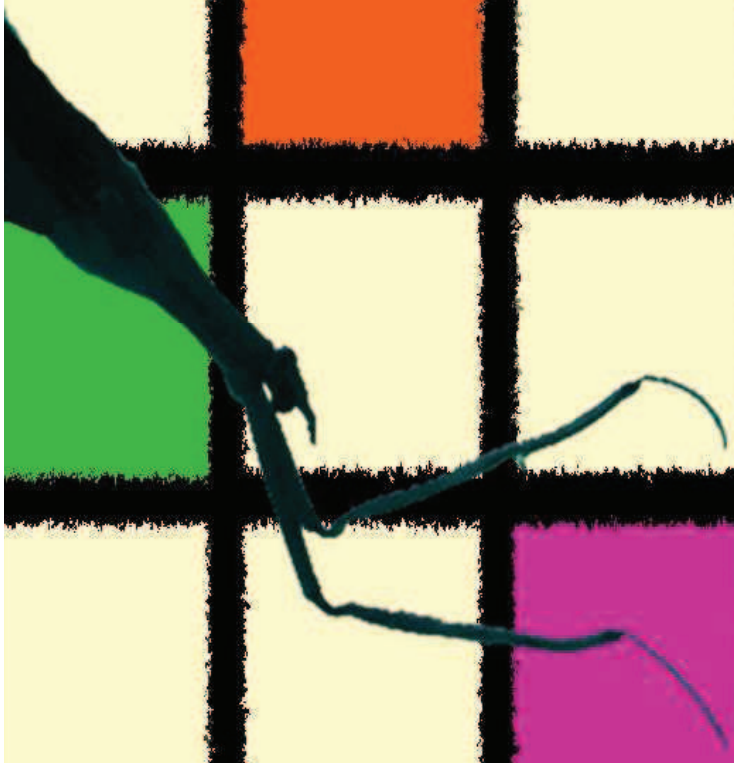
PREDICTING THE RESPONSE OF AQUATIC INVERTEBRATES TO STRESS USING SPECIES TRAITS AND STRESSOR MODE OF ACTION.



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Predicting the response of aquatic invertebrates to stress using species traits and stressor mode of action.

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Predicting the response of aquatic invertebrates to stress using species traits and stressor mode of action.

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Für Hilde und Volker.

CONTENTS

The back cover image is in orginal a piece from Banksy. Colours changed.

Saltlake with flamingos, Atacama, Chile, 2007

GENERAL INTRODUCTION

The ecosystems of the earth are encountering increasing threats from human activities and a growing world population, and in some cases there are already examples of at least partly irreversible environmental degradation (e.g., depletion of fisheries stocks, loss of natural forests, over-exploitation of freshwater resources). The ecosystem's ability to face further challenges without additional permanent damage is to date still relatively unpredictable, not only due to the complexity of multiple stress regimes, but also due to their dynamics. Ecosystem health is crucial for the world's societies, not only from an aesthetic and moral viewpoint, but also in order to sustain ecosystem goods and services that are critical for human welfare such as food and fresh (Millennium Ecosystem Assessment 2005). Anthropogenic stressors like urbanization, habitat loss and fragmentation (Fahrig 2003), and anthropogenic pollutants, coupled with dramatic changes in natural stressors due to anthropogenic activity (such as the increasingly evident climate change) (IPCC 2007) perhaps represent the biggest threats. Combinations of anthropogenic and natural stressors are often the reality, but the extent to which such combinations of stressors may affect the world's ecosystems is poorly understood.

In order to protect ecosystems while maintaining the growing demand for world food production and water supply, legislation in many countries regulates the use of chemicals and other anthropogenic activities by assessing risk to the environment. For instance the European Union implemented REACH (EC 2006) into the law in 2007, a new regulation for the safe use of chemicals in terms of environmental and human health which requires an environmental risk assessment (ERA) to be conducted in the registration process.

Pesticides are an important group of chemicals that in some cases can pose risks to aquatic and terrestrial ecosystems (due to their inherent design to be biologically active and control pests, diseases and weeds in crops). The ERA of pesticides in Europe is currently evaluated under Council Directive 91/414/EEC (EC 1991) and follows a tiered approach. This tiered approach begins with acute and chronic effects assessments in standardized test species (algae, waterfleas, fish) and worst-case short and long term exposure estimates. For compounds which have the potential to bioaccumulate, the potential risk of secondary poisoning is also assessed. If a pesticide fails these preliminary assessments, higher-tier studies or exposure calculations are performed. Such higher-tier studies test effects on more species to estimate the variation in sensitivity and/or at higher levels of biological organization, such as the population and community level (e.g., microcosms and mesocosms). Acceptable environmental concentrations for the pesticide in question are then derived from these test results by applying additional assessment ('safety') factors, which decrease at higher tiers in the registration process, since these represent more realistic scenarios of field conditions. This approach to assessing risk is based on pragmatic empirical experience rather than a fundamental understanding of the underlying processes.

In recent years, many researchers from academia, industry and governmental institutions have invested in the development of predictive and more ecological and mechanistic approaches based on the understanding of ecosystems and toxicity in order to improve the protectiveness of ERA and reduce the uncertainty associated with current practices (Van Straalen 1994; Chapman 2002; Van Straalen 2003; Van Den Brink 2008; Forbes *et al.* 2009). For instance, the realization that toxicity is time dependent led to differentiation between external exposure and the toxicologically relevant dose (McCarty & Mackay 1993) and to the development of mechanistic models which predict the time course and distribution of internal concentrations based on the external exposure via the surrounding medium and via the diet (Jager *et al.* 2006; Ashauer *et al.* 2007b; Barber 2008). Population, meta-population models and food web models have also been developed for some ecotoxicological key species and types of ecosystems (Arnot & Gobas 2004; Preuss *et al.* 2009a; Galic *et al.* 2010).

While these approaches address higher organizational levels with the lowest resolution of the individual, another branch of ecotoxicological research has investigated effects of chemicals on the molecular level. This led to discoveries of molecular mechanisms of action (mode of action) causing damage in the cell (Escher & Hermens 2002). Complex responses of cells to stressors in several model species were researched in great detail, leading to the development of biomarkers which can be used to indicate chemical induced stress in organisms (Korsloot *et al.* 2004; Domingues *et al.* 2010). The pool of knowledge from these different lines of research on effects and mechanisms of toxicity has not yet been made holistically available as a tool to prospective ERA (Baird & Van den Brink 2007).

In ecology and retrospective ERA, species properties (or 'traits') have been considered for almost 100 years, beginning with Thienemann's evaluation of species assemblages in response to environmental 'harshness' (Thienemann 1918). This was followed by the introduction of the habitat templet theory (Southwood 1977), which was further developed by several other researchers (Statzner & Higler 1986; Townsend & Hildrew 1994; Statzner *et al.* 1997; Usseglio-Polatera *et al.* 2000a; Bonada *et al.* 2006; Poff *et al.* 2006; Bonada *et al.* 2007; Horrigan & Baird 2008) due to the growing realization that a solely taxonomic description of natural ecosystems can limit our understanding of the system's stress responses. The use of traits (the phenotypic consequences of genetic information in single functional entities) can improve understanding of stress responses, and may help to facilitate the prediction and diagnosis of disturbances. Traits are therefore a complimentary tool to taxonomy in ERA. With this concept in mind, the development of traits-based approaches for ecotoxicology is attractive and has potential to integrate the existing molecular, ecological and process oriented pool of knowledge into a framework which may lead to more meaningful and knowledge based ERA.

In this thesis, to begin with (1st chapter) I will introduce the reader to some of the lively debate that is currently underway concerning the use of traits for ERA. In the second chapter I propose how traits can facilitate prospective ERA conceptually by bringing together traits and mechanistic effect models. A conceptual framework for traits-based prediction is introduced and also a trait inventory relevant for external exposure, intrinsic sensitivity of organisms and population sustainability is developed. The trait inventory includes an evaluation of the data availability and a ranking of the

weight of each mechanistic process-trait link. This publication is one out of the five publications resulting from the SETAC-TERA workshop on traits-based ecological risk assessment, which was held in Burlington, ON, Canada in 2009 (Van den Brink *et al.* in press a).

In the remaining chapters of the thesis I explore the potential use of traits in the proposed conceptual framework from an empirical perspective. In the third chapter, in order to help frame the research questions, I mined existing insecticide toxicity and trait data, and linked these to each other in order to find empirical relationships between intrinsic sensitivity of freshwater arthropods to three different classes of insecticides with two different modes of action. From the results of this study, I then focused the remainder of my experiments and modeling on the effects of the insecticide chlorpyrifos on freshwater arthropod species. Furthermore, I decided to target my research on the mechanism of toxicity at the organism level and its relationship to traits in order to evaluate the potential for using traits to predict intrinsic sensitivity. In Chapter 4, I experimentally investigated the dynamics and variation in sensitivity in a set of 14 trait-diverse freshwater arthropods to chlorpyrifos and discuss the results in relation to current ERA practices. In Chapter 5, I report the results of 17 bioconcentration studies with the same set of freshwater arthropods, which were performed in order to parameterize a one compartment toxicokinetic model, describing the variation of uptake and elimination across the tested organisms. Chapter 6 is central to this thesis and describes how a toxicokinetic - toxicodynamic model was parameterized for three different arthropods of the previous set by means of long term survival experiments under pulsed exposure. In Chapter 7, I first introduce a new trait data set which was created with the aims of this thesis in mind, and mostly consists of morphological and physiological, and also ecological traits. In the second part of this chapter I link the previous datasets from Chapters 4, 5 and 6 to this trait data set quantitatively and qualitatively. In doing so, I indentify relevant traits and evaluate their potential for prediction of intrinsic sensitivity. In the last part of the chapter these results are discussed in the context of the conceptual approach presented in chapter two and their implications for ERA are outlined along with future research recommendations.

Ranatra linearis and Anax imperator specimen samples, 2006

1. CHAPTER

Letter to the Editor: Trait-based ecological risk assessment (TERA): The new frontier?

This chapter has been previously published as a letter to the editor in Integrated Environmental Assessment and Management (2008) **4**: 2-3.

Donald J. Baird, Mascha N. Rubach & Paul J. Van den Brink

DEAR SIR:

Traits, and their relevance in ecological risk assessment

Traits are the physiological, morphological and ecological attributes of species, or other taxonomic entities, which describe their physical characteristics, ecological niche, and functional role within an ecosystem. Trait-based approaches are now being introduced into the field of Ecological Risk Assessment (ERA) and bioassessment of ecological quality (biomonitoring) of aquatic ecosystems e.g. (Usseglio-Polatera *et al.* 2000a; Poff *et al.* 2006; Baird & Van den Brink 2007). This is a consequence of our realization that taxonomy-based descriptions of natural systems place limitations on our ability to describe ecological responses to stress. Whereas taxonomy can be regarded as a higher-level expression of the genetic composition of organisms, traits can be seen as their functional consequence. The conventional view that taxonomic species are the building blocks of ecosystems can be challenged by the fact that different life stages of the same species can have radically different ecological functions and roles within food webs (e.g. expressed by traits such as size, feeding type and dispersal ability). On the other hand, different taxonomic species may have similar roles within the ecosystem and be interchangeable from a functional standpoint (functional redundancy). Therefore, if communities are expressed as combinations of trait characteristics rather than combinations of species, a more complete description of ecosystem structure and function can be obtained.

The use of species traits to characterize the functional composition of benthic invertebrate communities is well established in the ecological literature (Poff *et al.* 2006). One of the 1st attempts to include species traits in an ecological assessment system was made by (Peeters & Garderniers 1994), who developed an assessment system for Dutch lowland streams in which they used macroinvertebrate community composition to measure deviations from a reference state, based on current velocity and substrate preferences of the benthic community as well as saprobity, trophic status, and composition of functional feeding groups. Their approach was diagnostic in the sense that deviations from the reference situation were reflected in the quality classes obtained for the aspects

mentioned above. The number of traits included in their system was limited and others have indicated the need to include larger numbers of traits in biomonitoring studies e.g. (Dolédec *et al.* 1999). (Charvet *et al.* 2000) assessed the usefulness of species traits of benthic invertebrates from seminatural reference sites as a potential benchmark for large-scale monitoring. They found that community structure based on ecological and biological traits was more consistent along an environmental gradient than one based solely on taxonomic composition.

Pros and cons of applying trait-based approaches in ecological risk assessment

A major advantage of trait-based over taxonomy-based assessment is that it 'adds value' to taxonomic data by revealing species attributes. As traits are generic descriptors across community types, they offer a major advantage in that they permit data aggregation at any spatial scale. Traits thus offer a solution to the problem of extrapolation of bioassessment data across geographic regions, currently not possible using taxonomic data, which are spatially constrained by biogeographic boundaries. Their use also explicitly facilitates the incorporation of scale into ecological risk assessment.

Traits also link prediction of ecological function (as defined by trait combinations) and mechanisms of action of different stressors (both natural and anthropogenic) at different levels of organization. This could be done by correlating traits to parameters describing the toxicokenetics and toxicodynamics of a certain compound in a certain species, e.g. using the model described by (Ashauer *et al.* 2007b). In the case of natural stressors, traits linked to habitat suitability could explain the presence or absence of certain species (e.g. body form, mode of respiration). However, since trait attributes are often assigned at the species level, the problem of intraspecific variation in trait states (e.g. juveniles and adults occupying different ecological niches) remains unresolved. Attempts to overcome this problem of uncertainty by the use of fuzzy coding methods (Usseglio-Polatera 1991; Tachet *et al.* 2000) have led to more complex and less precise methods of data analysis. Increased uncertainty in trait states also occurs when trait attributes are derived from higher levels of taxonomic resolution. A further problem associated with trait-based ecological assessment is their inter-correlation. This can arise through different traits expressing different facets of the same phenomenon (e.g. 'possessing gills' and being a 'water breather') or because they are part of a co-adapted gene complex. At the present time we lack an adequate understanding of these inter-correlations and how this lack of independence among some traits, reflecting evolutionarily based phylogenetic constraints, may affect their use in risk assessment (Poff *et al.* 2006).

Why we need a unique trait-based ecological risk assessment approach

Considering the potential of trait-based ecological risk assessment (TERA) as well as the problematic facts connected to trait based approaches, the benefit of a unique, unifying TERA approach becomes apparent. The development of TERA presupposes the existence of trait information to map on to existing taxonomic data. A number of research teams around the world have

constructed and published trait databases which link taxonomic entities (generally genera or species) to their trait attributes (Heneghan *et al.* 1999; Usseglio-Polatera *et al.* 2000a; Poff *et al.* 2006). However, trait definitions are inconsistent among studies, and their selection is often poorly linked to their potential application in ecological risk assessment. The former problem commonly arises in the area of informatics, where project-specific definitions of data attributes or 'tags' are collectively described as 'folksonomies' (Wikipedia 2007). The need for a broadly applicable set of trait definitions - a so-called 'collabulary' (Wikipedia 2007) with application in risk assessment is a necessary next step. This could be achieved either through a mechanistic linkage of traits with individual stressors via experimentation or through accumulated evidence from scientific literature, obtained by data-mining. In the longer-term, linkage to other bioinformatics platforms (e.g. genomics, DNA barcoding) could further enrich the quantification of trait attributes for diagnostic purposes.

What is clear, however, is that the harnessing of trait-based approaches in ecological risk assessment will require a substantial, coordinated international effort to realise its full potential. The task of trait definition, linkage of traits to specific environmental drivers and the extraction of trait information by observation, experiment and literature data-mining is a task beyond individual research teams, and for that reason, a new research paradigm is needed.

The role of SETAC in fostering trait-based ecological risk assessment

As a society, Society of Environmental Toxicology and Chemistry (SETAC) is unique in the international, multi-disciplinary nature of its members, and the science to which they contribute. SETAC has always fostered an open approach to science, encouraging members from academic, governmental and industrial research cultures to work together to produce research of benefit to all members of society. Developing trait-based ecological risk assessment would greatly benefit from strong leadership through a scientific society like SETAC, where members could be encouraged to share their data and knowledge more widely. Linking traits to stressors will require access to as much observational and experimental data as possible, since informatics approaches of this kind are extremely data hungry. The creation of an international, consistently defined and openly accessible set of trait definitions linked together with an associated database of taxon-trait information would be a significant research endeavour, but one which would also pay rich dividends. The use of Web 2.0 technologies (Anderson 2006; Weinberger 2007) to achieve large-scale user-generated content via the internet offers a timely solution to the problem of how to populate such a database, once it is constructed. The involvement of a scientific society such as SETAC as both host and gatekeeper in this process could ensure data quality, and the database produced would be a landmark contribution to complement the burgeoning number of web-based taxonomic information sites (e.g. Species 2000 [www.sp2000.org], the Encyclopedia of Life [www.eol.org], the Barcode of Life [www.barcodinglife.org]) currently being developed. This is truly a major challenge for the science of ecological risk assessment, and one which, while keeping us busy for many years to come, could unlock the full potential of the historical data generated by ecologists and ecotoxicologists over the past century.

The TERA workshop, Canada, 2009

2. CHAPTER

A Framework for Traits-based Assessment in Ecotoxicology.

This chapter has been accepted by Integrated Environmental Assessment and Management.

Note: As this chapter is one publication out of a series of five about the TERA workshop 2009, currently 'in press' cross references to the other workshop publications are made by 'this issue, in press'. These manuscripts are available on request.

Mascha N. Rubach, Roman Ashauer, David B. Buchwalter, H.J. De Lange, Mick Hamer, Thomas G. Preuss, Katrien Töpke, Stephen J. Maund

ABSTRACT

A key challenge in ecotoxicology is to assess the potential risks of chemicals to the wide range of species in the environment on the basis of laboratory toxicity data derived from a limited number of species. These species are then assumed to be suitable surrogates for a wider class of related taxa, e.g., *Daphnia* spp. are used as the indicator species for freshwater aquatic invertebrates. Extrapolation from these datasets to natural communities poses a challenge, because the extent to which test species are representative of their various taxonomic groups is often largely unknown, and different taxonomic groups (and chemicals) are variously represented in the available datasets. Moreover, it has been recognized that physiological and ecological factors can each be powerful determinants of vulnerability to chemical stress, thus differentially influencing toxicant effects at the population and community level. It has been proposed recently that detailed study of species traits might eventually allow better understanding (and thus prediction) of the potential for adverse effects of chemicals to a wider range of organisms than those amenable for study in the laboratory. This line of inquiry stems, in part, from the ecology literature, where species traits are being used to better understand how communities are constructed, and how communities might respond to and recover from disturbance (see other papers in this workshop series). In this paper, we develop a framework for the application of traits-based assessment. The framework is based on the population vulnerability conceptual model of Van Straalen (1994) in which vulnerability is determined by traits that can be grouped into three major categories - external exposure, intrinsic sensitivity and population sustainability. Within each of these major categories, we evaluate specific traits and how they could contribute to the assessment of the potential effects of a toxicant on an organism. We then develop an example considering bioavailability to explore how traits could be used mechanistically to estimate vulnerability. A preliminary inventory of traits for use in ecotoxicology is included, and this also identifies the availability of data to quantify those traits, plus an indication of the strength of linkage between the trait and the affected process. Finally, we propose a way forward for the further development of traits-based approaches in ecotoxicology.

INTRODUCTION

Ecological risk assessment (ERA) of chemicals aims to determine the probability and extent of an adverse effect occurring to an ecological system with the ultimate goal of protecting the long-term viability of populations, communities and ecosystems. Since it is not feasible to test all species and chemical combinations, a major challenge for ERA is to extrapolate the population vulnerability *sensu* (Van Straalen 1994) - see below - of different species based on the available exposure and effects data. Usually, extrapolation between species or populations is based on taxonomy, i.e., it is assumed that a given laboratory model species is representative of a broader faunal group. However, it has been recognized that several factors (ecological, physiological) are also important in determining vulnerability to chemical stress, and play a key role in influencing effects at the population and community level. It has been proposed recently that incorporating more information about how a given species' traits might contribute to its susceptibility may eventually allow better prediction of the potential for adverse effects to a broader range of species (Baird *et al.* 2008). This does not imply replacement of taxonomy based approaches, rather, building on them and improving existing knowledge and methods using a different perspective. This is of particular importance since ecotoxicological data are generally collected based on taxonomy (Culp *et al.* this issue, in press), and subsequent analysis using traits may help to elaborate these data. In the near future, molecular techniques using next generation sequencing, such as DNA barcoding (Hajibabaei *et al.* 2007) will facilitate the combination of species taxonomy with traits.

In this paper, we propose a framework for incorporating species traits in ecotoxicology and risk assessment. Here we define a trait as a phenotypic or ecological character of an organism, usually measured at the individual level but often applied as the mean state of a species. The ideas for this framework were established at the SETAC Traits-based Ecological Risk Assessment (TERA) workshop held in Burlington, Ontario, Canada in September 2009.

In order to structure the presented framework, we used the vulnerability conceptual model of Van Straalen (1994) in which the ecotoxicological effects of a chemical toxicant on a population are described in three categories (Figure 1) namely:

(i) external exposure - the extent to which organisms are exposed to the toxicant;

(ii) intrinsic sensitivity - the potential of the toxicant to affect an organisms ability to survive, develop and reproduce. Two subcategories are included that of toxicokinetics (including bioaccumulation, distribution and transformation) and toxicodynamics (including target site considerations and compensation mechanisms;

(iii) population sustainability - the potential for a population to recover from any toxic effect. Two subcategories of demography and recolonisation are included.

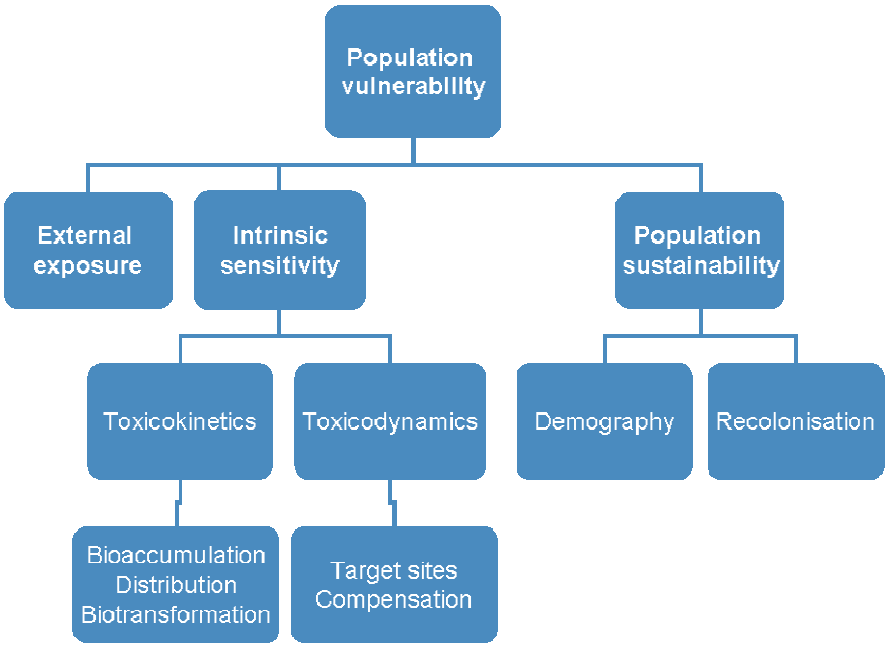

Figure 1: Categories for use of traits in ecotoxicology, after Van Straalen (1994). The categories external exposure, intrinsic sensitivity and population sustainability mechanistically contribute to the vulnerability of a population (species). The categories can each be broken down into a series of processes and sub-processes. To each category or process groups of traits can be related.

For each of these categories, we discuss the potential application of traits-based approaches in the sections below. This is not meant to be an exhaustive review, but rather an attempt to exemplify where traits could fit within the existing ERA paradigms. We evaluate a potential mechanistic approach for linking traits with vulnerability, and provide potential options for the future development of the field. Where trait types are mentioned in the text below, we use italics to highlight them to the reader (e.g. *habitat choice*).

EXTERNAL EXPOSURE

External exposure is the first step (category) in the sequence that determines whether an organism will be affected by a toxicant in the environment. Clearly if there is no exposure, there can be no direct effect, whereas increasing levels of exposure are likely to lead to potentially growing levels of impact. After entering the environment, the distribution of the toxicant in various environmental matrices will be determined by a variety of fate processes resulting in a range of environmental concentrations in the various potential habitats in the ecosystem. This can be predicted using the properties of the toxicant and landscape characteristics by means of fate models. The traits of a species will then determine the potential likelihood and magnitude of external exposure of an organism, which will subsequently lead to an internal dose. Therefore, traits can be used to evaluate whether a species is likely to come into contact with the toxicant by describing where, when and at what life-stage, exposures may occur in a contaminated habitat. Traits such as food or habitat preference are clearly the two key factors in determining likely exposure routes either by uptake

through food or uptake through the ambient matrix (water, soil, sediment, air, et cetera). In the section below we elaborate further on how various traits may influence external exposure.

Habitat choice

Habitat choice of a species is a major determinant of whether a species will come into contact with a toxicant. For instance, soil exposure will be less relevant for species that live exclusively on vegetation or aerial species, due to limited contact with the contaminated soil. Furthermore, the *habitat choice* of a species may also vary at different stages of the life-cycle, and this needs to be accounted for when evaluating potential risks. *Habitat choice* has been used in a number of case studies to explain differences in exposure, for example in evaluating the potential risks of cadmium for the little owl *Athene noctua* in a river floodplain (Kooistra *et al.* 2005) and mercury poisoning in the loon *Gavia immer* (Nacci *et al.* 2005). Furthermore, the duration and intensity of direct contact with a toxicant may vary with the *life span* and *home range* of an organism. The longer an organism lives, the greater the duration of exposure that can be expected, assuming exposure persists. An animal's home range size can have a major influence on the exposure to a toxicant, if the toxicant is heterogeneously distributed throughout the habitat, because exposure varies widely from none to significant levels, but may be of less importance for homogenously distributed toxicants, for which exposure is ubiquitous and uniform. The habitats may also vary in characteristics that influence toxicant bioavailability, for example pH and organic matter content are important variables determining metal accumulation (Van den Brink *et al.* 2010). Habitat preference on a smaller scale (e.g. preference for hedgerows with lower pH versus preference for open fields with higher pH) may therefore be an important factor determining metal exposure.

Food choice

Food choice or preference can determine exposure to toxicants. As some substances accumulate in the food chain, such as selenium in aquatic ecosystems and PCBs in aquatic and terrestrial ecosystems (Maul *et al.* 2006), predators can be exposed to higher concentrations of a toxicant, even when there is limited exposure through habitat. Similarly as with the trait *habitat choice*, *food choice* may also vary with life stage of a species. If food is the main exposure route, the *daily food intake* determines the extent of exposure to a toxicant. This can add complications since many traits will be correlated e.g., *daily food intake* is related to the *metabolic rate* of a species, and both are correlated with body mass (Nagy 2005). Other life-cycle traits like *hibernation*, *aestivation* or *migration* may alter external exposure through a seasonal change in habitat, which could either reduce or increase external exposure. For instance, some species of mammals, birds, fish and insects exhibit migratory behavior resulting in long-distance journeys in order to feed or reproduce in a specific habitat which may influence the amount and frequency of external exposure.

A range of other behavioral traits can also affect the external exposure of a species. Active *avoidance* of contaminated media or food will reduce external exposure, particularly if there is a spatially heterogeneous contamination and the organism can move to less contaminated areas. Avoidance behavior can be triggered by internal receptors in the context of chemotaxis, and the resulting behavioral avoidance leads to decreased contact with the toxicant and therefore to

decreased external exposure. Avoidance behavior has been shown for invertebrates avoiding contaminated sediment (De Lange *et al.* 2006), soil (Natal-da-Luz *et al.* 2008) or water (Schulz & Liess 2001), for fish, such as zebrafish avoiding copper and acid mining drainage (Moreira-Santos *et al.* 2008) and for mammals and birds through avoidance of contaminated food (Linder & Richmond 1990).

INTRINSIC SENSITIVITY

Traits can contribute significantly to intrinsic sensitivity differences among species. These traits (with the exception of size) are not usually considered by ecologists, but can be of significant toxicological importance. Here, we summarize a range of traits that contribute to the acquisition of contaminants and the abilities of organisms to cope with chemical exposure.

Toxicants generally elicit direct effects by interacting with a certain biological compartment in the organism (which varies depending on the toxicant) that then causes physiological consequences in the organism, potentially resulting in toxicity. The intrinsic sensitivity of an organism to a toxicant partly depends on the concentration of toxicant that is able to reach the target site (Meador *et al.* 2008). Even if there is external exposure, differences in traits may mean that in some species the toxicant is unable to reach the target site, whereas in others it may do so easily. Thus internal concentrations at the target site or whole body concentrations (as a surrogate of target site concentration) may provide a better basis for linking traits to intrinsic sensitivity than external concentrations alone.

The toxicological processes of bioaccumulation, biotransformation and the internal distribution of toxicants between organs and tissues determine the internal concentration at the target site. This has also been called the biologically relevant dose (Paustenbach 2000). Hence any trait that affects these processes may also help explain the sensitivity of the organism. Mechanistically, intrinsic sensitivity of an organism to a chemical stressor is a result of processes that can be grouped into toxicokinetics and toxicodynamics. Toxicokinetics describe the time-course of uptake, distribution, biotransformation and elimination of the toxicant, i.e., all processes that together determine the concentration of the toxicant at the target site. Toxicodynamics describe the time-course of the actual toxic action at the target site, its physiological consequences, and how sub-organism level effects manifest themselves in organism level consequences. Toxicokinetic-toxicodynamic effect models (TKTD models) have recently been reviewed by (Ashauer *et al.* 2006a; Ashauer & Brown 2008) and are valuable because they provide a methodology for linking traits to the processes of toxicity.

Toxicokinetics – Bioaccumulation

Whether considering organic or inorganic contaminants, the literature is rich with examples of sympatric species varying (sometimes by orders of magnitude) in contaminant body burdens. Intuitively, it makes sense that such inter-specific differences in bioaccumulation are a function of species traits. Organisms accumulate contaminants directly from their ambient media (e.g., water, air, soil, sediments) and from their diets, and morphological and physiological traits will be fundamental drivers of these bioaccumulation processes. For example, in the case of metals, biodynamic modeling approaches (Luoma & Rainbow 2005) have demonstrated that species-specific physiological traits related to iono-regulation and digestive processes drive bioaccumulation differences among taxa.

Similarly, accumulation of organic contaminants has been shown to be related to organism size and lipid content (Hendriks *et al.* 2001), of which the latter is discussed in more detail below.

For organic contaminants in particular, *body size* and related *surface area to volume relationships* can exert a profound influence on the bioconcentration of contaminants. Several authors have reported that bioconcentration is inversely proportional to the *volume* or *weight* of different species (Preuss *et al.* 2008). Baird and van den Brink et al. (2007) included *dry mass* as one of five characteristics that could together describe approximately 79% of the inter-specific variability in sensitivity to several different compounds. While biomass is easy to measure, it is possible that the *surface area to volume relationships* may be more powerful predictors of bioconcentration rate differences between species. However, *surface area to volume relationships* are significantly more difficult to measure than biomass, particularly for small invertebrates with complex three-dimensional gill surfaces. In addition to size and/or body mass differences among species, the *nature of body surfaces* may be radically different among species. Crustaceans, for example, tend to have calcium rich integuments while insects may be soft and membranous or heavily armored with chitin. The means by which different integument types or biological barriers affect the diffusion rates of organic contaminants remain still poorly understood (Boudou *et al.* 1991), though methods do exist. Further progress through genomics and proteomics approaches will likely be made in this area in the near future.

Respiratory strategies (e.g., having surfaces such as gills) can also be important determinants of bioaccumulation. Indeed, *respiratory strategy* was also among the four key explanatory traits used by Baird and Van den Brink (2007) as described above. Buchwalter et al. (2002) found that water-breathing (dissolved oxygen breathing) insects tended to be more permeable to water and had faster chlorpyrifos uptake rates than comparably sized air-breathing species. Across all species in that study, water permeability was strongly correlated with chlorpyrifos uptake rates. In both *respiratory strategies*, body size still exerted first order control of overall permeability. However the use of morphological traits as predictors of physiological processes should be treated with some caution. More research is needed to identify the importance of morphological traits in relation to bioaccumulation and to develop quantitative relationships between traits and their affected processes. For example, the *presence or absence of gills* may be less informative than the *relative permeable surface area* of a water breathing species. Inter-segmental membranes in some insect species may serve as gaseous exchange surfaces and can be similarly permeable to external gills. Therefore other characteristics, such as the *degree of sclerotization* may not be good approximates for integument permeability, as some species with membranous integuments can be quite impermeable, despite common perceptions to the contrary (Buchwalter *et al.* 2002).

In contrast to traits that focus on the interface of the organism and its environment, the internal composition of species can vary considerably as well. Perhaps the best understood trait in relation to the bioaccumulation of organic compounds is *lipid content* (Hendriks *et al.* 2005). Here we make a distinction between the overall quantity of lipid (% lipid) and the qualities of various lipid pools at the organism level. Although the initial critical body residue concept did not explicitly consider the role of lipids in toxicity (McCarty & Mackay 1993), later (Di Toro *et al.* 2000) identified the membrane lipid fraction (*polar lipids*) in the organism as the generic site of toxic action for contaminants eliciting

baseline toxicity such as organic compounds, uncouplers, inhibitors of photosynthesis or ATP synthesis (Escher & Hermens 2002; Hendriks *et al.* 2005).

Storage lipids on the other hand may act as a transient sink for hydrophobic organic contaminants in organisms. The amount and composition of *storage lipids* within a given organism undergo dramatic seasonal fluctuations compared to *polar lipids* (Naesje *et al.* 2006) and depend on food quality and quantity (Goulden & Place 1993), density dependence (Cleuvers *et al.* 1997) and life-stage (Bychek & Gushchina 1999). As the composition and distribution of lipids within an organism may modify intrinsic toxicity, insights into lipid dynamics and the relationship to contaminant partitioning could therefore provide a stronger basis for understanding toxicity of tissue residues and predicting effects. Because hydrophobic organic chemicals preferentially partition into lipids, it has become common practice to normalize bioaccumulation data to the lipid content of the sample (Escher & Hermens 2002). Furthermore, the partitioning of hydrophobic contaminants in tissues follows predictable patterns. For instance fugacity-based approaches are increasingly used to understand and predict bioaccumulation differences among species. Species' *lipid content* and *body size* are the most important traits included in quantitative bioaccumulation models (Arnot & Gobas 2004; Hendriks *et al.* 2005). Nevertheless, the role and influence of lipid should be interpreted with caution, particularly when comparing different studies as there are uncertainties associated with lipid measurement. For instance, a variety of analytical methods using different solvent combinations and ratios generated differences in lipid concentrations for the same sample (Smedes 1999; Manirakiza *et al.* 2001).

Depending on the contaminant and the species, *diet* may be a primary exposure pathway and may therefore alter toxicity (Fisher & Hook 2002). For terrestrial vertebrates, *diet* is assumed to be the major source of exposure for many contaminants. Diet can also be the primary route of exposure for many metals to aquatic invertebrates (Martin *et al.* 2007a), although it is generally accepted that dietary uptake of organic chemicals is less important than uptake from ambient media (Gomes *et al.* 2004). In those cases where direct uptake from water by animals appears relatively unimportant (e.g. Se), food web dynamics may drive bioaccumulation differences among species (Stewart *et al.* 2004; Conley *et al.* 2009). In this example, both *food choice* and *digestive processes* drive bioaccumulation differences among species.

Differences in the dietary assimilation of metals appear to be profoundly different among species and diets. Dietary assimilation efficiencies of cadmium in predatory stoneflies ranged from (85-90%) (Martin *et al.* 2007a), whereas they could be as low as 5% in zebrafish (*Danio rerio* Hamilton) fed daphnids (Liu *et al.* 2002), and 3% in silversides (*Menidia* sp.) fed copepods (Reinfelder & Fisher 1994). Dietary uptake models for organic contaminants in fish have been reviewed by Barber (2008), where *assimilation efficiencies* ranged from 40 to 100%. *Assimilation efficiencies* of chemicals must be distinguished from *assimilation efficiencies* of food components such as lipids. The extraction of lipids from food during the gut passage in fish for example, increases the fugacity of organic contaminants in the gut, which in turn leads to further transfer of organic contaminants from the gut into the fish (Gobas *et al.* 1999). This provides the mechanistic explanation for biomagnification, i.e., an increase of fugacitiy in food chains (Kelly *et al.* 2004). *Assimilation efficiencies* of organic contaminants in several taxa have been reviewed by (Hendriks *et al.* 2001) who also established a quantitative relationship to organism body weight. Dietary uptake of toxicants (metals or organics) depends on the *food choice*,

ingestion rate and *assimilation efficiency*, as well as on the concentration within the food source, which is in turn triggered by the traits of the food source itself. While there is a growing body of literature associated with the dietary assimilation of metals in aquatic organisms, this remains a relatively difficult trait to measure with precision, because assimilation can vary with diet type, ration, and concentration.

Bioaccumulation of contaminants is a function of both uptake (directly from surrounding media and diet) and loss. Mechanistically, much less is known about the traits that drive loss rate differences among species for a given contaminant, but numerous studies have measured profound differences among species. The rapid elimination of metals by the caddis fly *Hydropsyche* (Cain *et al.* 2006) may help explain the observed metal tolerance of this genus. Furthermore, in the case of cadmium in aquatic insects, it appears that elimination capacity of species is not arbitrary, but seems to cluster phylogenetically (Buchwalter *et al.* 2008). Thus, more comparative studies in different faunal groups may eventually be a means of predicting a taxon's ability to eliminate toxicants based on phylogenetic considerations. The relationship between elimination rates of organic compounds and *body size* has been quantified for several taxa (Hendriks *et al.* 2001; Kooijman *et al.* 2004). Another important process which can shift elimination rates of organic compounds is biotransformation (see below). Further traits triggering elimination of a compound include *ventilation rate*, *fecal egestion*, *growth dilution* and *reproduction* (maternal transfer) and the influence of these traits on elimination are reviewed and discussed in detail elsewhere (Barron 1990; MacKay & Fraser 2000), but will be addressed briefly in the following section.

Several other traits might be expected to play roles in differential contaminant bioaccumulation, but have received significantly less attention. For example, species with high *growth rates* may be aided in limiting bioaccumulation relative to slower growing species via the process of growth dilution. Slow *growth rates* may contribute to the relatively high bioaccumulation tendency of some freshwater mussels for example, but high *water filtration rates* may be a more important trait in this regard. The ability to *close the operculum* in mollusks might be advantageous for avoiding exposure in the short term, but is unlikely to be beneficial in chronic exposure scenarios (Cope *et al.* 2008). Also other processes may result in reduction of contaminant burdens in tissues. For example, the transfer of some contaminants such as selenium to eggs is known to be important to fish (Coyle *et al.* 1993), daphinds (Lam & Wang 2006) and insects (Conley *et al.* 2009). For such contaminants, traits such as *fecundity*, which are known to vary tremendously among taxa may play a modifying role. However, this trait is likely to be more important in determining population recovery and resilience (see below). Other contaminants such as cadmium and manganese may be lost during the molting process, as was shown for a shrimp species (Keteles & Fleeger 2001), and a mayfly (Cid *et al.* 2010) and *molting frequency* can also vary widely across species.

Toxicokinetics – Internal distribution

Once a toxicant is taken up into an organism its internal distribution is either driven by partitioning through passive diffusion or active biological transport, which emphasises the relevance of *body size* as a trait. Partitioning depends on the toxicant's physico-chemical characteristics, and for organic compounds partition it may be determined by the fugacity capacities of the compartments

(tissues, organs) involved (Mackay 2004). Consequently the traits *lipid content*, *lipid distribution* and *lipid composition* (e.g. storage lipid, membrane lipids) will affect the partitioning processes of organic chemical stressors and, by doing so, alter the temporary concentration at the target site.

Aside from body size and lipid characteristics, there is little understanding of how basic 'Bauplan' affects the distribution of contaminants. For example, even the fundamentals of how different types of *circulatory systems*, presence/absence of *barriers* separating the site of action from the rest of the organism, or characteristics of specific *organs* affect the distribution of contaminants are unknown. Once further relevant traits and their relationship to internal distribution are identified these may be linked to pharmacokinetic models (Physiologically-Based Pharmaco-Kinetic (PBPK) and Adsorption, Distribution, Metabolism, Excretion (ADME) models) (Barber 2008) or dynamic energy budget models (DEB) (Kooijman & Bedaux 1996), which are well-developed for some model systems, but are rarely applied to non model species due to their high data demand. On the other hand, processes of active biological transport may be very suitable to incorporate into these types of models. For instance processes like the sequestration of metals into cell specific *granules*, e.g. cuprosomes for copper (Joosse & Verhoef 1987) or *transport* of xenobiotics across membranes, which may also be inhibited by other organic compounds (Epel *et al.* 2008) can influence the distribution of toxicants significantly, but are yet difficult to combine with available modelling approaches.

Toxicokinetics - Biotransformation

Biotransformation (defined as enzymatic conversion of a toxicant to a structurally different product with altered chemical and toxicological properties) is one of the major confounding factors in the prediction of toxicokinetics and toxicodynamics of organic chemicals. Birds, mammals, fish, and many aquatic invertebrates are able to extensively metabolize a range of organic toxicants (Stegeman & Kloepper-Sams 1987; Boon *et al.* 1997; Livingstone 1998), though this ability appears to be species specific (Chambers & Carr 1995). Biotransformation includes direct chemical changes in the structure of the parent compound (phase I reactions) together with the conjugation of the parent compound with hydrophilic groups (phase II reactions) to facilitate excretion. Various enzymes and/or proteins are involved in these biotransformation pathways, e.g. cytochrome p450, mixed function oxidase, methallothioneines (see also below) and glutathione-S-transferase. The presence and translation rates of all these enzymes reflect biotransformation potential and can differ even between closely-related species (Rust *et al.* 2004). Biotransformation can lead to the formation of compounds that can be either more or less toxic than the parent, depending on the compound/enzyme combination (Perkins & Schlenk 2000). Both metabolites and conjugates have been shown to persist in several invertebrates and fish (Preuss *et al.* 2008), although their contribution to toxicity has yet to be determined. Biotransformation may dominate toxicokinetics, and if biotransformation is faster than efflux then toxicokinetics is no longer a simple partitioning process between two phases (Preuss *et al.* 2008). Hence any trait that determines biotransformation is important for explaining the variability in intrinsic sensitivity between species and could therefore be described in general as *biotransformation potential* (see below). Biotransformation rates are also related to the substance's structural properties (e.g. structure, functional groups present). For contaminants that can be biotransformed, failure to address biotransformation can lead to either over- or underestimation of toxicological risk depending on the

toxicity of the transformation product. Applying a traits-based approach to this topic could be done in a very detailed way, measuring the presence and amount of all possible enzymes within all species. However, this approach would come up with thousands of new traits, defined by the type and subtype of an enzyme and since this approach would likely take an unreasonable amount of time, other approaches will be necessary.

One possible alternative might be to use the *biotransformation potential* of a species as trait. For fish it was demonstrated that *in vitro* tests can be used to determine the *biotransformation potential* of a species/compound combination (Fitzsimmons *et al.* 2007; Dyer *et al.* 2009). Nevertheless confounding factors for the use of biotransformation rate as traits are many, especially since biotransformation rates, just as sensitivity strongly depend on the species/compound combination and the physicochemical conditions (Fitzsimmons *et al.* 2007). Before biotransformation can be used within a traits-based approach further research in this area is needed, as well as data mining algorithms to fill the gaps in mechanistic knowledge and to consolidate this information.

Toxicodynamics – Target Sites

As described above the effect caused by a toxicant is mediated by its mode of action (MOA) and concentration at the target site (Escher & Hermens 2002). Target sites can be polar lipids in membranes, receptors, transporters, ion channels or enzymes, and also DNA or intracellular systems which steer cell division (European Centre for Ecotoxicology and Toxicology of Chemicals, ECETOC 2007). Significant conceptual progress has been made in recent years with the establishment and classification of toxicants based on their target sites, even though sometimes only incomplete mechanisms of action are known (Verhaar *et al.* 1992; Verhaar *et al.* 1996; Escher & Hermens 2002; ECETOC 2007). As an example of how traits may be applied to a specific site of action, the estrogen receptor is discussed in Box 1.

ECETOC (2007) has reviewed the concepts and availability of data on MOAs across biota in the context of developing intelligent testing strategies for toxicants (ECETOC 2007). In principle, as soon as a biologically active molecule has reached its site of action, which in case of agrochemicals is often fairly well-defined, their interaction will initiate an effect chain, which may eventually lead to damage of tissue or system function. This toxicological effect may originate from either antagonistic or agonistic behaviour of the toxicant (ECETOC 2007).

Despite the highly complex nature of toxicant/target site interactions, it may be possible to identify traits that can serve as reasonable descriptors. The mode of action approach, which links chemical types with certain target sites, provides a good start for this, although knowledge is currently restricted to a few model organisms at relatively high levels of taxonomic resolution (kingdom, phyla and subphyla). The *presence or absence of the site of action* can be considered as a trait for an individual organism that will influence its sensitivity to a particular toxicant. This may be relatively easy to define if the toxicant is a pesticide or another chemical with a well-known mode of action (e.g. for a xenoestrogen or a acetylcholinesterase inhibitor the trait modality would be presence/absence of estrogen receptor or acetylcholinesterase enzyme, respectively). Although this detailed knowledge about sites of action is only available for a few model organisms, for the short term it might be useful to

assume that closely-related species share these since a range of studies suggest that drug target sites are evolutionarily well-conserved (Gunnarsson *et al.* 2008).

Intrinsic sensitivity will also be affected by the *binding affinity* of the toxicant to the site of action (due to differences in steric constitutions) and its *reversibility* (i.e. reconstitution of receptors), rate of de *novo synthesis* of the site of action (up-regulation of gene expression), the *activity of enzymes* and the *speed of ageing* of the receptor. It is unclear to what extent the sites of toxic action vary in their constitution on species level; nott many comparative studies have evaluated this, let alone the other complex processes described above. In the future these issues might be addressed in comparative studies using molecular techniques, including genomics and proteomics.

Box 1: The estrogen receptor as a site of action. The relevant toxicants for the estrogen receptor are xenoestrogens, a class of chemicals which act on the estrogen hormone system. This class of chemicals produces mostly sublethal effects at low concentrations, i.e. below baseline toxicity. Once in the tissue of an organism, the potency of these organic chemicals is directly related to their affinity, activity, and reversibility (see main body of text for explanation) at the receptor site. Ligand binding leads to activation or deactivation of the receptor, producing agonistic or antagonistic response within the biological system (ECETOC 2007). Estrogenic potency of substances can be measured with various in vitro assays, from estrogen receptor (ER) binding assay to reporter gene and proliferation assays. These assays differ in the ER used (e.g. Erα, ERβ), the organism the receptor is derived from (e.g. human, rat, fish) and the cellular surrounding (e.g. yeast cells, human, rat or fish cell lines or primary cell culture). Binding to the ER does not necessarily provide information on the estrogenic potency of the substance as the MOA may be partially estrogenic or anti-estrogenic. Estrogenic potency varies between receptor subtypes (Katzenellenbogen *et al.* 2000) and the organism from which the receptor is derived (Segner *et al.* 2003). Another important factor influencing the activity of the receptor-ligand complex is the cellular surrounding (Katzenellenbogen *et al.* 2000). While xenoestrogens lead to very specific effects in vertebrates (which express the estrogen receptor), they act mostly as baseline toxicants in arthropods (which lack the estrogen receptor). However, the presence/absence of the estrogen receptor is often not known at the species level, and, as for other sites of action, has only been investigated in a few model species.

Toxicodynamics – Compensation

Numerous compensatory abilities have been described that help organisms overcome chemically induced stress and stabilise the cell's homeostasis. Knowledge about stress compensation in organisms mostly relates to the sub-organism level while little information is available at the organ or whole body level. On sub-organism level mechanisms range from (a) sequestration and/or metabolisation (covered in the section 'biotransformation') of the offending agent through; (b) complex cellular machinery to combat the secondary oxidative or toxic challenge that the organism is facing to; (c) repairing and/or regenerating damaged macromolecules and finally; (d) controlled cell death (apoptosis) can also be considered a mechanism of compensation.

We shall briefly discuss how compensatory or coping mechanisms may fit into a traits context. Korsloot et al. (2004) describe existing stress response systems and their specificity, which is used here to give an overview of the existing knowledge base (Table 1). The cellular stress response system as a whole is a very complex network of different single specific response systems, each itself consisting of a complex network of interacting enzymes and messengers, leading to different gene expression profiles. Some single stress response systems can be linked to various types of stressors, such as methallothioneines (MTs) or anti-oxidant capabilities for metals, although these stress systems are also induced by other stressors. The induction of MTs or metallothioneine-like proteins

(MTLPs) protects cellular machinery from offending metals by complexation of metals, which was shown to vary considerably across species in the extent to which metals are associated with MTs or MTLPs (Buchwalter *et al.* 2008). Similarly, some faunal groups are known to store metals in extracellular granules with are considered physiologically inert. Thus, the *extent to which species can sequester* their metal body burdens can be considered a highly relevant trait in determining their susceptibility. The *oxidative stress response systems* of animals can play a major role in ameliorating chemically-induced oxidative stress. These antioxidant systems are highly conserved, a relatively small suite of tools is necessary to make meaningful measurements across a wide variety of taxa. Although the MTs, MTLPs and the oxidative stress responses are not entirely specific, other stress response systems such as the basal signal transduction and the general stress protein system are even less specific for certain stressors and therefore more difficult to apply in TERA. Similarly to the *biotransformation potential* described above, the ability to repair macromolecules damaged by a toxicant is an important factor which can affect the sensitivity of an organism (Marquis *et al.* 2009). Techniques such as the COMET assay may be a means of better understanding the susceptibility of different faunal groups to DNA damage, or perhaps to compare their repair capacity. Additionally, other avoidance mechanisms such as *dormancy* exist, whereby the stress response leads to a change in physiology and/or behavior (Joosse & Verhoef 1987). Although a great deal of fundamental knowledge about compensatory mechanisms exists for certain model species, their variability is not well known, because they are mostly studied only in model organisms. Again, comparative toxicology research programs would be needed to take what is known in these areas and apply it across different faunal groups. At this point however, our understanding of truly toxicological traits is extremely limited.

Table 1: Stress response systems and their position in the sequence of stress compensation [a]

Stress Response System	Involved molecules, enzymes systems or processes	Specificity for stressors
Basal Signal Transduction Systems (B)	Second messenger such as cAMP, Ca2+/calmodulin, PKC, MAPK-cascade	Unspecific, all stressors
General stress protein system (B)	Heat shock proteins (hsp70 and hsp90 families), ubiquitin, HSF	Protein denaturing stressors, quite unspecific
Oxidative stress response system (A)	ROS, SOD, GSH, CAT and many more	Unspecific
Metallothioneine system (A)	MTs	Metal stressors, very specific
Mixed function oxigenase (MFO) system (A)	Cytochrome p450, AHH, AhR, hemoproteins, several oxidases	Quite specific for organic pollutants, in insects
Cellular response system (development of tolerance) – general	HSP, MT, MFO, INA, AFP	Quite specific, also natural stressors, organic pollutants
Cell aging/death response (D)	Oxidative stress responses, MAPK, ATP, ETC, Ca^{2+}	All stressors, especially oxidative stressors
DNA repair (C)	DNA polymerases	Genotoxic and mutagenic stressors

(A = primary, B = secondary, C = repair, D = apoptosis)
Note: cAMP = cuclic adenosine monophosphate, PKA = protein kinase A, MAPK = mitogen-activated-protein-kinase, HSP = heat shock proteins, HSF = heat shock (transcription) factors, ROS = reactive oxygen species, SOD = superoxide dismutase, GSH = glutathione, CAT = catalase, MTs = metallothioneins, AHH = aryl hydrocarbon hrydroxylase, AhR = aryl hrdrocarbon receptor, INA = ice nucleating agents, AFP = anti freeze proteins, ATP = adenosine triphosphate, ETC = electron transport chain
[a] adapted from (Korsloot et al. 2004)

POPULATION SUSTAINABILITY

As outlined above, a population's vulnerability to a toxicant not only includes its intrinsic sensitivity to the toxicant, but is also influenced by population- and community-level processes (Van Straalen 1994). ERA generally aims to preserve the long-term viability of populations (Ratte 1996; Forbes *et al.* 2009) – what we consider here to be population sustainability (Figure 1). To that end, higher-tier approaches have been developed that include semi-field or field studies at the population and community level (e.g., aquatic and terrestrial model ecosystem studies, non-target arthropod and earthworm field studies, etc). The advantage of these approaches is that they include more realistic exposure to the toxicant and many of the ecological processes that may influence the responses of a population or community to toxicant stress. There are limitations to this methodology however, in that clearly not all circumstances or scenarios can be covered by such experiments (Grimm *et al.* 2009). Consequently, approaches that would allow extrapolation to other scenarios would be a useful development. To meet this need, ecological models have been developed to predict effects on populations and communities (Grimm *et al.* 2009), including simple population models and others considering food webs or metapopulation approaches. The input parameters for such models are fundamentally based on the organism's life history (Stark *et al.* 2004; Rowe 2008) along with the inherent sensitivity of the various life-stages, if appliacable (Preuss *et al.* 2009a). For the purpose of discussion here, we have categorized these life-history attributes into demographic and recolonization traits.

Demography

Demographic traits are those that influence the population growth rate (or intrinsic rate of increase), and ultimately drive population densities and age distributions (and these are also key for recovery considerations – see below). Key demographic traits include *life span/survival*, *generation time* (the interval between reproductive events), *voltinism* (the number of reproductive events per year), and the *number of offspring* (or clutch size) per reproductive event. The combination of these traits to age-specific birth, growth, and death rates determines population growth rate (Sibly & Hone 2002), and the former depends on inter- and intra-specific competition for resources, predators, parasites, and so fourth, which in turn may depend directly or indirectly on population density. The importance of such traits for population growth rates, and the influence of toxicants upon them have been demonstrated in a large number of studies. In most population-level experiments, toxicant effects have been measured at relatively low population densities using life-table response experiments (Levin *et al.* 1996) and generally these have shown that population growth rate declines with increasing toxicant concentration, largely due to decreases in the *survival*, *fecundity* and/or a delay in *time to maturity* of individuals. However, some studies have shown that toxicant effects may differ at high densities (Forbes *et al.* 2001) due to buffering effects when resources were limited, presumably because toxicant-induced mortality resulted in more resources for the survivors, e.g. (Hooper *et al.* 2003). In other studies, however, toxicant effects were exacerbated when resources were scarce, for instance populations of the rotifer *Brachionus calyciflorus* was seven times more sensitive to

pentachlorophenol when tested under limited rations (Cecchine & Snell 1999). These considerations, therefore, have important consequences for risk assessments at the population level, because of differences between the density of test and field populations. Consequently, understanding the demographic traits of a species will be an important factor in developing a mechanistic understanding of effects and the population level, and allowing the development of models to predict effects over a range of population and environmental conditions.

