

Environmental Research
of the Federal Ministry for the Environment,
Nature Conservation and Nuclear Safety

UBA Projekt: „*Empfindlichkeitsvergleich der
Daphnien- und Fischtoxizität - wann kann auf einen
chronischen Fischtest verzichtet werden?*“

Project No. 27448

**Comparison of species
sensitivity of *Daphnia* and fish
in acute and chronic testing**

by

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ON BEHALF OF THE
FEDERAL ENVIRONMENT AGENCY

Hannover, 7th May 2014

Report Cover Sheet

Report No.	UBA-FB 27448
Report Title	Comparison of species sensitivity of <i>Daphnia</i> and fish in acute and chronic testing
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Funding Agency	Umweltbundesamt Postfach 14 06 06813 Dessau-Roßlau
Report Date (Year)	2014
Project No. (FKZ)	27448
No. of Pages	47
Supplementary Notes	
Keywords	Aquatic toxicity, environmental risk assessment, acute to chronic extrapolation, species sensitivity

Berichtskennblatt

Berichtsnummer	UBA-FB 00
Titel des Berichts	Vergleich der Sensitivität von <i>Daphnia</i> und Fischen in akuten and chronischen Studien
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Fördernde Institution	Umweltbundesamt Postfach 14 06 06813 Dessau-Roßlau
Abschlussjahr	2014
Forschungskennzahl (FKZ)	27448
Seitenzahl des Berichts	47
Zusätzliche Angaben	
Schlagwörter	Aquatische Toxizität, Umweltrisikobewertung, "acute to chronic extrapolation", Speziessensitivität

Abstract

Based on the animal welfare concept (Art. 13, Art. 25), the REACH Regulation (EC 1907/2006) provides several options to avoid the long term fish toxicity test. About 240 substances from the ECHA and ICS database including 73 pesticides are analysed for species sensitivity differences and acute to chronic ratios to evaluate whether and when chronic fish toxicity tests can be avoided without underestimating environmental risk. Only studies that have been conducted in line with guidelines recommended in the EU guidance documents are used for this study. Sensitivity comparison of fish and Daphnia toxicity indicates that none of both trophic levels is generally more sensitive in acute or long term testing. Based on the finding that the sensitivity in chronic testing is associated with sensitivity in acute testing a classification scheme for acute sensitivity comparison was proposed to contribute the integrated testing strategy. The categorization system can be applied independently of the physicochemical properties water solubility and octanol-water partitioning. Based on the data evaluation the chronic fish test is required for about 13 % of the substances. For substances being 5x more sensitive to one trophic level long term testing of the respective trophic level should be conducted. Additionally, the results show that an assessment factor of 100 for acute to chronic extrapolation, as implied by the European guidance documents, is protective for >90 % of the industrial chemicals. Moreover, a relation between Kow and increased ACR values and species sensitivity can not be confirmed suggesting that the Kow does not represent a determinant indicating the requirement of chronic fish testing. Identification of chemical structures that are associated with significantly increased potential for a high ACR or fish sensitivity in chronic testing leads to the derivation of structural alerts.

Kurzbeschreibung

Auf der Grundlage des Tierschutzgedankens (Art. 13, Art.25) bietet die REACH-Verordnung (EG 1907/2006) verschiedene Optionen, um auf den chronischen Fischtest für die Umweltrisikobewertung zu verzichten. Über 240 Stoffe aus der ECHA und ICS-Datenbank, darunter 73 Pflanzenschutzmittel, werden in dieser Studie auf die Speziesempfindlichkeit und das Verhältnis zwischen akuten zu chronischen Effektwerten ausgewertet, um zu untersuchen unter welchen Bedingungen auf den chronischen Fischtest verzichtet werden kann. Für die Studie werden ausschließlich Studien verwendet, die entsprechend den Leitlinien der EU empfohlen werden. Der Empfindlichkeitsvergleich von Fischen und Daphnia zeigt, dass keine der beiden trophischen Ebenen in akuten oder langfristigen Tests systematischer empfindlicher ist als die andere. Die Ergebnisse deuten darauf hin, dass die Empfindlichkeit einer Trophieebene in chronischen Tests mit der Empfindlichkeit in den akuten Tests verknüpft ist. Für die integrierte Teststrategie wird daher ein Klassifikationsschema für den Empfindlichkeitsvergleich akuter Daten vorgeschlagen, welches unabhängig von den physikalisch-chemischen Eigenschaften Wasserlöslichkeit und Oktanol-Wasser Verteilung angewendet werden kann. Basierend auf den Daten dieser Studie ist der chronische Fischtest in etwa 13 % der Fälle notwendig und in erster Linie angezeigt für Substanzen, die im akuten Test >5x toxisch gegenüber dem Fish als Daphnien sind. Weiterhin kann gezeigt werden, dass ein Extrapolationsfaktor von 100, wie er in der Europäischen Union für die Extrapolation der chronischen Toxizität aus Daten von Kurzzeitstudien verwendet wird, für >95 % der Chemikalien hinreichend protektiv ist. Darüber hinaus kann kein Zusammenhang zwischen dem Kow Wert als Determinate und einem erhöhten ACR-Wert sowie einer bestimmten Speziessensitivität bestätigt werden. Die Identifikation von chemischen Strukturen mit deutlich erhöhtem Potenzial für einen hohen ACR oder einer stark ausgeprägten Sensitivität von Fischen resultiert in der Ableitung von „structural alerts“.

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List of Abbreviations

ACR = Acute to Chronic Ratio

AF = Assessment Factor

CESAR = Canada's Existing Substances Assessment Repository

EC = Effect Concentration

ECHA = European Chemicals Agency

EU = European Union

FELS = fish early life stage

ICS = Information System Chemical Safety database

Kow= partition coefficient (1-octanol/water)

LC = Lethal Concentration

MoA = Mode of action

NOEC = No Observed Effect Concentration

OECD = Organisation for Economic Co-operation and Development

PEC = Predicted Environmental Concentration

PNEC = Predicted No Effect Concentration

Qa = LC50 (fish, acute) / EC50 (Daphnia, acute)

Qc = NOEC (fish, chronic) / NOEC (Daphnia, chronic)

Qc* = 5 x NOEC (fish, chronic) / NOEC (Daphnia, chronic)

QSAR = Quantitative structure–activity relationship

REACH = Registration, Evaluation, Authorisation and Restriction of Chemicals (European Union Regulation)

SIDS = Screening Information Data Set

TTC = Threshold of Toxicological Concern

US EPA = US Environmental Protection Agency

Summary

Based on the animal welfare concept and to avoid animal experiments (Art. 13, Art. 25), the REACH Regulation (EC 1907/2006) provides several options for waiving the chronic fish toxicity test. The present study addresses the question whether the integrated testing strategy (ITS) approach is adequate and applicable for environmental risk assessment regarding the need of long term vertebrate testing. In particular, it is evaluated whether and when chronic fish toxicity can be extrapolated from acute data or from other trophic levels.

The dataset is based on data from the OECD eChemPortal and from the Information System Chemical Safety database (ICS) of the German Federal Environmental Agency. In total about 240 compounds are considered including 169 industrial chemicals and 73 pesticides. The majority represents organic substances that are underlain by an FELS test. Animal testing can be avoided for the majority of chemical substances since chronic Daphnia and fish toxicity levels are related to each other, and fish toxicity can be estimated to a certain degree from chronic Daphnia test results. Based on this data evaluation the chronic fish toxicity test is required for about 13 % of the substances for risk estimation and can not be estimated from chronic Daphnia data in a protective manner in these cases.

The statistical findings of the sensitivity comparison of Daphnia and fish shows in average a rather similar sensitivity between fish and invertebrate toxicity in acute and chronic testing with Daphnia being slightly more sensitive than fish. The study further shows that the more sensitive trophic level in chronic testing is associated with its sensitivity in acute testing. Thus, chronic species sensitivity could be estimated from acute testing. To estimate chronic test requirements the study proposes a classification system for an acute sensitivity ratio to support the ITS. The result suggests that the classification system can be applied independent of physicochemical properties. Since chronic testing strategies are usually based on an initial evaluation of acute data the here presented classification scheme may contribute to a scientifically justified testing strategy. For substances being 5x more sensitive to one trophic level in acute testing long term testing of the respective trophic level is required. A chronic fish toxicity test should usually not be requested unless fish are 2x more sensitive than Daphnia in acute testing. The presented categorization scheme differs from the current guidance documents which consider a threshold of 10 for sensitivity distinction between trophic levels (ECHA, 2012). Only 10 % of the evaluated substances were 10x more sensitive towards one trophic level. Quantitative sensitivity analysis of chronic testing further shows that the chronic Daphnia test is considered sufficiently protective for more than 85 % of the analysed substances. Fish toxicity can be adequately evaluated from chronic Daphnia test for substances that are comparable or more toxic to Daphnia in acute testing. By contrast, the chronic fish test is already required for substances being 5x more sensitive to fish than to Daphnia. In this case, extrapolation from chronic invertebrate data is not adequate and a threshold of 10 might underestimate chronic fish sensitivity. Thus, adaption of the current ITS is suggested by this data analysis proposing a reduction of the sensitivity factor from 10 to 5.

Analysis of acute to chronic ratios is an important tool to derive acceptable No-Effect levels and to re-evaluate and support current risk assessment approaches. This analysis evaluates ACRs from different trophic levels of a comprehensive data set in the context of an individual substance. The evaluation of existing data on Daphnia and fish toxicity testing shows that acute to chronic extrapolation represents a sound approach for environmental risk estimation. For chemicals median ACRs of 12.2 for fish and 8.8 for Daphnia as well as 90 percentiles of 68.0 and 50.2 were determined. The ACR for the most sensitive aquatic species (ACRaqu) is derived by comparing the lowest acute and chronic effect value. The median was determined to 9.9

and the 90 %-percentile was 58.5. Based on these data an ACR of 100 as implied by the European guidance documents is protective for more than 90 % of the substance regarding Daphnia and fish toxicity of industrial chemicals. In addition an AF of 1000 applied on the lowest acute effect level of three trophic levels was not exceeded in any case. Subsequently, chronic data usually improves or knowledge on toxicity levels and contributes to a refinement of the PNEC. In contrast the current extrapolation approach is not protective for pesticides since pesticides exhibit median ACRs of 17.2 for fish and 11.1 for Daphnia and 90 percentiles of 154.2 and 109.4, respectively.

The physicochemical properties water solubility and the octanol-water partitioning are mentioned in the REACH Regulation and the corresponding guidance documents as determinants that indicate the need of chronic data or the requirement of a chronic fish toxicity test for risk assessment. The results of this study indicated that a predictive value on the need for chronic test can not be proven for both properties since the chronic fish toxicity test is only required for <20% of the substances with a log Kow > 3 and for <10% of the substances with a poor water solubility. The results rather show that both acute to chronic extrapolation and species sensitivity of a substance can be assessed independently of the water solubility and Kow. The results suggest that both the chronic toxicity level as well as species sensitivity can be adequately derived from the effect values of acute toxicity testing. A differentiated analysis for substances only shows that a high Kow may be a trigger for a chronic fish toxicity test if fish were 2x to 5x more sensitive than Daphnia in the acute tests. Thus, the physicochemical properties water solubility and the octanol-water partitioning have a supporting character if certain conditions are met, but do not seem to be good indicators for the requirement of long term or vertebrate tests as implemented in the REACH Regulation and the corresponding guidance documents.

For a number of substances that exhibit an increased sensitivity to fish in chronic testing a structural relationship is determined. For example, para-substituted phenols represent a group of substances that is identified with an increased probability of a pronounced toxic effect to fish in the chronic test. Thus, analysis of structural alerts in the context of the ITS could provide an opportunity to evaluate the need of chronic fish toxicity tests and to determine exceptions from the applied approach. However, it has to be taken into account that the ratio of false positive results by structural alert analysis can be quite high and that a majority of identified "structural alert" already show an increased sensitivity towards fish in acute testing.

Zusammenfassung

Auf der Grundlage des Tierschutzgedankens (Art. 13, Art.25) bietet die REACH-Verordnung (EG 1907/2006), verschiedene Optionen, um auf den chronischen Fischtest zu verzichten. Die vorliegende Studie befasst sich mit der Frage, ob die ITS zutreffend und ausreichend für die Umweltrisikobewertung in Bezug auf die Notwendigkeit von chronischen Wirbeltierversuchen ist. Für die Analyse wird ein Datensatz von 240 chemischen Substanzen der sowohl Industriechemikalien als auch Pflanzenschutzmittel umfasst, in Bezug auf die Speziesempfindlichkeit und das Verhältnis zwischen akuten zu chronischen Effektwerten ausgewertet, um zu untersuchen unter welchen Bedingungen auf den chronischen Fischtest verzichtet werden kann. Der Datensatz basiert auf Daten des OECD eChemPortal Informationssystems und des Informationssystems Chemikaliensicherheit (ICS) des Umweltbundesamtes und umfasst in zum Grossteil Datensätze von organischen Substanzen bei denen die chronische Fischtoxizität mittels des FELS test untersucht wurde. Die Ergebnisse zeigen, dass Tierversuche für die meisten chemischen Substanzen vermieden werden können, da die chronische Daphnia- und Fischtoxizität in Beziehung zu einander stehen. Somit kann die Fischtoxizität bis zu einem gewissen Grad anhand des chronischen Daphnien-Testergebnisse abgeschätzt bzw mittels eines angemessenen Sicherheitsfaktors abgedeckt werden. Basierend auf den Daten dieser Studie und unter Einbeziehung eines AF von 50 auf den chronischen Daphnientest ist ein chronischer Fischtest für etwa 13% der analysierten Substanz für die Risikoabschätzung erforderlich. Dies gilt insbesondere für Substanzen die bereits im akuten Test eine hohe Sensitivität für Fische zeigen.

Die statistische Auswertung des Empfindlichkeitsvergleich von Fischen und Daphnia zeigt, dass im Durchschnitt keine der beiden trophischen Ebenen in akuten oder langfristigen Tests empfindlicher ist als die andere. Die Studie zeigt weiterhin, dass die Speziessensitivität in chronischen Tests mit der Sensitivität in akuten Tests verbunden ist und die Empfindlichkeit einer Art im chronischen Test aus den Ergebnissen der akuten Tests abgeschätzt werden kann. Im Rahmen des Projekts wird ein Klassifizierungssystem für den Vergleich der akuten Empfindlichkeit von Fischen und Daphnien vorgestellt, um die Testanforderungen an chronischen Studien abzuschätzen. Das Klassifikationssystem kann unabhängig von physikalisch-chemischen Eigenschaften und außerdem für Stoffe, die einen spezifischen Wirkungsmechanismus durch die Interaktion mit bestimmten Rezeptormolekülen (MoA4) aufweisen, angewendet werden. Da die Teststrategie in der Regel auf einer Auswertung der akuten Daten basiert, bietet das hier vorgestellte Klassifikationsschema einen empirisch begründeten Ansatz für die integrierte Teststrategie und die Abschätzung der Notwendigkeit von chronischen Fischtests für die Risikobewertung. Die quantitative Sensitivitätsanalyse der chronischen Tests zeigt weiterhin, dass der chronische Daphnia Test unter Einbeziehung eines AF von 50 als ausreichend protektive für mehr als 85% der untersuchten Substanzen angesehen werden kann. Entsprechend der statistischen Ergebnisse sollte ein chronischer Fischtest in der Regel nicht gefordert werden, insofern Fische im akuten Test nicht 2-fach empfindlicher sind als Daphnien. Im Gegensatz dazu ist der chronische Fischtest schon für Substanzen erforderlich, die 5-fach empfindlicher gegenüber Fischen als Daphnien sind. In diesem Fall ist der Extrapolation von Daten aus chronischen Invertebraten-Tests nicht ausreichend protektiv. Das vorgeschlagene Klassifizierungsschema unterscheidet sich von den aktuellen Europäischen Leitlinien, die eine Schwelle von 10 für die Empfindlichkeit zwischen trophischen Ebenen (ECHA, 2012) berücksichtigen. Auf Basis diese Datenanalyse wird gezeigt, dass bereits bei einem Sensitivitätsunterschied von >5x die Testung des sensitiveren trophischen Levels notwendig ist. Daher wird eine Überprüfung des aktuellen Schwellenwerts für den Empfindlichkeitsvergleich vorgeschlagen und eine Verringerung des Faktors von 10 auf 5 empfohlen.

Die Analyse des „acute to chronic ratios“ (ACR) ist ein wichtiges Instrument, um No-Effect Konzentrationen (NOEC) abzuleiten. Diese Studie untersucht ACR von verschiedenen trophischen Ebenen und für die jeweils sensitivsten Arten in akuten und chronischen Test (ACRaqu). Die Auswertung der vorhandenen Toxizitätsdaten für Daphnien und Fische zeigt, dass die Ableitung der chronischen Toxizität durch Extrapolation des akuten Effektwerts einen robusten Ansatz für die Umweltrisikobewertung darstellt. Für Industriechemikalien wird ein Median des „acute to chronic ratio“ (ACR) von 12,2 für Fische und 8,8 für Daphnia sowie eine 90. Perzentile von 68,0 und 50,2 ermittelt. Der ACR für die empfindlichsten aquatischen Arten (ACRaqu) wird durch den Vergleich des niedrigsten akuten und chronischen Effektwerts abgeleitet. Der Median ACRaqu ergibt einen Wert von 9,9 und eine 90. Perzentile von 58,5. Somit ist ein Extrapolationsfaktor von 100, wie er in der Europäischen Union verwendet wird, für mehr als 90 % der Chemikalien für Daphnia und Fische hinreichend protektiv. Außerdem wird ein Extrapolationsfaktor von 1000 der auf den niedrigsten akuten Effektwert aller drei trophischen Ebenen angewendet wird in keinem Fall für den ACRaqu überschritten. Hingegen kann der gegenwärtige Extrapolationsansatz als nicht hinreichend protektiv für die Bewertung von Pflanzenschutzmitteln angesehen werden, die einen Median ACR von 17,2 für Fische und 11,1 für Daphnia und eine 90. Perzentile von 154,2 und 109,4 aufweisen.

Die physikalisch-chemischen Eigenschaften Wasserlöslichkeit und der Oktanol-Wasser-Verteilungskoeffizienten werden in der REACH Verordnung und den entsprechenden Leitfäden mehrmals als Determinanten genannt, die auf die Erfordernis von chronischen Daten oder die Notwendigkeit eines chronischen Fischtests für die Risikobewertung hinweisen. Die Ergebnisse dieser Studie zeigen, dass ein Einfluss beider Eigenschaften auf die Vorhersage der Notwendigkeit von chronischen Fischtests nicht vorhanden ist, da ein chronischer Fischtest nur in <20 % der Substanzen mit einem $K_{ow} > 3$ und in <10 % der schlecht wasserlöslichen Substanzen notwendig ist. Weiterhin zeigen die Daten, dass auch die Höhe des ACR unabhängig von der Wasserlöslichkeit und des Oktanol-Wasser-Verteilungskoeffizienten einer Substanz ist und somit bei hohen log K_{ow} oder geringer Wasserlöslichkeit die Wahrscheinlichkeit für einen relevant erhöhten ACR nicht gegeben ist. Eine differenzierte Analyse für Stoffe, die 2 bis 5x sensitiver gegenüber dem Fisch verglichen zur Daphnia sind, zeigt lediglich, dass ein hoher K_{ow} ein Indikator für eine Notwendigkeit eines chronischen Fischtoxizitätstests darstellen kann, insofern Fische bereits in akuten Tests 2x bis 5x empfindlicher als Daphnia waren. Daher spielen die physikalisch-chemischen Eigenschaften Wasserlöslichkeit und der Oktanol-Wasser-Verteilungskoeffizienten eher eine untergeordnete Rolle bei der Abschätzung von chronischen Testanforderungen und sollten nicht, wie im Moment in den „Guidance documents“ der EU verankert, als generelle Determinanten verwendet werden, die allgemeingültig und unabhängig die Notwendigkeit chronischer Tests anzeigen. Für eine Reihe von Substanzen, die eine erhöhte Sensitivität von Fischen im chronischen Test aufweisen, kann eine strukturelle Ähnlichkeit gezeigt werden. So stellen beispielsweise para-substituierte Phenole eine Stoffgruppe dar, die eine erhöhte Wahrscheinlichkeit für eine ausgeprägte toxische Wirkung im chronischen Fischtest zeigen. Somit könnte die Analyse von „structural alerts“ im Rahmen der integrierten Teststrategie eine Möglichkeit bieten, um die Notwendigkeit von chronischen Fischtests abzuschätzen und um Ausnahmen von der angewandten Methode zu erkennen. Allerdings bleibt zu berücksichtigen, dass der Anteil an falsch positiven Ergebnissen recht hoch sein kann und, dass die Mehrzahl der identifizierten „structural alerts“ bereits im akuten Test eine erhöhte Sensitivität gegenüber Fischen zeigt.

