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Review and Enhancement of New Risk Assessment Concepts under REACH

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Review and Enhancement of New Risk Assessment Concepts under REACH

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Summary

REACH foresees the possibility to waive testing provided that the available information from related testing and/or non-testing sources is sufficient to draw a reliable conclusion on the endpoint of concern. Concepts how to evaluate this information are described as Non-Testing (NT) approach in the REACH guidance document R.6 and as Weight-of-Evidence (WoE) approach in R.7b. Since the REACH guidance documents were developed and published before the first registration phase began, experience on practicability and usefulness was still lacking. In order to verify and improve the NT and WoE concepts, the German Federal Environment Agency (Umweltbundesamt; UBA) initiated the present research project.

In this project, the guidance documents should be verified using the example of potentially endocrine disrupting (ED) chemicals. Three substances were selected mainly based on *in silico* (2,4,6-tribromophenol), *in vitro* (benzanthrone) and *in vivo* data (benzophenone-2). In order to evaluate the potential for endocrine disruption, several additional *in vitro* tests and one *in vivo* test were performed for two of the selected substances. For all three selected substances, an integrated assessment of potential endocrine activity in aquatic vertebrates was performed. Results of this assessment are presented in a non-public annex to this report.

Besides the endpoint endocrine disruption, the standard endpoints on short-term toxicity to fish, daphnids and algae were also considered within the project in order to gain experience with the NT and WoE approach.

Data on structural alerts, mode of action (MoA), grouping, read-across and QSARs were generated predominantly by applying the OECD (Q)SAR Application Toolbox and the ChemProp software developed by the Helmholtz Centre for Environmental Research (UFZ). These data were used for supporting the evaluation of the acute endpoints following the WoE approach for the three selected substances.

The endpoint ‘acute toxicity to fish’ of benzanthrone was used as an example for this evaluation. For this endpoint, results of a standard test (OECD test guideline [TG] 203) were available Moreover it is known from non-standard studies that aquatic toxicity of benzanthrone is enhanced by UV radiation (phototoxicity). The predictions using the non-testing methods were partly in good agreement with results of the standard test. However, since none of the above-mentioned models considers phototoxicity, the predicted values were clearly higher than the photo-induced LC_{50}. In addition, benzanthrone is out of the domain of a number of *in silico* models. The consequences and the resulting limitations are discussed.

Taking into account that for substances with a tonnage band of more than 100 t/a, which was assumed for benzanthrone, long-term testing has to be considered, the application of the non-testing methods would not have resulted in the avoidance of an *in vivo* test, unless it could clearly be shown that in acute tests daphnids are more sensitive than fish by more than one order of magnitude. This could not be assessed, partly due to uncertainties regarding the relevance of the phototoxicity of benzanthrone under a regulatory point of view.

Based on the experience with the guidance documents gained during application and review, the guidance documents were commented. Suggestions for improvements were developed for the NT approach in R.6 and for the WoE approach in R.7b. Although both sections describe the important steps needed for the evaluation, a restructuring is recommended at some points in order to lead the applicant in a more helpful way through the necessary steps.

Regarding the NT approach, which is an essential part of the WoE concept, a proposal to modify the eight-step workflow (steps 0 – 7) of R.6 has been developed. The revised scheme contains mode-of-action and effect-level classification as second step followed by an initial assessment, and applies chemical categories before employing read-across and QSAR models. For all steps, more detailed guidance has been developed, covering considerations for alternative routes, the availability of pertinent non-testing tools and instruments for assessing the confidence in predictions – the applicability domain and its major components as well as consensus modelling strategies. The approach is illustrated through its detailed application with the above-mentioned concrete example.

The WoE concept should be divided into three evaluation phases: (1) collection and preliminary evaluation of available information (Phase I: Minimum information level), (2) an extended data search and evaluation including WoE (Phase II: Extended information level) and – optionally – (3) developing of test proposals considering integrated testing strategies (ITS) (Phase III: Testing proposal level). In the current version of the WoE approach, the single steps for data collection, i.e. compiling available substance information (e.g. physico-chemical properties, results from *in silico, in vitro* and *in vivo* methods) are arranged in a successive way. It is suggested to rearrange this approach so that it becomes obvious that the information derived from the different sources can be collected independently from each other. Furthermore, some additional information and updates on the state of the art regarding useful methods is provided. For instance, the fish threshold approach was mentioned as the only example for an integrated testing strategy (ITS), which helps reducing the number of fish used in aquatic toxicity testing. Meanwhile, some more ITS with respect to aquatic toxicity testing have been developed and published. The recent OECD proposals have been included in the ITS section of the revised WoE approach.

With regard to Appendix R7.8-5, some restructuring is recommended so that an overview of the whole assessment is given before the single evaluation steps and, then, tests are presented. For some of the steps, more guidance is required to more effectively instruct the user of the guidance document. Further guidance should, for example, be provided on how to evaluate information derived from mammalian studies with regard to endocrine activity in aquatic organisms. Moreover, guidance on evaluation of *in silico* and, especially, *in vitro* screening data is rather limited, although such data are likely to represent the majority of the available data on possible endocrine disrupting potential. Further issues that deserve some attention are possible metabolites with potential endocrine activity, and endocrine effects, which are observed at substance concentrations that are in the range of or only slightly below concentrations causing general toxic effects.

1 Introduction

In order to support the implementation of the new European chemicals policy REACH (EU Regulation EC 1907/2006), several guidance documents for industry and authorities were prepared and published by the European Chemicals Agency (ECHA) in May 2008.

For a successful registration of a substance under REACH, the applicant has to provide reliable information on substance properties, e.g. physico-chemical properties, environmental behaviour, ecotoxicology and toxicology. The amount of information, i.e. the number of endpoints to be addressed, depends mainly on production volumes and is described in the Annexes VII to X of the regulation. Generally, these standard requirements are to be fulfilled by laboratory tests for effects on the specific endpoints. However, it is also intended to avoid unnecessary testing, especially of vertebrates. Therefore, REACH foresees the possibility to waive such testing provided that the available information from related testing and/or non-testing sources is sufficient to draw a reliable conclusion on the specific endpoint.

With respect to aquatic ecotoxicology, a *Weight-of-Evidence* (WoE) concept is presented in the guidance document R.7b *Endpoint specific guidance* (ECHA, 2008a). Amongst others the WoE concept allows to consider information on analogous substances as well as QSAR results and other non-testing information. The detailed assessment of QSAR models, structural alerts and chemical categories is laid down as a *Non-Testing* (NT) approach in the guidance document R.6 *QSARs and grouping of chemicals* (ECHA, 2008b).

Following article 57f of the REACH regulation, endocrine disrupting compounds (EDCs) may be included in Annex XIV. This annex lists *Substances of Very High Concern* (SVHC), i.e. dangerous substances which are not to be placed on the market unless their use is explicitly authorised. The evaluation of potentially endocrine disrupting chemicals and the handling of related information are laid down in Annex R.7.8-5 of guidance document R.7b, including a scheme for a stepwise approach for an integrated assessment.

1.1 Aim of the project

Since the guidance documents were developed and published before the first registration phase began (December 2008), experience on practicability and usefulness was still lacking. As it is important to keep the guidance documents on the best level of knowledge and practicability, the German Federal Environment Agency (Umweltbundesamt; UBA) initiated a research project with the objective to verify and improve the guidance documents R.6 and R.7b, especially of the NT and WoE concepts.

The guidance documents should be verified using the example of potentially endocrine disrupting chemicals. Therefore, candidate substances were screened and three representative substances were selected on the basis of defined criteria (for details see next section).

In order to evaluate the potential for endocrine disruption (ED), several additional *in vitro* tests and one *in vivo* test were performed for two of the selected substances.

Besides the endpoint endocrine disruption, the standard endpoints on short-term toxicity to fish, daphnids and algae were also considered within the project in order to gain experience with the NT and WoE approach.

1.2 Structure of the project

The responsibilities within the project were shared between the two cooperating institutes *ECT Oekotoxikologie GmbH* (*ECT*) and *Helmholtz-Centre for Environmental Research, Department of Ecological Chemistry* (*UFZ*).

While the investigations of the *UFZ* concerned those parts of the guidance documents which deal with *in silico* methods, i.e. addressed in particular the Non-Testing approach in R.6, *ECT* dealt with the above-mentioned parts of R.7b, especially the WoE approach. *ECT* also evaluated *in vitro* and *in vivo* information with respect to endocrine disruption and acute aquatic toxicity.

The project consisted of five different phases:

- Phase 1: Identification and selection of candidate substances
- Phase 2: Extended data search on selected substances
- Phase 3: Application of the guidance documents on the selected endpoints
- Phase 4: *In vitro* and *in vivo* experiments on endocrine disrupting potential
- Phase 5: Elaboration of suggestions for improvement of the guidance documents

The project phases are described in more detail in the following section.

2 Description of the project phases

2.1 Phase 1: Identification and selection of candidate substances

In the first project phase, the main objective was to identify candidate substances, which were suspected to have endocrine disrupting effects. The main focus of the project was on substances with suspected estrogen or androgen receptor agonistic activity in fish. The candidate substances should fit into the following three categories:

Category 1: Effect on estrogen / androgen axis suspected based on *in silico* data,

Category 2: Effect on estrogen / androgen axis suspected based on *in vitro* data,

Category 3: Effect on estrogen / androgen axis suspected based on *in vivo* data.

As a starting point and basis for the selection of the candidates, two databases with potentially endocrine disrupting chemicals were used:

- The EU database of potential endocrine disruptors (last update 2007; reports and a download file of the database are available online at http://ec.europa.eu/ environment/ endocrine/strategy/substances_en.htm#priority_list)
- The UBA database of potential endocrine disruptors, which is based on *in vitro* results. This database is not publicly available; it was provided by UBA.

2.1.1 Category 1

The two lists were combined by *ECT* into one list. The EU database comprised 593 substance data sets and the UBA database 852 data sets. After combining both lists, duplicates and substances indexed as “excluded” in the EU list were removed. The resulting combined list comprised 1109 substance datasets.

For 955 compounds from this list of category 1 candidate compounds, information on the following endpoints was compiled by *UFZ*:

Physico-chemical properties

- log K_{ow}, predicted and experimental (as far as available) employing EPISuite v4.0 (US EPA, 2008);
- Log S_w (water solubility), predicted using the *UFZ* software ChemProp (Kühne et al., 2006; more details can be found at http://www.ufz.de/index.php?en=6738).

Aquatic toxicity

- Experimental data for acute toxicity towards daphnids (300 compounds), algae (269 compounds) and fish (692 compounds), using in-house databases of the *UFZ*;
- Predicted excess toxicity towards daphnids (von der Ohe et al., 2005), algae and fish (structural alert models, developed from experimental data by *UFZ*) including associated chemical domain information;

- ECOSAR model predictions for acute toxicity towards daphnids, algae and fish

Endocrine disruption

- Predicted estrogen receptor agonistic activity based on a yeast-based reporter gene assay, employing the Netzeva/Saliner model (Saliner et al., 2006) as implemented in ChemProp;
- Predicted androgen receptor antagonistic activity according to a reporter gene assay with Chinese hamster ovary cells, employing the Vinggaard model (Vinggaard et al., 2008) as implemented in ChemProp.

