Quantitative Structure-Activity Relationships for soil Ecotoxicity

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Chapter 3

QSAR development and bioavailability determination: The toxicity of chloroanilines to the soil dwelling springtail *Folsomia candida*

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Abstract

Quantitative structure-activity relationships (QSARs) are an established tool in environmental risk assessment and a valuable alternative to the exhaustive use of test animals under REACH. In this study a QSAR was developed for the toxicity of a series of six chloroanilines to the soil-dwelling collembolan *Folsomia candida* in standardized natural LUFA2.2 soil. Toxicity endpoints incorporated in the QSAR were the concentrations causing 10% (EC_{10}) and 50% (EC_{50}) reduction in reproduction of *F. candida*. Toxicity was based on concentrations in interstitial water estimated from nominal concentrations in the soil and published soil-water partition coefficients. Estimated effect concentrations were negatively correlated with the lipophilicity of the compounds. Interstitial water concentrations for both the EC_{10} and EC_{50} for four compounds were determined by using solid-phase microextraction (SPME). Measured and estimated concentrations were comparable only for tetra- and pentachloroaniline. With decreasing chlorination the disparity between modelled and actual concentrations increased. Optimization of the QSAR therefore could not be accomplished, showing the necessity to move from total soil to available concentration measurements.

Introduction

With increasing anthropogenic activity and use of chemicals, the risk of soil pollution becomes an increasing threat to ecosystem function. To assess the risk for an estimated 101,000 compounds traded and used in member states (Rovida and Hartung, 2009), regulatory authorities of the European Union approved the framework for the registration, evaluation, authorisation and restriction of chemicals (REACH) (EU, 2006) for all compounds with a minimal annual production volume above one tonne per year. But the costs of individual toxicity tests, the numbers of test organisms and time necessary to determine toxicity or risk for all ecosystems and key species are out of proportion. Statistical models like quantitative structure-activity relationships (QSARs), linking physical-chemical properties to the effect of a compound, provide a time and resource efficient alternative (Cronin et al., 2003). QSARs have been successfully developed over the last 25 years in soil ecotoxicity (Van Gestel and Ma, 1988) and provide an important tool for risk assessment and environmental protection.

The greatest challenge in the toxicity assessment of organic compounds in soil rises from the disparity between total concentration and the bioavailable fraction. Van Gestel and Ma (1988) introduced a three phase model from soil matrix to interstitial water and from interstitial water to organism, describing toxicity in soil as a dynamic process governed by the freely dissolved concentration. The bioavailable concentration is determined from the total soil concentration using the compound-specific soil-water partition coefficient ($\log K_{oc}$) and the known organic carbon fraction of the soil (f_{oc}). The uptake from the interstitial water into the organism is driven by the compound-specific lipophilicity, generally defined with the octanol–water partition coefficient ($\log K_{ow}$). QSARs are therefore based on interstitial soil water concentrations.

One of the most advanced techniques to measure the freely dissolved concentration of compounds in the interstitial water is solid-phase micro-extraction (SPME) (Ter Laak et al., 2006a; Van der Wal et al., 2004a). Placed into soil-water slurry, freely dissolved organic compounds diffuse in a bio-mimetic polymer coated fiber, avoiding exhaustive extraction of the total soil by organic solvents. Fast uptake kinetics and equilibration time within the medium proofed SPME fiber measurement in diverse matrices to

be a fast and reproducible tool in the determination of freely dissolved compounds (Jonker et al., 2007).

In this study QSARs were developed for the toxicity of aniline and five chlorinated analogues, with $\log K_{ow}$ ranging from 0.9 to 5.08, to the parthenogenetic soil-dwelling collembolan *Folsomia candida* in LUFA2.2 natural standard soil. Aniline and chlorinated anilines are substituted monocyclic aromatic compounds widely used in industrial production chains for e.g. azo-dyes or pesticides and mainly entering the environment as direct spillage or as degradation products of pesticides (Giacomazzi and Cochet, 2004; Tixier et al., 2001). Basically, (chloro-) anilines induce toxicity through membrane disruption. However, because of their ionisable amino group, which theoretically enables interaction with polar groups and proton translocation (Sixt et al., 1995), they are classified as polar narcotizing compounds with potential additional effects on the respiratory chain (Veith and Broderius, 1990; Verhaar et al., 1996). Data on the toxicity of chloroanilines to terrestrial invertebrates such as *F. candida* are scarce (Janssens et al., 2011; PayaPerez and Mannone, 1997; Van Gestel and Ma, 1993). With respect to QSAR development, (chlorinated) anilines, from non-chlorinated to fully chlorinated, offer the opportunity to test compound series over a wide lipophilicity range. QSARs were based on effective concentrations for 10% (EC_{10}) and 50% (EC_{50}) reduction in reproduction. Subsequently, soil was spiked with estimated nominal concentrations for the respective EC_{10} and EC_{50} for di-, tri-, tetra- and pentachloroaniline. Interstitial soil water concentrations of the freshly spiked soil were determined by polyacrylate coated SPME fibers. The obtained measured concentrations were compared with estimated concentrations with the focus on QSAR optimisation.