2 Introduction

With the registration of chemicals under the REACH Regulation (EC 1907/2006) manufacturers, importers and downstream users take responsibility for the safe use of their chemicals. The information required for registration depends on the produced or imported quantity of the chemical. Depending on the tonnage column 1 of Annexes VII to X of the REACH Regulation defines each of the experimental data requirements (physico-chemical, toxicological and ecotoxicological studies) that must be supplied by the registrant. Column 2 of the respective Annexes states the options for possible deviations from the standard testing regime (EU, 2006).

The basic idea to allow those deviations is the intention of the REACH Regulation, to avoid animal/ vertebrate testing as far as possible and to use non-animal test methods. In Article 25 the study of vertebrates is only considered as a last resort in the data collection and Article 13 suggests other methods such as QSAR and read across approaches. The applicability of the deviations that are intended to reduce the number of (animal) tests is carefully examined on the basis of the criteria set out in Annex XI. Data waiving or a selected alternative study needs to be fully/explicitly and transparently justified (EU, 2006).

For the decision on the need of ecotoxicological tests on animals ECHA guideline R7b and R10 are applied (ECHA, 2008; ECHA, 2012). In particular guideline R7b provides guidance for the assessment of ecotoxicological endpoints and includes an integrated testing strategy (ITS). Initially, aquatic short term studies on three different trophic levels (algae, invertebrates and fish) are the basis for the ecotoxicological evaluation of a chemical. Depending on the tonnage, as well as substance properties and effect data, further studies may be necessary to refine the risk assessment. These additional studies may comprise long term/chronic studies on invertebrates or fish. On the basis of the animal welfare concept and to avoid animal experiments (Art. 13, Art. 25), the ITS provides several options for waiving the chronic fish toxicity test. According to the usual procedure conducted by the ECHA a chronic fish toxicity test is basically required for substances with a tonnage >100 t/a, or if fish is likely to be at least a factor of about 10 less sensitive than other trophic levels or if a risk is identified by using the PEC / PNEC ratio based on the result of the chronic Daphnia test (ECHA, 2012). Additionally, QSAR, read across and other studies should be taken into account for decision making.

An environmental risk assessment of chemical substances (industrial chemicals, biocides, pesticides, pharmaceuticals) is based on the comparison of the Predicted No effect Concentration (PNEC) and the predicted environmental concentration (PEC). The PNEC represents a concentration below which unacceptable effects are not expected and is usually derived from laboratory effect studies by applying an assessment factor on the lowest determined effect concentration or the No Observed Effect Concentration. The size of an assessment factor should cover several uncertainties such as intra- and interspecies variations, short- to long-term toxicity extrapolation and intra- and inter-laboratory variation. Moreover, some chemicals may show different modes of action (MOAs) under short- and long-term conditions. According to European guidance documents an assessment factor of 1000 is applied on the lowest effect value for acute testing from the three trophic levels of algae, invertebrates and fish if only data from acute testing is available. This factor can be refined to 100, 50, or 10 if the values of No Observed Effect Concentrations (NOEC) are available from long-term tests covering one, two, or three trophic levels, respectively (ref)(EC, 1996; ECHA, 2008). Although not explicitly mentioned the procedure implies an acute to chronic extrapolation factor of 100 based on the difference of the assessment factor from acute and chronic testing of three trophic levels. This approach includes first extrapolation from the EC50 to the NOEC and

second extrapolation from short term to long term exposure (EC, 1996; ECHA, 2008; ECHA, 2012).

Extrapolation from acute effect values to chronic toxicity on the basis of publicly available data has already been analysed previously by several studies (Ahlers et al., 2006; ECETOC, 2003; Heger et al., 1995; Lange et al., 1998; Mayer et al., 1994; Raimondo et al., 2007; Roex et al., 2000). However, previous studies usually derived ACR for single trophic level, but chemical substances have not been evaluated in the context of existing data from all relevant trophic levels as demanded by the current risk assessment approaches in the EU and the US.

Furthermore, the data basis compiling experimental data for ACR extrapolation was limited in these publications and the reliability of some literature data could not be tracked. Moreover, a lot of data refer to chemicals with a high toxicity or a specific mode of action such as pesticides and metals, and usually ACRs of different trophic levels for the same substance were not compared. Especially, experimental data on chronic fish toxicity is still limited and an increased variability in ACR is expected as fish studies cover different species, exposure times and endpoints. For invertebrates in particular *Daphnia magna* is established as standard test organism and meanwhile exposure time and endpoints are standardized for acute and chronic testing in the official test guidelines. An ACR for algae represents the difference between the EC50 and the chronic effect value, either the NOEC or the EC10 from the same study.

Therefore, the lowest ACR compared to the other trophic levels is usually found for algae (Ahlers et al., 2006). The most recent studies on ACRs determined average ACR values of about 10 for fish and 8 for *Daphnia* (Ahlers et al., 2006; Raimondo et al., 2007) being in general agreement with the current extrapolation approaches in the EU and the US (ECHA, 2008; Zeemann, 1995).

3 Objectives

With regard to the limited data of chronic fish toxicity tests and the waiving options for chronic fish toxicity testing; conclusions on aquatic risk estimation need to be evaluated and the question arises whether the chronic toxicity of fish can be adequately derived from acute data or from other trophic levels to avoid animal testing. In particular species specific mechanisms of long term toxicity such as effects on developmental stages of fish cannot be covered by acute tests on fish or by chronic studies on invertebrate or algae. The aim of this study was to investigate the ACR of Daphnia and fish studies for each compound and to analyse the currently proposed hazard evaluation according to the ITS on the basis of already existing toxicity tests. Based on species sensitivity comparison the results of the data analysis provide information on whether and when chronic fish toxicity tests can be avoided without underestimating environmental hazards. Furthermore, the physicochemical properties solubility in water and octanol-water partitioning and their predictive value for the risk assessment for the aquatic environment are analysed.

4 Materials and Methods

Data sources and data collection

Data were obtained by using the OECD eChemPortal and the Information System Chemical Safety database (ICS) of the German Federal Environmental Agency. The OECD eChemPortal database accesses a multitude of participating data sources, such as the ECHA data base of registered substances, EPA data sets or Canada's Existing Substances Assessment Repository (CESAR), as well as the OECD SIDS. The OECD eChemPortal further allows a variety of search options by which the aim of this study could be purposefully processed. Database analysis was conducted in July 2013. The dataset was retrieved by the OECD eChemPortal and throughout based on the ECHA database. Data entries in the ECHA database were generated for the chemical registration under REACH by the registrant. Data from the ECHA database is provided by the registrants is confidential and thus the primary data source could not be evaluated. Additionally, data was extracted from the ICS database comprising dataset of pesticide and new registered chemicals. Data of the ICS database were peer reviewed by the German authority.

Only substance entries with acute and chronic studies on both *Daphnia* and fish were considered. Selection of data is based on studies that were conducted according to the recommended guidelines in the EU, and thus met comparable test conditions. Selection criteria for comparison were conformity in species, endpoints investigated and test duration as well as a documentation of the concentrations.

Specification of fish data

Short term fish toxicity was ascertained from the 96 h LC₅₀ of tests performed according to OECD 203 or comparable design (OECD, 1992a). For long term tests on fish the Fish early-life stage test (FELS) according or equivalent to OECD 210 was considered (OECD, 1992b). Equivalent studies comprise tests conducted according to the EPA guideline OPPTS 850.1400 and EPA OPP 72-4. In addition, OECD 212 (Fish toxicity test on embryo and sac-fry stages as well as OECD 215 (Fish juvenile growth test were considered to evaluate the chronic toxicity (OECD, 1998b; OECD, 2000). Non-guideline studies were considered only if a well-documented study comprised early life stage, juvenile growth or embryo or sac fry stages. Furthermore, substance concentrations should have been verified or at least in one test it should be documented that the measured concentration corresponds to the nominal concentration and that the substance is stable under the test condition. Read across and studies on adult fish according to OECD 204 were not considered (OECD, 1984). If more than one study was documented for an endpoint the lowest effect concentration was considered, in general, as this is usually relevant for risk assessment. Fish studies were primarily conducted on the recommended freshwater species *Pimephales promelas*, *Danio rerio*, *Oncorhynchus mykiss* and *Oryzias latipes* and in particular case on others such as *Jordanella floridae* or *Cyprinus carpio*.

Specification of *Daphnia* data

For short term test on invertebrates studies conducted according or equivalent to the OECD Guideline 202 (*Daphnia* acute test) using the 48 h EC₅₀ value were considered (OECD, 2004). Chronic *Daphnia* tests according or equivalent to OECD 211 (*Daphnia* chronic test) were considered using the 21 d NOEC (OECD, 1998a). Studies on invertebrates were mostly conducted on *D. magna* and in particular special cases on *D. pulex*. Other invertebrate species were not considered.

Specification of the data set

The intersection search comprising both acute and chronic guideline studies on aquatic invertebrates and fish resulted in about 167 industrial chemicals and 73 pesticides entities. Within the data set 27 poorly water-soluble substances and 34 substances with a log Kow > 4.5 were identified. Organic chemicals exhibit the most comprehensive group including hydrocarbons, aromatic hydrocarbons, halogenated compounds, polycyclic dyes, amino and nitro compounds, alkyl sulfate and sulfonyl derivatives as well as phenol derivatives. ACR could be derived from 133 organic chemicals and 71 pesticides that were considered within the scope of this study.

Data analysis

To calculate acute to chronic ratios ACR the lowest EC50 or LC50 from acute tests for Daphnia and fish were divided by the respective chronic studies, where the NOEC is determined from the most sensitive endpoint of hatchability, survival, growth, or reproduction, for example. Open ended toxicity values (> 100 mg/L or <1 mg/L, for example) were not included in the data analysis to determine acute to chronic ratios. Separate analyses of trophic levels (invertebrates and fish) were conducted. ACR for the aquatic compartment (ACRaqu) were derived using the most sensitive species in acute and the most sensitive trophic level in chronic testing, respectively. The resulting ACR distribution deviated from normal distribution and was analysed by non-parametric test using Statistica.

Visual inspection of the chemical structures was used to identify structural alerts in terms of sub-structural features that are associated with the occurrence of high ACRs. A comparative analysis of structural moieties further resulted in the identification of potential structural alerts for an increased probability of high ACR for fish. As described previously by Ahlers et al. (2006), an ACR of 30 was used to separate between low and high ACR for structural alert analysis (Ahlers et al., 2006).

For sensitivity comparison in acute and chronic testing the following quotients were generated:

$$\text{Quotient}_{\text{acute}} (\text{Qa}) = \text{LC50 (fish, acute)} / \text{EC50 (Daphnia, acute)}$$

$$\text{Quotient}_{\text{chronic}} (\text{Qc}) = \text{NOEC (fish, chronic)} / \text{NOEC (Daphnia, chronic)}$$

$$\text{Quotient}_{\text{chronic}}^* (\text{Qc}^*) = 5 \times \text{NOEC (fish, chronic)} / \text{NOEC (Daphnia, chronic)}$$

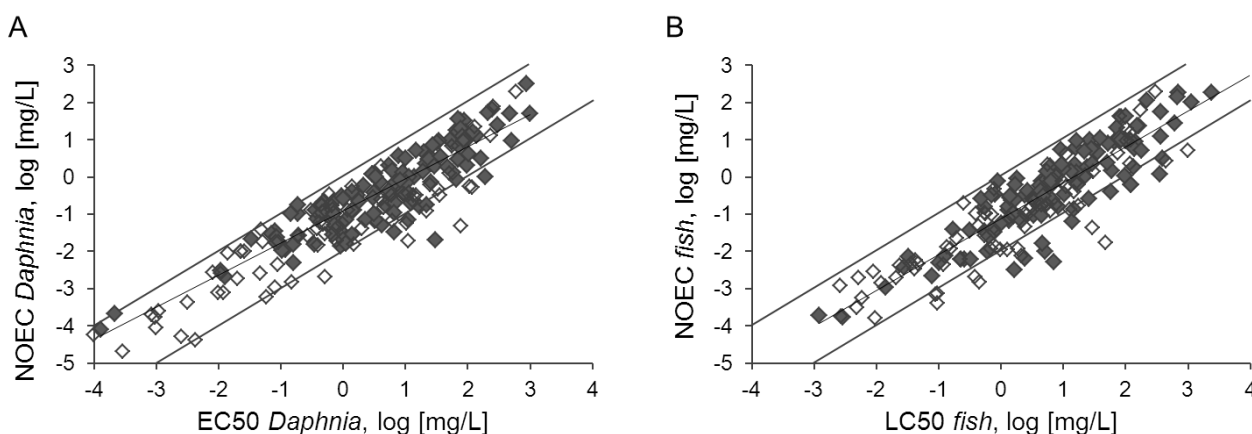
Statistical data analysis was determined by non-parametric test using Statistica. The statistical result was characterized by the median and 90%-ile value as well as by their minimum and maximum values. For interpreting of regression data it was assumed that a regression coefficient R^2 of >0.6 corresponds to a moderate correlation whereas a value of about 0.8 and more corresponds to a reliable prediction.

5 Results and Discussion

5.1 Correlation of acute and chronic effect values within one trophic level

Data search for ACR evaluation comprising acute and chronic guideline studies on aquatic both invertebrates and fish resulted in 202 chemical substance entities including 133 chemicals and 69 pesticides that were considered within the scope of this study. First, the correlation of the acute values, either the EC50 for *Daphnia* or LC50 for fish, with the respective NOEC from the chronic study was evaluated for industrial chemicals and pesticides (Fig 1A and B). A clear relationship between acute and chronic effect values for fish and *Daphnia* could be demonstrated. A simple regression for the compounds achieved a regression coefficient R^2 of about 0.8 for both species. A correlation was also given for the individual data sets of industrial chemicals or pesticides. Thus, the chronic NOEC correlated with the acute effect value and a close relationship between acute and chronic toxicity was indicated.

Figure 1: Relationship between acute and chronic effect data.



(A) Relationship between the acute (EC50) and chronic (NOEC) effect level of *Daphnia* for industrial chemicals (filled symbol) and pesticides (open symbol). The regression line is indicated in black. Regression of data resulted in a regression coefficient of $R^2 = 0.82$. The upper gray line indicates a similar acute and chronic effect value whereas the lower gray line indicates a 100 fold lower chronic effect value compared to the acute effect value. (B) Relationship between the acute (LC50) and chronic effect level of fish (NOEC) for industrial chemicals (filled symbol) and pesticides (open symbol). Regression of data resulted in a regression coefficient of $R^2 = 0.80$.

5.2 Acute to chronic ratios (ACR)

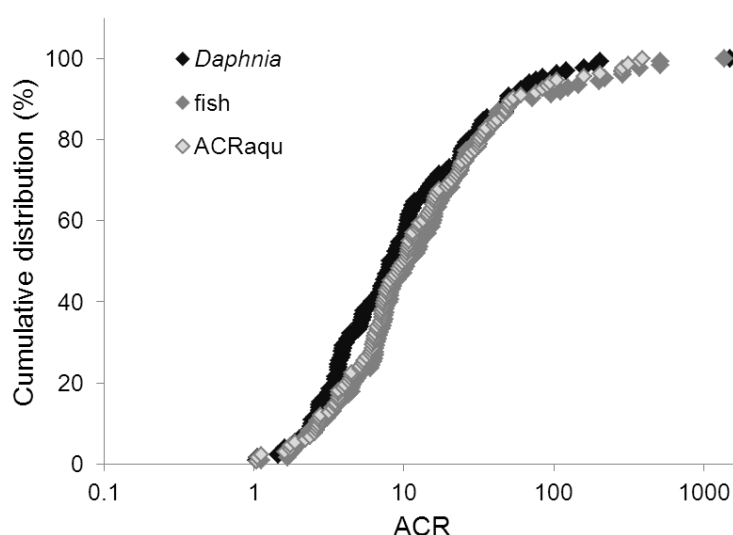
Above results confirmed the relation between acute and chronic effect data. For industrial chemicals, the acute to chronic ratios (ACR) ranged from 1.1 to 1370 (median 12.2) for fish and from 1.1 to 1500 (median 8.8) for *Daphnia* (Fig. 2). The 90 %-ile of ACRs accounted to 50.2 for *Daphnia* and to 68.0 for fish (Table 1). For *Daphnia* 4.5 % of the substance entries had an ACR >100 and 8.9 % had an ACR >100 for fish.

According to the REACH guidance the hazard of a compound is usually derived from the lowest value of test from three trophic levels. Therefore, ACR for the aquatic compartment (ACRaqu) were derived using the most sensitive species in acute and the most sensitive species in chronic testing, respectively. The ACRaqu ranged from 1.1 to 390 with a median of 9.9 for chemicals. The 90 %-ile value for the aquatic compartment was determined to 58.5. These finding indicated that an extrapolation factor of 100 is protective for more than 95 % of the industrial chemicals since <5 % of the substances of this data set showed an ACRaqu >100. Interestingly, values above 1000 were only observed for species specific ACR (one for fish and one for *Daphnia*), but not for ACRaqu.

Pesticides are assumed to have a distinct mode of action (MoA) and are allocated to MoA4 for specifically acting chemicals (Verhaar et al., 1992). Therefore, ACRs of pesticides were separately evaluated. For pesticides a median ACR of 11.4 for *Daphnia*, 16.8 for fish, and 11.1 for ACRaqu was determined (Table 1). A 90 %-ile ACR of 154.2 for fish, 109.4 for *Daphnia* and 138.6 for the most sensitive trophic level was calculated. Hence, an acute to chronic extrapolation factor of 100 is not protective for 90 % of the pesticide entries.

In summary, the results indicated that acute to chronic extrapolation represents a sound approach and a protective measure by using an adequate assessment factor. The ACR for industrial chemicals differed in their profile from pesticides as ACRs of pesticides were increased compared to chemicals in terms of median, 90%-ile value and maximum and exceeded the proposed assessment factors used for chemical registration according to the European guidance documents. Based on these statistical findings and the 90%-ile value, chemicals can, hence, be evaluated in a protective manner by the current acute to chronic extrapolation approach, but for pesticides the approach has to be adapted.

Figure 2: Cumulative distribution of chronic effect values



Cumulative distribution of ACR of *Daphnia* (black), fish (grey) and ACRaqu (non-filled) was superimposed.