For models with published training sets information on the chemical domain can be generated. For the Vinggaard model, the training set definition was ambiguous from the paper; here, *UFZ* used data reasonable guess set possible to implement the chemical domain of the model.

The resulting data-enriched list of possible category 1 substances was further processed by ECT in order to select suitable candidate substances. Substances were removed from the list, which

1) had no alert for endocrine disruption;
2) had an alert for endocrine disruption, but were outside the chemical domain of the two QSAR models,
3) are not predominantly used as industrial chemicals (e.g. pesticides, hormones);
4) are not pre-registered under REACH,
5) had no clear identification (i.e. no CAS numbers),
6) were considered as candidates for category 2 or 3.

Twenty-one potential candidates for K1 remained of the list.

2.1.2 Category 2

The UBA list (*in vitro* results) was used as a starting point. From this list substances were removed, which

1) had no estrogen / androgen receptor agonistic activity in *in vitro* tests,
2) are not predominantly used as industrial chemicals (e.g. pesticides, hormones),
3) are not pre-registered under REACH,
4) had no clear identification (i.e. no CAS numbers),
5) were considered as candidates category 3.

Forty-two substances remained for the K2 category, with 39 substances suspected to have estrogenic receptor agonistic activity and 3 suspected to have androgen receptor agonistic activity.

2.1.3 Category 3

The EU database was used by *ECT* as a starting point together with results from the UBA project "Gewässerrelevanz endokriner Stoffe und Arzneimittel" (Moltmann et al., 2007). Substances were removed from the list, which

1) had no estrogenic / androgenic effects on fish,
2) are not predominantly used as industrial chemicals (e.g. pesticides, hormones etc.),
3) are not pre-registered under REACH,
4) had no clear identification (i.e. no CAS numbers).

Thirty-three potential category 3 candidates remained.

2.1.4 Candidate proposals

From the three candidate lists, substances were successively selected by *ECT* for further data search. This search comprised information on uses, production volumes, classification & labelling, physico-chemical properties, environmental fate and aquatic ecotoxicity. In addition, it was checked whether the substance is commercially available and whether a method for chemical analysis is available or could be established at the DVGW-Technologiezentrum Wasser (TZW) with reasonable effort.

For the data search, tools like EPISuite (v4.0) and an Excel sheet for logD calculation from log K_{ow} and pK_a were used.

The following websites were searched for information on the substances:

- ECB: http://ecb.jrc.ec.europa.eu/esis/
- OECD: http://cs3-hq.oecd.org/scripts/hpv/
- ECOTOX: http://cfpub.epa.gov/ecotox/
- TOXLINE: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?TOXLINE
- HSDB: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB
- NITE: http://www.safe.nite.go.jp/english/kizon/KIZON_start_hazkizon.html
- Scorecard: http://www.scorecard.org/index.tcl
- SPARC: http://ibmlc2.chem.uga.edu/sparc/
- Chemexper: http://www.chemexper.com/
- Google: http://www.google.de/
- WIKIPEDIA (en): http://en.wikipedia.org/

Literature on *in vitro* and *in vivo* test results on endocrine disrupting effects of the candidate substances was reviewed. Table 1 gives an overview of the 5 to 6 pre-selected candidate compounds for each category and indicates a priority setting for each category.

Table 1. Overview of production volume, use, most relevant physico-chemical and fate properties, ecotoxicity and potential for endocrine activity for the pre-selected candidate substances for categories K1, K2 and K3.

No.	Substance, CAS	Substance class, production volume, use	Water solubility, volatility [1]	Biodegra-dability	Availability of ecotoxicity data, toxicity	Potential for endocrine activity [2]	Candi date rating	Remarks
Category 1								
1	2,4,6-Tribromophenol CAS 118-79-6	Brominated phenol HPV; flame retardant, biocide, intermediate	Moderate water solubility	Bio-degradable	Acute fish, *Daphnia*, algae available: very toxic Chronic *Daphnia* available (NOEC: 0.1 mg/L)	*In silico*: estrogenic *In vitro*: anti-estrogenic, anti-androgenic, effects on aromatase and transthyretin	++	–
2	4-Nitrophenol CAS 100-02-7	Phenol derivative HPV; various uses in organic synthesis, precursor, intermediate	Good water solubility	Not readily bio-degradable	Acute fish, *Daphnia*, algae available: toxic. Chronic fish and *Daphnia* available (NOECs ca. 1 mg/L)	*In silico*: estrogenic	+	Ecological relevance questionable (precursor, intermediate)
3	1-Naphtol, CAS 90-15-3	Bicyclic hydrocarbon HPV; precursor of dyes, perfumes and agrochemicals	Good water solubility	Readily bio-degradable	Acute fish, *Daphnia*, algae available: very toxic	*In silico*: estrogenic *In vitro*: not estrogenic	+	Ecological relevance questionable (precursor, intermediate)
4	Benzoic acid, CAS 65-85-0	Monocyclic benzene derivative HPV; important precursor, food preservative; used in pharmaceuticals	Good water solubility	Readily bio-degradable	Acute fish, *Daphnia*, algae available: harmful	*In silico*: estrogenic, not anti-androgenic *In vitro*: not anti-androgenic *In vivo*: contradictory results regarding uterotrophic effects in rodents	+	–
5	Terephthalic acid CAS 100-21-0	Monocyclic benzene derivative HPV; starting compound for PET; feed preservative	Low water solubility (15 mg/L at 10°C)	Readily bio-degradable	Acute fish, *Daphnia*, and algae available: not harmful	*In silico*: estrogenic *In vitro*: not anti-androgenic *In vivo*: no endocrine effect in rat multi-generation study	±	–
Category 2								
1	Benzanthrone, CAS 82-05-3	PAH LPV; used e.g. as dyestuff intermediate, photosensitizer	Low water solubility	Not readily biodeg.	Acute fish, *Daphnia* available: very toxic. No data on algae	*In silico*: anti-androgenic *In vitro*: estrogenic, androgenic	++	–

No.	Substance, CAS	Substance class, production volume, use	Water solubility, volatility [1]	Biodegradability	Availability of ecotoxicity data, toxicity	Potential for endocrine activity [2]	Candidate rating	Remarks
2	Biphenyl CAS 92-52-4	Biphenyl HPV; various uses (incl. pesticide), preservative, precursor, intermediate	Low water solubility (7 mg/L at 25°C). High volatility	Readily biodegradable	Acute fish, *Daphnia* available: toxic/very toxic No data on algae Chronic fish and *Daphnia* available (NOECs ca. 0.2 mg/L)	*In silico*: not estrogenic *In vitro*: estrogenic	+	–
3	Bisphenol F CAS 2467-02-9	Bisphenol LPV; start product for epoxy resins; used in liners, lacquers, plastics, coatings	Good water solubility	Not readily biodegradable	No data	*In silico*: estrogenic (but domain: critical) *In vitro*: estrogenic; androgen receptor binding, anti-androgenic *In vivo*: controversial results regarding estrogenic effects in rodents	+	–
4	Methyl parabene CAS 99-76-3	Paraben LPV; used as preservative in the cosmetic and pharmaceutical industry	Good water solubility	Possibly readily biodegradable	Acute *Daphnia* available: harmful No data on fish and algae	*In silico*: estrogenic *In vitro*: estrogenic, not anti-androgenic *In vivo*: controversial results regarding estrogenic effects in rodents	+	–
5	2-tert-Butyl-anthraquinone CAS 84-47-9	PAH Neither LPV nor HPV	Practically insoluble in water	Not readily biodegradable	Acute fish available: toxic No data on *Daphnia*, algae	*In silico*: not estrogenic *In vitro*: androgenic	±	Ecological and EU market relevance questionable
6	Dibenzo[a,h]-anthracene CAS 53-70-3	PAH Neither LPV nor HPV	Practically insoluble in water	Not readily biodegradable	Daphnia available: very toxic No data on fish, algae.	*In vitro*: androgenic, not anti-androgenic	±	Probably carcinogenic; ecological and EU market relevance questionable
Category 3								
1	Benzophenone-2 CAS 131-55-5	Benzophenone LPV; used as UV-filter	Moderate water solubility	Not readily biodegradable	No data	*In silico*: estrogenic *In vitro*: estrogenic, not anti-estrogenic	++	–

No.	Substance, CAS	Substance class, production volume, use	Water solubility, volatility [1]	Biodegra-dability	Availability of ecotoxicity data, toxicity	Potential for endocrine activity [2]	Candi date rating	Remarks
						In vivo: vitellogenin induction		
2	Benzophenone-1 CAS 131-56-6	Benzophenone LPV; various uses in organic synthesis, UV-filter	Practically insoluble in water	Not readily bio-degradable	No data	*In silico*: estrogenic *In vitro*: estrogenic, not anti-estrogenic *In vivo*: vitellogenin induction	+	–
3	3-Benzylidene camphor CAS 15087-24-8	Vinyl/allyl ketone LPV, UV-filter	Practically insoluble in water. Moderate volatility.	Not readily bio-degradable	No data	*In silico*: not anti-androgenic *In vitro*: estrogenic, anti-androgenic *In vivo*: vitellogenin induction, effects on reproduction	+	–
4	4-tert-Pentylphenol CAS 80-46-6	Phenol derivative LPV, germicide, fumigant, in matrix of oil resins	Good water solubility. Moderate volatility.	Not readily bio-degradable	Acute fish available: toxic No data on *Daphnia*, algae	*In silico*: estrogenic *In vitro*: estrogenic, controversion results regarding anti-androgenic effects *In vivo*: vitellogenin induction, inhibited spermatogenesis and other effects on gonads	±	–
5	n-Butylparaben CAS 94-26-8	Paraben HPV; used e.g. as preservative	Moderate water solubility	Readily bio-degradable	No data	*In silico*: estrogenic *In vitro*: estrogenic *In vivo*: vitellogenin induction	±	Main use as biocide
6	Triethylene phosphoro-triamide (TEPA) CAS 545-55-1	Organophosphate Neither LPV nor HPV; used e.g. as chemosterilant, pesticide, alkylating agent	Good water solubility	Not readily bio-degradable	Acute fish available: not harmful No data on *Daphnia*, algae Chronic fish available (NOEC: 1 mg/L)	*In vivo*: testicular atrophy and reduced male fertility in fish	±	Not clear if specific endo-crine effect Ecological and EU market relevance questionable

[1] Only indicated in case of high volatility.

[2] *In silico* data based on the model of Saliner et al. (2006) for estrogenic activity, and on the model of Vinggaard et al. (2008) for anti-androgenic activity, both implemented in the ChemProp software; *in vitro* data based on (non-public) UBA database of potential endocrine disrupters as provided by UBA; *in vivo* data based on EU database of potential endocrine disrupters.

After discussion with the UBA, the following substances were selected:

- **Category 1: 2,4,6-Tribromophenol** (*2,4,6-TBP*, CAS 118-79-6)
- **Category 2: Benzanthrone** (*BA*, CAS 82-05-3)
- **Category 3: Benzophenone-2** (*BP-2*, CAS 131-55-5)

2.2 Phase 2: Extended data search on selected substances

Phase 2 of the project comprised an extended literature and data search on the selected substances including a preliminary identification of possible analogue compounds. For each of the three selected substances, UFZ selected 13-15 analogue substances by applying an atom centred fragment (ACF) method as implemented in the ChemProp database. For each analogue, data on acute toxicity were extracted from the database. Moreover, structural alerts on potential endocrine disrupting effects were identified for each analogue using the Netzeva/Saliner model and the Vinggaard model.