Material and Methods

Test compounds

Test compounds included aniline (AN; Aldrich, 99%), 4-chloroaniline (4-MCA; Aldrich,99%), 3,5-dichloroaniline (3,5-DCA; Aldrich, 98%), 2,3,4-trichloroaniline (2,3,4-TrCA; TCI Europe, 98%), 2,3,5,6-tetrachloroaniline (2,3,5,6-TeCA; Chemos, 99%), and pentachloroaniline (PCA; Pestanal, 99%). Physical-chemical properties are given in Table 1.

Table 1. Selected test compounds and relevant properties: Chemical Abstract Service number; molecular weight; organic carbon-water partition coefficient (log K_{oc}); octanol-water partition coefficient ($logK_{ow}$); and melting point (mp; °C).

Chemical	CAS no.	MW	$logK_{oc}$ [1]	$logK_{ow}$ [1]	mp [2]
Aniline	62-53-3	93.13	1.41	0.94	-6
4-chloroaniline	106-47-8	127.57	1.96	1.88	68-71
3,5-dichloroaniline	626-43-7	162.02	2.49	2.90	49-53
2,3,4-trichloroaniline	634-67-3	196.46	3.03	3.68	65-67
2,3,5,6-tetrachloroaniline	3481-20-7	230.91	3.94	4.46	110
Pentachloroaniline	527-20-8	265.35	4.62	5.08	206

[1] Sablic et al. (1995)

[2] suppliers

Maintenance and culture of test organism

Continuous *Folsomia candida* (strain "Berlin") cultures were maintained in climate chambers (20°C, 12h dark /12h light, 75% humidity) in pots with a moist plaster of Paris base. Animals were fed dry baker's yeast (Dr Oetker). Toxicity tests were performed with synchronised 10-12 day old animals according to ISO guideline 11267 (ISO, 1999).

Test soil

Standardized natural LUFA2.2 soil (Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA), Speyer, Germany) was dried and defaunated at 60°C for 24 hours. LUFA2.2 is classified as a sandy loam (particle size distribution: 50-2,000 µm, 75.3%; 2-50 µm, 16.6% and < 2 µm, 8.1%) with 2.2±0.2 % organic carbon, and pH 5.5±0.4. Maximum water holding capacity (WHC) was around 44 % (w/w).

Soil treatment and experimental set-up

Range-finding tests were performed to determine appropriate concentration ranges for concentration-response analyses. For the final tests with AN, 4-MCA, and 3,5-DCA, concentrations of 1.25, 2.5, 5, 10, 20, and 40 mg/kg dry soil were used. For 2,3,4-TrCA, 3.75, 7.5, 15, 30, 60, and 120 mg/kg dry soil and for 2,3,5,6-TeCA and PCA, 5, 10, 20, 40, 80, and 160 mg/kg dry soil were applied. All tests were performed with five replicates and controls for water and the carrier solvent acetone (HPLC-grade, Sigma-Aldrich) were included. Stock solutions for each compound and each concentration were prepared in acetone and were used within one day. Subsequently, the total spiking volume was equalised for each treatment by pure acetone addition in order to minimize effects of the acetone on the soil matrix, 10 percent of the total soil mass for each test concentration was pre-spiked. Spiking solutions were added directly onto the soil in a glass container, with a thin layer of acetone covering the soil. The containers were then closed with Teflon lids. Soil-acetone-compound slurries were shaken (150 rpm) for one hour and stored overnight in the dark. To allow slow acetone evaporation, containers were opened for around 10% of the lid area and placed in a fume cupboard for 24 hours. When no acetone could be smelled anymore, the remaining 90% of dry LUFA2.2 soil was added to the container, which was then mixed thoroughly. Mixed dry soil was moistened to 50% of the WHC with demineralised water. Thirty grams of moist spiked soil were transferred into 100 ml acetone-cleaned glass test jars. The jars were closed with methanol-cleaned screw caps and stored in the dark for maximal 16 hours (over night).

Ten individual 10-12 day old *F. candida* were brought into each test jar and a few grains of dried baker's yeast were added as food to each jar. During the test, food was only replenished after the second week when no grains were visible on the soil surface. The jars were opened once a week for aeration. Water loss through evaporation was controlled once a week and compensated with demineralised water when necessary. Jars were incubated under a 12:12 h light:dark regime at 20°C and 75 % humidity. The tests were terminated after 28 days by flushing soil and animals into 400 ml glass beakers, using 100 ml of water such that surviving adults and juveniles floated on the water surface. Pictures were then taken from the surface using a digital camera (C-5060, Olympus). This was done in triplicate to

compensate for animal movements and the photos were analyzed with the digital counting program Cell$^{\wedge D}$ (Olympus). The mean juvenile and adult numbers counted on three pictures were used for further calculation.