Table 1: Acute to chronic ratios of chemicals and pesticides

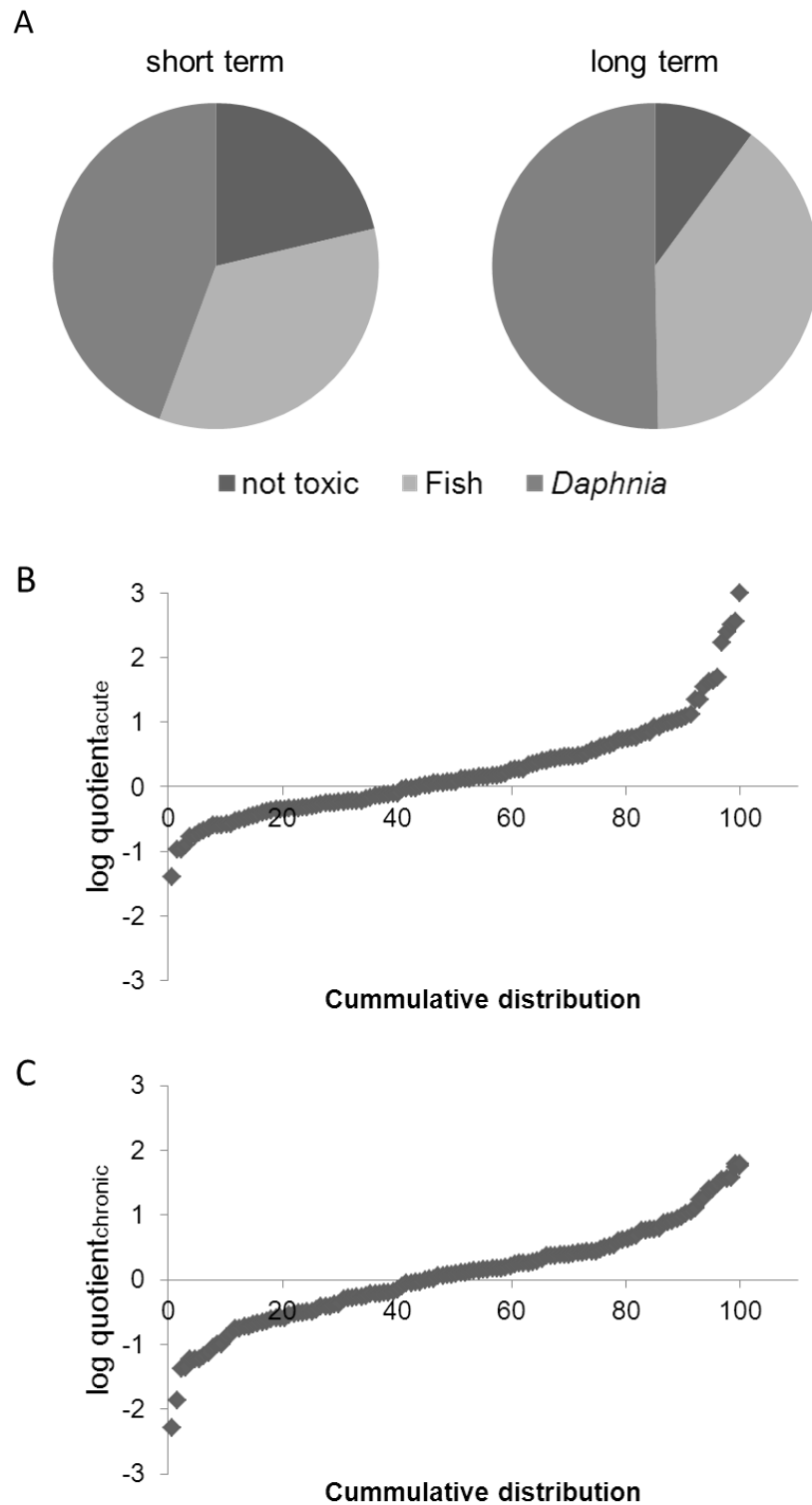
Parameter	Chemicals			Pesticides			total		
	Daphnia	Fish	ACRaqu	Daphnia	Fish	ACRaqu	Daphnia	Fish	ACRaqu
No. of values	130	123	133	67	68	69	197	191	202
Min.	1.1	1.1	1.1	1.2	1.7	1.2	1.1	1.1	1.1
Median	8.8	12.2	9.9	11.4	16.5	11.1	9.3	13.8	10.0
90 %-ile	50.2	62.0	58.5	109.4	154.2	138.6	76.5	112.8	94.4
Max.	1500.0	1370.6	390.0	1661.5	659.1	1661.5	1661.5	1370.6	1661.5

5.3 Sensitivity between *Daphnia* and fish

5.3.1 Evaluation of the Sensitivity between *Daphnia* and fish

Sensitivity differences between fish and *Daphnia* were qualitatively analysed for acute and chronic testing. In acute testing about 40 % of the substances were more toxic to fish and about 40 % of the substances were more toxic to *Daphnia*, whereas no toxicity was determined in about 20 % of the cases (Fig. 3A). An almost comparable result was achieved for chronic tests with *Daphnia* appearing in average slightly more sensitive than fish (Fig.3A). To quantitatively compare species sensitivity of individual substances in acute and chronic testing a quotient between the effect value of the *Daphnia* and fish study was derived referring to quotient_{acute} (Qa) and quotient_{chronic} (Qc). A quotient > 1 indicates that *Daphnia* are more sensitive compared to fish and a quotient < 1 indicated that fish is more sensitive than *Daphnia*, subsequently. The median of the ratio was 1.2 for acute testing and the 10 %-ile and the 90 %-ile values ranged from 0.26 to 12.1 (Fig. 3B). The median for chronic testing was 1.35 with a 10 %-ile and the 90 %-ile value ranging from 0.15 to 25.0 (Fig 3C). Together, these statistical findings suggested a rather similar sensitivity between fish and invertebrate toxicity in acute and chronic testing with *Daphnia* being only slightly more sensitive than fish. Hence, the results indicated that none of both investigated trophic levels is generally more sensitive than the other.

Figure 3: Sensitivity distribution between *Daphnia* and fish in acute and chronic testing



(A) The more sensitive trophic level was qualitatively determined for acute and chronic testing. The quantitative range of sensitivity distribution was determined for acute (B) and chronic (C) testing using $quotient_{acute}$ and $quotient_{chronic}$. The values were sorted starting with highest value that indicated a stronger susceptibility of *Daphnia* compared to fish. A log value > 0 (quotient > 1) indicates that *Daphnia* was more sensitive compared to fish and a log value < 0 (quotient < 1) indicated that fish was more sensitive than *Daphnia*, subsequently.

5.3.2 Acute sensitivity classification (ASC)

To classify acute sensitivity distribution the quotient_{acute} was grouped in four categories that were considered relevant for regulatory purposes and testing strategies to estimate chronic toxicity for further testing requirements. Initially, Cat 1: < 0.1; Cat 2: 0.1 – 0.2; Cat 3: 0.2 – 10; Cat 4: >10 was suggested in the kick-off meeting by the UBA. However, the analysis of the results suggested an adaptation of the boundaries due to three mayor shortcoming:

- 1) More than 80 % of the substance entries were initially assigned to Cat.3. Therefore, a differentiated evaluation of the dataset appeared to be not feasible. Furthermore, the initial consideration of the system envisaged that a chronic fish test should not be indicated for substances in Cat.3 and Cat.4 and may be only considered in exceptions. However this would result in an underestimation of fish toxicity in several cases.
- 2) An outer value of 0.1 and 10 for Cat.1 and Cat.4 appeared to be rather high and not protective. Only four substances were >10x more sensitive to fish. 17 substances were >10x more sensitive towards Daphnia. Therefore, these values were adapted to 0.2 and 5.
- 3) Cat.2: 0.1 – 0.2 appeared to be rather small with only eight substance entries. Furthermore the lower boundary of 0.2 for Cat.2 appeared to be not protective for fish since several substances being 5x more sensitive towards fish than Daphnia in chronic testing were determined at lower quotients.

The four categories as applied in this study were based on discussion by the project partners and were classified as follows: Cat 1: < 0.2; Cat 2: 0.2 – 0.5; Cat 3: 0.5 – 5; Cat 4: >5 (Table1). Cat 3 was based on the sensitivity with fish being up to 2x more sensitive than Daphnia or Daphnia are up to 5x more sensitive than fish. Cat 4 represents datasets where Daphnia appeared to be > 5x more sensitive than fish and Cat 1 comprises datasets with sensitivity differences of fish being > 5x more sensitive than Daphnia. The range of the quotient for Cat 3 was, hence, defined as 0.5 – 2. Cat 2 referred to sensitivity ratio where fish are between 2 to 5x more sensitive than Daphnia. The classification system was compiled to be conservative regarding fish toxicity and to avoid animal testing since two categories were established for substances being more sensitive to fish in acute testing. A value of 0.5 between Cat 2 and Cat.3 was chosen assuming that a range 0.5 to 2 represents equal sensitivity so that Cat.1 and Cat.2 includes all data where fish is more sensitive than Daphnia in acute testing. A value of 0.2 and 5 was chosen to define Cat.1 and Cat.4 where one trophic level is significant (in terms of 5x) more toxic than the other since a limit of 10 as implicated in the guidance R7b for sensitivity distinction appeared to be rather high as discussed below.

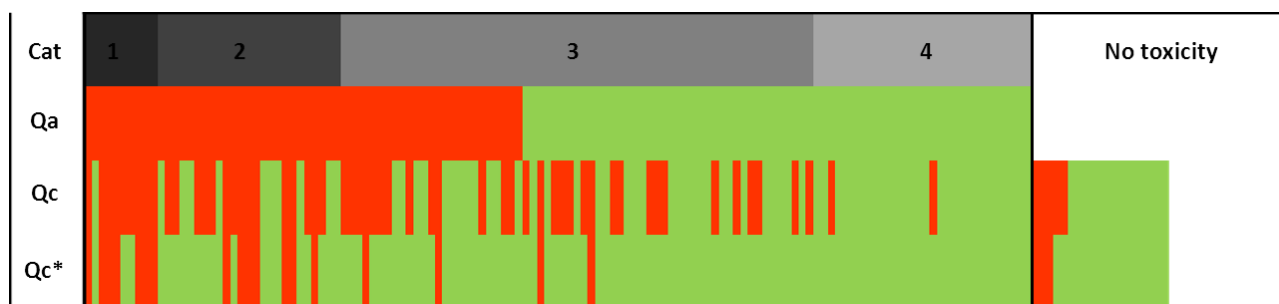
Table 2: Classification of acute sensitivity between *Daphnia* and fish

Classification	Cat 1	Cat 2	Cat 3	Cat 4	No toxicity observed
Ratio	≤ 0.2	0.2 to 0.5	0.5 to 5	≥5	-
Description	Fish is 5x more sensitive than Daphnia	Fish is more sensitive than Daphnia	<i>Daphnia</i> are comparable or more sensitive than fish	<i>Daphnia</i> are more 5x sensitive than fish	no acute effects were determined

Findings	fish toxicity would NOT be covered by 70 % by the chronic Daphnia test and an AF of 50	fish toxicity would NOT be covered by 28% by the chronic Daphnia test and an AF of 50	chronic fish toxicity would be covered by 94 % by the chronic Daphnia test and an AF of 50	chronic fish toxicity would be covered by 100% by the chronic Daphnia test and an AF of 50	
ITS	Test on chronic toxicity to fish required and sufficient	Test on chronic toxicity to <i>Daphnia</i> required, and additionally on fish if log Kow >3	Test on chronic toxicity to <i>Daphnia</i> indicated	Test on chronic toxicity to <i>Daphnia</i> required and sufficient	Test on chronic toxicity to <i>Daphnia</i> indicated

To investigate the sensitivity in acute and chronic testing quotients for an individual substance were depicted in a heat map diagram (Fig 4). The dataset was sorted by quotient_{acute} (Qa) starting with the lowest Qa value indicating increased fish sensitivity. The classification system as described in table 1 as well as quotient_{chronic} (Qc) was allocated to quotient_{acute} (Qa) for the respective substance. Green coloured entries in row 2 (Qa) and 3 (Qc) indicated that *Daphnia* are more sensitive compared to fish and red coloured entries indicated that fish are more sensitive than *Daphnia*, subsequently.

According to the European guidance documents an assessment factor of 50 instead of 10 can be applied on the most sensitive species if chronic data on fish is not available (ECHA, 2012; EC., 1996). To address the question whether the extrapolation approach from *Daphnia* was protective to cover chronic toxicity of fish it was assumed that *Daphnia* data but not fish data is available for a substance. The difference between both assessment factors of 5 was included in the quotient_{chronic}* (Qc*). Subsequently, red coloured entries in column 4 indicated that fish is > 5x more sensitive than *Daphnia* in chronic testing, and that fish toxicity was not adequately covered by the chronic *Daphnia* test and an AF 50. In total, the chronic fish toxicity test was required for 13 % of the substances using this approach. About 14 from 18 (78 %) of the chemicals that were 5x more sensitive to fish in chronic testing were already >2x more sensitive to fish in acute testing. The observation suggested that acute and chronic species sensitivity were related to each other, and that fish tests were usually only indicated if fish was 2x more sensitive in acute testing. The finding was corroborated by plotting the ratio of quotient_{acute} and quotient_{chronic} (Fig. 6). In general, substances that were more toxic to fish than to *Daphnia* in acute testing exhibited a relevant toxicity to fish in chronic testing.

Figure 4: Sensitivity categorization between *Daphnia* and fish for individual substances

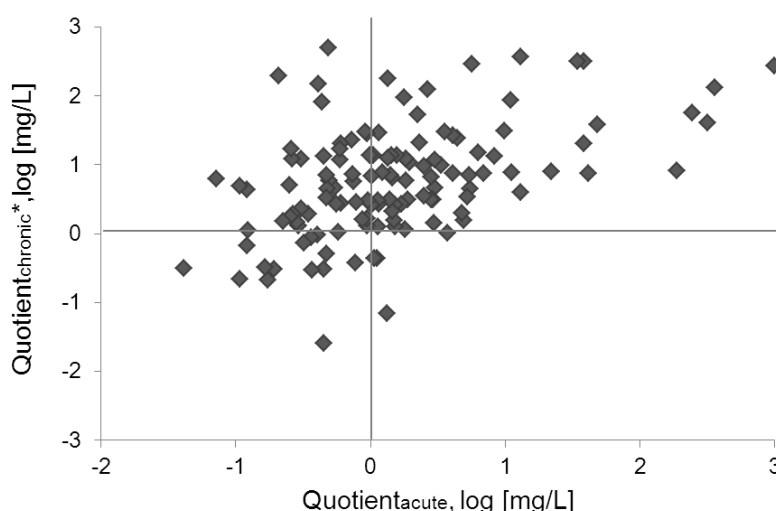
Cat: Quotient_{acute} was allocated to the classification system as described in Table 2.

Qa: To compare the species sensitivity in acute testing a quotient between the effect value of the *Daphnia* and fish study was derived referring to quotient_{acute} (*Qa*). A quotient >1 (green) indicates that *Daphnia* are more sensitive compared to fish and a quotient <1 (red) indicated that fish are more sensitive than *Daphnia*, subsequently. The dataset was sorted starting with the highest quotient_{acute} value.

Qc: To compare the species sensitivity in chronic testing a quotient between the effect value of the *Daphnia* and fish study was derived referring to quotient_{chronic} (*Qc*). A quotient >1 (green) indicates that *Daphnia* are more sensitive compared to fish and a quotient <1 (red) indicated that fish are more sensitive than *Daphnia*, subsequently.

*Qc**: Quotient_{chronic} was multiplied by 5. Substance entries that were subsequently $>5x$ more sensitive to fish in chronic testing were coloured red.

Sensitivity distribution between *Daphnia* and fish was elucidated for individual substances by a heat map diagram. The dataset was obtained from the ECHA database and the ICS database. Each column represents a single chemical entry ($n=167$). Sensitivity differences were calculated by quotient_{acute} and by quotient_{chronic}, and substance entries sorted by quotient_{acute} starting with the lowest value. Species sensitivity was coloured green for *Daphnia* and red for fish being more sensitive to a chemical in acute and chronic testing, respectively. Substance entries exhibiting no toxicity in the respective test setting were listed on the right (not coloured). Quotient_{acute} was allocated to the classification system described in table 1. Substance entries were allocated to the classification system that is based on quotient_{acute} and described in table 1. The percentage of substances being more toxic to fish in chronic testing and exhibiting a quotient_{chronic} <1 was evaluated for each classification class (black) for chemicals.

Figure 5: Relationship between *Qa* and *Qc**

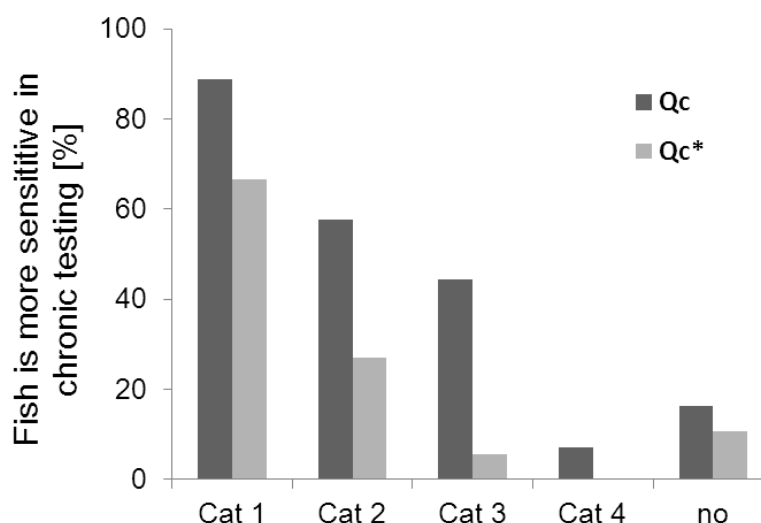
Qa was correlated with *Qc**. Substances that were $>5x$ sensitive to fish in chronic testing were depicted in the lower quadrants 3 and 4.

Next, the percentage of substances with a $Q_c < 1$ (dark grey) that were more sensitive to fish and substances with a $Q_c < 0.2$ (grey) that were more than 5x more sensitive to fish were analysed for each class (Fig.6). In the following several observations are shown:

- 1) The evaluation showed a relationship between acute and chronic sensitivity as the sensitivity of fish in chronic testing decrease with increased *Daphnia* toxicity in acute testing.
- 2) Including a factor of 5 on the chronic *Daphnia* test showed that chronic fish toxicity would have been covered by 95 % of the cases for substances with a $Q_a > 0.5$ (Cat 3 (94%) and Cat 4 (100 %)). Four substances of 64 (6 %) in Cat 3 required a chronic fish toxicity test.
- 3) Fish was in neither case more sensitive to *Daphnia* in chronic testing ($Q_c < 0.5$) if *Daphnia* was 5x more sensitive in acute testing (Cat 4). This finding suggested that fish was not more sensitive in chronic testing than *Daphnia* if *Daphnia* was 5x more sensitive in acute testing. Hence, based on the data analysis the AF on the chronic *Daphnia* test may be reduced to 10 for substances in Cat 4 and a chronic fish toxicity test was not required.
- 4) For substances in Cat.1 inclusion of a factor of 5 on the chronic *Daphnia* test was not protective since fish toxicity was not covered in about 60 % of the cases. Hence, a chronic fish test is required for Cat.1.
- 5) Regarding Cat.2 about 28 % of the substances were not properly covered by the *Daphnia* test and the extrapolation approach. Nevertheless, the statistical analysis showed that in the majority of cases (72 %) the chronic fish test is indeed not necessary in Cat.2.

Moreover, the dataset comprised about 20 % substances that did not show acute toxicity to *Daphnia* or fish because no effect (LC50, EC50) was determined up to highest tested concentration for both trophic levels due to the performance of a limit test or because the water solubility limit was exceeded. Therefore, a sensitivity comparison was not appropriate for these substances. About 50 % of the substances also showed toxicity in chronic testing. Three substances (8 %) with a $Q_c < 0.2$ were determined where fish was substantially more sensitive in chronic testing than *Daphnia* and would not be covered by an extra AF. However, in most cases *Daphnia* was more sensitive than fish in chronic testing. Thus, chronic fish toxicity testing would have been underestimated for less than 10% of the evaluated substances that showed no acute toxicity if chronic fish toxicity has not been tested (Fig. 4).

Figure 6: Fish sensitivity of chemicals in each category



Substance entries of chemicals were allocated to the classification system. The percentage of substances being more toxic to fish in chronic testing and exhibiting a quotient_{chronic} <1 was evaluated for each class (black), and the percentage of substances that were >5x more sensitive to fish in chronic testing and exhibited a quotient_{chronic} <0.2 were evaluated for each class (grey).