ECT conducted an extended literature search on the three candidate substances including mammalian toxicity data. Relevant literature on ecotoxicology and endocrine disrupting potential (*in vitro* / *in vivo* results) for each of the three selected substances was evaluated.

Furthermore, a data search on endocrine disrupting potential was performed for each of the analogue substances in the UBA database (*in vitro* results) as well as in the EU database (*in vitro* and *in vivo* results). The research results including the analogue data provided by UFZ were documented in the substance data sheets (see Annexes 2 and 3A of this report). This extended data search formed the basis for the next project phase.

2.3 Phase 3: Application of the guidance documents on selected endpoints

In this phase the guidance documents R.6 (Chapter 6.7.1: NT approach) and R.7b Chapters R.7.8.1-7.8.5: WoE approach and Appendix R.7.8-5: Evaluation of endocrine effects) were reviewed, applied and commented. Shortcomings of the guidance documents which were identified during their application were documented. In total, more than 120 points (of editorial, technical, structural or specific nature) were commented. The comments on the guidance documents can be found in Annex 1 to this report.

The main target in phase 3 was the application of the Non-Target (NT) approach outlined in the REACH Guidance Document (GD) R.6 and of the Weight-of-Evidence (WoE) approach outlined in GD R.7b.

From a total of 12 endpoints (acute toxicity to *Daphnia*, algae and fish and endocrine activity for each of the 3 substances), 4 endpoints were considered to be adequately covered by available experimental results. The remaining 8 endpoints were considered in the phase 3 evaluations. In addition, the endpoint ‘fish acute toxicity of benzanthrone’ was evaluated with the NT and WoE approach (Table 2). This was considered useful due to the known special mode of action (phototoxicity) and the relevance of fish as protected animals.

Table 2. Availability of experimental data for the evaluated endpoints.

Endpoint	2,4,6-Tribromophenol	Benzanthrone	Benzophenone-2
Fish acute toxicity	covered	covered / evaluated *	evaluated
Daphnia acute toxicity	covered	evaluated	evaluated
Algae growth inhibition	covered	evaluated	evaluated
Endocrine disrupting potential	evaluated	evaluated	evaluated

* Covered by experimental results, but also evaluated (for details see section 4.1).

The evaluation of the 9 endpoints was performed following the NT and WoE approach as outlined in the guidance documents R.6 and R.7b.

The NT concept was followed by UFZ mainly by using the ChemProp software and the OECD Toolbox. ECT conducted the WoE approach based on the provided *in silico* data from UFZ and considering the collected information on phys.-chem. properties, *in vitro* and *in vivo* results. The overall evaluation of the results including *read-across* was evaluated in co-operation between the project partners. Details on the proceedings and results of the evaluation can be found in section 4.

2.4 Phase 4: *In vitro* and *in vivo* experiments on endocrine disrupting potential

Experimental studies were performed for the category 1 substance, 2,4,6-tribromophenol, and the category 2 substance, benzanthrone. In order to obtain further evidence on possible estrogenic and anti-estrogenic activity, E-screens with the MCF-7 breast cancer cell line (Soto et al., 1991, 1995; Körner et al., 1999, 2001) were performed for both compounds. For benzanthrone, the yeast androgen screen (YAS; Routledge & Sumpter, 1996, Sohoni & Sumpter, 1998) was used to detect androgen receptor agonistic activity and yeast anti-androgen screen (YAAS; Routledge & Sumpter, 1996) was employed to detect androgen receptor antagonistic activity.

A short-term fish screening assay for endocrine effects according to OECD test guideline 230 (OECD, 2009a) was performed with 2,4,6-tribromophenol.

2.5 Phase 5: Elaboration of suggestions for improvement of the guidance documents

This last project phase was predominantly dedicated to the development of suggestions for improvement of the NT and WoE chapter of the guidance document R.6 and R.7b, respectively. The corresponding proposals can be found in the following section.

3 Proposed improvements of the REACH Guidance Documents

In the following sections proposals for improvements of the NT (by *UFZ*) and WoE (by *ECT*) approach are presented. The proposals were developed on the basis of the practical experiences with the REACH guidance documents. New and old parts of the guidance documents are not marked since due to many structural and content changes, this would have been confusing.

3.1 Proposals for R.6 (Non-testing approach, NT)

R.6.1.7 Stepwise approach for the use of non-testing data

R.6.1.7.1 Meeting regulatory requirements with computational tools

In Section R.6.1.8, the most commonly used QSAR tools are reviewed. Generally, some but not all of the existing tools will be useful to address the requirements of REACH. Moreover, some useful tools may not be generally available due to their proprietary nature, some are currently under development, and some may need to be developed in the near future.

Due to the limited availability of freely-accessible QSAR software, there is a need to develop a range of transparent tools to be eventually available to all stakeholders involved in the REACH process (especially industry, governmental authorities and the European Chemicals Agency). The essential functionalities needed for implementing REACH should ideally be available in the form of a Decision Support System (DSS) in which different needs (functionalities) are addressed by different (but mutually compatible) component tools. The different components of such a DSS should enable the user to generate non-testing information within the context of a structured workflow, and to obtain guidance on the applicability of the information for the regulatory goals of REACH.

This chapter presents possibilities how different commercially and publicly available tools, including those described in Section R.6.1.8, could be integrated into a DSS for the generation of non-testing data for REACH. The intent of this chapter is to illustrate how a diverse range of different tools can be used in the context of a single workflow. The development and evaluation of this workflow represents work in progress. At this time, prototype DSS available include the QSAR Application Toolbox and the ChemProp OSIRIS Edition. These DSS are intended to be broadly applicable in the international context. They will nevertheless take into account as far as possible the specific needs of national/regional legislations, including REACH.

R.6.1.7.2 Structured workflow for the generation and use of non-testing data

The workflow proposed for the generation and use of non-testing data comprises a sequence of operations exploiting the functionalities of a wide array of Information Technology (IT) tools and databases. The description of the workflow in this chapter tries to identify useful tools that could be applied in association with different steps of the process, but due the large number of available applications, only some of them can be mentioned.

The proposed stepwise approach is intended to guide the registrant in meeting the general requirements for using non-test methods laid down in REACH Annex XI (e.g. a QSAR prediction for a substance should fall within the applicability domain of the QSAR model, and appropriate documentation of the applied method should be provided).

The workflow is summarized in Figure 1.

The details of the various steps of the workflow are explained below in separate sections. As the user proceeds through the workflow, results can be stored in form of a database, which may be called *Working Matrix*. Besides standard software such as Excel, DSS *in silico* tools as mentioned above can be used for this purpose. Different rows store information for different compounds, and different columns refer to specific types of information (e.g. a physico-chemical property).

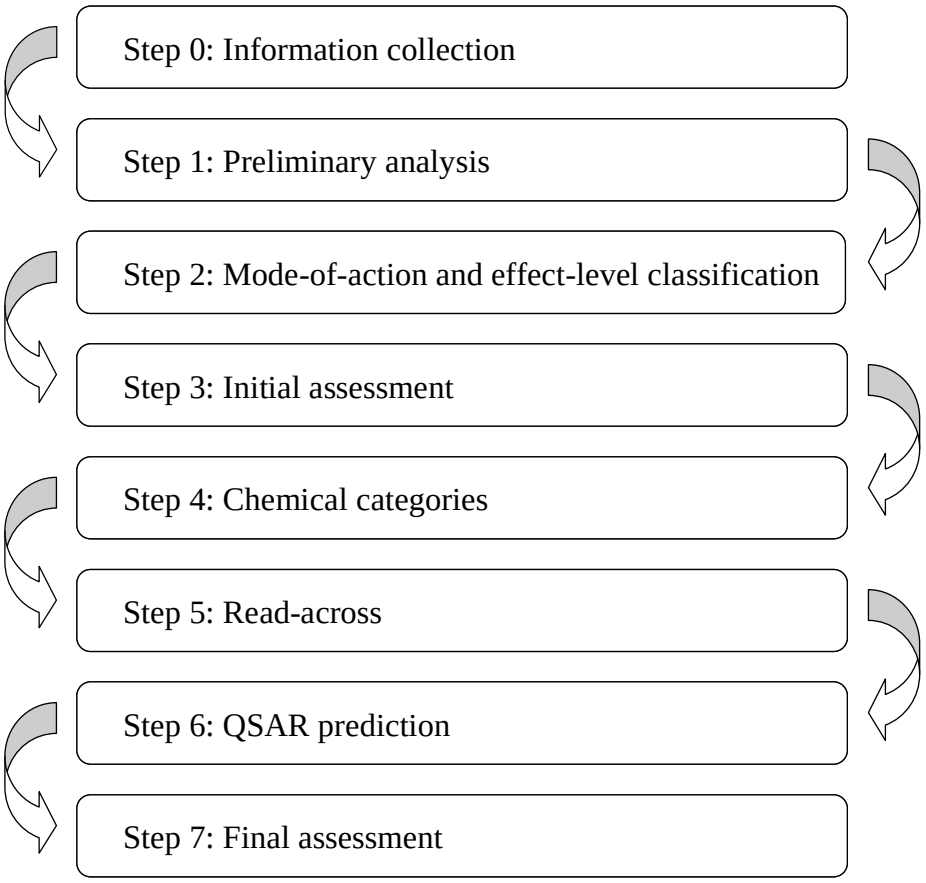


Figure 1. Flowchart for the use of non-testing approaches in the regulatory assessment of chemicals

It is emphasized that the workflow is intended to be flexible, so that it can be adapted to meet the specific and context-dependent needs of the user. For example, depending on the substance, endpoint of interest and regulatory purpose, it might be more efficient to omit certain steps or to perform them in a different order. Generally, it is recommended to consider all steps because this will increase the confidence in the overall assessment.

The guidance below is based on the assumption that each chemical is subject to potential transformation (either biotic or abiotic), independent of whether it actually transforms under a defined set of conditions. The term *parent compound* is introduced to distinguish between the main chemical of interest (the *parent*) and its potential transformation products.

In the starting step, information on experimental data is collected, existing data gaps are identified and the endpoint of interest is defined. Step 1 involves a preliminary analysis of the environmental reactivity, uptake and fate profile expected for both the substance of interest and its (chemical or metabolic) transformation products. Step 2 solicits further information on the likely biological activity of the compound using classification schemes concerning modes or mechanisms of action and effect levels (narcosis-level vs. excess toxicity). Step 3 provides a preliminary assessment of the expected uptake, toxicity and fate profile. In Step 4 compounds are classified, followed by a search for analogous compounds, whereas Step 5 uses read-across and Step 6 uses QSARs. Finally, an overall assessment is carried out in Step 7. Depending on the particular substance, endpoint of interest and regulatory purpose, certain steps may be omitted, or performed in a different order.