Recolonization

Recolonization of a habitat can be described by several traits. Firstly, a species needs to be able to reach a new habitat, and this is determined by aspects such as *dispersal capacity* and *dispersal mode*. Once in the new habitat, a population then needs to be established. This involves *mode of reproduction* and other demographic traits described in the previous section.

Dispersal capacity describes the ability of a species to disperse to a new area. Populations of species with a low *dispersal capacity* have a higher risk of local extinction due to the presence of contaminants than species with a high *dispersal capacity*, which can easily move to colonize new habitats. Species with a high *dispersal capacity* will tend to have a longer dispersal distance. The distance travelled is partly related to *body size*, e.g. active dispersal of aquatic invertebrates might occur on a smaller scale than dispersal of birds or mammals. Nonetheless, some invertebrates can travel impressive distances, such as the Monarch butterfly (Altizer *et al.* 2000). Also, the *species distribution* in an area affects the potential for recolonization. Species with a patchy distribution have a smaller ability for colonization to new areas or spreading in their biotope than other species with a dense distribution. This means that suitable patches in their biotope will be empty for longer, reducing their recolonization potential. Similarly, *territorial behavior* limits a species' ability to move freely in available space, thereby reducing its recolonization potential. A species that does not show this behavior, on the contrary, can settle down wherever it is able to because it is not threatened by conspecifics and is able to colonize new habitats or places in its biotope more easily. *Territorial behavior* can occur at all life stages, or only during breeding. Whether a species can actually settle in a new habitat at a certain point in time is also determined by its *trophic level*. Recolonisation of an empty habitat follows certain arrival rules. In general, early colonizers create a more favorable habitat for subsequent arrivers (Lake *et al.* 2007), so lower *trophic levels* need to arrive first before higher *trophic levels* (predators) can be sustained.

Dispersal mode can be active or passive. Active dispersal is mostly used by vertebrate species, but also by some invertebrates such as butterflies. Passive dispersal is used by mostly invertebrate species, by means of water flow (hydrochory), air (anemochory) or attached to other animals (zoochory). *Dispersal mode* can change with different life stages. Active dispersal capacity is generally higher in adults than juveniles. Passive dispersal is sometimes best developed for resistant egg stages, such as zooplankton resting eggs (ephippia) attached to waterfowl (known as zoochorous dispersal) or earthworm cocoons transported by water (known as hydrochorous dispersal). The *dispersal mode* is related with the *locomotion type* of a species, the way it moves in its environment. For example, aquatic invertebrates can be crawlers or active swimmers, with the latter offering more potential for active dispersal. Drift is a passive mode of dispersal for aquatic invertebrates.

Downstream drift from upstream or tributary areas is by far the most frequently cited mechanism for re-colonisation within lentic systems (Wallace 1990). Higher drift can occur due to disturbance, chemical exposure, parasites or pesticide application (Schulz & Liess 2001).

Mode of reproduction has an influence on the speed of recolonization of a new habitat. For parthenogenetic reproducing species, a single individual is enough to start a new population. For species that reproduce sexually, at least one male and one female need to be present to start a new population. A combination of a *resistant stage* with good *dispersal capacity* (ephippia, cocoon) and *parthenogenetic reproduction* (Daphnia, earthworm species) gives a species an advantage. In general, species that have a short generation time and produce large clutches (typical r-strategists) will be able to establish a new population quickly.

TOWARDS A MECHANISTIC LINK BETWEEN TRAITS AND VULNERABILITY

Population vulnerability is the aggregated endpoint of external exposure, intrinsic sensitivity and population sustainability (Figure 1). To address population vulnerability quantitatively, it can be broken down into a series of (semi)mechanistic models which could predict how various traits in these categories contribute to population vulnerability. Although we do not attempt here to match each of the conceptual categories in Figure 1 with corresponding mechanistic models, it is evident that a set of mechanistic models could be used to describe the main aspects of population vulnerability (Pastorok *et al.* 2003; Grimm *et al.* 2009). The advantage of these (semi-)mechanistic models is two-fold: they divide population vulnerability into several, inter-linked processes and so facilitate the identification of traits which affect these processes; and they consist of equations or algorithms which quantify the effect of chemicals on organisms, populations or communities based on process related parameters.

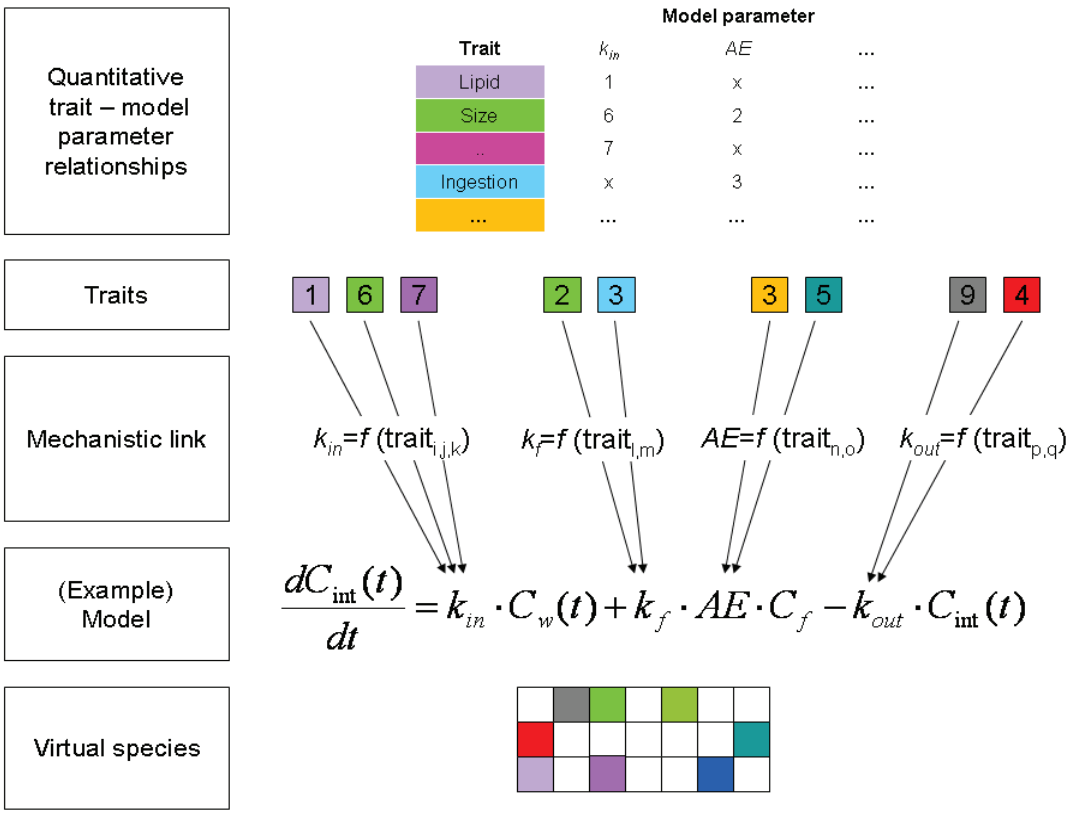

Figure 2: Mechanistic link between species traits and population vulnerability using mechanistic models. The framework is illustrated using a bioaccumulation model as example (see text for details). The table on top represents the hypothesised quantitative relationships between model parameters and traits. These relationships are further propagated into the models since model parameters are viewed as functions of trait suites. Traits that modify parameter values of the model are indicated with arrows. In this way, effects on species with unknown sensitivity, but a known trait composition, so called 'virtual species', can be predicted by a re-combination of quantitative trait information and linking this to mechanistic effect model parameters.

Here we illustrate a framework (Figure 2) for linking species traits quantitatively with aspects of population vulnerability described as a series of mechanistic effect model components. Figure 2 uses a simple bioaccumulation model – a process leading to intrinsic sensitivity - as an example to illustrate this link. The model equation describes the change in internal toxicant concentration (C_{int}) as a sum of uptake from water (uptake rate constant k_{in}, toxicant concentration in water C_w) and food (food intake rate constant k_f, toxicant concentration in food C_f, assimilation efficiency AE) as well as elimination processes (elimination rate constant k_{out}). Previously quantitatified relationships between traits and model parameters are represented in the table on top in Figure 2 using the traits *lipid* and *size* in combination with the model parameters uptake rate constant (k_{in}) and assimilation efficiency (AE) as illustrative examples. Parameters of mechanistic effect models are then viewed as functions of a series of species traits. Each model parameter can depend on multiple traits and each trait can modify multiple parameters. The crucial step lies in the establishment of quantitative relationships between model parameters and traits on basis of fundamental mechanistic knowledge and empirical data, which classically involves (i) *a priori* hypothesis about trait/parameter relation (mechanistic link) (ii) verification of the hypotheses leads to establishing the mechanistic link and (iii) empirical quantification of the mechanistic link (trait/parameter correlation). Once these relationships or links between traits

and model parameters are established, the traits of a species with unknown sensitivity or vulnerability can be used to generate a (semi-)mechanistic estimate of those effects. Theoretically the population vulnerability of a "virtual species" can be constructed from quantitative relationships between species traits and mechanistic effect models (lower part of Figure 2), where the "virtual species" stands for any of the many untested species. Similarly, these predicted effects on a species can subsequently be compared to measured data in order to test the established trait-parameter relationships and hence validate these traits-based models. Such studies to test and evaluate the predictive power of the traits-based approach to ecotoxicology are a pre-requisite for use in a regulatory context. For bioaccumulation, chosen here, excellent examples of quantitative trait/parameter relationships exist already. For instance (Hendriks *et al.* 2001) related toxicokinetic rate constants quantitatively to *weight*, *lipid content* and *trophic level* of organisms. Model parameters for this aspect of population vulnerability can then be generated for untested species, provided that the relevant traits are known. The outlined approach for extrapolation of toxicant effects to untested species based on their trait composition implies that traits may be more suitable for this purpose when quantified on a numerical scale. The use of categorical trait descriptions results in larger uncertainty for extrapolation. Hence, wherever possible traits quantified on a numerical scale are preferred.

The framework for a mechanistic link between traits and population vulnerability can be used with all kind of mechanistic models and at different levels of biological organisation. The traits described in the previous sections and in Table 2 outline the possibilities, but do not claim to be a comprehensive collection of relevant traits. Another example of well-established quantitative links between traits and aspects of population vulnerability are population models. Simulation of population dynamics using individual based models for example, is based explicitly on species traits. These might be life cycle traits to predict population dynamics (Preuss *et al.* 2009a) or a mix of life-cycle and ecological traits to predict recovery potential (Van Den Brink *et al.* 2007). Currently, there are many activities undertaken to promote the use of mechanistic models within environmental risk assessment of chemicals (Grimm *et al.* 2009). Hence it can be expected that approaches linking traits and models will receive increasing attention in the near future, especially in view of the potential for inter-species extrapolation when linked to traits.

KNOWLEDGE STATUS FOR USE OF TRAITS IN ERA

In the sections above, we have explored how and which traits can be mapped onto an established ecotoxicological framework for assessing population vulnerability (Figure 1). To build further on this, in Table 2 we evaluate the availability of trait data and strength of the trait linkage to ecotoxicological processes (i.e., how well the trait and the affected process could be mechanistically linked). The list of traits presented is not exhaustive, but our intention is to provide a preliminary inventory (and we welcome future elaboration) that can be linked to the toxicological processes leading to a population's vulnerability. From this analysis, it appears that traits linked to external exposure are mainly ecological traits such as life-history, habitat and food type related traits. Traits that map to intrinsic sensitivity are primarily morphological and physiological. Strong linkages between processes and traits were identified for bioaccumulation and biotransformation, and data availability for

bioaccumulation is good, while in the majority of cases biotransformation data is very limited (although it has a strong mechanistic link). Internal distribution is strongly linked to sensitivity, but available knowledge (probably due to practical considerations) is limited and focused on larger organisms (e.g. mammals). There is a lack of information about target site traits and traits describing compensatory mechanisms even though there are some strong mechanistic linkages. Population sustainability is also influenced by a range of ecological traits with a strong focus on life-history traits. There were strong links between traits and population sustainability, and in many cases large amounts of data are available.

Table 2 (continued on next page): Availability[1] and linkage[2] of traits to processes which affect population vulnerability in ecotoxicology.

Vulnerability factor	Affected process	Trait	Availability	Linkage
External exposure	Likelihood and magnitude of exposure	Habitat choice	xxx	xx
		Homerange	xx	x
		Food choice	xx	xxx
	Magnitude of exposure	Life span	xx	xx
		Migration	xxx	xx
		Ingestion	x	xx
		Active avoidance	x	xxx
	Likelihood of exposure	Hibernation	xx	xx
Intrinsic sensitivity	Bioconcentration	Mode of respiration	xx	xx
		Size of organism	xxx	xxx
		Surface area: volume	x	xx
		Integument permeability	x	xx
	Bioaccumulation	Assimilation efficiency	x	xxx
		Type of lipid	x	xx
		Lipid content	xx	xx
		Toxicant elimination ability	x	xxx
		Egestion rate	x	xx
		Transporters in membranes	x	xx
		Somatic growth rate (dilution)	xx	xxx
		Reproduction (transfer to offspring)	x	x
		Molting loss	x	x
	Internal distribution	Type/amount/distribution of lipid	x	xxx
		Target site distribution	x	x
		Circulatory system	xxx	x
		Sequestration	x	xxx
	Biotransformation	Biotransformation potential/conjugation	x	xxx
	Site of action	Presence of target	x	xxx
		Binding affinity	x	xx
	Compensation mechanisms	Anti-oxidant capabilities	x	x
		Heat shock proteins	x	x
		DNA repair mechanism	xx	x
		Metallothioneins	x	xxx
		Mixed function oxygenase	x	xxx

Demography	Intrinsic recovery rate	Generation time	xxx	xxx
		Life span	xx	xxx
		Voltinism	xxx	xxx
		Clutch size	xx	xxx
		Survival to reproduction	xx	xxx
Recolonisation	Dispersal	Dispersal mode	xx	x
		Locomotion	xxx	x
		Drift	xx	xx
		Distance travelled	xx	x
		Territorial behavior	xxx	x
		Anadromy	xxx	x
		Trophic level	xx	x
		Resistance stages	xxx	xx
		Reproduction mode	xxx	xx

[1] *Availability:* data are already in existence or can be easily acquired. Categories low = x (minimal data, limited taxa), medium = xx (good review available, some taxa), high = xxx (database, lots of taxa)

[2] *Linkage:* the link between the trait and affected process. Categories unknown/proxy = x (plausible but not proven), hypothesised = xx (some evidence for some taxa), established = xxx (relationships available for several taxa)

Note: The assignment of the categories varies greatly depending on the taxa, and this analysis is designed to give a broad overview based on the experience of the authors as consensus and preliminary judgment on fish, aquatic invertebrates, aquatic plants, bird and mammalian wildlife.

CURRENT CHALLENGES AND THE WAY FORWARD

Whilst the use of traits in ecotoxicology offers great potential for the future development of ERA, we recognize that there are some significant challenges that will have to be overcome for traits-based ERA to fulfill its potential. The broader challenges that confront the field, for instance on how to combine taxonomy based data with traits are also addressed in the other papers in this issue (Baird *et al.* in press; Culp *et al.* in press; Van den Brink *et al.* in press b) and here we highlight those that are specific for the field of ecotoxicology. In order to be able to develop the mechanistic approaches described above, it will be important to further identify and elaborate the relevant traits (suites) for use as model parameters. An adjacent challenge will be establishing means of translating fundamental ecological knowledge on traits into a form in which it is suitable to be used within the modeling framework. For instance despite the fact that there is often a good deal of fundamental knowledge about target site/compound interactions for certain model species, the suitability of such model species for making generalizations about other taxa is currently unknown. It would be possible for TERA to bridge this gap, if meaningful and predictive traits for target sites presence/absence could be formulated based on practical experimental observations and herewith establish a mechanistic link between mode of action and target sites. Similarly, developing the mechanistic approach would also call for a better integration and combination of the different effect models that can be used to estimate population vulnerability. The existing bioaccumulation, TKTD models, PBPK or ADME models and population models cited above offer a good starting point for this initiative. Modeling will also help us to prioritize which traits to quantify for a large number of species. For example sensitivity analysis can

pinpoint those traits in a given model or sub-model of population vulnerability that have the greatest influence on the predicted endpoints.

The linkage, or lack of linkage, of traits through phylogeny is also an important consideration and an area for future research. Traits are not purely independent entities, so before we can arbitrarily assign organisms into groupings based on shared traits, we need to better understand their linkages to each other. For example, the need to make contact with atmospheric air results in the tendency for many air breathing aquatic insect taxa to have well developed swimming abilities as evidenced in the Hemiptera. Thus, well developed swimming capacity and air-breathing often co-occur in species. Similarly, certain predation styles lend themselves to higher crawling mobility as seen in predatory stoneflies. Little is known about trait correlation with respect to contaminant susceptibility. Poff et al. (2006) use the term "trait syndromes" to describe traits that tend to co-occur, and observed that different phylogenetic groups tended to occupy different 'morphospace'. The tendency for closely related species to have similar traits is pervasive (Blomberg *et al.* 2003), and yet there are also many examples of related species being significantly different to each other for a given trait. For aquatic organisms, we currently lack a fundamental understanding as to which traits are more likely to "follow" phylogeny, and which are likely to be more labile. Poff et al. (2006) provide an excellent summary of trait lability in aquatic insects for a suite of traits associated with life history, mobility, morphology and ecology. They further suggest that the most phylogenetically labile traits are the most informative, because they are most responsive to local selection. Buchwalter et al. (2008) provide a different perspective, by examining the extent to which physiological traits "follow" phylogeny. In this example, physiological traits that contribute to cadmium sensitivity were compared across several insect species and found to be tightly linked to phylogeny. From an experimentalist's perspective, the consideration of phylogeny is attractive because it raises the possibility of predicting or extrapolating trait states on the basis of phylogeny, and having a rational framework for testing these predictions. In contrast, mechanistic and empirical relationships between traits and processes can exist and the role of phylogeny remains unclear as is the case e.g. for size related traits. Therefore, a major area needed to explore the use of traits for ecological assessments is to better understand the linkages among traits both within an individual taxon and across taxa. In this area and also for the characterization of many physiological and morphological traits rapid progress will be made in the coming years with techniques such as next generation sequencing and bioinformatics approaches as many genomes will gradually become available. A more detailed discussion of how this can contribute to TERA is discussed in Baird et al. (in press).

For each of the relevant fields or categories that we have identified in the framework, it could be useful to establish 'think tanks' or organized communities to further elaborate the ideas developed here and to also explore which approaches could be applied from other disciplines. For example, are there aspects of resistance management of pesticides that could assist our learning with developing our understanding of compensation mechanisms in non-target organisms? However, 'speaking the same language' is often a challenge in a multi-disciplinary science like ecotoxicology, and the same will certainly be the case for TERA. We therefore see it as critical that the traits-community comes together to develop an ontology that can be used to allow effective and efficient information exchange (Baird *et al.* 2008).

How then should we to move forward with TERA? In this paper, we have shown how traits could be used in ecotoxicology to enhance ERA. There are a number of key activities that are needed to build on the ideas presented here. Firstly, it is clear that traits-based approaches rely on the availability of good quality data for the trait in question. These data need to be stored and organized in a systematic way, so that data sharing and exchange is simplified. To this end, we recommend the development of traits ontologies and databases, and further discussion of options for these are discussed in a related paper (Baird *et al.* this issue, in press). Despite the challenges that remain, we believe that traits-based approaches have the potential to enable ecotoxicologists to develop a more mechanistic approach to understanding the reasons for differences in the vulnerability of populations to toxicants. Ultimately this enhancement of understanding should allow an improvement in our ability to extrapolate between species, providing more effective risk assessment methodologies.

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Moving the Procambarus spec. and Neocaridina denticulata cultures, 2008

3. CHAPTER

A new method for ranking mode-specific sensitivity of freshwater arthropods to insecticides and its relationship to biological traits.

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ABSTRACT

The problem of how to deal with species sensitivity differences to toxic substances has been addressed successfully with the species sensitivity distribution (SSD), yet this has not increased understanding about the underlying mechanisms of sensitivity. Other researchers have identified the mode of action of chemicals and also biological traits of species as determinants for sensitivity, yet no systematic approach combines these factors. To achieve this, first existing data on organophosphate, carbamate and pyrethroid toxicity and mode of action and also species trait information were mined. Second, we linked taxon sensitivity to their traits at the family level in order to generate empirical and mechanistic hypotheses about sensitivity-trait relationships. In this way, a mode-specific sensitivity (MSS) ranking method was developed, and tested at the taxonomic level of family and genus. The application of several quality criteria indicated overall confidence in rankings, but confidence in exact taxon rank was less certain, due to data insufficiency for certain groups. The MSS rankings were found to be applicable for trait-based approaches and were successfully linked to existing trait data in order to identify traits with predictive potential. Although this empirical analysis cannot test causality relationships between traits and sensitivity, testable hypotheses were generated, for further experimental investigation. Single traits as well as combinations of traits can be used to predict laboratory sensitivity to the substances tested, although associations were not as strong as in previous studies. We conclude that existing trait data are not suitable for every trait-based research question and that important traits remain to be identified and quantified in relation to the processes of toxicity, i.e., the toxicokinetics and toxicdynamics.

INTRODUCTION

In the ecological risk assessment of toxic substances, the search for the most sensitive indicator species has been pursued by many researchers, with little progress to date (Vaal *et al.* 2000). The lack of progress can at least partially be attributed to phylogenetic constraints on species sensitivity (Buchwalter *et al.* 2008), and to other factors, such as stressor mode-of-action and environmental variability (Escher & Hermens 2002), but not least to a sheer lack of empirical

observations on most taxa. We are thus limited to empirical data from a small number of species exposed as individuals in standardized toxicity tests, or as assemblages in microcosms or mesocosms. These data have been collated in toxicity databases like the U.S. Environmental Protection Agency (U.S. EPA) ECOTOX database (www.epa.gov/ecotox) and the Dutch National Institute for Public Health and the Environment (RIVM) Ecotox database (De Zwart 2002). These databases facilitate data mining to discern patterns of species sensitivity to individual stressors (Wogram & Liess 2001), allowing the ranking and grouping of species according to their vulnerability. For example, the SPEAR index combines data on intrinsic sensitivity originating from laboratory studies with voltinism, a life-history trait important in determining the population recovery potential (Liess & Von Der Ohe 2005). Although promising, the SPEAR approach does not consider pesticide mode of action (MoA), which strongly influences sensitivity (Vaal *et al.* 2000; Escher & Hermens 2002; Baird & Van den Brink 2007). Moreover, Liess and co-workers use the median lethal concentration (LC50) of *Daphnia magna* to account for inherent differences in toxic potency between chemicals. Although, *D. magna* is potentially a good choice for sensitivity centering because it is the most studied species in the field of ecotoxicology (Wogram & Liess 2001), its use poses the risk of over-reliance on toxicity information from a single species, which may not always be the most sensitive. Thus there is a clear need for a ranking method which would eliminate reliance on the exemplary properties of a single species and could incorporate knowledge of substance MoA.

A widely used tool in environmental risk assessment to estimate the variation in sensitivity among species, is the species sensitivity distribution (SSD) concept (Posthuma *et al.* 2002). However, this is a purely empirical and probabilistic approach that ignores mechanism, is based on inductive logic, and has likely hindered research on mechanistic understanding to predict species sensitivity. Biomonitoring and other retrospective assessment methods for aquatic environmental management (Rosenberg & Resh 1993) are strongly reliant on macroinvertebrate species presence/absence or abundance data. These approaches suffer from the difficulty in defining pristine ecological status for specified ecosystems, to allow identification of reference sites (Barbour *et al.* 2000). As a result, retrospective assessment cannot yet diagnose the cause of disturbances, such as pesticide run-off events. While a loss of species within a community at a certain site in time can be detected, the reason for this loss often remains uncertain since mechanistic and systematic understanding of sensitivity and vulnerability of species towards particular stressors is lacking. Therefore, not only prospective but also retrospective risk assessment benefits could be obtained from species being ranked on the basis of their intrinsic sensitivity to particular types of stressor.

Phylogeny alone does not determine sensitivity: Two species of the same genus may show large differences in sensitivity (Buchwalter *et al.* 2008), while convergent evolution may lead to similar sensitivity among taxonomically unrelated taxa. For this reason, it is not surprising that various authors have argued that taxonomy is not an inherently informative descriptor for prospective or retrospective risk assessment of stressors, but that phylogenetically-related aggregations of certain species traits could fit this purpose (Townsend & Hildrew 1994; Tachet *et al.* 2000; Poff *et al.* 2006; Barnett *et al.* 2007). Recently, Baird and Van den Brink (2007) have hypothesized that species sensitivity to stressors could be a predictable function of various species properties or traits, such as mode of respiration, size and life-cycle duration. We hypothesize that phylogeny in combination with

physiological, morphological and ecological traits of the organism, MoA, and physico-chemical properties of the stressor, and prevailing environmental conditions determine intrinsic species sensitivity (susceptibility) both in the laboratory, and also in the field. If this hypothesis continues to be supported, key traits could be defined for monitored species in threatened ecosystems, and their community-level variation assessed. By doing so, biomonitoring measurement endpoints (community composition defined by relative abundance of species) could be replaced by a more meaningful assessment endpoint, in mechanistic terms: the trait composition of the local community. In terms of prospective risk assessment, prediction of sensitive taxa could be made prior to experimentation, while for biomonitoring, species traits which are over- and/or underrepresented at putative impact sites can be used to indicate the presence and relative strength of individual stressors within a multi-stress regime (Ducrot *et al.* 2005; Horrigan & Baird 2008). Although various authors have indicated the potential of single traits for ecotoxicology, the systematic use and investigation of traits as explanatory variables or predictors of sensitivity is a new field of investigation (Baird & Van den Brink 2007; Buchwalter *et al.* 2008).

Here, the approach developed in Baird and Van den Brink (2007) was further extended, by exploring a larger dataset, focusing on a smaller group of compounds: organophosphate, carbamate and pyrethroid pesticides. By employing a series of online trait data sources, the number of traits available was widened in order to examine the influence of key taxon traits on acute sensitivity to these compounds, based on a large number of laboratory observations. Moreover, an alternative method for ranking intrinsic sensitivity is introduced, focusing on chemical classes or MoAs, which does not rely on a single species as a benchmark for sensitivity, its suitability for use in predictive modeling of taxon sensitivity to toxic substances is tested.

MATERIALS AND METHODS

Ranking

Toxicity data for the analysis were obtained from (De Zwart 2002). Only data entries that referred to acute toxicity (i.e., LC50 or median effective concentration [EC50] with 24 to 96h exposure, of which 79% (acetylcholinesterase- inhibitors) and 65% (pyrethroids) referred to lethality or immobilization as endpoint) of carbamates, organophosphates and pyrethroids to freshwater arthropods at the taxonomic genus level were retained, however no information on life stage of the tested animals was given. This resulted in a data set comprising 301 entries for carbamates, 1098 for organophosphates and 289 for pyrethroids. To these data, information on chemical class (here, carbamates, organophosphates, pyrethroids) was added, based on matching Chemical Abstracts Service numbers using the Pesticide Action Network PAN Pesticide database (Kegley *et al.* 2005) http://www.pesticideinfo.org/) and the Pesticide Manual (Tomlin 2003). In order to perform sensitivity ranking at different taxonomic levels, every species in the dataset was complemented with taxonomic information on associated genus, family, order and phylum as named in the Integrated Taxonomic Information System ([ITIS], retrieved on 10.02.2006/ 28.11.2008 for carbamates and organophosphates and on 09.02.2009 for pyrethroids at http://www.itis.gov.). To ensure taxonomic consistency, only one information system on taxonomy was used leading to a further reduction of the

datasets since 15 datapoints were excluded from further analysis because no corresponding genus entry existed in ITIS. This reduced the datasets to 296 (carbamate), 1093 (organophosphate) and 284 (pyrethroid) entries. Of the data retained, 2.3% of all entries were renamed at genus or species level following ITIS taxonomy, whereas for 5.6% no species entry, but valid genera were found in ITIS and included in the analyses (step 1 in Fig. 1).

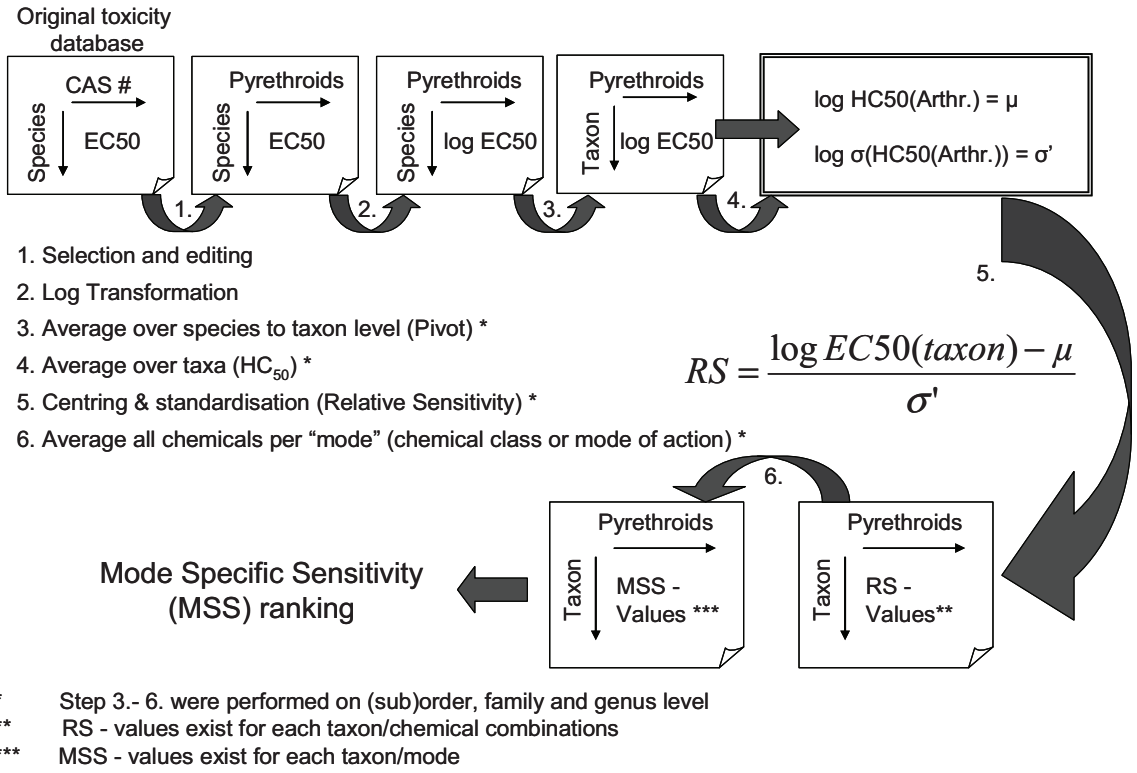

Figure 1: Stepwise data processing for mode-specific sensitivity (MSS) ranking with pyrethroids as an example. Note that data for input have to be controlled and corrected for consistent taxonomy. CAS = Chemical Abstracts Service; EC50 = median effective concentration; HC50 = median hazardous concentration.

The logEC50 was calculated for each record from the reduced data set (step 2 in Fig. 1) and averaged if multiple values were available for a chemical/species combination. Subsequently, the average of all data points for each chemical/taxon combination was calculated for each of three taxonomic levels (genus, family and order, step 3). In order to average across substances belonging to the same mode (step 6), the geometric mean of the toxicity values (median hazardous concentration [HC50], i.e., μ in Fig. 1) of all arthropod taxa and the spread of the log-normal distribution (the difference between the HC2.5 and HC97.5, i.e., ~ 2 x σ', the standard deviation of the population mean indicated in Fig. 1) of the toxicity data were calculated (step 4). For each taxon (at the order, family and genus level), all available log EC50 data were centered and standardized using μ and σ' as shown in Figure 1 (step 5), giving a relative sensitivity (RS) for each chemical/taxon combination. The μ accounts for inherent differences in toxicity while σ' standardizes data in terms of magnitude of differences in sensitivity for each substance. At this point, all chemicals with less than four taxon RS values were excluded from the analysis to minimise the error associated with imprecise estimates of μ

and σ' as a result of small numbers of observations. Small data sets can contain unrepresentative taxa due to sampling error, potentially skewing the distribution of sensitivities. Datasets of chemicals were omitted, if they did not contain both values for a sensitive and an insensitive taxon, i.e., taxa that were more or less sensitive than the average of all toxicity data available for that chemical class. This was done to ensure that the sensitive or insensitive part of the unknown sensitivity distribution of a particular chemical was represented in the data. The RS values obtained were averaged per taxon over all chemicals belonging to carbamates, organophosphates or pyrethroids separately, resulting in an empirical mode-specific sensitivity (MSS) value for each taxon per chemical class. If the MSS value was negative, the taxon was more sensitive than the average of all arthropods in the database at the taxonomic level of concern. If the value was positive, the taxon was less sensitive than the average arthropod, which has an MSS value of zero by definition.

Linking MSS ranking to traits

Two trait databases were linked to the MSS rankings at family level to extract trait-sensitivity relationships for carbamates, organophosphates and pyrethroids separately. To ensure taxonomic consistency with the toxiocity data all arthropod taxa were aligned in both trait databases with ITIS as well (retrieved 17/18.02.2009 from http://www.itis.gov/) and taxonomically invalid entries deleted, in addition to entries for which no MSS value was available. In order to limit the number of variables, traits which were either redundant, because they were present in both data bases, clearly irrelevant with regard to the toxicity data used (i.e., a short term toxicity test system) such as "sabrobity, trophic status, substrate preferendum" or had no mechanistic relation to intrinsic sensitivity (e.g., "waterbody type, aquatic stages, resistance forms, biogeographic regions, altitude, longitudinal distribution, transversal distribution, locomotion and substrate relation") were excluded from the analyses (see Table 1 for complete list of included and excluded traits). All definitions of traits and categories (values, modalities) were adopted from the original sources. The Pond-FX database is publicly available (http://ipmnet.org/PondFX/pondlife_main.htm; retrieved on 28.04.2006) and contains trait information for 86 Arthropod taxa, mostly at the family level (Heneghan *et al.* 1999), from which nine taxa were deleted from the database due to inconsistent taxonomy. Unfortunately, for the families Asellidae, Atyidae, Cambaridae, Chydoridae, Cyclopodidae, Daphniidae, Diaoptomidae, Gammaridae, Moinidae, Palaemonidae, Pontoporeiidae, Streptochephalidae and Talitridae only order (Amphipoda, Copepoda, Decapoda, Isopoda) or class (Branchiopoda) level trait data were available. All other trait-sensitivity matches were made at the family level. The second trait database - hereafter referred to as the Tachet database, was kindly provided by Henri Tachet and Phillipe Usseglio-Polatera (Usseglio-Polatera 1991; Usseglio-Polatera *et al.* 1999; Tachet *et al.* 2000) and contains trait information for 365 arthropod taxa mostly at the genus, but also at species level. This data set was reduced in the same way as Pond-FX; 10 traits were retained and 84 taxa were deleted due to inconsistent taxonomy. In contrast to the Pond-FX database, direct family level matches between MSS values and traits were possible in this case. The trait information in the Tachet database was fuzzy coded at the genus and species level. For example, size was assigned to different classes using percentages, meaning that a taxon can belong 50% to size class 1 and 25% to size class 2 and 3; for further details see (Chevenet *et al.* 1994). In order to create trait-MSS matches at the family level, the fuzzy coded trait information

was aggregated at the family level using the median of the original fuzzy codes of all taxa belonging to that family. If available, higher weighting was given to genera that were also present in the toxicity dataset used to calculate the MSS values. By doing so, trait information was linked in a close match between taxonomy and toxicity information. The categorized trait information at the family level (binary in Pond-FX and fuzzy in the Tachet database) of both databases had to be recoded from categorical coding to continuous or factorial variables to be able to perform multiple regression analysis. Where possible, the included traits were converted to continuous variables by calculating weighted averages of the different categories or modalities (for the above example this would yield ((1·0.5)+(2·0.25)+(3·0.25))/(0.5+0.25+0.25) = 1.75). If this was not possible as for respiration type, the classified traits were treated as factors and the category with the highest percentage was used. If more than one class had the same highest percentage (e.g., 30% gill respiration and 30% tegument respiration) a missing value was recorded. This resulted in two matching matrices for each chemical class investigated, one containing the families and their MSS values as response variables and the other the families and their trait information (16 traits of which nine were expressed as continuous variable and seven as factors) as explanatory variables (all input matrices are available as supplementary data).

Table 1 (continued on next page): Selected and adopted traits from Pond-FX and Tachet databases. For excluded traits see footnotes [a,b]. Numbers for trait categories were added for recoding in this study (see text for further details on how this was performed).

Acronym	Original Database	Trait	Original categories	Type of variable after recoding
Pvolti	Pond-FX	Number of generations per year (voltinism)		
		1	Semivoltine	Continuous
		2	Univoltine	
		3	Bivoltine	
		4	Multivoltine	
		5	Strongly multivoltine	
Pasexu	Pond-FX	Self-fertile or asexual reproduction		
		1	Yes	Continuous
		2	No	
		3	Occasionally	
Pmxage	Pond-FX	Maximum age (years)		
		1	< 0.1 years	Continuous
		2	0.1 – 0.5 years	
		3	0.6 – 0.9 years	
		4	1 - 1.9 years	
		5	2 - 4 years	
		6	> 5 years	
Pfetyp	Pond-FX	Feeding type		
		1	Herbivore	Factor
		2	Carnivore	
		3	Detritivore	
		4	Omnivore[c]	
			External parasite[d]	
			Internal parasite[d]	
Psooxy	Pond-FX	Source of oxygen		
		1	Water	Factor
		2	Atmosphere	
		3	Both[c]	
Pmobil	Pond-FX	Mobility		Factor
		1	Affixed sedentary	

		2	Free moving	
		3	Both[c]	
Tmsize	Tachet	Maximum potential size		
		1	Maximal potential size: ≤ 0.25 cm	Continuous
		2	Maximal potential size: > 0.25 - 0.5 cm	
		3	Maximal potential size: > 0.5 - 1 cm	
		4	Maximal potential size: > 1 - 2 cm	
		5	Maximal potential size: > 2 - 4 cm	
		6	Maximal potential size: > 4 - 8 cm	
		7	Maximal potential size: > 8 cm	
Tlcydu	Tachet	Life cycle duration		
		1	Life cycle duration: ≤ 1 year	Continuous
		2	Life cycle duration: > 1 year	
Tcynoy	Tachet	Potential number of life-cycle per year		
		1	Potential number of cycles per year: < 1	Continuous
		2	Potential number of cycles per year: 1	
		3	Potential number of cycles per year: > 1	
Tdispe	Tachet	Dispersal		
		1	Dispersal: aquatic passive	Factor
		2	Dispersal: aquatic active	
		3	Dispersal: aerial passive	
		4	Dispersal: aerial active	
TrespT	Tachet	Respiration type		
		1	Respiration: tegument	Factor
		2	Respiration: gill	
			Respiration: plastron[d]	
		3	Respiration: spiracle	
			Respiration: hydrostatic vesicle[d]	
TfeedH	Tachet	Feeding habit		
			Feeding habits: absorber[d]	Factor
		1	Feeding habits: deposit feeder	
		2	Feeding habits: shredder	
		3	Feeding habits: scraper	
		4	Feeding habits: filter-feeder	
		5	Feeding habits: piercer	
		6	Feeding habits: predator	
			Feeding habits: parasite[d]	
TveloP	Tachet	Current velocity preferendum		
		1	Current velocity: null	Continuous
		2	Current velocity: slow (< 25 cm/s)	
		3	Current velocity: medium (25-50 cm/s)	
		4	Current velocity: fast (> 50 cm/s)	
TsaliP	Tachet	Salinity preferendum		
		1	Salinity: freshwater	Continuous
		2	Salinity: brackish water	
TtempP	Tachet	Temperature preferendum[e]		
		1	Temperature: eurytherm with a slight preference for cold (< 15°C)	Factor
		2	Temperature: eurytherm with a slight preference for warm (> 15°C)	
		3	Temperature: eurytherm, no preference	
TpHpef	Tachet	pH – preferendum		
		1	pH: ≤ 4	Continuous
		2	pH: > 4-4.5	
		3	pH: > 4.5-5	
		4	pH: > 5-5.5	
		5	pH: > 5.5-6	
		6	pH: > 6	

[a] Excluded traits from Pond-FX: eggs, pupa, adult, dispersal ability, waterbody type, habitat type, and water depth.
[b] Excluded traits from the Tachet database (Henri Tachet and Phillipe Usselgio-Polatera): traits aquatic stages, reproduction, resistance forms, locomotion and substrate relation, food, transversal distribution, longitudinal distribution, altitude, biogeographic regions, substrate preferendum, trophic status and sabrobity.
[c] Category did not exist in original database, but was added for recoding.
[d] Category not present in the data subsets with mode-specific sensitivity values, category deleted.
[e] All taxa in data subsets were eurytherms, but some had a preference for > or < 15°C, in which case categories were modified.

To analyse the relationship between MSS ranking of families per chemical class and family-level traits, a linear regression selection method was performed, employing the RSEARCH procedure in GenStat Release 11.1 (Payne 2007). First, simple linear regressions were performed with single traits and MSS values for every chemical class separately. Thereafter, all possible combinations of two, three, or four traits were analyzed using general linear models. Candidate regression models (with $p < 0.1$ for each explanatory variable) were investigated separately, employing a multiple regression approach. An unconventionally high critical p value of 0.1 was chosen due to an expectation of high background noise in both the toxicity data as well as the trait data, and with a view towards generating hypotheses rather than testing them in these preliminary analyses. For the analyses, closed data sets with no missing data were necessary. Since trait information for some MSS-trait combinations was missing, data sets were further reduced using three different optimization criteria: MSS optimized (traits were deleted, so Pond-FX traits (P-traits) and Tachet traits (T-traits) were analyzed separately), trait optimized (all 16 traits were kept, deleting MSS/taxa), intermediate (some traits and some MSS values were deleted). These three optimisation criteria were used to extract maximum insight from the data. With these three resulting datasets the multiple regression analyses were performed separately for each chemical class. Before analysis, traits deemed to be obviously aliased were excluded from the multiple regressions (e.g., for the intermediate analysis "mobility, dispersal and feeding habit" were dropped).

RESULTS

Ranking

At the order level, for carbamates the most sensitive groups by rank were Cladocera, Plecoptera, Amphipoda, and Diptera and the most insensitive were Anostraca, Coleoptera, Isopoda and Decapoda. Calanoida, Cladocera, Amphipoda and Podocopida ranked as most sensitive towards organophosphates, while in contrast to carbamates, Plecoptera only ranked as the sixth most sensitive. More consistently with carbamates Anostraca, Isopoda, Coleoptera and Anisoptera ranked as most insensitive. The orders most sensitive (Amphipoda, Isopoda, Ephemeroptera, and Plecoptera), and insensitive (Heteroptera, Coleoptera, Trichoptera, and Anisoptera) to pyrethroids showed only a limited overlap with carbamate and organophosphate rankings, while Cladocera ranked with an MSS value of -0.005, in the middle.

At family level, the groups most sensitive to carbamates were Atyidae, Perlodidae, Pteronarcyidae, and Chironomidae, with Haliplidae, Cambaridae, Dytiscidae, and Streptocephalidae as most insensitive (Fig. 2). Daphniidae ranked as the fifth most sensitive family. For organophosphates the most sensitive (Atyidae, Chydoridae, Diaptomidae, and Caenidae) and insensitive (Streptocephalidae, Belostomatidae, Brachycentridae, and Hydrophilidae) families differed substantially from carbamates. Daphniidae again ranked as fifth most sensitive family (Fig. 2). The most sensitive families to pyrethroids were Baetidae, Ephemeridae, Pteronarcyidae, and Gyrinidae, while Hydrophilidae, Corixidae, Haliplidae, and Hydropsychidae were the most insensitive.

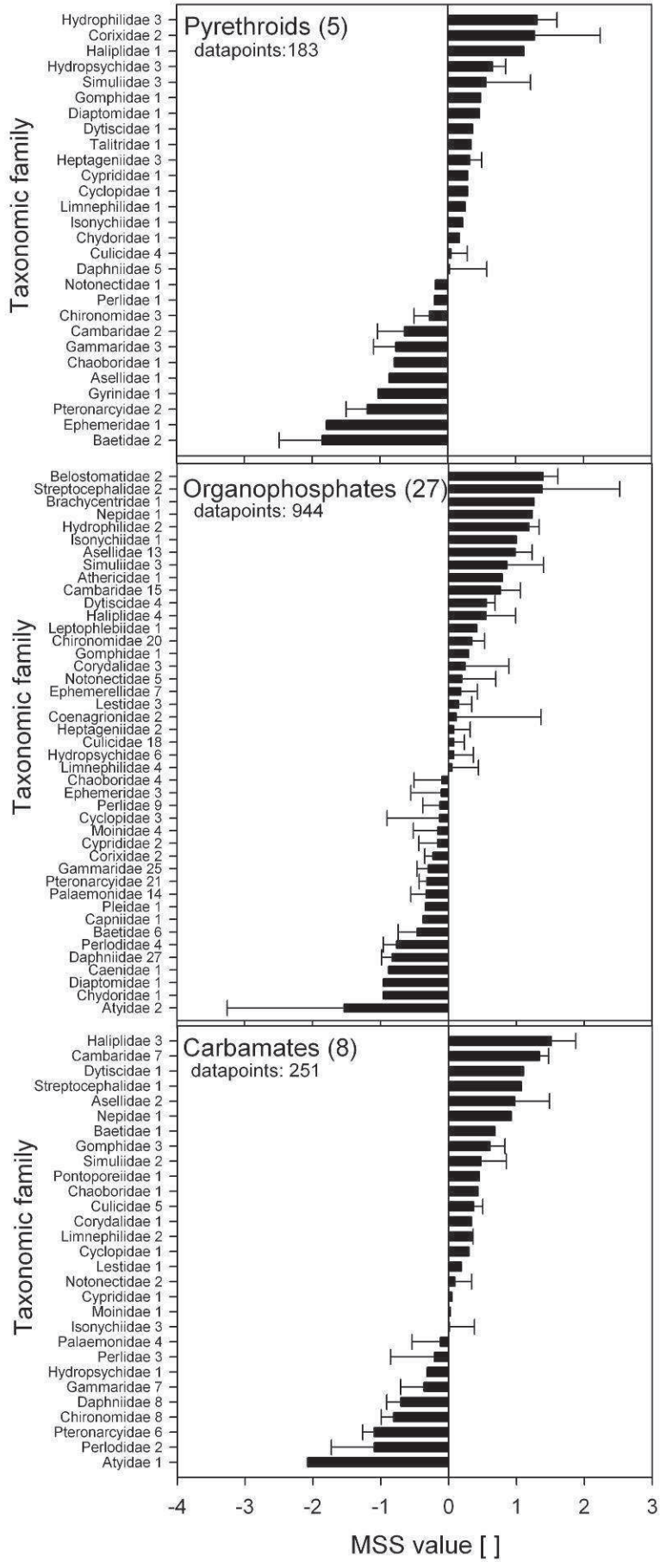

Figure 2: Mode-specific sensitivity (MSS) ranking of families for pyrethroids, organophosphates and carbamates. Negative MSS values indicate a more sensitive, and positive MSS values a less sensitive family compared to the average freshwater arthropod family in the database. Accordingly sensitive taxa are placed on the lower end of the Y axis and insensitive at the upper end. Numbers behind families indicate the number of unique chemical substances included in the MSS rank and the error bars indicate the standard error of their mean. The numbers in brackets denote the number of unique chemical substances included in the overall ranking. Datapoints reports the total number of underlying lower taxon values contributing to the overall ranking.

At genus level, most sensitive to carbamates were *Caridina*, *Skwala, Claassenia, and Ceriodaphnia* while *Peltodytes, Tanypus*, *Procambarus, and Eretes* ranked as the most insensitive genera. *Caridina*, *Ceriodaphnia, Isoperla, and Simocephalus* were the most sensitive genera to organophosphates, while *Mesocyclops, Streptocephalus, Ceratopsyche,* and *Ranatra* were ranked most insensitive to organophosphates. The ranking on genus level for organophosphates can be seen in Figure 3, where the taxonomic consistency of genera ranking in relation to family ranking is also illustrated. The most sensitive (*Hexagenia*, *Baetis*, *Cloeon,* and *Pteronarcys*) and insensitive (*Procambarus*, *Sigara*, *Hydrophilus,* and *Anopheles*) genera to pyrethroids showed no overlap with carbamates and organophosphates. All ranking results are listed in the supplementary data file.

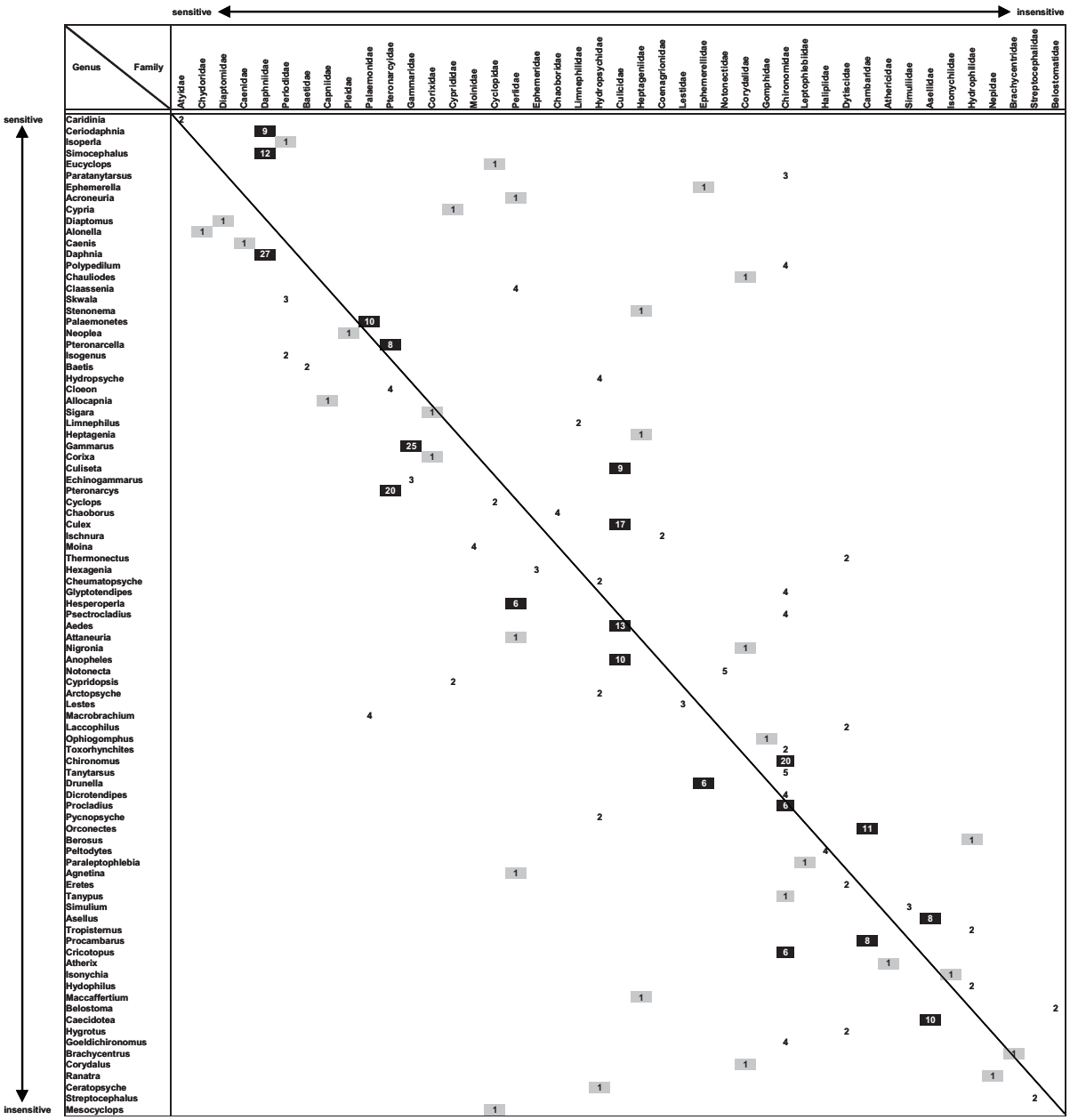

Figure 3: Tabular illustration of taxonomic representation in mode-specific sensitivity (MSS) rankings for organophosphates. Families on the top are listed according to their MSS ranks from left (sensitive) to right (insensitive). Genera are listed on the left as they appeared in the MSS ranking from the top (sensitive) to the bottom (insensitive) and number cell entries reflect taxonomic relations. The numbers itself reflect the number of relative sensitivity (RS) values (= chemicals) contributing to the MSS value of a genus. Black cells with white numbers are based on more than six chemical RS values and grey cells with black numbers indicate MSS ranks based on a single chemical. The line represents the optimal taxonomic representation of familie's sensitivity versus genera's sensitivity and is well represented by MSS ranks calculated on basis of six unique organophosphates.

Linking ranking to traits

The results of the regression analyses for organophosphates, carbamates, and pyrethroids for up to four interaction terms are summarized in Table 2 and described per chemical class in the following, with traits both from the Pond-FX (P-traits) and the Tachet (T-traits) database. The traits "temperature preferendum (TtempP), feeding type (Pfetyp) and current velocity preferendum (TveloP)" alone were significantly related to the MSS sensitivity of families to carbamates. As for clusters of traits the pair of Pfetyp and "dispersal" (Tdispe), the triple of Pfedtyp, Tdispe, and "maximum age" (Pmxage) and the two trait quads of "voltinism" (Pvolti), Pmxage, Pfetyp, Tdispe and "maximum potential size" (Tmsize), Tdispe, "respiration type" (TrespT) and "feeding habit" (TfeedH) were correlated significantly with carbamate MSS rankings at the family level. These results were all obtained from the regression analyses with the trait optimized dataset and T-traits. The analyses with the intermediately optimized and P-trait optimized datasets did not yield significant results.

Table 2: Significant ($p < 0.1$) single and multiple linear regressions with statistical details[a] for linkage of family mode-specific sensitivity (MSS) rankings to recoded trait information (for trait acronyms see Table 1).

	Explanatory variables	Organophosphates	Carbamates	Pyrethroids
Linear Regression	Single traits	Pasexu (0.033) {0.098} [37]	Pfetyp (0.098) {0.148} [25]	NS
		TtempP (0.008) {0.247} [30]	TveloP (0.077) {0.124} [19]	
			TtempP (0.04) {0.247} [19]	
Multiple Regressions (analyzing combinations of traits)	Pond-FX traits	NS [37]	NS [25]	NS [25]
	Tachet traits	TtempP (0.076) + TveloP (0.078) {0.357} TveloP (0.008) + TsaliP (0.044) + TfeedH (0.068) {0.526} [16]	Tmsize (0.022) + Tdispe (0.057) + TrespT (0.037) + TfeedH (0.068) {0.695} [12]	Tlcydu (0.019) + TrespT (0.021) {0.554} [11]
	All traits (trait optimized)	CP [17]	Pfetyp (0.041) + Tdispe (0.047) {0.446} Pfetyp (0.047) + Tdispe (0.052) + Pmxage (0.079) {0.571} Pvolti (0.017) + Pmxage (0.007) + Pfetyp (0.012) + Tdispe (0.004) {0.773} [16]	Pasexu (0.005) + Pvolti (0.049) {0.4} [17]
	Intermediate analysis	NS [27]	NS [17]	TrespT (0.061) + TtempP (0.079) {0.37} TPsooxy (0.063) + TtempP (0.072) {0.366} TrespT (0.039) + TveloP (0.039) + Pasexu (0.058) {0.402} [17]

[a]Abbreviations: NS = no significant result in all regressions; CP = computation not performed due to strong alias between traits; (p) gives the actual p-value ; {r^2} indicates the adjusted coefficient of determination; [n] the number of response units (MSS values).

