Quantitative Structure-Activity Relationships for soil Ecotoxicity

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Chapter 1

General Introduction

Soils represent an essential component of terrestrial ecosystems and play a key role in global nutrient cycles affecting the total biosphere on our planet (Mosier, 1998). They serve as substrate for agriculture production, are an effective buffer and filter system for groundwater and are a major sink for the excessive carbon dioxide production through human activity. Hence, nearly all food and drinking water is derived directly or indirectly from the functioning of soil processes. Yet, soils and terrestrial ecosystems are under pressure by increasing human activity and effects of globalization that continuously challenge ecosystem structure and function (Cardinale et al., 2012; Hooper et al., 2012). Physical impacts, such as compaction, surface sealing and agricultural land use, eutrophication, acidification and contamination with heavy metals and organic compounds are threatening the soil. With growing awareness and concerns of the public regarding protection of human and environmental health deriving from pollution and contaminated ecosystems, European governments and the European Commission are starting to assess and manage the potential effects of industrial chemicals. Therefore, the European Commission implemented legislations for the registration, evaluation and authorization of chemicals (REACH) (EU, 2006), for an estimated 101,000 compounds (Rovida and Hartung, 2009). Though the main focus of REACH is to assess risks for human life and freshwater safety, terrestrial ecosystems were also included.

To ensure that all aspects of human and environmental risks are evaluated, REACH promotes the use of Intelligent Test Systems (ITS), as a collective term for the replacement of animal testing and use of existing available data. *In silico* methods, such as read across (Hurdzan et al., 2011) comparing similar compound structures or quantitative structure-activity relationships (QSARs) relating toxicity to chemical properties of a series of compounds (Schultz et al., 2003; Verhaar et al., 1994), make use of established datasets to extrapolate potential risk for new developed untested compounds. Advancements in software and statistical tools promote the application of any *in silico* method, as reproducible and comparable

techniques which can be relative effortless employed by small and medium sized companies. Furthermore, in vivo methods, tests with cell lines or molecular bioassays, such as microarrays, are employed to identify species specific reactions and sensitivities to toxicants on gene response level. However, in general performing new tests on organisms, e.g., to assess sensitive key species or ecosystems for which data a lacking, are seen as a last resort.

QSAR in environmental risk assessment

Generally, QSARs are statistical models that relate defined toxic endpoints, physiological response of the animal, compound test series to their inherent structure or physical-chemical properties. In order to derive QSARs, first chemical descriptors have to be determined and selected, which have significant influence on their behaviour in the test system and their potential toxic effect on the organism. To ensure applicability and reliability QSARs are based on the empiric results derived from actual animal toxicity tests. New compound structures are then later integrated into the model. Therefore, QSARs became one of the most prominent ITS to evaluate risk to the environment (Fenner et al., 2006) and analyse large datasets with increasingly sophisticated computer technology.

The development of QSARs for purposes of environmental risk assessment (ERA) started with a study by Könemann on the hydrophobicity dependent toxicity of 50 industrial compounds, in which he described a correlation of the median lethal concentration to fish (LC_{50}) with the compounds partition coefficient between n-octanol and water ($\log K_{ow}$) (Könemann, 1981). This pattern was found and adopted for other species and aquatic and terrestrial ecosystem (Chiou, 1985; Van Gestel and Ma, 1988). Toxicity tests use concentration-response analysis do derive mean concentrations for the effects on survival (LC_{50}) and reduction of reproduction (EC_{50}), or the respective 10% reduction of reproductive success (EC_{10}). When compared with the $\log K_{ow}$ of a set of organic compounds, linear QSAR regression models can be developed, in which the L/ECx concentration expressed on a molar base (mol/l) decrease with increasing lipophilicity. This general patter is independent from the used test organism.

Reversible interaction with lipophilic molecules and disturbances of the cellular membranes are assumed to be the mode of action of organic