SPME measurements

A second batch of LUFA2.2 soil was spiked with chloroanilines in concentrations corresponding with the calculated EC_{10} and EC_{50}, based on the initial nominal concentrations (see below), using the same setup as described above. Concentrations of 3,5-DCA, 2,3,4-TrCA, 2,3,5,6-TeCA, and PCA in the interstitial water were determined with SPME. Within 12 hours after finishing the spiking procedure, 2 grams of soil and 6.5 ml of Millipore water containing 0.01 M calcium chloride and 25 mg/l sodium azide were mixed in 7 ml amber-colored vials. For each compound and concentration four replicates were performed. After shaking the soil slurries, one 2 cm long 30 µm thick polyacrylate-coated SPME fiber (Poly Micro Industries, Phoenix, USA) was transferred into each vial. SPME fibers were incubated for 4 weeks on a "Rock-and-Roller" apparatus (Snijders Scientific) in the dark. At the end of the equilibration period, fibers were removed, cleaned with Millipore water wetted tissue, and transferred into vials containing cyclohexane. Internal standard solution was then added (1 mg/l PCB31 in cyclohexane) and concentrations in the fiber extracts were determined by GC-ECD (GC 8000 gas chromatograph; Fisons Instruments, Milan, Italy), equipped with a CombiPAL autosampler system (CTC Analytics, Zwingen, Switzerland), a Rtx-5 amine column (30 m length, coating thickness 25 µm, internal diameter 0.25 µm), a deactivated fused silica pre-column (length 2 m), and a ^{63}Ni electron capture detector. Chromatographic data were analyzed with Chromcard 1.21 (CE Instruments).

Concentrations in fibers (C_f) were used to calculate freely dissolved chloroaniline concentrations in the soil interstitial water (C_w^{meas}), using polyacrylate fiber-water partition coefficients (K_{fiber}), which were determined according to the method described in Muijs and Jonker (2009). For this measurement seven 100 ml full glass, brown bottles were filled each with Millipore water containing 0.01 M calcium chloride and 25 mg/l sodium azide. Then, 21 cm of SPME fiber was added to each bottle, and the systems were spiked with a mixture of the chloroanilines in acetone. After six weeks

of equilibration on a shaker at 20 °C, the fibers were removed and placed in autosampler vials with 200 µl of cyclohexane in an insert. Fifty ml of the water phase was then extracted three times with 10 ml of n-hexane, after which the solvent phases were pooled, concentrated, and exchanged to 0.5 ml of cyclohexane. All extracts finally received the internal standard and were analyzed as described above. Resulting data were corrected for blank and recovery determinations (both in fivefold).

Data analyses

Concentrations needed for effects on survival (LC_{50}) and reproduction (EC_{10} and EC_{50}) were estimated by applying a log-logistic model in SPSS (Version 15.00; SPSS Chicago, US). Curves were fit following the equation described by van Brummelen et al. (1996), based on the initial nominal concentrations:

$$y = k / (1+\exp (\ln 9((\log x - \log EC_{50})/(\log EC_{50} - \log EC_{10}))))$$

Where y is number of juveniles or survivors, respectively, x is the test concentration, and k the number of juveniles or survivors in the control group. Corresponding concentrations in the soil interstitial water were calculated using the organic carbon content (f_{oc}) of the soil and the organic carbon-water partitioning coefficients (K_{oc}) reported Sablic et al. (1995). Calculated concentrations were used for linear structure-activity relationship regression analysis. Derived QSARs for both estimated EC_{10} and EC_{50} values were tested for their significance with F-distribution analysis and compared with a generalized likelihood ratio test in SPSS (Version 15.00; SPSS Chicago, US).

Results and Discussion

Toxicity of chloroanilines

The performed toxicity tests met quality standards with an average survival >80% in the water and solvent control and total number of juveniles of 728 ± 103 (n=5) in the solvent control. Results of the solvent control were used in the subsequent analysis. All six applied (chloro-) anilines affected *F. candida*. Estimated EC_{10} and EC_{50} values, for the effect on survival and reproduction in mg and µmol/kg dry soil, based on initial nominal concentrations, are summarized in Table 2. Based on effects on survival the series can be divided in three groups; (1) the non-chlorinated AN and 4-MCA, followed by (2) 3,5-DCA together with 2,3,4-TrCA and 2,3,5,6-TeCA for which the LC_{50} (in µmol/kg dry soil) was roughly 2-3 times higher compared with the first two compounds, while PCA separated individually. The pattern changed for effects on reproduction to group AN, 4-MCA and 3,5-DCA with EC_{50}s between 75 and 78 µmol/kg, while for the other compounds EC_{50} increased with increasing $\log K_{ow}$. The LC_{50} and EC_{50} values of AN and 4-MCA showed no significant difference. The EC_{10}s in contrast showed less difference for the different compounds. Differences in the steepness of the concentration-response curves might be the reason for this deviating result.

Table 2. Chloroaniline concentrations causing 10% and 50% reduction of reproduction (EC_{10}, EC_{50}) and 50% mortality (LC_{50}) of *Folsomia candida* exposed for 28 days to LUFA2.2 soil. Concentrations are given as µmol/kg dry soil with 95 % confidence intervals.