5.3.3 Considerations for the categorization limits

The limits of each category are based on discussion by the project partners. It should be noted that adaption of these limits is assumable. In particular, the limit between Cat.3 and Cat.4 was discussed. Here, the following three options are assumable (table and Figure :

A: Cat.3: 0.5 to 2; Cat.4 >2

In this case Cat.3 represents a range of comparable sensitivity between fish and Daphnia. Cat.4 was applied for all substances that were more sensitive to Daphnia in acute testing. The advantage is that in Cat.4 the number of substances increases and no substance was 5x more toxic to fish compared to Daphnia in chronic testing. On the other less than 10 % of the substances in Cat.3 required a chronic fish test to avoid underestimation of fish toxicity by extrapolation from chronic Daphnia data.

B: Cat.3: 0.5 to 5; Cat.4 >5

This categorization represents the system as applied in this study. Cat.3 is based on a range of comparable sensitivity between fish and Daphnia. But the boundary between Cat.3 and Cat.4 is expanded from 2 to 5 to emphasis the value of 5 for sensitivity distinction in the categorization system as it is also used between Cat.1 and Cat.2. Furthermore, the value of 5 may be of regulatory value since the data of this study suggest that the AF on the chronic Daphnia test may be reduced from 50 to 10 for Cat.4 in this case but not if a limit of 2 is used between Cat.3 and Cat.4. Moreover, the percentage for Cat.3 is reduced compared to A resulting in 6% of the substances in Cat.3 required a chronic fish test to avoid underestimation of fish toxicity by extrapolation from chronic Daphnia data.

C: Cat.2 0.2 to 1; Cat.3: 1 to 5 ; Cat.4 >5

In this case Cat.2 is expanded from 0.5 to 1. This measure could be interpreted as a more conservative approach regarding fish toxicity since the number of substances in Cat.3 that required a chronic fish test is reduced from four to two substances compared to B. However,

the adaptation to a more conservative evaluation regarding chronic fish toxicity may be considered as marginal since Q_c was statistically reduced from 49.2 to 43.9 and Q_p from 6.2 to 4.9. On the other hand the number of substances in Cat.2 is almost doubled from 25 to 49. Thus, the adaptation of the limit from 0.5 to 1 between Cat.2 and Cat.3 may be considered as inefficient since the number of substances in Cat.2 and, hence, the number of possible fish test strongly increase whereas the statistical increase of safety in terms of a conservative hazard assessment remains almost unchanged.

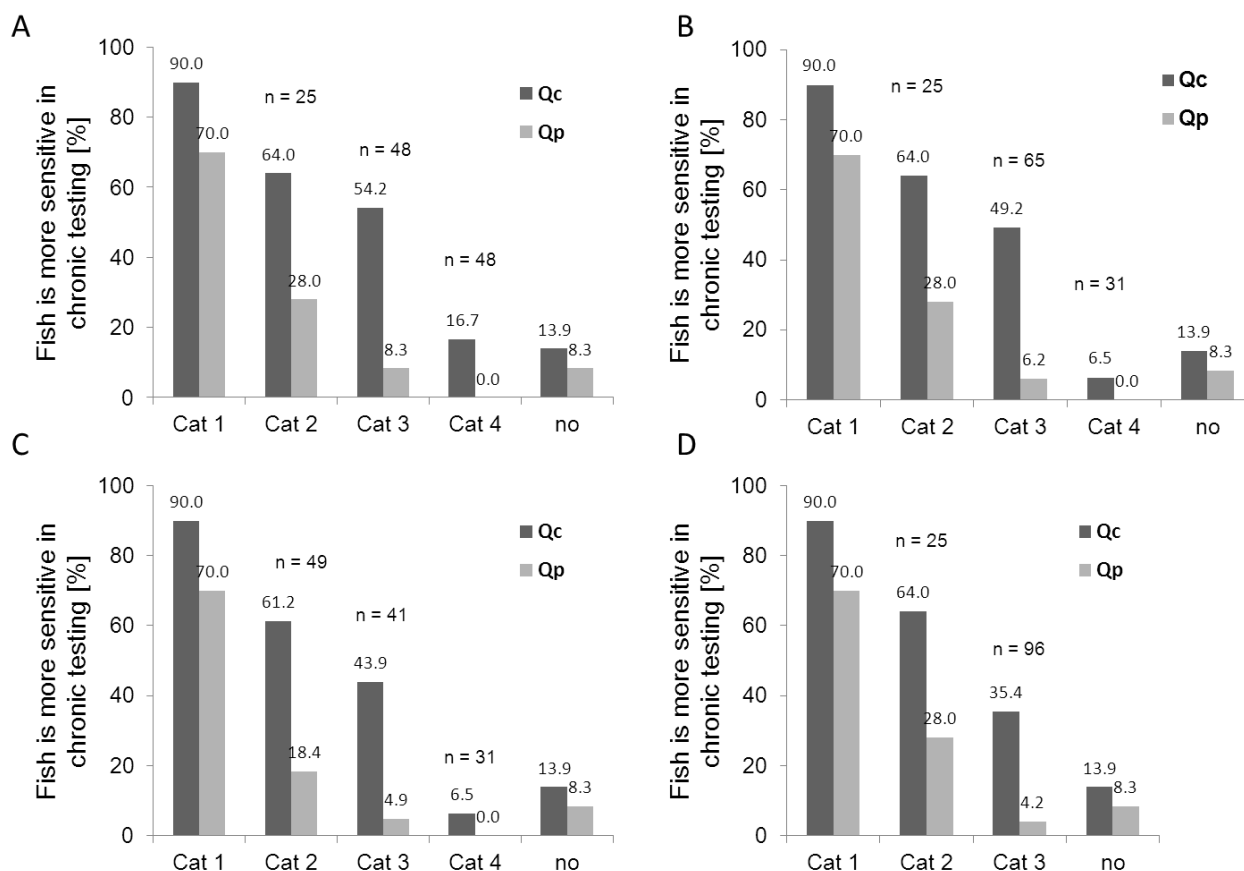
D: Merge of Cat.3. and Cat.4 to one category

From the statistical point of view the chronic Daphnia test is indicated for > 95 % of the substances if Daphnia Cat.3 and Cat.4 were merged. Due to the reduction of categories this adaptation may represent a simplification since the substances could be classified into substances that require a chronic fish test (Cat.1) or a chronic Daphnia test (Cat.3) as well as in substances for that both the chronic fish test and the chronic Daphnia test should be considered.

Table 3: Different discussed categorization systems

Figure	Cat 1	Cat 2	Cat 3	Cat 4
A	≤ 0.2	0.2 to 0.5	0.5 to 2	≥ 2
B	≤ 0.2	0.2 to 0.5	0.5 to 5	≥ 5
C	≤ 0.2	0.2 to 1	1 to 5	≥ 5
D	≤ 0.2	0.2 to 0.5	≥ 0.5	-

Figure 7: Analysis of different categorization systems

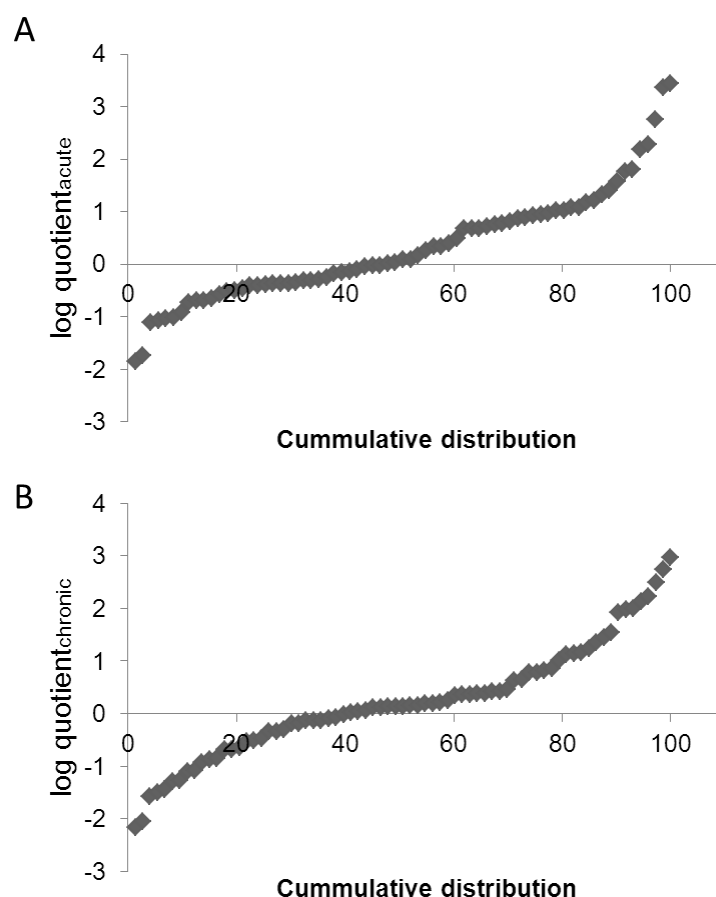


Different categorisation systems as displayed in Table 3 were analysed as described in 6. For Cat.2 to Cat.4 the number of substance is depicted. The percentage of substances being more toxic to fish in chronic testing and exhibiting a quotientchronic <1 was evaluated for each class (black), and the percentage of substances that were >5x more sensitive to fish in chronic testing and exhibited a quotientchronic <0.2 were shown.

5.4 Evaluation of the applicability of the categorisation system for pesticides

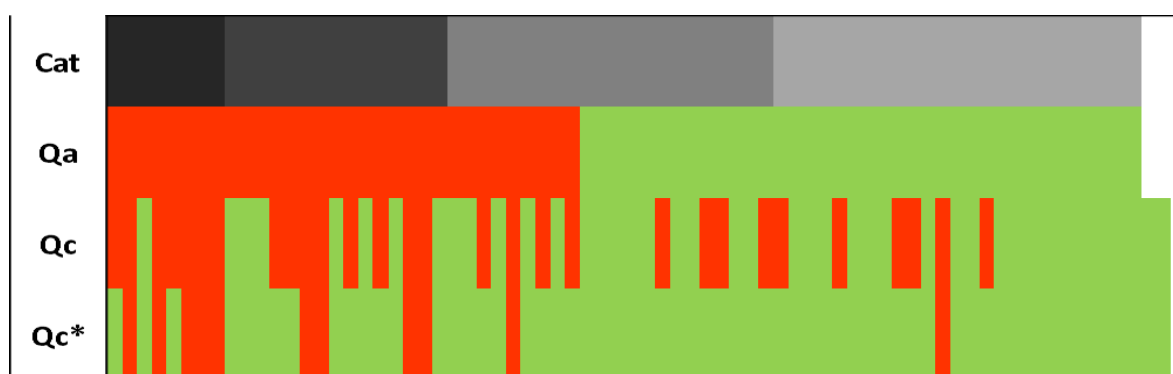
73 pesticide entries were analysed for sensitivity distribution. Essentially, comparable results for pesticides and chemicals were observed (Fig 7). The median of the sensitivity ratio was 1.2 for acute testing and the 10 %-ile and the 90 %-ile values ranged from 0.18 to 38. The median for chronic testing was 1.4 with a 10 %-ile and the 90 %-ile value ranging from 0.08 to 82.7 (Fig. 7).

Figure 8: Sensitivity distribution between *Daphnia* and fish for pesticides in acute and chronic testing



The quantitative range of sensitivity distribution was determined for acute (A) and chronic (B) testing using quotient_{acute} and quotient_{chronic}. The values were sorted starting with highest value that indicated a stronger susceptibility of *Daphnia* compared to fish. A log value > 0 (quotient > 1) indicates that *Daphnia* was more sensitive compared to fish and a log value < 0 (quotient < 1) indicated that fish was more sensitive than *Daphnia*, subsequently.

Furthermore, sensitivity of one trophic level in acute testing was associated with a comparable sensitivity in chronic testing towards the same trophic level for pesticides. More than 90 % of the pesticides being more sensitive to fish in chronic testing were already >2x more sensitive to fish in acute testing (Fig. 8). Pesticides in Cat 1 and Cat 2 were in about 90 % and 50 % of the cases

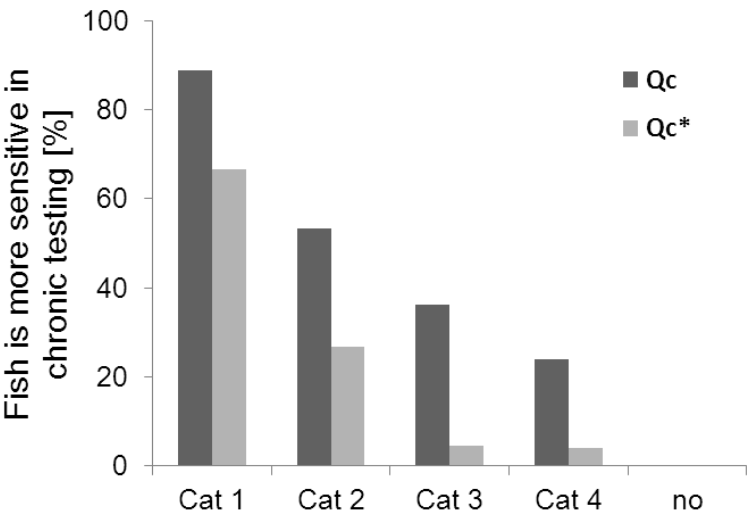


more toxic to fish. Upon application of a factor of 5 on the chronic Daphnia test, substances in Cat 1 were in about 70 % of the cases also more toxic to fish and substances in Cat 2 were in about 30 % of the cases more toxic to fish in chronic testing (Fig 9). Substances in Cat 3 and Cat 4 were in > 90 % of the cases more toxic to Daphnia in chronic testing upon inclusion of a factor of 5 on the chronic Daphnia test. Hence, the chronic Daphnia test was considered as protective for pesticides that were classified to Cat 3 and Cat 4. In total about 11% of the evaluated pesticides compared to 13 % of the evaluated chemicals were 5x more toxic to fish in chronic testing and would have required a chronic fish toxicity test. Together, the findings showed that sensitivity distribution of chronic toxicity was comparable between chemicals and pesticides and that the sensitivity classification approach may also be applicable for pesticides.

Figure 9: Sensitivity distribution between *Daphnia* and fish of pesticides

Sensitivity distribution between Daphnia and fish of pesticides was elucidated by a heat map diagram as described in figure 4.

Figure 10: Fish sensitivity of pesticides in each category



Sensitivity of fish was allocated to the classification system. The percentage of substances being more toxic to fish in chronic testing and exhibiting a quotient_{chronic} <1 was evaluated for each classification class (black), and the percentage of substances that were >5x more toxic to fish in chronic testing and exhibited a quotient_{chronic} <0.2 were evaluated for each class (grey).

5.5 Octanol-water partitioning coefficient

5.5.1 Relationship between species sensitivity and Kow

The octanol-water partitioning coefficient of a substance may be relevant for chronic fish toxicity as a high Kow is considered to be related with the potential of bioaccumulation (EC, 1996; ECHA, 2012; EU, 2006). 61 substances exhibiting a $\log Kow > 3$ and 34 substances exhibiting a $\log Kow > 4.5$ were identified within the data set (Fig. 11). The evaluation showed that sensitivity of one trophic level in acute testing was also associated with the sensitivity in chronic testing for substances with a high $\log Kow$. Based on Qp and considering an AF of 50 the chronic fish toxicity test was not indicated for substances in Cat 4 and for only two substances in Cat.3 (8 %), whereas a chronic fish toxicity test was required for substances in Cat.1 (Fig. 10). Hence, chronic sensitivity could be estimated by the classification approach independent of the octanol-water partitioning coefficient. The result further showed that the chronic fish test is not in general indicated for all substances with a $\log Kow > 3$ and can often be avoided since the chronic fish toxicity test is required for less than 20% of the substances with a $\log Kow > 3$. Thus a chronic fish test is not generally required for substances with a $\log Kow > 3$.

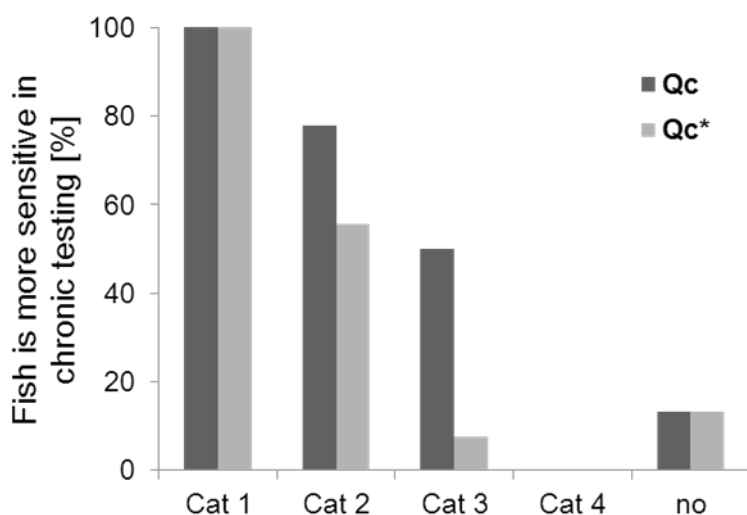
For substances in Cat.2 the challenge arises in which case a chronic fish test is necessary. Interestingly, 55 % of the substances in Cat.2 (compared to 28 % of the complete dataset) were 5x more sensitive to fish (Fig. 10). Moreover, five of the seven substances (72%) from the complete data set that were classified in Cat 2 and that were 5x more sensitive to fish in chronic testing had a $\log Kow > 3$ (Fig. 4). Three of these substances even had a $\log Kow > 4.5$. This observation suggested that a $\log Kow$ value of > 3 or > 4.5 may represent a trigger for chronic fish toxicity testing for substances classified in Cat 2. Right positive were five out of seven (71 %, sensitivity) substances with a $\log Kow > 3$ ($\log Kow > 3$; $Qc < 0.2$). False positive values for this trigger value were indicated in the case that Daphnia was more sensitive in chronic testing than fish and exhibited a $\log Kow > 3$ ($\log Kow > 3$; $Qc > 0.2$). False negative were indicated in the case that fish was 5x more sensitive in chronic testing than Daphnia but exhibited a $\log Kow < 3$. False negative ($\log Kow < 3$; $Qc < 0.2$) amounted to 12 % (two of 16) of the substances with a $\log Kow < 3$. Subsequently, the negative predictive value was 88 %. Moreover, 78 % of the substances in Cat.2 that were more sensitive to Daphnia in chronic test had a $\log Kow < 3$ (specificity). The accuracy was, hence, 76 %. Taken together, a $\log Kow > 3$ or > 4.5 was not confirmed as determinant indicating in general the requirement of chronic fish toxicity testing. However, a $\log Kow > 3$ or > 4.5 may support the ITS as trigger to indicate the requirement of chronic fish toxicity testing for substances in Cat.2.

A high Kow is considered to be related with the potential of bioaccumulation in fish. Subsequently, it was assumed that increased exposure times result in bioaccumulation and increased toxicity in fish for substance with a high Kow. To address this question it was hypothesized that the sensitivity of fish should increase in chronic testing compared to Daphnia with increasing Kow. To analyse the hypothesis a ratio between Qa and Qc was derived for the complete dataset or only for FELS test and correlated with the $\log Kow$ (Fig. 12). The result showed that the ratio was comparable over the evaluated Kow range and did not increase in average as indicated by the slope. Furthermore, the intercept of about one indicated a rather comparable sensitivity between Daphnia and fish. The regression coefficient of < 0.1 for the complete dataset and for FELS test showed that no relationship between $\log Kow$ and increase fish sensitivity compared to Daphnia can be established. Thus, fish sensitivity in relation to Daphnia toxicity did not increase in chronic testing with increasing $\log Kow$. This finding suggested that fish sensitivity in relation to Daphnia did not increase upon prolonged exposure times.