compound described as baseline toxicity (Figure 1). All compounds have an influence on the membrane as the first barrier to cross when taken up by an organism. However, numerous compounds excite their toxicity at lower concentration than baseline toxicity predicts, indicating additional factors and affected target sides in the organism. A classification system, identifying four distinct groups of mode of action (MOA), was established based on the baseline toxicity and the deviations from it (Hermens, 1995; Oberg, 2004; Ren, 2002; Verhaar et al., 1996; Verhaar et al., 2000; Verhaar et al., 1992) (Figure 1). The first category (Class I) contains non-polar compounds, defining baseline toxicity, acting only on the membrane, also named narcosis I. Class II compiles polar compounds with the chemical potential to form hydrogen bonds and interfere with electron transfer (narcosis II). Though the exact mechanism and sites of interactions remain unspecified, the higher toxicity of polar chemicals is explained by these potential yet unspecific reactions. The other two classes summarize reactive compounds, which potentially cause phosphorylation and other reactions (Class III), and compounds that target specific biochemical pathways or molecules in the organisms (Class IV) (von der Ohe et al., 2005). As shown in figure 1 the later classes may contain chemicals that are much more toxic than baseline toxicity, however, this is also dependent on the tested organisms and test systems. The majority of compounds (60%) under REACH fall into the first two categories. Toxicity of non-polar, such as chlorobenzenes or polycyclic aromatic compounds (PAC) (Belfroid et al., 1993; Hurdzan and Lanno, 2009) and polar compounds, e.g. anilines and phenols (Aruoja et al., 2011; Ramos et al., 1997), can be modelled using the $\log K_{ow}$ as single descriptor.

QSAR for soil ecotoxicity

Soils are highly complex and heterogeneous ecosystems containing an inorganic mineral fraction derived from weathering processes, and an organic fraction deriving from the debris of decomposed biological material (humic substances) and combustion material (soot). In addition, the soil matrix includes gaseous pockets (porosphere) and the pore water with dissolved organic matter (DOM) and ions. It is generally assumed that the bioavailability of pollutants for soil dwelling organisms is governed by the freely dissolved porewater concentration, while the remaining compounds are (ir)reversibly bound to the particulate or dissolved fraction of the test

matrix and therefore not accessible to the test organisms (Chiou et al., 1998; Domene et al., 2010). Therefore, QSARs use the molar concentration of the freely dissolved, also called bioavailable, fraction in the soil interstitial water (Bauerlein et al., 2012). For this a soil sorption coefficient (Kd) is determined, applying compound soil-specific organic carbon-water partition coefficient ($logK_{oc}$) and the fraction of the organic matter (foc) of a given soil (Bollag and Loll, 1983; Cole and Mackay, 2000; Doucette, 2003; Rayne and Forest, 2009; Rutherford et al., 1992; Sverdrup et al., 2002c).