	LC_{50}		EC_{50}		EC_{10}	
Compounds	mg/kg	µmol/kg	mg/kg	µmol/kg	mg/kg	µmol/kg
Aniline	7 (6.5; 8.7)	**82**	7 (5.8; 8.2)	**78**	3 (2; 5.6)	**41**
4-Monochloroaniline	9 (8.2; 10.4)	**73**	9 (7.5; 11.3)	**75**	7 (6.5; 8.9)	**55**
3,5,-Dichloroaniline	27 (23.4; 32)	**171**	12 (10.5; 15.4)	**77**	6 (5.5; 6.9)	**39**
2,3,4-Trichloroaniline	29 (24.1; 33.7)	**147**	20 (17.1; 22.8)	**102**	12 (10.2; 14.5)	**63**
2,3,5,6-Tetrachloroaniline	46 (37.8; 55.5)	**202**	27 (24.9; 30.6)	**120**	11 (10.3; 12.9)	**50**
Pentachloroaniline	92 (83;102.6)	**350**	43 (40.1; 46.8)	**164**	18 (16.5; 19.2)	**67**

Lethal and effective concentrations in mg/kg dry soil were positively correlated with the $logK_{ow}$ (Table 2). Earlier findings in soil toxicity tests with structurally related chlorobenzenes (Giesen et al., 2012) and other organic compounds (Droge et al., 2006) always showed a decrease of the toxicity values with increasing lipophilicity, when expressed as mg/kg dry soil. Furthermore, LC_{50} and EC_{50} values based on total soil concentrations expressed as mmol/kg dry soil significantly increased with increasing $logK_{ow}$ (Figure 1).

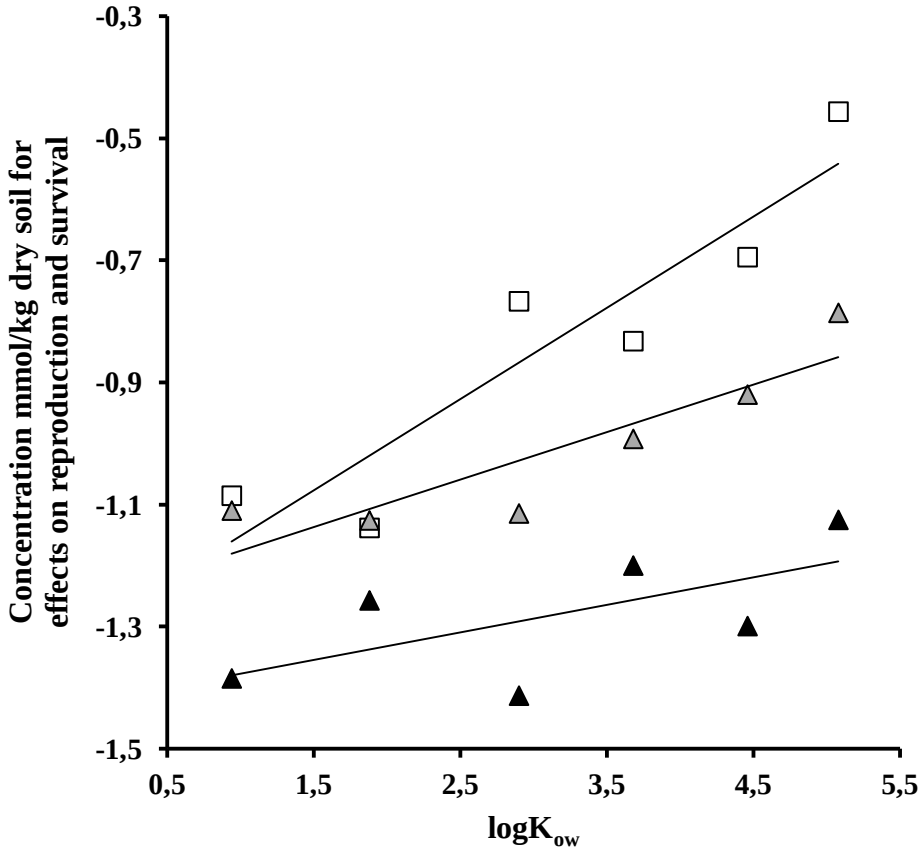

Figure 1: Relationships between LC_{50}, EC_{50} and EC_{10} values for the effect on survival and reproduction of *Folsomia candida* after 28 days of exposure to chloroanilines based on mmol/kg and $logK_{ow}$. LC_{50}s are plotted with white squares, EC_{50} with grey triangles and EC_{10}s with black triangles. Effect on survival and the 50% reduction on reproduction are significantly positively correlated with $logK_{ow}$ (LC_{50} p=0.008; EC_{50} p=0.017)

This is in contradiction with the general hypothesis that based on molar concentrations toxicity is not dependent on the lipophilicity of the compounds (Connell and Markwell, 1990). Similar experiments (PayaPerez and Mannone, 1997; Van Gestel and Ma, 1993) using different isomers, omitting aniline and dichloroaniline, and testing the earthworm species *Eisenia andrei* and *Lumbricus rubellus*, showed no negative correlation of LC_{50} values with lipophilicity. For *F. candida* and the two earthworm species pentachloroaniline appeared to be the least toxic when based on total soil concentrations, with the collembolan being 12-14 times more sensitive than the earthworms (Van Gestel and Ma, 1993). In acute toxicity tests with AN, 4-MCA, 3,5-DiCA and 2,3,4-TrCA, Dom and co-workers (2011) found similar results in tests with the freshwater crustacean *Daphnia magna*. EC_{50} values in mg/l and µmol/l for the anilines were positive correlated with the $logK_{ow}$ while a QSAR based on ECOSAR database values predicted a decrease in EC_{50} values with increasing $logK_{ow}$. Effective concentrations for algae and fish in the same study, however, were negatively correlated with the $logK_{ow}$. Together with the studies of van Gestel and Ma (1993) and findings in this study it appears that aniline and its chlorinated analogues have an exceptional effect on invertebrates, regardless of the applied test system. None of these studies, however, reflects on the uptake site, uptake/elimination kinetics or potential biotransformation. Soil invertebrates may be more exposed to compounds because their whole body surface can be involved in the uptake, while for fish the gills and for mammals ingestion may have stronger effects. Also, especially for soil dwelling organisms the elimination and transformation capacity should not be underestimated as structurally similar compounds derived from e.g. lignin decomposition naturally occur in their habitat. As a consequence, biotransformation activity may be higher, as has been shown for polycyclic aromatic hydrocarbons in Collembola (Howsan and Van Straalen, 2003) and isopods (Stroomberg et al., 2004a; Stroomberg et al., 2004b).