The traits "self-fertile, asexual reproduction" (Pasexu) and TtempP alone were significantly correlated with MSS ranking of family sensitivity to organophosphates. In addition the trait pair TtempP and TvelopP and the trait triple TveloP, "salinity preferendum" (TsaliP) and TfeedH showed a significant relationship to organophosphate MSS ranking of families and these results originate from the analysis with the T-trait dataset (MSS optimized). All other analyses either did not reveal significant results or were not performed due to strong alias between traits.

Family's MSS rankings for pyrethroids could not be related significantly to any single trait, but to a number of trait clusters. The four trait pairs Pasexu and Pvolti, TrespT and TtempP, "life cycle duration" (Tlcydu) and TrespT and "source of oxygen" (Psooxy) and TtempP were significantly related to the pyrethroid MSS rankings of families. Also, one trait triple, composed of TrespT, TveloP, and Pasexu explained the MSS ranking of families for pyrethroids significantly. Most of the significant results for pyrethroids originated from the analyses with the intermediately optimised dataset, while the P-trait optimised dataset did not reveal any significant result.

In the analysis with separated traits (MSS optimized) a general difference was seen between P- and T- traits. The P-traits were only significant in combination with T-traits, while the latter also appeared in significant clusters without P-traits. However when P- and T-traits are pooled the computation was not possible for the largest dataset (organophosphates) due to strong aliasing between traits. Furthermore, analyses with the intermediately optimized dataset only yielded significant results for pyrethroids, but not for carbamates and organophosphates. The output of all regression analyses are compiled in the supplementary data file.

DISCUSSION

Ranking

Many studies have attempted to elucidate general mechanisms explaining differences between taxa in laboratory sensitivity to chemical stressors (Vaal *et al.* 2000; Escher & Hermens 2002; Posthuma *et al.* 2002). Most have suggested that the MoA of the chemical stressor could explain the largest part of the variation in sensitivity, while the remainder could be related to intrinsic sensitivity differences among species (Vaal *et al.* 2000). The MSS ranking derived here yields results that are consistent with our knowledge of the laboratory sensitivity of various freshwater Arthropoda to pesticides (Schroer *et al.* 2004; Maltby *et al.* 2005). A detailed discussion on every taxon, however, would be beyond the scope the present study. Within macroinvertebrates, EPT (Ephemeroptera, Plecoptera, Trichoptera) taxa are considered to be highly vulnerable to anthropogenic stressors, and their presence in streams is an indicator of good ecological status and water quality (Roy *et al.* 2003). In addition to insects, amphipods and isopods, our analysis also includes zooplankton and larger crustaceans such as crayfish, some of which are intrinsically more sensitive than EPT taxa. Therefore, intrinsic sensitivity and recovery potential, which together define vulnerability, of a wider range of taxa should be emphasised in biomonitoring studies. For instance, Amphipoda and Isopoda, here ranked as highly sensitive towards pyrethroids, might not be vulnerable taxa in the field due to their high recovery potential (Beketov & Liess 2008c; Maltby & Hills 2008). This distinction between intrinsic

(physiological) sensitivity at the organism level and field related vulnerability is important for risk assessment.

Mode-specific sensitivity rankings obtained here at the order level can be compared with order level rankings towards organic compounds from (Wogram & Liess 2001), which were further refined by (Von Der Ohe & Liess 2004) and used to calculate SPEAR values (Liess & Von Der Ohe 2005). The presented MSS and SPEAR rankings do not always agree; large differences were observed in the ranking for organophosphates (especially Plecoptera and Decapoda) and smaller differences for carbamates (mainly Diptera, Trichoptera and Decapoda), despite the fact that both datasets have a large overlap (i.e., through the U.S. EPA ECOTOX database). These differences in rank can be explained by the different standardization approaches employed by the two methods (i.e., using *D. magna* (SPEAR) versus all arthropods (MSS)), by the differences in quality criteria and data selection, which are discussed in more detail below and finally by the fact that MSS rankings are separated by MoA or specific chemical classes. This latter difference is crucial, since it has been shown that the MoA of chemicals to a large extent determines the range in sensitivity differences across species compared to intrinsic inter-species differences (Vaal *et al.* 2000; Maltby *et al.* 2005). (Von Der Ohe & Liess 2004) discuss the dilemma of deciding between decreased quantity of data and aggregation of taxa and chemicals, choosing to aggregate chemical substance as organic pollutants. Although this choice is well discussed in the paper our results show that it is crucial to include MoA into the ranking, and that relatively stable rankings are still possible when MoA is considered. This is clearly indicated by the dissimilarity in ranking between MSS and SPEAR (Wogram & Liess 2001; Von Der Ohe & Liess 2004). The SPEAR approach omits outliers, defined by a substance-by-species value differing more than a factor of 30 from the next nearest observation in a group of at least three independent observations. This criterion for discarding variation is questionable, however, since sensitivity differences of up to two orders of magnitude between clones of *D. magna* have been reported before (Baird *et al.* 1991), and sensitivity differences between life stages have also been widely demonstrated (Medina *et al.* 2002). In this context, the choice of *D. magna* as benchmark is unfortunate, since it is a clonal organism with known variation in sensitivity. Also, for our purposes, since all species have a unique trait composition, using a single species as benchmark introduces bias into the ranking when a linkage to traits is intended.

The development of any ranking method for taxon sensitivity requires: justification of data selection, taxonomic consistency and other quality criteria, representativeness of taxomomic levels, display and sources of variation, and applicability for risk assessment/biomonitoring and shortcomings. These are discussed in the following: Regarding the justification of data selection, the present study followed previous recommendations in data selection used for the construction of SSDs (Maltby *et al.* 2005; Baird & Van den Brink 2007). Saltwater arthropods were excluded from the data selection due to a dominant bias for Crustaceans in their taxa composition, which may alter their sensitivity distribution (Maltby *et al.* 2005). As to taxonomic consistency and other quality criteria, in order to further reduce error and improve the quality of the MSS values, several quality criteria were applied when selecting data, i.e., at least four taxon RS values had to be present for one chemical of which one taxon had to be a sensitive and another an insensitive taxon to avoid a skewed sensitivity distribution. The check for consistent and valid taxonomy using ITIS was found to be crucial; the ranking of some taxa obtained

before (results not shown) and after this check changed quite substantially. This indicates that consistent taxonomy is necessary when examining sensitivity due to constraints arising from phylogeny. With reference to the representativeness of taxonomic levels, the range of MSS values, the variation of RS values and the representativeness of taxonomic levels are helpful measures to analyse which taxonomic level is most appropriate for the environmental risk assessment of chemicals. Any taxonomic sensitivity ranking should be a good representation of reality, and should aim for the highest taxonomic resolution possible. For our research question, taxonomic family level has been a good compromise, but could be a poor descriptor in another context. Various authors have suggested working at the species or, at least, genus level to estimate sensitivity to stressors and to monitor the environment, since large differences between closely-related species are known to occur (Buchwalter *et al.* 2008); however, this might be more of an exception than a general rule. The highest taxonomic resolution possible in our analysis was genus level, since only a few toxicity or trait data were available at the species level. Some RS values obtained for families were based only on a single genus, resulting in an estimate with no confidence interval. Order level resolution yielded poorer results, since families and genera within orders had very different MSS ranks. A good example of this is provided by the genus *Caridina,* which belongs to the family Atyidae and the order Decapoda. In the organophosphate MSS ranking *Caridina* and Atyidae are ranked as the most sensitive genera and family, respectively. Decapods, however, are ranked the sixth most insensitive order out of 17. Also inconsistencies are common between family and genus level rankings, especially for the family of Chironomidae, whose species are very diverse and variable in sensitivity, while overall showing an average sensitivity (MSS value of 0.02). Good examples for this variation are the genera *Paratanytarsus* and *Polypedilum*, which are ranked as quite sensitive genera (5th and 14th out of 90) in contrast to *Goeldichironomus* and *Tanypus*, which are ranked as insensitive (85th and 76th out of 90). This generates an interesting hypothesis for future testing: that more ecologically diverse taxonomic groups are more likely to vary in their sensitivity to stressors. For cases where more than six chemicals (RS values) contributed to an MSS value, both order and family level representation of species sensitivity proved consistent with expectations (Fig. 3). However, MSS values based on only one chemical tended to deviate significantly from the ideal representation of taxonomic relations (Fig. 3). This again emphasises the problem of a lack of sufficient data for modelling sensitivity (Baird & Van den Brink 2007), in addition to shortcomings in data quality and in terms of accuracy in taxonomy. Although the data used here represented the largest available quality-assessed data set for carbamate, organophosphate, and pyrethroid pesticides, the ranking of taxa obtained should be cautiously interpreted for the reasons stated above. Ultimately, the present study was compromised in terms of trade-offs among data type, data availability and data quality, and we would strongly encourage future studies to strive to use taxonomic descriptors of the highest resolution (i.e., species) when data permit. Concerning the display and sources of variation, Figure 2 presented the standard error of the MSS values as a measure of variation. Sources of variation in sensitivity of taxa to toxic substances will reflect differences between laboratories, studies and places, the precision of the estimation, and the potential secondary and tertiary MoAs of the single chemicals (Escher & Hermens 2002; Casida & Quistad 2005). For example, it is possible that substances grouped by MoA or chemical class do not always act as similarly across taxa, as is suggested by the MoA classification

(Vaal *et al.* 2000), which is discussed below in more detail. Another potential source of variation in species sensitivity could be related to the development of genetically implemented resistance in test populations after long term sublethal or pulsed exposure to chemicals and mixtures of chemicals (Bard 2000; Brausch & Smith 2009). In terms of applicability and shortcomings, the presented ranking is a neutral and flexible way of expressing sensitivity differences to toxic substances at different taxonomic levels and for different classes of toxicants and therefore suitable for a wide range of applications in environmental risk assessment. The need to differentiate between MoAs and/or chemical classes becomes apparent when the different MSS rankings are compared. For instance, the orders Plecoptera and Diptera show comparable differences in rankings for carbamates and organophosphates, when compared to the ranking for pyrethroids. In contrast, order MSS rankings for pyrethroids classify Isopoda and Ephemeroptera as sensitive next to Amphipoda and Plecoptera, while Isopods were among the most insensitive orders for both classes of acetylcholinesterase inhibitors (AChE-I) and Ephemeroptera ranked as moderately sensitive. On the other hand Trichoptera are very insensitive to pyrethroids, while they exhibit moderate sensitivity to both classes of AChE-I. These results indicate that the MSS ranking method is able to express sensitivity differences of taxa for different MoA and chemical classes, however differences in ranks are more transparent and distinct between MoAs. This emphasizes the significance of MoA based environmental risk assessment and biomonitoring, although the high data needs, the requirements for taxonomic consistency and other quality criteria will restrict applicability of this ranking method due to sparseness of data for other chemical stressors. When data are sparse the exact ranking results also strongly depend on in/exclusion criteria of taxa and chemicals. Therefore the exact data needs and quality criteria used in this ranking method should be further evaluated by application to more datasets, including different toxic substances, phyla, classes and habitats. Another critical point of the presented ranking is its inability to take variability in sensitivity on lower taxon levels (e.g., species) into account. This variation can be substantial; sometimes species LC50/ EC50 can vary by a factor of 500 within one genus for reasons which remain unclear. Inclusion of this variability into the calculation, however, would blur the results because the resulting overall variability cannot be assigned to a clear source. Despite these limitations, the MSS ranking method enables us to link sensitivity data with traits, and is therefore a key first step towards the development of trait-based approaches in environmental risk assessment and biomonitoring.

Linking of MSS-ranking to traits

The results indicate that the ranking of sensitivity of families to carbamates and organophosphates is empirically related to particular single traits (Pasexu and TtempP for organophosphates; Pfetyp, TveloP, and TtempP for carbamates), while combinations of up to four traits were also statistically related to MSS rankings for all three chemical classes (e.g., Tasexu and Tvolti). These two key findings emphasize the predictive potential of traits for sensitivity as previously proposed by Baird & Van den Brink (2007). Since the results presented here are empirical relationships, they can only serve as hypotheses at this time, as no underlying mechanism was posited. Mechanistically, sensitivity to toxic substances is a complex interaction between phenotypic characters and toxic modes of action. For AChE-I (i.e., the two chemical classes of organophosphates

and carbamates studied here), the trait TtempP was significantly related to the sensitivity ranking as single trait, but in the case of organophosphates also in combination with TveloP. The original trait from Tachet for TtempP was slightly modified for our analysis, since all taxa in our dataset were eurytherms, but only had slight preferences for either colder or warmer than 15°C. Uptake of chemicals strongly depends on temperature, as has been shown for chlorpyrifos (Buchwalter *et al.* 2003). Temperature variation across studies could be the underlying reason for this result if temperature conditions in the toxicity studies were highly variable, which were here not checked in the original publications, given the nature of our analysis. Also other traits related to preferences and stress showed significant relationships (TveloP and TsaliP), indicating that underlying physiological status of an organism is likely related to stress in general. The results also indicate that trait assemblages are more often significantly related to stress than single traits. The ranking and subsequent linking was also performed with a dataset with AChE-I (pooled data on carbamates and organophosphates, results shown in supplementary data) and employed an intermediate optimisation procedure. Remarkably, the trait quad of TveloP (0.02), TsaliP (0.01), TtempP (0.04) and TpHpref (0.033) and the combination of the five traits TrespT (0.099), TveloP (0.011), TsaliP (0.002), TtemP (0.028), and TpHpref (0.06) explained a significant part of sensitivity (adjusted coefficient of determination was 0.49 for both). This analysis was performed ignoring the strong aliasing between these traits and both the aliasing and the significant result support the hypothesis that particular trait assemblages are important in determining sensitivity. Aliasing in data sets can either be due to co linearity of traits or to bias within the data set. Traits are co linear with each other due to trait syndromes, i.e. traits occur together in species due to phylogenetic canalization (Poff *et al.* 2006). Bias in toxicity as well as the trait databases also occurs, due to practicality and pragmatism in choice of test organism. For instance, the data subsets for organophosphates consisted of 37% Diptera, 24% Cladocera, 13% Amphipoda, 9% Plecoptera, and 6% Decapoda, the rest of the 12 orders contributed with less than 2.5% each. For pyrethroids the situation is even more biased, since 42% of the data points referred to sensitivity of Cladocera and 34% to Diptera, while the remaining 12 orders contributed each less than 5.6 %. The carbamates dataset was dominated by 28% Cladocera, 27% Diptera, 12% Amphipoda and Decapoda, and 10% Plecoptera, while all other 11 orders contributed less than 2% to the data. For P-trait descriptors, families belonging to Cladocera, Amphipoda, Copepoda, Isopoda, and Decapoda were only described at the class/order level, which are the most common taxa in toxicity databases. The impreciseness of P-traits is likely a reason for the lack of significant relationships found (Table 2). In addition, there was no clear pattern in specific traits or clusters of traits appearing throughout the different regressions, nor in relation to MoA. Of all 16 traits investigated, only 2 traits (Pmobil and Tcynoy) were never significant in at least one of the regression analyses, and all other traits appeared either singly or in combination at least once. Despite the fact that looking at the frequency of significant appearance of traits throughout all analyses is not really a meaningful measure due to prior exclusion of aliased traits, it is worth noting their relative occurrence: TtempP (8), TveloP (7), Pfetyp (5), TrespT (5), Tdispe (5), TsaliP (4), Pasexu (4), TfeedH (4), Tmxage (2), Tlcydu (2), Pvolti (2), TpHpref (2), Tmsize (2), and Psooxy (1). Also a pattern in trait significance and MoA was observed: for organophosphates and carbamates, traits which were derived from physico-chemical preferences of taxa were mostly significant, while for pyrethroids and, to a lesser extent, carbamates, mostly biological traits were

significant. Summarizing all regression analyses, and considering non-significant, but 'interesting' traits, the important biological traits were generally related to asexual reproduction, respiration type/ source of oxygen and feeding type and habit. For the relationship between asexual reproduction and sensitivity, a possible hypothesis could be that r-strategy trades off costly detoxification or repair and recovery mechanisms, which makes an organism less susceptible to chemical stressors. They might be less well developed in organisms with (occasional) asexual reproduction in the course of evolution. In contrast, *D. magna* shows sophisticated detoxification abilities in various studies with various chemicals (Kashian 2004) and therefore its high intrinsic sensitivity might be related to other traits or processes. The relation of respiration type/ source of oxygen and sensitivity has been discussed before (Buchwalter *et al.* 2002) and already been implemented in quantitative structure activity relationships (Hendriks 2007). The underlying hypotheses mainly refer to increased uptake rates for water breathers in comparison to atmospheric breathers. Large uptake surfaces, such as gills, also enhance uptake in relation to body size, which is best expressed as surface area/volume ratio (Hendriks 2007). Unfortunately, this analysis cannot support the trait combination of body size and respiration type empirically. A reason for this could be that it was not possible to differentiate the toxicity data by life stages and therefore could not include this as a trait in our analysis. For the trait feeding type/habit we hypothesize that a specific diet leads to specific fatty acid compositions and total lipid content (Hanson *et al.* 1985), which could both be better descriptors for a trait than feeding habit or type in relation to sensitivity.

In the study of Baird & Van den Brink (2007) it was possible to explain 71% of the variation in sensitivity with four traits: respiration, life cycle, body mass and taxonomic group using multivariate statistics, but their trait data set was specifically compiled for their research question. The present study tried to follow this line of research by using a much larger toxicity database, but in this case with existing trait information and univariate methods to investigate MoAs separately, at the level of taxonomic family. In marked contrast, our approach here did not deliver as clear and robust results as in the previous study, suggesting both that a good selection of toxicity data is needed at the species level and/or a more targeted selection of traits is required, i.e., ones that have a clear mechanistic relationship to the MoA investigated and the mechanism of toxicity. Although our results highlighted the importance of a few biological and physico-chemical preference traits, these are not likely to be the fundamental determinants of intrinsic sensitivity. Next to an applicable sensitivity ranking for use in future trait-based approaches, the present study nevertheless has generated useful hypotheses on potential mechanistic trait-sensitivity relationships, which can be the subject of further study. In conclusion, existing trait information complied by ecologists was not found to have a strong relationship with laboratory sensitivity. For this reason, new, improved traits, better aligned with mechanistic hypotheses urgently need to be defined, categorized, measured and related to the mechanistic processes of toxicity such as toxicokinetics and toxicodynamics, and this will constitute the next important step in the development of trait-based ecological risk assessment.

SUPPLEMENTARY INFORMATION

PDF file containing (A) the origin of used dataset and documentation of the performed quality check by the RIVM; (B) contact information to obtain the used dataset; (C) Tables reporting the MSS values, errors and number of chemicals for all analyses at the taxonomic (Raymond *et al.*)order, family and genus level; (D) Tables reporting the chemicals included in the analyses; (E) the input data for linear and multiple regression analyses and (F) the output data of linear and multiple regression analyses. Online available at:
http://www3.interscience.wiley.com/cgi-bin/fulltext/122675420/sm001.pdf?PLACEBO=IE.pdf

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Single species tests, Michalis and Maria, 2006

4. CHAPTER

Variability in the dynamics of mortality and immobility responses of freshwater arthropods exposed to chlorpyrifos.

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ABSTRACT

The Species Sensitivity Distribution (SSD) concept is an important probabilistic tool for Environmental Risk Assessment (ERA) and accounts for differences in species sensitivity to different chemicals. The SSD model assumes that the sensitivity of the species included is randomly distributed. If this assumption is violated, indicator values such as the 50% hazardous concentration can potentially change dramatically. Fundamental research, however, has discovered and described specific mechanisms and factors influencing toxicity and sensitivity for several model species and chemical combinations. Further knowledge on how these mechanisms and factors relate to toxicological standard endpoints would be beneficial to ERA. For instance, little is known about how the processes of toxicity relate to the dynamics of standard toxicity endpoints and how these may vary across species. In this paper we discuss the relevance of immobilization and mortality as endpoints for effects of the organophosphate insecticide chlorpyrifos on 14 freshwater arthropods, in the context of ERA. For this, we compare the differences in response dynamics during 96 h of exposure with the two endpoints across species using dose response models and SSDs. The investigated freshwater arthropods vary less in their immobility response than in their mortality response. However, differences in observed immobility and mortality were surprisingly large for some species even after 96 h of exposure. As expected immobility was consistently the more sensitive endpoint and less variable across the tested species and may therefore be considered as the relevant endpoint for population of SSDs and ERA, even though an immobile animal may still potentially recover. This is even more relevant since an immobile animal is unlikely to survive for long periods under field conditions. This and other such considerations relevant to the decision-making process for a particular endpoint are discussed.

INTRODUCTION

Decades of ecotoxicological testing have repeatedly revealed large differences in the response of species towards toxicants, but have not resulted in the identification of a ‘most sensitive species’ (Cairns 1986), which is now widely accepted as non-existent, although some indications for generally more sensitive groups exist (Dwyer *et al.* 2005). Across chemicals, it is primarily the mode

and mechanism of action of a toxicant that determines organism response to exposure (Thurston *et al.* 1985; Escher & Hermens 2002; Jager *et al.* 2007), but even for a single toxic compound, large differences in species sensitivity have been found (Rubach *et al.* 2010). Differences in sensitivity across species are a source of uncertainty for the process of environmental risk assessment. In the lowest tier of environmental risk assessment, this uncertainty is often accounted for by using safety factors to derive threshold values for acceptable environmental concentrations (Van Leeuwen & Vermeire 2007). Despite their importance to the improvement of risk assessment, surprisingly little is known about the underlying mechanisms driving differences in sensitivity. Hence, most higher tier interspecies extrapolations are performed using probabilistic approaches like the species sensitivity distribution (SSD) concept (Posthuma *et al.* 2002) or by performing multispecies tests. It is well known that differences in uptake and elimination of a compound into the body or organs cause differences in sensitivity and these can be reduced when risk assessment is based on internal concentrations (McCarty & Mackay 1993). Also, numerous studies have indicated that differences in sensitivity can also be explained by physiological factors, such as differences in target enzyme constitution, detoxification or compensation abilities e.g. (Heckmann *et al.* 2008). In this context, the comparison of toxic effects measured with different endpoints in bioassays can hold useful information for ERA when interpreted with regard to the processes of toxicity such as toxicokinetics and toxicodynamics. For instance, time dependency of toxicity and differences in the toxicity response for different endpoints indicate that major differences in the processes of toxicity exist across species (Verhaar *et al.* 1999b). Although suitable methods, such as time to event analysis (Newman & McCloskey 1996), time independent sensitivity values (Mayer *et al.* 2002) and the dynamic energy budget theory (DEB) (Kooijman & Bedaux 1996) have been developed, dynamics of effects have been largely ignored in ERA. Often lethal and effective concentration values for different time points are treated equally without distinction, both for practical reasons and for lack of more specific data. The consequences of this ignorance are difficult to estimate at this point, but may lead to arbitrary conclusions. For instance, for an organophosphorous compound such as chlorpyrifos which affects the nervous system but does not lead to immediate mortality large differences in effect endpoints could exist between species, which is also relevant to risk assessment of time-variable exposure scenarios. Despite widespread activities to establish sub-lethal endpoints in risk assessment not only for chronic, but also for acute toxicity, literature lacks publications discussing the relevance of particular endpoints for the ERA of pesticides (Baas *et al.* 2010). The only exception are endocrine disruptors, for which the debate for the most relevant endpoint has developed further (Rhind 2009).

This study aims at evaluating how well the two endpoints, mortality and immobility reflect the toxicity of the organophosphorous insecticide chlorpyrifos in a variety of freshwater arthropods in terms of their effect dynamics and the their variability across species. Experimental data were collected by means of 96 h toxicity tests with a range of exposure concentrations. These data were used to calculate both 50 %-effective and lethal concentrations ($L(E)C_{50}$s) with which SSDs per investigated time point and endpoint were populated. The influence of endpoint choice on risk indicators such as ($L(E)C_{50}$s) and the 5 % hazardous concentration (HC_5) is discussed. Furthermore, we discuss the context in which toxicity information on different endpoints can improve understanding of differences in

toxic processes among investigated species and how this can lead to more mechanism-based risk assessment.

MATERIALS AND METHODS

Chemicals and stock solution

For the 96 h toxicity experiments, chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate, 99 % purity, CAS 2921-88-20, lot 51205) purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) was used. To avoid the use of a solvent carrier, aqueous solutions of chlorpyrifos were prepared using the principle of generator columns (Devoe *et al.* 1981) as described in the supporting information of (Rubach *et al.* in press b). The effluent of the generator column delivered a stable concentration of parental chlorpyrifos of approximately 1250 µg/L. Before use, each such obtained stock solution was measured by liquid/liquid extraction of subsamples with n-hexane followed by gaschromatography (GC) and electron capture detection (ECD) to determine its exact concentration of chlorpyrifos and dosing schemes were subsequently adapted.

Gas chromatography

Gas chromatography of aqueous stock solutions and test concentrations for the 96 h short term toxicity testing was performed with an Agilent 6890N GC equipped with a micro EC detector and 7683 Series B Injector (Agilent Technologies, Inc., Santa Clara, CA, USA). The injection volume was 3 µl at a temperature of 250 °C with a split ratio of 10. As stationary phase a DB5 medium bore column of 30 m length and with a 0.32 mm internal diameter was used, while the mobile phase was created with a constant flow of 3.1 mL of helium per minute. The oven temperature was set to 200 °C in isotherm mode. The temperature of the ECD was 300 °C and N_2 was used as make-up gas with a flow of 30 mL/ min. The retention time of chlorpyrifos using this method was 3.8 minutes. For each test a specific limit of detection (LOD) was calculated (Table 1) due to differences in the sample extraction on basis of the detection limit of the apparatus (0.1 µg/L) and the highest respective concentration factor, used for the lower concentration levels per species.

Test species and test media

The 14 freshwater arthropod species used in the present study, their origin, life stage and sampling date are reported in Table 1. Species identity was determined by trained staff according to established protocols using 6-10 individuals sub-sampled from the catch. Most species were collected at the experimental field station of Alterra, “The Sinderhoeve” (Renkum, The Netherlands) where they were sampled from untreated cosms, ditches and storage systems, but *Molanna angustata* originated from the field and *Daphnia magna*, *Procambarus* spec. and *Neocaridina denticulata sinensis* were cultured at Alterra as reported in the supporting information of (Rubach *et al.* in press b). The species *Procambarus* spec., here tested in adult and juvenile stage, is better known as the ‘Marmorkrebs’ (marbled crayfish), a parthenogenetic freshwater crayfish species belonging to the Cambaridae showing only female phenotypes, at least under culture conditions (Scholtz *et al.* 2003; Vogt *et al.* 2004; Martin *et al.* 2007b). In a recent study, this species has been used in an ecotoxicological test

(Vogt 2007). The species *Neocaridina denticulata sinensis* var. red is a tropical shrimp, also called the 'sherry red shrimp' and although particularly popular with hobby aquarists, has rarely been employed in ecotoxicology as a test species. All test animals, including the cultured species were transferred into 0.45 µm membrane pressure filtered and 24 h aerated water pumped from the groundwater horizon of 'The Sinderhoeve' and supplied with appropriate food to acclimatize to the test medium for at least 3 days before testing. The same water was used to prepare either the test media or inter dilutions by spiking and homogenizing the filtered and aerated water with the adapted volumes of stock solution after the exact concentration of chlorpyrifos in the stock solution had been determined.

Table1: Test species and methodological details of 96h toxicity tests including LODs.

Order	Species	Life Stage	Origin [a]	Sampling date	Test system [b]	Intended Concentration [µg/L]	Measured Concentration [µg/L]	Replication (n)	# of animals/ replicate	LOD [µg/L]
Anisoptera	*Anax imperator*	Larva [c]	A	01.03.2007	***** c	0.9; 1.9; 4; 8.4; 17.6	0.8; 1.6; 3.5; 7.3; 14.3	2	5	0.05
Isopoda	*Asellus aquaticus*	Adult	B	06.12.2006	**** g	0.2; 0.5; 1.2; 2.7; 6.2	0.2; 0.6; 1.3; 2.9; 6.1	3	10	0.01
Diptera	*Chaoborus obscuripes*	larva	A, B	02.02.2007	****	0.2; 0.5; 1; 2; 4.2	0.3; 0.9; 1.7; 3.3; 3.9	3	10	0.05
Diptera	*Cloeon dipterum*	larva	A, B	23.04.2007	**** g	0.03; 0.07; 0.17; 0.41; 1	0.04; 0.06; 0.12; 0.3; 0.8	3	10	0.01
Cladocera	*Daphnia magna*	neonate [d]	C	27.11.2006	*	0.01; 0.03; 0.1; 0.3; 1; 3	0.01; 0.03; 0.1; 0.3; 0.9; 2.5	4	5	0.01
Amphipoda	*Gammaurs pulex*	adult	A, B	24.11.2006	***** c , g	0.02; 0.05; 0.11; 0.25; 0.6	0.02; 0.04; 0.1; 0.24; 0.4	2	9	0.01
Trichoptera	*Molanna angustata*	larva	D	09.08.2007	** g	0.1; 0.4; 1.6; 6.4; 25.6	0.3; 0.5; 1.9; 7.3; 29.1	3	5	0.01
Decapoda	*Neocaridina denticulata*	adult/juvenile	C	12.09.2008	*** g	5; 13.3; 35.1; 93.1; 246; 653	5; 11.7; 34.2; 88.1; 228; 735	4	5	0.04
Heteroptera	*Notonecta maculata*	adult	B	11.09.2008	*** c	1; 2.7; 7.29; 19.7; 53.1	0.8; 2.1; 6.8; 15.6; 50.2	4	5	0.04
Lepidoptera	*Paraponyx stratiotata*	larva	B	17.06.2007	** g	0.2; 0.9; 4.1; 18; 82	0.4; 2.6; 5; 16; 23	2	5	0.05
Heteroptera	*Plea minutissima*	adult/nymph	A, B	01.03.2007	****	0.9; 1.9; 4; 8.4; 17.6	0.8; 1.8; 3.8; 8.9; 13.5	3	5	0.05
Decapoda	*Procambarus spec.*	juvenile	C	20.07.2007	**** g	0.2; 0.9; 4.1; 18.2; 82	0.4; 0.9; 3.2; 14.1; 107	3	5	0.05
Decapoda	*Procambarus spec.*	adult	C	20.07.2007	***** c , g	5; 10; 20; 40; 80	4; 7; 16; 30; 42	2	4	0.05
Heteroptera	*Ranatra linearis* [e]	adult	B, E	01.03.2007	***** c	3.3; 6.5; 13; 26; 52	NA	2	5	NA
Megaloptera	*Sialis lutaria*	larva	A	03.08.2007	* g	0.2; 1; 5; 25; 125; 625	0.3; 1; 5; 27; 65; 327	10	1	0.1

NA = not available, samples were lost.

[a] *Origin of test animals:* A = Sinderhoeve ditches, B = Sinderhoeve cosms, C = culture at Alterra laboratories, D = Lake Groene Hoevel (sandy oligotrophic), Wijchen, The Netherlands, E = Sinderhoeve storage basin

[b] *Test system:* * = 100 mL screw cap glass vials, ** = 250 mL SCHOTT flasks, *** = 600mL beaker, **** = 1.5 L WECK beakers, ***** = aquaria; g = stainless steel gauze for structure, c = compartmented

[c] Larvae stadium 3

[d] Neonate daphnids were ≤ 24h

[e] A substantial number of parasitic mites were removed 1 week before start of experiment

Toxicity experiments

To address differences in sensitivity and species specific requirements, such as prevention of cannibalism, the specific test design of toxicity experiments varied slightly among tested species (Table 1). Cannibalistic species were either tested in 4.2 L aquaria divided into the necessary number of compartments with inlets of stainless steel gauze, singly in 100 mL screw cap glass beakers or in 600 mL borosilicate beakers, which were divided into four compartments with stainless steel gauze. Expected non-cannibalistic species were either tested in 250 mL SCHOTT flasks, 1.5 L WECK beakers or 600 mL borosilicate beakers. When required for a particular species, these test systems were provided with stainless steel hook-shaped gauze pieces to provide a structural element.

To maintain constant temperature the aquaria, WECK beakers and the 600 mL beakers were kept in a water bath, whereas the 100 mL screw-cap glasses, the 250 ml SCHOTT flasks and the aquaria (for the experiemtn with *Procambarus* spec. adults), were kept in an incubator cabinet (Sanyo MIR 552). All experiments were carried out at the same light dark regime (16/ 8 h) with an average light intensity of 13 µmol·sec^{-1}·m^{-2} (min - max: 10.5 - 15.5 µmol·sec^{-1}·m^{-2}), however species, known to be stressed by light were shaded using aluminium foil to reduce stress. The experiments were carried out at a temperature of 17 ± 3 °C and an average pH of 7.61 ± 0.41 (measured with electrode pH 323B/set, WTW Germany) and at an average dissolved oxygen level of: 8.8 ± 1.8 mg/L (measured with electrode Oxi330/set, WTW Germany). These parameters were measured at 0, 48 and 96 h in at least one replicate per treatment. To reduce the losses of chlorpyrifos through evaporation, the test vessels were covered with parafilm or cling foil during exposure. However, if atmospheric breathers were tested beakers were only covered with nylon gauze to prevent escape of the organisms. All experiments were run in a static exposure regime with initial peak dosage at the start of the experiment. After insertion of the test animals (t = 0) with appropriate forceps or low volume pipettes, water samples were taken from the test systems to determine the measured nominal concentrations. The water samples were extracted with n-hexane (99 % pure) in graduated glass tubes by horizontal shaking for three minutes, subsequent layer separation for 10 minutes, after which approximately 1 mL of the upper (n-hexane) layer was transferred into amber GC vials and capped using lids with teflon-lined septa. Samples were stored at -20 °C until GC-ECD analysis. Water samples were taken after 0.5 (= 0), 48 and 96 h of application of chlorpyrifos. The intended and verified concentrations (t = 0) are reported in Table 1 together with the replication of treatments, number of test animals per replicate and the test system used.

Investigated endpoints of toxicity in each test were mortality and immobilization at 24, 48, 72 and 96 h of exposure. At these time points the number of dead and immobile animals were counted in each replicate. For every species clear criteria were set beforehand to distinguish between immobilization and death. In detail, test animals showing abnormal movement (paralyzed limbs, inability to walk, missing reflexes) in comparison to control animals after repeated agitation with forceps, were classified as immobile. Subsequently, if immobile animals did not show any visible movement within 30 seconds after repeated agitation, these were classified as dead. To distinguish between death and immobility, immobile specimen of some species (*Asellus aquaticus*, *Chaoborus obscuripes*, *Cloeon dipterum*, *D. magna, G. pulex*, *M. angustata*, *N. denticulata, Parapoynx stratiotata*, *Plea minutissima and Sialis lutaria)* were investigated using a binocular microscope, while the other

species were controlled for effects macroscopically. *D. magna* was classified in the second step as dead if no heartbeat could be detected within 30 seconds. *R. linearis* was classified as dead if no movement was detected after removing it from the water and putting it back upside down on the water surface, because mobile animals immediately turned themselves back and immobile specimen would show at least limb movements in this position.

Data analysis

For analysis of the effect data first the total number of dead and immobile animals was calculated per replicate and observation time point, while dead animals were also counted as immobile. All subsequent calculations were performed on basis of the measured initial concentrations of each single replicate (for rationale see below). The total number of dead and immobile animals per replicate, together with the initial number of test animals was used to calculate EC_{50s} and LC_{50}s respectively. The calculation of EC_{50} and LC_{50} values was carried out for every endpoint and every observation time by means of log-logistic regression using the software GenStat 11th edition (Laws Agricultural Trust, 2009 by VSN International Ltd., Oxford, UK) and equation one with 'y' being the fraction of dead or affected test animals (dimensionless), 'conc' the applied dose in µg/L on basis of the measured concentrations at t = 0 and the parameters a (ln EC_{50}), b (slope in L/µg) and c (fraction of background effect), all of which were fitted.

$$y(conc) = c + \frac{1-c}{1+e^{-b\cdot(\ln conc - a)}} \qquad (1)$$

For both mortality and immobility of species exposed to chlorpyrifos SSDs (Posthuma et al. 2002) were constructed per observation time point. For this the $E_TX^{2.0}$ program (Van Vlaardingen *et al.* 2004) was used, which fits a log normal model to the data. For each SSD the geometric mean of the log transformed toxicity data (log HC_{50} SSD data), their standard deviation (σ' SSD data) and 95% confidence interval (CI SSD data) were used as indicator and uncertainty measures for the variation in sensitivity observed (Aldenberg & Jaworska 2000). Furthermore the median 5 and 50 % hazardous concentrations (HC_5 and HC_{50}) and their confidence limits were calculated. The goodness-of-fit was tested using three different test for normality, the Anderson-Darling-test, the Kolmogorov-Smirnoff-test and the Cramer van Mises-test.

RESULTS AND DISCUSSION

Exposure

A prerequisite for the correct interpretation of effects of chemicals on biota is the confirmation of intended exposure regimes in experimental studies: Table 1 summarizes the measured concentrations of chlorpyrifos, at the start of the experiments. The intended concentrations were well achieved with an average coefficient of determination of 0.97 ± 0.04, average slope = 0.86 ± 0.22 and average intercept = -0.55 ± 2.77 for linear regression across all experiments of intended and measured concentrations at t = 0. As expected, in the course of the experiment the measured concentrations of chlorpyrifos, in most of the test media decreased (Table 2). The experiments with the different species however showed differences in dissipation of chlorpyrifos, ranging from 55.2 to 111.7 % remaining

after 48 h exposure and 21.9 to 120.7 % after 96 h (Table 2). Although no consistent pattern could be found when comparing species or treatments the interplay of factors such as animal size, bioconcentration, evaporation and degradation could explain these differences. For further calculation of $L(E)C_{50}$ values and SSDs the initial concentrations in the static systems were used, because of the short test duration and the relatively long organism recovery-time shown for organophosphates (Ashauer *et al.* 2007a).

Table 2: Dissipation of chlorpyrifos in test media in the course of the experiment relative to initially measured concentrations.

	Remaining chlorpyrifos after 48 and 96h of exposure					
	A: Second highest treatment			B: Highest treatment		
Species	0h [µg/L]	48h [%]	96h [%]	0h [µg/L]	48h [%]	96h [%]
Anax imperator	7.3	88.5	empty	14.3	81.1	empty
Asellus aquaticus	2.9	70.1	40.4	6.1	67.4	38.9
Chaoborus obscuripes	3.3	81.6	37	3.9	75.7	74.4
Cloeon dipterum	0.3	95.2	92.4	0.8	96.6	87.4
Daphnia magna	0.9	84.3	62.3	2.5	86.7	64.5
Gammaurs pulex	0.24	60.8	22	0.4	72.3	57.8
Molanna angustata	7.3	87.5	63.2	29.1	69.4	51.7
Neocaridinia denticulata	228	55.4	39.5	735	58	34.9
Notonecta maculata	15.6	76.2	63.3	50.2	80.7	40.5
Paraponyx stratiotata	16	70.1	52.4	23	111	49.4
Plea minutissima	8.9	86.9	82.8	13.5	111.7	120.7
Procambarus spec. (adults)	30	85.1	21.9	42	71.4	empty
Procambarus spec. (juveniles)	3.2 a	85.3	49.8	14.1 [a]	empty	empty
Ranatra linearis	NA	NA	NA	NA	NA	NA
Sialis lutaria	65	80.3	70	327	78.1	65.8

NA not availabe, samples were lost
[a] Highest treatment (107 µg/L) induced 100% mortality (denoted émpty') after 24h, therefore in A the third highest and in B the second highest treatments are shown

In general, concentrations of chlorpyrifos in control replicates were below the respective LODs, but occasionally chlorpyrifos was measured in single control samples (*C. obscuripes*, *D. magna*, *N. denticulata*, *P. stratiotata*, *Procambarus* spec. juveniles and *S. lutaria*), which explains the high variability in the intercept reported above. These exceptions are related to cross contamination of controls with chlorpyrifos due to its high volatility and associated contamination routes, especially when experiments with high exposure concentrations were performed. In all the controls showing cross contaminations, immobilization or mortality was below 10 % and therefore tolerated in the presented study. In the experiment with *M. angustata* all control replicates were contaminated with on average with 0.143 µg/L chlorpyrifos and immobility was induced in 1 to 2 control animals per replicate (20 - 40%) leading indirectly to cannibalism and as a result control mortality and increased mortality in the lower concentrations was observed during the experiment. Cannibalism was also observed in the these lower concentrations, but not in the intermediate and high concentrations, where no mortality, but full immobilisation was induced in all test animals and prevented cannibalism. Due to a current lack of data on the effects of chlorpyrifos for this species, it was decided to rather correct the total number

of immobile or dead animals for further analysis, instead of excluding the species. The data correction was performed by setting both concentrations of the contaminated control replicates and the immobility/mortality at 24h in the first two treatments to zero.

Effects on mortality and immobility

Effects of chlorpyrifos induced in a range of freshwater arthropods by short term exposure in simple laboratory test systems are presented as concentration response relationships for mortality and immobility (Table 3 and 4). Respective concentration-response parameters are reported – according to Equation 1 – next to L(E)C_{50} values and their confidence limits. At least the highest test concentrations of chlorpyrifos induced 100 % immobility within the 96 h of exposure in all species except in *C. dipterum*, where 93 % of all test animals were immobile at the end of the experiment. In *P. stratiotata* all initial test animals in the highest concentration were immobile at 72 h of exposure, but subsequent recovery occurred resulting in 70 % immobilization at the end of the experiment. In contrast, at least the highest concentrations induced 100 % mortality only in six of the 14 experiments (*Anax imperator*, *G. pulex*, *P. minutissima*, *Procambarus* spec. adults and juveniles and *Ranatra linearis*) and for some species no mortality (*S. lutaria*, *M. angustata*) or very low mortality (*Asellus aquaticus*) was observed within the time of exposure, even in the highest concentrations. For the remaining five species, we found between 70 and 87 % mortality (*C. obscuripes*, *C. dipterum*, *D. magna*, *N. denticulata*, *Notonecta maculata* and *P. stratiotata*) in their respective highest concentrations. Figure 1 illustrates that for some species independent of time the differences between lethal and sublethal effect concentrations are substantial, while others show a relatively good match.

The observed effects on mortality and immobilisation for the tested species is in the range reported in literature, which is up to three to four orders of magnitude for both lethal and sub-lethal effects for up to 96 h (Van Wijngaarden *et al.* 1993; Maltby *et al.* 2005; Rubach *et al.* 2010). Also, L(E)C_{50} values for particular species agree well with previous findings, with the exception of *C. obscuripes*, for which an LC_{50} 22 times higher (6.6 µg/L, 96 h exposure) was previously determined (Van Wijngaarden *et al.* 1993). These authors also observed differences in the concentrations inducing mortality and immobility for some arthropod taxa (*A. aquaticus*, *Proasellus coxalis*, *G. pulex*, *C. dipterum*, *C. horaria*, *C. obscuripes*) and again no mortality was induced within 96 h of exposure for *A. aquaticus* and also *Caenis horaria* in their study. Although the experiments of Van Wijngaarden et al. (1993) were either performed in flow-through or semi-static test systems and therefore under constant exposure, the congruence of results with our study, shows that for chlorpyrifos exposures up to 96 h the initial (peak) concentrations are equally representative for effects induced within this period of time.

Table 3: Lethal effects of chlorpyrifos on freshwater arthropods in 96 h. Next to the 50% lethal concentrations (LC_{50}) and their confidence intervals [lower - upper] the equation parameters of equation 1 are given (a, b, c); if no value for c is reported it was 0.

Species	24 h exposure		48 h exposure		72 h exposure		96 h exposure	
	LC50 in µg/L [CI 95%]	Parameters	LC50 in µg/L [CI 95%]	Parameters	LC50 in µg/L [CI 95%]	Parameters	LC50 in µg/L [CI 95%]	Parameters
Anax imperator	6.93 [6.34 - 7.56]	(1.94, 22.37)	3.29 [2.97 - 3.66]	(1.19, 20.64)	2.35 [1.77 - 3.13]	(0.854, 5.57)	1.98 [1.5 - 2.63]	(0.685, 4.31)
Asellus aquaticus	> 7		NC		7.64 [6.53 - 8.93]	(2.03, 9.24, 0.033)	8.58 [4.63 - 15.9]	(2.15, 2.34, 0.037)
Chaoborus obscuripes	2.47 [1.38 - 4.42]	(0.903, 0.664)	1.13 [0.76 - 1.68]	(0.118, 0.943)	0.61 [0.35 - 1.08]	(-0.494, 0.822)	0.30 [0.13 - 0.7]	(-1.21, 0.815)
Cloeon dipterum	1.11 [NC]	(0.102, 10.15)	0.81 [NC]	(-0.211, 22.36)	0.58 [0.45 - 0.74]	(-0.549, 2.5)	0.36 [0.29 - 0.45]	(-1.02, 2.93)
Daphnia magna	889 [0.005 - 1.7 *10^8]	(6.79, 0.289)	27.43 [0.66 - 1145]	(3.31, 0.459)	4.37 [0.87 - 21.96]	(1.474, 0.55)	0.82 [0.44 - 1.51]	(-0.202, 0.971, 0.014)
Gammaurs pulex	3.10 [0.06 - 158]	(1.13, 0.703)	0.43 [0.21 - 0.87]	(0.85, 1.12)	0.23 [0.18 - 0.29]	(-0.463, 4.99, 0.07)	0.23 [0.2 - 0.25]	(-1.49, 22.31, 0.097)
Molanna angustata	> 34.2		> 34.2		NP		NP	
Neocaridina denticulata	1103 [540 - 2256]	(7.01, 2.03)	660 [453 - 960]	(6.49, 2.33)	477 [342 - 667]	(6.17, 2.61, 0.009)	457 [317 - 658]	(6.12, 2.34, 0.018)
Notonecta maculata	> 16		23.9 [15 - 38.2]	(3.18, 1.65)	11.6 [7.83 - 17.1]	(2.45, 1.72)	7.97 [5.34 - 11.9]	(2.08, 1.76)
Parapoynx stratiotata	55.1 [6.13 - 496.3]	(4.01, 0.94)	29.4 [NC]	(3.38, 1.21)	31.6 [5.57 - 179]	(3.45, 0.73)	27.2 [2.94 - 252]	(3.3, 0.47)
Plea minutissima	11.2 [8.95 - 13.93]	(2.41, 3.48)	5.94 [4.026 - 8.765]	(1.78, 1.65)	2.9 [1.92 - 4.39]	(1.07, 1.45)	1.98 [1.32 - 2.99]	(0.684, 2.14, 0.058)
Procambarus spec. (adults)	44.7 [27.33 - 73.12]	(3.8, 2.91)	34.81 [24.48 - 49.5]	(3.55, 2.95)	13.8 [9.61 - 19.9]	(2.6, 2.95)	12.9 [9.13 - 18.2]	(2.56, 3.65)
Procambarus spec. (juveniles)	4.05 [2.44 - 6.74]	(1.399, 5.16, 0.045)	2.75 [1.78 - 4.25]	(1.01, 3.79, 0.063)	1.88 [1 - 3.54]	(0.154, 1.2)	1.55 [0.78 - 3.06]	(0.968, 1.84, 0.129)
Ranatra linearis	22.5 [15.3 - 33.2]	(3.12, 3.58, 0.067)	11.97 [NC]	(2.48, 22.4, 0.145)	5.02 [3.29 - 7.68]	(1.61, 3.46, 0.115)	4.48 [2.39 - 8.41]	(1.5, 3.13, 0.318)
Sialis lutaria	> 327		> 327		NC		21700 [0.26 - 1.7*10^9]	(9.98, 0.242)

Note: NC = not calculated , NP = not performed

Table 4: Sub-lethal effects of chlorpyrifos (immobility) on freshwater arthropods in 96 h. Next to the 50% effective concentrations (EC_{50}) and their confidence intervals [lower – upper] the equation parameters of equation 1 are given (a, b, c); if no value for c is reported it was zero.

Species	24 h exposure		48 h exposure[a]		72 h exposure		96 h exposure	
	EC50 in µg/L [CI 95%]	Parameters	EC50 in µg/L [CI 95%]	Parameters	EC50 in µg/L [CI 95%]	Parameters	EC50 in µg/L [CI 95%]	Parameters
Anax imperator	5.16 [NC]	(2, 96.1)	3.13 [NC]	(1.14, 18.9)	1.66 [1.55 - 1.77]	(0.505, 24.3)	1.63 [NC]	(0.49, 20.1)
Asellus aquaticus	7 [NC]	(1.95, 17.8)	6.16 [4.89 - 7.76]	(1.82, 3.41, 0.033)	5.27 [4.07 - 6.82]	(1.66, 2.59, 0.035)	3.43 [2.75 - 4.26]	(1.23, 3, 0.05)
Chaoborus obscuripes	0.86 [NC]	(-0.149, 1.77)	0.44 [0.32 - 0.59]	(-0.826, 1.96)	0.32 [0.22 - 0.47]	(-1.13, 1.92)	0.18 [0.07 - 0.43]	(-1.74, 1.44)
Cloeon dipterum	0.88 [NC]	(-0.131, 16.39)	0.76 [NC]	(-0.271, 20)	0.41 [0.33 - 0.50]	(-0.89, 3.14)	0.31 [0.26 - 0.38]	(-1.16, 3.56)
Daphnia magna	6.91 [1.06 - 45.1]	(1.93, 0.607)	0.48 [0.34 - 0.69]	(-0.726, 2.81, 0.028)	0.25 [0.19 - 0.32]	(-1.38, 3.55, 0.036)	0.17 [0.12 - 0.23]	(-1.76, 6.52, 0.05)
Gammaurs pulex	3.10 [0.06 - 158]	(1.13, 0.703)	0.38 [0.2 - 0.7]	(0.97, 1.21)	0.24 [0.04 - 1.34]	(-1.44, 34, 0.097)	0.23 [0.2 - 0.25]	(-1.494, 22.3, 0.097)
Molanna angustata	1.86 [1.56 - 2.22]	(0.619, 52)	1.86 [1.56 - 2.22]	(0.619, 52)	NP	NP	NP	NP
Neocaridina denticulata	410 [NC]	(6.02, 1.36)	327 [NC]	(5.79, 1.9)	237 [147 - 381]	(5.47, 1.39)	171 [NC]	(5.15, 1.81)
Notonecta maculata	19.5 [12 - 31.8]	(2.97, 12)	9.07 [7.18 - 11.5]	(2.21, 5.05)	6.06 [4.46 - 8.31]	(1.81, 7.44)	2.78 [NC]	(1, 141)
Parapoynx stratiotata	5.88 [NC]	(1.77, 2.62)	2.94 [1.65 - 5.24]	(1.08, 1.67)	3.87 [2.14 - 6.92]	(1.3, 1.48)	2.86 [1.17 - 6.97]	(1.05, 0.795)
Plea minutissima	5.35 [4.19 - 6.83]	(1.68, 5.47)	2.65 [2.06 - 3.39]	(0.973, 5.29, 0.031)	1.55 [NC]	(0.436, 3.57)	1.29 [0.92 - 1.8]	(0.253, 5.71, 0.069)
Procambarus spec. (adults)	40.6 [26.6 - 61.9]	(3.7, 3.03)	20.7 [14.7 - 29.2]	(3.03, 2.88)	12.9 [9.1 - 18.2]	(2.56, 3.65)	10.7 [7.64 - 15]	(2.37, 4.43)
Procambarus spec. (juveniles)	3.59 [2.6 - 5]	(1.28, 4.46, 0.044)	1.7 [1.03 - 2.8]	(0.521, 4.1, 0.064)	1.29 [0.82 - 2.01]	(-0.069, 1.56, 0.002)	0.20 [0.75 - 1.93]	(0.181, 2.75, 0.139)
Ranatra linearis	19.9 [13.3 - 29.9]	(2.99, 7.53, 0.072)	12 [NC]	(2.48, 22.4, 0.145)	4.4 [2.8 - 7.1]	(1.49, 2.8, 0.11)	3.33 [2.95 - 3.76]	(1.2, 23.3, 0.3)
Sialis lutaria	3.19 [1.7 - 6]	(1.16, 2.31)	1.55 [0.25 - 9.58]	(0.437, 8, 0.05)	1.07 [0.96 - 1.2]	(0.066, 21, 0.1)	0.96 [NC]	(-0.046, 12.5, 0.204)

NC = not calculated , NP = not performed

[a] taken from Rubach *et al.* (in press b)

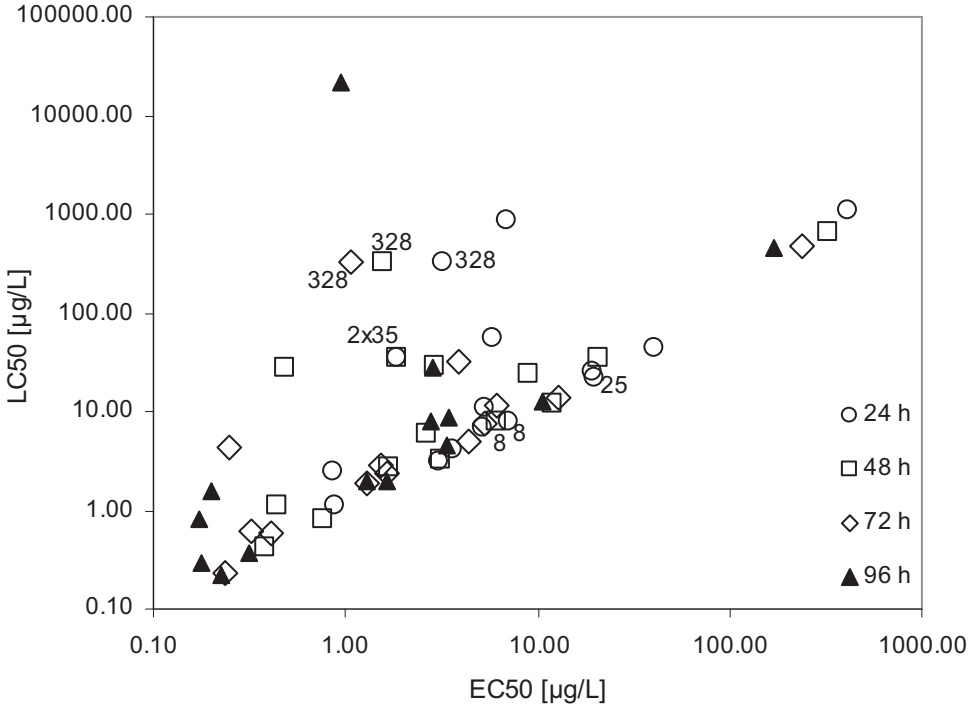

Figure 1: Measured LC_{50} versus EC_{50} values for chlorpyrifos estimated for 14 species freshwater arthropods under constant exposure of 24, 48, 72 and 96 h. If zero/ low mortality was observed in the experiments, no LC_{50} value was calculated and numbers in the plot indicate which substitute value was used.