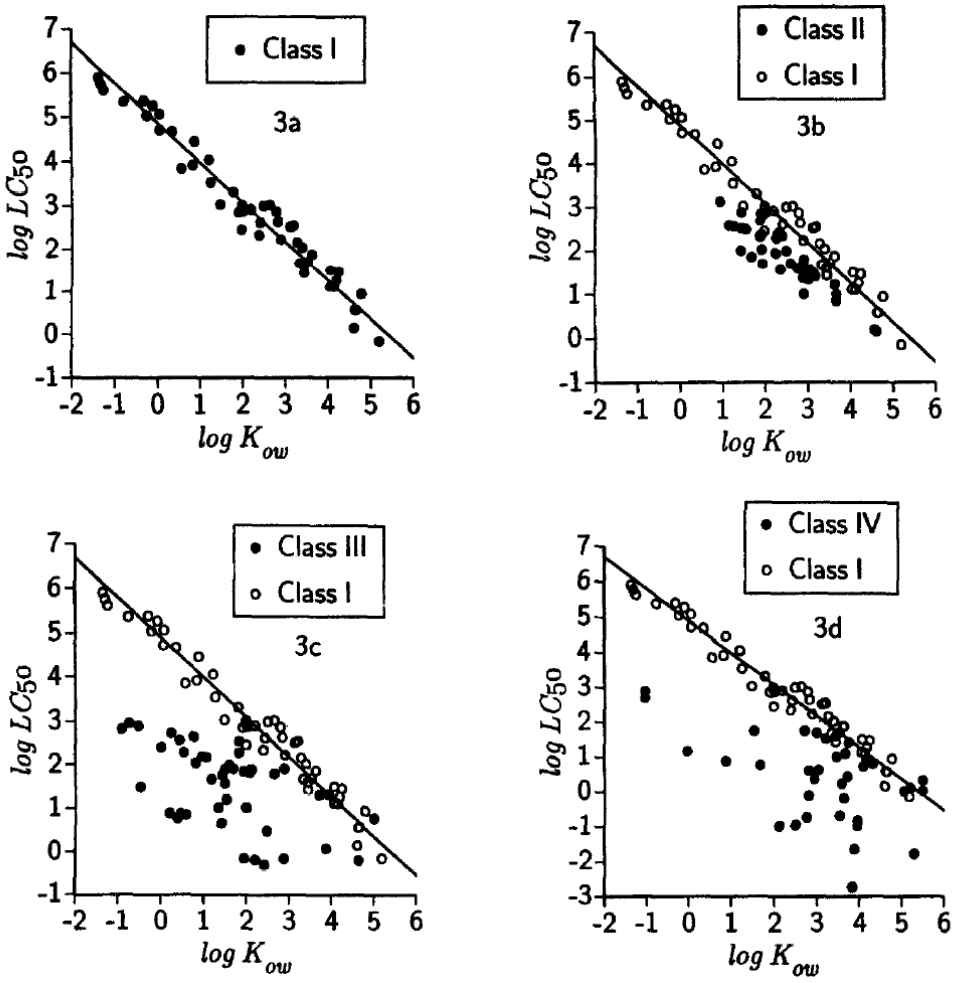

Figure 1. Comparison of four classes of compounds, defined by chemical descriptors and classification according to Verhaar, summarized by Hermens (1995). Acute toxic effects (LC_{50}) to guppy (*Pocecilia reticulate*) of organic chemicals versus their lipophilicity ($logK_{ow}$). Class I inert non-polar compounds; Class II compounds with substitution; Class III reactive compounds; ClassIV compound with specific mode of action.

$Kd = K_{oc} * foc$

Mechanisms of interactions of organic compounds with different compartments in the soil matrix are:

Non-reactive (Van Brummelen et al., 1996)

- Neutral sorption to the organic matrix of the soil
- Neutral adsorption towards inorganic surfaces driven by van der Waals interactions
- Formation of hydrogen bonds with the organic or mineral surfaces
- Adsorption driven by ionic or electrostatic interaction with surfaces
- Change of the ionic strength for compounds with polar groups with potential precipitation as salts with metal complexes

Reactive (Thorn et al., 1996)

- Covalent (ir)reversible integration by covalent binding to the soil organic matter
- Non-specific reaction with extracellular enzymes and incorporation into the soil organic matter

With this soil sorption factor, nominal concentrations corresponding to ECx values derived from toxicity tests are normalised and interstitial water concentrations estimated (Belfroid et al., 1995; van Brummelen and van Straalen, 1996).

ECx (mol/l) = ECx (mol/kg soil) / Kd (l/kg)

The final uptake of compounds into the organism from the aqueous phase is described traditionally by a descriptor of their lipophilicity, the n-octanol-water partition coefficient ($logK_{ow}$), which acts as a surrogate for the bilayer membrane of a cell.

Freely dissolved bioavailable concentrations have been notoriously difficult to measure and hence QSARs are based on the estimated molar concentrations in the soil interstitial water. This first involves assessment of the total soil concentrations using extractions of the total soil with bulk

organic solvent combined with heat or pressure, followed by the necessary calculations. A direct approach to assess freely dissolved concentrations in soil pore water is favourable as it indeed reflects actual concentrations and avoids the statistical uncertainties and differences in chemical behaviour due to variable sorption or fate mechanisms (Van der Wal et al., 2004a). Passive sampling techniques such as solid-phase microextraction (SPME) that uses partitioning of compounds from gaseous or aqueous phases into polymers, can be applied to measure freely dissolved concentrations in various complex matrices (Muijs and Jonker, 2009; Ouyang and Pawliszyn, 2006; Styrishave et al., 2008; Ter Laak et al., 2006b; van der Wal et al., 2004b). The effective amounts taken up by the polymers are negligible and do not disturb the (pseudo)equilibrium concentration of a compound in the substrate. When the compound concentration in the fiber reaches equilibrium with the surrounding medium, it is subsequently extracted and prepared for analysis. The freely dissolved concentration can then be calculated using determined partition coefficients for fiber-water equilibrium (Hurdzan and Lanno, 2011; Verbruggen et al., 2000).

Validation and optimizing QSAR

The predictive and descriptive power of QSARs depends heavily on the quality of the data that define the toxic endpoints. Although this appears well documented, comparable studies in soil ecotoxicology including relevant sets of chemicals, soil types and species are scarce. Toxicity data in literature and databases generally is based on initial nominal concentrations of the dose-response test (Sverdrup et al., 2001; van Brummelen and van Straalen, 1996). Chemical analysis of the test medium, if performed at all, often includes only the total soil extraction applying various techniques, which ultimately need further calculations. Hence, the QSARs in the literature describing large variety of compounds in various ecosystems using predicted ECx without addressing the freely dissolved concentration directly all are based only on the assumption that the pore water-hypothesis is correct (Van Gestel and Ma, 1993). As a consequence, they may carry the potential risk of systematic errors. Uncertainties could multiply when polar and non-polar compound test sets are compared with each other (Vaes et al., 1998). Inert compounds have fewer possible pathways to interact with the environmental and biological matrix then compounds with polar substitutes

(see above). The application of SPME to assess concentrations in soil interstitial water is enhancing the accuracy of ECx values. Combining passive samplers to measure predicted ECx with QSAR offers for the first time the possibility for researchers to actually validate estimated concentrations covering multiple soils and other substrates.