R.6.1.7.3 Step 0: Information collection

Assess information requirements under REACH

The workflow begins by considering the information requirements under REACH, which are largely tonnage-dependent and specified in Annexes VII-X.

Select a representative structure for the assessment

The composition of the substance (main chemical component, other components, impurities) should be clearly defined, and the appropriate specific compound is selected for the study. This operation is necessary because predictions from QSAR methods and category/read-across approaches are generated by feeding them with a single well-defined structure (e.g. the two-dimensional structural formula in the form of a SMILES code). The purity/impurity profile might be useful at a later stage to explain discrepancies between experimental and non-testing data, which also can help to find a suitable model.

In the case of multi-component substances (mixtures), it may be necessary to model two or more structures if a single representative structure is not considered sufficient. This also includes interaction effects of mixture components. If mixture components are known to interact independently, the independent action approach may be used. Otherwise it may be necessary to consider concentration addition approaches. The selection of relevant models will depend on the particular substances, endpoints of interest and regulatory issues. The complex issue of rosk assessments for mixtures cannot be addressed in the current project, but has been studied in detail in the EU Integrated Project NoMiracle (NoMiracle 2009). A review on mixture toxicity has been given by Altenburger et al. (2003)

Verify the chemical structure of the parent compound

If the parent compound is only known by CAS or EC number or by name, it is essential to obtain its chemical structure (e.g. in the form of the SMILES code) to be used in predictions. This can be achieved using a *Structure Generator* tool. If the structure is known, it is important to verify that the structural information agrees with the CAS number or with the name. If a compound can appear in different tautomers it is necessary to consider which ones are relevant (Thalheim et al., 2010). Some software tools that can be employed at this step are:

Non-commercial software

- The EPISUITE contains libraries to obtain a structural form (SMILES) from CAS.
- The QSAR Application Toolbox contains libraries to obtain a structural form (SMILES) from CAS. There is basic support for tautomers when retrieving data.
- ChemProp OSIRIS Edition contains a database to obtain a structural form (SMILES, InChI, XML, formats containing the full 3D structure) from CAS. It also allows inspecting possible tautomers. Support of searching for generic compounds and mixtures is currently under development. The software will be available through a free-of-charge license after the end of the OSIRIS project in October 2011. For regulatory agencies, a preliminary version can be provided on request.
- Ambit (Idea Consult Ltd) contains a database and can be used to obtain a structural form (SMILES) from CAS.

Non-commercial online tools

- eMolecules (eMolecules, Inc.), a large database which contains more than 7 Mio molecules that can be used to validate CAS number, chemical name and the corresponding possible structure.
- ChemID (National Library of Medicine), which can be used to check the CAS number, the chemical name, and to identify the corresponding possible structure.

Commercial software

- CAS SciFinder (commercial), which is a definitive source of CAS registry numbers matched with chemical name and structure information.

Retrieval results need to be examined carefully for possible errors. In particular, online resources are known to contain a considerable amount of incorrect structures.

<u>Collect available and reliable information for the parent compound</u>

Available chemical information (including physico-chemical properties and toxicity data) about the parent compound can be taken from literature or suitable databases. Important non-commercial tools to derive information, based on databases and suitable estimation methods, are:

- EPISUITE
- QSAR Application Toolbox

- ChemProp OSIRIS edition

A list of additional useful external databases is provided in Section R.6.1.8.

EPISUITE contains QSAR models mainly for physico-chemical properties as well as abiotic and biotic transformation rates, but also some toxicity endpoints (ECOSAR models). Further more a database with experimental physico-chemical data is included.

The QSAR Application Toolbox contains QSAR models, chemical categories as well as a database of QSAR predictions. In the 2.0 Edition of the QSAR Application Toolbox, EPISUITE models are included.

ChemProp OSIRIS Edition contains QSAR and read-across methods for physico-chemical properties with particular emphasis on partitioning, transformation rates as well as toxicological and ecotoxicological endpoints. Furthermore it contains database tools including advanced substructural searching.

Substance information can also be retrieved from **ESIS**, the European chemical Substances Information System, accessible from the European Commission's Joint Research Centre (JRC) website (ESIS also includes the QSAR Application Toolbox). Moreover, the following databases are planned to be implemented for queries through ESIS:

QSAR Model Database (QMDB): this database is planed to be an inventory of robust summaries of QSARs that can be searched, for example, by endpoint or by chemical. The search by chemical could provide information on whether the chemical in question is present among the training and test sets of some models. The QMDB will provide information on evaluated models documented in the form of QSAR Model Reporting Formats (QMRFs);

QSAR Prediction Database (QPDB): the models that are documented in the QMDB can be used to generate predictions for various chemicals. These predictions are planed to be stored in the QSAR Prediction Database, so that each prediction is associated with a robust summary of the model used to generate it. For individual predictions, the QMDB is planed to provide links to the appropriate QSAR Prediction Reporting Formats (QPRFs);

Chemical Categories Database: an inventory of existing categories is planned to be useful to apply category/read-across approaches. This database is planned to include all the information necessary to adequately document the use of a specific category for generating predictions.

Relevant physico-chemical and fate properties for evluation of aquatic toxicity

The most relevant physico-chemical and fate properties for evaluating the behaviour of a substance in water-sediment systems are:

- Water solubility
- Evaporation potential (Henry's Law Constant)
- Dissociation constants (pK_a)
- Partition coefficients/adsorption behaviour (log K_{ow}/K_{oc})

- Stability (Hydrolysis, reactivity, biodegradability)
- Bioaccumulation behaviour (BCF)

This information is needed in order to be able to assess the stability, bioavailability and potential for bioaccumulation of the substance in aquatic systems and organisms, respectively.

Arrange information and identify information gaps

All pieces of information collected in the previous phases can be stored in a Working Matrix (see above), typically in one of the software tools already mentioned. Then it should be possible to identify information gaps by comparing the REACH information requirements and the collated information. If necessary, the search for existing information is refined through taking into consideration specific information gaps.

At this point, the endpoints of interest, i.e. endpoints with information gaps, should be defined. An endpoint for which non-testing data is seeked for and which can be generated by means of QSAR methods and category/read-across approaches is then selected, and one or more of Steps 1-7 are followed to obtain the non-testing data along with guidance on how to interpret the data in the regulatory context. In addition, non-testing data not specifically referred to in the Information Requirements may still be useful for contributing to the overall regulatory assessment.

R.6.1.7.4 Step 1: Preliminary analysis of transformation potential, uptake and fate

The preliminary analysis of the environmental reactivity, uptake and fate of the substance of interest is based on existing information as well as inferences made by using physico-chemical data.

Collect information on the transformation potential of the parent compound

At this stage, information on the environmental reactivity of the parent compounds is collected or generated. Environmental reactivity means the ability of a substance to undergo transformation reactions under environmental conditions. A high environmental reactivity of a compound (i.e. a high degradation rate) implies that potential transformation products have also to be taken into account.

Compounds can be transformed abiotically or biotically (Schüürmann et al., 2007). The most important abiotic transformation reactions under environmental conditions are photolysis, hydrolysis and redox reactions. Biotic transformation can be microbially mediated (biodegradation) or occur in the species of interest (biotransformation). Generally, both detoxification and toxification may take place.

Information on abiotic and biotic reactions involving the parent compound can be retrieved from the peer-reviewed literature and from available prediction tools (Kühne et al., 2007; Schüürmann et al., 2007; US EPA, 2008) and databases, including the following resources:

Non-commercial

- EPISUITE with AOPWIN, HYDROWIN, BIOWIN, BioHCwin
- QSAR Application Toolbox with EPISUITE models
- ChemProp OSIRIS Edition
- KEGG

Commercial

- CAS SciFinder
- Catabol developed by LMC, University of Bourgas, Bulgaria
- MDL Reaction database
- METEOR, Lhasa
- META, MCASE
- TIMES developed by LMC, University of Bourgas, Bulgaria

Not many freeware software applications are available for analysing the metabolic fate of chemicals. EPIWIN from **EPISUITE** (this model is also included in the **QSAR Application Toolbox**) and **ChemProp OSIRIS Edition** estimate biotransformation half-lives in fish. Furthermore the **QSAR Application Toolbox** contains maps of estimated metabolic pathways for a large number of chemicals.

If a structure leads to the assumption that phototoxicity is relevant, the Mekenyan criteria (AM1 HOMO-LUMO gap of 7.1 +/−0.4 eV; Mekenyan et al., 1994) can be checked. Respective quantum chemical software is included in the **QSAR Application Toolbox** and also available separately (e.g. MOPAC, Gaussian).

The collated information, including additional information on metabolites and transformation products, can be used as basis for the following steps.

Collect Information on bioavailability and uptake

Partition coefficients

Several partition coefficients provide information about the bioavailability of a compound in aquatic bioassays, thus allowing evaluating the potential reduction of bioavailability through sorption or volatilization from aqueous solution. Bioavailability triggers the uptake of componds into organisms.

Sorption can be evaluated through the logarithmic octanol/water partition coefficient (log K_{ow}), which quantifies the thermodynamic partitioning of a substance between octanol and water. Octanol is used as model substance for lipids in the cell membrane. If no experimental value is available, it is currently recommended to estimate log K_{ow} via KOWWIN from

EPISUITE. The **OECD Application Toolbox** and **ChemProp OSIRIS Edition** also provide respective models.

A further issue concerning bioavailability is water solubility. If the nominal concentration of an experimental test result is above the water solubility of the test compound, the experimental setting used would require further investigation. In case of the additional presence of a solvent for solubilizing the test chemical, a respective control demonstrating the absence of the solvent effect on the experimental outcome would be required.. In case no solubilizer had been used, the nominal test result is less confident and should not be taken into account without appropriate correction according to the water solubility of the test chemical. Thus, the water solubility may be taken as the maximum test concentration.

Volatilization from aqueous solutions can be evaluated based on Henry's law constant (i.e., the air/water partition coefficient) and on the vapour pressure, both of which can be estimated by the **EPISUITE** or alternatively through the **ChemProp OSIRIS Edition**. Other important partition coefficients are the sorption coefficient K_{oc} (partitioning between soil/sediment organic matter and water) and the octanol/air (K_{oa}) partition coefficient. Respective models are also available in these software systems.

A potential pitfall in this step is the ionization of a compound. A compound can be already ionized in pure form, or may dissociate or become protonated upon dissolution in water. The unionized and ionized compound fractions depend on the pK_a of the compound (which in case of a base refers to its conjugated acid) and on the solution pH. Concerning the sorption coefficient in terms of log K_{oc}, first models for acids and bases have become available that take into account the compound pK_a and the Henderson-Hasselbalch equation (Franco et al., 2008, 2009) and are also implemented in ChemProp. However, their applicability appears to be still limited (e.g. for bases only for a fixed pH of 4.5).