Dynamics of effects and their variability

As expected, the effects of chlorpyrifos on both mortality and immobility increase in time in all tested species, which can be seen from the overall decrease in $L(E)C_{50}$ values (Fig. 2 and Table 3 and 4). Chlorpyifos mainly acts on the nervous system by inhibition of the enzyme acetylcholinesterase, leading to a synaptic block and therefore inhibiting electric signal transmission. This first leads to sub-lethal intoxication symptoms and subsequent death is likely caused by final respiratory failure (Eaton *et al.* 2008). Hence as expected, observations of immobility consistently resulted in lower EC_{50} values in time when compared to their respective LC_{50} values, although the difference between these endpoints decreased in the course of the experiment, especially for *D. magna* for which the LC_{50}/EC_{50} ratios decrease from 128.6 to 4.75 in 72 h (Fig 2). In general it is logical that the effect concentrations for immobility and mortality will converge to the same value in time, however it is evident that this does not occur with the same speed for all the tested species (Fig. 2). For some species the differences between LC_{50} and EC_{50} even stay relatively constant within the 96 h of test duration. However, for the species *A. imperator*, *C. dipterum*, *G. pulex*, *Procambarus* spec. and *R. linearis,* a good match between effective and lethal concentrations was observed right from the start of the experiments

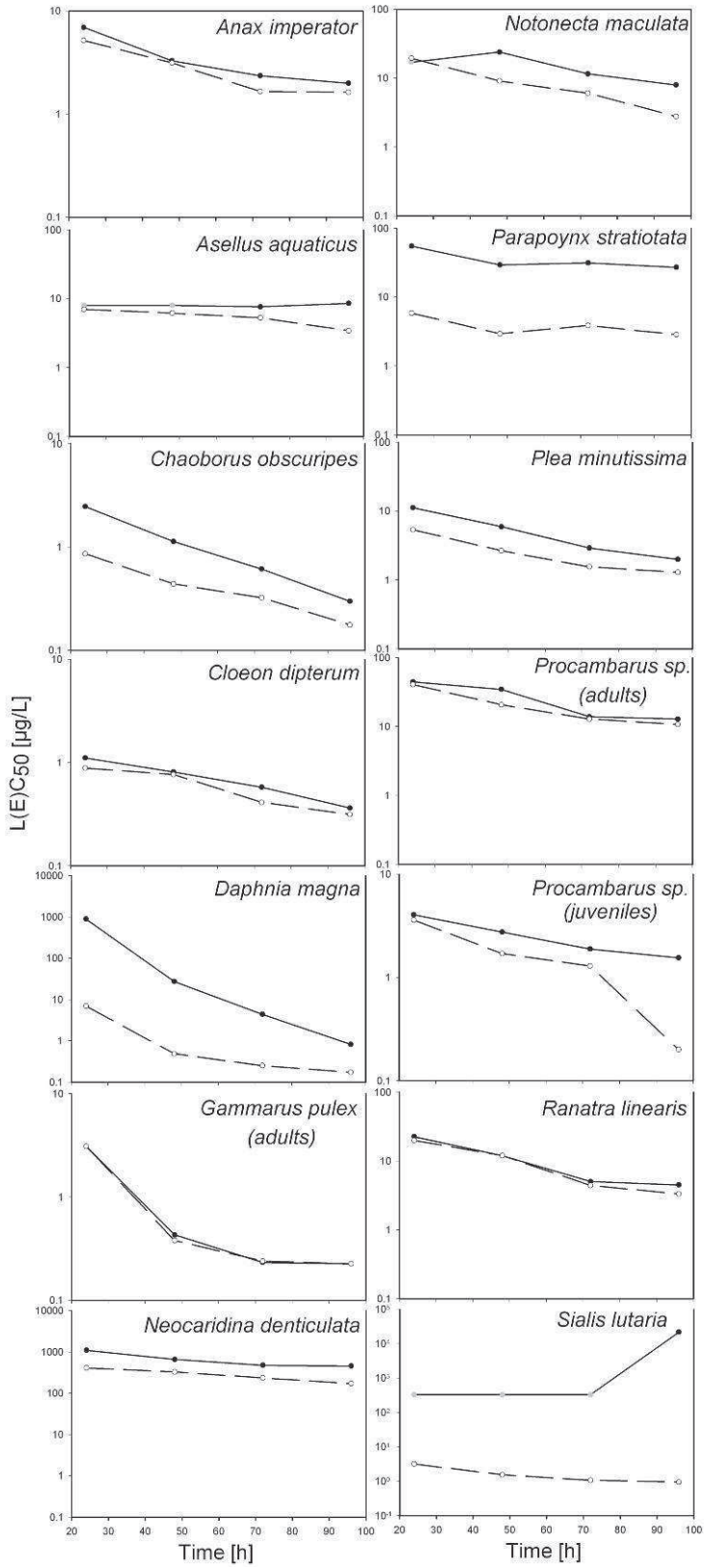

Figure 2: Dynamics of 50% lethal and effective concentrations for each species. Filled symbols and solid lines represent mortality (LC_{50}) and empty symbols and dashed lines represent immobility (EC_{50}). Grey symbols show the surrogate values for no or low observed mortality as shown in Fig 1. The species *Molanna angustata* is not shown, because only 24 and 48 h observations were available and zero mortality was observed.

(estimated LC_{50}/EC_{50} ratios around 1; see also Fig 1 and Fig 2). In contrast, for a third group of species (*N. denticulata*, *N. maculata* and *P. stratiotata*), the difference between EC_{50} and LC_{50} values for a particular species does not necessarily change in time (LC_{50}/EC_{50} ratios were constantly around 2, 2 and 9, respectively). Also, the extent to which LC_{50} and EC_{50} differ for certain time points seems rather species-specific – especially for *S. lutaria* and *M. angustata* – where no significant incipient mortality was induced by the applied concentrations, but where immobility was induced at quite low concentrations (Fig 2). This is interesting in the sense that these species-specific differences in incipient mortality or immobility can be either due to differences in the toxicokinetics and/or toxicodynamics. For instance, on the one hand, *S. lutaria*, *M. angustata* and *A. aquaticus* could have the ability to either reduce or regulate uptake and/or elimination of chlorpyrifos, biotransform chlorpyrifos slower to the chlorpyrifos-oxon or detoxify the latter very fast and therefore delay incipient mortality significantly, which would relate to differences in toxicokinetics. On the other hand, differences in the species responses might be caused by other processes belonging to the toxicodynamcis, e.g. differences in the interaction of chlorpyrifos and acetylcholinesterase (target enzyme) or in the ability to compensate or repair damage. For details on toxicokinetics and toxicodynamics see (Ashauer *et al.* 2006a). Rubach *et al.* (in press b) measured uptake and elimination kinetics of ^{14}C labeled chlorpyrifos in the same species and indicated that up to 38 % of the variation in sensitivity (EC_{50}, immobilisation in 48 h, same data) may be explainable by uptake and up to 28% by elimination kinetics. Interestingly, *S. lutaria*, *A. aquaticus* and *M. angustata*, which responded with a remarkable concentration difference between incipient immobility and mortality in this study, show high bioconcentration factors (9625, 3242 and 5331 $\mu g/kg_{ww}$ respectively). Because their uptake rates are moderate to high and immobility is effectively induced at much lower concentrations, differences in uptake itself can be excluded. More likely are differences in biotransformation rates (either bioactivation or detoxification) or a highly efficient compensatory gene regulation ability. The most insensitive of the investigated species, *N. denticulata,* shows high uptake and high elimination rates and therefore a moderate bioconcentration, which partly explains its insensitivity.

Clearly, the extent of variation in observed sensitivity to chlorpyrifos across species highly depends on the endpoint under consideration, which is already evident from the concentration-response relationships, but also from the SSDs shown in Figure 3. The SSDs also indicate by the 'left shift' of both mortality and immobilisation that effects increase in time. The slopes of the SSDs for immobility do not seem to be significantly different, however the variability increases slightly in time, indicated by an increase in σ' (Table 5).

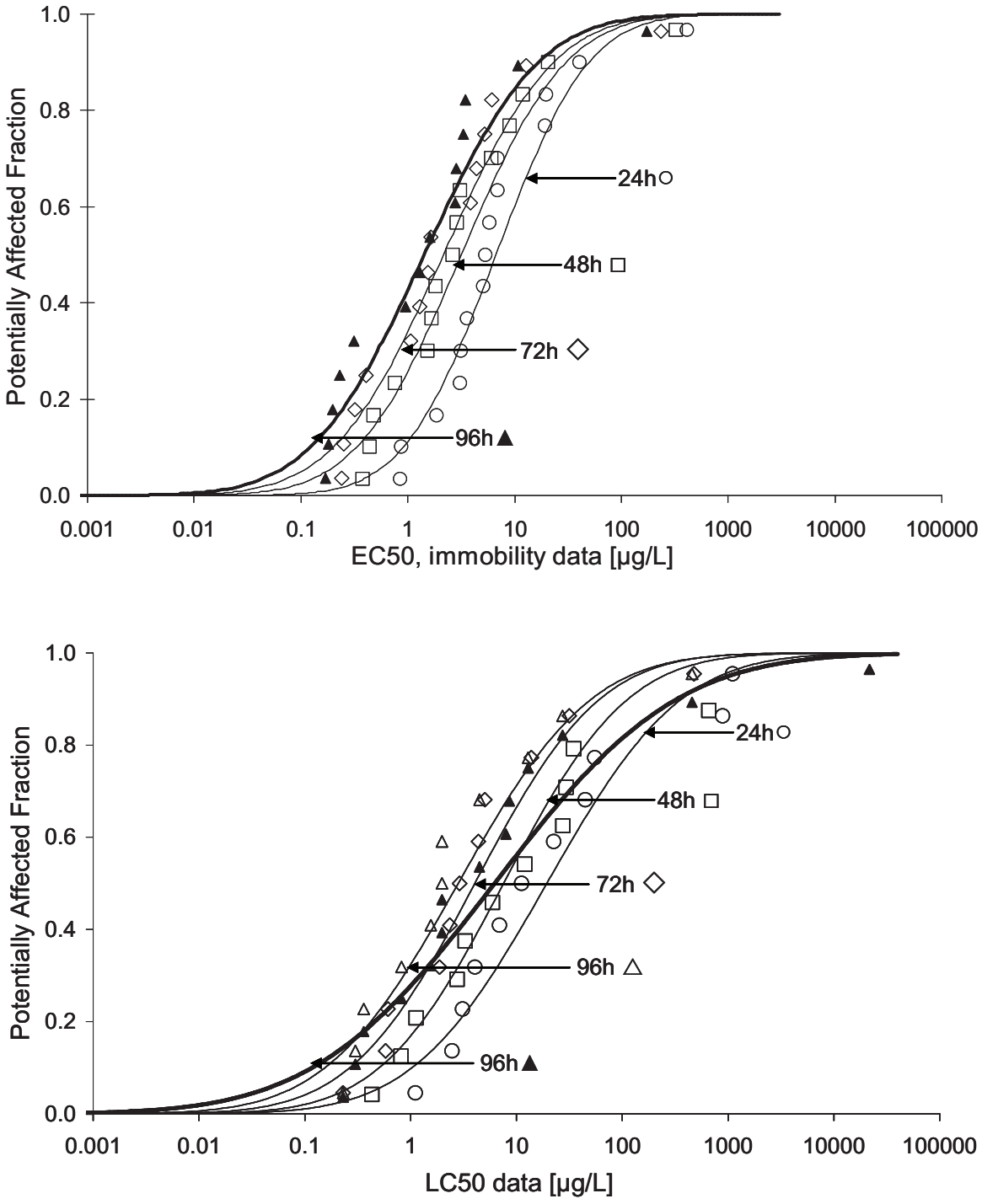

Figure 3: Species Sensitivity Distributions (SSDs) for freshwater arthropod species under 24, 48, 72 and 96 hours of exposure to chlorpyrifos constructed from observations of immobility (upper panel) and mortality (lower panel). For mortality SSDs were calculated from 24 – 96 h using the same closed data set (minimum number) of species (k = 11, empty symbols) and for 96 h using the maximum number of species available (k = 14, filled symbols) to illustrate the influence of species selection. In Table 5 the SSD indicator values and statistical details are given.

Table 5: Species Sensitivity Distribution (SSD) characteristics.

	SSD characteristic	24 h	48 h	72 h	96 h [1]
Immobility	n (number of species)	15	15	14	14
	log HC_{50} (SSD data)	0.827	0.501	0.333	0.155
	σ' (SSD data)	0.678	0.766	0.806	0.834
	CI (SSD data)	0.343	0.388	0.423	0.437
	HC_5 [µg/L]	0.49	0.16	0.09	0.06
	lower CI	0.12	0.03	0.02	0.01
	upper CI	1.18	0.44	0.28	0.17
	HC_{50} [µg/L]	6.71	3.17	2.15	1.43
	lower CI	3.30	1.42	0.90	0.58
	upper CI	13.66	7.07	5.19	3.54
Mortality	n (number of species)	11	11	11	14/ 11
	log HC_{50} (SSD data)	1.287	0.917	0.607	0.794/ 0.45
	σ' (SSD data)	0.99	0.879	0.923	1.345/ 0.983
	CI (SSD data)	0.587	0.519	0.545	0.705/ 0.581
	HC_5 [µg/L]	0.40	0.21	0.17	0.03/ 0.06
	lower CI	0.03	0.02	0.02	0.002/ 0.005
	upper CI	1.19	0.84	0.55	0.21/ 0.27
	HC_{50} [µg/L]	19.38	7.57	4.61	6.23/ 2.82
	lower CI	5.55	2.39	1.74	1.44/ 0.98
	upper CI	67.70	24.02	12.18	26.99/ 9.7

Note: HC_5 and HC_{50} denote the 5 % or 50 % hazardous concentration; log HC_{50}: is the; geometric mean of the log SSD data; σ' denotes the standard deviation of the log SSD data; CI denotes 95% confidence interval of the log SSD data.
[1] for the 96 h mortality data two SSDs were calculated: maximized dataset/ minimum dataset for comparison

Nevertheless, the confidence intervals of the SSD (Table 5) show a strong overlap and therefore this trend cannot be confirmed reliably and species variation in immobilisation might still be relatively constant in time. The variation in mortality is generally much higher and also relatively constant in time (σ', Table 5) until 72 h of exposure after which a sharp increase in species variability was observed. This is evident from the high σ' (Table 5) and low slope of the SSD for 96 h, but only if all available species are included in the SSDs (Fig. 3). This impact on the slope is an artefact caused by the species selection and the close-to-zero mortality in *A. aquaticus*, *M. angustata, S. lutaria* for which no LC_{50} could be calculated and which were thus not included in the SSDs for exposure times up to 96 h. If these insensitive species (with high LC_{50} values) had been included for all time-points, the slopes of these mortality SSDs would have been similar in time or even lower than the one for 96 h of exposure and σ' would have indicated an even bigger variation. Therefore, the variability in immobility is generally lower than for mortality, however both are relatively stable in time, if based on the same selection of species. The ratio of HC_{50} (mortality) / HC_{50} (immobility) derived from Table 5 decreases very slowly in time (2.9, 2.4, 2.1, 2.0) when based on the same species selection, but, similarly, if all 14 species are included in the 96h mortality SSD this ratio is 4.4. This shows that strong differences in time dependent toxicity exist between species and that the SSD can only account for this, if species selection is restricted to similarly reacting groups.

Choice of endpoint for ERA

Until now, from the perspective of the individual sensitivity response the presented data support the assumption that immobility is the better endpoint when investigating a neurotoxic substance such as chlorpyrifos, because paralysis is the first visible symptom and a species-specific time lag until incipient mortality was found for several species. In addition, also from a population sustainability viewpoint immobility is almost just as relevant as mortality. Where on the one hand, mortality is unilateral in the sense that a dead specimen cannot become alive again, any immobile or otherwise sublethally affected specimen may become mobile again and thus be able to contribute to a population's sustainability. On the other hand, an immobile specimen is likely going to be outcompeted, starved, drifted or predated quickly under field conditions and also more prone to multiple stress regimes.

However, when the risk assessment is based on SSDs rather than on safety factors, including only data based on immobility illogically does not always yield the most conservative threshold, but may deliver a more confident estimate of the HC_5 due to less variation in the species selection. Steep SSDs with higher confidence, as calculated here for immobility, will deliver less conservative HC_5 values than shallower SSDs, such as shown here for mortality after 96 h of exposure. This is especially the case if the lower end of the curve shows a bad fit with the data (as the 96 h mortality SSD for all species in Fig. 3). Such SSDs can however be excluded using goodness-of-fit measures, especially the Anderson-Darling test, which is sensitive for the quality of fit in the lower concentration range (Van Vlaardingen *et al.* 2004). All presented SSDs passed the three performed goodness-of-fit tests with $p < 0.001$, except for the 96 h mortality SSD with all species included, which did not pass the Anderson-Darling test and the Cramer van Mises test (both $p < 0.1$). The lower confidence limit of the HC_5 (LLHC5) derived from an SSD may serve as a protective threshold in higher-tier risk assessment (Maltby *et al.* 2009; Brock *et al.* 2010). This value can differ substantially between different observation-times and different endpoints (see Table 5) depending on the species selection. Although in general a rather conservative threshold, the protectiveness of the LLHC5 highly depends on the incipient time of the effect, the endpoint included (which correlates to the incipient of effects) the extent to which the species included in the SSD vary in their sensitivity and other quality criteria as reviewed in (Brock *et al.* 2010). Our results show that the most confident estimates can be derived with an SSD when immobility after sufficient time of exposure is chosen as an endpoint for the SSD. Herewith, if these 'quality criteria' are addressed the convolution caused by inclusion of insensitive species does not touch the usefulness of the SDD as a tool for ERA, especially if the most sensitive group for one chemical is well represented in the SSD (Van Den Brink *et al.* 2006), however it also clearly shows that a SSD does not necessarily represent the true existing variation in sensitivity.

Other endpoints, in addition to mortality and immobility, describing population sustainability, like reproduction, can be derived from chronic testing, but cannot be deduced from short term tests. In order to derive a good and conservative proxy for population sustainability, another sub-lethal endpoint – such as post-exposure feeding-inhibition – should be considered for short term testing (McWilliam & Baird 2002; Satapornvanit *et al.* 2009). If a specimen is not able to feed within a given time period of 24 h after the end of a short-term exposure, it is rather unlikely that it is able to contribute to a

populations sustainability. This, however, is yet far from being taken into account in current risk assessment practices. Another problematic issue for the selection and definition of an appropriate endpoint for ERA when comparing effects on species are the criteria which have to be set for this particular endpoint. For instance, in this study, transparent species could be observed for heartbeat and thus had a good criterion by which to distinguish death from immobility. Non-transparent and highly-sclerotised species, however, do not provide such clear-cut criteria to determine clinical death. In the present study, endpoint criteria for each species are rather well defined, thus minimizing such described difficulties.

In order to improve ERA, the identification of the best endpoint for assessing the risk of a certain group of chemicals must be based on its exposure scenario, its functional relevance for the mode or mechanism of action, its toxicity in time or on other prior knowledge and the ecological consequences of a given endpoint.

CONCLUSIONS

The presented dataset demonstrates that freshwater arthropod species can be highly variable in their dynamic response towards a particular stressor. What exactly causes these differences in sensitivity within such a narrow group of taxa in response to chlorpyrifos – an insecticide designed to affect this particular test group, remains mostly unclear. Hypothetically, the differences in effects among tested species are partly related to differences in bioconcentration, but biotransformation and/or differences in the amount of internally caused damage and/or differences in their abilities to recover or repair the induced damage must also play a major role. However, clear mechanistic explanations remain open, considering the current lack of knowledge on how these processes differ in the tested arthropods. Furthermore, presented findings illustrate the importance of considering an appropriate endpoint for a protective risk assessment based on knowledge about the mode of action of a particular group of compounds. In general, but surely for neurotoxic compounds such as organophosphates, immobilization in favour of mortality may be the appropriate endpoint to be propagated into further risk assessment. Mortality and/or immobility however might not be the appropriate endpoints for other non-neurotoxic compounds such as growth- or molting-inhibitors, endocrine-disruptors and mutagenic or genotoxic substances. For other mode of actions the most relevant endpoints for further ecological risk assessment still need to be defined on basis of the function(s) affected by the mode of action and the relevance of these function(s) in a field scenario. The test-length of short term toxicity tests for some of those types of compounds is not sufficient, but more realistic approximations of acute risks may be derived from short-term tests if a post-exposure feeding assay is performed after a 96 h exposure. However, sub-lethal effects are sometimes reversible, meaning that recovery even on individual level is possible. In ERA, probabilistic approaches such as the SSD provide useful tools to derive protective threshold values, however they do not necessarily account for the true variation in sensitivity. In contrast to the mechanistic effect models described, these tools are not based on the processes of toxicity and, therefore, extrapolation of information from one chemical to the other is difficult. Future research may be able to relate certain

species characteristics to these processes and also to mode-of-action-specific sensitivity and thus provide a more mechanistic understanding on which to base and evaluate risk assessment.

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Bioconcentration studies, 2007

5. CHAPTER

Toxicokinetic variation in 15 freshwater arthropod species exposed to the insecticide chlorpyrifos.

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ABSTRACT

Recent advances in modelling the processes of the toxicity of chemicals - toxicokinetics (TK) and toxicodynamics (TD) - are improving environmental risk assessment (ERA) through prediction of effects from time-varying exposure. This has been achieved by linking chemical fate and toxicological effects mechanistically, based on internal concentrations, through the tissue residue approach. However, certain questions remain, for instance how do TK and TD differ among species and how does this relate to differences in species sensitivity? In a series of experiments, we studied the TK of [^{14}C]-chlorpyrifos in 15 freshwater arthropod species, two of which were studied in juvenile and adult life stages. Uptake (k_{in}) and elimination (k_{out}) rate constants were fitted using a one-compartment single first order kinetic model. The application of two complementary parameter estimation methods facilitated the calculation of bioconcentration factors (BCF) with prediction intervals and 95% depuration times (t_{95}) for all tested species. Extremely slow elimination was observed in some species as well as high overall variation in k_{in}, k_{out}, BCF and t_{95} across the tested aquatic arthropod species. This variation has implications for the development of TKTD approaches in ERA, including assessing fluctuating exposure concentrations and the interpretation of observed toxicity responses in the laboratory and in the field.

INTRODUCTION

Ecotoxicological studies with a range of aquatic species and types of chemicals have revealed considerable variation in species sensitivity. This has been shown in a variety of studies ranging from short-term, single-species tests to higher-tier tests such as micro- and mesocosm studies (Maltby *et al.* 2005; Baird & Van den Brink 2007). A key challenge in environmental risk assessment (ERA) is how to account for these differences in sensitivity between species. Traditionally, this has been done by adding safety or uncertainty factors to the lowest endpoint for the relevant taxon tested. More recently probabilistic tools such as the species sensitivity distribution (SSD) (Posthuma *et al.* 2002) have been extensively applied, evaluated and discussed (Maltby *et al.* 2005). Although for a range of insecticides, such tools were found suitable for the protection of aquatic life (Maltby *et al.* 2005), a systematic understanding of the reasons for variation in sensitivity across species is still lacking (Baird & Van den

Brink 2007; Van Den Brink 2008). While it has been shown that a large part of the variation in species sensitivity towards toxicants can be explained by their physico-chemical properties and their mode of action (Vaal *et al.* 2000), the remaining variation must lie in differences between species themselves. Conceptually, the toxicity of a chemical can be divided into two processes: its toxicokinetics (TK) and its toxicodynamics (TD). Uptake of the chemical into the animal, biotransformation and elimination of the parent and its metabolites are integrated as TK, whereas the damage and subsequent effects induced by the chemical as well as internal recovery and repair processes are included in TD. Existing models and concepts of TKTD approaches have been reviewed recently by Ashauer et al. (2006). Since toxicity is driven by these processes, variation in sensitivity across species must be systematically related to these processes. The first objective of the present study was to measure uptake and elimination in 15 different freshwater arthropod species of varying sensitivity to the organophosphate insecticide chlorpyrifos. Uptake and elimination of total radioactivity of a labelled compound characterizes the first step in the toxicological process. Other processes such as biotransformation, which could lead to either more or less toxic metabolites, might also influence toxicity, and are also discussed here. The second objective was to measure the variation in TK by estimating uptake (k_{in}) and elimination (k_{out}) rate constants from the experimental data using a one compartment single first order kinetic model. Variation in sensitivity across species with respect to species differences in uptake and depuration are discussed. Toxicokinetic and toxicodynamic modelling has been identified as a promising risk assessment tool at the SETAC European Workshop on Linking Aquatic Exposure and Effects in the Registration Procedure of Plant Protection Products (ELINK) (which aimed to provide guidance on evaluating the effects of time-varying exposure) (Brock *et al.* 2010). The third aim of the present study was to calculate 95%-depuration times (t_{95}) and bioconcentration factors (BCF) with prediction intervals, and discuss the consequences of the observed variation across freshwater arthropods for ERA.

MATERIALS AND METHODS

Chemicals and stock solutions

The radiolabelled insecticide [ethyl-1-^{14}C]chlopyrifos (98.3% purity, 30.1 Ci/mol, batch XVI/1) was purchased from IZOTOP, Institute of Isotopes (Budapest, Hungary). To avoid the use of a solvent carrier, aqueous stock solutions of [^{14}C]-chlorpyrifos were prepared by means of a generator column as described in the supplemental data.

Test species and test media

Seventeen experiments were conducted with 15 freshwater arthropod species, of which two species were tested in both juvenile and adult life stages (Table 1). Most species were either collected from outdoor mesocosms (www.sinderhoeve.org) or from the field over the period May to November 2007, while others were cultured in the laboratory (Table 1, for details on culturing see supplemental data). For each mesocosm- or field-collected species, six to ten sub-sampled individuals were taxonomically identified by trained staff according to regularly used protocols. However for the species, here named *Culex pipiens,* one individual in a subsample of 50 could not be definitively identified beyond Family:

Culicidae. All test animals, including the cultured species were transferred into filtered and aerated groundwater after collection and supplied with appropriate food (Table S1) to acclimate to the test medium for at least 3 d before testing. The groundwater was pumped from the groundwater horizon below the Alterra field station The Sinderhoeve in Renkum, The Netherlands and was filtered through a 0.45 µm membrane using pressure filtration and aerated for at least 24 h prior to usage. The filtered and aerated groundwater was spiked with dosage volumes of aqueous stock solutions of [^{14}C]-chlorpyrifos and homogenised before insertion of test animals.

Table 1. Details on species used, intended and measured chlorpyrifos concentrations (based on total [^{14}C]) in the 17 uptake and elimination studies (n = 5). SD = standard deviation.

No	Order	Species	Life Stage	Origin[a]	Sampling date	Fresh weight ± SD [mg][b]	Lipid content [% fresh weight, n = 1]	EC50, 48 h [µg/L][c]	Intended Concentration [µg/L]	Measured Concentration [µg/L]
1	Anisoptera	*Anax imperator*	Larva[d]	A	20.09.2007	1411.9 ± 181.7	2.49	3.13	0.4	0.39
2	Isopoda	*Asellus aquaticus*	Adult	B	06.07.2007	11.5 ± 4.8	0.85	6.16	0.5	0.44
3	Diptera	*Chaoborus obscuripes*	Larva	A, B	20.09.2007	17.5 ± 5.3 *	1.04	0.438	0.03	0.03
4	Ephemeroptera	*Cloeon dipterum*	Larva	A, B	20.09.2007	7.7 ± 3.0 *	7.21	0.763	0.04[e]	0.04
5	Diptera	*Culex pipens*	Larva	C	06.08.2007	3.4 ± 1.3 *	0.70	not tested	0.10[e]	0.1
6	Cladocera	*Daphnia magna*	Adult	D	07.10.2007	13.5 ± 22.3 *	0.94	0.484	0.1	0.08
7	Amphipoda	*Gammarus pulex*	Adult	E	22.06.2007	19.1 ± 9.6	1.36	0.379	0.10[f]	0.09
	Amphipoda	*Gammarus pulex*	Juvenile	E	22.06.2007	4.7 ± 3.0	0.49	not tested	0.1	0.09
8	Trichoptera	*Molanna angustata*	Larva	F	09.08.2007	23.5 ± 9.3	2.93	1.86	0.15[g]	0.16
9	Decapoda	*Neocaridina denticulata*	Adult/juvenile	D	07.10.2007	39.9 ± 19.6	1.25	327	30	29.99
10	Heteroptera	*Notonecta maculata*	Adult	B	06.08.2007	133 ± 13.9	3.81	9.07	0.50[g]	0.47
11	Lepidoptera	*Paraponyx stratiotata*	Larva	B	06.08.2007	33.8 ± 10.6	4.52	2.94	0.16	0.16
12	Heteroptera	*Plea minutissima*	adult/nymph	A, B	06.07.2007	4.8 ± 2.2 *	7.61	2.65	0.6	0.53
13	Decapoda	*Procambarus* spec.	Juvenile	D	27.07.2007	25.9 ± 19.4	1.97	20.7	0.19	0.19
	Decapoda	*Procambarus* spec.	Adult	D	07.10.2007	2794 ± 1206	1.16	1.7	2.85	2.75
14	Heteroptera	*Ranatra linearis*	Adult	A, E	25.07.2007	146.6 ± 35.7	0.96	12	1.20[g]	0.99
15	Megaloptera	*Sialis* lutaria [h]	Larva	A	10.08.2007	39.7 ± 13.6	1.92	1.55	0.2	0.19

[a] *Test animals originate from* A = Sinderhoeve ditches, B = Sinderhoeve cosms, C = rainwater collector in private garden, D = culture at Alterra laboratories, E = Sinderhoeve storage basin, F = Lake Groene Heuvel (sandy oligotrophic), Wijchen, The Netherlands.
[b] Fresh weights denote averages of all tissue samples (n = 50) during one experiment and correspond to one individual or 3 individuals (*); exceptions: *C. obscuripes* and *C. dipterum* both n = 100, *C. pipiens* n = 49, *D. magna* n = 48, *S. lutaria* n = 39.
[c] For methodological details and results of toxicity tests see supplemental data. Value for *R. linearis* is based on intended concentrations in the toxicity test.
[d] Larva was in third instar.
[e] = value based on data extracted from the Etox database (De Zwart 2002).
[f] = value chosen from Ashauer *et al.* (2006b) for comparability.
[g] = value based on intended concentration of EC50 test.
[h] Replication was n = 4.

Total lipid in test animals

After the start of the experiment ($t \cong 6$ h), specimen sub-samples were taken (and were frozen at -20°C until analysis) from most of the test animal populations (with the exception of *Daphnia magna*, *Neocaridina denticulata* and *Molanna angustata*) to determine their total lipid content. The three other species were sampled from cultures or the field under comparable conditions at a later date. Total lipid was determined gravimetrically with a method adapted from (Smedes 1999; Smedes & Askland 1999) which is described in more detail in the supplemental data.

Uptake and elimination experiments

The uptake and elimination studies were designed in general accordance with (Ashauer *et al.* 2006b), but the test animals were not fed to avoid uptake via the diet. The experiments are summarised below, with further details described in the supplemental data. All experiments comprised a 48 h exposure phase in which the test medium was spiked initially with a sub-lethal dose (Table 1), and a subsequent 120 h elimination period for which the animals were transferred into untreated medium (Figure 1). The sub-lethal exposure levels were around 10% of the 48 h EC_{50} for immobilisation as determined in toxicity experiments (supplemental data), which was equivalent to an effect level of < EC1 for all of the test species. For *C. pipiens,* 10% of the average LC_{50} of all entries in the Etox data base was chosen (De Zwart 2002) and the experiment with *Gammarus pulex* employed the same concentration as in Ashauer et al. (2006b) to test reproducibility. Sampling of test solutions and animals was carried out at the same points in time in order to determine concentrations of [^{14}C]-chlorpyrifos in water and tissue on the basis of total radioactivity. Test solutions were sampled immediately after insertion of animals at 0 h and then at 3, 10, 24 and 48 h after the start of the experiment during the exposure phase and at 49, 52, 59, 73, 97, 121 and 169 h during the elimination phase. From each replicate 5 ml of test medium were sampled, 10 ml scintillation cocktail (Ultima Gold^{TM} LSC Cocktail, Perkin Elmer) was added and radioactivity was quantified with liquid scintillation counting (LSC). Test animals were sampled at 3, 10, 24, 48, 52, 73, 97, 121 and 196 h after the start of the experiment and survival was also recorded. In general, one individual test animal was randomly sampled per time point from each replicate. However in experiments with smaller species three test animals per replicate were sampled (supplemental data). After removal from the beaker, the test animals were blotted dry, weighed on a precision balance and stored in screw cap glass vials equipped with Teflon septa at -20°C until digestion. All experiments were carried out in 600 ml borosilicate beakers filled with 500 ml test medium at 17 ± 1°C; this temperature is acceptable for all investigated species and also corresponds to temperatures in the spring/summer period in the field in the Netherlands. The test systems were not aerated during the experiments to minimise and equalize evaporation of [^{14}C]-chlorpyrifos in all experiments, and pH (average: 7.91 ± 0.44; electrode pH 323B/set, WTW Germany) and dissolved oxygen (average: 7.05 ± 2.4 mg/L; electrode Oxi330/set, WTW Germany) were measured daily. Additionally, a chemical control without test animals was employed during the exposure phase in four experiments. In the experiments with *G. pulex*, *Asellus aquaticus*, *Cloeon dipterum*, *M. angustata*, *Procambarus* spec., *Sialis lutaria*, *Parapoynx stratiotata* and *N. denticulata* one to three stainless steel hook-shaped gauze pieces were inserted into the

beakers to provide a physical substrate for the animals. The same gauze was hung in the water column to provide substrate for *Plea minutissima*. If more than one predatory animal was tested in the same beaker (*Ranatra linearis*, *Anax imperator*, *S. lutaria* and *Notonecta maculata*) rectangles of stainless steel gauze were inserted vertically into the beakers to separate them, and provide substrate for the animals. Beakers were covered either with parafilm, or with nylon gauze for air-breathers, the latter to ensure air exchange but prevent escape of test animals. The experiments were conducted in true treatment replicates of five plus one control replicate. The exact design differed slightly between species due to the size and cannibalistic behavior of some species (for details see supplemental data).

Digestion of sampled test animals was carried out slightly differently for each species due to differences in body sizes as described briefly in the following (for details see supplemental data). After defrosting, individuals of large-bodied species were cut systematically into a certain number of pieces in the glass vial itself, while first legs and antennae were separated from the thorax and nerve tissues such as eyes and ganglia were opened. Subsequently different volumes of tissue solubiliser (Soluene-350, PerkinElmer) were added to the whole or dissected animals after which the samples were stored at 60°C for different periods of time to digest (see supplemental data). After digestion, samples were cooled, and 1.5 ml of the homogenate mixed with 8.5 ml scintillation cocktail (Ultima Gold™ LSC Cocktail, Perkin Elmer). The exoskeletons were removed from the homogenate and rinsed twice with 3.5ml Ultima Gold. All fractions were pooled and counted with LSC. Due to their large body size, homogenates of *A. imperator* and *Procambarus* spec. adults had higher volumes and were thus sub-sampled with 1.5 ml and this directly mixed with 15.5 ml Ultima Gold. The vials and exoskeletons of the 48 h samples of the other species were repeatedly extracted to check for potentially remaining radioactivity. If radioactivity above control activity was detected, one replicate per time point was re-extracted and the measured radioactivity added to the previous results. In contrast, internal concentrations of neonates of *D. magna* released during the experiment and sieved after 48 h and 196 h were measured separately per replicate, but not added to the internal concentrations of sampled adult daphnids. Deposited eggs (*N. maculata* and *R. linearis)* and feces were neither removed nor analyzed.

Liquid Scintillation Counting

Liquid Scintillation Counting for stock solutions, water samples and tissue samples was carried out with a Tri-Carb 2100 TR Liquid Scintillation Analyser, Packard Instrument Co. Inc., 1994, USA. Aqueous [^{14}C]-chlorpyrifos concentrations in the stock solutions were determined immediately before the experiments by sampling 100 µL stock solution (n = 5), adding 4.5 ml of Ultima Gold and counting each sample once for 10 min or until 10000 counts were reached. The counting was corrected internally for background activity measured simultaneously in MilliQ controls (n = 3). Subsequently, actual concentrations of [^{14}C]-chlorpyrifos in the stock solutions were adjusted and dosing schemes were re-calculated based on total radioactivity and the specific activity. Water samples from the experiment were counted for ten min after addition of Ultima Gold twice for 30 min or until 10000 counts were reached. The counting was internally corrected with background activity measured in blank samples with groundwater (n = 3). Tissue samples were left to settle for one h before counting,

then counted twice for thirty min or until 10000 counts were reached. The counting was internally corrected with background activity measured in blank samples with 1.5 ml Soluene-350 and Ultima Gold (n = 3). Also, all samples were corrected for color quenching using two different quench curves supplemental data. Additionally, all samples were manually corrected for activity measured in the water or tissue of control treatments during the data analysis. The background and control radioactivities, limits of detection and quantification are documented in the supplemental data. However, values below the LOD were not set to zero in the subsequent data analysis because the determined LODs for internal concentrations are based on averages over a few of the tested species, which would impose a larger error on some species than on others; and generally the measured radioactivity will likely be closer to the real radioactivity than a value of zero and should thus be included as such in the modelling.

Data Analysis

Measured total radioactivity in test media and test animals was expressed as concentrations of [^{14}C]-chlorpyrifos in µg/L and µg/kg$_{wet\ weight}$ and measured control concentrations in test media and test animals were subtracted from each replicate treatment value. Water concentrations from the experiment with *A. aquaticus* and *P. minutissima* were corrected as described in the supplemental data, due to their accidental external contamination after the experiment. The data sets for internal concentrations of all species were explored per species and time point for extreme outliers (> 3 time the interquartile range of internal concentrations) with SPSS 15.0.1.1 for Windows (SPSS Inc., 2007) and the Grubb's test (alpha = 0.05, GraphPad software Inc, 2002 – 2005: http://www.graphpad.com/quickcalcs/Grubbs1.cfm and when both tests confirmed an outlier, the data point was excluded from further analysis and modelling (out of the 936 data points, 22 were excluded as outliers). For the water concentrations, arithmetic means (n = 5) were calculated for model input, whereas internal concentrations were used as individual replicate data points.

Modelling

The uptake and elimination rate constants were estimated using one compartment, first-order kinetic model (1) for all species, because the measurement of bioconcentration into specific organs was impossible for the small and medium sized test species.

$$\frac{dC_{\text{int}}}{dt} = k_{in} \cdot C_w(t) - k_{out} \cdot C_{\text{int}}(t) \qquad (1)$$

with t = time [time]; C_{int} = internal concentration [amount · mass^{-1}]; C_w = concentration in the water [amount · volume^{-1}]; k_{in} = the uptake rate constant [volume · mass^{-1} · time^{-1}] and k_{out} = the elimination rate constant [time^{-1}]. The model was implemented in OpenModel v.1.1 (University of Nottingham, 2009) and fitted to the internal concentration data for each species using the measured water concentration as a driving variable (input files are available in the supplemental data). The parameters k_{in} and k_{out} were estimated using two complementary methods in sequence: first, least-squares curve fitting with the Levenberg-Marquardt (L-M) algorithm; and subsequently Bayesian calibration with Markov Chain Monte Carlo simulation (MCMC) based on the Metropolis-Hastings algorithm, where the results of the L-M estimation served as prior distributions. For further detailed explanation and

rationale see supplemental data. From the k_{out} estimated with the MCMC method, 95% depuration times were calculated using the formula: $t_{95} = -\ln(0.05)/k_{out}$. BCFs were calculated by setting the water concentration to one and simulating the internal concentration using the first-order kinetic model for 100 d to ensure steady state and deriving the BCF and its prediction interval from the simulated internal concentration at steady state.

RESULTS AND DISCUSSION

Modelling methodology

The first order kinetic model was successfully fitted to all 17 experimental datasets using the two complementary parameter-estimation methods. The estimated toxicokinetic parameters k_{in} and k_{out} are listed in Table S4 and Table 2 for all species, and both the L-M and the MCMC estimated parameters, respectively. Solving the model numerically allowed the use of the time course of measured aqueous concentrations as model input, even when concentrations were variable, as was the case here. In combination with parameter-search algorithms such as the L-M algorithm, both parameters were fitted simultaneously to both experimental phases at once, thus all the derived information was efficiently used in a single analysis. Moreover, using these results subsequently as prior distributions for Bayesian model calibration resulted in estimates with prediction intervals. Coefficients of variation (CV) for k_{in} across all species for the L-M fits were on average 6.8 ± 3.0 % ranging from 2.9 to 14.0 %. For the MCMC fits, less variation was found, with an average CV of 4.9 ± 2.4 % ranging from 2.2 to 10.8 %. The CVs of the estimated k_{out} were highly variable for the L-M fits (on average 248 ± 853.1 %, ranging from 1.2 to 3538.3 %) due to the extremely low elimination rates of *S. lutaria* and *C. pipiens,* but were much less variable for the MCMC fits (15.9 ± 10.5%, ranging from 6 to 39.5 %). Hence, the complementary use of least-squares fitting with L-M and MCMC proved more useful than L-M estimates alone. The model efficiency of both methods was comparable, indicated by the goodness-of-fit estimates (R^2 and mean % error) provided in the supplemental data. These showed no major difference in the two fitting methods, although the mean percentage error was slightly lower for the MCMC fits. Due to these findings, the MCMC parameter estimates were employed for further analysis.

Measured and modelled toxicokinetics

The experimental data for all 17 experiments on water concentrations and internal concentrations are presented in Figure 1 (total measured radioactivity and MCMC fits with 95% prediciton intervals). In all experiments, the concentrations of [^{14}C]-chlorpyrifos in the water phase decreased during the uptake phase. There was no decline observed in any of the chemical control runs (decline appeared to be related to the presence of the animals themselves, through adsorption and uptake).

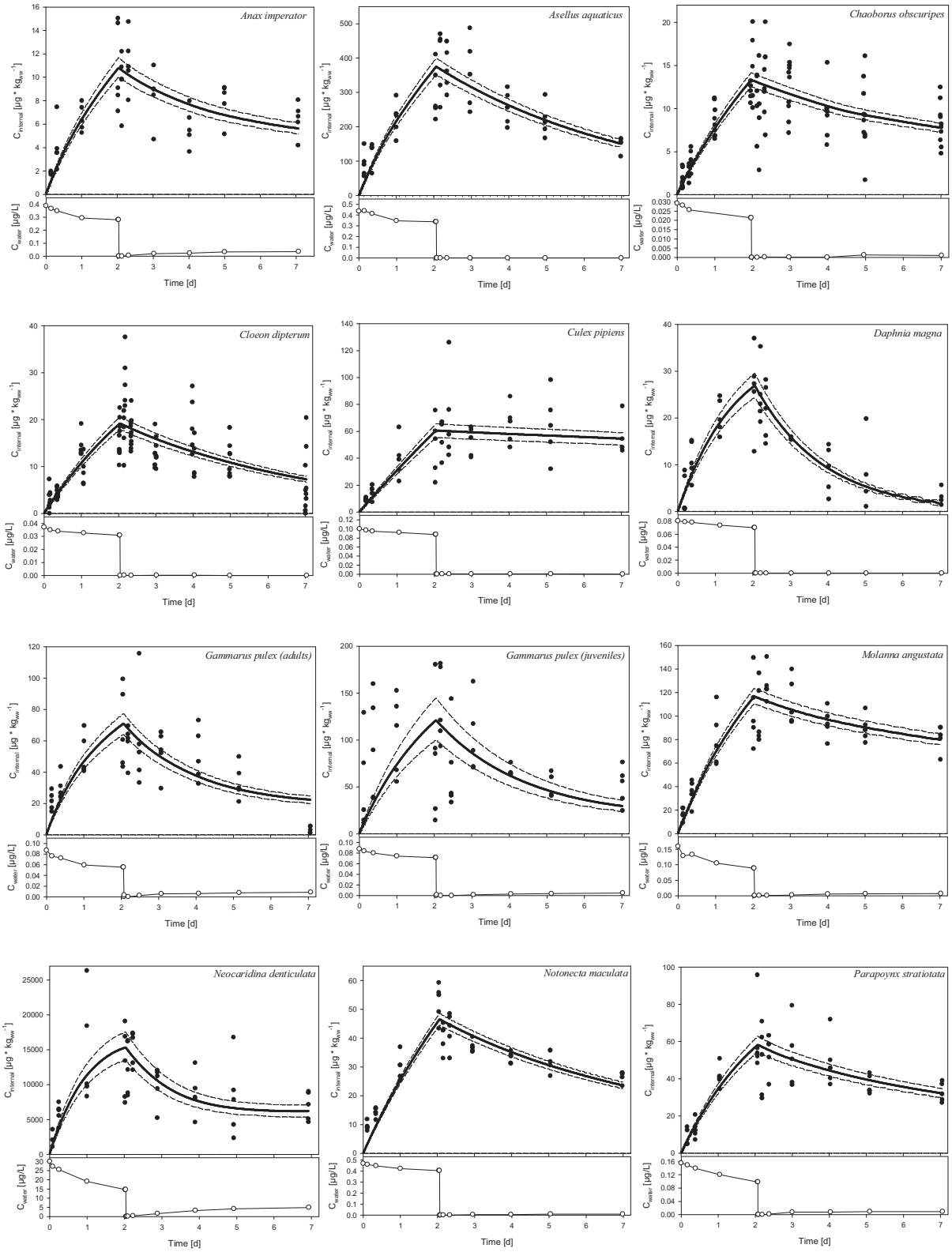

Figure 1 (continued on next page): Uptake and elimination kinetics of [^{14}C]-chlorpyrifos in 15 freshwater arthropod species. Per panel both water (lower, open circles, averages, n = 5) and internal (upper, closed circles, 1 data point represents 1 replicate) concentrations are shown, calculated on basis of total measured radioactivity. The solid bold line illustrates the mean internal concentration from the forward Monte Carlo simulation based on the parameter sample from the Markov chain Monto Carlo fit and the dashed lines represent the upper and lower 95% prediction intervals.

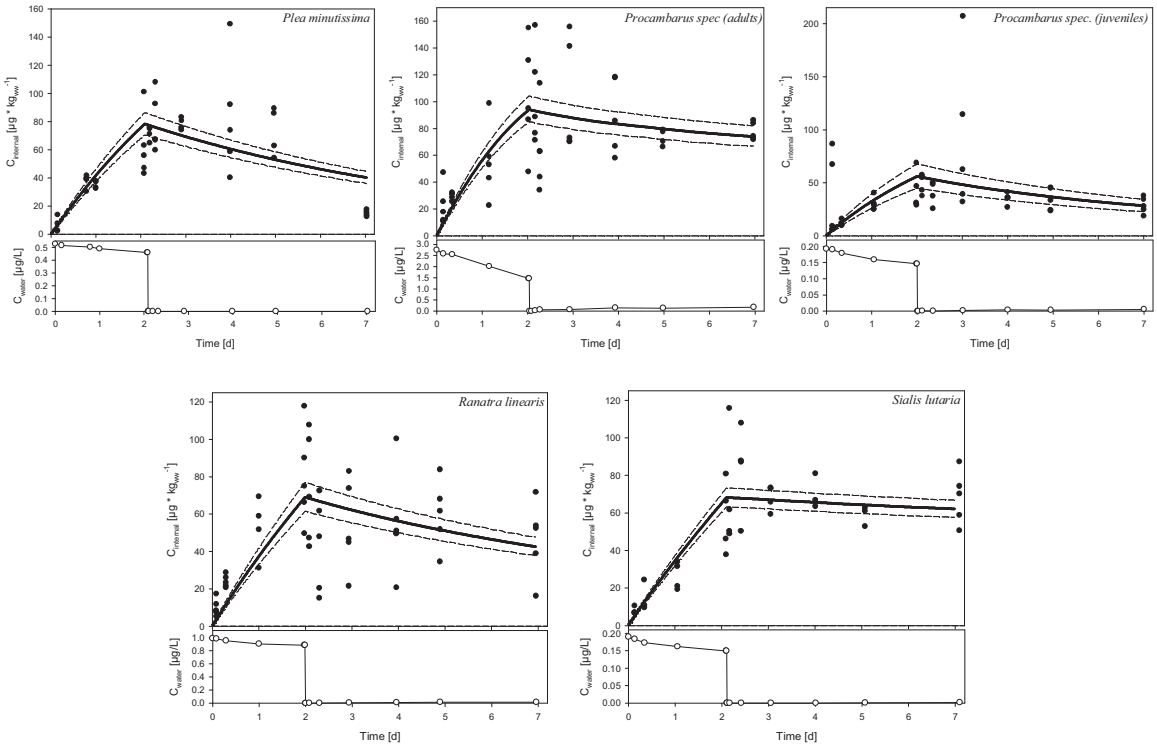

Figure 1 (continued): Uptake and elimination kinetics of [^{14}C]-chlorpyrifos in 15 freshwater arthropod species. Per panel both water (lower, open circles, averages, *n* = 5) and internal (upper, closed circles, 1 data point represents 1 replicate) concentrations are shown, calculated on basis of total measured radioactivity. The solid bold line illustrates the mean internal concentration from the forward Monte Carlo simulation based on the parameter sample from the Markov chain Monto Carlo fit and the dashed lines represent the upper and lower 95% prediction intervals.

Conversely, internal concentrations increased during this time period, indicating uptake of the chemical into the body tissue and/or absorption to the body surface of the animal. After transfer of the contaminated animals into clean water, increasing concentrations in the water phase were observed together with decreasing internal concentrations, showing that the toxicant was being eliminated. Despite this general pattern, there were large differences in the shapes of the model fits in Figure 1, indicating differences in the kinetics between species, i.e. the speed of the uptake and the speed of the elimination of the chemical. In four of the tested species (*S. lutaria*, *M. angustata*, *N. maculata*, *R. linearis*) residues of [^{14}C]-chlorpyrifos, ranging between 0.127 to 7.903 µg/kg$_{wet\ weight}$ were found in the re-extracted exoskeletons and added to the concentrations from the previous extraction. Further details on control and background [^{14}C]-chlorpyrifos concentrations are documented in the supplemental data. The results presented here clearly show that the applied experimental methodology is applicable to a broad range of freshwater arthropod species.

Although differences in the experimental data and the shape of the model fits between the different species are apparent, these differences can be compared quantitatively using the estimates for the model parameters k_{in} and k_{out} as reported in Table 2. These parameters are independent of the different initial sub-lethal exposure concentrations during the experiments and reveal large differences in both uptake (by a factor 50) and elimination rates (by a factor 26) across the investigated species.

Table 2. Uptake (k_{in}) and elimination rate (k_{out}) constants ± standard deviations in 15 freshwater Arthropod species using Markov-Chain-Monte-Carlo fitting with the Metropolis Hastings algorithm and correlation between parameters.

Species	k_{in} [$L*kg_{ww}^{-1}*d^{-1}$]	k_{out} [d^{-1}]	Correlation
Anax imperator	21.2 ± 0.796	0.212 ± 0.0199	0.55
Asellus aquaticus	596 ± 19.5	0.185 ± 0.0168	0.45
Chaoborus obscuripes	318 ± 9.21	0.131 ± 0.0127	0.45
Cloeon dipterum	349 ± 11.9	0.196 ± 0.0171	0.47
Culex pipens	328 ± 13.6	0.0240 ± 0.0098	0.35
Daphnia magna	295 ± 13.8	0.546 ± 0.0390	0.64
Gammarus pulex adults	812 ± 46.2	0.398 ± 0.0371	0.61
Gammarus pulex juveniles	1110 ± 120	0.360 ± 0.0600	0.51
Molanna angustata	579 ± 15.4	0.109 ± 0.0110	0.53
Neocaridina denticulata	617 ± 40.8	0.478 ± 0.0509	0.7
Notonecta maculata	61.9 ± 1.34	0.152 ± 0.0091	0.53
Paraponyx stratiotata	275 ± 9.53	0.171 ± 0.0163	0.5
Plea minutissima	88.2 ± 4.39	0.135 ± 0.0203	0.36
Procambarus spec. adults	24.2 ± 1.22	0.0860 ± 0.0189	0.47
Procambarus spec. juveniles	199 ± 20.3	0.154 ± 0.0398	0.38
Ranatra linearis	42.1 ± 2.4	0.107 ± 0.0243	0.45
Sialis lutaria	203 ± 7.27	0.0210 ± 0.0082	0.32

All species clearly differed from each other in their uptake and elimination rates. The species with the highest uptake rate constants for total [^{14}C]-chlorpyrifos were juveniles of *G. pulex* followed by *G. pulex* adults and then *N. denticulata*, *A. aquaticus* and *M. angustata*. The lowest uptake rate constants were determined for *A. imperator*, followed by *Procambarus* spec. adults, *R. linearis* and *N. maculata*. A few species show only small differences in their elimination rate constants, especially *Procambarus* spec. (adults and juveniles), *P. minutissima*, *R. linearis*, *N. maculata*, *C. obscuripes* and *M. angustata*. The highest elimination rate constants were estimated for *D. magna*, followed by *N. denticulata* and then *G. pulex* adults and juveniles. *S. lutaria* and *C. pipiens* showed extremely low elimination rate constants (0.021 d^{-1} and 0.024 d^{-1}, respectively) compared to all other investigated species, where elimination rate constants ranged from 0.086 d^{-1} to 0.546 d^{-1}. Overall, the observed range of toxicokinetic parameters is in general accordance with literature data (Serrano *et al.* 1997; Lotufo *et al.* 2000; Nuutinen *et al.* 2003), but uptake rate constants of chlorpyrifos for fish are higher (Deneer 1993). A previous study by Buchwalter et al. (2002), where uptake of chlorpyrifos within 12h was measured for 10 different aquatic insects, reported uptake rates between < 1 to 14 ng · g^{-1} · h^{-1} at a single and unique exposure concentration of 240 ng/L. On basis of the calculated k_{in} here, the arthropods characterized in the present study would show uptake rates between 0.212 and 11.1 ng · g^{-1} · h^{-1}, if exposed to the water concentration used by Buchwalter el al. (2002), confirming their findings. In the field, highly species-specific bioaccumulation has also been found for other chemical groups, e.g.

polychlorinated biphenyls (Bazzanti *et al.* 1997), which supports the presented findings. Previously, Ashauer et al. (2006b) performed the same type of experiment with *G. pulex* and chlorpyrifos. Although the design of their experiment was slightly different (usage of ethanol as solvent carrier, aeration of test systems, feeding of test animals). The results of the present study (k_{in} = 812 L · kg^{-1} · d^{-1} and k_{out} = 0.398 d^{-1}) agree well with their findings (k_{in} = 747 L · kg^{-1} · d^{-1} and k_{out} = 0.45 d^{-1}), which gives confidence in the consistency of the methods used. Additionally, this shows that TK approaches can be robust at the species level and are not highly variable despite small differences in experimental design. More importantly for ERA, differences between populations of test species also varied to a lesser extent than differences between species.