The predictive and descriptive power of QSARs also depends on the quality and origin of the applied physicochemical descriptors employed for model development (Bintein and Devillers, 1994). Soil organic carbon sorption coefficients ($\log K_{oc}$) used in the calculation to estimate freely dissolved ECx values are highly dependent on sorbent and sorbate properties (van Wezel and Opperhuizen, 1995). In the past, however, QSARs used ECx data that derived from calculations with non-soil-specific K_{oc} values. To ensure that estimated concentrations do reflect actual conditions in the soil this parameter would need to be determined for all soils in a study. A second step to reduce uncertainties in the QSAR development is the search and determination of alternative partition coefficients. Though octanol provides a standardizable lipid-surrogate since three decades other compound properties such as olive oil-water partition coefficient or artificial membrane partition have been applied in toxicity models and QSARs (Escher et al., 2002; Escher and Hermens, 2002; Vaes et al., 1998; van der Heijden and Jonker, 2009).

It is critical that physico-chemical properties, toxicity data and applied tools fulfil minimum quality standards to be employable for QSARs as the statistical tools.

Bioassays in soil toxicology

Next to a an optimization of statistical tools and their integration with environmental conditions, new available in vivo methods, like microarrays assessing gene-responses on the transcriptomic level (the total of messenger RNA), are part of the ITS evaluation process. In this approach specific nucleotide probes, complementary to different genes, of the total known transcriptome of a species are covalently bound to a glass surface and labelled with dyes. For ecotoxicogenomic studies, transcriptomes of an organism that was exposed to a toxicant, usually for only two days, to an ECx value determined in a chronic study, is compared to a control group (Gong et al., 2007; Owen et al., 2008; Snape et al., 2004). The resulting pattern of up or down regulated genes elucidates species-specific reactions to

a certain toxicant (Nota et al., 2009; Nota et al., 2008). A drawback in their application is that changing soil properties, like soil texture (de Boer et al., 2010), and test conditions, such as temperature (Bone et al., 2011; de Boer et al., 2010), alter gene-response. Additionally, in their standardized approach they relate to afore performed toxicity tests and resulting ECx values, without consulting if the effect occurred on the adult level, or in younger stages like eggs or juveniles. Nevertheless, integrating these methods into ecotoxicological routine may help to unravel specific pathways of compound transformation and physiological pathways, in which species react to the stress of a pollutant. Transcriptome profiles assist in biologically assessing the mode of action of compound series replacing the physicochemical categorisation (Aruoja et al., 2011; Dom et al., 2011; Janssens et al., 2011). A yet not fully explored possible application of bioassays is the species to species extrapolation. When conserved genes, within taxa or on higher levels of organisation, involved in the reaction to a toxicant are identified risk evaluations for a higher number of organism and ecosystem effects could be optimized.

Test compounds

For the experiments conducted in this thesis two structurally similar compound series were chosen: nine inert non-polar chlorobenzenes and six chloroanilines (figure 2).

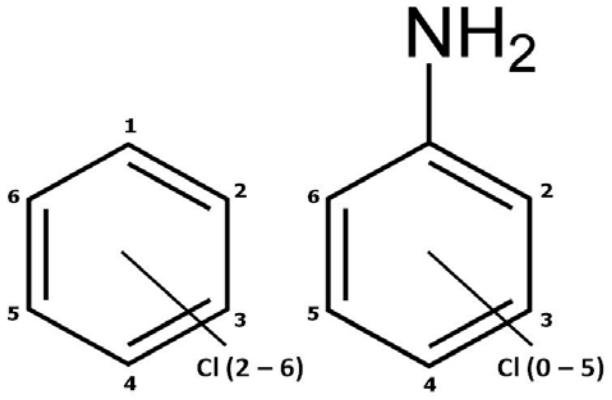

Figure 2. Test compounds used in standardized soil toxicity tests. Basic structure is a benzene ring with varying chlorination (Cl (2-6) for chlorobenzenes tested in chapters 2, and 4-6; and Cl (0-5) for chloroanilines tested in chapters 3-6). Numbers indicate positions for chloride substitution for isomer identification.