Table 4: Evaluation of log Kow value of 3 as trigger value to suggest the indication of a chronic fish test for substance allocated to Cat.2.

	Fish is 5x more sensitive than Daphnia		
	positive	negative	
Log Kow >3	True positive 5	False positive 4	Pos. predictive value 56 %
Log Kow <3	False negative 2	True negative 14	Neg. predictive value 88 %
	Sensitivity 71 %	Specificity 78 %	

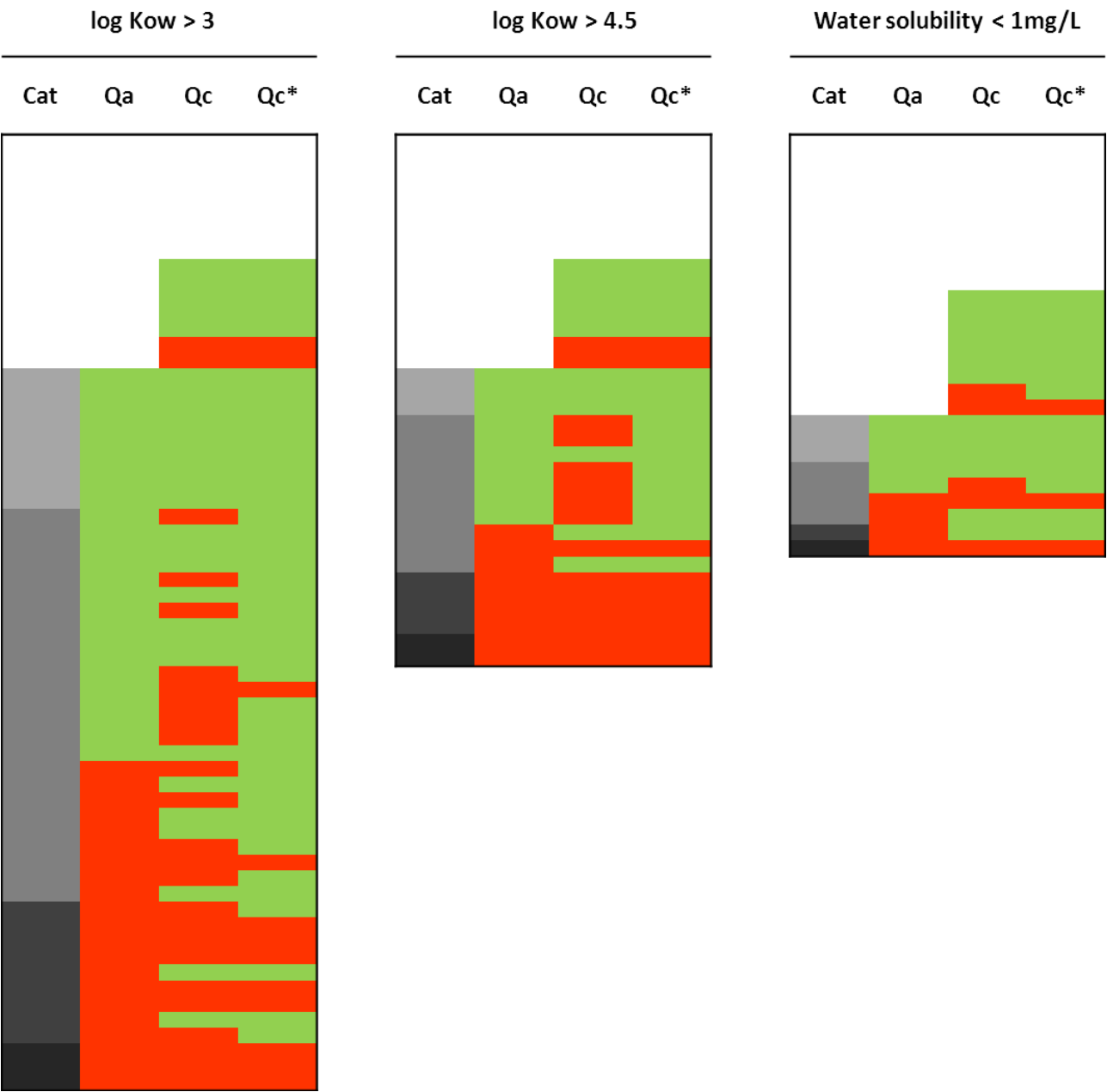
Figure 11: Relationship between physicochemical properties and fish sensitivity in each class



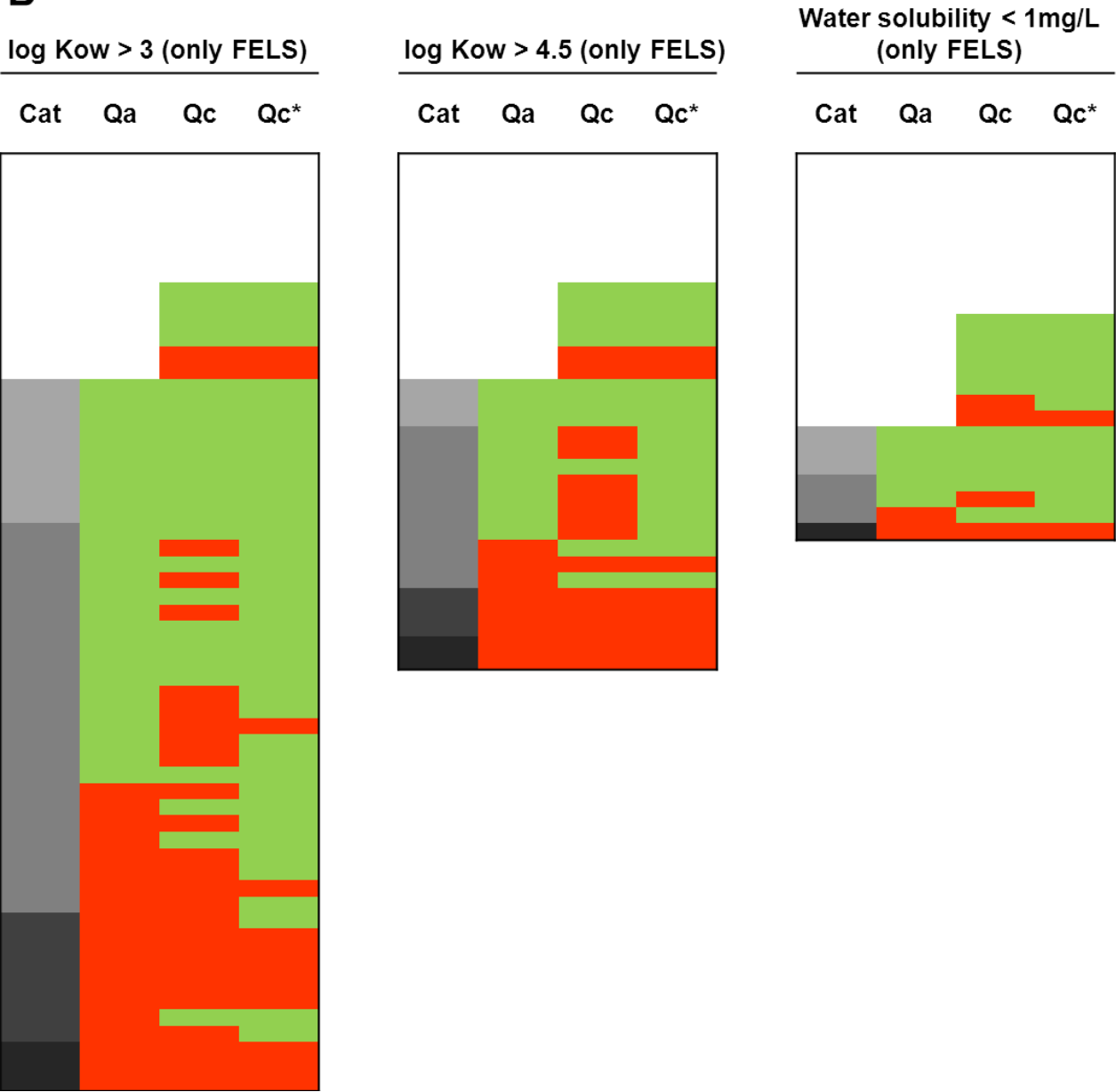
Sensitivity distribution of the data from FELS tests only of chemicals with a log Kow >3 were allocated to the classification system. The percentage of substances being more toxic to fish in chronic testing and exhibiting a quotientchronic <1 was evaluated for each classification class (black), and the percentage of substances that were >5x more sensitive to fish in chronic testing and exhibited a quotientchronic <0.2 were evaluated for each classification class (grey).

Figure 12: Relationship between physicochemical properties and sensitivity distribution

A

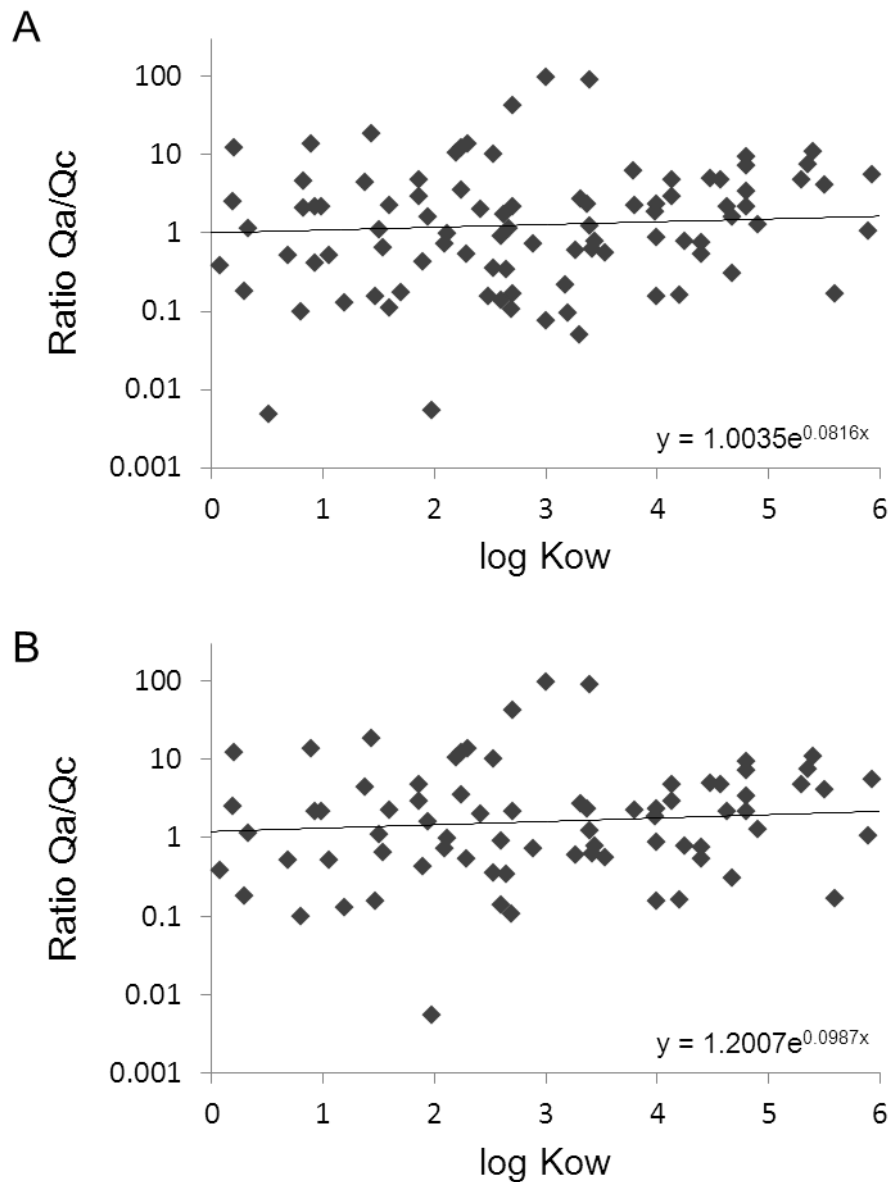


B



Sensitivity distribution between *Daphnia* and fish of substances with a log Kow >3 or >4.5, or of substances with poor water solubility (<1 mg/L) was elucidated by a heat map diagram as described in figure 4 for (A) the complete dataset and for (B) data only underlaid by FELS test.

Figure 13: Relationship between species sensitivity and octanol-water partitioning in acute and chronic testing.



The ratio between Q_a and Q_c was derived for the complete dataset (A) and for FELS tests only (B), and correlated with the $\log K_{ow}$. The slope of the trend line of almost 0 and the intersection point of about 1 were determined for both evaluations.

5.5.2 Correlation of ACR and octanol-water partitioning coefficient

A high octanol-water partitioning coefficient $\log K_{ow} > 3$ and a $\log K_{ow} > 4.5$ is considered to be associated with the potential of bioaccumulation or bioconcentration, respectively. Therefore, it has been suggested that low concentrations of a substance with high K_{ow} may accumulate in the organism and may result in toxic effects when reaching a steady state upon long term exposure (ECHA, 2012). The median ACR for both trophic levels was allocated to the indicated $\log K_{ow}$ classes each representing one K_{ow} magnitude (Fig. 13). Although slightly increased ACR for fish and were determined at $\log K_{ow} < 1$ and $\log K_{ow} > 4$ compared to the $\log K_{ow}$ from 1 to 4 no significant differences could be determined for a $\log K_{ow}$ class as the p-values of each class compared to the remaining dataset were determined to be > 0.05 by the Mann-Whitney U-test using Statistica. Thus, the result in figure 13A showed that the median ACR for fish and Daphnia was almost comparable for all classes. Furthermore, correlation of single data points in figure 13 B and C showed that a relationship for the data set could not be established since the slope of the regression line almost equaled 0.

Statistical evaluation of substances exhibiting a $\log K_{ow} > 4.5$ resulted in a median of 4.2 and a 90%-ile value of 24.6 for Daphnia. This result indicated that the hazard assessment applying an ACR of 100 is protective in any case and that an ACR of 100 may overestimate chronic Daphnia toxicity. For fish the median ACR of 16.0 was increased by a factor of about 1.5 compared to the complete dataset. Compared to the remaining dataset the increase of the median ACR was not significant and a p-value of > 0.05 was determined compared to the remaining dataset by the Mann-Whitney U-test using Statistica. Furthermore, the 90%-ile value of 42.6 was decreased and did not exceed 100 in any case (Table 3). Again the result suggested that the hazard assessment for fish and substances exhibiting a $\log K_{ow} > 4.5$ is protective in any case by using an ACR of 100.

For substances exhibiting a $\log K_{ow} > 3$ the ACR evaluation resulted in a median of 6.3 and a 90%-ile value of 47.1 for Daphnia as well as in a median of 10.5 and a 90%-ile value of 67 for fish. Compared to the complete dataset the median value and the 90%-ile value for fish were almost comparable and no significant differences were determined by the Mann-Whitney U-test using Statistica. Taken together, no relationship between K_{ow} and ACR could be established. This observation is in line with results reported by Ahlers et al. (2006). Since the EU guidance documents use an ACR of 100 as safety factor the 90%-ile value can be considered as relevant statistical threshold. The ACR at high K_{ow} appeared to be reduced compared to the ACR of the complete dataset (Table 3). The results further suggested that the hazard assessment for fish and substances exhibiting a $\log K_{ow} > 4.5$ is protective in any case and that an ACR of 100 may, hence, overestimate chronic toxicity in this case.

According to the guidance documents, it has been suggested that with a potential to bioaccumulate ($\log K_{ow} > 3$), the need for long-term testing is more compelling (TNG, page 180) and that long-term toxicity testing shall be considered [...] for substances in quantities > 10 t with $\log K_{ow} > 3$ (R7B, page 53)(EC, 1996; ECHA, 2012). This evaluation showed that increased ACR were probably not associated with high K_{ow} values. Thus, it is indicated that long term testing is not generally required for substances with a $\log K_{ow} > 3$.

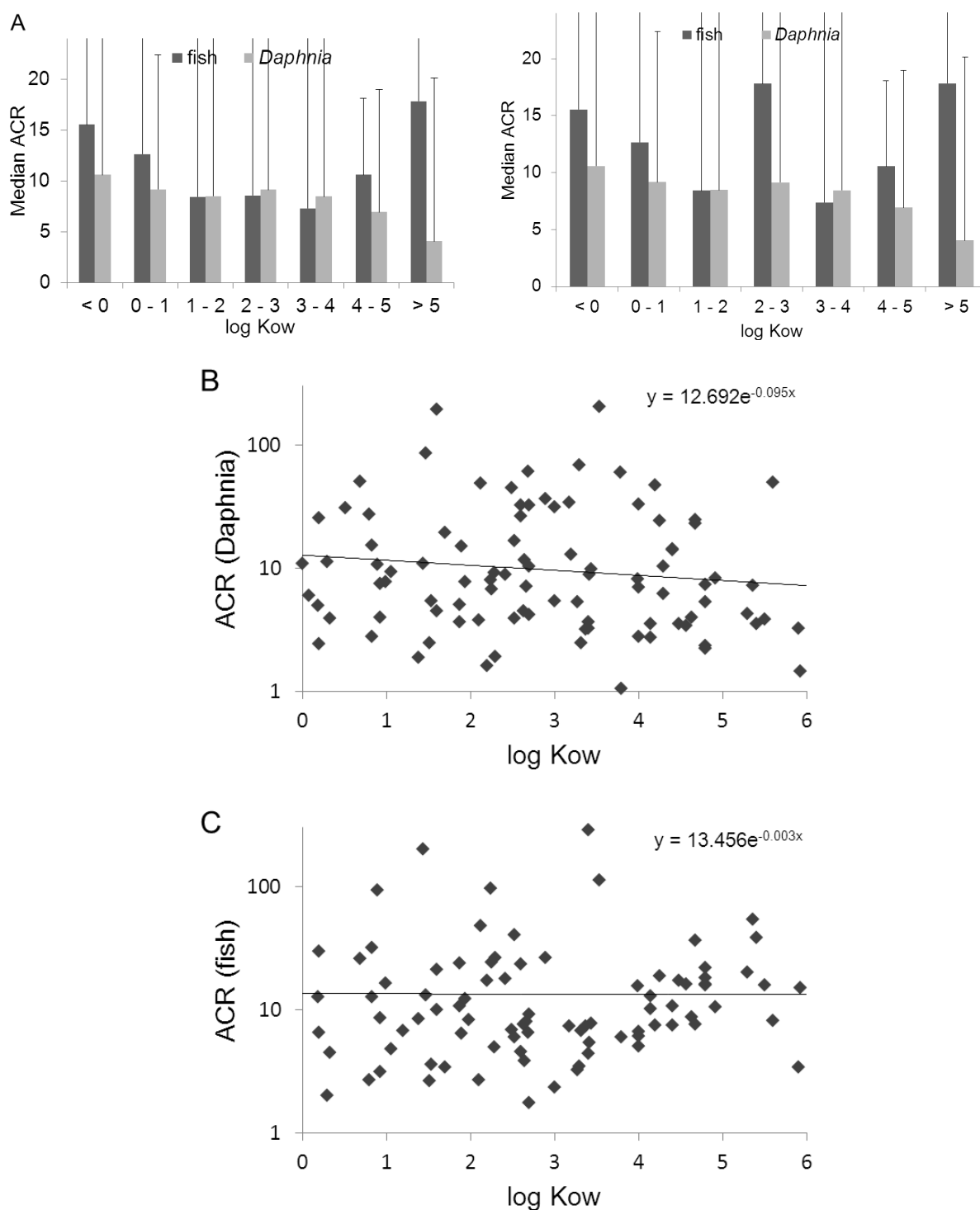
Increased ACRs ($ACR > 100$) were preferentially observed for a $\log K_{ow}$ range of 2 to 3.5 and for $\log K_{ow}$ values < 1 . Substances with medium $\log K_{ow}$ may exhibit specific distribution properties as these substances partition in the water phase, but also in the lipophilic membrane. In contrast, substances with either high or low $\log K_{ow}$ accumulate in the respective compartment (lipophilic or water). Lipophilic substances may exert toxic effects already in short-term testing due to narcosis and subsequently increased chronic toxicity is not

expected due to this mode of action upon long term exposure. Substances with a low log Kow may not be able to pass the cell membrane. Hence, it can be assumed that active transport processes or an increased exposure time are required for substances with a low log Kow.