Table 3. Bioconcentration factors (BCF) at steady state with lower (LPI) and upper (UPI) 95% prediction intervals and 95% depuration times (t_{95}) of chlorpyrifos in 15 freshwater arthropod species.

Species	BCF_{ww} [L · kg_{ww}^{-1}] [a]	LPI [a]	UPI [a]	BCF_{lipid} [L· kg_{lipid}^{-1}] [b]	t_{95} [d]
Anax imperator	100	93	109	4021	14.1
Asellus aquaticus	3242	3121	3456	382956	16.2
Chaoborus obscuripes	2428	2282	2582	234140	22.9
Cloeon dipterum	1782	1652	1912	24699	15.3
Culex pipens	13930	12710	15050	1999644	124.8
Daphnia magna	541	489	595	57437	5.5
Gammarus pulex adults	2039	1800	2279	149919	7.5
Gammarus pulex juveniles	3083	2547	3690	627029	8.3
Molanna angustata	5331	5068	5645	181901	27.5
Neocaridina denticulata	1291	1153	1409	103599	6.3
Notonecta maculata	407	397	424	10679	19.7
Paraponyx stratiotata	1601	1496	1730	35458	17.5
Plea minutissima	654	587	720	8592	22.2
Procambarus spec. adults	280	253	311	14220	34.8
Procambarus spec. juveniles	1295	1042	1570	111332	19.5
Ranatra linearis	392	349.5	437	40891	28.0
Sialis lutaria	9625	8921	10320	500412	142.7

[a] BCF_{ww}, LPI and UPI were calculated by forward Monte Carlo simulation based on the parameter sample from the MCMC fit.
[b] BCF_{lipid} were calculated using the BCF_{ww} and species lipid content [% ww] listed in Table 1.

The listed BCFs (Table 3) describe how much chlorpyrifos accumulates at steady state in an aquatic species considering both its uptake and elimination abilities, and are mostly in accordance with ranges given in literature (Barron & Woodburn 1995). However, the investigated species rank in their BCF_{ww}s from unexpected extremely high values for *C. pipiens* (13930) and *S. lutaria* (9625), through high values for *M. angustata* (5331) and *A. aquaticus* (3242), to low values for *Procambarus* spec. adults (280) and *A. imperator* (100). The prediction intervals of BCF_{ww}s only overlap for seven pairs and one triplet of species indicating similar BCF_{ww}s for those groups and overall this applies to species

showing BCF_{ww}s between 392 and 3242. This surprisingly large variation (coefficient of variation: 0.762) in the bioconcentration of an insecticide within arthropods (a taxonomically relatively narrow scale) might be reduced when normalized to the available lipid information. (Table 3). Indeed the CV indicates a reduced variability of 0.546 and the ranking of species changes somewhat, especially for *G. pulex* juveniles, *M. angustata*, *C. dipterum* and *P. minutissima*. However the exact species rank should be interpreted with care, because the employed methodology to determine total lipid, has several limitations as discussed below.

Factors influencing toxicokinetics

The reasons for the observed differences in the elimination of chlorpyrifos are very likely related to differences in biotransformation routes and enzyme specification and kinetics, leading to different detoxification abilities as is well known for humans (Eaton *et al.* 2008), but other factors such as size and lipid content are likely to be important too. Also, high k_{out} values might hypothetically be related to high physiological activity leading to a high metabolic turnover. Also, in the present study slow elimination could either originate from a decrease in fresh weight during the time course of the experiment or to increasing bound or sorbed [^{14}C]-labeled residues. A decrease in fresh weight however can be excluded, since a one way ANOVA with parametric (Tukey) and non-parametric (Tamhane T2) post hoc tests showed that *A. aquaticus*, *C. pipiens., D. magna*, *P. minutissima*, *Procambarus* spec. juveniles showed significant differences in fresh weights at different time points per species, but none showed a significant trend in fresh weight decrease over time. Therefore, elimination rates were likely not underestimated for these species as a result of starvation, and slow elimination must therefore be related to bound residues. Following the reasoning described in the limitations section below, these [^{14}C]-labelled residues are most likely bound or incorporated to either the acetylcholinesterase as the target enzyme itself, other macromolecules or stored in the lipid compartment of the animal. Despite these general considerations, allocation of the label residues to specific molecules or compartments is not possible with the method used here and a detailed characterisation of metabolic pathways of chlorpyrifos in the different species would be required for more detailed interpretation. This can theoretically become very complex and different for each species, as numerous possibilities exist to enhance or reduce elimination through metabolism. For instance, resistance in cockroaches to chlorpyrifos was previously explained by increased A-esterase activity detoxifying the quickly produced chlorpyrifos-oxon to 3,5,6-trichloro-2-pyridinol (Roberts 1998), which enhances elimination of the active ingredient. In contrast, the bioactivation step of parental chorpyrifos to the chlorpyrifos-oxon which would delay elimination could vary largely across species due to variable enzymatic configurations in the mixed-function-oxygenase system, i.e. cytochrome p450s (Korsloot *et al.* 2004).

The extent of variation in uptake rate constants across species might be explained by various factors including body size (Hendriks *et al.* 2001) and respiratory strategy (Buchwalter *et al.* 2002). Another factor, which may lead to large differences in both uptake and elimination between species is lipid content since the lipid content of animals highly determines the bioconcentration of lipophilic substances (Verhaar *et al.* 1999a). In the present study, total lipid was quantified mostly from sub-samples of the test animals as listed in Table 1 and values varied within a factor of 15. This agrees

with a literature review (see supplemental data) indicating that freshwater arthropods vary in their total lipid content by a factor of 20. Surprisingly, the total lipid contents measured in the present study did not show a significant correlation with uptake or elimination rate constants in linear regressions. Furthermore, the BCFs corrected for lipid content (Table 3) did not show such a reduced variability across the tested species as it was expected (see above). Moreover, the observed uptake rate constants vary within a factor of 50, supporting previous conclusions that other biological factors, such as size related descriptors or respiration type, determine uptake of chemicals as well (Hendriks *et al.* 2001; Buchwalter *et al.* 2002). This is also supported by the present study, since juveniles of *G. pulex* and *Procambarus* spec. differ significantly in their size from their adult counterparts and also show much higher uptake rates. Also, atmospheric oxygen breathers, with the only exception of *C. pipiens*, showed relatively low uptake rates.

Lipid content

The quantification of lipid content entailed several difficulties especially associated with the limited amount of tissue available, low sample size (n = 1), and the presence of extractable non-lipids (hairs and other body parts, which largely differ among species). Another reason for the low explanatory power of lipid content could be that on such a relatively small taxonomic gradient of organisms and small number of species, single factors have less explanatory power than combinations of factors, as indicated in (Rubach *et al.* 2010). Clearly, ERA approaches that incorporate lipid content need further discussion in the future, since large seasonal and individual variation has been observed for aquatic invertebrates (Hanson *et al.* 1985; Meier *et al.* 2000), and variation in lipid content may alter organism sensitivity by temporally affecting internal bioavailability. A biologically active molecule which is temporarily stored in the lipid reservoir may not lead to damage due to low internal bioavailability. However, if lipid levels fall, circulating concentrations of the chemical may rise, potentially leading to damage at a later point in time. Furthermore, the variety of analytical methods to determine lipid content leads to quite variable results with different methodological limitations (Manirakiza *et al.* 2001). Also, for some of the these methods it is unclear to what extent and in what manner these methods extract biologically relevant compartments such as membrane, storage or other types of lipids and/or also other molecules which are actually not part of a lipid fraction. Although understanding these links is important, such methodological problems might limit the use of this type of data as explanatory or predictable variables.

Limitations

The approach presented to evaluate TK in various freshwater arthropod species has several limitations. Firstly, differences in experimental design across species were minimized to increase comparability, resulting in a general test design representing a compromise between species differences, their requirements and experimental practicalities. Thus, testing occurred under sub-optimalconditions for some species and therefore inevitably to inconsistent multiple stress regimes across the different species. This resulted in mortalities > 20% for some species as reported in the supplemental data, which were tolerated in the present study, since only survivors were sampled and the research question addressed uptake and elimination kinetics rather than effects of chlorpyrifos.

Furthermore, sub-optimal experimental conditions may alter behavior, which in turn may alter the kinetics. This and also the feedback on behavior induced by sub-lethal exposure itself as hypothesized by (Cid Montañés *et al.* 1995), were tolerated in the presented study. However, even if sub-lethal exposure levels affected uptake and elimination, they likely did so consistently in the present study, as all species were exposed to 10% of their respective EC_{50}. Another limitation important for interpretation is that all measurements are based on total [^{14}C] radioactivity, meaning the fractions of parental chlorpyrifos and its metabolites were not characterized separately. To estimate the uncertainty associated with this approach, metabolic pathways of chlorpyrifos were reviewed in Racke (1993), ATSDR (1997), Roberts (1998), Eaton *et al.* (2008) and at the Dow website (http://www.dowagro.com/chlorp/na/science/. As a result, the identified major and minor metabolites with and without [^{14}C] label are specified in the supplemental data. According to (Roberts 1998), four molecules are mainly related to degradation routes of chlorpyrifos in insects, of which 3,5,6-trichloro-2-pyridinol does not carry the [^{14}C] label after metabolic biotransformation, and therefore parental chlorpyrifos, chlorpyrifos-oxon and diethyl-thiophosphate are the [^{14}C]-labelled molecules potentially contributing to the here measured total [^{14}C] radioactivity. Quantitative-structure-activity-relationships (QSARs) were applied to all possible metabolites in order to estimate their hydrophobicity (log P value) and so characterize the molecule's partitioning behavior. The QSARs used were ALOGPs (Tetko *et al.* 2001; Tetko 2002; Tetko *et al.* 2005), ALOGP (Viswanadhan *et al.* 1989) and XLOGP3 (Cheng *et al.* 2007), selected on the basis of a benchmarking study of Mannhold *et al.* (2009) and QSAR calculations were performed with the VCCLAB webtool (VCCLAB, Virtual Computational Chemistry Laboratory (http://www.vcclab.org)) based on Tetko *et al.* (2005). For plausibility average QSAR estimations on log P were compared with average experimental log K_{OW}s from the LOGKOW© databank, if available (Sangster 1997, online at the URL www.gdfdatabanks.ro/sangster.PDF,, http://logkow.cisti.nrc.ca/logkow/). From the estimated log P values it was concluded that total internal [^{14}C] radioactivity is composed mainly of parental chlorpyrifos and chlorpyrifos-oxon, because dietyl-thiophosphate is four orders of magnitude less hydrophobic and therefore likely to be excreted much faster than parental chlorpyrifos and the bioactive chlorpyrifos-oxon. In addition, the chlorpyrifos-oxon is fifty times less hydrophobic than chlorpyrifos, but may be more likely to remain in the organism due to its higher affinity to the acetylcholinesterase. Accordingly, total [^{14}C] in the water is most likely composed of dietyl-thiophosphate and parental chlorpyrifos. Minor or unknown metabolites and conjugates may add further uncertainty to the composition of measured total [^{14}C]. Partitioning into the lipid phase as well as binding to macromolecules does not change the composition of the here measured total [^{14}C] concentrations, but may affect the representativeness of the latter for the concentration at the target site, which must be taken into consideration when discussing toxic effects based on internal concentrations as has been discussed above.

Importance for ERA

The most important implications and also potential application of toxicokinetic variation for ERA lies within the calculation of BCFs, t_{95} and other risk indicators. In general, at present various ERA guidelines currently use BCFs as trigger values for additional risk assessment of compounds with bioaccumulation potential, e.g. for secondary poisoning, e.g. a fish BCF > 1000 L/kg for compounds

which are readily degradable in the 91/41/EEC (EC 1991) [38], http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1991L0414:20070201:EN:PDF.

Another relevant value for ERA derived from the toxicokinetic data is the t_{95} (Table 3) or the time it would take the organism to eliminate 95% of the accumulated toxicant when returned to clean water (equivalent to the time to reach the steady state). This value depends on the elimination rate constants only and consequently follows the same highly variable species pattern as k_{out} itself. The depuration time is important because it defines the minimum length of the interval between repeated exposure events required for the organisms to recover. Therefore, the highly variable elimination abilities found in the present study have implications for the risk assessment of time-varying exposures, especially in the light of very slow elimination for some species. These species clearly require long periods of time for full elimination and potential recovery, or further exposure will have enhanced effects (Ashauer *et al.* 2007a, b). Management practices derived from ERA approaches could use this information in order to deal with fluctuating exposures. However, further research is needed on how the limitations and assumptions of experimental studies of this kind influence the confidence with which measured [^{14}C] activity represents the toxicologically relevant dose. These uncertainties could be quantified and reduced in future studies, which should focus on species identified as exhibiting particularly interesting or unexpected TK patterns. Future research should also focus on identifying biological factors that determine uptake and elimination in relation to sensitivity of the investigated species using the presented dataset. Regardless of the specific reasons for the observed variation in uptake and elimination, it is of interest for ERA how this variation in the toxicokinetics relates to differences in sensitivity. In general, high uptake rates correlate with high sensitivity and low elimination rates with insensitivity; both these relationships were significant in their respective linear regressions (Figure 2), indicating that up to 38% of the variation in sensitivity may be explainable by uptake and up to 28% by elimination. It was not accounted for the sensitivity differences between juveniles and adults of *D. magna* and *G. pulex*, although it is well known that sensitivity differences exist between life stages and might increase the explanatory power of k_{in} for sensitivity.

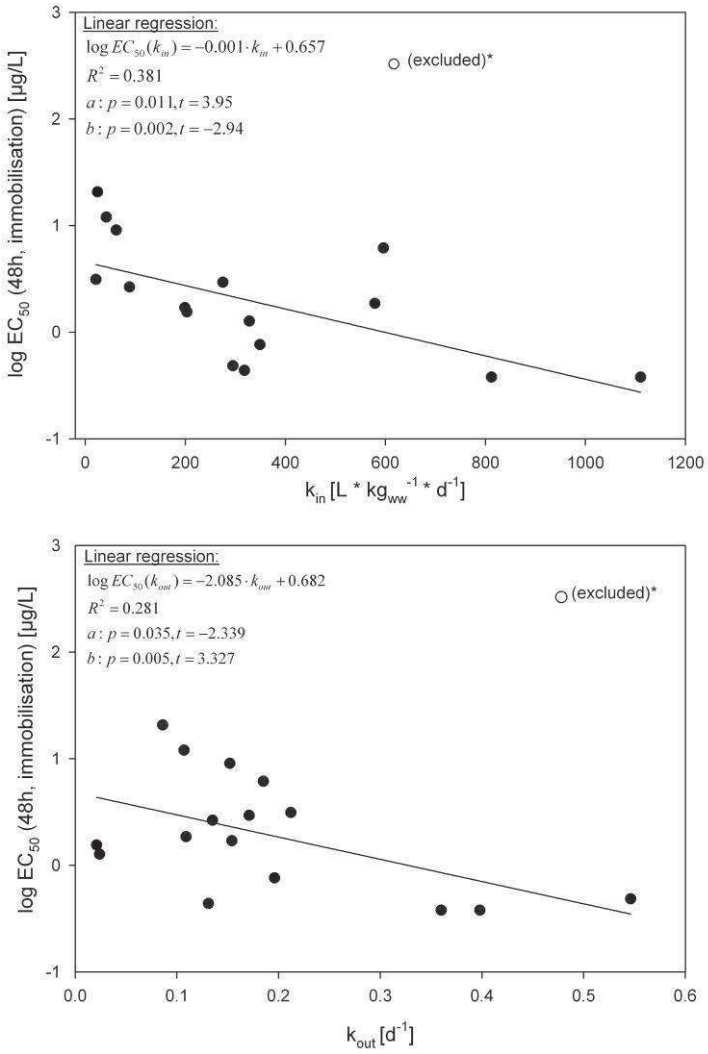

Figure 2: Linear regressions of sensitivity against toxicokinetic parameters k_{in} (upper panel) and k_{out} (lower panel) with equations, R^2, *p* values and *t*-statistics given. *From both analyses one datapoint (empty circle, species *Neocaridina denticulata*) was excluded as an outlier (Grubb's test).

In contrast, the BCF_{ww} was not significantly correlated with sensitivity (R^2 = 0.054). Apparently, TK cannot fully explain the differences in sensitivity indicating that other processes, such as biotransformation, internal damage/hazard and internal recovery and repair may influence sensitivity. Actual recovery from chlorpyrifos exposure may even take longer than the depuration time alone (Ashauer *et al.* 2007a, b) in order to repair and recover from potentially induced damage, which are both toxicodynamic processes. Toxicokinetic parameters are required for investigations of the toxicodynamics and consequently for a full understanding of the dynamics of effects and organism recovery. If uptake and elimination rate constants are known, then toxicodynamic parameters for damage, internal repair and recovery, as well as thresholds can be estimated and the time course of toxicant effects and organism recovery can be simulated (Ashauer *et al.* 2007b) in order to assess how these processes vary across species. Hence depuration times and toxicokinetic parameters provide important information for ERA of chemicals, especially for estimation of effects of fluctuating or sequential pulses of toxicants.

SUPPLEMENTAL DATA

Supplemental data includes details on preparation of aqueous stock solutions of [^{14}C]-chlorpyifos, cultivation of three test species, methodology and results of determination of EC_{50} values, methodological details of uptake and elimination studies including LOD/LOQs, control and background activities, quench corrections, data correction due to external contamination, experimental control and treatment mortality, determination of lipid content, modelling methodology and model settings, input data and goodness of fit measures and details on considerations about metabolite partitioning and lipid literature review. The supplemental data can be downloaded from as 2 files (.xls and .doc) from:
http://www3.interscience.wiley.com/journal/123489257/suppinfo

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Sampling and collection of test animals, 2008

6. CHAPTER

Toxicodynamics of chlopyrifos in freshwater arthropods.

Mascha N. Rubach, Roman Ashauer, Steven J.H Crum, Stephen J. Maund and Paul J. Van den Brink

ABSTRACT

One of the major dilemmas that ecotoxicologists face is that in standard laboratory tests, exposure concentrations are maintained to establish the inherent hazard properties of a compound, whereas in the environment, exposure to toxicants varies in time. Predicting environmental effects of variable concentrations on the basis of maintained laboratory exposure concentrations is therefore a significant challenge. Recent research has seen the development of toxicokinetic and toxicodynamic (TKTD) models whose aim is to provide a better understanding of the likely effects of time-varying exposure to toxicants. In this study, a TKTD model (the Threshold Damage Model, TDM) was parameterized for three freshwater arthropod species based on data concerning their sensitivity to and toxicokinetics of the organophosphorus insecticide chlorpyrifos (collected in previous experimental studies). The TDM was fitted to survival data derived from experiments with aquatic invertebrates exposed to comparable pulsed exposure designs, allowing for consideration of damage accrual, complete elimination and potential recovery. In so doing, killing rate constants, recovery rate constants and internal thresholds for the three species were estimated for comparative purposes. The analysis posed a number of difficulties, particularly with regards to the robustness of parameter estimates, which are fully discussed. Nonetheless, the combination of experimental and modeling data allowed inferences to be drawn about the differences in toxicodynamics of chlorpyrifos in these species which relate to their apparent sensitivity to the insecticide. The water hoglouse *Asellus aquaticus,* for instance, was relatively insensitive to chlorpyrifos, and although significant bioconcetration was observed, its resilience indicates that it is likely to have a sophisticated physiology that enables detoxification and/or damage repair, compensation and recovery. For the phontom midge larva *Chaoborus obscuripes* low threshold and recovery tolerances were found. The experiments revealed that chlorpyrifos may only act as a baseline (narcotic) toxicant in the shrimp *Neocaridina denticulata*, instead of acting specifically by acetylcholinesterase inhibition, the classical mode of action of organophosphates, which perhaps explains its extremely low sensitivity. However, atyid shrimps are known to be very sensitive to organophosphates, so further work is needed to establish species-sensitivity relationships for this family.

INTRODUCTION

Environmental risk assessment (ERA) faces several major challenges in dealing with the complexity of ecosystems and species composition, but also within time and chemical composition resulting in variable exposures in the environment (Reinert *et al.* 2002). In recent years the predictability of time-variable and mixed exposures in the environment has advanced through various dispersion, transport and fate models (Dubus & Surdyk 2006; Brock *et al.* 2010). Additionally, links to possible biological effects have been established through laboratory scale experiments and effect models (Legierse *et al.* 1999; Escher & Hermens 2004; Ashauer *et al.* 2006a; Brock *et al.* 2010). Among the latter, toxicokinetic-toxicodynamic models (TKTD models) have received significant attention, because of their mechanistic nature and the potential to use them to analyze and predict the effects of sequential exposures of different chemicals and chronic effects (Lee *et al.* 2002; Reinert *et al.* 2002; Schuler *et al.* 2004; Jager *et al.* 2006; Ashauer *et al.* 2007b). TKTD models predict survival on the basis of the processes contributing to toxicity, which are uptake, biotransformation and elimination (summarized as toxicokinetics, TK), but also on basis of induced damage and its repair, leading to damage threshold levels which need to be exceeded before an effect is visible (summarized as toxicodynamics, TD). These models vary in their explicit formulations and underlying concepts (Ashauer *et al.* 2006a; Ashauer & Brown 2008) and have been parameterized for several chemicals, mostly pesticides, but only for one model species in each case. Another challenge for ERA is that species can vary up to several orders of magnitude in their sensitivity towards the same (non-narcotic) compound (Vaal *et al.* 2000; Rubach *et al.* 2010). This variation is mainly addressed using assessment factors or using probabilistic methods such as the species sensitivity distribution (SSD) concept. Due to a lack of comparative studies, it is largely unknown how much of the variation in sensitivity is attributable to species differences in toxicokinetics and toxicodynamics. Rubach *et al.* (in press b) have concluded that although a large part of the variation in sensitivity can be explained by uptake (32 %) and elimination (28 %), the remaining part might be related to differences in biotransformation or the toxicodynamics. The toxicokinetics, whose concepts and models relate to bioaccumulation and bioconcentration, have been better explored than the toxicodynamics. Processes of toxicodynamics are also time dependent, include damage, recovery or repair and effect thresholds, and can therefore be researched by studying the effects of well designed pulsed treatments of toxicant on individuals (Ashauer *et al.* 2007b; Baas *et al.* 2010). Experimental data on how different species respond to time-variable exposures combined with information about the toxicokinetics in those species can increase the understanding of the underlying reasons for the observed large variation in intrinsic sensitivity for certain compound classes or mode of actions. Such knowledge is pivotal to facilitate the development of interspecies extrapolation approaches for future ERA.

The first objective of the present study was to investigate experimentally how freshwater arthropods with different sensitivities and toxicokinetic characteristics respond to sequential pulses of chlorpyrifos in their survival and their mobility. As a second objective the experimental survival data were used to fit a TKTD model. For the first two objectives, the results of Rubach *et al.* (in press b), who characterized the toxicokinetics of chlorpyrifos for 15 different freshwater arthropod species, were used. The TKTD model used, i.e. the Theshold Damage Model (TDM) has been successfully

parameterized to describe the response of *Gammarus pulex* to several substances (Ashauer *et al.* 2007c, a, b; Ashauer 2010; Ashauer *et al.* 2010). The final objective was to derive biologically meaningful toxicodynamic model parameters in order to compare the tested species quantitatively in their damage, recovery and repair and internal effect thresholds characteristics with the final aim to explain their sensitivity differences.

MATERIALS & METHODS

In order to compare the toxicodynamic characteristics of chlorpyrifos in a range of freshwater arthropods survival experiments with pulsed exposures were performed with three different freshwater arthropod species. In a second step these data were used to fit the TDM.

Experimental

Analytical chemistry

For the survival experiments chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate, 99% purity, CAS 2921-88-20, lot 51205) was purchased from Dr. Ehrenstorfer Gmbh (Augsburg, Germany) and aqueous solutions of chlorpyrifos were prepared using the principle of generator columns (Devoe *et al.* 1981) as described in the supporting information of Rubach *et al.* (in press b). The generator column was built on 10.09.2008 with 309.8 mg chlorpyrifos diluted in 5.1 g of acetone by mixing this with 16.9 g fine glass beads. The effluent of the generator column delivered a stable concentration of parental chlorpyrifos of approximately 600 µg/L. Before use, each such obtained stock solution was measured by liquid/liquid extraction of subsamples with n-hexane followed by gas chromatography (GC) and electron capture detection (ECD) to determine its exact concentration of chlorpyrifos and dosing schemes were accordingly adapted. The GC-ECD was carried out as described in Rubach *et al.* (in press c). Also, all water samples taken during the experiment to characterise the time-variable exposures were extracted with n-hexane and measured by means of GC-ECD as described in Rubach *et al.* (in press c) in more detail. The resulting limits of detection (LODs) are given in Table 1.

Test species and test media

In the present study three freshwater arthropod species were tested and Table 1 shows their origin, life stage and sampling date. The species identity of *Asellus aquaticus* and *Chaoborus obscuripes* sampled from cosms at the experimental field station of Alterra, The Sinderhoeve (Renkum, The Netherlands), was determined by trained staff according to established protocols using 6-10 individuals sub-sampled from the catch. The species *Neocaridina denticulata sinensis* variation red is a tropical shrimp, also called the 'sherry red shrimp' and although particularly popular with hobby aquarists, has rarely been employed in ecotoxicology as a test species; it was cultured at Alterra as reported in the supporting information of Rubach *et al.* (in press b). All test animals were transferred into water (0.45 µm membrane pressure filtered and aerated for 24 h) pumped from the groundwater horizon of 'The Sinderhoeve' and supplied with appropriate food (see Table 1) *ad libitum* to acclimatize to the test medium for at least three days before testing. The same water was used to prepare the test

media by spiking and homogenizing the filtered and aerated water with the adapted volumes of stock solution after the exact concentration of chlorpyrifos in the stock solution had been determined.

Table 1: Methodological details of the survival experiments under pulsed exposure

	Species		
	Asellus aquaticus	*Chaoborus obscuripes*	*Neocaridina denticulata*
Life stage	adult	larva	adult/juvenile
Origin [1]	A	A	B
Species sampling date	09.10.2008	09.10.2008	27.10.2008
LOD [µg/L] [2]	0.04	0.01	2
Duration of experiment [d]	31	34	20
Temperature (ºC) [3]	17.3 ± 1.48	17.6 ± 1.57	16.4 ± 2.04
Oxygen [mg/L] [3]	11.2 ± 1.36	10.7 ± 1.63	9.5 ± 1.91
pH [3]	7.3 ± 0.66	7.4 ± 0.63	7.0 ± 0.55
Light regime [4]	14:10 shaded	14:10 shaded	14:10 non-shaded
t_{95} [d] [5]	16.4	23.2	6.3
T1 (intended concentration [µg/L], day pulse1|2|3) [6]	(12; 0|8|23)	(0.7; 0|8|31)	(458; 0|5|16)
T2 (intended concentration [µg/L], day pulse1|2) [6]	(15.4; 0|16)	(2.5; 0|17)	(600; 0|8)
T3 (intended concentration [µg/L],day pulse1|2) [6]	(15.4; 0|23)	(2.5; 0|31)	(600; 0|16)
Aeration	non-exposure periods	no	non-exposure periods
Food (regime) [7]	4 *Populus x canadensis* leaf disks	< 20 *Daphnia magna* juveniles	4 pieces carrot

[1] Origin: A = Sinderhoeve cosms; B = culture
[2] Derived from concentration factor of the respective extraction procedure for T1 and the limit of detection of the used apparatus 0.1 µg/L.
[3] Temperature and pH measured with pH 323B/set and oxygen measured with Oxi330/set, both WTW Germany.
[4] Tropical daylight lamps: JBL Solar tropic, 30W T8.
[5] Taken from Rubach *et al.* (in press b)
[6] Treatment 1(T1): 3 x LC_{30}; T2 and T3: 2 x LC_{50}; taken or calculated from Rubach *et al.* (in press c)
[7] Diameter of leaf disks was 1.7 mm, pre-soaked for > 1 week with tap water; *D. magna* juveniles were cultured; pieces of carrot were pre-cooked, defrosted and 3-5 mm.

Survival experiments

All three survival experiments were in principle designed in a similar fashion as described by Ashauer *et al.* (2007b). The general methodology described in the following is detailed out for each species in Table 1, including the physico-chemical conditions under which the experiments were carried out. Each species was tested in three different pulsed exposure regimes with chlorpyrifos, each replicated five times and one control treatment in triplicate. During the course of the experiment, water concentrations were measured and mortality and immobility were recorded daily. The duration of one pulse was 24 h for *A. aquaticus* and *C. obscuripes* and 48 h for *N. denticulata* and at the end of the pulse the animals were transferred into uncontaminated water. The first treatment (T1) consisted of three pulses, each intended to induce 30 % mortality within 24 h (*A. aquaticus*, *C. obscuripes*) or 48 h

(*N. denticulata*). The second (T2) and third (T3) treatments were comprised of 2 pulses, each intended to lead to 50% mortality in 24h (*A. aquaticus*, *C. obscuripes*) or 48 h (*N. denticulata*). In order to determine appropriate intended exposure concentrations for the given effect levels, the concentration-response relationships of Rubach *et al.* (in press c) were used. These authors and also others (Van Wijngaarden *et al.* 1993) however were not able to determine a 24 h or 48 h concentration-mortality relationship for *A. aquaticus*. Therefore the 72 h mortality and immobility data from Rubach *et al.* (in press c) were compared and a factor of 1.45 derived to describe the dose difference in endpoints per time point. This factor was applied to the EC_{50} for 24 h of exposure in (Van Wijngaarden *et al.* 1993) and thereby the extrapolated lethal doses were derived. All intended exposure concentrations are listed in Table 1. Furthermore, the treatments differed in the intervals between pulses, in order to experimentally simulate three scenarios: damage accrual (T1), full elimination (T2) and potential organism recovery (T3). For this the 95 % depuration times (t_{95}) from (Rubach *et al.* in press b) were used, i.e. the species specific time it takes the organism to eliminate 95 % of the accumulated amount of chlorpyrifos. In T1 the interval between the first and the second pulse was shorter than the t_{95} and therefore should induce damage accrual, whereas in T2 the interval between pulses matched exactly the t_{95}, just allowing for full elimination and in T3 the interval was longer than t_{95} and therefore allowing for potential organism recovery. The test animals were given appropriate food only during non-exposure times in order to avoid uptake via the food pathway and to factor out differences in animal condition resulting from different food levels. In order to minimise evaporation of chlorpyrifos during the pulses, only during non-treatment times were the test systems of *A. aquaticus* and *N. denticulata* aerated (delivered through Pasteur pipettes with a gentle filtered oxygen flow). The experiments were carried out in 600 mL tall Pyrex glass beakers, filled with 500 mL test medium, containing 15 animals per species and treatment replicate, and were covered with Parafilm to reduce the evaporation of chlorpyrifos and the likelihood of cross contamination. The species *N. denticulata* and *A. aquaticus* were given two to three pieces of hook shaped stainless steel gauze as a structural substrate. The test media in controls and in treatment systems during non-pulse periods were changed at the latest after eight days. Water samples in all experiments were taken from each replicate before a water change or a new pulse, immediately after a water change, at the end of a pulse and as well every other four days. The number of animals, and the survival and mobility of the animals was recorded daily per replicate. For *C. obscuripes* also pupation and emergence were recorded. Animals were classified as immobile if abnormal movements compared to control animals were observed (if necessary under a binocular microscope) within 10 seconds after repeated gentle agitation with forceps. If no movement was detected the animals were classified as dead and only then removed from the test system.

Data analysis

In order to characterize the chlorpyrifos exposure the concentrations measured by means of GC-ECD in the hexane extracts were recalculated to concentrations in the test media and converted to nmol/ml. This was done per test replicate and time point, but for the purpose of modeling and further analysis, averages were calculated (replicated data are available on request). The fraction of survival and mobility was calculated per replicate and time point. First, the cumulative number of dead and

immobile test animals was calculated (dead animals were also included into the number of immobile) and then expressed as a fraction of the number of animals at the start of the experiment (in total 15 per replicate and 75 per treatment). For modeling and further analysis averages were calculated. We included pupae or emerged specimen of *C. obscuripes* in the analysis, but we did not account for cannibalism.

Modeling

TKTD model

The experimental survival data were used to parameterize the second part of the TDM (Ashauer *et al.* 2007b) to describe toxicodynamic processes for the three tested species and model their survival. The TDM employs a two step approach, of which the first step describes the time course of uptake and elimination of a chemical into an animal (toxicokinetics) using a one compartment, first order kinetic:

$$\frac{dC_{\text{int}}}{dt} = k_{in} \cdot C_w(t) - k_{out} \cdot C_{\text{int}}(t) \quad (1)$$

with t as time [time], C_w [amount · volume^{-1}] as the water concentration, C_{int} [amount · body weight^{-1}] as the internal (whole body) concentration and the two parameters k_{in} [volume · body weight^{-1} · time^{-1}], the uptake rate constant and k_{out} [time^{-1}], the elimination rate constant. This first step, the toxicokinetics had been parameterized previously by Rubach *et al.* (in press b) and their estimated parameters (Table 1) were used here to predict internal concentrations resulting from the pulsed exposures using Equation 1. The predicted time course of internal concentrations, driven by the applied water concentrations is subsequently used to estimate damage in the animal again by employing a one compartment, first order kinetic:

$$\frac{dD}{dt} = k_k \cdot C_{\text{int}}(t) - k_r \cdot D(t) \quad (2)$$

with D [-] as damage, k_k [body weight · amount^{-1} · time^{-1}], the killing rate constant and k_r [time^{-1}], the recovery rate constant as parameters. In the next step the time course of internal damage D is compared to an internal threshold and if the latter is reached the hazard increases:

$$\frac{dH}{dt} = \Theta \cdot \max[D(t) - threshold] \quad (3)$$

with H [-] as hazard, Θ [time^{-1}] as a proportionality constant and the parameter '*threshold* [-]. The hazard at a given time t is in principle a probability of dying at that given time and used in the next step to transform the hazard rate dH/dt into a survival probability:

$$S(t) = e^{-H(t)} \cdot S_{background}(t) \quad (4)$$

with $S(t)$ [-] being the survival probability for a given time t and $S_{background}$ the survival in control treatments to account for background mortality. The background survival is accounted for by fitting the background survival to:

$$S_{background}(t) = e^{-h_b \cdot t} \quad (5)$$

with h_b being the background hazard rate [time^{-1}]. For more information and evaluation of the TDM and its toxicodynamic assumptions the reader is referred to (Ashauer *et al.* 2007b, a; Ashauer & Brown 2008).

Implementation

In order to compare the toxicodynamic parameters k_k, k_r and *threshold* for several species, their respective parameter estimates need to be both biologically meaningful and estimated using the same method and criteria.

The model was implemented in OpenModel (OM) release 11 (Crout 2008), retrieved from the website of Nottingham University (http://www.nottingham.ac.uk/environmental-modelling/OpenModel.htm) on 11.11.2009. Parameter estimation was carried out per species by employing non-linear least squares curve fitting using the built-in Levenberg-Marquart algorithm (LMA) and estimations were tested for robustness as described below.

Parameter estimation for TD

We fitted the three parameters k_k, k_r and *threshold* for each species separately to the measured survival data of all treatments and using the LMA. To test the robustness of the fits we used 34 combinations of starting values for the estimation of the three parameters. These combinations of starting values were partly generated using prior knowledge, i.e. the robust toxicodynamic parameter estimations for chlorpyrifos in *Gammarus pulex* (Ashauer *et al.* 2007c). We varied these parameter values each singly with the factors 0.001, 0.01, 0.05, 0.1, 1, 5, 10, 100, 1000, 10000, subsequently permuted them, deleted the redundant combinations and were so left with 34 combinations. If a combination of starting values crossed the experimental data when evaluated with the model it was used as starting value for the actual estimation procedure. In a second step obviously bad estimates were identified and removed on basis of a visual inspection of the model fit, the actual parameter values and their standard deviations, the minimum residual sum of squares (RSS), the Chi^2 and the R^2 statistic in comparison to the other estimations. The criteria for included fits for each species are detailed out in Table 2. For the final estimation we used the average parameter estimates of all valid estimations and employed user constrains derived as follows: For instance, k_r values < 0.002 d^{-1}, which correspond to recovery times longer than the maximum lifespan of a species (e.g. 1 year for *C. obscuripes*) do not correspond to our experimental design. Thus this value was used as the lower limit for parameter estimation. Furthermore, due to the fact that we observed organism survival only daily the fastest recovery time was assumed to be 8.6 minutes (k_r = 500 d^{-1}) and used as the upper limit for parameter estimation. The consequences of these user constraints are discussed below.

RESULTS

Experimental

Analytical chemistry

The average measured exposures of all treatments to chlorpyrifos are displayed in Figure 1 for all experiments. Throughout the experiment, concentrations measured in blank groundwater samples

(n = 33) were below the detection limits. Likewise concentrations of chlorpyrifos in all control treatments were below the respective species' LODs (on average 0.004, 0.0007, 0.045 µg/L for *A. aquaticus*, *C. obscuripes* and *N. denticulata* respectively). Over all experiments, 87.9 ± 4.5 % of the intended concentrations were on average verified in the treatments during the exposure peaks (83.4, 87.2, 93.2 % for *A. aquaticus*, *C. obscuripes* and *N. denticulata* respectively). Before transfer into clean water, on average 78.5 ± 11.2 % of these achieved concentrations remained in the exposure media. For *N. denticulata* only 65.2 % of the concentration at t = 0 was found remaining after 48 h, whereas for *A. aquaticus* and *C. obscuripes* 80.4 and 89.7 %, respectively was measured after 24 h. The chlorpyrifos concentrations found in between pulses in the groundwater before a water change within a maximum period of 8 days were below the detection limits for *A. aquaticus* and *C. obscuripes*, but on average 12.6 ± 10.6 µg/L (minimum: 1.4 µg/L, maximum: 46.5 µg/L) chlorpyrifos was found in the test media of *N. denticulata*.

Survival

The experimental results on survival and mobility of the three tested freshwater arthropods are presented in Figure 1 and described below. For all three species the mortality and immobility in control treatments was on average less than 20 %, whereas the survival and mobility responses to the different pulsed exposure treatments differed between species.

For the water hog louse *A. aquaticus*, the first chlorpyrifos peak in all treatments led to the exact intended effect of approximately 30 % (T1) and 50 % (T2) mortality within two days, after which the effect returned to background mortality. The second peaks in all treatments, however, led to delayed effects. Survival in T1 continuously decreased to 20% in the following 14 days until the next peak and did not further stabilize to background mortality. The survival in T2, which received the second peak later than T1, i.e. after a period allowing for 95 % elimination of previously accumulated chlorpyrifos was delayed in a similar way. In treatment T3, which received the second pulse even later than T2, after an extended period for potential organism recovery, survival was still delayed, but was not as slow as in the other two treatments. At the end of the experiment and after a third dosage in T1, which led to a further decrease in survival, all treatments showed a similar effect on survival (9.3 – 17 % survival). The mobility responses of *A. aquaticus* to the first peaks showed immediate effects within 24 h, which were slightly higher (44 % and 64 %) than the intended lethal effect dose. The maximum effect was reached after two days and in all treatments 10 % organism recovery of immobile test animals occurred within two to three days after the transfer into clean water. The following peaks also induced immediate immobility in all treatments showing enhanced effects in T1, where the second peak induced an additional 50 % immobility, but slightly reduced effects in T2 and T3, where only 35 and 39 % additional immobility was caused by the second peaks, respectively. Organism recovery was also occasionally observed after the second pulses.

The survival response of the larval stage of *C. obscuripes* showed even longer delayed effects. In all treatments only 10 % mortality was observed after 24h, however survival decreased continuously until stabilizing at background mortality only on day nine for T1 and day 19 for T2 and T3. These extremely delayed effects resulted in 53 % mortality in T1 and 90 % in T2 and T3, which was much higher than the anticipated 30 and 50 %, respectively. The second peak in T1 did lead to a

further decrease of 33 % in survival, which represents the intended effect in this case and after seven days survival stabilized to background mortality. In T2 only a very small decrease in survival was observed after the second peak. Surprisingly the third peak in T1 and the second peak in T3 did not show any effect on survival until termination of the experiment. In contrast to the substantially delayed lethal effects, immediate inhibition of mobility was observed for *C. obscuripes* within 24h. The amplitude of effect was 35% immobilisation in T1, which corresponds to the intended effect, but the 74 and 79 % effect in T2 and T3 respectively were not expected. During the following day the effect also intensified by about 10-20% after which mobility stabilized at background level. In general the effect on mobility stabilized after 1-2 days to background immobility. The second peak in T1 induced further 22 % immobility and another 8 % in T2, however it had no effect on the survivorship in T3. The third peak in T3 did induce a slight increase in immobility of approximately 1 %, corresponding to one affected animal, before the experiment was terminated. This affected animal was floating on the water surface, whilst being immobile, whereas all the other survivors were submerged in the water column. In general, it was a qualitative but consistent observation that immobile and/or dead larvae floated on the water surface. Pupal stages however were never found floating when dead or immobile. Furthermore, no recovery was observed for *C. obscuripes* throughout the duration of the experiment.

The third species investigated, the shrimp *N. denticulata,* showed a significant decrease in survival, however well below the intended effect. In fact it induced comparable mortality of 20-35% in all three treatments, despite the differences in dosages. Remarkably, the survival did not stabilize at background mortality anymore in any of the treatments, although the continuous decrease in survival was slow. Another surprising result was that further peaks, given at different time intervals did not produce any differences in the responses between treatments. At the end of the experiment the survival in the three treatments was still 56 (T1), 43 (T2) and 41 (T3) %. Similarly, the mobility response did not differ between the treatments until day 17, after which the organisms in T1 and T3 reacted with an increased immobility of 23 %, whereas mobility in T2 stabilized. For this species recovery was frequently found within 24 h. An additional surprising observation was that mobility and survival responses did not differ when compared to each other.

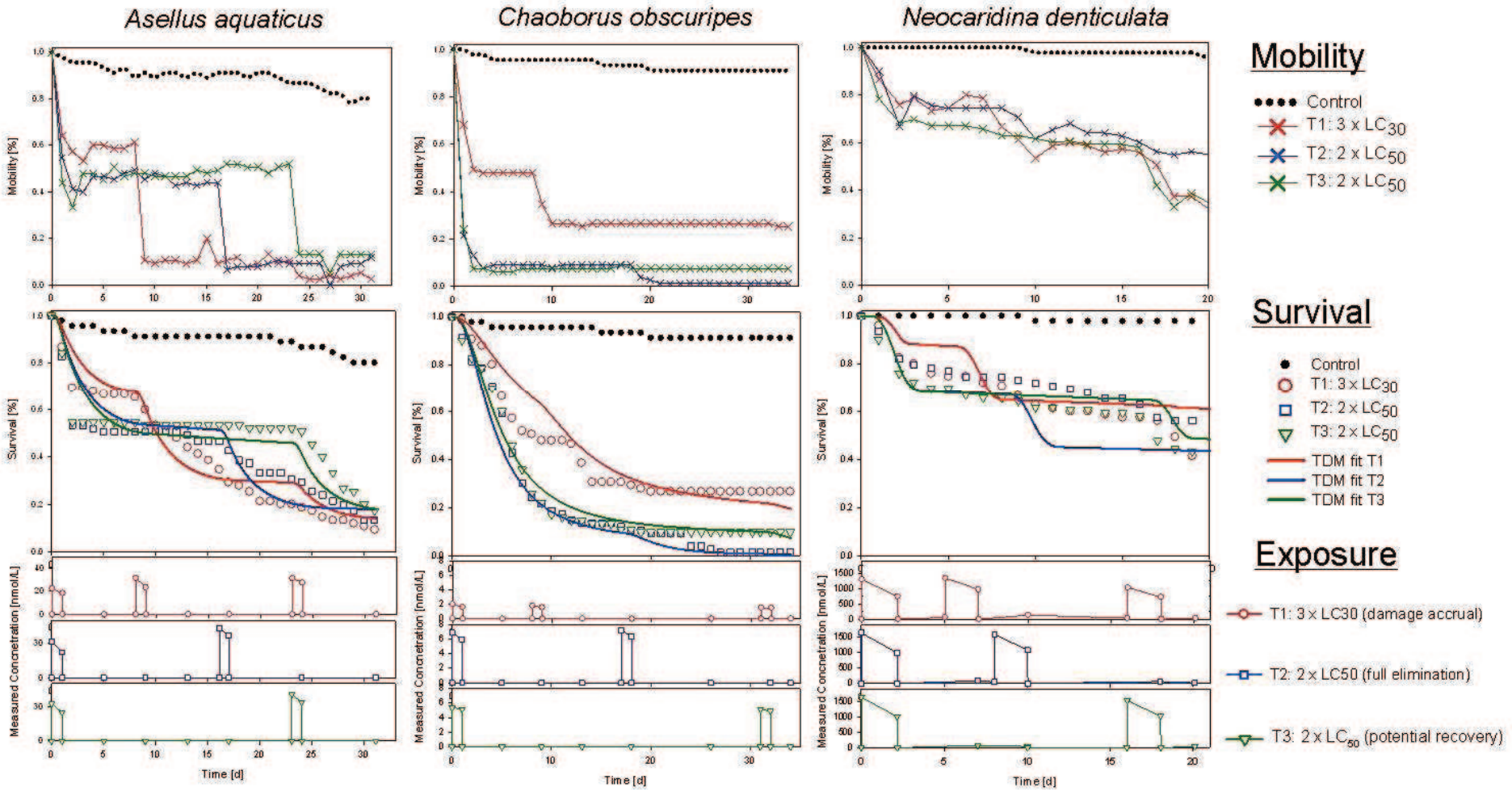

Figure 1: Experimental survival and mortality of three freshwater arthropods to pulsed exposure of chlorpyrifos. Upper panels display the fraction of mobile test animals in time, panels in the middle display the fraction of survivors in time (symbols) and the Threshold Damage Model fit using the final parameter estimates (lines, averages from test of robustness used as starting values). The three lower panels illustrate the pulsed exposure designs T1, T2 and T3 on basis of measured concentrations in the test media. All presented data represent averages of 3 (controls) and 5 (treatments) experimental replicates, error bars were omitted here for clarity. In total 75 test animals were used per treatment.

Modeling

The TDM could be fitted quite well to the experimental data sets for the three investigated species using the OM implementation of the model (Figure 1, middle panel and Table 3). The fits in Figure 1 were generated with parameter values, which are listed in Table 3 together with their goodness of fit measures. Killing rate constants ranged from 0.088 to 7.072 $mL \cdot g_{ww}^{-1} \cdot d^{-1}$ with *C. obscuripes* showing the lowest k_k and *A. aquaticus* the highest k_k. The recovery rate constants k_r ranged from 0.518 to 500 d^{-1} (due to user constraint) with *C. obscuripes* showing the slowest recovery rate and *A. aquaticus* and *N. denticulata* the fastest recovery rates, both reaching the limits of the user constraint. The threshold parameter varies from $3.3 \cdot 10^{-8}$ to 0.362 with *C. obscuripes* having the lowest threshold and *N. denticulata* the highest threshold. The goodness of fit measures indicated a good quality of fit for *A. aquaticus* and *C. obscuripes* and to a lesser extent for *N. denticulata*. The parameter values in Table 3 represent TDM estimations, which employed the averages for parameter values derived from the test for robustness (Figure 2, Table 2).

Table 2: Robustness of parameter fitting with the LMA algorithm in the OM implementation. (*n*) = number of valid estimations, their minimum, maxium, average and standard deviation are given.

		Parameter				Goodness of fit			
Species		k_k [$g_{ww} \cdot nmol^{-1} \cdot g^{-1}$]	k_r [d^{-1}]	threshold [-]	h_b [-]	Chi^2	R^2	min RSS	Mean% error
Asellus aquaticus (16)	Min	0.146	13.01	0.022	0.006	0.560	0.937	0.124	12.81
	Max	56.4	3931	0.105	0.006	0.603	0.945	0.537	14.45
	Average	6.33	455	0.049	0.006	0.569	0.941	0.483	13.50
	Stdev	14.1	1008	0.017	1.4 ·10-5	0.014	0.002	0.096	0.33
Chaoborus obscuripes (16)	Min	0.075	0.424	2.2 ·10-8	0.0035	0.331	0.978	0.309	15.42
	Max	0.149	0.923	2.2 ·10-4	0.0037	0.414	0.983	0.385	16.58
	Average	0.088	0.539	3.3 ·10-5	0.0036	0.374	0.980	0.337	16.01
	Stdev	0.017	0.109	5.6 ·10-5	6.3 *10-5	0.034	0.002	0.021	0.40
Neocaridina denticulata (17)	Min	0.00013	1.52	2.2 ·10-7	0.0018	0.427	0.842	0.411	8.82
	Max	10.5	122690	0.016	0.0037	0.496	0.865	0.487	9.66
	Average	0.841	9856	9.5 ·10-4	0.0028	0.435	0.861	0.430	9.00
	Stdev	2.52	29594	3.8 10-3	0.0004	0.017	0.007	0.024	0.24

Table 3: Threshold Damage Model parameters for four freshwater arthropods.

		Species			
	Parameter [unit]	*Gammarus pulex*	*Asellus aquaticus*	*Chaoborus obscuripes*	*Neocaridina denticulata*
Toxicokinetics [1]					
uptake rate constant	k_{in} [$mL \cdot g_{ww}^{-1} \cdot d^{-1}$]	812	596	318	617
elimination rate constant	k_{out} [d^{-1}]	0.398	0.185	0.131	0.478
Toxicodynamics		*(Ashauer et al. 2007c)*[a]	*this study*[a]	*this study*[a]	*this study*[a]
killing rate constant	k_k [$g_{ww} \cdot nmol^{-1} \cdot d^{-1}$]	0.047	7.072 *	0.088	0.269 *
recovery rate constant	k_r [d^{-1}]	0.169	> 493.5 *	0.518	> 496 *
threshold	thr [-]	0.022	0.048	$3.3 \cdot 10^{-8}$ *	0.32 *
background mortality	h_b [-]	0.01	0.006	0.004	0.004
R^2		NA	0.942	0.984	0.738
Mean%error		NA	13.43	15.41	11.91

NA = not available.
* lack of robustness (see text and Figure 2)
[1] Toxicokinetic parameters from Rubach *et al.* (in press b), for *Gammarus pulex* raw data from Ashauer *et al.* (2006 b) used.
[2] Toxicodynamic parameters for *Gammarus pulex* from Ashauer *et al.* (2007 c)

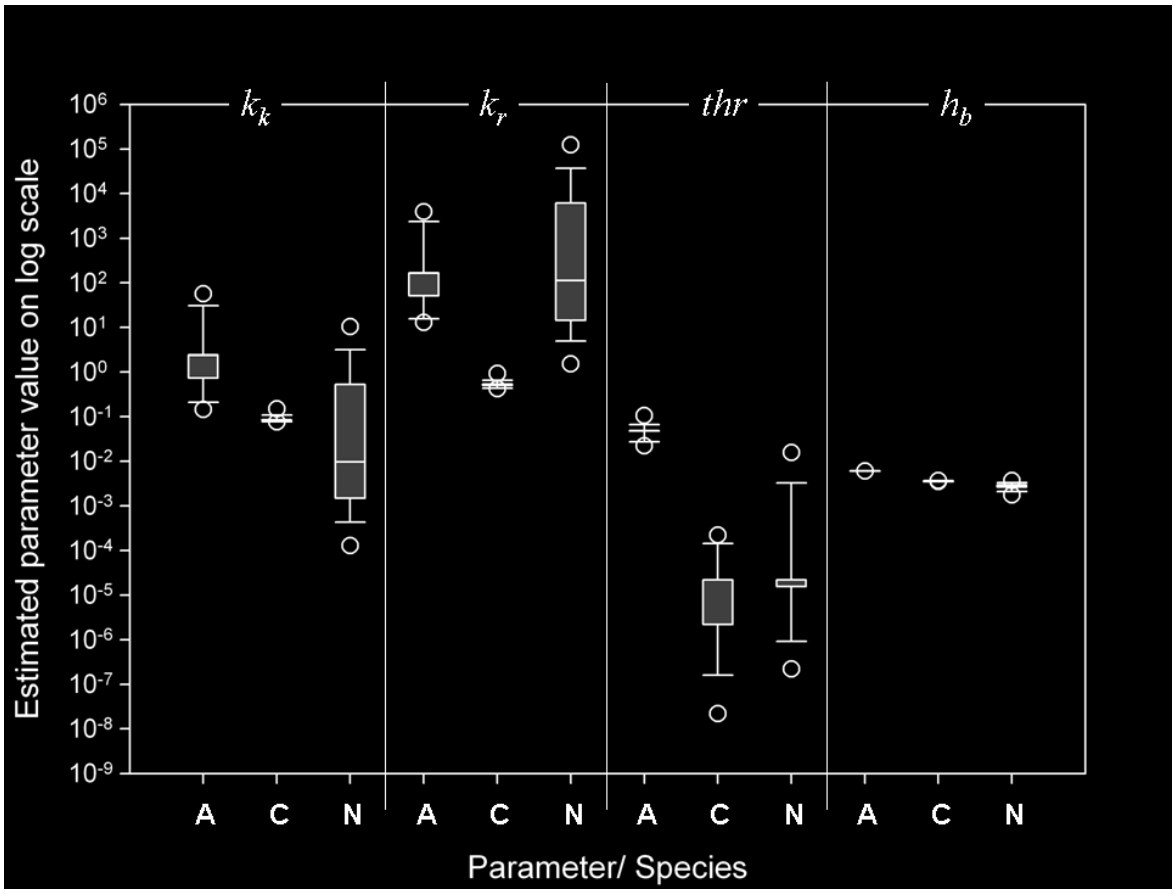

Figure 2: Robustness of toxicodynamic parameter estimates for *Asellus aquaticus* (A), *Chaoborus obscuripes* (C) and *Neocaridina denticulata* (N) using the Threshold Damage Model. Selected estimated parameter values are displayed (n = 16 for A,C and n = 17 for N out of 56) as box plots with outliers on log scale for the killing rate constant (k_k), the recovery rate constant (k_r) and the threshold (*thr*) and background death rate (h_b). Selection criteria were comparable goodness of fits.

Figure 2 illustrates the variability of the estimated parameters depending on the choice of starting values, while the plotted population of parameter estimates represents comparatively good fits. The background death rate h_b was estimated for all three species robustly, whereas the robustness of the other three parameters varied by species and parameter. For *C. obscuripes* both k_k and k_r were estimated very robustly, but the threshold parameter showed high variability, however only unidirectional approaching zero. In contrast, for *A. aquaticus* the threshold parameter could be estimated with confidence, whereas k_k and k_r showed a lack of robustness with a variability of three orders of magnitude. The estimations for *N. denticulata* did not show any robustness, in fact the observed variability was in the range of five orders of magnitude.