For modelling the hydrophobicity of ionogenic compounds with consideration of the degree of dissociation or protonation, two standard approaches introduced so far are to either assume that only the neutral compound fraction is relevant, or to take into account both the unionized and ionized compound fraction (Fujita, 1966; Scherrer & Howard, 1977; Escher & Schwarzenbach, 1996; Schüürmann, 1998). In both cases, the resultant property is called distribution coefficient D_{ow} (rather than partition coefficient K_{ow}), acknowledging that more than one molecular species (acid and anion, or neutral and protonated form of a base) is subject to partitioning between aqueous and non-aqueous phases. When only the neutral compound fraction f_u is considered to be bioavailable, D_{ow} is obtained through respective multiplication of K_{ow}:

$$D_{ow} = f_u \cdot K_{ow}$$

In case both the unionized and ionized compound fractions, f_u and f_i, contribute to the activity of interest, D_{ow} as composite measure of the overall hydrophobicity is then given by

$$D_{ow} = f_u \cdot K_{ow} + f_i \cdot (K_i + K_{ip})$$

where K_i denotes the octanol/water partition coefficient of the ionized molecular species, K_{ip} the respective distribution between ion pairs in octanol and their (dissociated) components in water, and f_u and f_i are defined for acids and bases through

$$f_{\mathrm{u}}^{\mathrm{acid}} = f_{\mathrm{i}}^{\mathrm{base}} = \frac{1}{1+10^{\mathrm{pH}-\mathrm{p}K_{\mathrm{a}}}}$$

$$f_{\mathrm{i}}^{\mathrm{acid}} = f_{\mathrm{u}}^{\mathrm{base}} = \frac{1}{1+10^{\mathrm{p}K_{\mathrm{a}}-\mathrm{pH}}}$$

with pK_a for bases referring to their conjugated acids (Schüürmann, 1998). Depending on the target property of interest, the one or other approach is preferred, and there is no generally preferred way how to address dissociation or protonation in the QSAR context. In fact, QSARs for predicting partition coefficients typically refer to the neutral compound form, ignoring dissociation and protonation.

Recent literature informs about the performance of QSAR methods for predicting log K_{ow}, water solubility, Henry's law constant including its temperature dependence, log K_{oc}, vapour pressure, pK_a and further physico-chemical properties (Schüürmann et al., 2006a, 2006b, 2007; Kühne et al., 2005, 2006; Yu et al., 2010). Generally, attention should be paid to assessing the applicability domain of *in silico* models. In this context, ChemProp OSIRIS Edition provides respective means to estimate model uncertainties, and to test the physico-chemical and structural applicability domain.

Molecular size

Besides partition coefficients, other properties such as molecular size and structure may affect the uptake. Size can be described by molecular weight, which can be easily obtained from databases or calculated from the molecular formula. Molecular structures may be too bulky to penetrate biological membranes, thus hindering their uptake into organisms. More detailed analyses require three-dimensional molecular geometries and means to quantify molecular diameters.

Preliminary analysis of transformation potential, uptake and fate

A preliminary assessment of the expected potential for environmental transformation reactions, uptake and fate is performed on the basis of the information for the abiotic and biotic reactions involving the parent compound. The following considerations should be taken into account for a first screening:

- What types of reactions are expected for the parent compound?
- Which environmental transformation products and metabolites are generated?
- In which environmental compartments (air, water, sediment, soil) are the substances expected?
- Are there other parameters influencing uptake and fate?
- Have the substances a significant potential for bioaccumulation?

Select suitable query compound(s)

Preliminary analysis of uptake and fate is used to determine which compound(s) (parent compound and/or metabolites produced in humans and animals and/or abiotic and biotic degradation products produced in the environment) are suitable for modeling the endpoint of interest. Having identified the suitable query compounds according to these criteria, Steps 2-6 are applied for each compound.

R.6.1.7.5 Step 2: Mode-of-action and effect-level classification

In this step, information on the potential biological activity of the compound of interest is investigated.

Aquatic toxicity

At present, there are two types of classification schemes available that both draw conclusions from substructural features of the compounds. On the one hand, the compound is allocated to one or more of several pre-defined modes of action such as narcosis, oxidative uncoupling, protein binding, or mutagenicity. (Modes of action are based on biochemical mechanisms of action but more generalising and more effect related than the latter, e.g., may include precursors). On the other hand, the compound is allocated to one of two (or possibly more) effect levels such as narcosis level and excess toxicity. Excess toxicity is defined as a toxicity enhancement of a factor of typically 10 or 100 as compared to the baseline toxicity predicted from the log K_{ow}. Baseline toxicity is the minimum toxicity already caused by narcosis only (von der Ohe et al., 2005). Other modes of action often but not always increase the observed toxicity. With respect to both of these considerations, compounds with observed toxicities within the baseline range are also denoted as compounds exerting *narcosis-level* toxicity.

In aquatic toxicity, both mode-of-action and effect-level classification schemes are available.

Mode-of-action classification

For acute aquatic toxicity, several classification schemes have been developed. Some of them are implemented in non-commercial software packages:

- **OECD Application Toolbox**
  - Verhaar et al., 1992 (acute fish toxicity) [R10, Tab. R10.14]
  - OASIS acute aquatic toxicity model [R10, Tab. R10.15]
  - Protein binding profiler (not literally a mode of action model, but useful as additional piece of information)
- **ChemProp OSIRIS Edition**
  - Lipnick rules (Lipnick, 1991; acute fish toxicity) [R10, Tab. R10.16]
  - Verhaar et al., 1992 (acute fish toxicity) [R10, Tab. R10.14]
  - Russom et al., 1997 (acute fish toxicity) [R10, Tab. R10.14]

Effect-level classification

For the (typically but not necessarily acute) aquatic toxicity, structural alerts have been developed to discriminate narcosis-level from excess-toxic compounds. At present, the following non-commercial software implementation is available:

- **ChemProp OSIRIS Edition** [R10, Tab. R10-15]
  - Von der Ohe et al., 2005 (acute daphnid toxicity)
    [also R10, Tab. R10.16]
  - Structural alerts for algae, *Daphnia* (updated)

Human and mammalian toxicity

Concerning human toxicity, the presently available classification schemes are usually confined to informing about the potential presence or absence of a certain mode of action such as carcinogenicity or endocrine disruption. For most human and mammalian endpoints it is relevant whether a compound has a certain effect or not. Accordingly, classification schemes addressing human and mammalian toxicity draw conclusions from the presence or absence of certain substructural features (structural alerts) about the molecular disposition for exerting certain hazardous effects such as mutagenicity, carcinogenicity, oral toxicity, skin and eye irritation or corrosion, and endocrine disruption.

Several non-commercial and commercial software programs are available to perform this kind of analysis:

Non-commercial software

- **OECD Application Toolbox**
  - Cramer rules (Cramer et al., 1978)
  - Protein binding
  - Carcinogenicity, mutagenicity, oncologic primary classification rules, DNA binding, ER binding
  - Skin and eye irritation and corrosion
- **ChemProp OSIRIS Edition**
  - Cramer Rules (Cramer et al., 1978)
  - Carcinogenicity, mutagenicity, estrogenicity, androgen receptor antagonism
  - Skin and eye irritation and corrosion

Commercial software

- **Derek, Lhasa**
  - HERG channel inhibition, hepatotoxicity

- Carcinogenicity, mutagenicity, chromosome damage, genotoxicity
- Teratogenicity
- Irritancy, ocular toxicity, respiratory sensitisation, skin sensitisation

- **MCASE**
  - Acute toxicity in mammals, cytotoxicity
  - Carcinogenicity, genetic toxicity
  - Developmental toxicity, teratogenicity
  - Skin and eye irritations as well as allergies
  - ADME, adverse effects in humans
- **Leadscope**
  - Neurotoxicity
  - Carcinogenicity
  - Developmental toxicity, reproductive toxicity
  - Adverse cardiological effects, adverse hepatobiliary effects, adverse urinary tract effects

Evaluation of the outcome of classification schemes

For the application of both types of classification schemes, it is essential to take into account information about their applicability domain with regard to the compound of interest. This holds in particular if the compound of interest has no substructural feature associated with a certain mode of action, and/or no structural alert associated with excess toxicity. Only if the chemical domain of such a compound is covered by the model applied, the respective model result provides relevant information (Kühne et al., 2009).

Accordingly, the application domain of the classification scheme of interest is crucial for an appropriate assessment of the level of confidence of the model outcome. The presently available software packages differ in the presence and type of tools for addressing the application domain of mode-of-action and effect-level classification schemes. Non-commercial software covering such tools includes the OECD Application Toolbox and ChemProp OSIRIS Edition.

A further potential pitfall concerns biotransformation. If in Step1 the compound of interest is identified to be likely metabolized in the organism, the resultant metabolite or metabolites could have a significantly different potential for exerting a given mode of action or effect level as compared to the parent compound. While some classification schemes have built in explicit predictions for metabolites, others address this issue indirectly through allocating – in the training set used for the model derivation – the final toxicological or ecotoxicological outcome to the parent compound, thus incorporating the potential contribution of the metabolites. Depending on the type of training set data used, however, it is also possible that metabolism is neither directly nor indirectly accounted for. An example of the latter would be a model basedon *in vitro* data generated from a cellular system without metabolic capacity. A further possibility is that the model derivation was based on data generated

using a test organism with limited metabolic capacity as compared to the target organism under evaluation, resulting in an only partial (direct or indirect) account of metabolic pathways. It follows that for the proper use of classification schemes, information about the underlying experimental data as compared to the target organism of interest should be taken into account.

The overall assessment of the acute mode of action should take the following questions into account:

- Does the chemical contain structural alerts? (e.g. R.10, Table R.10.16 [p. 35] for daphnids and fish)
- Is the characterisation using different tools consistent with respect to the mode of action? (see also R.10, Tab. R.10.15 [p. 53f] and Tab. R.10.14 [p. 52])
- If the results of different classification schemes differ, is there a reasonable explanation?
- Can additional information be derived from the results?

In many cases it will be difficult to detect a specific mode of action such as inhibition of photosynthesis. Therefore, the evaluation should focus on the question whether the substance is likely to show baseline toxicity or if it is likely that it will exceed baseline toxicity. The answer to this question will be helpful for the evaluation of QSAR predictions as well as for the assessment of the reliability of experimental data and for the assessment of the relative species sensitivity. For the assessment the following considerations might be helpful.

The presence of a structural alert gives a strong indication, that the toxicity of the substance under investigation exceeds baseline toxicity with respect to the acute endpoint under investigation (e.g. acute fish toxicity). On the other hand the absence of a structural alert does not mean that the substance can be classified as baseline toxic.

Consistence of different schemes for the characterisation of the mode of action

The algorithm of different characterisation schemes and the outcome (identification of specific mode of actions or identification of excess toxicity) differs. With respect to the question if the substance shows baseline toxicity, different tools should be combined.

It can be assumed that the characterisation of a substance as being baseline toxic is reliable if different tools, based on different algorithms characterise the substance as baseline toxic and if no structural alerts could be identified. For a high reliability it is important that characterisation tools were included that are able to actively identify baseline toxicity (e.g. according to Verhaar et al., 1992). However it should be carefully assessed if the overall assessment considers all parts of the molecule or if substructures are present that were not evaluated, e.g. the possibility of phototoxic effects should be considered by checking the HOMO-LUMO gap according to the Mekenyan critera (Mekenyan et al., 1994).