QSAR

Contrasting the above described exceptional behaviour, when expressed on the basis of molar interstitial water concentrations a negative correlation of toxicity values and lipophilicity was found. On the basis of the estimated molar concentrations significant (F-distribution $p>0.001$) QSAR linear regression models were developed for EC_{10} and EC_{50} (Figure 2).

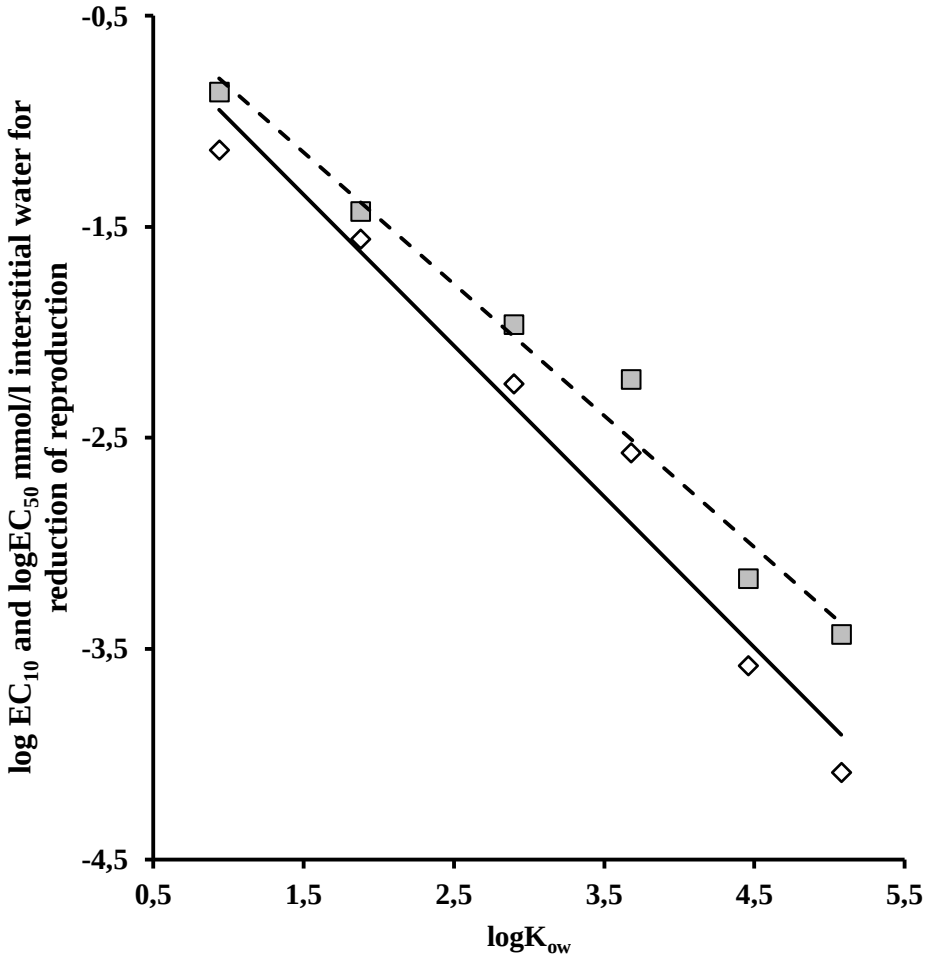

Figure 2: Relationships between EC_{10} (white rhombi; full trendline) and EC_{50} (grey squares; dashed trendline) values for the effect of chloroanilines on reproduction of *Folsomia candida* and the $logK_{ow}$ in LUFA2.2 soil.