DISCUSSION

Experimental exposure and effects

Exposure

The interpretation of the presented effects data can only be done meaningfully by integrating the measured exposure in the different experiments. Intended and achieved concentrations were compared. The intended concentrations were achieved in the range of 83-93 % (lowest: *A. aquatiucs* < *C. obscuripes* < *N. denticulata* highest). There seems to be species or experiment specific differences. These differences can either be related to differences in applied concentrations or adsorption to and uptake into the test animals, and possibly differences in biotransformation rate. Before the transfer of the test animals into clean water, the percentage of remaining concentration in the test media was very similar for the three species. In the test systems for *C. obscuripes*, the least dissipation was measured, while the test systems for *A. aquaticus* (measured and transferred after 24h exposure) and *N. denticulata* (measured and transferred after 48 h) showed very similar dissipation. The differences in dissipation measured in the present study may be related to differences in uptake rate constants, which are similar for *A. aquaticus* and *N. denticulata* and somewhat lower for *C. obscuripes* (Table 3) (see Rubach *et al.* in press b). In general the dissipation found in this study was less than previously measured dissipation rates in the field, which are concentration and treatment dependent (50 % dissipation times (DT_{50}) between 3.1 and 18.4 d were found by (López-Mancisidor *et al.* 2008). In order to fulfil the objectives of this study it is not only essential to compare the intended and measured exposure concentrations, but also to fully characterize the pulsed exposure pattern using measured data (Fig. 1). The first pulses per treatment in the experiment with *A. aquaticus* consistently had a 10 nmol/L lower amplitude than the respective subsequent pulses, for which the reason remains unclear. In contrast, the intended concentrations of the second and third pulses (34.2 nmol/L for the LC_{30} in T1 and 42.8 nmol/L for the LC_{50} in T2 and T3) are exactly matched by the measured concentrations (Fig. 1). This however was not observed in the experiments with the two other species, only the third T1 pulse in the experiment with *N. denticulata* was slightly lower than the previous pulses. Also, for the latter species increasing chlorpyrifos concentrations were detected during non-exposure periods between the pulses, ranging from 4.0 – 132.6 nmol/L. This is likely related to the high exposure concentrations themselves in combination with the high uptake and high elimination rate constants previously measured for this species (Table 3, see Rubach *et al.* in press b). The large amount of chlorpyrifos taken up by the test animals in the exposure phase was transferred into the clean media together with the test animal where it eliminates the parent compound as detected by GC-ECD. These measured external exposure concentrations are factored into the interpretation of the effects on survival and mobility below.

Effects

The three investigated freshwater arthropod species reacted with surprisingly different survival and mobility patterns to the pulsed exposure designs. For *A. aquaticus* no LC_{50} was available at 24 h and therefore the intended exposure was extrapolated using combined knowledge from previous studies (see above). Although the observed survival of this species matched the intended effect, the

measured exposure revealed that the first peaks in all treatments were of a 20 – 36 % lower amplitude than the subsequent peaks, the latter of which actually matched the intended concentration. This explains the enhanced effects on survival and mobility for the second T1 peak, which was however not observed for the second T2 and T3 peaks, which induced another 40 % effect. Potentially, this can be interpreted as the ability of *A. aquaticus* to detoxify chlorpyrifos and all related toxic metabolites and/or repair from induced damage within the time needed to eliminate 95 % of the bioconcentrated chlorpyrifos. Hence, from these results an experimental estimate of a 24 h LC_{50} can be derived and is expected to be approximately 31 nmol/L (~ 11 µg/L). Although the verification of actual exposure might explain the slightly enhanced effects, this cannot explain the delayed mortality observed for this species in response to the second pulses in all treatments. Delayed (or latent) effects have been observed before, e.g. for *D. magna* depending on the intervals given for recovery (Van Der Hoeven & Gerritsen 1997; Naddy & Klaine 2001), but also for other chemicals and species (Beketov & Liess 2008a). In contrast to survival, the effect on mobility was not delayed for any of the pulses. This comparison and the fact that the delay was reduced when the recovery interval between pulses increased suggests that these delayed effects may be related to either effective biotransformation or recovery and repair abilities in this species. The use of a toxicokinetic parameter set derived from experiments without characterization of metabolites (Rubach *et al.* in press b) does not allow a clear distinction between these processes, based on model parameters. However it is more likely that this delay in effects is related to mitigation from compensatory abilities, because the non-toxic metabolites of chlorpyrifos are likely to be eliminated due to low partition coefficients (log P) and have therefore likely been captured by the radiochemical measurements (Rubach *et al.* in press b). This in turn leads to the hypothesis that *A. aquaticus*, with relatively slow elimination abilities, has a well developed compensatory physiology (in terms of recovery and repair abilities), resulting in rapid organism recovery which was also observed in this experiment. Interestingly, organism recovery from immobilisation was observed after the first peak in all treatments and not so frequent after the subsequent pulses. Also, these first peaks induced immediate instead of delayed mortality in contrast to the subsequent pulses. This supports the individual effective dose theory (Gaddum 1953) with the underlying hypothesis that individuals have a specific tolerance towards chemical exposure. For the second pulses, however, immobility was immediate, whereas mortality was delayed, which suggests that stochastic principles govern this pattern, potentially related to the organisms physiological compensation abilities. The dilemma between the concepts of individual tolerance and stochastic mortality has been discussed before and (Zhao & Newman 2007) also concluded as well that both individual tolerances and stochasticity are important for survival.

The effects of chlorpyrifos on the survival of the larvae of *C. obscuripes* were also delayed and enhanced (i.e. higher percentage of immobility and mortality than intended) after the first pulses in all treatments, however the concentrations measured in the test media were close to the intended LC_{30} and LC_{50}. The enhanced immobility was induced immediately, leading to a proportional enhanced mortality after 9 and 19 d for T1 and T2/T3, respectively. This can be explained by a combination of factors. First, the sensitivity of *C. obscuripes* depends very much upon the endpoint chosen to describe it, i.e. their 24 h LC_{50} and 24 h EC_{50} differ. This gap in endpoint dependent sensitivity, which leads to both the delay and enhancement of effects, can be explained by the larvae being equipped

with hydrostatic vesicles (swim bladders). These vesicles are used to suspend the organism in the water column, while lurking for prey. A low sub-lethal dose, which does not yet itself affect the mobility of the organism (twitching, reflexes and movement are still possible) may negatively effect the control of these hydrostatic vesicles via the central nervous system (due to the mode of action of chlorpyrifos acting on nerve transmission) and therefore inhibit the suspension in the water column. As a result the animals surface and begin to float. This in turn reduces further uptake, but the float is not reversible and the organism finally dies. This also explains why no recovery was observed for *C. obscuripes*. Another explanation for the enhanced effect on mobility and survival could be a seasonal difference in sensitivity as has been hypothesized and observed before, also for other stressors (Winner *et al.* 1990; Pérez-Landa *et al.* 2008), but this does not necessarily directly explain the observed latency in effects. Surprisingly the second pulses did not have enhanced effects on the experimental groups. In fact, in T1 the exact intended effect was caused by the dose with a somewhat shorter delay and in T2 and T3 the doses showed less than the expected effect. This again supports the hypothesis of individual tolerance distributions for both endpoints. An effect which is less than intended and exhibits a reduced latency indicates, firstly, that the survivors have a higher tolerance than the previously eliminated specimen and, secondly, that the survivors possess a better physiology to detoxify the toxicant and compensate the induced stress. Unfortunately, the number of remaining organisms in T2 and T3 was not sufficient to confirm this observation.

The survival and mobility pattern observed for *N. denticulata* did not show any differences in response, neither to the different concentrations and pulsed designs, nor between endpoints. Furthermore the effects were less than intended, despite verification of intended exposure concentrations. This result is remarkable, because it suggests that this species does not respond at all to the specific mode of action of acetylcholinesterase inhibition, although crustaceans and especially atyid shrimps are known to be highly sensitive to organophosphates (Rubach *et al.* 2010). The 50 % internal lethal concentration (ILC_{50}) of 4.06 mmol/kg_{ww} derived from the LC_{50} (24 h) value of 1103 µg/L for this species (Rubach *et al.* in press c) together with their BCF of 1291 L/kg_{ww} (Rubach *et al.* in press b) confirm that effects are probably caused by narcotic action, because in the 2 - 8 mmol/kg_{ww} range of ILC_{50}s, baseline toxicity can be expected for narcotics (Escher & Hermens 2002). Again, the insensitivity of this species towards chlorpyrifos might be related to highly efficient biotransformation (and therefore detoxification), well developed compensatory physiology and/or an incompatible stereotypic constitution of the (acetyl)cholinesterases in this species. The high elimination rate constant determined by (Rubach *et al.* in press b) hints at sophisticated detoxification abilities. This, however, can still not explain the lack of differences in responses to the treatments, doses and endpoints, because the experiments were designed using the time the animal required to eliminate 95% of the bioconcentrated chlorpyrifos. Therefore it seems most likely that this species also has a good compensatory physiology (i.e. a highly efficient stress response to acetylcholinesterase inhibitors and/or narcotics), which is supported by the frequently seen recovery in the present study. Survival hardly stabilized at background mortality during the experiments, but in fact slowly, but constantly decreased (with the exception of T2, which potentially stabilized at the end of the experiment). Questions however remain, because normally narcotic compounds are not known to cause latent effects. Nontheles, this survival pattern might still be interpreted as delayed effects, related to internal

damage caused by e.g. oxidative stress and disrupted membranes in response to chlorpyrifos acting as a baseline toxicant or toxicant with a specific mode of action in this species.

Modeling

Next to understanding the differences in basic survival response patterns of different freshwater arthropod species to pulsed chlorpyrifos exposure, these experiments were also carried out in order to provide further calibration data sets for the TDM for additional species. The latter enables us to compare species on basis of their TKTD parameters with respect to their experimentally determined sensitivity. The first step to fit the TDM to the experimental data sets discussed above was in general successful for all three species (Fig.1). However the provided fits lacked robustness for some parameters and species (Fig 2). Therefore a systematic approach was chosen to constrict the range of possible parameter values for each species (results of this are shown in Table 3). The lack of robustness is displayed for each parameter and species (Fig 2) thereby indicating their uncertainty. The estimated parameter values and their robustness are discussed below with regard to the nature of the underlying experimental data, the TDM itself and the estimation methodology.

In all fitting procedures, the parameter estimation for the background death rate h_b was very robust. The threshold parameter however could only be determined robustly for one species, i.e. *A. aquaticus*. In the TDM the threshold parameter is dimensionless and can therefore not be related to any process directly, as well as the damage and the hazard itself, both of which are also dimensionless. However, when comparing the threshold parameters between species, *C. obscuripes* has a consistently low threshold (frail estimations occure only in the direction of smaller values approzimating zero), whereas for *N. denticulata* such an observation was not made. A modification of the TDM in which damage, hazard and threshold would be related to measureable processes or units might solve this lack of robustness in fitting. It is however not clear if such parameters would be equally difficult to identify, while at the same time a reparameterisation would also imply a loss of model generality and herewith be unsuitable for a comparative approach. The problems encountered when estimating the threshold parameter also touch upon the theoretical problem of individual tolerance distributions versus stochastic death. As described above in the experimental section, both processes are likely to occur in reality. For instance the first pulse eliminates individuals with a lower tolerance from the population and the survivors mortality after the second pulse then follows stochastic death. The TDM, however, as a pure hazard model assumes stochastic death (Ashauer 2010), potentially leading to the misfit for the first half of the dataset and herewith also to parameter estimation problems. How this relates to the uncertainly in estimations for the threshold parameter needs to be investigated in future research. The situation was similar for the killing rate constant k_k, which could only be determined robustly for *C. obscuripes*, but not for the other two species. This parameter however is less indistinct than the threshold parameter, because it is embedded in the damage term of the model and has a unit. The recovery rate constant k_r is the parameter which can be related to its respective process the best. It is also, like k_k embedded in the damage term and has inverse time as a unit and is therefore a direct rate. This is the reason why fitting was finally performed under a user defined constraint for this parameter, while the other parameters were not constrained. This user constraint for k_r might be unrealistic since enzymes may react much faster and in theory recovery and repair may be

completed within seconds or minutes on molecular level, while the recovery of an immobile organism may take days to be observed and the influence of recovery and repair abilities on survival may be represented through delays in the lethal response. A linear regression of k_k and k_r for each species shows that for *A. aquaticus* (R^2 = 0.995, n = 16) and *N. denticualta* (R^2 = 1, n = 17) these parameters are highly correlated and less for *C. obscuripes* (R^2 = 0.508 when one outlier causing a high leverage was excluded n = 15, versus R^2 = 0.944 when the outlier was included, n = 16). This also supports the need for a reparameterisation of the TDM in order to improve robustness of estimations, because the ratio k_k/k_r seems constant for two of the three species and therefore single parameters might not be informative here. Other reasons for the labile parameter estimation might be found, e.g. in the choice of the minimization function (least squares), the choice of fitting algorithm (Martin *et al.*), the biological variability in the experimental data and/or the design of the experimental set up. Especially the latter might be important, because the different treatment survival curves crosses each other at some point (*A. aquaticus*) or many points (*N. denticulata*) in time, although the driving variable, i.e. the water concentrations, are different at these times. This could be a possible cause for the multiple minima (lack in robustness).

However, the LMA, as a standard technique for non-linear curve fitting is considered as an algorithm that is well suited to finding the global minimum, because it balances the use of the steepest descent (when the current solution is far from the measured value) with the Gauss-Newton method (when the solution is close to the measured value). Nevertheless, other fitting methods and minimizing functions might reduce the encountered estimation problems and its uncertainty due to different numerical solutions and have to be applied and evaluated with these datasets in the future.

We have also tried to fit the TDM to the mobility data, which was not successful (data not shown). This was expected, because the TDM is a hazard model, explicitly addressing mortality, a non-reversible process opposite to immobility, which may be reversible. Furthermore the mathematical formulation of the current TDM is not compatible for incorporation of immobility as a process without adding two to three parameters. Doing this would be counterproductive since the estimation problems described above already show that the model is over-parameterized given the amount of calibration data available from the described experiments.

Species comparison on basis of toxicodynamic parameters

The three freshwater arthropod species tested in the present study showed very different effect responses to the same principal experimental exposure design. By employing a TKTD modeling approach, differences in species were able to be accounted for. In order to compare the species in their toxicodynamic characteristics, the systematically estimated toxicodynamic parameters (Table 3) will be considered. This needs to be done with caution with respect to the fitting difficulties and their lack of robustness described above, since for interspecies comparisons, parameters are required that are not only biologically meaningful, but also estimated with the same modeling methodology and absolute robustness. The observation that chlorpyrifos exhibits mostly narcotic action in *N. denticulata* is coherent with the observation of its extremely low sensitivity (Rubach *et al.* in press c). Therefore the estimated parameters for this species also mostly relate to the baseline toxicity of chlorpyrifos in this species, but all parameter estimates lack robustness. The only parameter estimate with some

relevance for interpretation is the high recovery rate of > 496 d^{-1}. This estimate indicates good detoxification and/or compensatory abilities, although it was estimated under constraints and is, therefore, probably much higher. The high *threshold* value for the shrimp is also related to the baseline toxicity of chlorpyrifos and could represent a realistic value. The killing rate constant k_k is, however, surprisingly high and might be related to the high correlation between k_k and k_r. The same accounts for *A. aquaticus*, which shows the highest k_k, a high k_r (which are both correlated and lack robustness) and a relatively high *threshold*. The high recovery rate, together with the rather high threshold, could finally explain its relatively low sensitivity (Rubach *et al.* in press c), because the TK parameters resulting in a high bioconcentration as described in (Rubach *et al.* in press b) did not explain this very well.

For *C. obscruripes* the very low (but not robustly estimated) threshold, together with a very low k_r compared to the two other species in this study, reflects that this species is the most sensitive species tested in this group. However, the k_k is much lower than the rate constants found for the two other species, but the latter are both not robust and highly correlated with the species k_r and therefore less reliable. If the TD parameters for *C. obscrupires* are compared with a TDM parameter set for *Gammarus pulex* (Ashauer *et al.* 2007c) (Table 3), which is more sensitive to chlorpyrifos (Rubach *et al.* in press c), k_r and k_k are both higher in *C. obscrupies* than in *G. pulex*. It is possible to speculate that the higher k_r in *C. obscuripes* compensates for the higher k_k but does not do so in *G. pulex*. To our knowledge this is the first time an attempt has been made to compare estimated toxicodynamic parameters quantitatively across species in order to explain differences in their sensitivity and correlate these to their physiological abilities. This comparison of species with each other and with previous similar estimations illustrates that we are currently not able to fully explain differences in sensitivity using these parameters, however the approach has been useful to generate additional hypotheses which can be tested in future research.

The problems described above illustrate the need for an in-depth evaluation and robustness testing of TKTD models such as the TDM for different species and chemicals. They also clearly indicate that a unification of models and underlying concepts and assumptions is needed to provide a model which can account for the apparent differences and variety in species responses. At the 1st Eawag toxicokinetic-toxicodynamic (TKTD) modelling workshop in Kastienbaum, Switzerland in May 2010 this issue was discussed (workshop summary available at http://www.eawag.ch/about/personen/homepages/ashauero/TKTD_workshop_summary). Despite certain drawbacks, this study illustrates the usefulness of such models and types of experiments for future ERA as has been suggested previously (Reinert *et al.* 2002; Jager *et al.* 2006; Baas *et al.* 2010). Additionally, TKTD approaches might finally allow comparisons of toxicity across different species quantitatively and thereby contribute to better understanding of the factors that contribute to sensitivity differences. Improving such models is an essential step towards a more predictive approach, moving ecotoxicology from a phenomenological discipline ('dose it and see'), to a more mechanistic and predictive science. Further contributions to developing our understanding of sensitivity differences is also likely to arise from a better understanding of basic biological differences between species such as their morphological, physiological and ecological characteristics (Rubach *et al.* in press a).

Relevance for Environmental Risk Assessment

The survival experiments presented here with pulsed exposure designs were resource intensive. Investing such efforts needs to be justified and (Reinert *et al.* 2002) have listed decision criteria for situations and scenarios when assessment of effects of pulsed exposures should be considered. Therefore, there is a need to discuss the increase in knowledge they achieve, their applicability and potential difficulties with methodologies. The presented experimental methodology proved to be suitable for the different, non cannibalistic freshwater arthropods with different respiratory traits and food requirements. The intermittent food and aeration regime enabled us to characterize mortality caused by bioconcentration of chlorpyrifos in these test organisms. Other suitable designs, which include both uptake via the food and from the surrounding media are also possible, but models will need to be adapted and the toxicokinetics will need to be parameterized for these routes as well. In all experiments major insights originated from the first two pulses, while the third pulse applied in T1 did not really result in gained knowledge. For future experimental designs, two pulses with different intervals would probably suffice. This would also allow to apply an LC_{50} instead of an LC_{30} in all treatments and improve the comparability.

The difference in intended and observed effects for the species *C. obscuripes* indicate that several problems related to the choice of the intended dose exist. In order to design these type of experiments, prior experimental knowledge on time dependent toxicity of the compound in the species of interest and its elimination kinetics is necessary. The usual uncertainty associated with experimental limitations, seasonal and spatial variability of test populations and unexpected specific conditions or characteristics of species add a priori uncertainty to the experimental outcome as was seen here. Nevertheless, when performed with care and including experimentally verified exposures, these types of experiments enhance our understanding of toxicity and are therefore useful for the further development of effect models, such as TKTD models and their linkage to organism characteristics. The latter is especially important, because performing such types of experiments can serve as a filter to identify morphological, physiological and even ecological traits (seasonal variability) which relate to sensitivity, e.g. having a hydrostatic vesicle as demonstrated for *C. obscuripes*. The investigation of sublethal endpoints, such as immobility proved to be very informative and actually pivotal. For the mode of action of acetylcholinesterase inhibition, immobility of an organism proves that the chlorpyrifos has reached and acted on the target enzyme, whereas mortality was in this case less informative since it reflects also secondary damage and only occurs with a long species-specific delay. Because both survival and mobility were simultaneously observed, this experiment revealed that chlorpyrifos does not appear to act via acetylcholinesterase inhibition in *N. denticulata*. In order to investigate this species in the future mechanisms of resistance in pest insects can be used as a starting point. The delay in effects also illustrates the importance of selecting the relevant endpoint for an ERA with regard to the mode of action of the assessed chemical (Rubach *et al.* in press c). As an additional sublethal endpoint, future experiments could also record the feeding success after the pulses throughout the experiment, once transferred into clean water. This would allow the characterization of the post-exposure-feeding inhibition as an endpoint which is likely to extrapolate well to the population level, since an organism that cannot feed will unlikely be able to reproduce.

The rationale for the application of several pulses with variable duration in toxicity experiments can be enlightening from several perspectives. As previously demonstrated, pulsed designs with at least two pulses can reveal sequence-dependent damage accrual, also for different chemicals of the same mode of action (Ashauer *et al.* 2007c, a, b). As demonstrated here with *A. aquaticus* and *C. obscuripes,* pulsed designs can collect evidence for both the different concepts of individual tolerance distributions and stochastic mortality and thereby emphasize the need for a unified model approach.

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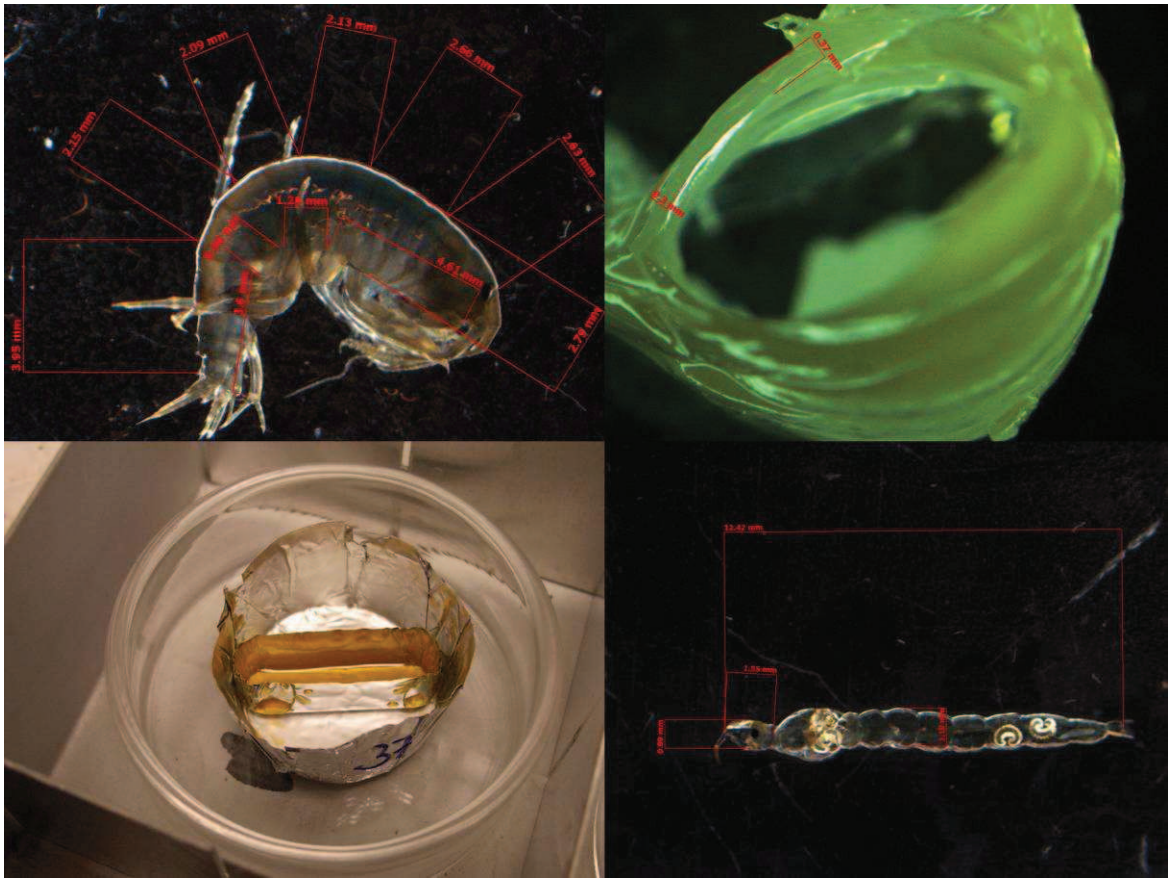

Trait measurements (size related traits, exoskeleton thickness, lipid content), 2009

7. CHAPTER

Traits as predictors for intrinsic sensitivity – a synthesis and discussion.

ABSTRACT

Ecological risk assessment (ERA) has developed on the basis of a taxonomy-based approach, making the assumption that related species will show similar sensitivity to toxicants, and using safety factors or species sensitivity distributions to extrapolate from tested to untested species. In ecology it has become apparent that taxonomic approaches may have limitations for the description and understanding of species assemblages in nature. Therefore it has been proposed that the inclusion of species traits in ERA could provide a useful and alternative description of the systems under investigation. At the same time, there is a growing recognition that the use of mechanistic approaches in ERA, including conceptual and quantitative models, may improve predictive and extrapolative power. Purposefully linking traits with mechanistic effect models could add value to taxonomy-based ERA by improving our understanding of how structural and functional system facets may facilitate inter-species extrapolation. Here, we explore whether and in what ways traits can be linked purposefully to mechanistic effect models to predict intrinsic sensitivity using the available data on the sensitivity, toxicokinetics and toxicodynamics of a range of freshwater arthropods exposed to chlorpyrifos. The results of a quantitative linking of seven sensitivity endpoints and traits demonstrate that whilst it is possible to establish meaningful quantitative links between traits and/or trait combinations and process based (toxicokinetic) model parameters, predicting classical sensitivity endpoints is more elusive. Future research needs to include a quantitative linking of toxicodynamic parameter estimations and physiological traits, and requires further consideration of how mechanistic trait-process/parameter links can be used for prediction of intrinsic sensitivity across species for different substances in ERA.

OVERVIEW CHAPTER 7

INTRODUCTION

Traits have been used in ecology for almost 100 years and have increased the understanding and analysis of species assemblages in relation to their habitat (Thienemann 1918; Southwood 1977; Statzner & Higler 1986; Townsend & Hildrew 1994; Poff *et al.* 2006). This has led to the systematic application of traits in biomonitoring and retrospective environmental and conservation risk assessment (Usseglio-Polatera *et al.* 2000b; Gayraud *et al.* 2003; Bonada *et al.* 2006; Barnett *et al.* 2007; Beketov & Liess 2008b; Horrigan & Baird 2008; Mattila *et al.* 2008). In ecotoxicology, traits have also been used in some quantitative structure activity relationship (QSAR) models (Escher & Hermens 2002), the SPEAR index (Liess & Von Der Ohe 2005), in corrections of certain risk indicators for specific traits (e.g. lipid correction of bioconcentration factors), effect analysis and modeling using life table and life history traits (Arnot & Gobas 2004; Preuss *et al.* 2009a) and in test battery optimization (Ducrot *et al.* 2005). However, since rarely more than one or two traits have been incorporated in these approaches, and the acquisition and use of traits have been made somewhat unsystematically, the potential of traits for prospective environmental risk assessment has not really yet been fully explored (Baird *et al.* 2008). Prospective risk assessment, which is based on probabilistic assumptions and empirical data linked to mechanistic knowledge, could benefit from traits-based approaches, particularly when combined with predictive models (Rubach *et al.* in press a, Chapter 2). The application of effect models for ecotoxicology and ecological risk assessment (ERA), including toxicokinetic (TK) - toxicodynamic (TD) models and individual based (population) models (IBMs), has been and is being explored in various publications, (e.g. Barnthouse 1992; Pastorok *et al.* 2003; Van Den Brink *et al.* 2007; Galic *et al.* 2010; Schmolke *et al.* 2010), workshops (Forbes *et al.* 2009; Brock *et al.* 2010; Van den Brink *et al.* in press a) advisory groups (Preuss *et al.* 2009b) and mostly recently in the EU project 'Mechanistic Effect Models for Ecological Risk Assessment of Chemicals' - CREAM (Grimm *et al.* 2009). The combined potential of effect models for ERA and traits-based approaches could be significant, as it would offer a potential means to facilitate interspecies extrapolation, one of the major challenges facing the future refinement of ERA (Rubach *et al.* in press a, Chapter 2). The exploration of such an approach will be highly dependent on the establishment of mechanistic links between the relevant traits and processes and the availability of suitable data (Rubach *et al.* in press a, Chapter 2). In order to parameterize and test a traits-based effect model exploring the intrinsic sensitivity of freshwater arthropods to a model compound (chlorpyrifos), the previous chapters of this thesis were concerned with the collection of appropriate calibration data. Intrinsic sensitivity is the integrated organism-level result of several internal processes, i.e., uptake, biotransformation and elimination (summarized as TK) and damage/hazard, internal recovery and thresholds (summarized as TD) (Ashauer *et al.* 2007b). The aforementioned TKTD models link external exposure and survival effects by describing dynamically the processes of TK and TD. Data mining of existing toxicity and trait data in order to establish empirical links between traits and sensitivity led to the conclusion that existing data are not suitable for calibration of traits-based effect models and further experimental work on the processes of toxicity and also a more purposeful collection of trait data should be explored (Rubach *et al.* 2010, Chapter 2). Rubach *et al.* (in press c, Chapter 4) characterized the variation in

sensitivities of a set of freshwater arthropods to chlorpyrifos in form of 24 – 96 h L(E)C_X values (x % lethal or effective concentrations) and also addressed species differences in response dynamics at lethal and sub-lethal levels. Rubach *et al.* (in press b, Chapter 5) used this information to perform bioconcentration experiments under sub-lethal exposure to chlorpyrifos with the same test species in order to parameterize the processes of uptake and elimination (TK) by means of a one-compartment, first order kinetic model for a range of freshwater arthropod species, which varied in their sensitivity and trait composition. These two studies delivered a comprehensive dataset on classical and toxicokinetic (process based) sensitivity endpoints which are linked here to a collection of traits in order to explore the conceptual model proposed by Rubach *et al.* (in press a, Chapter 2) in more detail, to establish links between the toxicokinetic processes (uptake and elimination) and identify relevant traits that may be useful as predictors in a bioconcentration model. To achieve this end, we first populated a traits database composed of an *a priori* selection of traits. As a second step, we linked classical sensitivity (L(E)C_{50}) and toxicokinetic (uptake and elimination rate constants, bioconcentration factors) sensitivity endpoints to these traits by means of principal component analysis (PCA) and linear regression models. These quantitative links are discussed both from a trait and from a process based perspective. In Chapter 6 of this thesis, a TKTD model was parameterized (as far as possible) for a reduced set of species that had been used to generate the previous tested set. Due to uncertainty in model parameter estimation and a lack of relevant trait data links between toxicodynamic processes and traits could only be discussed qualitatively in terms of their predictive potential. Finally these results are used to outline future predictive traits-based research needs and to discuss the implications of traits-based approaches for environmental risk assessment in the context of the present thesis.

POPULATION OF TRAIT DATA

Criteria and strategy for trait selection

Empirical traits-based approaches face the significant scientific challenge of requiring an *a priori* selection of traits to be included or excluded from an analysis. Traits definition and selection are both somewhat subjective processes, and the proliferation of different trait databases in the literature reflects the extent of this challenge. Trait selection appears to often mirror existing schools-of-thought and is frequently constrained by the need to make biological assumptions of varying degrees of certainty. At present, the lack of a clear framework for traits selection and measurement in ecotoxicology has meant that mechanistic hypotheses usually cannot be tested with traits approaches and can therefore never be completely verified. It is, however, still possible with these approaches to collect and incorporate empirical evidence and trends in the form of traits, adding knowledge and reducing uncertainty, as has been done similarly in the field of epidemiology. For the present study, in order to provide a rationale for the *a priori* selection of traits, we used the expertise of several scientists in the field of ecotoxicology, and thereby only including traits which fulfilled the following criteria:

(i) Traits for which mechanistic hypotheses linked to the processes of interest exist and/or previous empirical evidence for these has been found;

(ii) Traits which are possible to measure, and which could have a plausible relationship to sensitivity, although they might not be the best descriptor for these underlying physiological characteristics;

(iii) Traits, which fulfill (i) and (ii) and additionally relate to the experimental design of the study.

A priori trait selection was made in a series of brainstorming and re-evaluation sessions among four experts in the field. Additionally, the information and hypotheses gained in previous studies about traits and sensitivity, especially the studies of Baird & Van den Brink (2007) and Rubach *et al.* (2010, Chapter 3) were considered in making the selection. Following this approach, traits were evaluated in terms of our ability to quantify them using existing literature and databases or by direct measurement from the same populations and/or sub-samples of the specimen used in the previous experimental chapters of this thesis. The selected traits and their modalities are listed in Table 1, including their abbreviated names, their coding type and the literature sources used for their quantification. Some traits which were originally intended to be included and quantified were not successfully addressed in this study due to an absence of information at the required level of detail in the literature. These traits are also briefly discussed below. In the following sections, the traits used and their modalities are introduced. *Trait modalities* will be printed in italics and/or represented by their abbreviated names.

Table 1: Populated trait(s)/ groups, their quantifications/ modalities, short-form names and origin.

Short-form names	Trait (group)	Quantification/ Modality	Unit	Type of variable	Reference
Biovol	Size related	Biovolume	mm^3	metric	this study
SurfArea		Surface area (without gills)	mm^2	metric	this study
AVratio		Surface area/ volume ratio	mm^{-1}	metric	this study
Length		Body length	mm	metric	this study
DryMass		Dry mass	mg/ individual	metric	this study
WatCont	Water content	Water content	%	metric	this study/ (Rubach *et al.* in press b)
ExoTh	Exoskeleton thickness	Exoskeleton thickness	mm	metric	this study
LipFW	Lipid content	% lipid of wet weight	% wet weight	metric	(Rubach *et al.* in press b)
LipDW		% lipid of dry weight	% dry weight	metric	(Rubach *et al.* in press b)
LipTot		Total lipid content	mg/ individual	metric	(Rubach *et al.* in press b)
ResConf	Respiratory regulation	Conformer	-	binary	(Welch 1922; Wingfield 1939; Mill & Hughes 1966; Wichard 1978; Babula 1979; Steele & Steele 1991; Taylor & Taylor 1992; Maltby 1995; Ueno *et al.* 1997; Pirow *et al.* 1999b; Freire *et al.* 2008; Merritt *et al.* 2008)
ResInt		Intermediate	-	binary	
ResReg		Regulator	-	binary	
SOatm	Source of oxygen	Atmospheric oxygen	-	binary	
SOdiss		Dissolved oxygen	-	binary	
ResMocut	Mode of respiration [a]	Cutaneous	-	binary	
ResMosip		Siphon	-	binary	
ResMoCoG		Compressable gill	-	binary	
ResMoExG		External gills	-	binary	
ResMoInG		Internal gills	-	binary	
ResMoPig		Respiratory pigments	-	binary	
TroDetr	Trophic relation	Detritivore	-	binary	(Brown 1960; Williams 1962a, b; Hickin 1967; McShaffrey & McCafferty 1990; Jalihal *et al.* 1994; Schuh & Slater 1995; Gupta & Stewart 2000; Yee *et al.* 2004; Locklin *et al.* 2006; Merritt *et al.* 2008)
TroHerb		Herbivore	-	binary	
TroCarn		Carnivore	-	binary	
TroOmni		Omnivore	-	binary	
SclPoor	Degree of sclerotization	Poor (< 10%)	-	binary	this study; (Poff *et al.* 2006) (trait armouring); (Merritt *et al.* 2008)
SclGood		Good (10 - 90%)	-	binary	
SclComp		Complete (> 90 %, carapace)	-	binary	
BauBox	Bauplan	(Rectangular) box shapes	-	binary	this study
BauCyl		Cylindroid	-	binary	
BauSphe		Spheres and ellipsoids	-	binary	
BauCone		Cones and half cones	-	binary	
Ladult	Life stage	Adult	-	binary	this study
Llarny		Larva/ nymph	-	binary	
Ljuv		Juvenile	-	binary	
PhylRES [b]	Phylogeny	Rank species (lowest rank = oldest)	-	ordinal	this study, based on (Maddison & Maddison 1996)
PhylEQ [c]		Rank taxon (lowest rank = oldest)	-	ordinal	

[a] Species tested did not account for the modes of respiration 'plant breather' and 'incompressible gill', which are therefore not listed as modalities here.
[b] Based on phylogenetic tree of 'Tree of life web project' (Maddison & Maddison 1996), retrieved on 01.09.2009: counted nodes of lowest taxonomic resolution possible (family/ (sub)/(infra)-order) back to common ancestor for arthropods (see text)

Traits obtained from literature and databases

Traits that were successfully quantified using existing literature and databases were related to respiration (respiratory regulation (Res), mode of respiration (ResMo) and source of oxygen (SO)), trophic relation (Tro), degree of sclerotization (Scl) and phylogeny (Phy).

For respiration, the literature detailed in Table 1 was reviewed and all relevant information related to respiration was documented. According to Merritt *et al.* (2008), all aquatic insects, independent of the nature of their respiratory organs, are able to control their oxygen uptake to a certain extent by behavioral patterns such as undulations, swimming abilities, using natural water flow, beating of gills, leg contractions (push-ups), movements and regulating a rectal pump. Therefore, the species were categorized according to their regulatory abilities, following the classification by Merritt *et al.* (2008) and adding the knowledge gained from the literature and from direct observation in our previous experiments. A species was categorized as a *respiratory regulator* (ResReg), if it had good regulatory abilities (breathing only atmospheric oxygen or breathing dissolved oxygen, but possessing at least two methods to regulate), as an *intermediate respiratory regulator* (ResInt) if one strong method of regulation was found (e.g. rectal pump or being a good swimmer) and *respiratory conformer* (ResConf) when only one (weak) or no method was found for that species (e.g. swimming, but being a bad swimmer). The modalities for the trait 'mode of respiration' described below were also based on Merritt *et al.* (2008) and characterized at the species level with the literature listed in Table 1. *Cutaneous mode of respiration* (ResMocut) denotes that an animal breathes through the exoskeleton, carapace or a closed tracheal system with apneustic spiracles, and only by diffusion through the cuticle. This occurs in many species as a supplement to gill breathing and could be limited by exoskeleton or cuticular thickness as well as chemical properties (types/layers of oils and waxes) or fugacities (Mackay 2004), which also may change the permeability. We have assumed that cutaneous respiration occurs in all arthropods by default, and this trait would only be meaningful if the respiratory surface area and its permeability were measured precisely. As ResMocut was consequently the same for all species in our study, it was excluded from the quantitative analyses. *Respiration via a siphon* (ResMosip) describes respiration via a tube that connects the atmosphere with an oligopneustic spiracle (open tracheal system) and therefore denotes simple air breathers. Plant breathers pierce macrophytes with modified open tracheal spiracles to tap the plant's specialized air channels (occurs in syrphids, ephydrids, curculionids and some beetle larvae) As none of the investigated species fell into this category this trait was not listed. *Respiration via a compressible gill* (ResMoCoG) describes a temporary air storage carried along in form of a bubble or plastron (an air film created by hydrofuge hairs). Dissolved oxygen diffuses into this storage, but is consumed quickly and/or the storage is small in relation to the activity levels of the animal, requiring frequent surfacing to replenish atmospheric oxygen (open tracheal system, mostly with polypneustic spiracles). Similarly, an incompressible gill is also a plastron or a spiracle with which the animal can dive, but no reloading with atmospheric oxygen is needed. Hydrofuge hairs or other structures create a permanent air storage in the form of a gaseous film where dissolved oxygen constantly diffuses at the rate proportional to oxygen consumption (open tracheal systems with poly- or oligopneustic spiracles, common in some Coleoptera, Hemiptera and the lepidoteran family Pyraloidea and some families of Diptera). It was not clear whether the species

Parapoynx stratiotata, a pyraloid moth, makes respiratory use of a plastron (Thorpe 1950) in addition to functional tracheal external gills (Welch 1922). Therefore this trait modality was not included in the database. *Respiration through external gills* (ResMoExG) describes very thin-walled and highly tracheated appendices and outgrowths connected to apneustic spiracles (closed tracheal system). External gills significantly increase the permeable surface area available for diffusion through the cuticle. The functionality of external gills for oxygen uptake is a subject of great debate in many species and these features are generally assumed to be more important for ion exchange and the creation of currents to enhance respiration through the body surface area. *Respiration via internal gills* (ResMoInG) denotes functional gills situated in internal rectal or lateral branchial chambers. Some insect taxa (Notonectidae: Anisops and Buenoa, Chironomidae) and most crustaceans exhibit *respiratory pigments* (ResMoPig), such as haemoglobine and haemocyanine (Crustacea) in their haemolymphe, thereby increasing the oxygen transport. Maltby (1995) found that *Asellus aquaticus* is five times less sensitive to hypoxia than *Gammarus pulex,* and related this finding to differences in blood characteristics. In this study, all Crustaceans were categorized as using respiratory pigments because no detailed information was available on species level. More simply (but perhaps more meaningfully for bicconcentration than the previous categorizations), is the differentiation of species in relation to their use of *atmospheric or dissolved source of oxygen*. In order to categorize species into atmospheric (SOatm) and dissolved oxygen (SOdiss) breathers, the information collated above was used.

For quantification of feeding-related traits, no distinction was made between the various modes of feeding (e.g. scraping, collecting, filtering, piercing, engulfing), because all but one test species fell into the category of collectors. However, a distinction was made between preferred food types, here called the trophic relation. Of the species studied, 90% of the species were observed to ingest algae, biofilms and detritus attached to preferred food items (Merritt *et al.* 2008). This 'passive omnivory' is not used further here but categorizations are made on the basis of an organisms' active food selection: *detritivore* (TroDetr), *herbivore* (TroHerb), *carnivore* (TroCarn) and omnivore (TroOmni) *trophic relation*. To categorize the species, we used the literature reported in Table 1 and also our own experience from the experimental work reported in the previous chapters.

The degree of body sclerotization was assessed by using the trait ‘armouring’ from the USGS database (Poff *et al.* 2006) supplemented with photographs collected in this study for the measurement of morphometric traits (see below). A *poor degree of sclerotization* was noted if sclerites were ≤ 10% or non-existent (SclPoor), a *good degree of sclerotization* was scored if coverage was between 10 to 90 % (SclGood), and *complete sclerotization* was indicated if 90 to 100% of the body surface was sclerotized or the species was equipped with a carapace (SclComp).

The trait 'bauplan' was quantified with close consideration of the trait surface area/ volume ratio (AVratio) described below. For the calculation of the latter, geometric shapes were assumed for each species, and the shapes were used to categorize the bauplan. The categories used were: *box shape bauplan* (BauBox) for rectangular shaped species that appear to be dorso-ventrally or laterally flattened; *cylindrical shape bauplan* (BauCyl) included all circular and elliptic-cylindrical formed shapes; *spherical shape bauplan* (BauSphe) included ball and ellipsoid-like species; and *cone shape*

bauplan (BauCone) was used for fully conic and dorso-ventrally-flattened conic or semi-conic organisms.

The life stage of each species investigated was categorized as *adult* (Ladult), *larval or nymph* (Llarny) or *juvenile* (Ljuv) *life stage*, based on the life-stage used in experiments described previously.

In order to address the evolutionary descent of the investigated species, their relative phylogenetic position was estimated. For this the Tree of Life web project (Maddison & Maddison 1996) was used (retrieved from http://tolweb.org on the 01.09.2009) to construct a phylogenetic tree to the highest taxonomic resolution possible for each species, which were Anisoptera, Asellota, Chaoboridae, Ephemeroptera, Culicidae, Cladocera, Amphipoda, Molannidae, Caridea, Notonectidae, Crambidae, Pleidae, Cambaridae, Nepidae and Sialidae. For the *highest resolution phylogeny* (PhyRES), the number of nodes to the common arthropod ancestor were counted back from these taxa. For the *equalized phylogeny* (PhyEQ) the number of nodes from the order level equivalents for each species were counted back to the common arthropod ancestor. For both quantifications, the number of nodes were transformed into ranks: 1 (1 node, oldest) to 11 (23 nodes, youngest) for PhyRES and ranks 1 (1 node, oldest) to 7 (12 nodes, youngest) for PhyEQ. It has to be noted that this quantification of phylogeny does not entirely describe how similar the taxa are, because the phylogenetic 'between taxa distances' were not accounted for.

All of the traits addressed above, except the phylogenetic quantifications, were binary coded, meaning that the database entry was 0, if a trait modality was non-existent in a species and 1 if it was existent, while it was possible to be assigned a 1 for multiple trait modalities of one trait. PhyRES and PhyEQ were ordinal variables, which corresponded to the rankings described above.

Experimentally quantified metric traits

The traits experimentally quantified in this study were all body-size related traits (biovolume, length, surface area, AVratio, dry weight), water content, lipid content and exoskeleton thickness. For lipid content, sub-samples from the toxicokinetic experiments were mostly used, and the methodology and detailed results for this trait are reported and discussed in Rubach *et al.* (in press b, Chapter 5). For the current study, lipid content was expressed as *total lipid content* (LipTot), *% lipid in fresh weight* (LipFW) and *% lipid in dry weight* (LipDW). For most of the other measurements, animals were collected from the field as reported in Table 2 (date, origin), together with information on several methodological details. In order to investigate representative samples with respect to the toxicity, toxicokinetic and toxicodynamic studies described in the previous chapters or due to a lack of presence, some species (denoted by the condition 'dead' in Table 2) traits were measured using surplus or sub-samples of previous samples stored at -20°C.

For the quantification of *surface area* (SurfArea), *biovolume* (Biovol), *surface area/volume ratio* (AVratio) and *length* (Length), species-specific distance measures were taken according to previously assigned geometric bodies and their respective formulas for surface area and biovolume, for which the length was always a necessary measure. This approach has been previously applied for phytoplankton (Hillebrand *et al.* 1999), but has been considered difficult to apply to aquatic invertebrates (Magnusson *et al.* 2003). Table 2 informs on the geometric bodies assigned to the species and Figure 1 shows the used equations and formulas for the geometrical bodies.

Table 2 (continued on next page): Methodological details on the experimentally measured traits.

Species	Sampled	Origin	Life stage	Measured	*n* (size)	*n* (dry weight)	*n* (ExoTh)	Size method	Condition	Geometric formula	TK sub-sample	TK match
Anax imperator	06.11.2006	SH ditches	Larva [a]	5/8.7.2007	37	37	11	Ruler	dead	elliptic cylinder	no	very good
Asellus aquaticus	15.06.2009	SH cosms	Adult	15.06.2009	20	20	10	Camera/software	alive	rectangular box	no	fair
Chaoborus obscuripes	10.03.2009	SH cosms	Larva	10.03.2009	20	19	4	Camera/software	alive	circular cylinder	no	very good
Cloeon dipterum [b]	14.05.2009	SH cosms, ditches	Larva	14.05.2009	10	25	3	Camera/software	alive	rectangular box	no	very good
Culex pipiens	09.6.2009	SH tank	Larva	09.6.2009	9	20	4	Camera/software	alive	circular cylinder+prolate sphere+sphere	no	very good
Daphnia magna	01.05.2009	Culture	Adult	01.05.2009	20	19	5	Camera/software	alive	ellipsoid + elliptic A	no	very good
Gammarus pulex (AD) [c]	22.06.2009	SH storage basin	Adult	22.06.2009	20	20	5	Camera/software	alive	2 circular cylinders subtracted	no	very good
Gammarus pulex (JU) [c]	16.06.2009	SH storage basin	Juvenile	16.06.2009	20	20	6	Camera/software	alive	2 circular cylinders subtracted	no	very good
Molanna angustata	03.07.2009	Groene Heuvel	Larva	03.07.2009	20	3	3	Camera/software	alive	elliptic cylinder	no	very good
Neocaridinia denticulata [c]	31.03.2009	Culture	Adult	31.03.2009	20	20	12	Camera/software	alive	rectangular box	no	very good
Notonecta maculata	06.08.2007	SH cosms	Adult	23.06.2009	17	3	11	Camera/software	dead	cone	yes	very good
Paraponyx stratiotata [d]	06.08.2007	SH cosms	Larva	09.07.2009	20	3	6	Camera/software	dead	elliptic cylinder	yes	very good
Plea minutissima	25.03.2009	SH cosms	Adult	25.03.2009	20	20	16	Camera/software	alive	elliposid	no	fair
Procambarus spec. (AD)	08.06.2009	Culture	Adult	08.06.2009	40	20	6	Ruler	alive	elliptic cylinder	no	very good
Procambarus spec. (JU)	08.06.2009	Culture	Juvenile	09.06.2009	20	20	11	Camera/software	alive	elliptic cylinder	no	very good
Ranatra linearis	06.11.2006	SH ditch 20	Adult	5/8.7.2007	38	38	5	Ruler	dead	rectangular box	no	very good
Sialis lutaria	22.06.2009	SH ditch 16	Larva	23.06.2009	14	18	11	Camera/software	alive	half cone + triangle	no	very good

[a] Larvae stage three
[b] 20 specimen measured (10 solely in top view and the other 10 solely in lateral view, measures merged to 3D measures of 10 animals)
[c] Length measured in fragments for bend lines, (upper bend and lower bend measured separately, sum of fragments averaged)

[d] Length measured in fragments for bend lines

Note:
'*n*' denotes the sample size. 'ExoTh' refers to the trait 'Exoskeleton thickness'.
'SH' denotes the experimental field station of Alterra, 'The Sinderhoeve', Renkum, The Nethelands.
'TK' refers to the toxicokinetic experiments (Rubach *et al.* in press b, Chapter 5)
'Groene Heuvel' is a sandy oligotrophic lake in Wijchen, The Netherlands.
The equations for the geometrical bodies used are given in Figure 1.
Alive animals were anaesthetized for measurements.

Some species were assigned only one geometric form, but for others combinations (summations and subtractions of different geometrical bodies) were used. For instance, the surface area of *Daphnia magna* was described using the surface area of an ellipsoid body in addition to the surface area of an ellipse, the latter of which stands for the folded inner part of the carapace, which is exposed to the outer medium (Pirow *et al.* 1999b, a). In general, all appendices, such as antennae, legs or siphons were not included in the measures. To obtain the required measures for the larger sized animals, a ruler or millimeter paper was used, while the smaller animals were placed under a stereo microscope (Olympus SZX 10) and digital pictures of the specimen were taken in dorsal and lateral views using a digital camera (Axiocam ICc3, Carl Zeiss, The Netherlands). Subsequently, to extract the required distance measures, the pictures taken were analyzed with the AxioVision 4.8 software (Carl Zeiss, The Netherlands), which was previously calibrated to the magnifications used for each photograph. The calculations for the size-related traits were made individually for the measured specimen and the average value for each species was incorporated into the trait database.

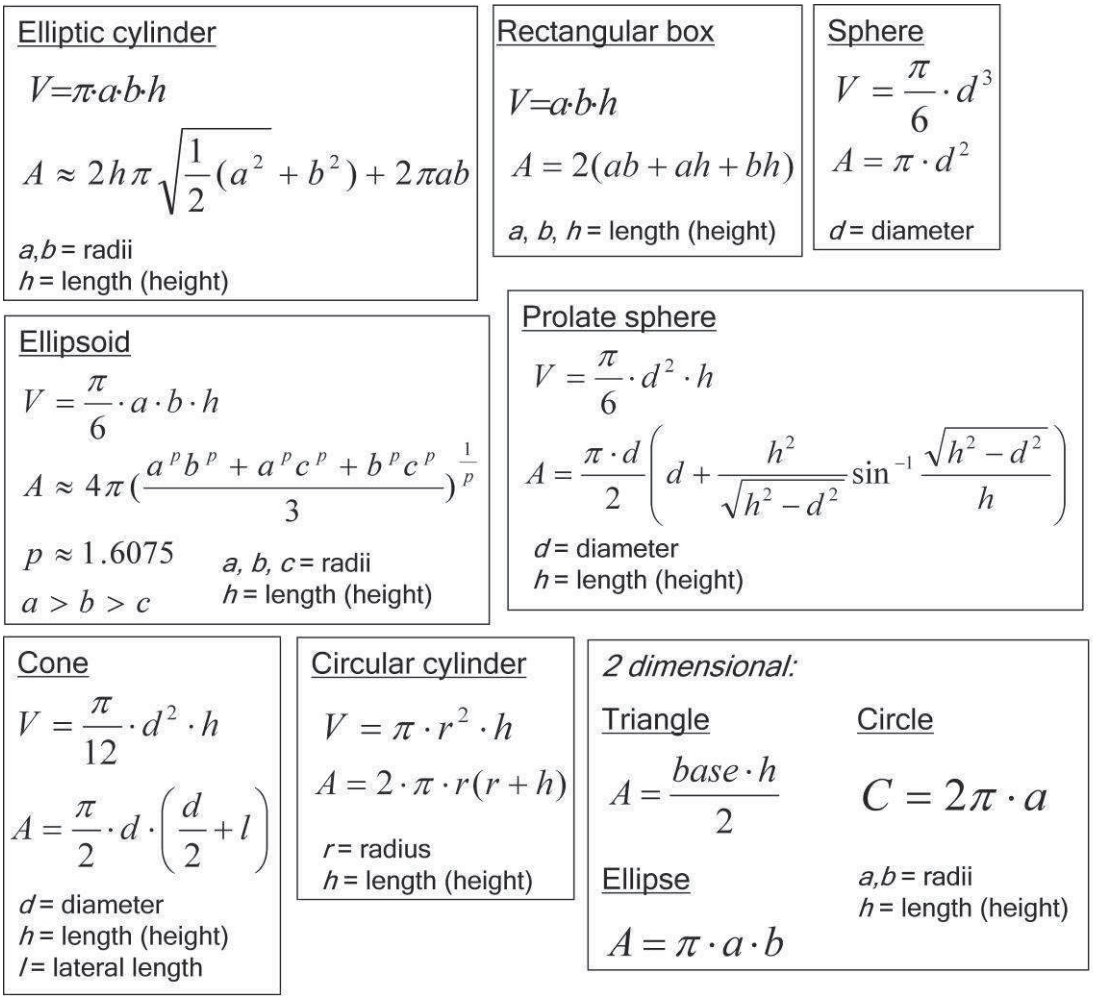

Figure 1: Equations and formulas for geometrical bodies used to quantify the size related traits for the different species. Formulas and approach were taken from Hillebrand *et al.* (1999), Wikipedia at http://en.wikipedia.org/wiki/Ellipsoid retrieved on 14.08.2009 (surface area of ellipsoid) and efunda at http://www.efunda.com/math/solids/IndexSolid.cfm (retrieved on 13.08.2009). *V* = (bio)volume; *A* = surface area; *C* = circumference. An organisms length mostly corresponded either to *h* (height) or a specific diameter, while the width and thickness were mostly diameters. Table 2 shows which combinations of geometrical bodies were used for each species.