Table 5: ACR evaluation of *Daphnia* and fish for chemicals with log Kow > 3 and > 4.5

Parameter	Log Kow >3			Log Kow >4.5		
	Daphnia	fish	ACRaqu	Daphnia	fish	ACRaqu
No. of values	45	42	46	17	17	18
Min	1.1	1.8	1.1	1.5	3.4	2.2
Median	6.3	10.5	10.3	4.2	16.0	11.1
90 %-ile	47.1	67.0	55.5	24.6	42.6	46.4
Max	203	514	390	49.3	53.8	53.8

Figure 14: Relationship between ACR and octanol-water partitioning



(A) The ACRs of fish and *Daphnia* were analysed for the indicated classes referring to one Kow magnitude for the complete data set (left) and only FELS test (right). Standard deviation was analysed using Excel and shown for each column. Single data points using only data from FELS test are shown for *Daphnia* (B) and fish (C). Linear regression of the data resulted in a slope that almost equaled 0 for *Daphnia* and fish.

5.6 Poor water solubility

5.6.1 Relationship between species sensitivity and poor water solubility

The data set included 27 substances exhibiting poor water solubility (< 1 mg/L) (Fig. 10). It is important to note that poor water solubility was often associated with a $K_{ow} > 3$ (80 % coincidence). The majority of substances showed no toxic effect in acute testing but 50 % of these substances exhibited a toxic effect in chronic testing (Fig. 10). This result confirmed that chronic testing should be considered for substances that show no acute toxicity up to the solubility limit and if a relevant exposure with a $PEC > 1/100$ th of the water solubility is expected (ECHA, 2008). Second, it was confirmed that a test on invertebrate is protective and should normally be conducted according to the guidance (ECHA, 2008) since only in one case ($< 5\%$) a chronic fish toxicity would have been underestimated if a chronic fish test was not conducted.

Nine of the poor water soluble substances showed acute toxicity (Fig. 11). The evaluation represented a limited data set and conclusions should be handled with care until further data will be available. However, the results supposed that the categorization system is applicable since chronic fish toxicity test is indicated for Cat 1 substances, but likely not required for substances in Cat 3 and 4. In total, the chronic fish test was indicated for 3 from 27 substances. Thus, poor water solubility was in general not confirmed as determinant indicating the requirement of chronic fish toxicity testing since chronic testing of fish was only indicated in about 11 % of the cases.

5.6.2 Correlation of ACR and poor water solubility

Acute and chronic toxicity was determined for 9 substances for *Daphnia* and for 6 substances for fish (Table 4). The acute to chronic ratio for low water soluble substances ranged from 1.5 to 68.8 (median 5.1) for *Daphnia* and from 1.9 to 26.0 (median 3.5) for fish. Using the most sensitive trophic level the ACRAqu ranged from 1.6 to 30.0 (median 6.2). 90 %-ile ACRs were determined to 24.6 for *Daphnia*, 8.7 for fish and 7.6 for the most sensitive trophic level (Table 4). Thus, poor water solubility was not associated with increased ACRs. Indeed, the ACR were markedly reduced compared to the average ACR of the complete dataset. In neither case an AF of 100 was exceeded and would have covered the NOEC from the chronic study. This finding supposed that an acute to chronic extrapolation is protective for poor water soluble substances if acute toxicity was determined.

Taken together, the evaluation represents a limited data set. Nevertheless, poor water solubility was in general not confirmed as determinant indicating the requirement of chronic fish toxicity testing since chronic fish toxicity testing was indicated in less than 10 % of the cases and species sensitivity as well as ACR could be adequately evaluated.

Table 6: ACR evaluation of *Daphnia* and fish for chemicals with water solubility < 1 mg/L

Parameter	low water solubility (<1 mg/L)		
	<i>Daphnia</i>	fish	ACRaqu
No. of values	9	6	9
Min	1.5	1.9	1.6
Median	4.3	6.1	6.2
90 %-ile	n.a.	n.a.	n.a.
Max	68.8	26.0	30.0

5.7 Quality criteria of fish chronic testing

The data for fish toxicity represented a heterogeneous dataset with a variety of species and chronic study types. Therefore, quality criteria were defined and tests were subdivided into FELS studies (n=129) and other chronic study types representing the non-FELS studies (n=40) within the dataset. FELS studies represent the vast majority in the data set and thereby dominate the overall results. The result showed that the type of chronic test affected the average sensitivity distribution. While sensitivity of *Daphnia* and fish was now comparable if only FELS tests were considered for fish, substance entries underlaid with non-FELS tests exhibited in average an increased sensitivity to *Daphnia* in chronic testing (Fig. 14 and Fig.15). This result suggested that the type of chronic test effected the average sensitivity distribution. However, the result shown in figure 14 B was almost comparable to the result of the complete dataset in figure 6. The applicability of the categorization system was also confirmed if only FELS tests were considered since the chronic *Daphnia* test was still indicated for Cat.3 and Cat.4 whereas the fish test was required for Cat.1 to avoid underestimation of fish toxicity by extrapolation from chronic *Daphnia* data. Again a chronic fish test was not indicated for Cat.4 and in 7% of the cases for Cat.3. In total, a confidence interval of 95 % was determined for substance with a $Q_a > 0.5$ in Cat.3 and Cat.4 since the chronic fish test was indicated for 5 % of the substances. Moreover, the fish test was required in Cat.2 for about 28 % for the substances of the complete dataset compare to about 33 % of the substances for Cat.2 if only FELS test were considered.

For ACR evaluation different quality criteria were defined corresponding to the methodology applied previously (Ahlers et al., 2006). The first (ACR1) included all data across all fish species and test types (Table 5). The second (ACR2) included all test types but was confined to data with acute and chronic results from the same species whereas the third (ACR3) was confined to data from different species. The fourth (ACR4) was restricted to chronic toxicity testing conducted according or equivalent to OECD 210 and the fifth (ACR5) included all chronic studies that were not conducted according or equivalent to OECD 210 representing the non-FELS studies within the dataset. ACR6 only included FELS test on the same species in acute and chronic testing. ACR7 and ACR8 included FELS tests from organic chemicals only.

ACR1, ACR2 and ACR3 showed comparable median values indicating that statistical ACR medians did not differ between evaluations using the same species and evaluations with species differentiation. The 90%-ile value of ACR2 species specific ACRs was reduced compared to ACR1 and ACR3 whereas ACR3 was increased compared to ACR1 and ACR2. Furthermore, a 10%-ile value was 3.4 for the ACR2 whereas a value of 2.7 was determined for inter-species ACRs (data not shown). Thus, the inter-species ACRs showed a 41 fold range between the 10%-

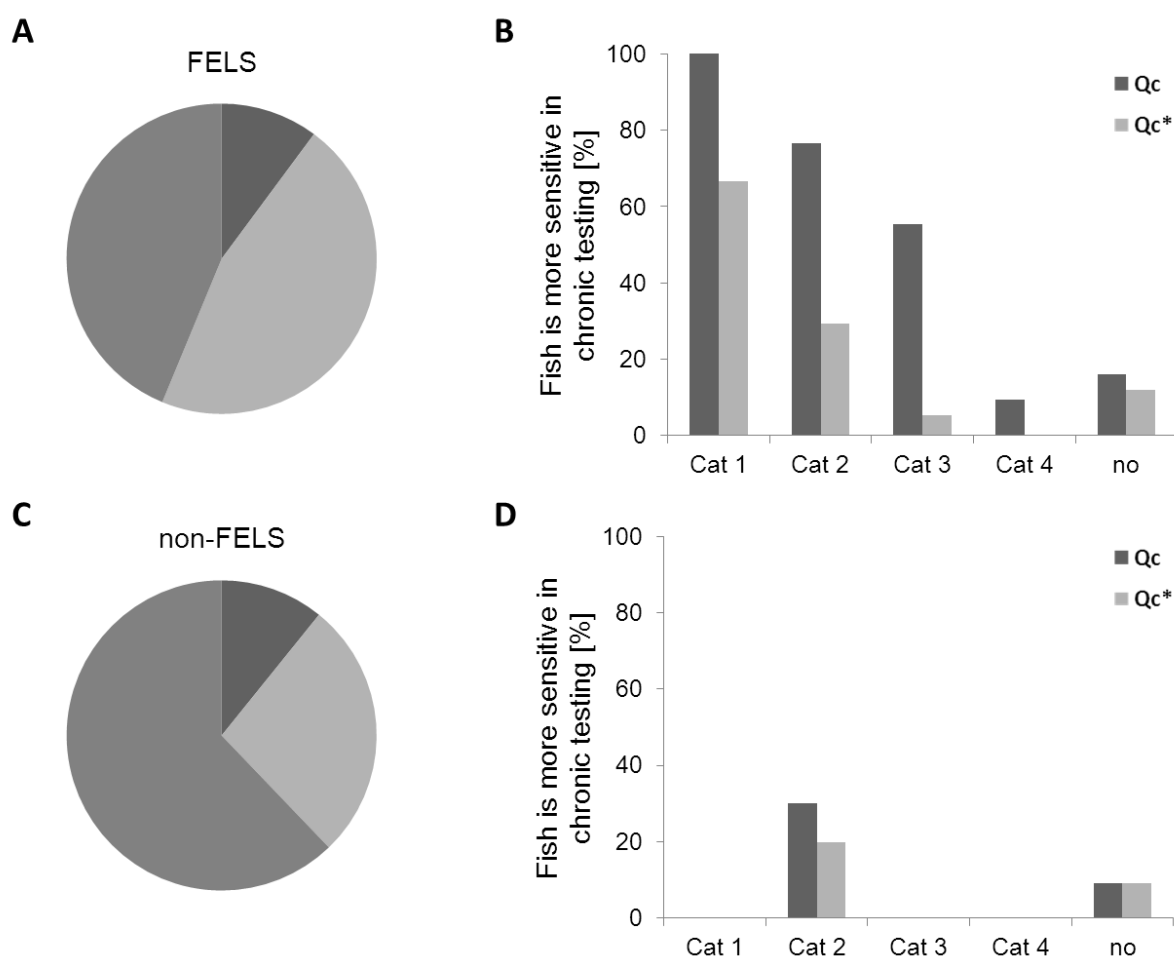
ile and the 90%-ile value whereas species specific ACRs showed a 19 fold range. This observation suggested that the variance in data distribution is increased using evaluations of different species.

The study type of chronic testing appeared to have an effect on the ACR, as well. FELS based studies showed a median ACR4 of 13.0 and a 90%-ile value of 81.8 (Table 5). The median ACR5 of 7.2 and the 90%-ile value of 36.6 of non-FELS studies were reduced compared to the FELS based studies. Hence, ACR of FELS studies was in average about 1.8 fold larger than ACR of non-FELS studies. Thus, FELS based studies appeared to be more sensitive to determine chronic toxicity compared to other studies types and may be considered as more conservative.

However, the result on non-FELS studies is based on a limited dataset (n= 23) and interpretation should be handled with care and on a case by case evaluation since different tests types were not compared for the same substance.

ACR6 and ARC8 were considered as data with the highest statistical data quality since only FELS test that were confined to data with acute and chronic results from the same species were included. The median was determined to 12.2 and the 90%-ile was calculated to 63.5 for ACR6. ACR8 only included FELS tests from organic chemicals. The median was determined to 10.4 and the 90%-ile was calculated to 63.5. 5% of the substance had an ACR >100.

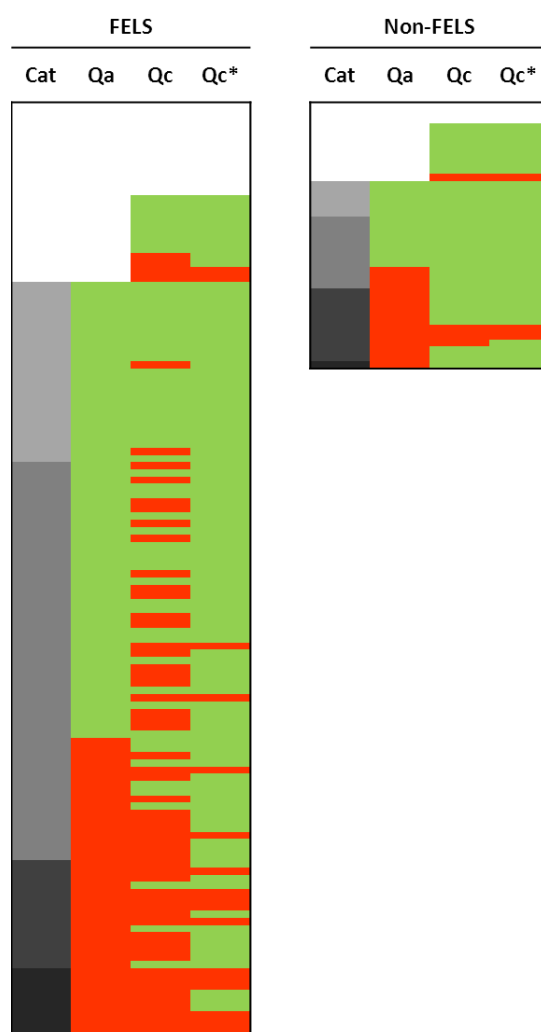
Figure 15: Overview of results of chemicals for fish by different study quality criteria



6

Sensitivity distribution of *Daphnia* and fish toxicity was visualized in a pie diagram for substance entries underlain with data from chronic toxicity testing conducted equivalent to OECD 210 (A) or with data from non-FELS studies (C). Substance entries were allocated to the classification system that is based on quotient_{acute} and described in table 1. The percentage of substances being more toxic to fish in chronic testing and exhibiting a quotient_{chronic} <1 was evaluated for each classification class (black). Substances that were >5x more sensitive to fish in chronic testing and exhibited a quotient_{chronic} <0.2 was evaluated for each classification class (grey). (B) Data was restricted to chronic toxicity testing conducted equivalent to OECD 210. (D) Data was included from all chronic studies that were not conducted equivalent to OECD 210 and represent the non-FELS studies.

Figure 16: Sensitivity distribution by different test quality criteria of fish studies



Sensitivity distribution between *Daphnia* and fish was elucidated by a heat map diagram as described in figure 4 for substances underlain by a FELS or by non-FELS studies.

Table 7: ACR evaluation of chemicals for fish by different study quality criteria

Parameter*	ACR1	ACR2	ACR3	ACR4	ACR5	ACR6	ACR7	ACR8
No. of values	123	75	48	100	23	63	89	55
Min.	1.1	1.8	1.3	1.1	1.7	1.9	1.1	1.9
Median	12.2	10.7	12.4	13.0	7.4	12.2	12.1	10.4
90 %-ile	68.0	63.5	111.1	81.8	36.6	63.5	96.6	63.5
Max.	1370.6	1370.6	514.0	1370.6	375.0	1370.6	1370.6	1370.6

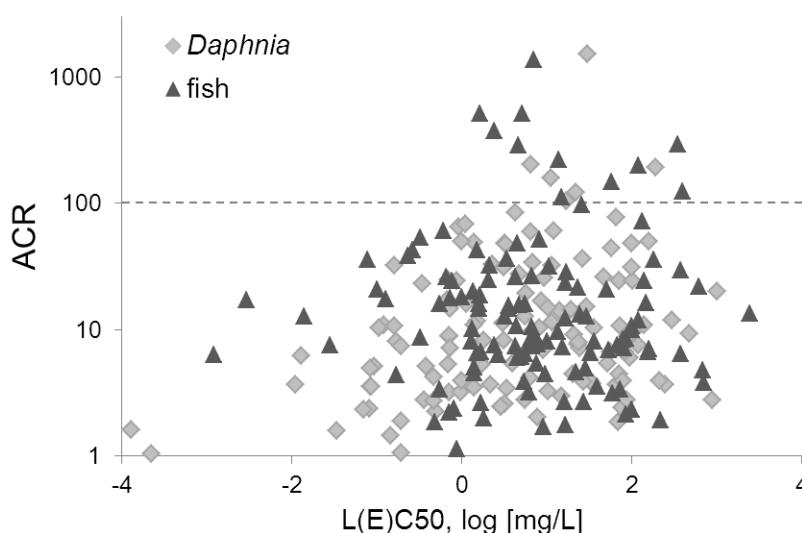
ACR1 included data across all fish species and test types. ACR2 included all test types but was confined to data with acute and chronic results from the same species whereas ACR3 was confined to data from different species. ACR4 was restricted to chronic toxicity testing conducted according or equivalent to OECD 210 representing FELS studies. ACR5 included all chronic studies that were not conducted according or equivalent to OECD 210 and represent the non-FELS studies. ACR6 included all tests that were confined to data with acute and chronic results from the same species and where the chronic study was conducted according to

FELS. ACR7 included FELS tests from organic chemicals only. ACR8 included FELS tests from organic chemicals only that were confined to data with acute and chronic results from the same species.

5.8 Relationship between acute toxicity and ACR

Ahlers et al. (2006) hypothesized that highly acute toxic compounds have little room to increase through prolonged exposure times (Ahlers et al., 2006). Moreover, it can be supposed that some chemicals showing less acute toxicity may address non-lethal endpoints covered by chronic tests resulting in increased ACRs. To investigate the relationship between acute effect data and ACR toxicity levels *Daphnia* and fish were correlated with the respective ACR (Fig. 16). ACR values above 100 were not observed for substances exhibiting acute values of $L(E)C_{50} < 1\text{mg/L}$ (Fig. 15). Distribution analysis showed that 90%-ile values of 24.3 for *Daphnia* and 38.0 for fish were determined for substances exhibiting acute values of $EC_{50}/LC_{50} < 1\text{mg/L}$, whereas a 90%-ile value of 60.5 for *Daphnia* and 111.1 for fish was determined for substances exhibiting acute values of $EC_{50}/LC_{50} > 1\text{mg/L}$ (data not shown). These results led to the conclusion that highly acute toxic compounds with $EC_{50}/LC_{50} < 1\text{mg/L}$ have a lower probability for substantially increased chronic effect values.

Figure 17: Relationship between acute effect value and ACR

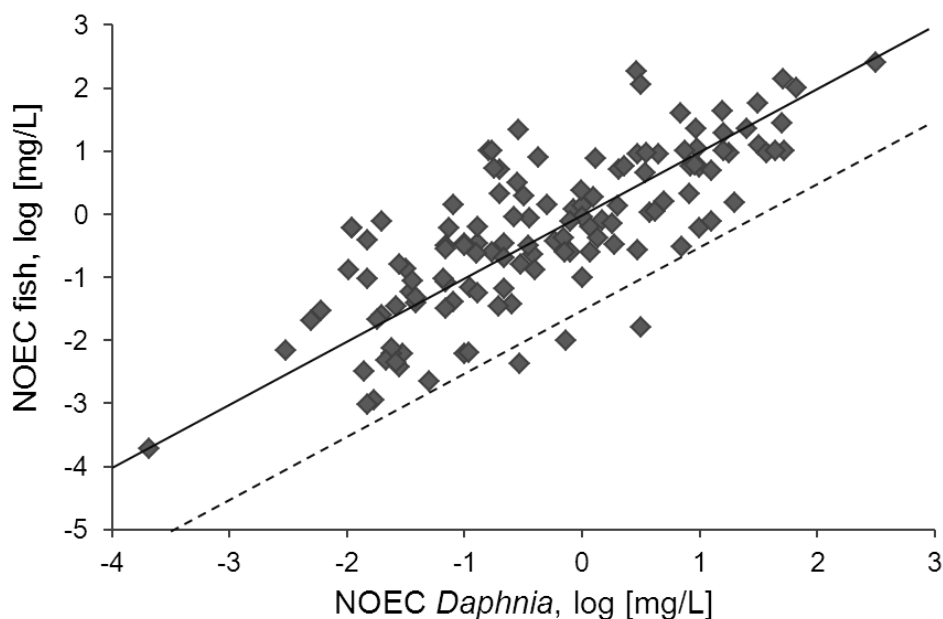


The ACR of fish (dark grey) and *Daphnia* (grey) was correlated with the acute effect concentration (EC_{50} , LC_{50}) for chemicals.

