslag liggen aan de toxiciteit van reactieve chemicaliën.
- Nieuwe benaderingen ontwikkelen waarmee voorspellende toxicologische modellen op een meer fysiologische basis gefundeerd zijn.
- Voorspellende modellen voor de toxiciteit van reactieve chemicaliën ontwikkelen, in het bijzonder voor aquatische organismen.

Het onderzoek

Voornamelijk twee groepen reactieve chemicaliën werden tijdens het onderzoek als model-stoffen gebruikt om de bovengenoemde doelen te bereiken: α,β-onverzadigde carboxylaten (acrylaten en methacrylaten) en organofosfaat-esters (OP-esters). Om meer zicht te krijgen in de fysiologische aspecten van de toxiciteit van de carboxylaten werd glutathion, een belangrijk onderdeel van het metabolisme in cellen, nader onderzocht. Levende vissen, primaire cel culturen, sub-cellulaire *in-vitro* proeven en computer programma's zijn hierbij toegepast.

In **hoofdstuk 1** werden structuren, toxiciteit en enkele blootstellingssituaties voor een aantal voorbeelden uit deze groep besproken. Voor reactieve chemicaliën geldt, dat kleine verschillen in hun chemische structuur grote verschillen in de werkingsmechanismen en daardoor in hun toxische effecten kunnen veroorzaken.

In **hoofdstuk 2** werd een model ontwikkeld om de chemische reactiviteit van een groep van onverzadigde carboxylesters te beschrijven. Empirische parameters en quantum-chemische parameters, die verkregen werden met behulp van computer-berekeningen, werden gecorreleerd met gemeten chemische reactiesnelheden. Dit resulteerde in kwantitatieve structuur-activiteits relaties (QSAR's) voor de reactiviteit met glutathion en voor de hydrolyse-snelheid.

In **hoofdstuk 3** werd de chemische reactiviteit van acrylaten en methacrylaten vergeleken met hun acute toxiciteit voor vissen. Daarbij werd een model gepresenteerd dat de depletie van glutathion in de viskieuwen relateert aan de acute toxiciteit. Verder werd voor enkele stoffen uit de geteste groep een afwijkend werkingsmechanisme vastgesteld (narcose).

De verschillen in werkingsmechanismen van reactieve stoffen zijn verder uitgewerkt aan de hand van literatuur in **hoofdstuk 4**. Het bleek dat structurele gelijkenis, een veel gebruikt criterium om stoffen te groeperen voor een QSAR, tot misleidende conclusies kan leiden. Informatie over werkingsmechanismen blijkt een betrouwbaarder criteria voor een dergelijke groepering te zijn.

Een niet aquatisch systeem, namelijk primaire ratte-hepatocyten, werden gebruikt om combinatie effecten te bestuderen (**hoofdstuk 5**). Om te onderzoeken of het effect van onverzadigde carboxylesters bij blootstelling aan verschillende chemicaliën additief is, moeten een grote aantal dosis-effect relaties bepaald worden. Er is gekozen voor het gebruik van een in-vitro systeem omdat dit het aantal proefdieren tot een minimum beperkt. De uitgevoerde proeven lieten duidelijk zien dat bij blootstelling aan een mengsel van acrylaten en methacrylaten het effect van elke aparte stof bijdraagt aan de totale glutathion depletie in de cellen. Deze resultaten kunnen gebruikt worden bij risicoschattingen voor mengsels met een bekende samenstelling.

In **hoofdstuk 6** werd voor één van de geteste stoffen, ethylacrylaat, een computermodel (physiologically based pharmacokinetic and pharmacodynamic model of kortweg PBPK-PD model) opgezet om de opname en verdeling van die stof in de regenboogforel, en het effect van ethylacrylaat op de kieuwen van de vis te simuleren. Gegevens uit blootstellings experimenten met vissen en uit sub-cellulaire *in-vitro* systemen werden in het model verwerkt. Het model werd gebruikt om bestaande kennis te verenigen en te visualiseren en om experimentele gegevens te verklaren. Met zo een PBPK-PD model is het mogelijk verschillende blootstellings scenario's te simuleren en te vergelijken.

In **hoofdstuk 7** werd een algemeen model gepresenteerd dat op een simpele manier fysiologische een chemische eigenschappen combineert. Dit EPD-model (elementary

pharmacodynamic model) werd ontwikkeld op basis van literatuur over de toxiciteit van OP-esters in aquatische organismen. Het is in het bijzonder geschikt om tijd-effect relaties van irreversibele werkingsmechanismen te voorspellen. Bij een langere blootstellingsduur wordt een toenemende toxiciteit verwacht. Verder geeft het EPD-model een realistische verklaring voor het optreden van drempelwaarden, waaronder geen toxische effecten waargenomen worden. Het model laat bovendien parallellen zien met de wet van Haber, die stelt dat het produkt uit blootstellingsconcentratie en tijd constant is (cxt=constant).

Hoofdstuk 8 bestaat uit een algemene discussie, conclusie en een samenvatting. Voor acrylaten een methacrylaten zijn de belangrijkste werkingsmechanismen in vissen geëvalueerd. Dit zijn narcose, irreversibele binding aan proteïnen in de kieuwen en lever toxiciteit. Verder wordt in dit hoofdstuk een algemene discussie over de waarde en de toepasbaarheid van voorspellingsmethoden in de toxicologie gevoerd. Uit dit onderzoek kan geconcludeerd worden dat farmacokinetische en farmacodynamische modellen beter geschikt zijn dan de tot nu toe in de aquatische toxicologie veel gebruikte chemische modellen. De farmacologische modellen zijn vooral beter, omdat ze de interactie tussen stof en organisme kunnen beschrijven terwijl chemische modellen zich vaak beperken tot een beschrijving van de stof. Uit de hoofdstukken 2 tot 7 blijkt, dat voor een risico-evaluatie zowel theoretische (computer) als praktische (*in-vivo* en *in-vitro*) modellen toepasbaar zijn. Terugkomend op het inleidende citaat van Francis Bacon kan worden geconcludeerd, dat een intelligente combinatie van theorie een praktijk het meeste succes oplevert.

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Curriculum vitae

Andreas Freidig werd geboren op 28 april 1969 te Thun, Zwitserland. Hij behaalde zijn Matura-examen aan het Gymnasium in Interlaken in 1988. Een jaar later begon hij met een studie "Umweltnaturwissenschaften" aan het Swiss Federal Institute of Technology (ETH) in Zürich. Na twee jaar basis studie, die elk met het successvol behalen van een "Vordiplom" afgesloten werden, begon hij 1991 aan de bovenbouwstudie "Umwelthygiene". In het academische jaar 92/93 studeerde hij met een ERASMUS-beurs aan de biologische faculteit van Lund, Zweden. De studie aan de ETH werd in 1995 afgesloten met een "Diplomarbeit" verricht tijdens een stage bij het Research Institute of Toxicology (RITOX) van de Universiteit Utrecht. Vanaf 1996 was hij werkzaam als onderzoeker in opleiding bij het RITOX onder begeleiding van Dr. Joop Hermens. In 1999 was hij tevens 6 maanden werkzaam als onderzoeker aan het Swiss Federal Institute of Environmental Sciences (EAWAG) in Dübendorf bij Prof. Dr. René Schwarzenbach. Tijdens zijn werkzaamheid als promovendus volgde hij de Postdoctorale Opleiding Toxicologie en nam hij deel aan diverse workshops van de onderzoeksschool Milieuchemie en Toxicologie.

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a.f.

Que ça rate, que ça réussisse, après tout, c'est secondaire.
Alberto Giacometti