Resulting model equations are:

EC_{10} (Eq.1)
Log EC_{10} (mmol/l) = -0.71 * logK_{ow} – 0.272
r^2= 0.968 F= 120.85 n= 6 df= 4

EC_{50} (Eq.2)
Log EC_{50} (mmol/l) = -0.62 * logK_{ow} – 0.212
r^2= 0.975 F= 155.94 n= 6 df= 4

By evaluating the regressions for the EC_{10} and the EC_{50} it is important to notice the sudden drop when moving from 2,3,4-TrCA to the two higher chlorinated compounds for which toxicity values are over one log-unit lower. Applying a preliminary gene-expression response analysis with the same compound series, an additional mode of action for the two highest chlorinated forms was already described for *F. candida*. In a multivariate analysis AN, 4-CA, 3,5-DiCA and 2,3,4-TrCA formed a distinct group together with the non-polar 1,2,3,4-tetrachlorobenzene. For this group gene-expression response only indicated an effect on the membrane but no more specific modes of action. PeCA separated from this cluster, however, not strong enough to be significantly different to be indentified as an uncoupling compound. Only 2,3,5,6-TeCA was clearly identified as proton chain inhibitor (Janssens et al., 2011). On the other hand the developed QSAR is significant with effective concentrations correlating with logK_{ow} and the different modes of action did not have strong effects on this outcome. Gene-response analysis was tested at a concentration corresponding with the EC_{50} derived from a 28-d standard toxicity test, but it used 25-26 d old animals exposed for only 2 days followed by RNA extraction. These age and endpoint differences might explain why the gene-expression response and the QSAR seem to differ. However, for the development of QSARs one should consider the possibility of a “toxic-mimicry” that underlying physiological reactions vary within a group of compound analogues. Compared with models based on tests with non-polar chlorinated benzenes (Giesen et al., 2012), chlorinated anilines induce toxicity at lower concentrations based on their logK_{ow}, so they are more toxic (Figure 2). In the differentiation system for aquatic toxicology developed by Verhaar et al.

(1996), chloroanilines are described as polar narcotics to discriminate them from non-polar organic compounds. Adoption of this system for soil toxicology has proven to be sufficient to describe differences suggesting the $\log K_{ow}$ is a suitable descriptor. Nevertheless, octanol is only a rough surrogate for biological bilayer membranes. Lipids consist of two long fatty acid chains, with the extra potential of double bonds, and a largely polar end with e.g. phosphate or sulphur groups. Polar and non-polar compounds could therefore interact at different positions on the membrane, however, partitioning may be similar. Vaes et al. (1998), determining the partitioning to artificial membranes, showed that it is possible to achieve indiscriminative QSARs for polar and non-polar compounds. It should therefore be seen as an urgent task to determine optimized lipid-mimicking substitutes. In addition, the interaction of organic pollutants with proteins, e.g. trans-membrane, or other biopolymers, e.g. chitin of the exoskeleton, should be investigated. Especially for arthropods the exoskeleton appears to be a yet not well understood potential site of action. The better understanding of the more biological matrix driven partitioning of compounds would greatly help in the further optimization of QSARs and would give a more realistic picture of the compound behaviour in the environment.

Statistical models in ecotoxicological studies, such as QSARs, heavily depend on which $\log K_{ow}$ and $\log K_{oc}$ values are selected for each individual substance. In some instances the values for either partition coefficient can vary over several log units depending which source or type of measurement is used, e.g. comparing different HPLC columns, the $\log K_{oc}$ of 3,5-DiCA varied between 1.64 and 2.64 (Szabo et al., 1999). For the development of the QSARs presented in this study however, it was of more importance to select data for octanol-water and organic carbon partitioning derived and generated by using comparable methods (Sabljic et al., 1995). With this approach the possible differences in sorption were at least consistent over the whole compound series. Using coefficients from other sources might change toxicity data and hence affect the model remotely. Additionally, the uncertainty deriving from using data from a random or per compound hand-picked reference can be excluded.

SPME measurements

Polyacrylate fiber-water partition coefficients ($logK_{fiber}$) determined for 3,5-DCA, 2,3,4-TrCA, 2,3,5,6-TeCA and PCA are presented in Table 3. Due to their relatively short half-life time in the thin polymer coating of the fiber, compared to the time needed for the cleaning and extraction process, AN and 4-MCA were excluded from SPME analysis. K_{fiber} values are, though slightly lower, proportional to octanol-water partition coefficients ($logK_{ow}$) (Figure 3).