Explanation of differences

If the reliability of the outcome of the assessment is low because the outcome of the different schemes differs, the following considerations might be helpful:

- Can the difference be explained by different algorithms of the tools? For instance if the characterisation as baseline toxic is based on tools that do not actively identify baseline toxicity a higher uncertainty can be assumed because of the possibility that the substance simply can not be characterised by the scheme (e.g. ECOSAR).
- Can the difference be explained because different parts of the molecule were considered for the assessment? In this case, the characterisation should generally be based on the most conservative result (e.g. excess toxicity rather than baseline toxicity).

R.6.1.7.6 Step 3: Initial Assessment of transformation routes, uptake, toxicity and fate

This step requires expert judgment. A preliminary assessment of the expected profile of the parent compound concerning transformation, uptake, fate and toxicity is performed, using the outcomes of Steps 1-2 applied to all relevant query compounds.

The preliminary analysis in Step 1 (physico-chemical properties, transformation products, metabolites) may help to assess the likelihood of exposure of the organism (or tissue) or the environmental compartment of interest.

The application of Step 2 may help to focus the assessment on the assumed prevalent modes of action and effect levels. This information is useful for triggering the further design of the assessment procedure, regarding both the potential consideration of experimental investigations and the selection of appropriate non-testing (*in silico*) methods.

In Step 3, the query compound or compounds in terms of molecular structures selected for representing the chemical substance(s) of interest are fixed, and the endpoints to be taken into account are selected. This includes the possibility of considering endpoints beyond the initial selection performed in Step 0 when analyzing the direct REACH requirements.

A respective example is phototoxicity that may have turned out to be relevant in Step 1 when evaluating the HOMO-LUMO gap according to the respective Mekenyan criterion (see description of Step 1 above). A further example is endocrine disruption, if the initial analysis of environmental toxicity has revealed a respective potential.

In Step 3 the information gaps to be subsequently addressed using non-testing methods should be determined, thus further shaping the analysis framework for the subsequent Steps 4-7.

At the end of Step 3, the following issues of the subsequent analysis should be fixed, preferably through an update of the *Working Matrix* (see above) of the evaluation procedure:

- Molecular structure(s) of the compound(s) of interest to represent the chemical substance under evaluation

- Endpoint(s) of interest according to the envisaged use pattern of the substance and the associated REACH requirements
- Potentially additional endpoint(s) of significant relevance due to information gained during the initial analysis
- Endpoint(s) sufficiently addressed for the final evaluation through the availability of appropriate experimental information
- Endpoint(s) in need of further information for their final evaluation according to the REACH requirements
- In case of evaluation-relevant information gaps: Non-testing method(s) options as far as available for addressing the remaining information needs

R.6.1.7.7 Step 4: Chemical categories

The goal of this step is to identify, for the substance of interest, sufficiently similar compounds called *reference compounds* with preferably experimental data so that an interpolation from the data for these reference compounds to the respective property or effect of the substance of interest becomes possible (*read-across*).

A chemical category is a group of chemicals, of which the physico-chemical, toxicological or ecotoxicological properties are likely to be similar or follow a regular pattern as a result of chemical similarity. Chemical grouping means to allocate a compound to existing or newly formed chemical categories.

Chemical similarity may be assessed from different viewpoints and in a context-specific manner, and is generally understood to concern different aspects, so-called components or domains (Dimitrov et al., 2005), such as

- Physico-chemical domain
- Structural domain
- Descriptor domain
- Reaction mechanism domain
- Metabolism domain

The **physico-chemical domain** includes physico-chemical properties such as molecular size, log K_{ow}, water solubility, Henry's law constant, pK_a and bioavailability of the compound.

The **structural domain** characterizes the structural composition of the compound in terms of substructural features. It may include atom and bond types and further measures of the structural complexity (Schüürmann et al., 2006a), the presence or absence of pre-defined functional groups, and a characterization in terms of atom-centered fragments (Kühne et al., 1996, 2009). When focusing on a specific endpoint, the presence or absence of structural

alerts associated with certain modes of action of effect levels may also serve as similarity measure.

The **descriptor domain** concerns the property profile of the compound of interest with regard to its values of the descriptor or descriptors needed for a relevant *in silico* model. Taking the excess aquatic toxicity of certain organic electrophiles such as α,β-unsaturated Michael acceptors as example, the QSAR-relevant descriptors could be log K_{ow} and the logarithmic rate constant of the reaction with glutathione as soft thiol surrogate, as well as log k_{GSH} (Böhme et al., 2009; 2010; Schwöbel et al., 2010). In this case, descriptor-space similarity would mean similar values for both log K_{ow} and log k_{GSH}, assuming that similarity concerning the effect-relevant properties (the descriptor space or descriptor domain) is expected to translate into a similar biological activity.

The **reaction mechanism domain** characterizes the disposition of the substance of interest to undergo certain types of transformation reactions. An example would be the readiness of aldehydes to undergo Schiff-base formation (Dimitrov et al., 2004b), and their sub-class of α,β-unsaturated aldehydes with Michael-addition reactions as additional pathway (Böhme et al., 2010; Schwöbel et al., 2010).

The **metabolic domain** accounts for biotransformation reactions to be expected for the target organisms, preferably covering both the phase-I and phase-II metabolism. Here, similarity refers to prevalent metabolic pathways or to the metabolites formed or to both, considering implications for the toxification or detoxification after uptake into the organism (Dimitrov et al., 2005).

A general scheme for grouping is given in Figure 2.

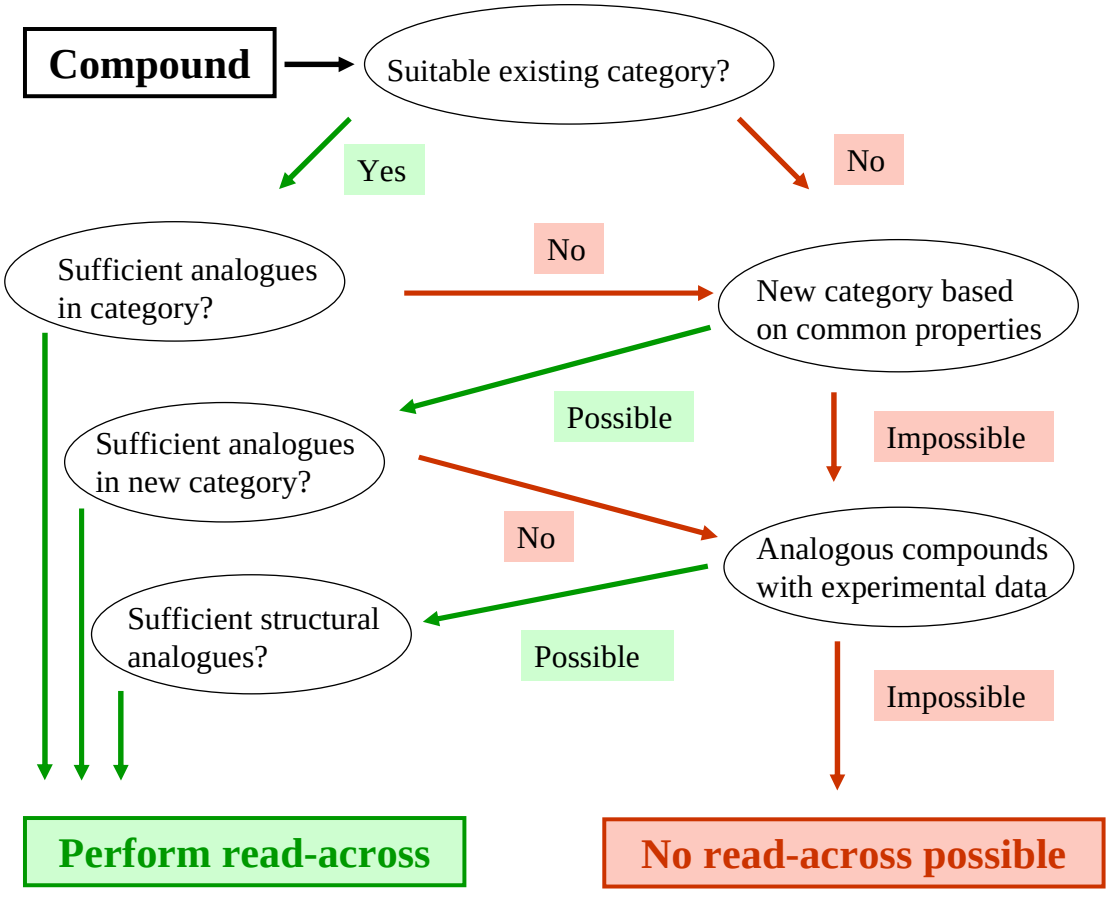


Figure 2. General grouping approach

Step 4a. Chemical grouping according to existing categories

A straightforward way to find analogues of the query compound is to browse existing categories where the compound may be listed as a member. In such a case, the properties associated with the existing category can directly be applied to the compound of interest. Nevertheless, it needs to be clarified whether the category information is useful and applicable for the endpoint under investigation.

It is also possible to apply expert knowledge to link the compound in question to an existing category even though the compound is not explicitly listed as a member. In this case, a similarity assessment is required, covering one or more of the above-discussed similarity domains. A recommended starting point would be an analysis of the atom types and functional groups, followed by an ACF (atom-centered fragment) assessment of the structural similarity (Kühne et al., 2009), which in turn may require the initial generation of the ACF domain of the reference compounds forming the category.

The availability of a database of existing categories is useful for this phase. The **QSAR Application Toolbox** contains pre-defined compound class categories developed for and applied to substances of the OECD HPV Chemicals program and the HPVC Challenge program of the US-EPA.

If a compound belongs to a category, it is necessary to check whether this category is suitable for that compound in the context of the endpoint evaluation to be undertaken. If this is the case, experimental data of the reference compounds from a given category may serve as basis for subsequent read-across. In this way, the endpoint of interest for the substance under investigation can be interpolated from compounds with respective experimental information that belong to the same category.

If the compound of interest does not belong to or cannot reasonably be associated with any existing category, categories may be formed *ad hoc* from databases or otherwise available compounds through application of certain criteria, followed by an assessment of their chemical similarity with the compound of interest (Step 4b). In addition, it is possible to start directly with searching for chemically similar compounds, and then check the pool of selected reference compounds for potentially relevant properties concerning the endpoint under evaluation (Step 4c).

Step 4b. Chemical grouping according to newly formed categories

New categories can be formed through application of structural, property-related or effect-related criteria that appear useful in the context of the compound and endpoint under analysis.

Examples would be the *ad hoc* characterization of database compounds concerning their potential for protein binding through application of a respective *in silico* tool, and the identification of all compounds from a given set that are known experimentally to exert mutagenicity.

Once a respective category has been established and populated with suitable compounds (reference compounds), the potential similarity of these compounds with the substance under investigation may be assessed.

Depending on the number of the reference compounds, their structural overlap with the substance of interest in terms of atom and bond types and functional groups can be assessed by manual inspection. For these and more complex criteria such as the number of jointly occurring atom-centered fragments (ACFs), *in silico* tools such as implemented in the **OECD Application Toolbox** and in the **ChemProp OSIRIS** Edition can be employed. In any case it is recommended to at least initially consider the above-mentioned major domains of chemical similarity as far as relevant and applicable.

If the substance of interest can be allocated with sufficient confidence to one or more of the categories formed, their characteristics can be inferred to apply also for this compound. Collection of relevant experimental data of the category compounds may then serve as basis for subsequent read-across, thus filling the data gap for the substance of interest through interpolation from data of category-related compounds.