The combination allows assessment over a large scale of chemical descriptors and a comparison of non-polar and polar toxicity. Long distant transport (Vorkamp et al., 2004) and persistence (Sharer et al., 2003) in the ecosystem and transfer through the food web have been described for compounds belonging to both groups of chemicals. Combined with their annual production and use as intermediate products in chemical production chains these compounds became high priority compounds under REACH.

Animals

The chosen test organism to perform soil toxicity tests was the non-pigmented invertebrate *Folsomia candida* (Collembola, Isomatidae). Regarded as a cosmopolitan species it lives in the top layer of soils feeding mostly on fungal hyphae. Populations consist exclusively of parthenogenetic individuals, which allows toxicity tests excluding potential gender effects toxicological studies and molecular analysis (Timmermans et al., 2008). The short reproduction time and simple culturing made them perfect study objects not only for toxicity tests (Droge et al., 2006; Mayer and Holmstrup, 2008) but also for other stressors and their combinations (Fountain and Hopkin, 2005).

Picture 1. *Folsomia candida* in different life stages (photo by Hopkin) (Fountain and Hopkin, 2005)

Aim and objectives

The aims of this thesis are 1) to develop QSARs and optimize their predictive power for soil ecotoxicology and 2) to integrate complementary bio-based test methods in order to assess the compounds mode of action of selected toxicants.

The following objectives have been set to fulfil these aims

- To determine the toxicity of non-polar and polar compounds in two soils to the soil dwelling springtail *Folsomia candida*
- To develop QSARs for the toxicity of non-polar and polar compounds to *F. candida* by applying $\log K_{ow}$ as descriptor to determine the general pattern of toxicological expectation and to compare them with former studies
- To validate predicted toxic endpoint concentrations (EC_{10} and EC_{50}) and QSARs based on estimated interstitial water concentrations by applying SPME-fibers
- To unify QSARs developed for two soils and two sets of compounds separately by determining soil-specific organic carbon water partition coefficients and liposome-water partition coefficients
- To newly classify non-polar and polar compounds based on the assessment and comparison of differences in molecular responses

Outline of this thesis

QSARs for soil ecotoxicology based on endpoints derived from chronic toxicity tests commonly are based on interstitial water concentrations, which are estimated from the initial nominal concentrations in the soil using the $\log K_{oc}$ values. However, predicted endpoint concentrations of chronic toxicity test for soils are uncertain due to soil unspecific sorption descriptors and thus exposure concentrations are unknown. Therefore, in *chapter two* the concentrations causing 10 % (EC_{10}) and 50% reduction of reproduction (EC_{50}) of *Folsomia candida* caused by nine chlorobenzenes, were determined by dose-response analysis. The freely dissolved concentrations corresponding with these endpoints were i) estimated and ii) measured, and used to develop models that were compared

to factual concentrations. *Chapter three* uses a similar approach with chlorinated anilines.

Compounds of a test series for QSARs undergo chemico-physical and biological processes, such as degradation, sorption and incorporation in the soil with different rates. Thus, the bioavailable concentrations organisms are exposed to in the soil are dynamic rather than static. In order to incorporate the losses over the duration of standardized toxicity tests (28 days) in the QSAR models, in *chapter four*, freely dissolved concentrations were analysed with SPME at days 0, 14 and 28. The geometric means of the measured concentrations for each compound were used to develop QSARs.

In *chapter five* a single unified QSAR model for two soils and a set of polar and a set of non-polar compounds is presented. Soil-specific $\log K_{oc}$ and compound-specific liposome-water partition coefficients ($\log K_{lipw}$) were determined, and SPME were used to determine freely dissolved concentrations in the interstitial water of two soils corresponding with the respective EC_{50} values of 11 chlorinated compounds.

QSARs for biological endpoints cannot precisely distinguish between the physiological interactions in a test organism, but present only the resulting reaction. Therefore, in *chapter six*, the transcriptional responses toward acute exposure to concentrations corresponding with the pre-determined EC_{50} of polar, amino-group substituted chlorinated, anilines and a non-polar chlorobenzene were determined and compared to define differences in the mode of action.

With further optimization, QSARs will remain one of the keystones in environmental risk assessment and regulatory toxicology. Adequate implementation and the necessary integration with other available methods to support predicted chemical behaviour are discussed in chapter seven, focusing on limitations and applicability of recent developments.