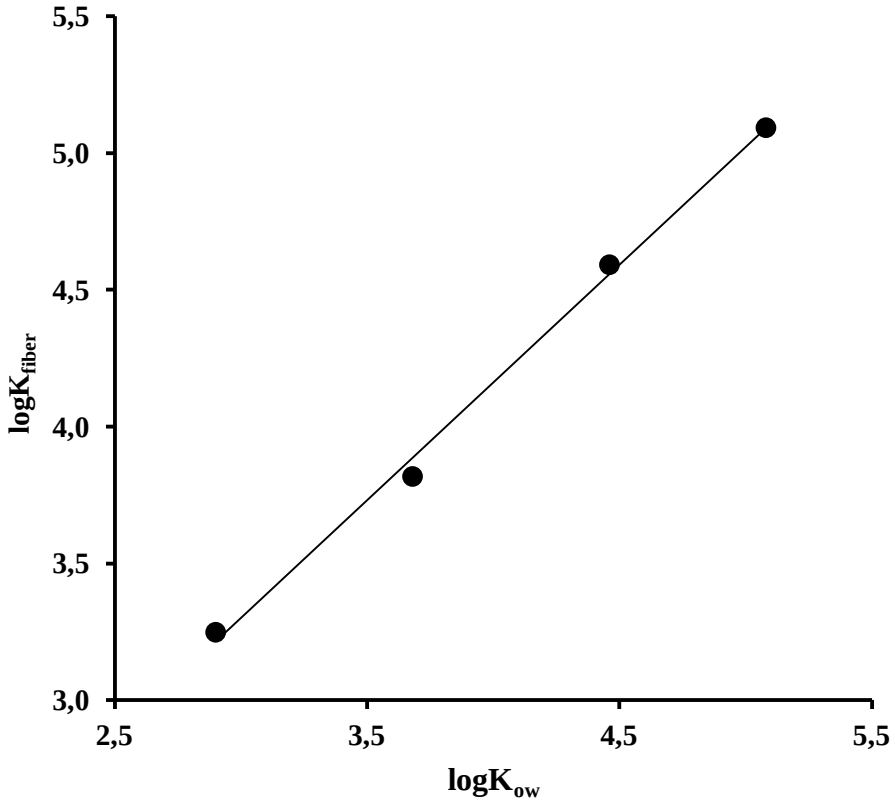

Figure 3: Relationship between polyacrylate fiber-water partition coefficients ($logK_{fiber}$) and octanol-water partition coefficients ($logK_{ow}$) for chloroanilines

Obtained values were used for the subsequent determination of chloroaniline concentrations corresponding with the EC_{10} and EC_{50} values in interstitial water of LUFA2.2 soil: Table 3.

Table 3. Polyacrylate fiber-water partition coefficients ($logK_{fiber}$) for selected chloroanilines and measured concentrations in interstitial water of LUFA2.2 soil at EC_{10} and EC_{50} levels for the effect on the reproduction of *Folsomia candida.* Concentrations are given as µg/l with standard deviations (n=4).

			SPME LUFA2.2			
			EC_{10}		EC_{50}	
Chemical	$logK_{fiber}$	stdev	µg/l	stdev	µg/l	stdev
3,5-dichloroaniline	**3.25**	0.02	**167.8**	9.6	**434.6**	10.6
2,3,4-trichloroaniline	**3.82**	0.02	**284.3**	15.0	**481.1**	27.6
2,3,5,6-tetrachloroaniline	**4.59**	0.02	**60.2**	2.3	**187.2**	7.6
Pentachloroaniline	**5.09**	0.02	**49.7**	2.0	**104.6**	5.0

In Figure 4 the EC_{10} and EC_{50} based on estimated interstitial water concentrations, used for QSAR development, and the respective values based on SPME measurements are compared. Predicted concentrations for 2,3,5,6-TeCA and PCA are similar to measured concentrations for both the EC_{10} (Figure 4a) and the EC_{50} (Figure 4b). For the other two chlorinated anilines the difference between predicted and measured effect concentrations increased with decreasing $logK_{ow}$ from 2,3,4-TrCA to 3,5-DCA. Biodegradation (Gullotto et al., 2008; Loidl et al., 1990; Susarla et al., 1997a; Susarla et al., 1997b) or unspecific enzymatic reaction in the soil (Park et al., 1999) of lower chlorinated anilines have been described, but these can be excluded as a reason for the high loss of 3,5-DCA. Any biological reaction was stopped by the added sodium azide and the soils were spiked with the test compounds no more than 12 h before the SPME measurement started. Persistent soil bound residues may also be formed by lower chlorinated anilines, leading to an irreversible integration into the soil organic matter (Bollag and Loll, 1983; Park et al., 1999, 2000).