Step 4c. Chemical grouping with structural analogues

Besides starting with reference compounds that belong to a certain category in terms of a property or biological activity, an alternative way is to identify, for the substance of interest, similar compounds from databases or other sources.

Again, the different similarity domains as discussed above may be taken into account. In this case, however, the typical starting point would be structural similarity. Depending on the endpoint of interest and the non-testing methods envisaged for subsequent application, additional consideration of the physico-chemical similarity, the reaction mechanism similarity and the descriptor similarity may then be undertaken.

A potential pitfall is metabolic conversion, the predictive assessment of which requires expert knowledge or advanced software tools. An example is the pro-electrophilic class of primary and secondary propargylic alcohols that may be metabolized to α,β-unsaturated carbonyls (Michael acceptors) through enzymatic catalysis by alcohol dehydrogenase (Lipnick et al., 1985; Bradbury & Christensen, 1991). In this case, metabolic similarity in terms of resultant metabolites would allow drawing on potentially available ecotoxicity data of structurally similar Michael acceptors for characterizing the expected effect level of the propargylic alcohol provided the organism of interest is assumed to have the respective metabolic capacity.

Generally, the similarity-based search for analogues may result in an analogue-defined category, specified *ad hoc* through the applied similarity criteria and populated by reference compounds meeting these criteria. Subsequently, experimental data may be searched for these analogues. If sufficient data for the same test species (e.g. toxicity towards fathead minnow) are not available, information on the respective endpoint for related species (e.g. toxicity towards the guppy or rainbow trout) may be taken into account, keeping in mind the additional uncertainty associated with species-species extrapolation.

It is always helpful to perform a search for similarity based analogues (even if the chemical can be associated with existing category) since new and valuable information could be obtained. This step may lead to the identification of multiple analogues which might form the basis of a new category. Software tools to identify analogues include:

Non-commercial software

- QSAR Application Toolbox
- ChemProp OSIRIS Edition
- AMBIT (Ideaconsult Ltd)
- Toxmatch

Non-commercial online tools

- Analog Identification Methodology (AIM)
- ChemID Plus Advanced
- PubChem

Commercial software

- Leadscope

The **QSAR Application Toolbox** enables similarity searches concerning all major similarity domains outlined above (Dimitrov et al., 2005). The **ChemProp OSIRIS Edition** includes similarity searches concerning the physico-chemical and structural domain, the latter in terms of both substructural features (atom and bond types, functional groups, arbitrarily defined substructural units) and atom-centered fragments (ACFs; Kühne et al., 1996, 2006, 2007, 2009).

A potential pitfall concerns the choices to be made by the user with regard to the large variety of similarity-defining options. Recently, some guidance about standard ACF settings has become available (Kühne et al., 2009). However, more context-specific guidance on the appropriate choices of similarity criteria is needed. For the time being, the selection and specification of the similarity criteria to be applied for a given investigation should be based on expert knowledge and – as far as available and documented – on default settings of the respective software tools.

Evaluation of information gained from chemical grouping

The reliability of results obtained by grouping according to chemical categories depends on the selection of appropriate analogues and chemical classes. General guidance for the assessment of the reliability of grouping approaches is provided in Section R.6.2. With respect to aquatic toxicity, the following additional aspects should be considered:

- Are substances used for the grouping approach that are comparable with respect to substructural features (e.g. do they all contain / not contain structural alerts)?
- Can a similar mode of action be assumed for all substances of the category of interest?
- Are the reference compounds comparable with respect to physico-chemical properties that affect aquatic toxicity (e.g. comparable hydrophobicity)?
- Is information available about toxicity-relevant metabolic pathways of the reference compounds, and would this indicate similarity or dissimilarity concerning these pathways or the resultant metabolites or both?

Detailed guidance on grouping of chemicals can be found in the OECD documents

- Guidance on Grouping of Chemicals (OECD, 2007) and
- Guidance Document for using the OECD (Q)SAR Application Toolbox to develop Chemical Categories according to the OECD Guidance on Grouping of Chemicals (OECD, 2009b).

R.6.1.7.8 Step 5: Read-across

Read-across (see also R.6.2.1.2, p. 70, Fig. 3) means interpolation of the endpoint information for the substance of interest from respective experimental information of similar compounds. As such, read-across can be used for filling data gaps for the query compounds representing the substance of interest, the *target chemical*, using the information available for judiciously selected *reference compounds*.

The general principle of read-across is illustrated in Figure 3.1.3. In this scheme, compounds with associated endpoint data are shown as rows, and the respective endpoints as columns. Available experimental data are marked with an X, while data gaps are marked with an unfilled circle. Taking compound #2 (Cmpd 2) in Figure 3 as example, experimental data are available for endpoints 2 and 4, but missing for endpoints 1 and 3, respectively. The arrows in indicate opportunities for read-across. Taking endpoint 2 as example, an approach would be to interpolate the missing value for compound 3 through interpolation between the values for compounds 2 and 4.

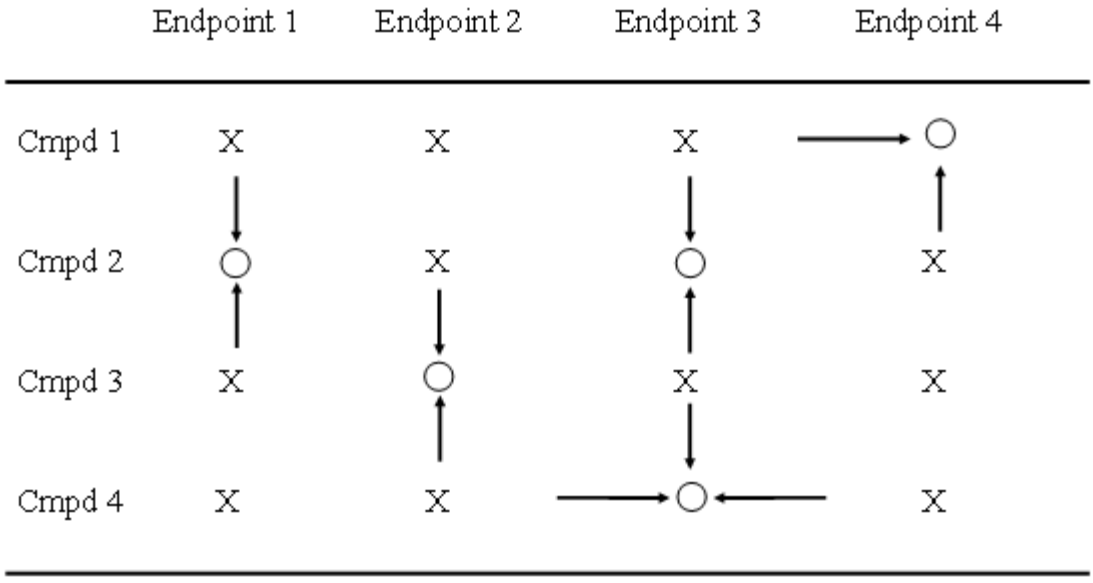


Figure 3. General read-across approach (cmpd: compound; X: available experimental data; circles: data gaps)

Both categorical and numerical information can be gained through interpolation, corresponding to *qualitative* and *quantitative read-across*. Examples of the former are the classification of compounds as being mutagenic or non-mutagenic, and as exerting narcosis-level or excess toxicity. Numerical information can be any appropriate quantification of a property or biological activity such as water solubility (e.g. S_w [mol/L]) or acute fish toxicity (e.g. LC_{50} [mol/L]). Because read-across as a variant of structure-activity relationships relies on the intrinsic activity of molecules, the quantification of the activity of interest must be in molar units such as mol/L. Mass-based units such as mg/L are not appropriate for this purpose, because any difference in molecular weight between the compounds under analysis would confound the information about the intrinsic molecular activity (the activity per molecule). It follows that before interpolating from the data of the reference compounds to the respective value of the target chemical, any mass-based data must first be converted to molar values, selecting one molar unit such as mol/L or mmol/L for all data of interest. Logarithmic molar units (e.g. log LC_{50} with LC_{50} quantified in mol/L) are also valid choices, provided that these are consistently applied throughout this part of the study.

In case of quantitative read-across, a further distinction is between reading across the data of reference compounds expected to exert the same mode of action and effect level, and assuming only the same mode of action. In the former case, the read-across result is gained as – possibly similarity-weighted – numerical average of the relevant reference compound data. The latter case forms a special variant of read-across that is sometimes also called *trend analysis*. Here, the reference compounds with experimental data are used for deriving an *ad hoc* QSAR model with one or few (preferably simple) descriptors. Provided this local *ad hoc* model appears convincing in terms of standard statistics and visual inspection, it is then

used for predicting the endpoint value for the target chemical based on its respective descriptor values.

Read-across is based on chemical similarity (Step 4). Accordingly, the prediction quality of this interpolation procedure depends on the way how similarity is defined and applied, and how many sufficiently similar compounds with pertinent experimental data can be found.

The distribution of the reference compound data provides further important information concerning read-across quality. In case of qualitative read-across or quantitative read-across through averaging (with or without similarity weighting) the reference compound data, the distribution of the latter forms a crucial indicator of the prediction quality to be expected. The more the relevant experimental value varies among the reference compounds, the smaller is their suitability as basis for reading across.

The presence of outlying data of one of more reference compounds may indicate differences in the quality of the experimental data, or differences in the experimental setting (e.g. with or without a contribution from phototoxicity). The standard read-across procedure is to use only data of the same experimental type. Taking acute fish toxicity in terms of LC_{50} as example, the same type usually means the same species (e.g. fathead minnow) and the same exposure time (e.g. 96 h). In case of lack of a sufficient number of respective data for the identified reference compounds, however, additional consideration of (preferably closely) related data such as similar exposure times and similar species may be undertaken. In such cases, both a respectively detailed documentation and consideration of appropriate extrapolation procedures such as between species or between exposure times – as far as possible and available – are mandatory.

In case of quantitative read-across through a trend analysis, the observed quality of the trend is an obvious indicator of its suitability for making the envisaged endpoint value inference for the target chemical. In case certain reference compounds yield outlying data, possible ways forward include the following options:

- Refine the similarity criterion and seek for mechanistically sound arguments to remove some or all of the outliers.
- Select different descriptors for the local *ad hoc* QSAR model, provided these appear meaningful from mechanistic consideration.

A typical starting point is to plot the reference compound data vs. molecular weight, log Kow or logarithmic water solubility. Other options include the use of frontier orbital energies (e.g. HOMO-LUMO gap in case of examining phototoxicity), and of electrophilic reactivity in terms of rate constants (Böhme et al., 2009, 2010; Schwöbel et al., 2010) or appropriate quantum chemical parameters (Wondrousch et al., 2010; Schwöbel et al., 2010; Mulliner et al., 2011) when covalent binding to nucleophilic sites of endogenous macromolecules is assumed to play a role. Generally, the trend analysis variant of read-across is expected to involve several interactive steps in order to find a reasonable local QSAR model as basis for filling the data gap through interpolation.