The determination of dry weight and water content was conducted in accordance with Wetzel *et al.* (2005) by placing individual specimen into clean previously weighed aluminum boats, reweighing these and drying at constant 103°C for at least 24 h to a constant dry weight. All weights were determined on a microbalance and from the loss measures, the dry weight and water content were calculated. Specimen of *Anax imperator*, *Cloeon dipterum*, *Molanna angustata*, *Notonecta maculata*, *Parapoynx stratiotata* and *Ranatra linearis* were defrosted and blotted dry before measurement. Due to loss of samples, the values in the database for the water content of *Culex pipiens* and *D. magna* originate from the parallel dry weight determination for lipid content, as described in Rubach *et al.* (in press b, Chapter 5). All individually calculated dry weights and water contents were averaged and these values are listed in the trait database.

Exoskeleton thickness was determined using frozen organisms from the samplings listed in Table 2 and again for the species *R. linearis*, *N. maculata*, *A. imperator* and *P. stratiotata* from subsamples of the toxicokinetic studies Rubach *et al.* (in press b, Chapter 5). Animals were cut into several pieces in such a way that the animals were opened appropriately to ensure tissue digestion and also to ensure that representative pieces of the animal were collected. For instance, in case of *R. linearis* a leg, a claw, part of the siphon, a cross cut piece of the abdomen, the thorax, the head and the wings were included into the vial for subsequent tissue digestion. The tissue digestion was carried out with different volumes of tissue solubiliser (Soluene-350, Perkin Elmer Inc.). The amount of Soluene-350 varied between 1.5 mL and 10 mL, depending on the amount of tissue and the time needed (varied between 24 h and 11 d) to fully digest the tissue in a water bath of 60°C. The remains of this digestion process were washed thoroughly with ethanol to remove the Soluene-350 and any tissue leftovers. Afterwards the exoskeletons were stored in ethanol until microscopic analysis. Before measurement of the thickness of the randomly collected parts of the exoskeleton, these were cut into cross sections which were then placed on a glass slide under a stereomicroscope. For some species, auxiliary means were used to stabilize the sectional planes. Also, exoskeletons of some species were easier to measure when the ethanol had evaporated and others needed the suspension in either water or ethanol. The following measurement of the exoskeleton thickness was conducted using the same set-up with the stereomicroscope, camera and associated software, as described for the size related traits above. The individual measures taken were averaged and included as such into the trait database.

Results and Discussion

The trait database is displayed in Table 3 and relates to the information compiled in Table 1. This database is a combination of experimentally-quantified traits and literature information and generally contains wholly new information not present in other existing trait databases. The matrix consists of 12 different traits, expressed as 37 trait modalities (and/ or different ways of quantification) in total, which were scored for 17 ‘species’ (15 taxonomic species of which two were quantified separately for juvenile and adult stages). The information in the database is coded in three different ways: metric, binary and ordinal. Previous trait databases have been either binary (Heneghan *et al.* 1999) or fuzzy coded (Chevenet *et al.* 1994; Usseglio-Polatera *et al.* 2000b), with the latter approach providing a higher resolution when assigning trait modalities to species, because one species can

have more than one trait modality, expressed as 'affinities' to a certain trait modality. The application of this approach facilitated the inclusion of trait modalities which were present in all species investigated (i.e. inclusion of ResMocut or omnivory). Nevertheless in the present study, the binary approach was preferred in order to reduce complexity, and this was required as the combined datasets were each expected to introduce high background variability.

Although, on the one hand, the database is not yet comprehensive, especially with regard to physiological traits (see discussion below), it already contains a large number of trait modalities compared to the number of species included. Hence the variance in the explanatory variables (traits) could exceed the variance in the response variables (sensitivity) when used in further analyses. For this reason, the number of traits was reduced systematically before using the database. For example, only the most relevant size-related traits were included with regard to each response variable in question. Nevertheless the database should in future be extended, since the missing physiological traits are of major importance with regard to intrinsic sensitivity, and are therefore important to be meaningfully quantified. Traits which were originally intended to be quantified but could not be addressed in the present study included the *complexity of the nervous system*, the *metabolic rate*, the *amount of target receptors* (acetylcholinesterase), and the *detoxification abilities* of a species. We were unable to quantify these traits from the literature because most work on these traits has been performed only for certain model species and hardly any comparative studies on freshwater arthropods exist to date. The other option, namely to quantify these type of traits experimentally was tempting, because of the expected strong relationships to the toxicodynamics and the possibly improved interpretation of the toxicokinetic patters observed by Rubach *et al.* (in press b, chapter 5). Although this was beyond the scope of this thesis, these traits are discussed further below.

In order to measure the *complexity of the nervous system* several approaches were considered. The general neuro-anatomy of insects consists of the central nervous system with brain, ventral nerve cord and ventral ganglia plus the peripheral nerve system. Interesting ways of approaching its complexity could have been to determine the number of different cell types in the nervous system, or extracting the complete central nervous system, counting the number of ganglia and fused ganglia and calculating their ratio. The more fused ganglia an insect has, the more complex the nervous system might be, and thereby the organism's nervous system might be either easier disrupted by neurotoxic compounds or have better compensatory abilities. The other option would be to base phylogeny on the nervous system (Harzsch 2006) and assume that complexity increases in evolution (Valentine *et al.* 1994; McShea 1996, 2000; McCarthy & Enquist 2005).

Table 3: The trait by species database, see Table 1 for the explanation of the short-form names and the units of the traits.

Species	Biovol	SurfArea	AVratio	Length	DryMass	WatCont	ExoTh	LipFW	LipDW	LipTot	ResConf	ResInt	ResReg	SOatm	SOdiss	ResMocut	ResMosip	ResMoCoG	ResMoExG	ResMoInG	ResMoPig	TroDetr	TroHerb	TroCarn	TroOmni	SclPoor	SclGood	SclComp	BauBox	BauCyl	BauSphe	BauCone	Ladult	Llarny	Ljuv	PhylRES	PhylEQ
Anax imperator	2141	12468	6.01	46.67	263.7	79.8	0.16	2.49	16.42	35.15	0	1	0	0	1	1	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	4	5
Asellus aquaticus	10.11	33.70	3.51	5.21	1.35	81.1	0.06	0.85	4.25	0.10	0	0	1	0	1	1	0	0	1	0	1	1	1	0	0	0	0	1	1	0	0	0	1	0	0	4	4
Chaoborus obscuripes	11.85	44.11	3.78	12.02	1.25	77.6	0.02	1.04	6.62	0.06	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0	8	7
Cloeon dipterum	2.98	16.17	5.55	4.60	0.84	71.5	0.01	7.21	10.84	0.18	0	1	0	0	1	1	0	0	1	0	0	1	1	0	0	1	0	0	1	0	0	0	0	1	0	1	2
Culex pipiens	5.25	17.77	3.39	4.40	0.38	80.4	0.03	0.70	5.75	0.01	0	0	1	1	0	1	1	0	0	0	0	1	1	1	1	1	0	0	0	1	0	0	0	1	0	8	7
Daphnia magna	2.86	13.27	4.81	2.81	0.14	94.4	0.03	0.94	16.12	0.04	1	0	0	0	1	1	0	0	1	0	1	1	1	1	1	0	0	1	0	0	1	0	1	0	0	1	1
Gammarus pulex (AD)	25.35	146.2	8.22	12.46	11.36	80.1	0.04	1.36	5.44	0.26	0	0	1	0	1	1	0	0	1	0	1	1	0	1	0	0	0	1	1	0	0	0	1	0	0	2	4
Gammarus pulex (JU)	3.14	27.71	10.90	5.26	1.26	30.9	0.03	0.49	2.27	0.02	0	0	1	0	1	1	0	0	1	0	1	1	0	1	0	0	0	1	1	0	0	0	0	0	1	2	4
Molanna angustata	33.79	71.78	2.19	9.47	5.49	82.2	0.11	2.93	16.42	0.69	1	0	0	0	1	1	0	0	1	0	0	0	1	1	0	1	0	0	0	1	0	0	0	1	0	9	7
Neocaridinia denticulata	76.47	149.5	2.09	16.25	9.66	73.5	0.04	1.25	6.13	0.50	0	0	1	0	1	1	0	0	1	0	1	1	1	0	0	0	0	1	1	0	0	0	1	0	0	3	3
Notonecta maculata	65.20	105.8	1.63	12.93	43.28	61.0	0.05	3.81	9.76	5.07	0	0	1	1	1	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0	9	6
Paraponyx stratiotata	41.91	91.38	2.24	12.30	7.63	72.2	0.23	4.52	16.21	1.53	1	0	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0	11	7
Plea minutissima	1.84	7.89	4.34	2.50	0.71	62.0	0.04	7.61	8.09	0.12	0	0	1	1	1	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0	0	10	6
Procambarus spec. (AD)	7304	25074	3.78	57.88	2615	64.2	0.30	1.97	7.04	55.06	0	1	0	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	0	1	0	0	1	0	0	6	3
Procambarus spec. (JU)	123.7	1595	15.84	13.91	17.02	84.1	0.05	1.16	5.92	0.30	0	1	0	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	0	1	0	0	0	0	1	6	3
Ranatra linearis	375.4	4780	12.88	34.34	63.70	56.0	0.08	0.96	3.11	1.41	0	0	1	1	0	1	1	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1	0	0	7	6
Sialis lutaria	8.22	40.94	5.11	14.34	9.26	82.0	0.51	1.92	8.82	0.76	0	1	0	0	1	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1	0	5	6

Note:
D. magna /ResMoCut: respiration is enhanced through the inner shell and the rostrum area.
A. aquaticus, *G. pulex*, *N. denticulata*, *Procambarus* spec. /ResMoExG: the external gills are probably covered with a cuticule, that shed with mold.
C. dipterum /ResMoExG: unclear if external gill is functional.
S. lutaria /ResMoExG : external gills are much more permeable than the rest of the body surface.
P. stratiotata/ ResMoExG: possibility that the external gills are dysfunctional and respiration occurs via a plastron (incompressible gill).
AD denotes adult specimen, JU juvenile specimen

The variety of methods available to quantify the *amount of target receptors* was manifold. A relatively straightforward approach would have been to measure enzyme activities of acetylcholinesterase as a biomarker in the test species under control conditions. It was unclear however, how comparable the data would be across species, since the used test animals were sampled from different sites/cultures, with different background history, at different times/ seasons and under different conditions (Domingues *et al.* 2010). Therefore we would always only obtain 'snapshots' of enzyme activity, which would not necessarily be specific to the species. From the body of literature reviewed, it was concluded that in order to interpret such biomarker data in a comparative approach, extensive experimental work would have to be performed. In more detail, to be able to relate this information systematically to differences in species sensitivity, it would have even been necessary to investigate differences in enzyme constitution, their affinities to chlorpyrifos in addition to concentration and time dependent differences in transcription rates and gene expression of the acetylcholinesterase in all species. Similar difficulties were encountered for the quantification of the detoxification abilities, however, here the situation turned out to be even more complex, because although many detoxification mechanisms are known, more enzymes and also the feedback loops of different stress response systems are involved (Korsloot *et al.* 2004). Feedback loops were also hypothesized to play a role for the quantification of target receptors due to receptor aging and *de novo* synthesis. In the future, research such as Heckmann *et al.* (2008) has conducted for *D. magna*, will enable comparative studies, because modern techniques such as next generation sequencing genomes of species will be rapidly and easily available. Also, other techniques such as DNA bar-coding (Valentini *et al.* 2009) will contribute to revisiting phylogeny and support the genetic description of traits (see also below).

LINKING TRAITS TO SENSITIVITY

In order to identify biological factors which cause differences in toxicokinetics and sensitivity across species and also explore how these relationships can be expressed quantitatively, the trait database described above was quantitatively linked to seven different sensitivity endpoints, which were characterized for the 17 species in the Chapters 4 (Rubach *et al.* in press c) and 5 (Rubach *et al.* in press b) of this thesis. Two of the seven different sensitivity endpoints are classical endpoints, namely the median lethal dose in 48 h constant exposure (LC_{50} 48 h), the median effective concentration for immobility in 48 h constant exposure (EC_{50} 48 h), both originating from Chapter 4 (Rubach *et al.* in press c). Furthermore as toxicokinetic sensitivity endpoints, the uptake rate constant (k_{in}) corrected for fresh weight, the uptake rate constant, but uncorrected for fresh weight (k_{in} *uncorr*), the elimination rate constant (k_{out}), the bioconcentration

Table 4: The seven sensitivity endpoints (five toxicokinetic and two general endpoints) used to link traits and sensitivity.

	Endpoints						
	k_{in} [a]	k_{in} *uncorr* [a]	k_{out} [a]	BCF_{ww} [a]	BCF_{lipid} [a]	48h LC_{50} [b]	48h EC_{50} [b]
Species	[$L \cdot kg_{ww}^{-1} \cdot d^{-1}$]	[$L \cdot d^{-1}$]	[d^{-1}]	[$L \cdot kg_{ww}^{-1}$]	[$L \cdot kg_{lipid}^{-1}$]	[$\mu g \cdot L^{-1}$]	[$\mu g \cdot L^{-1}$]
Anax imperator	21.2	0.02993	0.212	100	4021	3.29	3.134
Asellus aquaticus	596	0.00683	0.185	3242	382956	n.c. [c]	6.159
Chaoborus obscuripes	318	0.00555	0.131	2428	234140	1.13	0.438
Cloeon dipterum	349	0.00268	0.196	1782	24699	0.81	0.763
Culex pipiens	328	0.00112	0.024	13930	1999644	0.2	n.p. [e]
Daphnia magna	295	0.00398	0.546	541	57437	27.43	0.484
Gammarus pulex (AD)	812	0.01554	0.398	2039	149919	0.43	0.379
Gammarus pulex (JU)	1110	0.00520	0.36	3083	627029	n.p. [d]	n.p. [d]
Molanna angustata	579	0.01362	0.109	5331	181901	z.m.[f]	1.857
Neocaridinia denticulata	617	0.02459	0.478	1291	103599	660.1	327.2
Notonecta maculata	61.9	0.00823	0.152	407	10679	23.938	9.071
Parapoynx stratiotata	275	0.00930	0.171	1601	35458	29.41	2.94
Plea minutissima	88.2	0.00043	0.135	654	8592	5.94	2.645
Procambarus spec. (AD)	24.2	0.06762	0.086	280	14220	34.81	20.727
Procambarus spec. (JU)	199	0.00516	0.154	1295	111332	2.75	1.702
Ranatra linearis	42.1	0.00617	0.107	392	40891	11.97	11.97
Sialis lutaria	203	0.00806	0.021	9625	500412	z.m. [f]	1.548

[a] taken from Rubach *et al.* (n press b), k_{in} and k_{out} based on the Markov-Chain-Monte-Carlo estimates.
[b] taken from Rubach *et al.* (in press c)
[c] not computed, for analysis the 72 h LC_{50} was used: 7.639 $\mu g \cdot L^{-1}$.
[d] not performed, for analysis the values of *G. adults* was used (LC_{50} = 0.43 and EC_{50} = 0.379, both in $\mu g \cdot L^{-1}$)
[e] not performed, LC_{50} was used.
[f] zero mortality observed, for analysis value of 10000 was used.

factor (BCF) corrected for fresh weight (BCF_{ww}) and the BCF corrected for lipid content (BCF_{lipid}) were all taken from Rubach *et al.* (in press b, Chapter 5). These seven sensitivity endpoints and the values used are displayed in Table 4. These endpoints only address general systemic toxicity and toxicokinetics because the available datasets for them were sufficiently large for a quantitative analysis. The data on toxicodynamic parameters were partly uncertain and available for no more than four species, which only facilitated a qualitative discussion. Nevertheless, from the quantitative linking of traits with sensitivity and toxicokinetics together with a qualitative analysis of toxicodynamic patterns observed for the species tested, the importance of the toxicokinetics and toxicodynamics for intrinsic sensitivity can be evaluated.

Quantitative linking of sensitivity and toxicokinetics to traits

To quantitatively link sensitivity to traits a two step approach was chosen and both assume linear models for the relationships between traits and sensitivity. Firstly, a PCA with indirect gradient analysis was performed with the species trait and sensitivity data to explore how traits grouped with each other on basis of our species selection and how this variation relates to the variation in the sensitivity endpoints measured. In the second step, single and multiple linear regressions were performed with the same data with the following three objectives: Firstly, to evaluate the explanatory

potential of single traits and to identity their relevance for each sensitivity endpoint (single regressions). Secondly, to extract combinations of traits with explanatory potential for each sensitivity endpoint and to evaluate their relevance for the processes of toxicity (multiple regressions). Thirdly, to identify the best sensitivity endpoint(s) and the level of mechanistic detail needed to link traits and sensitivity quantitatively.

Principle Component Analysis

Before the PCA was performed, the metric traits were square root transformed and the toxicity data (sensitivity endpoints) were log transformed. The PCA was performed in Canoco for Windows Version 4.55 (2006) with the species/ trait data (species represented the 'sites/samples' in classical Canoco terminology and the traits were used as 'species') and then combined with an indirect gradient analysis using the sensitivity endpoint data as passive explanatory variables (environmental variables in classical Canoco terms).

The results are presented in Figure 2 as a PCA biplot with overlay of the explanatory variables. The first axis displays 21% and the second 19% of the variation in traits which were explained by the PCA. The first axis also explains 22% of the variation in sensitivity parameters, the second another 30% from the indirect gradient analysis. The PCA illustrates that our species selection was representative for the diversity in traits measured. Similarly, Rubach *et al.* (in press c, Chapter 4) have shown that these same species also represent the range in variation of sensitivity to chlorpyrifos in arthropods. These findings are a prerequisite for the extraction of traits and combinations of traits which may explain differences in sensitivity. The PCA furthermore indicates the possible correlations between traits which may either be due to the method of quantification (in case of the size-related traits), structural correlations (for instance the lipid content is naturally correlated to size related measures) or small phylogenetic distance, leading to 'trait suites or -syndromes', (Poff *et al.* 2006; Culp *et al.* in press). It is however difficult to interpret these correlations in a biplot.

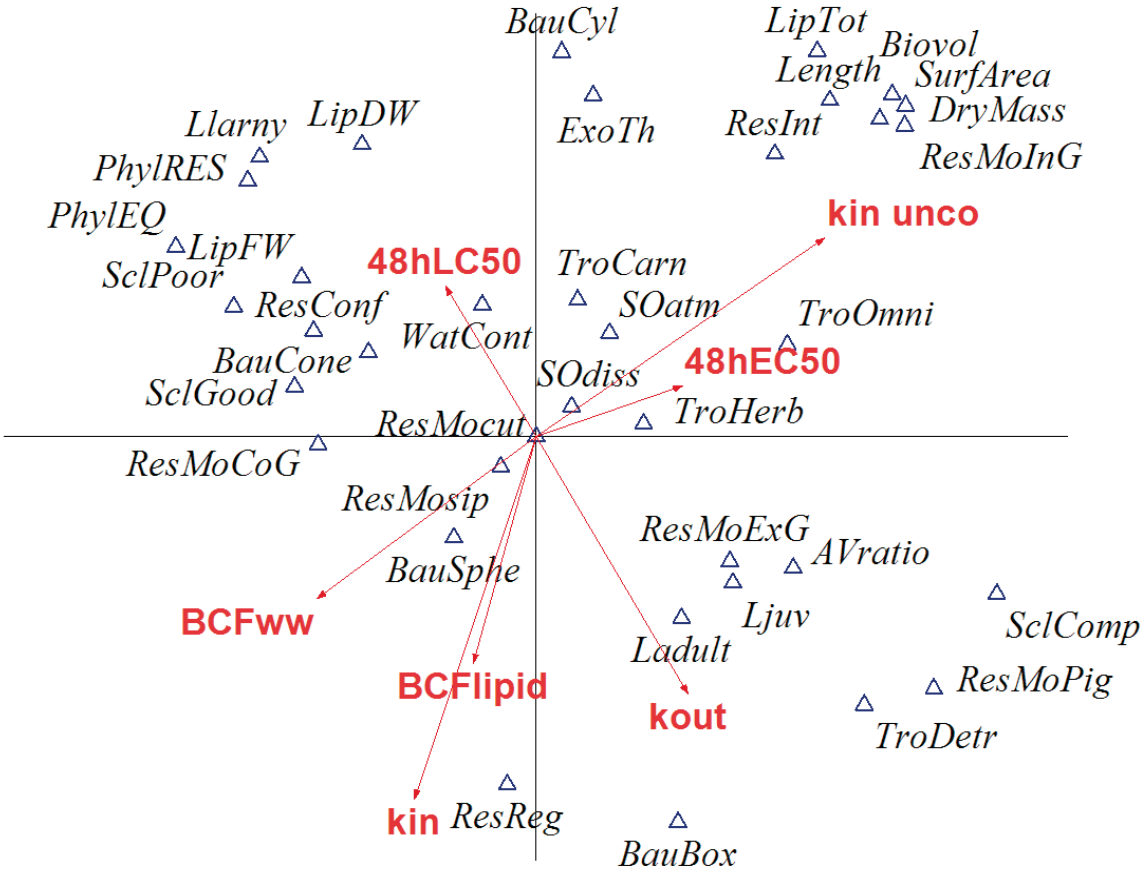

Figure 2: PCA biplot from the indirect gradient analysis using the species trait and species sensitivity databases compiled in this and previous studies (see supplemental data). Sensitvity data were log transformed and metric trait data were square root transformed. In classical Canoco terminology the species played the role of samples (sites) and the traits the role of species, while the sensitivity endpoints were used as explanatory variables (= environmental data). The first axis displays 21% of the total variation in traits between the taxa and 22% of the variation in sensitivity parameters, while the second axis another 19% of the variation in traits and 30% of the variation in sensitivity parameters. Abbreviations of the traits are explained in Table 1, those of the sensitivity parameters in Table 4.

The correlation analysis performed together with the regression analyses below are perhaps more informative. Although the overlay adds information to the PCA biplot, these results have to be interpreted with care, especially the variation explained by the axes. The overlay of the sensitivity endpoints shows that the fresh weight corrected k_{in} is indeed negatively correlated with the size related measures (with the exception of the AVratio, which is not correlated at all) in contrast to k_{in} *uncorr* which is positively correlated. These both indicate that size is a strong explanatory and therefore predictive variable for uptake. Similarly, clear correlations with uptake are indicated also for LipTot, ExoTh and ResInt. The endpoints for bioconcentration are both highly correlated with the corrected k_{in} and therefore in the same pattern related to the same traits. For the toxicokinetic endpoint k_{out}, the PCA suggests that adult and juvenile stages (as categorized here) have better elimination abilities than larval and nymphal stages. Also complete sclerotization, being a detritivore, and being equipped with respiratory pigments seems to be related with high elimination rate constants. In turn, many traits are negatively correlated with k_{out}, among which the lipid content, poor sclerotization and a high relative

rank in phylogenetic position (late separation from arthropod lineage) are found. Interestingly, good elimination abilities are negatively correlated with insensitivity for lethality (high 48 h LC_{50} values), but not at all correlated with immobility (48 h EC_{50}), which leads to the hypothesis that the elimination rate constant might be the dominant rate determining survival dynamics. Rubach *et al.* (in press b, Chapter 5) also correlated k_{out} in a single linear regression analysis with the 48 h EC_{50} and in contrast found a significant correlation, but only when the species *Neocaridina denticulata* was excluded. Remarkably, in terms of their correlation with traits, neither of the standard toxicity values, the LC_{50} or EC_{50} showed a strong explanatory potential in this analysis. This exploratory multivariate analysis is informative and provides a first good overview, but is unsuitable for determining significant single traits and combination of traits that are relevant for sensitivity. This will be addressed below by means of single and multiple linear regressions.

Linear regression analysis

In order to identify single traits and combinations of traits as factors with high explanatory and therefore predictive potential for toxicant sensitivity, a linear regression selection method was performed using the RSEARCH procedure (Payne 2007) in GenStat release 12.1 (2009). Subsequently for all traits, simple single linear regressions were performed with all trait quantifications/modalities and each of the seven sensitivity endpoints separately. Thereafter, from each of the 12 traits, the one quantification or modality which explained the largest variance in the respective sensitivity endpoint was selected for the forward multiple regressions, irrespective of significance. Also, trait modalities were omitted from an analysis if they were strongly correlated (e.g. although frequently significant in the single linear regressions, LipTot was removed from all subset analysis because correlation analysis indicated strong correlations with all size-related variables except the AVratio). The selected trait quantifications and modalities (always a set of 12 traits) were then used as candidate regression models (all possible combinations) that were then analyzed separately using a multiple regression approach employing both a threshold level of $p \leq 0.1$ and $p \leq 0.05$ separately. In order to give an overview about the relevance of each trait quantification/ modality investigated, the significance and coefficient of determination (adjusted R^2)

Table 5 (continued on next page): Results of single linear regressions (each sensitivity endpoint regressed against each trait modality/quantification).

Trait (modality)	k_{in} [L·kg$_{ww}^{-1}$ ·d^{-1}]		k_{in}*uncorr* [L·d^{-1}]		k_{out} [d^{-1}]		BCF$_{ww}$ [L·kg$_{ww}^{-1}$]		BCF$_{lipid}$ [L·kg$_{lipid}^{-1}$]		48h LC$_{50}$ [µg·L^{-1}]		48h EC$_{50}$ [µg·L^{-1}]	
	p	adj. R^2	*p*	adj. R^2	*p*	adj. R^2	*p*	adj. R^2	*p*	adj. R^2	*p*	adj. R^2	*p*	adj. R^2
BioVol	0.001**	0.471	0.005**	0.379	0.714	NC	0.011**	0.316	0.05**	0.182	0.842	NC	0.117	0.1
SurfArea	**< 0.001****	**0.56**	0.01**	0.326	0.727	NC	**0.004****	**0.391**	**0.041****	**0.2**	0.991	NC	0.144	0.079
AVratio	0.87	NC	0.682	NC	0.674	NC	0.656	NC	0.725	NC	**0.11**	**0.106**	0.291	0.012
Length	0.001**	0.474	**< 0.001****	**0.545**	0.646	NC	0.014**	0.298	0.074*	0.143	0.527	NC	**0.045****	**0.191**
DryMass	0.005**	0.384	0.008**	0.338	**0.59**	**NC**	0.036**	0.213	0.09*	0.125	0.752	NC	0.13	0.089
WatCont	**0.895**	**NC**	**0.788**	**NC**	**0.627**	**NC**	**0.643**	**NC**	**0.856**	**NC**	**0.326**	**0.002**	**0.751**	**NC**
ExoTh	**0.102**	**0.113**	**0.055***	**0.173**	**0.042****	**0.197**	**0.871**	**NC**	**0.603**	**NC**	**0.009****	**0.329**	**0.325**	**0.002**
LipFW	**0.297**	**0.01**	**0.317**	**0.004**	0.893	NC	0.379	NC	**0.006****	**0.37**	0.645	NC	0.743	NC
LipDW	0.428	NC	0.598	NC	**0.834**	**NC**	**0.373**	**NC**	0.066*	0.156	**0.119**	**0.098**	0.824	NC
LipTot [a]	< 0.001**	0.558	0.004**	0.397	0.734	NC	0.004**	0.392	0.012**	0.31	0.717	NC	**0.134**	**0.086**
ResConf	0.346	NC	0.935	NC	0.557	NC	0.642	NC	0.801	NC	0.318	0.004	**0.318**	**0.004**
ResInt	**0.077***	**0.14**	**0.253**	**0.025**	**0.272**	**0.018**	**0.401**	**NC**	**0.263**	**0.022**	0.756	NC	0.882	NC
ResReg	0.454	NC	0.256	0.021	0.626	NC	0.716	NC	0.427	NC	**0.253**	**0.025**	0.481	NC
SOatm	**0.017****	**0.278**	0.284	0.015	0.113	0.103	**0.321**	**0.003**	0.383	NC	0.454	NC	**0.524**	**NC**
SOdiss	0.493	NC	**0.23**	**0.034**	**0.064***	**0.157**	0.562	NC	**0.27**	**0.019**	**0.338**	**NC**	0.737	NC
ResMocut	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
ResMosip	0.493	NC	0.23	0.034	0.064*	0.157	0.562	NC	0.27	0.019	0.228	NC	0.737	NC
ResMoCoG	0.206	0.044	0.099*	0.116	0.938	NC	0.263	0.022	**0.059***	**0.166**	0.973	NC	0.572	NC
ResMoExG	0.027**	0.237	0.162	0.068	0.253	0.025	0.25	0.026	0.3014	0.008	**0.206**	**0.044**	0.827	NC
ResMoInG	**0.013****	**0.304**	**0.064***	**0.158**	0.897	NC	**0.029****	**0.231**	0.105	0.11	0.718	NC	**0.485**	**NC**
ResMoPig	0.187	0.054	0.198	0.048	**0.023****	**0.252**	0.811	NC	0.427	NC	0.642	NC	0.532	NC
TroDetr	**0.086***	**0.129**	0.878	NC	**0.202**	**0.046**	0.495	NC	**0.168**	**0.064**	**0.131**	**0.089**	0.767	NC
TroHerb	0.323	0.003	**0.743**	NC	0.854	NC	**0.426**	**NC**	0.541	NC	0.546	NC	0.534	NC
TroCarn	0.175	0.06	0.79	NC	0.274	0.018	0.623	NC	0.968	NC	0.803	NC	**0.128**	**0.091**
TroOmni	0.525	NC	0.852	**NC**	0.507	NC	0.897	NC	0.623	NC	0.5	NC	0.505	NC
SclPoor	**0.25**	**0.026**	0.378	NC	0.27	0.019	**0.055***	**0.172**	**0.337**	**NC**	0.766	NC	**0.126**	**0.092**

Table 5 (continued)

Trait (modality)	k_{in} [L·kg$_{ww}^{-1}$ ·d^{-1}]		k_{in}*uncorr* [L·d^{-1}]		k_{out} [d^{-1}]		BCF$_{ww}$ [L·kg$_{ww}^{-1}$]		BCF$_{lipid}$ [L·kg$_{lipid}^{-1}$]		48h LC$_{50}$ [µg·L^{-1}]		48h EC$_{50}$ [µg·L^{-1}]	
	p	adj. R^2	*p*	adj. R^2	*p*	adj. R^2	*p*	adj. R^2	*p*	adj. R^2	*p*	adj. R^2	*p*	adj. R^2
SclGood	0.283	0.015	0.196	0.05	0.146	0.078	0.983	NC	0.391	NC	**0.209**	**0.043**	0.738	NC
SclComp	0.824	NC	**0.063***	**0.16**	**0.025****	**0.244**	0.087*	0.128	0.83	NC	0.504	NC	0.264	0.02
BauBox	**0.087***	**0.128**	0.78	Nc	**0.073***	**0.145**	0.725	NC	0.435	NC	0.343	NC	**0.478**	**NC**
BauCyl	0.362	NC	0.348	NC	0.203	0.046	0.986	NC	0.996	NC	0.88	NC	0.591	NC
BauSphe	0.76	NC	**0.029****	**0.232**	0.335	NC	**0.34**	**NC**	**0.274**	**0.018**	0.997	NC	0.561	NC
BauCone	0.458	NC	0.849	NC	0.1	0.115	0.694	NC	0.946	NC	**0.089***	**0.12**	0.723	NC
Ladult	0.441	NC	**0.69**	**NC**	0.159	0.07	**0.081***	**0.135**	**0.215**	**0.041**	0.825	NC	**0.038****	**0.206**
Llarny	0.886	NC	0.863	NC	**0.048****	**0.186**	0.142	0.081	0.573	NC	0.618	NC	0.147	0.077
Ljuv	**0.328**	**0.001**	0.723	NC	0.468	NC	0.686	NC	0.302	0.009	**0.261**	**0.022**	0.392	NC
PhylRES	**0.217**	**0.039**	**0.425**	**NC**	0.043**	0.194	0.859	NC	0.61	NC	**0.456**	**NC**	0.7	NC
PhylEQ	0.671	NC	0.485	NC	**0.014****	**0.295**	**0.245**	**0.028**	**0.541**	**NC**	0.588	NC	**0.647**	**NC**

[a] Total lipid content was never selected for the forward multiple linear regressions in order to avoid artifact results in relation to body size.

Note:
- *p* value reports the F statistic, notation for significance: * = $p \leq 0.1$ and ** = $p \leq 0.05$.
- adj. R^2 = adjusted coefficient of determination, percent variance accounted for by trait combination.
- NC = not calculated, because residual variance exceeded variance of response variable.
- NP = not performed, because all species were quantified as cutaneous breathers.
- Boldface indicates the variable/ modality selected for the forward multiple linear regression analyses.

of all single linear regressions are shown in Table 5. In the table, boldface is used to indicate which trait variables were selected for the forward multiple regressions for which the results are displayed in Table 7. Below, firstly the result of the single regressions will be described and discussed, followed by the results and discussion of the multiple regressions.

Single regressions – single traits

In order to alleviate the interpretation of single trait-sensitivity relationships shown in Table 5, Table 6 lists the trait variables found significant in the single regressions for each sensitivity endpoint separately, also indicating the nature of relationships (negative or positive) and aspects of their qualitative analysis discussed in the following. Many of the more general observations from the PCA discussed above were confirmed by the single linear regressions.

Table 6: Nature and interpretation of relationship between single traits and sensitivity.

Senitivity endpoint	Biovol	SurfArea	Length	DryMass	ExoTh	LipFW	LipDW	LipTot	ResInt	SOatm	SOdiss	ResMoCoG	ResMoExG	ResMoInG	ResMoPig	TroDetr	SclPoor	SclComp	BauBox	BauSphe	BauCone	Ladult	Llarny	PhylRES	PhylEQ
k_{in} [L·kg^{-1}·d^{-1}]	-	-	-	-				-	-	-			+	-		+			+						
k_{in} *uncorr* [L·d^{-1}]	+	+	+	+	+			+				-		+				+		-					
k_{out} [d^{-1}]					-						+	+			+			+	+				-	-	-
BCFww [L·kg$_{ww}$$^{-1}$]	-	-	-	-				-						-			-	-				+			
BCF$_{lipid}$ [L·kg$_{lipid}$$^{-1}$]	-	-	-	-		-	-	-				-													
48h LC_{50} [µg·L^{-1}]					+																+				
48h EC_{50} [µg·L^{-1}]			+																			+			
Legend:	expected, known						size artefact					interesting, logical					unlogical								

For instance, the size-related traits (except AVratio) and LipTot showed highly significant positive relationships with uptake and bioconcentration of chlorpyrifos in freshwater arthropods, while the nature of the relationship corresponds as expected with whether the sensitivity variable was corrected (negative for k_{in}, BCF_{ww}, BCF_{lipid}) or uncorrected (positive for k_{in} *uncorr*) for fresh weight (Table 6). The BCF_{lipid} is also indirectly a correction for size due to the total lipid content. Thus the strong correlation between both BCFs and k_{in} also seen in the PCA above is not surprising. Of the size-related traits, SurfArea and Length were most significant, which is surprising. In fact, as hypothesized and also supported by previous data (Hendriks *et al.* 2001; Weiner *et al.* 2004; Preuss *et al.* 2008; Rubach *et al.* in press b) the AVratio was expected to have the highest explanatory potential for uptake and bioconcentration. There are three possible explanations as to why the AVratio was not found to be significant in this analysis. Firstly, the quantification technique as described above might not be sufficient to detect differences among the investigated species in their AVratio. This however is contradicted by a Kruskal-Wallis test (SPSS 15.0.1, 2007), which indicated significant statistical differences with $p < 0.01$ between the species AVratios measured. Secondly, the differences in AVratio between the species selected for investigation are not profound enough to be important to cause differences in uptake. Thirdly, in the toxicokinetic experiments the adsportion of chlorpyrifos to the external parts of the exoskeleton (surface area) could have been high and was methodologically included in the measured internal concentrations by Rubach *et al.* (in press b, Chapter 5). This can, however, only then dominate the analysis if the adsorption was so high that the uptake was no longer

important. This is highly unlikely as the exposure concentrations were proportional to species specific effect concentrations in the experiments of Rubach *et al.* (in press b, Chapter 5). Furthermore, other size-related traits, including biovolume, were also highly significant in the present study. Nevertheless, adsorption certainly does appear to play a role in this study, which is supported by the finding that the length but no other size related measurement was significantly correlated to the 48 h EC_{50}. Externally adsorbed chlorpyrifos probably did not induce any immobility in the toxicity experiments and therefore length, which does not describe the surface area of an organism, was apparently important for this relationship. Adsorption of metals to carapaces, although saturated in time, has previously been identified as an important factor to consider in bioconcentration studies (Robinson *et al.* 2003). The importance of the AVratio for uptake could not be verified in this study with the methods used, and therefore future traits-based approches interested in this relationship need to improve the quantification of this trait with more sophisticated, but easy and rapid techniques, which still need to be developed.

Of the traits describing lipid content, mostly LipTot was significantly related to the sensitivity endpoints. As previously mentioned this is a size artifact, also indicated by correlation coefficients between 0.854 and 0.955 stemming from independent measurements for size related measurements and the total lipid content. The fresh weight and dry weight corrected lipid contents were only significantly negatively related to the BCF_{lipid}, which is also a size artifact, but from the other direction and therefore negative (Table 6).

Among the single traits addressing respiration in one way or another, the strongest explanatory power was observed for traits that describe independence from dissolved oxygen (SOatm, ResMoCoG), which reduces uptake of chlorpyrifos and in turn is dependent on dissolved oxygen (ResMoExG, ResMoInG) enhancing uptake. However ResMoInG also showed negative relationships with k_{in} and BCF_{ww}. Some species with internal branchial chambers, such as *A. imperator* close their branchial chambers in order to facilitate ventilation (Mill & Hughes 1966), which if induced by exposure to chemicals may explain why this trait delivered inconsistent signals in the present study. In contrast to the patterns found for uptake, using dissolved oxygen for respiration enhances elimination abilities, which indicates that respiratory organs are also used for ion exchange, which is broadly known and accepted (Freire *et al.* 2008; Merritt *et al.* 2008; Fu *et al.* 2010). Interestingly, respiration regulatory abilities did not show strong relationships to sensitivity endpoints. Only having intermediate regulatory capabilities (ResInt) was found to decrease uptake.

Detritivore food preference was found to be the only trophic relation significantly related to the sensitivity endpoints analyzed, namely to increased uptake. As test animals were not fed during the experiments used to parameterize the toxicokinetic model, this cannot be related to detritus in general, but either to another underlying associated mechanism, such as physiological differences or ingestion behavior. More likely however this can be explained by intra-experimental opportunistic cannibalism, which was observed in the experimental treatments in Chapter 5 (Rubach et al. in press b), especially for the detritivore and omnivore test species.

The patterns seen for degree of sclerotisation are difficult to interpret. On the one hand, complete sclerotization seems to enhance uptake (k_{in} *uncorr*), maybe due to higher adsorption on sclerotized body parts than non-sclerotized body parts. On the other hand, contradictory negative

relationships were identified for both poor and complete sclerotisation. All these regressions were only moderately significant ($p \leq 0.1$), while complete sclerotisation slows elimination down more significantly ($p = 0.025$). The latter relationship seems logical for water soluble metabolites.

The trait bauplan was adapted from the geometrical bodies used for the quantification of the AVratio, but these variables are still not overly correlated (correlation coefficients between 0.106 and 0.309). Since the AVratio did not deliver a signal in our analysis, it is interesting to analyze bauplan instead. The box shaped modality was significantly ($p \leq 0.1$) related to both enhanced uptake and elimination, which is logical since being dorsoventrally flattened offers a lot of surface for exchange of molecules. Likewise, the cone shape positively correlated with higher 48 h LC_{50} values, however reasons for this remain unclear. A sphere-shaped body, offering less surface area to the surrounding media in comparison to the biovolume, correlated negatively with k_{in} uncorr ($p \leq 0.05$), which supports the hypothesis that the AVratio plays a role for uptake.

The juvenile trait modality of life stage did not show any significant relationships to sensitivity, which is most probably related to a lack of appropriate data, namely only two juvenile life stages were tested altogether, of which one was not differentiated for the 48 h $L(E)C_{50}$ values from their adult stage. For the latter sensitivity endpoint as well as for the BCF_{ww} the observed correlation with adult life stages is likely a size artifact as well, since adults of the tested species are larger. Other physiological traits, which can be different in juvenile and adult life stages may also be responsible for differences in the sensitivity observations. Larvae or nymphs of insects seem to be less well-able to eliminate ($p \leq 0.05$) than other life stages. However, this variable is correlated (correlation coefficients between 0.513 to 0.789) with seven other trait modalities (LipDW, ResReg, ResMoPig, SclPoor, SclComp, BauCyl, Ladult) and therefore this has to be recognized, but treated with caution.

The trait modalities PhyRES and PhyEQ, which were intended to approximate the phylogenetic position of the investigated test species both significantly ($p \leq 0.05$) correlate with k_{out}. This interesting result was already observed in the PCA and suggests that the earlier an arthropod clade separated from the common arthropod ancestor, the better the elimination abilities for chlorpyrifos of its associated recent species are. This result however has to be intrepreted with care, since the quantification of phylogeny as presented here lacks profound investigation and validation. Buchwalter et al. (2008) used phylogenetic signal (Blomberg *et al.* 2003) to relate species phylogenetic similarity to ecophysiological (toxicokinetic) traits, but for such an analysis a larger sample size (21 species) is necessary. It was decided to include a general indicator of phylogeny, rather than no information at all, into the presented approach, because the role of phylogeny as mentioned several time in the previous chapters and in various publications about traits-based approaches, is crucial for the understanding of clustering and importance of traits for faunal responses (Poff *et al.* 2006; Buchwalter *et al.* 2008; Rubach *et al.* in press a). Currently, it is difficult to reliably resolve the phylogeny of arthropods without qualified measurement, since the body of literature is immense and the number of approaches similarly large. Very specialized expert knowledge on phylogeny of arthropods and eventual additional measurements for the species investigated, e.g. 16S and/or 28S rRNA (Timmermans *et al.* 2008) would be necessary to finally solve this issue.

Exoskeleton thickness was significantly related to the k_{in} *uncorr*, the 48 h LC_{50} ($p \leq 0.1$) and k_{out} ($p \leq 0.05$), while for the first two a positive and the latter sensitivity endpoint a negative relationship

was observed. The correlation with the 48 h LC_{50} suggests that a thick exoskeleton is related with insensitivity, although the relationship with k_{in} *uncorr* indicated increasing concentrations with increasing exoskeleton thickness. Furthermore, it seems that a thick exoskeleton decreases the speed of elimination as perhaps might be expected. The trait water content was unexpectedly not significantly related to any of the sensitivity endpoints investigated for reasons that currently remain unclear. Potential inconsistencies in some of the discussed observations might be related to the consideration that trait combinations may be more informative than single traits (Rubach *et al.* 2010, Chapter 3).

Multiple regressions – combinations of traits

The results of the forward multiple regressions (Table 7) will firstly be described below in relation to the sensitivity endpoints, and combinations of up to four traits with high explanatory potential will be indicated. Subsequently, these results will be discussed together with the results and findings from the single regressions and the PCA. The observed significant trait combinations are numbered in Table 7 and the text refers to these numbers together with listing the relevant traits. As above, the term 'significant' is used in reference to an accepted 5% probability for type I errors ($p \leq 0.05$), while 'moderately significant' refers to 10% accepted error probability ($p \leq 0.1$) due to the anticipated high levels background variation in the combined datasets.

For k_{in}, eleven significant and two moderately significant trait combinations were found, of which seven were pairs, three were triplets, and another three were quadruplets. The highest explanatory potential (adj. $R^2 = 0.869$) was found for the quadruplet combination (11) with the traits SurfArea, TroDetr, SOatm and PhyRES. Also the triplet (8) and the pair (1) of the same first two or three traits alone explained 83 % or 73.1 % of the variance in k_{in}, respectively. Another combination (9) that explains 72.9% of the variance in k_{in} is composed of the SurfArea, LipFW and SOatm. The lowest amount of variance ($R^2 = 0.494$) in k_{in} was significantly explained by ResMoCoG and Ljuv. The trait SurfArea appears the most often in all combinations, followed by SOatm, TroDetr, Ljuv, ResMoCoG, ResMoInG, LipFW, PhyRES, WatCont, BauBox in this order.

For the uncorrected uptake rate constant k_{in} *uncorr*, eleven trait combinations were identified in three pairs, two triplets and six quadruplets. The quadruplets explained the highest variation, but were mostly only moderately significant. The highest explanatory, but only moderately significant potential was (20), the quadruplet of SOdiss, LipFW, Length and ResMoInG with an adjusted R^2 of 0.802. Similarly, although explaining less variance in uptake, but significantly affecting the outcome, it was found that when LipFW was substituted with a TroHerb (19) explanatory power increased. Length and SOdiss also explained 74.9 – 77.7 % variance when combined with ResInt and either BauSphe, TroHerb or PhyRES (22-24). The trait pair of ExoTh and SclComp (16) was found to be highly significant, but explained only 39.9 % of the variability in k_{in} *uncorr*. The traits influencing k_{in}*unorr* were dominantly Length and SOdiss, followed by BauSphe, LipFW, ResInt, SclComp, ResMoInG, TroHerb, and PhyRES in this order.

Table 7 (continued on next page): Results of multiple linear regressions (F statistic of combinations of trait modalities for each sensitivity endpoint,)

Sensitivity endpoint	Combination Nr.	Nr. of trait terms	Adj. R2	SurfArea	SOdiss	ExoTh	LipFW	Length	Leadlt	LipDW	TroDistr	SolComp	ResInt	ResMobHG	PhyIEQ	SOstrm	TroCom	SolPoor	BauSphe	BauBox	ResMobDG	Ljuv	ResMobPig	TroHerb	Llarmy	PhyIRES	WatCont	ResMobEG
k_{in} ($L*kg_{ww}^{-1}*d^{-1}$)	1	2	0.731 **	< 0.0001							0.006																	
	2		0.652 **	0.001												0.043												
	3		0.65 **	< 0.0001			0.045																					
	4		0.645 **	< 0.0001																						0.05		
	5		0.55 **								0.009			0.002														
	6		0.494 **											0.001								0.022						
	7		0.635 *																	0.063								
	8	3	0.83 **	< 0.0001							0.002					0.01												
	9		0.729 **	< 0.0001			0.043									0.042												
	10		0.712 *	0.001												0.018						0.068						
	11	4[a]	0.869 **	< 0.0001							0.001					0.002										0.046		
	12		0.798 **								0.01					0.005					< 0.0001	0.013						
	13		0.752 **													0.024					< 0.0001	0.001					0.04	
k_{in} uncorr. ($L*d^{-1}$)	14	2	0.67 **		0.021			< 0.0001																				
	15		0.408 **									0.034							0.017									
	16		0.399 **			0.019						0.022																
	17	3	0.755 **		0.003		0.031	< 0.0001																				
	18		0.734 *		0.008			< 0.0001											0.058									
	19	4	0.779 **		0.003			< 0.0001						0.032										0.032				
	20		0.802 *		0.001		0.015	< 0.0001						0.066														
	21		0.794 *		0.002		0.049	< 0.0001											0.088									
	22		0.777 *		0.003			< 0.0001					0.085						0.032									
	23		0.752 *		0.01			< 0.0001					0.058													0.068		
	24		0.749 *		0.005			< 0.0001					0.076												0.073			
k_{out} (d^{-1})	25	2	0.537 **										0.01		0.001													
	26		0.463 **		0.012	0.08																						
	27		0.427 **			0.03						0.019																
	28		0.418 **		0.034							0.015																
	29		0.383 **		0.021															0.023								
	30		0.4 *			0.077									0.027													
	31		0.39 *			0.055																	0.031					
	32		0.336 *		0.054																			0.041				
	33	3[b]	0.71 **		0.002	0.002						0.003																
	34		0.65 **		0.034								0.003		0.002													
	35		0.623 **		0.004							0.003	0.012															
	36		0.589 **			0.004				0.024		0.002																
	37		0.563 **							0.004										0.007				0.006				
	38		0.534 **			0.013				0.038													0.005					
	39	4	0.663 **			0.022				0.04										0.031			0.005					
	40		0.666 **			0.045				0.02										0.012				0.005				
	41		0.756 *		0.001	0.015						0.001	0.088															
	42		0.757 *		0.008	0.001				0.085		0.001																
BCF_{lipid} ($L*kg_{lipid}^{-1}$)	43	2	0.67 **	0.002			< 0.0001																					
	44		0.533 **				0.001											0.026										
	45		0.509 **	0.004																	0.006							
	46		0.467 **								0.002				0.003													
	47		0.46 *				0.003		0.083																			
	48	3	0.735 *	0.005			< 0.0001											0.056										
	49		0.733 **	0.002			< 0.0001		0.058																			
	50		0.597 **				0.035				0.013				0.007													
	51	4	0.798 **	0.003			0.002				0.011				0.007													
	52		0.752 **	0.005							0.004				0.002						0.008							
	53		0.781 *	0.001		0.077	< 0.0001											0.032										
	54		0.738 *	0.001			< 0.0001						0.065		0.087													
BCF_{ww} ($L*kg_{ww}^{-1}$)	55	2	0.521 **	0.001															0.041									
	56		0.469 **						0.015					0.006														
	57		0.482 *	0.005					0.077																			
	58	3	0.56 *	0.004					0.028	0.084																		
	59		0.538 *	0.015						0.065								0.041										
	60	4	0.736 **	< 0.0001		0.007				0.004								0.005										
	61		0.697 **						0.001	0.006				0.001													0.016	
	62		0.649 **						0.002	0.028				0.002										0.042				
48h LC_{50} ($\mu g*L^{-1}$)	63	2	0.47 **								0.003																	0.004
48h EC_{50} ($\mu g*L^{-1}$)	64	2	0.41 **					0.009									0.023											
	65		0.375 **					0.041	0.036																			
	66		0.309 **														0.031	0.031										
	67		0.304 *						0.033								0.1											
	68	3	0.596 **					0.005	0.017								0.011											
	69		0.531 *					0.016									0.06	0.052										
	70		0.404 *			0.087			0.015								0.062											
	71		0.404 *						0.016					0.09			0.038											
	Frequency of significance:			21	18	15	14	13	12	11	10	9	8	8	8	7	7	7	5	5	4	4	3	3	3	3	2	1

[a] no combinations with $p \leq 0.1$found

(continued from Table 7)

[b] only combinations with $p \leq 0.05$ shown, all analyzed subsets were significant.
Note:
- *p* value reports the F statistic, notation for significance: * = $p \leq 0.1$ and ** = $p \leq 0.05$.
- adj. R^2 = adjusted coefficient of determination, percent variance accounted for by trait combination.
- Never in a significant combination: Biovol, AVratio, Dry Mass, ResConf, ResReg, ResMoSip, TroOmni, SclGood, BauCyl, BauCone.
- LipTot and ResMocut, were not included in analyses (see text for explanation).
- Grey shaded areas depict the most significant combination for each sensitivity endpoint.

Out of the eighteen different trait combinations identified for k_{out}, thirteen were significantly and five moderately significantly related, explaining between 33.6 and 75.7 % of the variance in elimination rate constants. The highest explanatory power was found for one out of the four trait quadruplets (42; SOdiss, ExoTh, LipDW, SclComp) and the lowest was found for one out of the eight trait pairs (32; SOdiss, Llarny), while the six triplets showed adjusted R^2s between 0.534 and 0.71.

The trait pair SOdiss and ExoTh (26) together explained 46.3 % of the variance in elimination and each of these two traits appeared in one of the other significant pairs together with other traits (i.e. SclComp, PhyEQ, BauBox ResMoPig and Llarny), but they then explained less variance. The trait pair explaining the most variance was ResInt together with PhyEQ (25, R^2 = 0.537). The power of these traits increases when combined with SOdiss (34), although the triplet of SOdiss, ExoTh and SclComp (33) explains more variance (R^2 = 0.65 and 0.71) respectively. The trait LipFW emerges as important in combinations with three and four traits. Clearly, the dominant traits for k_{out} were ExoTh and SOdiss followed by SclComp, LipFW, ResInt, Bau Box, PhyEQ, ResMoPig and Llarny in this order.

For the BCF_{lipid}, eleven different combinations of traits (5 pairs, 3 triplets and 4 quadruplets) were found with eight being significant and four being moderately significant, explaining between 46 and 79.8 % of the variance in lipid corrected bioconcentration. The most explanatory power was again found for a quadruplet (51; Surf Area, LipFW, TroHerb, PhylEQ) and the least for a pair (47; LipFW, Ladult). SurfArea and LipFW appeared consistently in almost all significant combinations and are, although artifacts of size and lipid correction as discussed above, important, but not surprising. Other interesting combinations involve the pair (46) with TroDetr and PhyEQ, explaining 46.7 % variance and the other traits emerging throughout the analyses, which are SclPoor, ResMoCoG, ResInt and Ladult. Dominant traits for BCF_{lipid} were LipFW and SurfArea, followed by PhyEQ, TroDetr, Scl Poor, ResMoCoG, Ladult, ResInt and ExoTh.

For the BCF_{ww}, eight combinations of traits were found, of which five were significant and three were moderately significant. Also a trait quadruplet (60; SurfArea, ExoTh, LipDW, SclPoor) explained the most variance in wet weight based bioconcentration (73.6 %) , while a pair (56; Ladult, ResMoInG) explained the least (46.9 %). In addition to the SurfArea also Ladult and LipDW frequently appear in significant combinations. For instance, the quadruplet (61) with Ladult, LipDW, ResMoInG and WatCont explains 69.7 % of the variance significantly. If the water content in this combination is substituted with TroHerb, somewhat less variance can be explained (64.9 %). The dominant traits for BCF_{ww} were SurfArea, Ladult and LipFW followed by ResMoIng, SclPoor, ExoTh, WatCont, TroHerb and BauSphe in this order.

For the classical sensitivity endpoints, both the 48 h $L(E)C_{50}$s together nine combinations of traits were found to be significantly related, while only one pair of traits (63, TroDetr, ResMoExG) correlated with the 48 h LC_{50} significantly (R^2 = 0.47). The other eight combinations were four pairs or triplets of trait combinations explaining between 30.4 and 59.6 % of the variance in 48 h EC_{50} measured of which four trait combinations were significant and the other four moderately significant. The most variance by pairs is explained with the combination of Length and TroCarn (adj. R^2 = 0.41). Next to these two traits, also Ladult and SclPoor have explanatory potential in all possible combinations of these four traits. The highest correlation was detected for the triplet Length, Ladult and

TroCarn (adj. R^2 = 0.596) followed by the triplet Length, TroCarn and SclPoor, explaining 53.1 % of the variance, however, only moderately significant. Also the other traits listed for the trait pairs above emerge here again in combination with ExoTh and ResMoInG. The traits most dominant in relation to the 48 h EC_{50} were TroCarn followed by Ladult, Length,SclPoor, ExoTh and ResMoInG in this order.