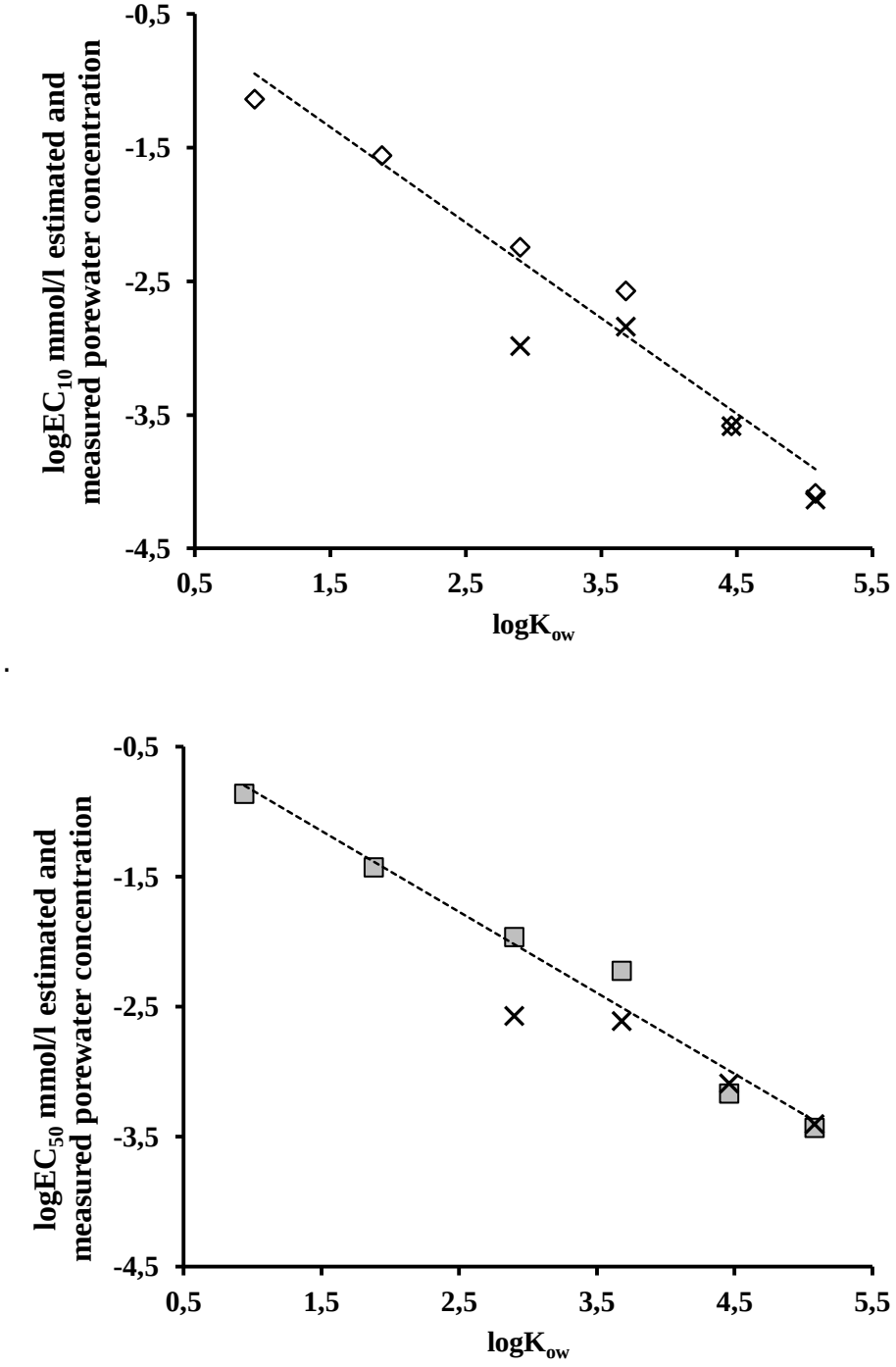

Figure 4: Comparison of the relationships between estimated (K_{oc}-derived) EC_{10} (a; white rhombi) and EC_{50} (b; grey squares) values and measured (SPME-derived) EC_{10} (a; black x) and EC_{50} (b; black x) values for the toxicity of chloroanilines to *Folsomia candida* in LUFA2.2 natural soil.

This might offer an explanation why only for 2,3,5,6-TeCA and PeCA the measured concentrations match the estimated ones used for the QSAR. As SPME-derived values reflect the actual interstitial water concentrations, $\log K_{oc}$ values derived from the literature may provide weak reflections of actual sorption, which has been observed for other chemicals (Hawthorne et al., 1999). Furthermore, the sorption to inorganic fractions of the soil is not included as long as sorption is not measured for each compound and soil specifically. That chloroanilines with their polar amino group do bind to the surface of clay particles has already been shown (Angioi et al., 2005; Polati et al., 2006), but to what extent this can have an influence on the bioavailable concentration remains unclear. Another more reasonable explanation for the lower concentration in the order 3,5-DCA< 2,3,4-TrCA < 2,3,5,6-TeCA / PeCA is the loss due to volatilisation. The higher chlorinated compounds have a lower Henry's Law constant (Table 1) and a lower chemical activity based on the melting point. Volatilization will already occur during the spiking procedures, for both the toxicity test as well for the SPME analysis, and is inevitable. Hence a optimisation of QSARs based on initial nominal concentration is not accomplishable.

Conclusion

Soil dwelling springtails, such as *F. candida*, are generally more sensitive towards organic contamination than other soil organisms like the earthworm species *E. andrei* or *L. rubellus*. Therefore, springtails represent an optimal animal model to evaluate potential threats of organic chemicals to terrestrial invertebrates and hence to ecosystem function, and should be favoured in the application of REACH. The particular effect of aniline and its chlorinated analogous to invertebrates needs future attention. As it appears to be hard to explain toxicity in mg/kg from the $\log K_{ow}$, it underlines the necessity to calculate bioavailable interstitial water concentrations as is done in risk assessments.

The QSARs developed using estimated EC_{10} and EC_{50} values based on concentrations in the interstitial water phase of the soil follow baseline toxicity but are lower compared to those for the non-polar chlorobenzene analogues (Giesen et al., 2012). However, though the QSARs are significant, the question of toxic-mimicry remains, as four out the six tested compounds

do excite their toxicity through membrane disruption (narcosis) while the two others have an indifferent effect or a proton chain uncoupling effect. The combination of QSAR development with gene-expression response analysis can identify and explain outliers and make them accessible to a more detailed risk assessment. Additionally, estimated EC_{10} and EC_{50} values were only confirmed for tetra- and pentachloroaniline by SPME analysis and therefore a validation of the developed QSAR was not achievable. The interstitial water concentrations of 2,3,4-TrCA and especially 3,5-DCA were clearly overestimated by the f_{oc} and the applied K_{oc}. Hence the prediction of toxicity in the applied compound series cannot be based solely on their lipophilicity. Nevertheless, QSARs will remain a very important tool for assessing potential risks of individual compounds and compound series. But their relevance may be further increased when supported with (SPME) measured available concentrations.