5.9 Relationship of effect levels between trophic levels in chronic testing

Predictions between trophic levels would be helpful for risk estimation and to avoid animal testing. Chronic effect values of *Daphnia* and fish were correlated (Fig. 17). A moderate relation between both trophic levels could be established. According to the guidance documents an assessment factor of 50 can be applied on the NOEC of the most sensitive species for chronic testing if chronic data on fish is not available. Hence, this approach includes a factor of 5 for interspecies variance if fish data is not available. In total, chronic fish toxicity was covered by the *Daphnia* test and an AF 50 except of three substances as indicated by the dotted line referring to 2 % of the evaluated substances (Fig. 17).

Figure 18: Relationship between chronic effect values of *Daphnia* and fish

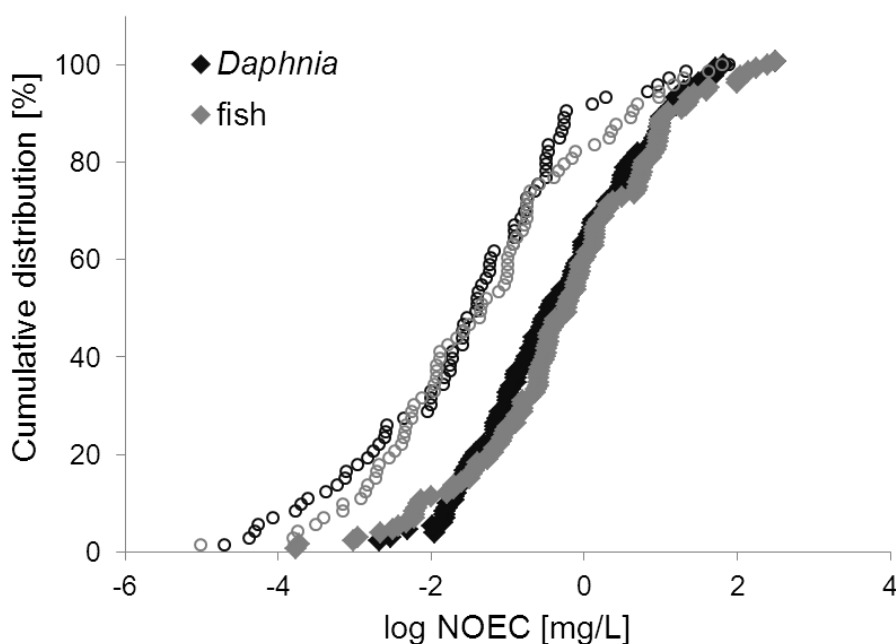


Chronic effect values of *Daphnia* and fish of chemicals were correlated. The filled line indicated comparable sensitivity. The dotted line includes an extrapolation factor of 50. For substances underneath the dotted line fish toxicity is not covered by a chronic *Daphnia* test and an AF of 50.

5.10 Toxicity levels of chemicals and pesticides

The distribution of individual chronic effect values of fish and *Daphnia* for industrial chemicals and pesticides is superimposed and depicted (Fig. 18). The result suggested a rather comparable distribution of chronic effect values between both species. In average NOECs of pesticides were about one magnitude lower than NOECs of chemicals. For chemicals the 95%-ile values were determined to 11 µg/L for *Daphnia* and to 4 µg/L for fish. By contrast, 95%-ile values were clearly reduced for pesticides and calculated to 0.06 µg/L for *Daphnia* and at 0.31 µg/L for fish. The lowest effect value of an organic chemical was determined to 0.001 mg/L.

Figure 19: Cumulative distribution of chronic effect values of *Daphnia* and fish



Cumulative distribution of chronic effect values of *Daphnia* (black) and fish (grey) were depicted for chemicals (filled rhombus) and pesticides (open circle).

5.11 Structural alerts

One important question for risk assessment and testing strategies is the knowledge which substance classes or structural properties are associated with specific chronic fish toxicity. Substances that were 5 x more toxic to fish than to *Daphnia* in chronic testing and that were, hence, assumed to show specific fish toxicity have been analysed. This concerns 21 out of 167 (13%) of all evaluated substances that would have required a chronic fish toxicity test ($Q_c < 0.2$). Additionally, seven substances that were > 2x more toxic to fish in chronic testing and exhibited a NOEC of < 0.01 mg/L were included in the evaluation for structural alerts. Together, 28 substances were evaluated comprising four metals and 24 organic chemicals. A comparative analysis of structural moieties of organic chemicals resulted in the identification of structural alerts for an increased probability of substantial sensitivity of fish in chronic toxicity testing. The results are summarized in Table 6. The most abundant group with six substances showing substantial fish toxicity referred to phenol derivatives. Within this group, four substances comprised para-substituted phenols. Thus, in particular para-substituted phenol derivatives exhibited a substantial chronic toxicity to fish. Structural alerts further comprised a halogenated nitrobenzene and two aminobenzenes from in total nine substances within this aminobenzenes group. Two organophosphorus compounds were identified. However, both compounds were substituted with a phenol derivative. Therefore, it is questionable whether the effect is based on the phosphorus moiety or attributed to the structure of the substituent. Several substances exhibited unique structural properties compared to the other substances and could, hence, not be allocated to certain groups of substances. These substances comprised a phthalate ester, a peroxy acid or a thiocarbamate, for example. In addition, 18 pesticides representing 25 % of the evaluated pesticides were determined that were either 5x more toxic to fish in chronic testing or that were 2x more toxic to fish in chronic testing and that exhibited a NOEC < 0.01 mg/L.

29 substances were identified showing an ACR >30 for fish toxicity. A comparative analysis of structural moieties resulted in the identification of potential structural alerts for an increased probability of high ACR for fish. The results are summarized in Table 7. Again the most abundant group of structural alerts represented phenol derivatives and in particular para-substituted phenol derivatives. Furthermore, thiocyanate and thiocarbamate derivatives, nitrobenzene and aminobenzenes derivatives as well as 2-ethylhexyl derivatives represented structural alerts. One organophosphorus compound out of five was identified with an increased ACR. However, this compound was substituted with a para-alkylate phenol derivative. Therefore, it is questionable whether the effect is based on the phosphorus moiety or attributed to the structure of the substituent. Furthermore, organic substances exhibit unique structural properties and were not grouped. Analysis of substances that showed an ACR >30 and that were 5x more sensitive suggested that para-substituted phenols protruded from the data set whereas other substances represented individual substances with unique structural properties (Table 10).

Together, the analysis for structural alerts showed that in particular pesticides, para-substituted phenols, thiocyanate and thiocarbamate derivatives and musk compounds protrude from the data set as substances that exert increased ACR or significant fish sensitivity in chronic testing. The result supposed that structural alerts analysis may support the ITS. However, structural alerts appear to be of moderate predictive value since several of the compounds with increased ACR and chronic sensitivity could not be identified or allocated to a certain group. Furthermore, false positive results in a group were increased in some groups such as amino- and nitro-benzene derivatives or phthalate derivatives, for example, and the categorization scheme already classified the majority of the identified structural alert in the category 1 or 2.

Table 8: Structural alerts associated with chronic fish sensitivity.

Structural moiety	Chronic fish sensitivity >5	No. of total	Ratio
Amino-/Nitro-benzene derivatives	2	9	22
Phenol derivatives	6	24	25
Para-substituted phenol derivatives	4	18	22
Musk compounds	2	2	100
Phosphorus derivatives	2	10	20
Phthalate derivatives	1	4	25
2-Ethylhexyl ester derivative	2	4	50
Thiocyanate/ Thiocarbamat derivatives	1	2	50
Peroxoic acid	1	2	50
Michael addition type (α,β unsaturated carbonyl)	3	4	75
Quaternary ammonium compound	0	5	0
Pesticides	18	73	25

Substance entries with substantial chronic fish toxicity were evaluated and grouped for comparable structural moieties. The dataset compiled substance entries that were either 5 x more toxic to fish in chronic testing or that that were > 2x more toxic to fish in chronic testing and that exhibited a NOEC of < 0.01 mg/L in addition. A comparative analysis of structural moieties resulted in the identification of structural alerts for an increased probability of substantial sensitivity of fish in chronic toxicity testing.

Table 9: Structural alerts associated with high ACR.

Structural moiety	ACR >30	total	ratio
Amino-/Nitro-benzene derivatives	4	9	33
Phenol derivatives	6	17	35
Para-substituted phenol derivatives	6	13	46
Phthalate derivatives	1	3	33
Organophosphorus derivatives	1	5	40
2-Ethylhexyl ester derivative	1	3	33
Thiocyanate/ Thiocarbamat derivatives	2	2	100
Peroxoic acid	1	2	50
Michael addition type (α,β unsaturated carbonyl)	1	4	25
Quaternary ammonium compounds	1	4	25
Pesticides	23	70	33

Substances showing an ACR >30 for fish toxicity were determined. A comparative analysis of structural moieties resulted in the identification of potential structural alerts. Within a group of structural alerts the number of substances with an ACR > 30 were allocated to substances with an ACR < 30 and the ratio of substance with an ACR >30 calculated.

Table 10: Structural alerts associated with chronic fish sensitivity and a high ACR.

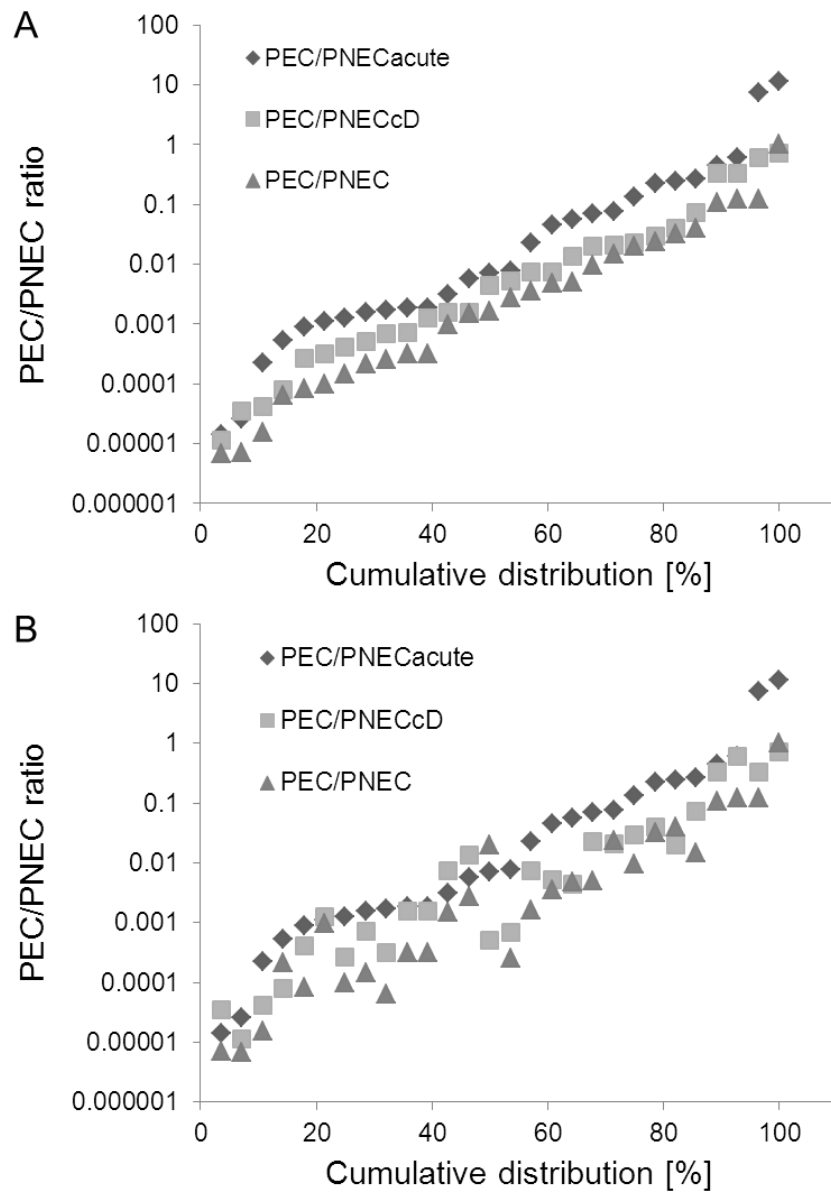
Structural moiety	total
Amino-/Nitro-benzene derivatives	1
Para-substituted phenol derivatives	3
2-Ethylhexyl ester derivative	1
Linear alcohol	1
Peroxoic acid	1
Michael addition type	1
Pesticides	3

Substances showing an ACR >30 for fish toxicity and that were 5x more sensitive to fish in chronic testing were determined.

5.12 Risk estimation for exemplarily substances

Exemplarily, a PEC/ PNEC comparison was conducted for 29 substances considering a PNEC derived from acute data and an AF1000 (PEC/PNEC_{acute}), a PNEC derived from chronic Daphnia data and an AF50 (PEC/PNEC_{CD}) and the PNEC derived from chronic data of the most sensitive trophic level and an AF10 (PEC/PNEC). Cumulative distribution of the PEC/PNEC ratio is depicted in figure 18A. The variance within the three ratios for one substance is shown in figure 18B. The PEC/PNEC ratio of 27 substances was <1 representing 92 % of the evaluated data set. Two substances showed a PEC/PNEC_{acute} >1. In both case chronic testing contributed to PNEC refinement. For one substance the PEC/PNEC ratio dropped from 7.5 to 0.6 (PEC/PNEC_{CD}) and 0.1 (PEC/PNEC). In the other case, the PEC/PNEC ratio dropped from 11.6 to 0.72 (PEC/PNEC_{CD}) and 1 (PEC/PNEC). As discussed above fish toxicity may be underestimated by the PEC/PNEC_{CD} if fish is >5x more sensitive than Daphnia or by the PEC/PNEC_{acute} in the case that the ACRA_{qu} is >100. However, an increase of the PNEC due to chronic testing of all three trophic levels was only observed in four cases. Thereby the increase was moderate and did not exceed a factor of 3. Thus, the result suggested that chronic data usually contributed to PNEC refinement. Furthermore, an early inclusion of the risk assessment may represent a helpful approach to estimate chronic testing requirements since the variance between both PEC/PNEC ratios derived from chronic data compared to the PNEC derived from acute data appeared to be low for the evaluated cases (Fig. 18B).

Figure 20: PEC/PNEC ratios of exemplary substances



PEC/PNEC ratios were calculated for exemplary substances based on only acute data and an AF of 1000 (PEC/PNECacute), the chronic Daphnia test and an AF of 50 (PEC/PNECcD), and on the NOEC of the most sensitive trophic level and an AF of 10 (PEC/PNEC). (A) Cumulative distribution of the respective PEC/PNEC ratios. (B) Cumulative distribution of PEC/PNECacute. PEC/PNECcD and PEC/PNEC were allocated PEC/PNECacute of the respective substance.

6 Conclusion

The study is the first data analysis of a comprehensive dataset on long term guideline studies on fish that compares sensitivity differences of Daphnia and fish for individual substance in acute and chronic testing. 240 entities from the ECHA and ICS database including 73 pesticides have been analysed within this study.

The dataset primarily comprises chemical substance entries of substances that are registered at ≥ 1.000 t/a under the REACH regulation or that are considered as substances of concern with regard to environmental toxicity. Due to the explicit coverage of substance of concern with regard to environmental toxicity within the database some substance classes may be overrepresented within this dataset. Indeed, chemical groups like para-substituted phenols, for example represented about 15% of the total chemicals whereas substances of low ecotoxicological concern may be underrepresented in this dataset as waiving of the chronic fish toxicity test is expected for these substances.

An uncertainty may arise by the fact that the data from the ECHA database is provided by the registrants and, thus, the primary data source could not be evaluated. For quality control only studies with a reliability of Klimisch score 1 (reliable without restriction) or Klimisch 2 (reliable with restriction) are considered. Re-evaluation of these studies by the ECHA or national authorities may still result in some changes. Data of the ICS database is validated by the German authority.

An uncertainty of the results and conclusions may further arise from selection of the data set. While often only one chronic study is available several studies are reported for acute testing. In this study the key study or the study with the lowest effect value was chosen since it is considered that these studies are relevant for hazard and risk assessment. Furthermore, the same species as in the chronic study was preferentially chosen in acute testing for fish to avoid a bias due to interspecies comparison. This approach corresponds to the approach conducted by Ahlers et al. (2006). However, different data evaluation approaches may be used and may affect the outcome. For example, the median of the toxicity levels can be used for data evaluation if three or more studies with high data quality are reported for one substance and one endpoint (ECETOC, 2003).

Although some uncertainties are associated with the data evaluation approach used in this study the comprehensive dataset is considered to provide a sound statistical basis.

6.1 ACR evaluation

The dataset which is confined to standardized tests that includes acute and chronic data on both fish and Daphnia for each substance entry allows the evaluation of ecotoxicological risk estimation within the context of the integrated testing strategy of the European Union chemical registration coincidentally considering different trophic levels. Previous studies resulted to some extent in very high ACRs that were calculated up to values of $> 10,000$ (ECETOC, 2003; Raimondo et al., 2007). However, these ACRs exhibit isolated values and were usually not related to additional ecotoxicological data of the respective substance. Therefore, these values do not consider whether the reported ACR of a species is relevant for environmental risk assessment or whether other trophic levels or species are more sensitive. The ACRaqu approach derives a value using the most sensitive species in acute and the most sensitive species chronic testing and can be considered as relevant for risk assessment.

Our results on species specific are basically comparable with previous reports, but interpretations differ in some points. Lange et al. (1998) and ECETOC (2003) both included organic chemical, metals and pesticides in their evaluation. Both datasets were based on the database EAT. ECETOC calculated median ACRs of 6.8 for *Daphnia magna* on the basis of 37 compounds including 7 pesticides and 3 metals. Median ACRs of 8.8 were reported for fish on the basis of 69 compounds including 19 pesticides and 11 metals. Lange et al. calculated median ACRs of 6.1 for invertebrates on the basis of 27 compounds including 9 pesticides and 9 metals. Median ACRs of 9.5 were reported for fish on the basis of 62 compounds including 24 pesticides and 8 metals. Based on a LC50 to LOEC evaluation of fish data Roex et al. (2000) determined a median ACR of 6.03 for chemicals. For specific acting chemicals a median ACR of 17.3 and a median ARC of 15.3 was reported for metals. Raimondo et al. (2007) investigated the variability of species specific ACRs including chemicals, pesticides and metals. Median ACR of 7.5 for invertebrates (n=195) and a median ACR of 9.3 for fish (n=261) as well as 90%-ile values of 68.3 for *Daphnia* and 90.0 for fish were reported (Raimondo et al., 2007). Ahlers et al. (2006) only included organic chemicals within the dataset and calculated median ACRs of 7.0 for invertebrates (102 substances), 10.5 for fish (32 substances) and 10.75 for ACRAqu (32 substances). In line with this study the 90th-percentile was determined to 41.5 for *Daphnia*, whereas a 90th-percentile values of 198.0 for fish and of 105.2 for ACRAqu were increased compared to this study. However, the ACR for fish and the ACRAqu calculated by Ahlers et al. (2006) are based on only 32 compounds. The differences between this and previous studies are probably related to the different number of evaluated compounds and the statistical data quality. Taken together, different approaches have been used for data evaluation, but mostly resulted in comparable results with median values ranging from 9.0 to 12.6 for fish and median ACRs ranging from 6.1 to 8.8 for invertebrates. Compared to previous studies the median ACRs of this evaluation were slightly increased for chemicals. However, the comprehensive data basis suggests that an ACR of 100 as implemented in the European risk assessment approach is protective for more than 90 % of the chemicals.