General discussion

The results described above and shown in the Figure 2 and Tables 5, 6 and 7 show that both single traits and combinations of traits have high explanatory potential for sensitivity, as predicted by Baird & Van den Brink (2007) and more specifically for process-related endpoints of sensitivity, such as the toxicokinetic parameters as hypothesized by Rubach *et al.* (2010, Chapter 3). The amount of variance in sensitivity endpoints that could be explained using single traits ranged between 0.1 and 56 %, whereas for the selected combinations of traits, much more (30.4 to 86.9 %) of the variance in sensitivity endpoints was explained. This is not due to simply adding one more variable, since for multiple regressions the adjusted R^2 was analyzed, which only increases when the variable itself increases the explanatory power of the combination. The highest adjusted R^2s were always seen for the trait quadruplets, whereas the lowest was observed for trait pairs, but among all significant trait combinations the number of interactions terms was variable and no dominance of pairs, triplets or quadruplets was found. In Table 7 the frequency of significant trait occurrence across all analyses is given for each trait in order to rank their importance as explanatory factor for sensitivity. Some traits, such as SOdiss, ExoTh, Ladult become more important overall when analyzed in combinations rather than as single traits, and others played a significant role only in combination (TroCarn, Ljuv, TroHerb, WatCont). These results illustrate that our understanding and therefore ultimately the predictability of sensitivity can be enhanced by combining species traits as predictors instead of using only one variable. This may sound somewhat trivial and intuitive because these results are due to the nature of the chosen analysis. However, it does show that the methods chosen reflect the intuitive understanding of how sensitivity differences emerge from trait patterns and therefore provide tools for describing this quantitatively, which can be integrated in effect models as outlined by Rubach *et al.* (in press a, Chapter 2). In order to avoid misinterpretation through statistical bias, the present study only focused on combinations up to four trait interaction terms, because the underlying dataset consisted only of 17 species. The explanatory potential of trait combinations could be enhanced in the future by extending such datasets and the identification of combinations of traits with five or more interaction terms.

Profound differences were seen between the general sensitivity endpoints and the toxicokinetic endpoints. Firstly, the maximum variance explained by single traits was the highest for the uptake rate constants (56% by SurfArea) and the lowest for the 48h EC_{50} (20.6% by Ladult), which was similar when combinations of traits were used (86% for k_{in} versus 47% for the 48h LC_{50}). Secondly, the number of (moderately) significant trait combinations was the highest for k_{out} (18 combinations) and the other endpoints related to the toxicokinetics (8 - 13) in contrast to the EC_{50} (8 combinations) and the LC_{50} (1 combination). Thirdly, the single traits, but also the combinations of traits, found to be significantly correlated with the general toxicity endpoints did not clearly corroborate the understanding of toxicity. Nevertheless, they do confirm the premise that size and life stage are

important factors for sensitivity and also point that detritivore and carnivore trophic relation, poor sclerotisation, exoskeleton thickness and respiration mode may play a role in sensitivity.

The correlation of traits with the toxicokinetic sensitivity endpoints establish a much more mechanistic understanding of how these traits influence these processes and therefore contribute to sensitivity. For instance, among the size-related traits, organism length explained uptake most successfully, however when uptake was corrected for fresh weight, surface area was a better predictor, indicating that another spatial parameter plays an important role in these processes and emphasizing adsorption. Uptake likely is a diphasic kinetic, consisting of a rapid adsorption isotherm coupled with a slow penetration of the toxicant across the cell membrane or interstices. Also the traits LipFW and LipDW, which were individually only correlated with the BCF_{lipid}, were found to be important in combinations of three or four traits for uptake, elimination and bioconcentration. Similarly, the source of oxygen for respiration or two modalities for the mode of respiration (ReMoCoG and ResMoInG) enhanced the explanatory potential, when combined with the traits previously mentioned (see combinations 9 and 21). These results are in accordance with literature as size has been proven to be an important factor for uptake (e.g. Hendriks *et al.* 2001; Arnot & Gobas 2004; Weiner *et al.* 2004; Hendriks 2007), as well as lipid content (e.g. Barron 1990; MacKay & Fraser 2000; Hendriks *et al.* 2005) and respiratory modalities (e.g. Buchwalter *et al.* 2002, 2003). Other traits which played a role in our analysis such as detritivore or herbivore trophic relation, spherical or box shaped bauplan, complete sclerotization and high resolution phylogeny may also play an additional role in combination with these traits, however further investigation of these traits should be supported by further empirical observation. Other important traits related to bioaccumulation and the food uptake path were not considered here, because test animals were not fed, however several authors indicate that this pathway and its related traits such as gut passage (Gaskell *et al.* 2007; Gergs & Ratte 2009) are important and need to be considered in ERA.

For the toxicokinetic process of elimination a very different trait pattern was observed. Size-related traits were not important for this process, neither indvidually nor in combination. Instead exoskeleton thickness, using dissolved oxygen for respiration, being completely sclerotized and the % lipid of fresh weight (combination 42) were of importance in addition to several other traits (BauBox, PhyRES, ResMoPig, ResInt and Llarny). Remarkably these traits appear significant in all different combinations and in this case likely interactions with more than four traits might be applicable and appropriate. Elimination abilities have yet not been subject to many comparative studies and are mostly related to physiological traits investigating detoxification, hence relating enzyme activities and effect responses with (Chambers *et al.* 1994; Chambers & Carr 1995; Printes & Callaghan 2004; Domingues *et al.* 2010). As no detoxification traits were included in this study (see above), it is difficult to compare the results found to existing knowledge in literature. It is quite likely that such traits would show high explanatory potential if meaningfully quantified (Chambers & Carr 1995; Eaton *et al.* 2008). The traits that were found to be important in this study however might also contribute to elimination, especially because the elimination rates measured are assumed to address the steps after detoxification. It is interesting that phylogeny as a single trait is highly correlated with elimination, because it might indirectly describe well conserved genes related to detoxification, which are known to exist for drug targets (Gunnarsson *et al.* 2008). This result is also in line with the findings of Buchwalter

et al. (2008), who showed that phylogeny can predict uptake, but even more so elimination of cadmium in Ephemeroptera, Plecoptera and Trichoptera. However also evidence for independent evolutionary invention of enzymes has been described (Galperin *et al.* 1998). Also, phylogeny will not be able to describe cases where uncommon enzymes, metabolic pathways or modifications cause lower sensitivity.

The traits which explain variations in both BCF endpoints are without exception a combination of the traits which were found also to be important predictors of uptake and elimination rate constants. As the BCF is the quotient of these two parameters, this result is not surprising and also provides confidence in the approach and the robustness of the methods chosen. As these toxicokinetic rate constants are dynamic in time, describe separate processes, and deliver more distinct trait patterns than both BCFs, they should be preferentially used for traits-based approaches.

The classical sensitivity endpoints, namely the LC_{50} and EC_{50}, were more difficult to relate to traits directly, either using individual traits or trait combinations. The individual variables only confirm size (Length, Ladult) as an important trait, being related to the 48 h EC_{50} and also identify exoskeleton thickness as being related to the 48 h LC_{50}. Combinations of traits did not improve the situation for the LC_{50}, where only one trait pair was found (TroDetr, ResMoExG), and this was not highly explanatory. For the 48 h EC_{50}, trait combinations could explain more of the variation found for this response variable. A carnivorous trophic relation, adult life stage, length, poor sclerotisation, an internal gill as well as the exoskeleton thickness appeared to be important explanatory factors in all possible combinations. This result is more informative than the results on individual traits and the results on the 48 h LC_{50}, although it still lacks the strong explanatory power of the process based sensitivity endpoints.

The demonstrated lack of suitability of the classical sensitivity endpoints to be related to traits is interesting also in comparison to Rubach *et al.* (2010, Chapter 3), who employed an analogous approach to link the largest available existing toxicity dataset, 24 – 96 h $L(E)C_{50}$ with existing ecological trait information for two mode of actions and three chemical classes. They were unable to extract consistent trait patterns using a similar methodological approach and concluded that other relevant traits need to be identified and quantified and the processes of toxicity must be considered separately in order to find relations of traits and sensitivity, which can serve as predictors in the future. In this study these conclusions were tested and fully supported.

Intrinsic sensitivity – a result of toxicokinetics, toxicodynamics and traits

As outlined in the previous chapters and briefly mentioned above, the intrinsic sensitivity response of a species is not only a result of the toxicant's toxicokinetics, but also its toxicodynamics in this species. Although substantial experimental and theoretical work with a strong emphasis on toxicokinetics and to a lesser extent on toxicodynamics is available in the literature, it is currently unclear if these processes are equally important for intrinsic sensitivity. Certainly, the situation is even more complex since the physico-chemical properties of a toxicant (Escher & Hermens 2002) and also its mode of action (Verhaar *et al.* 1992; Verhaar *et al.* 1996) are also strong determinants of sensitivity. For different chemicals, a large body of work has been carried out in the last two decades, and the integration of this is discussed in more detail later in this chapter. The present thesis experimentrally focused on one

model compound with a specific mode of action and aimed to quantify the variation in sensitivity, in toxicokinetics and in toxicodynamics across different species to finally link this to species traits in order to test their suitability as predictors for these processes and to learn more about the causes of differences in sensitivity. The experiments carried out to parameterize the toxicokinetics of chlorpyrifos in 17 freshwater arthropods showed that a large amount (50 – 60 %) of the variation in sensitivity (48h EC_{50}) can be explained by the toxicokinetics, i.e. by uptake (32%) and elimination (28%) (Rubach *et al.* in press b, Chapter 5). This estimation is associated with some uncertainty since biotransformation and therefore detoxification are not included. Therefore, toxicokinetics might have on the one hand even higher explanatory potential. On the other hand, the explanatory power might be somewhat overestimated due to adsorption on the outer body parts which both may not necessarily contribute to the concentration at the target site (see above). Despite this uncertainty these values can be used as an indication for the role of the toxicokinetic processes for intrinsic sensitivity. The remaining variation (40 – 50%) might therefore be attributed to biotransformation or toxicodynamics, namely to the amount of tissue and molecular damage induced, the ability to repair and recover from this damage and also being able to cope with a certain internal damage until a threshold is exceeded and effects become visible on the organism level. It has to be kept in mind that toxicodynamics are not independent from the toxicokinetics, since the concentration at the target site is needed to model these processes and therefore depend on an accurate estimation of the toxicokinetics. In Chapter 6 of this thesis, an evalution of the toxicodynamics of chlorpyrifos in three arthropods was made, for which the toxicokinetics had been parameterized before (Rubach *et al.* in press b, Chapter 5) and this information was used to design the study and also to model the toxicodynamics. Despite substantial difficulties with model parameter estimation, both the experimental and the modeling results show that major differences exist in the survival responses to pulsed exposures and the toxicodynamic parameters for these species. The major conclusions of this chapter with respect to the toxicodynamic variability observed for the tested species were that: (i) damage thresholds differ across species (low for *Chaoborus obscuripes* higher for others) and are likely to follow individual tolerance distributions (*Asellus aquaticus*), (ii) killing rates are different for the endpoint survival, but likely not so different for sublethal responses such as immobility (delays in survival responses are different per species, but not for immobility responses), if chlorpyrifos is acting via acetylcholinesterase inhibition, (iii) the recovery and repair abilities vary across freshwater arthropods (fast recovery in *A. aquaticus* and *N. denticulata*,, slow recovery in *G. pulex* and no recovery in *C. obscuripes*) (iv) in the crustacean *N. denticulata* chlorpyrifos likely does not exhibit acetylcholinesterase inhibition, but seems to act via narcosis. These findings can be explained and related qualitatively using processes and traits. The processes that play a role in explaining these differences include target site encounter rates, target site constitution (or binding affinity of toxicant to target site), induced (secondary) damage on target/ tissue/ DNA, biotransformation (belonging to the toxicokinetics; Phase I and II reactions; for chlorpyrifos leading to both biotoxification and detoxification) and compensation mechanisms (repair of disrupted enzymes, tissue and DNA; gene regulation, amplification and expression; aging; general and specific stress response systems). In our study the latter two processes cannot be clearly distinguished in reference to the effects measured, because metabolites were not characterized. Furthermore, stress response systems are very complex and interact with both processes of biotransformation and

compensation (Korsloot *et al.* 2004). For instance, part of a stress response is the up-regulation of genes for enzymes involved in Phase I reactions, such as monooxygenases (cytochrome p450 for chlorpyrifos) and glutathione-S-transferase (Korsloot *et al.* 2004), but also the up-regulation of genes to replace the inhibited target enzymes themself, induced by their own dysfunction (Raymond *et al.* 1989) or other even more complex mechanisms (Curtin *et al.* 2005). However, using the elimination rates provided by Rubach *et al.* (in press b, Chapter 5) despite their uncertainty, some conclusions on the dominant general processes can be made. For *A. aquaticus* and *C. obscuripes* moderate elimination rates were found, whereas *N. denticulata* and *G. pulex* showed high elimination rates (Rubach *et al.* in press b, Chapter 5). The former species of the two groups are insensitive to chlorpyrifos and the latter of the two groups are sensitive (Rubach *et al.* in press c, Chapter 4). Therefore the high and spontaneous recovery found for the two insensitive species (*A. aquaticus*, *N. denticulata*) in contrast to the sensitive species with lower or no recovery, could be related to differences in biotransformation (both bioactivating and/or detoxification) and/ or good compensation. Low or zero recovery rates however, might relate to other traits, which overrule physiology, such as the hydrostatic vesicle in *C. obscuripes*, where no individual recovery was found due to disruption of the function of this structure. The differences in latency (delay) of endpoint responses however clearly indicate that differences in compensation exist between these species and that they play a major role in their survival response, because the effect on the target has occurred when an animal is immobilized. If the hypothesis that chlorpyrifos does not exhibit acetylcholinesterase inhibition in *N. denticulata* is correct, a clear target site insensitivity may be the reason for insensitivity (Walsh *et al.* 2001) and the damage induced by narcosis is tackled with good compensation leading to high recovery. On the other hand, it is also possible that the biotoxification in this species does not occur via the oxon metabolite, due to the existence of different cytochrome p450 isoenzymes. Alternatively, the oxon is immediately detoxified by highly active A-esterases as known for resistant cockroaches (Roberts 1998). Many of the previous considerations originate from research that has been performed on resistance, which is attributed to mechanisms such as target site insensitivity, increased metabolic detoxification and sequestration or lowered availability of the toxicant induced on the molecular level by mostly point mutations in or amplification of relevant genes (Feyereisen 1995; Taylor & Feyereisen 1996; Hemingway *et al.* 2004; Enayati *et al.* 2005). The knowledge and research from resistance studies may help to direct future traits-based approaches in order to focus on certain molecular and physiological traits, which are known to cause resistance against pesticides. In combination with the development of rapid and readily usable molecular tools in the future the presence/absence of target sites or detoxification enzymes, their different constitutions or families and maybe also compensation and regulation abilities may be more easily measured in different species and then expressed as traits in a similar way as done in the present study. For instance, it might theoretically possible to define entities of the genome as a DNA barcode (Valentini *et al.* 2009) not for a species, but for phenotypic traits such as the presence/absence of an enzyme or isoenzyme identified to be important for resistance. As a next step, that kind of trait data can then be linked quantitatively to toxicodynamics and survival analysis and thereby to intrinsic sensitivity, which would increase the trait-based predictability of the latter. Arthropods and organophosphates are a good study system to develop such

techniques, because the body of literature on resistance as well as on effects is relatively comprehensive.

TRAITS AND THE MODE OF ACTION AS PREDICTORS FOR SENSITIVITY

As briefly mentioned earlier, not only species, but also chemicals vary in their properties and their mechanisms and modes of action, and this has to be taken into account in predictive approaches, since these are only useful if they reduce the amount of experimental testing needed to perform a risk assessment. In the previous chapters of this thesis, the experimental work highly focused on a single model compound in order to test the suitability and applicability of the theoretical concept described in Chapter 2 (Rubach *et al.* in press a). However, it is well known that the physicochemical properties of chemicals highly influence the toxicokinetics and the mode of action influences the toxicodynamics. Predictive approaches across chemicals for toxicokinetics and internal distribution, i.e. QSARs, Adsorption Distribution Metabolism Excretion (ADME) models and Physiologically Based Pharmacokinetic (PBPK) models are based on physicochemical properties (mostly the log K_{OW} and the molecular size) and are well established (Van de Waterbeemd & Gifford 2003). These type of models sometimes also include optional traits of the organism of interest, such as size, lipid content, organ structure and blood and as a result contain well-established links between some biological traits and physicochemical characteristics of toxicants. For many substances often the primary mode of action is known, especially for pesticides and therefore the molecular target can also be well defined. If the molecular target can be expressed as a meaningful trait, similar links between the mode of action and traits could be established. Together this would result in a matrix where both, the physicochemical properties and the mode of action of a substance are linked to traits. In addition to the matrix with quantitative trait – model parameter relationships (Rubach *et al.* in press a, Chapter 2), all toxicological relevant information for extrapolation across chemicals and species would be assembled and could be fed into effect models, either predicting organism (i.e. bioconcentration, bioaccumulation, survival) or population level responses. Another less mechanistic approach would be to construct an Artificial Neural Network (ANN), with at least three neuron layers: one for mode of action and physicochemical properties, one for traits and one or more for responses. The axons represent the relationships between the different layers, which would be partly known. The systematic use of traits in ecotoxicology is therefore not only attractive for the potential extrapolation between species alone, but also in combination with extrapolation between chemicals.

Another important aspect which needs to be addressed are the possibilities of how the observed relationships between traits and model parameters can be quantitatively established in such a way that they can be incorporated into effect models. Earlier in this chapter the explanatory power of single traits and combinations of traits has been evaluated on the basis of the R^2 and adjusted R^2, respectively and the significance of the relationship between the traits or trait combinations and the model parameter investigated. Since these measures can be understood as the amount of variance in the response variable explained by the single trait or that specific set of traits compared to the other tested sets of traits these values are potentially useful to be combined with the model parameters of the used effect model, however not directly. In order to be meaningful, the influence of the traits

involved need to be weighted, e.g. on basis of their significance in the combination. Another important parameter, which needs to be included is the nature of the relationship, which can be included using the slope of the respective single linear regression. How exactly the quantitative trait parameter relationships can be established is at this time unclear and should be the topic of future research.

IMPLICATIONS FOR ENVIRONMENTAL RISK ASSESSMENT

Environmental Risk Assessment focuses on the protection of the health of natural systems (i.e. air, water, land with fauna and flora) from potential risks. This involves several challenges because natural systems consist of many very different entities and levels of organization. Despite the ongoing debate about explicit protection goals, ERA and legislation aim at estimating and assessing ecosystem and population level risks and rarely consider the organism level, as is common practice in human health risk assessment. The research presented in this thesis has been performed at the individual organism level and has focused on the determination of intrinsic sensitivity, posing the question of how relevant such approaches are for ERA. Irrespective of the fact that current ERA practices, at least on the first and second tier, are based on empirical data gathered at the organism level, knowledge and research performed at lower levels of organization than the population, community and ecosystem level is pivotal for the identification of hazard mechanisms, which can be the only basis for truly protective ERA. In addition, the organism level is the main entity in individual population models (IBMs), which prove to be promising tools for the prediction of population level dynamics and effects (Galic *et al.* 2010). As a consequence, the better the organism level is explored, understood and described, the more realistic predictions of such population models will be. For instance, internal concentrations, which significantly determine the adverse effect (Meador *et al.* 2008) cannot be estimated for populations directly without considering the organism level. Therefore, knowledge about the intrinsic sensitivity at species level with its underlying processes and factors is important for a holistic and dynamic ecological risk assessment in the future.

A second aspect as to why predictable intrinsic sensitivity at the species level is attractive for ERA relates to the fact that, potentially, every chemical can be harmful for some species under certain conditions, but does not necessarily have to be harmful for every species. This means that simply too many chemical/species/conditions combinations exist, which potentially need to be tested. This is altogether under ethical, practical and financial considerations not acceptable and virtually impossible. If intrinsic sensitivity becomes meaningfully predictive by extrapolating adverse effects at the organism level on basis of species and chemical traits, then the amount of experimental toxicity testing needed and therewith the unnecessary use of test animals could be reduced substantially. In fact, when looking at higher organizational levels, such as the community or the ecosystem it is convenient to treat intrinsic sensitivity itself as a trait, which is done in several indices used in biomonitoring. A good example for this the SPEAR index (Liess & Von Der Ohe 2005), which uses experimentally-determined toxicity data standardized on *D. magna* in combination with life-history traits to indicate the proportion of species at risk at a specific field site. Therefore, for mechanistic ecotoxicology, prospective ERA, and also retrospective risk assessment (biomonitoring) and approaches which aim at diagnostics of

pertubations it would be a major advancemet in the field if intrinsic species-related sensitivity could be made more predictive.

The proposed approach for the use of traits in predictive ecotoxicology has several intriguing advantages, but also many shortcomings and disadvantages. The general shortcomings associated with traits are discussed in the Chapters 1,2 and 3 of this thesis (Baird *et al.* 2008; Rubach *et al.* 2010; Rubach *et al.* in press a) and in several other publications about traits-based approaches e.g. (Usseglio-Polatera *et al.* 2000b; Poff *et al.* 2006; Baird & Van den Brink 2007; Statzner & Bêche 2010; Baird *et al.* in press; Culp *et al.* in press; Van den Brink *et al.* in press b). Statzner & Bêche (2010) recently reviewed the limitations and potentials of biological traits as indicators (BTIs) for multiple stress responses, addressing both diagnostics of multiple stress in lotic systems and *a priori* predictions based on traits. Van den Brink et al. (in press b) report the results of a more general SWOT analysis (Strength, Weaknesses, Opportunities and Threats) during the SETAC-TERA workshop 2009 on traits-based ecological risk assessment (TERA). To address this here briefly, advantages of BTI and TERA are related to the improvement in prediction and diagnostics of effects through such methods, the addition and integration of (mechanistic) knowledge from different fields of science to management and risk assessment, the stability of traits across biogeographical regions, biomes and ecosystems and that these approaches can build on existing taxonomic data and do not require new sampling. The disadvantages are mostly associated with data management and data availability, unknown *a priori* relevancy or redundancy of some traits, quantification of physiological traits on molecular level, autocorrelation of traits (functional or phylogenetic) and that biodiversity cannot be protected directly with this approach, because traits describe functions. The approach for intrinsic sensitivity, presented and partly realized in this thesis shares these aspects, but has additional pros and cons. The most intriguing advantage is the potential for interspecies extrapolation of effects at different levels of organization using the properties of organism and chemicals in combination with process based effect models, especially IBMs as discussed above. This requires that these effect models are developed and improved in conjunction with research on traits and development of TERA, which is considered as an advantage. Furthermore, both traits and effect models can integrate knowledge from different fields of environmental sciences into tools for ERA. For instance molecular knowledge, 'omics techniques and biomarkers could be used not only retrospectively, but also for prospective risk assessment by quantifying this knowledge as traits. The presented concept therefore represents a truly interdisciplinary approach, which can bridge gaps between modelers, 'omics scientists, ecologists and ecotoxicologists. Such an approach may also be interesting from a risk assessment and regulators' perspective in the long run. This touches upon the major disadvantages of the presented approach, as the strongest points are mostly also the greatest weaknesses. The approach presented is ambitious, visionary, has several difficult, complex and unresolved issues and is relatively far away from realization or applicability, which makes it prone to criticism and dismissal for pragmatic reasons. Furthermore, interdisciplinarity, parallel development and integration of detailed knowledge is a big challenge. Therefore the realization and advancement in this field might not be an easy process and requires both a realistic view on possibilities, but also needs to accept some uncertainty and risk of failure. Despite these weaknesses and the long timeline until importance for regulators will emerge, this thesis has demonstrated that the approach has value. Apart from the

approach taken in this thesis many other ways of integrating traits into risk assessment are possible and attractive. Approaches at the (meta)population level using life-history traits of species have been in development for a while and are becoming more relevant for regulators (Galic *et al.* 2010). Also, Van Den Brink (2008) outlined how current risk assessment practices can become more meaningful and has also emphasized how traits can play a role in this, furthermore the use of phylogeny as a predictor in a traits-based context as demonstrated by Buchwalter *et al.* (2008) will be beneficial if incorporated into ERA.

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SUMMARY

One of the key challenges for environmental risk assessment (ERA) is to assess the potential risks of chemicals to the wide range of species in the environment on the basis of laboratory toxicity data derived from a limited number of sensitive standard test species. For prospective environmental risk assessment, these tested species are assumed to be suitably sensitive representatives for the various taxonomic groups, but uncertainty about potential differences in sensitivity is incorporated either through the use of safety factors or the application of species sensitivity distributions (SSDs). The (SSD) concept assumes that the sensitivity of different species can be described by a log-normal statistical distribution. SSDs integrate toxicity data on one chemical for a range of different species and allow estimations of the variation in sensitivity via the slope of the SSD. Effect thresholds can also be derived from this distribution, e.g. the concentration that is hazardous to 5 % of the exposed species (which field data indicate to be usually protective).

The SSD concept has served as a valuable tool for prospective ERA, but since its basis is essentially phenomenological (i.e. simply looking at the range of doses that cause effects), it does little to help improve our understanding of the underlying mechanisms of sensitivity. The toxicological mode of action of a chemical combined with the biological traits of species have been identified as the likely key determinants of sensitivity. However, yet no systematic approach has been developed to combine these factors to generate a more fundamental understanding as to why species differ in their sensitivity. Over recent years, explanations of some of the underlying processes of toxicity have increased through the development of mechanistic effect models. These processes include uptake, biotransformation, distribution and elimination of the toxicant (toxicokinetics), and also molecular mechanisms of internal damage, repair and compensation and effect thresholds (toxicodynamics).

The thesis starts with a prelude (Chapter 1), a letter to the editor of a journal, in which the potential use of traits in ERA is described along with the potential benefits, but also what difficulties need to be overcome to realize their full potential. In theory, traits have applications in both biomonitoring and predictive approaches in bioassessment because they describe function and overcome several limitations of taxonomic approaches. Due to several constraining factors associated with the description and quantification of traits, such approaches will only develop their full potential for bioassessment if a unified, internationally coordinated and substantial effort is made. The quantification and relevance of individual traits needs to be discussed openly on a platform such as Wikipedia where also historical data can be accessed and made available in a flexible way. This, however, requires a shift in research paradigms, data collection and data sharing culture.

The second chapter of this thesis proposes a conceptual framework for the application of traits-based assessment in ecotoxicology. The framework is based on the population vulnerability conceptual model of Van Straalen (1994) in which vulnerability is determined by traits that can be grouped into three major categories - external exposure, intrinsic sensitivity and population sustainability. Within each of these major categories, particular traits and how they could contribute to

the assessment of the potential effects of a toxicant are evaluated. It is then demonstrated (using bioaccumulation as an example model) how traits could be used mechanistically to predict vulnerability, by linking them to process-based model parameters. A preliminary trait inventory for ecotoxicology identifies the availability of data to quantify those traits and gives an indication of the strength of linkage between the trait and the affected process. Finally, the way forward for the further development of traits-based approaches in ecotoxicology is proposed.

The third chapter mines and links existing trait data and toxicity (EC_{50} and LC_{50}) data for three chemical classes with two different modes of actions (organophosphates, carbamates and pyrethroids) for freshwater arthropods in order to evaluate the value of readily available information and to set boundary conditions for the further study. Firstly, a mode-specific sensitivity (MSS) ranking method was developed and tested at the taxonomic levels of family and genus. The application of several quality criteria indicated overall confidence in the MSS rankings, but confidence in exact taxon rank was less certain due to insufficient data for certain groups. The MSS rankings were found to be applicable for traits-based approaches and were successfully linked to existing trait data to identify traits with predictive potential for sensitivity. Although this empirical analysis could not test causality of relationships between traits and sensitivity, testable hypotheses were nonetheless generated. Single traits as well as combinations of traits can be used to predict laboratory sensitivity to the substances tested, although associations were not as strong as in previous studies. It is concluded that existing trait data are not suitable for every traits-based research question and that important traits remain to be identified and quantified in relation to the processes of toxicity, i.e., the toxicokinetics (TK) and toxicodynamics (TD).

In order to explore more deeply the linkages between intrinsic sensitivity and traits, experimental work was focused on the insecticide chlorpyrifos and the phylum Arthropoda (selecting a range of species with a variety of sensitivities and a diversity of traits). The aquatic ecotoxicology of chlorpyrifos has been extensively studied at different levels of organization (molecular to ecosystem) and so was a suitable choice for the research because of the good understanding of the mode of action, and the availability of a range of data to help guide the research questions. Freshwater arthropods were chosen because whilst some species are known to be quite sensitive to chlorpyrifos, there is also quite a range of sensitivity (up to three orders of magnitude) from the most to the least sensitive. This therefore offered some intriguing possibilities for investigating whether a mechanistic traits-based approach could be used to explain these differences.

In Chapter 4, the first part of the experimental program is described in which 24, 48, 72 and 96 h $L(E)C_{50}$ (median lethal (mortality) concentration) and effective (immobilisation) endpoints were derived to describe the sensitivity of fourteen freshwater arthropod species to chlorpyrifos. The relevance of these two endpoints for ERA is then discussed by considering the observed differences in endpoint response dynamics during 96 h of exposure across the species using dose response models and SSD parameters. The variation in response of the freshwater arthropods was less for immobility than for mortality. However, observed differences in immobility and mortality were surprisingly large for

some species, even after 96 h of exposure. Furthermore, since immobility is consistently the more sensitive endpoint, it is the more relevant endpoint for ERA, even though an immobile animal may still potentially recover.

The results from Chapter 4 were expressed as external exposure concentrations (i.e., the concentrations in the test media). Although this is the standard approach in ecotoxicology, such experiments do not allow an assessment of the dynamics of intoxication because they do not express the effects as a dose in the organism, but rather as a concentration in the surrounding media. To this end, data on internal concentrations are needed to better describe toxicologically relevant doses resulting from exposure to the concentration in the surrounding media. Chapter 5 reports the results of seventeen bioconcentration experiments with fifteen species (of which two were tested with two different life stages) and ^{14}C labelled chlorpyrifos. These data were used to parameterize a one compartment, first order toxicokinetic model for each species, in order to investigate how uptake (k_{in}) and elimination (k_{out}) rate constants, bioconcentration factors (BCFs) and 95 % depuration times (t_{95}) vary across the selected species. Extremely slow elimination was observed in some species as well as high overall variation in k_{in}, k_{out}, BCFs, and t_{95} across the tested arthropods. The variation in uptake and elimination was significantly related to the species' EC_{50} values for immobilization at 48 h. Uptake and elimination explained up to 38 % and 28 % of the variation in sensitivity, respectively. The remaining variation in sensitivity is probably related to differences in biotransformation abilities (i.e., detoxification) or toxicodynamic (i.e., target site and repair) processes. These findings indicate that the further development of toxicokinetic - toxicodynamic approaches in ERA is likely to add significantly to our mechanistic understanding of ecotoxicology (e.g., by being able to better assess effects of fluctuating exposure concentrations).

Building on these data, Chapter 6 investigates the toxicodynamic processes related to sensitivity with experiments conducted under time variable exposure and the application of modelling techniques. Exposure of pesticides in the aquatic environment does not result in constant exposures (as used in standard test protocols) but is usually characterised by exposures that fluctuate greatly in concentration and time. Dynamic and mechanistic toxicokinetic-toxicodynamic (TKTD) models for ERA have been used to predict the survival effects of such exposure patterns, while at the same time parameters such as internal damage, repair and recovery, and effect thresholds can also be estimated. Chapter 6 describes how a TKTD model was experimentally parameterized for three freshwater arthropod species for which sensitivity and toxicokinetics data had been developed (as described above). Although there were considerable challenges to overcome, particularly with the robustness of parameter estimates, the combination of experimental and modelling data enabled differences in sensitivity to be explained by differences in the toxicodynamics of chlorpyrifos. The water hog louse *Asellus* for instance was relatively insensitive to chlorpyrifos, even though it showed high levels of bioconcentration. This indicates that *Asellus* is likely to have a sophisticated physiology for detoxification and/or damage repair, compensation and recovery. For the phantom midge larvae *Chaoborus*, a low survival threshold and low levels of recovery were found. Surprisingly, the experiments also revealed that chlorpyrifos may only act via narcosis (baseline toxicity) in the tropical

cherry red shrimp *Neocardinia*, instead of, as anticipated, by its specific mode of action of acetylcholinesterase inhibition. This may explain the observed very low sensitivity of *Neocardinia*, although other atyid shrimps are known to be very sensitive to organophosphates.

The final Chapter 7 of this thesis (the synthesis) brings together the datasets described in the previous three chapters and describes a newly constructed trait dataset that was established for the various arthropod species that were the focus of this research. The aim of this approach was to evaluate the applicability of the proposed traits-based prediction methodology, to instruct future research needs for this field, and to discuss the implications of these results for ERA. Firstly, the population of the trait data set is described and discussed. The traits that were collected were mainly of morphological and physiological nature, but also some ecological traits were quantified. Secondly, toxicokinetic endpoints (k_{in}, k_{out}, BCF_{ww}, BCF_{lipid}) and classical sensitivity endpoints (48 h $L(E)C_{50}$) were quantitatively linked to traits, in a similar manner as presented in Chapter 3, where existing data were mined. In addition, toxicodynamic endpoints were qualitatively discussed in relation to physiological traits, but these were not successfully quantified in this study. The results of the quantitative linkage of sensitivity and traits demonstrated that it is possible to establish meaningful quantitative, mechanistic links between traits and/or trait combinations and process based (toxicokinetic) model parameters. However, links between traits and classical sensitivity endpoints were less meaningful. Future research needs are outlined by a qualitative linking of toxicodynamic parameter estimations and physiological traits in addition to a discussion of how mechanistic trait-process/parameter links can be used for prediction of differences in sensitivity across species and chemicals in the future. The implications of the research presented in this thesis for both prospective and retrospective ERA are discussed, along with the advantages and challenges associated with these approaches. Finally, a rationale is given for the relevance and importance of predicting intrinsic sensitivity for ERA.

SAMENVATTING

Een van de belangrijkste uitdagingen voor de milieurisicobeoordeling (Ecological Risk Assessment; ERA) is het inschatten van potentiële risico's van chemische stoffen voor het brede scala van soorten in het milieu, op basis van laboratorium toxiciteitgegevens van een beperkt aantal standaard testsoorten. Voor prospectief ERA worden deze standaard testsoorten gezien als geschikte vertegenwoordigers van de verschillende taxonomische groepen. Met mogelijke verschillen in gevoeligheid wordt rekening gehouden door het gebruik van veiligheidsfactoren of door het toepassen van het soorten gevoeligheidsverdeling concept (Species Sensitivity Distribution; SSD). Het SSD-concept gaat ervan uit dat de gevoeligheden van verschillende soorten beschreven kunnen worden door een statistische verdeling, bijvoorbeeld een log-normale verdeling. Uit deze verdeling kunnen ook effectdrempels worden afgeleid, zoals bijvoorbeeld de concentratie die 95 % van de blootgestelde soorten beschermt, welke meestal als beschermend gezien wordt voor soorten en gemeenschappen in het veld.

Het SSD-concept is een waardevol instrument voor prospectief ERA, maar het concept helpt ons niet om de onderliggende mechanismen van gevoeligheid te begrijpen. Het toxicologische werkingsmechanisme van een chemische stof is, in combinatie met de biologische eigenschappen van soorten, geïdentificeerd als de belangrijkste determinant van gevoeligheid. Er is echter nog geen systematische aanpak ontwikkeld om deze factoren te combineren, om op die manier een fundamenteler inzicht te krijgen in de redenen waarom soorten verschillen in hun gevoeligheid. De afgelopen jaren is meer inzicht ontstaan in een aantal van de onderliggende processen van toxiciteit door de ontwikkeling van mechanistische effectmodellen. Deze processen omvatten opname, biotransformatie, distributie en eliminatie van de toxische stof (toxicokinetiek), en ook de moleculaire mechanismen van interne schade, reparatie en herstel en effectdrempels (toxicodynamiek).

Dit proefschrift begint met een voorbeschouwing (hoofdstuk 1). Dit is een brief aan de redacteur van een tijdschrift, waarin wij beschrijven welke voordelen het gebruik van de eigenschappen van soorten heeft voor ERA, maar ook welke problemen overwonnen moeten worden om alle mogelijkheden te benutten. Het gebruik van eigenschappen kan nuttig zijn voor zowel biomonitoring (retrospectieve ERA), als voor prospectieve ERA, omdat deze eigenschappen functies beschrijven en op deze manier een aantal verschillende beperkingen van taxonomische benaderingen kunnen overwinnen. Omdat het beschrijven en kwantificeren van eigenschappen door diverse factoren beperkt wordt, is er een internationaal gecoördineerde en substantiële krachtsinspanning nodig om het gebruik van eigenschappen voor ERA zo te ontwikkelen dat alle mogelijkheden optimaal benut worden. Informatie over het kwantificeren en de relevantie van individuele eigenschappen moeten openlijk beschikbaar worden gemaakt en bediscussieerd worden op een platform zoals Wikipedia, waar ook historische gegevens op een flexibele manier kunnen worden opgevraagd. Dit vereist echter een verschuiving van onderzoeksparadigma's en dataverzameling, en daarnaast een cultuurverandering met betrekking tot het delen van onderzoeksgegevens.

Het tweede hoofdstuk van dit proefschrift presenteert een conceptueel kader voor de toepassing van een op soortseigenschappen gebaseerde beoordeling in de ecotoxicologie. Het kader

is gebaseerd op het conceptuele model van Van Straalen (1994) dat de kwetsbaarheid van populaties beschrijft. Deze kwetsbaarheid wordt bepaald door eigenschappen, die kunnen worden onderverdeeld in drie hoofdcategorieën - externe blootstelling, gevoeligheid en de intrinsieke duurzaamheid van populaties. Binnen elk van deze drie categorieën worden specifieke eigenschappen geëvalueerd en wordt nagegaan hoe deze kunnen bijdragen aan de beoordeling van de mogelijke effecten van een schadelijke stof. Met bioaccumulatie als voorbeeld is aangetoond hoe eigenschappen mechanistisch ingezet kunnen worden om kwetsbaarheid te voorspellen, door ze te koppelen aan parameters die gebaseerd zijn op procesmodellen. In een eerste inventarisatie van het nut van deze benadering voor de ecotoxicologie is nagegaan in hoeverre gegevens om deze eigenschappen te kwantificeren beschikbaar zijn. Ook wordt een indicatie gegeven van de sterkte van het verband tussen de eigenschap en het aangetaste proces. Ten slotte geven wij aan hoe de op soortseigenschappen gebaseerde benaderingen verder ontwikkeld kunnen worden in de ecotoxicologie.

Het derde hoofdstuk gebruikt en koppelt bestaande gegevens over eigenschappen en gevoeligheden (EC_{50} en LC_{50}) van verschillende soorten zoetwater geleedpotigen voor drie chemische klassen met twee verschillende werkingsmechanismen (organofosfaten, carbamaten en pyrethroïden). Op die manier wordt de waarde van gemakkelijk beschikbare informatie over soortseigenschappen voor het voorspellen van gevoeligheden van soorten geëvalueerd en worden randvoorwaarden voor verdere studies bepaald. Allereerst is een methode ontwikkeld voor de rangordening van werkingsmechanisme specifieke gevoeligheid (MSS). Deze methode is getest op zowel het familie niveau als het genus niveau. De toepassing van een aantal kwaliteitscriteria verhoogde de betrouwbaarheid van de MSS-rangordening. Er was echter minder vertrouwen in de exacte rangordening van ieder taxon, omdat er onvoldoende gegevens voor bepaalde groepen bestaan. De MSS-rangordening kon succesvol gekoppeld worden aan bestaande gegevens over soortseigenschappen om op die manier eigenschappen te identificeren die de gevoeligheid kunnen voorspellen. Hoewel in deze empirische analyse de causaliteit van de relaties tussen eigenschappen en gevoeligheid van soorten niet getoetst kon worden, werden toch toetsbare hypothesen gegenereerd. Het bleek dat zowel individuele eigenschappen als combinaties van eigenschappen tot in zekere mate gebruikt kunnen worden om de gevoeligheid van soorten onder laboratorium condities voor chemische stoffen te voorspellen, hoewel de correlaties niet zo sterk waren als in eerdere studies. Geconcludeerd wordt dat de bestaande gegevens over eigenschappen onvoldoende zijn voor de ontwikkeling van een voorspellend mechanistisch model. Daarnaast zullen de soorts-eigenschappen die belangrijk zijn voor de toxiciteitsprocessen - toxicokinetiek (TK) en toxicodynamiek (TD) van stoffen, nog moeten worden geïdentificeerd en gekwantificeerd.

Om het verband tussen intrinsieke gevoeligheid en soortseigenschappen verder te onderzoeken, werden experimenten gedaan met het insecticide chloorpyrifos en een aantal soorten behorende tot het phylum Arthropoda, die verschillende eigenschappen hebben en een verschillende mate van gevoeligheid voor inseticiden hebben. Er is voor chloorpyrifos gekozen, omdat de aquatische ecotoxicologie van dit insecticide uitgebreid bestudeerd is op verschillende organisatieniveaus (moleculair tot ecosysteem), omdat er veel bekend is over het werkingsmechanisme en omdat er veel gegevens van beschikbaar zijn. Hiernaast is gekozen voor zoetwater geleedpotigen, omdat, vanuit het werkingsmechanisme verwacht mag worden dat deze

soorten erg gevoelig zijn voor chloorpyrifos, terwijl de groep ook een grote variatie in gevoeligheid heeft (tot drie ordes van grootte). Dit biedt daarom een aantal interessante mogelijkheden om te onderzoeken of een mechanistisch en op eigenschappen gebaseerde aanpak kan worden gebruikt om deze verschillen in gevoeligheid te verklaren.

In hoofdstuk 4 worden de experimenten beschreven, waarin voor chloorpyrifos de LC_{50} en EC_{50} (mediane letale en effect concentraties) na 24, 48, 72 en 96 uur bepaald zijn voor de eindpunten sterfte en immobilisatie voor veertien soorten zoetwater geleedpotigen. De relevantie van deze twee eindpunten voor de milieurisicobeoordeling wordt vervolgens besproken door de waargenomen verschillen in dynamiek in eindpunt tussen de soorten met behulp van dosis-respons modellen en SSD parameters te analyseren. De variatie in de respons van geleedpotigen was kleiner voor immobiliteit dan voor sterfte. Echter, de geobserveerde verschillen in immobiliteit en sterfte waren verrassend groot voor sommige soorten, zelfs na een blootstelling van 96 uur. Aangezien immobiliteit bovendien consequent het meest gevoelige eindpunt is, is dit eindpunt relevanter voor de milieurisicobeoordeling.

De resultaten van hoofdstuk 4 worden uitgedrukt als externe blootstellingsconcentraties (d.w.z.: de concentraties in de test-media). Hoewel dit de standaard aanpak in de ecotoxicologie is, maken dergelijke experimenten een beschrijving van de dynamiek van effecten niet mogelijk, omdat ze niet de interne dosis van het insecticide in het organisme aangeven. Daarom zijn gegevens over de interne concentraties nodig om de toxicologisch relevante doses ten gevolge van externe blootstelling beter te beschrijven. Hoofdstuk 5 beschrijft de resultaten van zeventien bioconcentratie-experimenten met vijftien soorten (waarvan twee soorten werden getest met twee verschillende levensfasen) en met radioactief gemerkte chloorpyrifos. Deze gegevens werden gebruikt om een eerste orde compartiment toxicokinetisch model voor elke soort te parameteriseren, waarmee onderzocht werd hoe de snelheidsconstanten opname (k_{in}) en eliminatie (k_{out}), de bioconcentratiefactoren (BCFs) en de 95 % reinigingstijden (t_{95}) variëren tussen de getoetste soorten. Sommige soorten vertoonden een extreem langzame eliminatie. Hiernaast werd een hoge totale variatie in k_{in}, k_{out}, BCFs, en t_{95} gemeten voor de geteste geleedpotigen. De variatie in opname en eliminatie was significant gecorreleerd met de EC_{50} waarden voor immobilisatie na 48 uur. Opname en eliminatie verklaren ieder respectievelijk 38 % en 28 % van de variatie in gevoeligheid. De resterende variatie in gevoeligheid is waarschijnlijk gerelateerd aan verschillen in mogelijkheden tot biotransformatie (dat wil zeggen: detoxificatie) of toxicodynamiek (dat wil zeggen: interne schade, reparatie en herstel en effectdrempels) processen. Deze bevindingen geven aan dat de verdere ontwikkeling van toxicokinetische en toxicodynamische benaderingen in ERA zullen bijdragen aan een verbetering van onze mechanistische kennis van de ecotoxicologie.

Voortbouwend op deze gegevens worden in hoofdstuk 6 de toxicodynamische processen die verband houden met de gevoeligheid onderzocht door middel van het parameteriseren van een toxicodynamisch model met behulp van resultaten van experimenten met een variabele blootstelling in tijd. Blootstelling aan bestrijdingsmiddelen in het aquatische milieu is niet constant, zoals het in standaard testprotocollen word getest, maar wordt meestal gekenmerkt door sterke fluctuaties in concentratie en tijd. Dynamische en mechanistische toxicokinetiek-toxicodynamiek modellen (TKTD-modellen) worden binnen de ERA van chemische stoffen gebruikt om sterfte-effecten door variabele blootstelling te voorspellen, terwijl tegelijkertijd parameters zoals interne schade, reparatie en herstel,

en effectdrempels geschat kunnen worden. Hoofdstuk 6 beschrijft hoe een TKTD-model experimenteel werd geparameteriseerd voor drie soorten van zoetwater geleedpotigen, waarvoor gegevens over gevoeligheid en toxicokinetiek bekend zijn (zoals hierboven beschreven). Hoewel er aanzienlijke problemen te overwinnen waren, met name wat betreft de robuustheid van de parameterschattingen, konden verschillen in gevoeligheid mogelijk verklaard worden door verschillen in de toxicodynamiek van chloorpyrifos. De waterpissebed *Asellus* was bijvoorbeeld relatief ongevoelig voor chloorpyrifos, hoewel deze een hoge bioconcentratie vertoonde. Dit geeft aan dat *Asellus* waarschijnlijk een verfijnde fysiologie voor detoxificatie en/of om schade te herstellen bezit. Voor de larve van de pluimmug *Chaoborus*, werden zowel een lage overlevingsdrempel als een lage mate van herstel gevonden. Verrassend is dat door de experimenten ook bleek dat chloorpyrifos in de tropische garnaal *Neocardinia* cherry red slechts via narcose (baseline toxiciteit) werkt, in plaats van, zoals verwacht, door zijn specifieke werkingsmechanisme van acetylcholinesterase blokkering. Dit zou de waargenomen extreem lage gevoeligheid van *Neocardinia* kunnen verklaren, hoewel andere atyide garnalen bekend staan als zeer gevoelig voor organofosfaten.

Het laatste hoofdstuk van dit proefschrift (de synthese) brengt de datasets die beschreven zijn in de vorige drie hoofdstukken samen en beschrijft een nieuw geconstrueerde dataset van eigenschappen van de verschillende soorten geleedpotigen die de focus van dit proefschrift waren. Deze aanpak had tot doel de toepasbaarheid van de voorgestelde op eigenschappen gebaseerde benadering te evalueren, suggesties te doen voor toekomstig onderzoek in dit veld, en de implicaties van de resultaten voor ERA te bespreken. Ten eerste wordt de constructie van de dataset van eigenschappen beschreven. De verzamelde eigenschappen zijn hoofdzakelijk morfologisch en fysiologisch van aard, maar ook een aantal ecologische kenmerken zijn gekwantificeerd. In de tweede plaats worden toxicokinetische eindpunten (k_{in}, k_{out}, BCF_{ww}, BCF_{lipid}) en klassieke gevoeligheidseindpunten (48 h LC_{50} en EC_{50}) kwantitatief gekoppeld aan de eigenschappen. Dit gebeurt op eenzelfde wijze als in hoofdstuk 3, maar met gegevens over gevoeligheid en soortseigenschappen die specifiek hiervoor gegenereerd zijn. Daarnaast worden toxicodynamische eindpunten kwalitatief besproken in relatie tot fysiologische eigenschappen, die niet succesvol gekwantificeerd zijn in deze studie. De resultaten van de kwantitatieve koppeling van gevoeligheid en eigenschappen toont aan dat het mogelijk is om zinvolle kwantitatieve, mechanistische verbanden af te leiden tussen eigenschappen en / of combinaties van eigenschappen en (toxicokinetische) proces modelparameters. Echter, het verband tussen eigenschappen en klassieke gevoeligheidseindpunten (LC_{50} en EC_{50}) gaf minder zinvolle informatie. De discussie over kwalitatieve koppelingen tussen toxicodynamische parameterschattingen en fysiologische eigenschappen biedt suggesties voor toekomstig onderzoek. Dit geldt ook voor de bespreking van hoe mechanistische relaties tussen soortseigenschappen en processen die de toxiciteit beschrijven (bijvoorbeeld toxicokinetsische) kunnen worden gebruikt voor het toekomstig voorspellen van verschillen in gevoeligheid tussen soorten voor dezelfde chemische stoffen. De implicaties van het onderzoek voor zowel prospectieve als retrospectieve ERA worden besproken, evenals de voordelen en uitdagingen van deze benaderingen. Tenslotte wordt de relevantie en het belang van het voorspellen van de intrinsieke gevoeligheid voor ERA beschreven.

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CURRICULUM VITAE

Mascha Nadine Rubach was born to Volker and Hilde Rubach on May 18th 1978 in Aachen, Germany and is sister to Malte Rubach. She graduated from the St. Ursula Gymnasium Aachen in 1997 and holds a diploma in biology from Aachen University since 2005. During her studies she specialized in environmental sciences with a strong interest for ecology, ecotoxicology and environmental hygiene, but also biotechnology and bionics. From 2002 to 2004 Mascha was involved in the quantification of estrogen active substances in wastewaters as a research student of the 'Aachen Research Training Group Elimination of Endocrine Disrupting Substances from Wastewater (AGEESA)' under supervision of Dr. Anja Coors. In her diploma thesis at the former Institute of Environmental Research – Biology 5 at Aachen University, supervised by Prof. Dr. Hans Toni Ratte and Dr. Thomas Preuß she investigated and modelled the effects of a nonylphenol isomer on populations of *Daphnia magna* using reproduction test data and individual based population models. During the course of her studies she worked all along as a student research assistant at the Institute of Environmental Research of the Aachen University or the 'Research Institute for Ecosystem Analysis and Assessment – gaiac', where she was involved in several toxicological studies, including mesocom experiments.

In 2006 she started her PhD project with Prof. Dr. Paul van den Brink at both the Department of Aquatic Ecology and Water Quality of Wageningen University and the Environmental Risk Assessment team of Alterra in The Netherlands. The original project proposal was awarded with the SETAC/ CEFIC-LRI award 2005 to Paul van den Brink. Its realisation was a joint endeavour with Syngenta and Environment Canada, involving Dr. Steve Maund and Prof. Dr. Donald Baird as co-supervisors. It aimed at exploring the potential of species traits as predictors for species responses to stressors in the environment and the findings of the research are summarized in this thesis. Mascha worked as guest researcher in the isotope laboratories of Plant Research International of the Wageningen UR, at Eawag in Switzerland in close collaboration with Dr. Roman Ashauer and at the University of New Brunswick in Fredericton, Canada together with Donald Baird. The project terminated with the beginning of the year 2010 and Mascha continued to work for Paul van den Brink by supporting other PhD students of his group in their work on community and population level effects and environmental risk assessment of aquacultures until July 2010.

Mascha is a member of the Society of Environmental Toxicology and Chemistry (SETAC) since 2005 and has co-organized the international SETAC TERA workshop on traits-based approaches, held in Canada in 2009. She also co-organized a series of 'SENSE research in Context' symposia. She is interested in the modelling of complex systems and interactions with an ecological background and the integration of different fields of environmental sciences for the further development of the applied environmental sciences. Soon, Mascha will start working for the Global Product Registration section of Syngenta in Basel, Switzerland.

In her free time Mascha trains and plays Capoeira, an Afro-brazilian art form, involving martial arts, dance, music, game and acrobatics and savours reading and nature.

LIST OF PUBLICATIONS

Rubach MN., Crum SJH., Van den Brink PJ. (in press c): Variability in the dynamics of mortality and immobility responses of freshwater arthropods exposed to chlorpyrifos. Archives of Environmental Contamination and Toxicology. Electronic open access publication ahead of print at http://www.springerlink.com/content/5170632144r1p0vh/fulltext.pdf

Rubach, MN., Ashauer R., Maund SJ, Baird DJ., Van den Brink PJ. (in press b): Toxicokinetic variation in 15 freshwater arthropod species exposed to the insecticide chlorpyrifos. Environmental Toxicology and Chemistry, Electronic publication ahead of print at http://www3.interscience.wiley.com/journal/123489257/abstract

Rubach MN., Ashauer R., Buchwalter DB., De Lange HJ., Hamer M., Preuss TG., Töpke K., Maund SJ. (in press a): A framework for Traits-based Assessment in Ecotoxicology. Integrated Environmental Assessment and Management. Electronic publication ahead of print at http://www3.interscience.wiley.com/cgi-bin/fulltext/123569542/PDFSTART

Rubach, MN., Baird DJ., Van den Brink PJ.(2010): A new method for ranking mode-specific sensitivity of freshwater arthropods to insecticides and its relationship to biological traits. Environmental Toxicology and Chemistry 29, (2), pp. 476 - 487.

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Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

C E R T I F I C A T E

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment (SENSE),
declares that

Mascha Nadine Rubach

born on 18 May 1978 in Aachen, Germany

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Wageningen, 7 October 2010

the Chairman of the SENSE board
Prof. dr. Rik Leemans

the SENSE Director of Education
Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN

The SENSE Research School declares that Ms. Mascha Nadine Rubach has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 51 ECTS, including the following activities:

SENSE PhD courses
- Environmental Research in Context
- Research Context Activity: "Co-organizing two SENSE Context meetings, on: (1) Sustainable Anaerobic Technology; now and in the future (Wageningen, 8th June 2007), and on: (2) Ethics in Environmental Science and Resilience in Environmental and Social Systems (Wageningen 20th November 2007)"
- The Art of Modelling

Other Phd and MSc courses
- Univariate and multivariate data analysis in ecology and ecotoxicology
- Dutch Self Study II
- Safe handling with radioactive materials and sources (ISO 9001)

Management Skills
- Co-organization of a technical SETAC workshop, on Traits-based Ecological Risk Assessment (TERA), held from 7th – 11th September 2009 in Canada.

Oral Presentations
- A trait-based study of arthropod sensitivity to pesticides, Society of Environmental Toxicology And Chemistry (SETAC) 5th World Congress, 3 – 7 August, 2008, Sydney, Australia
- Assessing organism level effects using toxicokinetics and – dynamic experiments and modelling, Short Course presentation at the Society of Environmental Toxicology And Chemistry (SETAC) 5th World Congress, 3 August 2008, Sydney, Australia
- Explanatory potential of traits for sensitivity of freshwater Arthropoda to pesticides, SENSE-EPCEM Symposium, 10 October 2008, Wageningen,The Netherlands
- Modelling effects of pulsed exposure in trait diverse freshwater Arthropoda and implications for interspecies extrapolation, Society of Environmental Toxicology And Chemistry (SETAC) Europe 19th annual meeting, 31 May – 4 June 2009,Gothenburg, Sweden
- Relating traits to intrinsic sensitivity of arthropods towards insecticides, SETAC workshop on traits-based ecological risk assessment (TERA), 7 – 11 September 2009, Burlington, Canada
- A framework for traits-based assessment in ecotoxicology, Society of Environmental Toxicology And Chemistry (SETAC) Europe 20th annual meeting,23 – 27 May 2010, Seville, Spain

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