Table 11: Overview of relevant literature on ACR evaluation.

	Year	Chemicals	Pesticides	Metals	total	ACR	Remark
Lange et al.	1998	30	24	8	62	9.5	EAT
Roex et al.	2000	"scarcity of ACRs for fish"					
ECETOC	2003	44	19	11	74	8.8	EAT
Ahlers et al.	2006	32	-	-	32	10.5	
Raimondo et al.	2007				< 261	9.3	

6.2 Sensitivity comparison

The evaluation of the dataset shows that the more sensitive trophic level in chronic testing can be predicted to a certain degree from the more sensitive species in acute testing. Thus, chronic species sensitivity could be estimated from acute testing. To estimate chronic test requirements the study proposes a classification system for an acute sensitivity ratio to support the ITS. The final arrangement of the applied categories of the categorization system was based on the initial concept of the kick-off meeting by the UBA, empiric results of the study and discussion by the project partners. For Cat.1 and Cat.4 it is suggested that the respective chronic test of the 5x more sensitive species in acute testing is required since omitting the chronic test of the more sensitive species by using another trophic level may result in an underestimation of the

environmental hazard. Although a test of the 5x more sensitive species is required to do not underestimate environmental hazard the term does not exclude that tests of other trophic levels or species may be considered for hazard assessment. For Cat.3 the results show that fish toxicity is covered by the chronic *Daphnia* test and an AF of 50 in more than 90 % of the cases. The result reveals that four substances in this category required the chronic fish test to avoid underestimation of the environmental hazard. This refers to 6% of the substances for the complete dataset or 8 % of the substances if only FELS studies are considered. A confidence interval of 90 % may be considered to give an adequate basis to suggest that the chronic *Daphnia* test is indicated for environmental hazard assessment whereas the chronic fish test can be avoided in general. Nevertheless, exceptions that require a chronic fish test to avoid underestimation of fish toxicity by extrapolation from chronic *Daphnia* data are possible if a comparable sensitivity is determined in acute testing.

While fish testing is indicated for substance in Cat.1 and *Daphnia* testing is indicated for substances in Cat.3 and Cat.4 substances Cat.2 appears to be associated with an uncertainty in predicting chronic testing requirements. Therefore, testing of both *Daphnia* and fish should be considered since no clear recommendation can be given based on the statistical result of this study. In the majority of cases (72 %) the chronic fish test is not necessary whereas 28 % of the substances are not properly covered by the chronic *Daphnia* test. Therefore, the chronic fish test should be considered unless substantial chronic fish toxicity can be excluded. However, a general indication to perform a chronic fish test for substances in Cat.2 results in a high number of animal tests that are retrospectively not necessary. Subsequently, the challenge arises in which case a chronic fish test is necessary and in which case the chronic fish test can be avoided. Interestingly, the Kow value appears to support decision making. It is proposed by the findings of this study that a high Kow can be applied as trigger to indicate chronic fish testing whereas the results suggest that a chronic fish test is not indicated for Cat.2 if the Kow is <3 and no structural alert is determined. However, this conclusion should be handled with care since no causal relationship exists between both determinants (classification and Kow) and due to a limited data set of substance in Cat2.

Finally, it is important to note that the classification is based on the empiric results of this study and refer to a statistical probability whether the chronic fish toxicity is covered by the chronic *Daphnia* test and an adequate assessment factor. The acute sensitivity comparison approach is designed to offer a method to avoid animal testing if possible and statistical reasonable. If a “no risk” approach should be followed and underestimation of fish toxicity should be excluded in any case chronic fish testing will be required for Cat.1, Cat.2 and Cat.3. Such an approach will result in animal testing for more than 60 % of the substances, subsequently, and it remains to be discussed whether this is compatible with the intention to avoid animal test.

6.3 Refinement of assessment factors based on acute sensitivity comparison

The results of this study suggest that *Daphnia* testing is indicated for substances in Cat.3 and Cat.4. The chronic fish test can be avoided in general in these categories. However, exceptions from a methodology can not be excluded and are already determined within this data set for substances that exhibit no or a low acute toxicity to fish in acute testing, but fish toxicity in chronic testing. Therefore, shortcomings of the methodology are assumed for substances that exert a mode of action in chronic fish toxicity testing which may not be derived from short term testing. In total, exceptions in Cat 3 and 4 that are not predicted to be more sensitive to fish in chronic testing by this method account to <5 % representing a confidence interval of >95 % for this approach, subsequently. Therefore, a chronic fish toxicity test should generally not be requested unless fish are 2x more sensitive than *Daphnia* in acute testing. However, four

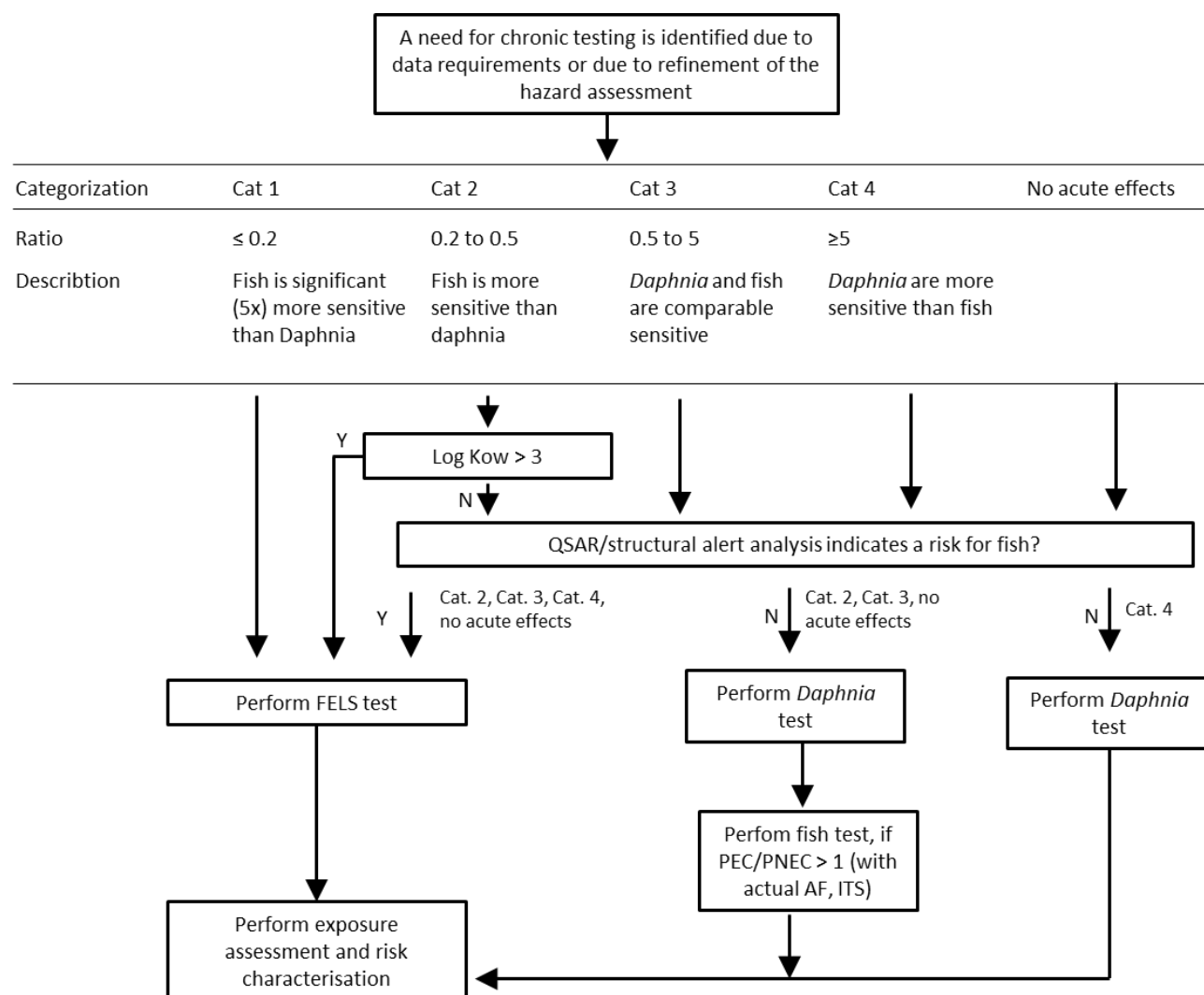
exceptions being more sensitive to fish in chronic testing were determined in the Qa range from 0.5 to 2 be referring to <10% of the total substances within this range. Physicochemical properties like water solubility and the octanol-water partitioning coefficient do not represent determinants to predict exceptions from the sensitivity classification methodology since the four substances in Cat 3 that were more toxic to fish showed a water solubility of > 1 mg/L and two have a log Kow <3. As discussed below application of structural relationship analysis as concomitant approach may be useful to verify the result of the sensitivity comparison. Indeed, two of the four exceptions represent para-substituted phenols and one a halogenated nitrobenzene. Furthermore, it should be noted that the remaining four substances exhibits NOEC > 1 mg/L. A PEC/PNEC comparison was conducted for this substance in section 5.11 resulting in a RCR of <0.001. Although a comprehensive risk assessment can not be conducted within the scope of this project this limited evaluation suggests that this substance is of less ecotoxicological concern and a chronic fish test is not necessarily required in this case.

6.4 Adaptation of sensitivity distinction as applied in the REACh guidance 7b

According to the European guidance document R7B, page 54, no further requirements for fish toxicity testing is indicated if there is compelling evidence to suggest that the fish value is likely to be at least a factor of about 10 less sensitive than invertebrates or algae (ECHA, 2012). The here presented classification system of acute sensitivity comparison differs from the current guidance documents which indicate a threshold of 10x sensitivity difference between trophic levels. First, the current approach initially results in an amount of about 95 % of the evaluated substances that may require a chronic fish toxicity test. Distribution analysis of quantitative sensitivity in chronic testing, however, shows that the chronic *Daphnia* test should be considered as sufficiently protective for more than 85 % of the analysed substances. Second, *Daphnia* was in neither case more 5x sensitive in chronic testing if fish was 5x more sensitive in acute testing. In this case *Daphnia* toxicity testing is not required. Third, fish was in neither case more sensitive in chronic testing if *Daphnia* was 5x more sensitive in acute testing. In this case fish toxicity testing is not required. In summary, the results of this study indicate that a threshold of 10x as used for sensitivity distinction in the current guidance documents should be reduced to 5x. Thus, adaption of the current ITS is suggested by this data analysis proposing a reduction of the sensitivity factor from 10 to 5. In the case that the value of *Daphnia* /fish is likely to be at least a factor of about 5 less sensitive than the other trophic levels, there are no further requirements for *Daphnia* /fish testing. For substances being 5x more sensitive to *Daphnia* in acute testing (Cat.4) 91 % of these substances are also more sensitive to *Daphnia* in chronic testing. Only three substances are slightly more sensitive to fish in chronic testing. However, in these cases the ratio Q_c was >0.5 . Thus, the substances can be considered to be comparable sensitive to *Daphnia* and fish in chronic testing since a range of data variance between 0.5 and 2 is assumed as comparable sensitive. Therefore, it is proposed that a fish toxicity test is not required and application of an AF of 10 on the chronic *Daphnia* is suggested to be protective for substances classified in Cat.4. The same applies for substances being 5x more sensitive to fish. 90 % of the substances in Cat.1 are more sensitive to fish in chronic testing. Only one substance is slightly more sensitive to *Daphnia* with a quotient of 3.2. Hence, toxicity of invertebrates is expected to be covered by the chronic fish test, and application of an AF of 10 on the chronic fish test is suggested to be usually protective for substances in Cat.1 while a chronic *Daphnia* test is not necessarily required. Taken together, the findings suggested that for substances being 5x more sensitive to one trophic level chronic testing is required for the respective trophic level. Chronic testing of the less sensitive trophic level from acute data is usually not indicated in this case. Application of an AF of 10 on the sensitive trophic level is further expected to be sufficient protective and thus may be applied for PNEC refinement for Cat.1 and Cat.4. For Cat.2 and Cat.3 an AF of 50 is still indicated and a reduction to 10 not applicable.

In the following a possible implementation of the categorization system in the ITS is proposed to estimate chronic testing requirements:

Figure 21: Proposed decision scheme for the conclusion on chemical safety assessment.



6.5 Physicochemical properties as determinants for the testing strategy

Physicochemical properties are included in the REACH Regulation (EC 1907/2006) and European guidance document as determinants for risk assessment and testing requirements. According to REACH Regulation (EC 1907/2006) and the European guidance document R7B, page 52, the long-term aquatic toxicity study on fish shall be considered if the substance is poorly water soluble. Furthermore, long-term toxicity testing shall be considered [...] for substances in quantities > 10 t with log Kow > 3 (R7B, page 53) and the need for long-term testing is more compelling for organic substances with a potential to bioaccumulate (log Kow > 3) (TNG, page 180). Based on the results of this data set a fish toxicity test is generally not necessary and can often be avoided for substances with a high log Kow or if the substance is poorly water soluble. Indeed, the chronic fish toxicity test is required for <20% of the substances with a log Kow > 3 and for <10% of the substances with a poor water solubility. Furthermore, the ACR of poor water soluble substances or substances exhibiting a log Kow > 4.5 was reduced compared to the result from the complete data set and does not exceed 100 in any case. This

suggests that an substantially increased toxicity is not expected in chronic tests compared the result from acute testing.

50 % of the poor water soluble substances exhibited a toxic effect in chronic testing although no toxicity was determined in acute testing up to the water solubility level. Therefore, chronic testing is required for low water soluble substances [...] that show no acute toxicity up to the solubility limit and if the PEC is $> 1/100$ th of the water solubility as stated in the guidance (ECHA, 2008). Based on this results and in line with the guidance documents the chronic Daphnia test is initially the study of choice and required to determine a No observed effect concentration. Even in the case of poor water soluble substances the log Kow does not generally represent a determinant that indicates the requirement of fish toxicity testing since more than 80 % of the evaluated poor water soluble substance exhibit a log Kow >3 .

In summary, acute sensitivity classification can be applied independent of the physicochemical properties water solubility and the octanol-water partitioning. Poor water solubility or a high Kow do not appear as predictors for species sensitivity upon long term exposure of Daphnia or fish. Both properties do not in general indicate the requirement of chronic toxicity testing or even the requirement of a chronic fish test. Furthermore, the physicochemical properties octanol-water partitioning and water solubility have a low predictive value regarding increased acute to chronic ratios. Therefore, it is proposed to adapt the guidance documents in this point as both physicochemical properties are not confirmed to be predictive for chronic testing requirements.

6.6 Structural alerts and QSAR modeling

Structural alerts and QSAR modeling will represent a valuable tool for risk estimation and to support testing strategies due to two reasons. First, the classification system was derived to provide a scientifically justified and reasonable approach for chronic effects estimation and further testing strategies. In general, exceptions from a methodology cannot be excluded and were already determined within this dataset for substances that exhibited no or a low acute toxicity to fish in acute testing, but fish toxicity in chronic testing. Therefore, shortcomings of the methodology are assumed for substances that exert a mode of action in chronic testing which may not be derived from short term testing. Physicochemical properties like water solubility and the octanol-water partitioning coefficient have been discussed in the literature and guidance documents to pose an additional hazard upon long term exposure. Both could not be confirmed as determinants by this study indicating the requirement of chronic fish toxicity testing. Furthermore, physicochemical properties do not represent determinants to predict exceptions from the methodology since the four exception in Cat 2 showed a good water solubility and a log Kow <3 . The analysis suggested that the most critical substances regarding fish toxicity are related to distinct structural properties like para-substituted phenols, thiocarbamates or musk compounds, for example. Therefore, it is proposed that exceptions can mostly be identified by structural alert or structural relationship analysis. Second, the data analysis shows that substances in Cat 2 that are more sensitive to fish in acute testing are in 72 % of the cases also more toxic to fish in chronic testing. In about 28 % of the cases chronic fish toxicity was considered to be not properly covered by the Daphnia test and the extrapolation approach. Hence, it is suggested that a chronic fish toxicity test should be considered and may be required for a protective approach unless substantial chronic fish toxicity could not be excluded. Nevertheless, the statistical analysis shows that in the majority of cases (72%) the chronic fish toxicity test is indeed not required and a general indication to perform a chronic fish toxicity test for substances in Cat 2 would result in an undesirable high number of animal

tests that are retrospectively not required. QSAR modeling, read across and structural alert prediction may provide an approach to support a testing strategy at this step.

General applied QSAR models like OECD QSAR Toolbox, are not validated or applicable for chronic data. Moreover, the guidance R10b Table 10-16 gives examples for structural alerts associated with enhanced toxicity in fish (and rat) and for Daphnia (ECHA, 2008). It is important to note that the structural alerts stated in the guidance document were identified as outliers from baseline toxicity QSAR of acute testing. In several cases these results are based on non-guideline studies. The applicability of the table to indicated structural alerts for fish sensitivity in acute testing needs to be evaluated, and the applicability of these structures for chronic predictions is questionable. The results of this study suppose that some possible structural alerts for chronic fish toxicity like para-substituted phenols are not included in this list whereas other structures included in the list do not appear as alerts for chronic fish testing within this study. Therefore, an uncertainty on the applicability of both current methods for chronic data (QSAR and structural alert) is envisaged and further research is required to investigate whether these structural alerts and QSAR models are suitable for chronic data and to predict species sensitivity. The predictive value for the here identified structural alerts refer mostly to less than 30% compare to the aim which is the identification of exceptions in Cat.3 and Cat.4 that represent 4% of the respective data set or 2% of the complete data set. Therefore, QSAR and structural alert analysis should be used very carefully to support decision making.

6.7 Data quality of fish tests

The results on data quality of chronic fish test suggest that the type of chronic test effects the average sensitivity distribution and has an effect on the ACR. The statistical finding supposes that fish toxicity might be underestimated by non-FELS studies compared to FELS studies. However, the result on non-FELS studies is based on a limited dataset (n= 22) and interpretation should be handled with care and on a case by case evaluation since different tests types were not compared for the same substance. Therefore, this evaluation is not applicable to confirmed whether the FELS test is more conservative than other test. Furthermore, it is important to note that the acute to chronic extrapolation approach is considered to be protective if only FELS tests are considered for data evaluation.

The median ACR for the different evaluated ACR in Table 5 ranged between 10.6 to 13.0 and the 90-% values between 63.5 and 111.1 (except of ACR5 that included only non-FELS studies and showed decreased values). Interestingly, data variance and increased ACR >100 are preferentially observed if acute and chronic data is based on different species (ACR3, 90 %-ile value of 111.1) whereas reduced 90%-ile values were observed if only the same species is compared (ACR2, 6, 8). This statistical finding supposed that the tested fish species in acute and chronic testing affects the result.

6.8 Outlook

- To re-evaluate the current results the data set should be complemented with additional data that is expected to be submitted to the ECHA database within the time schedule of the REACh regulation. Additional data may clarify how exceptions of the method can be identified and at which conditions a fish toxicity test may be requested for substances in Cat 2. Therefore, re-evaluation is suggested with the REACh deadline in 2018.
- The predictability of current QSAR model should be checked for the current data set in terms of effect levels, species sensitivity estimation and identification of structural alerts. In this line, the dataset may be used to evaluate whether chronic QSAR modeling is possible since no validated chronic QSAR models are available at the moment.
- Analysis of the mode of action (MoA) of the chemicals may contribute to a refinement of conclusions for different subgroups of chemicals.
- The dataset does not compile algae data since the ACR is expected to be lower for algae (Ahlers et al., 2006), and because it is expected that a chronic algae test is usually provided if chronic testing is required. However, to evaluate testing requirements the algae test should be considered assuming that in some cases algae represent the most sensitive species. Subsequently, the requirement of fish toxicity test may be further reduced by including algae data.

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