Overall, read-across includes a variety of options concerning the similarity criteria and with regard to potentially relevant molecular descriptors for performing trend analyses, and requires careful evaluation of the type and quality of data as basis for the ultimate interpolation. Accordingly, the actual read-across procedure may be complex, and may require a considerable amount of expert knowledge. Depending on the compound and data situation at hand, this procedure may fail to yield an appropriately predicted categorical or numerical endpoint value for the target chemical. Possible reasons of such a failure include:

- There are no sufficiently similar reference compounds
- The number of appropriate reference compounds is too low
- The reference compounds do not have a sufficient number of appropriate experimental data
- The variation of the reference compound data makes taking their (possibly similarity-weighted) average as interpolation value inappropriate
- The quality of the local *ad hoc* QSAR model derived through trend analysis is inappropriate for predicting the respective target chemical value

Non-commercial software enabling read-across includes:

- QSAR Application Toolbox
- ChemProp OSIRIS Edition

The **QSAR Application Toolbox** offers read-across as flexible interactive procedure, allowing the user to select in principle any endpoint of interest. It covers categorical and numerical endpoints, and for the latter similarity-weighted averaging and trend analysis. Reference compounds can be searched for from pre-defined and user-defined categories, the similarity criteria available cover all major domains as outlined above, and trend analysis includes fully automatized graphical display of the relevant data distributions (e.g. reference compound data vs. molecular descriptor) and the respective (multi)linear regression line for visual inspection.

The **ChemProp OSIRIS Edition** also covers categorical and numerical endpoints as well as similarity-weighted averaging, with trend analysis being currently under development. Here, special features are fully automatized quantitative read-across models for a variety of ecotoxicological and physico-chemical endpoints, including automatically generated information about their individual applicability domains. The currently available respective models include the following endpoints:

- Human toxicology endpoints:
  - Mutagenicity
  - Carcinogenicity
- Ecotoxicological endpoints
  - Acute fish toxicity (96-h log LC_{50}, fathead minnow, Schüürmann et al., 2011)

- Acute daphnid toxicity (48-h LC_{50}, *Daphnia magna*)
- log BCF (bioconcentration in fish)

- Physico-chemical endpoints
  - log K_{ow} (octanol/water partition coefficient)
  - log S_w (water solubility)
  - log K_{aw} (Henry's law constant)
  - log P_v (vapor pressure)
  - log K_{oc} (soil sorption coefficient)

Moreover, fully automatized semi-quantitative read-across models – again with also automatically generated information about the respective applicability domains – are available for predicting compartmental half-lives (Kühne et al., 2007):

- Fate-related endpoints
  - log $t_{1/2}$ (air)
  - log $t_{1/2}$ (water)
  - log $t_{1/2}$ (soil)
  - log $t_{1/2}$ (sediment)

Quantitative read-across models provide an alternative to conventional quantitative structure-activity relationship (QSAR) models for estimating numerical endpoint values. Correspondingly, qualitative read-across models provide an alternative to *in silico* models predicting categorical endpoints, the latter of which are also called qualitative structure-activity relationships (also abbreviated as QSAR).

Accordingly, read-across and QSARs may also be used as complementary tools of a consensus model approach, provided their mutual methodological independence translates into statistically independent prediction errors. In such a setting, a sufficient degree of agreement between the predictions resulting from the individual methods indicates an increased level of confidence, while significant disagreement would indicate a need for further investigation.

R.6.1.7.9 Step 6: QSAR

In this step, predictions for endpoints concerning human and environmental toxicity and bioaccumulation as well as for physico-chemical and fate-related endpoints are generated by using QSAR models or expert systems that incorporate such models (see also R.6.1.3 – 6.1.6, R.6.1.9, R.6.1.10).

The term QSAR is often understood as quantitative structure-activity relationship confined to quantitative endpoints. Examples are the logarithmic octanol/water partition coefficient, log K_{ow} (physico-chemical endpoint), the logarithmic biodegradation rate constant, log k_{biodeg}

(fate-related endpoint), and acute fish toxicity in terms of log LC_{50} (environmental toxicity endpoint).

However, the term QSAR may also cover qualitative structure-activity relationships predicting categorical (qualitative) endpoints such as carcinogenic vs. non-carcinogenic (human toxicology endpoint), and narcosis-level toxicity vs. excess toxicity (environmental toxicity endpoint). Because models for predicting categorical endpoints are also termed classification schemes and have already been addressed in Step 4, the focus is now on quantitative endpoints, and thus on environmental toxicology where most endpoints of concern are expressed in numerical values.

Application of QSAR Prediction database

It is planned by the ECB to establish a QSAR Prediction database, from which QSAR data can be directly retrieved along with the appropriate reporting formats - QSAR Prediction Reporting Formats and QSAR Model Reporting Formats. Furthermore, a QSAR Model Database is planned to be established in order to enable QSAR predictions for compounds which are not in the QSAR Prediction database. At this point in time, these databases are under construction and include already some models and QSAR data.

OECD criteria for QSAR models

For the application of QSAR models in the regulatory context, a set of quality criteria has been developed. These criteria have been published as OECD principles for QSAR models (OECD 2007a), and address the issues type of endpoint, algorithm definition, applicability domain, statistical measure, and mechanistic information.

Table 3 lists these OECD principles together with associated implications for aquatic toxicity endpoints.

Table 3: Specific aquatic toxicity aspects of the OECD validity criteria

OECD Principle	Specific considerations for aquatic toxicity assessment
Principle 1: a defined endpoint	A defined endpoint is assumed, if the QSAR model is based on experimental data with a) a single measured biological endpoint (e.g. mortality of a specific fish species) b) comparable exposure conditions (e.g. exposure duration, same age of test organisms) and c) a single statistically derived effect concentration (e.g. LC_{50})
Principle 2: an unambiguous algorithm	No specific considerations. Models based on linear regressions using log K_{ow} as sole descriptor are considered to have an unambiguous algorithm. General considerations for the scientific validation of QSAR models are described in Section R.6.1.3.
Principle 3: a defined domain of applicability	A defined domain of applicability can be based on a) definition of the descriptor domain of the model (e.g. range of log K_{ow} of the training set) b) definition of the structural domain of the model (e.g. description of fragments and functional groups covered by the model) c) definition of the mechanistic domain of the model
Principle 4: appropriate measures of goodness-of-fit, robustness and predictivity	No specific considerations for aquatic toxicity assessment. General considerations for the scientific validation of QSAR models are described in Section R.6.1.3.
Principle 5: a mechanistic interpretation (if possible)	A mechanistic interpretation is possible if the QSAR model is based on chemicals assumed to have the same mode of action (e.g. models for polar or non-polar narcosis) or on chemical classes with a known mode of action (e.g. carbamates).

Evaluation of the outcome of a QSAR prediction

Assessing the reliability of a QSAR prediction for aquatic toxicity endpoints is mainly connected with the question whether the target chemical is within the applicability domain of the QSAR model or not. Evaluation of the latter involves consideration of the chemical similarity as outlined in Step 4 above. Thus, the following components of the applicability domain require attention (Dimitrov et al., 2005):

- Physico-chemical domain
- Structural domain
- Descriptor domain
- Mechanism domain
- Metabolism domain

Further guidance for the assessment is provided in Section R.6.1.

In this context, it was recently demonstrated (Kühne et al., 2009) that the atom-centered fragment (ACF) approach provides pertinent information about whether and to what degree

a target chemical belongs to a given QSAR model applicability domain, which in turn is defined through the ACF space of all training-set compounds, possibly augmented by further compounds for which the model has been proven to provide confident predictions. Indeed, the degree to which a target chemical belongs to a model domain is fuzzy, ranging in principle from 0% to 100%. To account for this issue, the following four categories concerning the structural relationship between the target chemical and the applicability domain of the QSAR model of interest have been established (Kühne et al., 2009):

- Inside model domain
- Borderline inside
- Borderline outside
- Outside model domain

In this scheme, the applicability domain category "inside" indicates that the confidence in the QSAR prediction corresponds to the one obtained for the training set (which, in turn, should be known according to the OECD principles). By contrast, the domain check result "outside" indicates that the QSAR prediction would have no statistical confidence. Note that this does not mean that the QSAR prediction must be wrong. It means, however, that in this case the training set statistics are irrelevant for the QSAR model outcome, and that no level of confidence can be given.

The two intermediate categories "borderline in" and "borderline out" indicate intermediate situations. For target chemicals "borderline in", the QSAR prediction is expected to still yield reasonable results but with an average quality significantly below the training set statistics. Predictions for "borderline out" chemicals should be confined to screening-level applications, and would require additional information for any purpose beyond this level.

Generally, the issue of assessing the applicability domain of QSAR models requires further investigation and the development of further guidance.

Reliable QSAR results

The training set of an SAR integrated in a QSAR model should be of sufficient number. For instance, regarding the widely used ECOSAR model, several of the included SARs were developed with only very few training data. Therefore, the performance of the model is poor for some of the chemical classes integrated in ECOSAR (Kaiser et al., 1999; Reuschenbach et al., 2008).

However the following considerations might be helpful for the conclusion:

- At the present (2010) higher confidence is based on QSAR models for acute effects compared to QSAR models for chronic effects. Thus QSAR predictions should focus on acute effects, while QSAR results for chronic effects will be in most cases highly unreliable.

- In general, higher confidence is provided by QSAR predictions based on baseline toxicity compared to QSAR predictions based on specific modes of action or chemical classes that show more than baseline toxicity. Thus, if for a substance a highly reliable classification as baseline toxic and a valid QSAR model where the substance fits into the applicability domain is available, the confidence in the prediction might be high.
- Reliability of the result may increase if a close analogue is available and experimental results for this analogues fit to the QSAR prediction.

QSARs for aquatic toxicity

Non-commercial software with QSAR models for predicting ecotoxicological endpoints include:

- **EPISUITE ECOSAR module**
  - Acute and chronic fish toxicity
  - Acute and chronic *Daphnia* toxicity
  - Algae toxicity

- **QSAR Application Toolbox**
  - EPISUITE ECOSAR module (see above)
  - LMC models for the acute fish toxicity (fathead minnow)
    - M1: Narcosis and soft electrophilicity of aromatics (benzenes, anilines, phenols)
    - M2: Combined baseline and polar narcosis
    - M3: Baseline narcosis
    - M4: Polar narcosis

- **ChemProp OSIRIS Edition**
  - Baseline narcosis models for fish (guppy, fathead minnow), *Daphnia*, algae
  - Polar narcosis model for fish (fathead minnow)
  - ECOSAR QSAR models for fish, daphnids and algae toxicity
  - Abraham-type LSER models for nonpolar and polar narcosis (several species)
  - Read-across models for fish, daphnid and algae toxicity (see Step 5 above)
  - Narcosis-level vs. excess toxicity classification for fish, daphnids and algae (see Step 2 above)

A recommended approach is to first apply a classification scheme for discriminating between narcosis-level and excess toxicity (Step 2). Subsequently, QSARs for predicting narcosis-level toxicity may be applied to compounds identified as narcotics. For non-narcotic compounds, there are currently no general-purpose QSARs available except ECOSAR. Because ECOSAR covers also a QSAR for baseline narcosis toxicity and identifies narcotics automatically

through built-in rules, it can also be used for actually discriminating narcotics from compounds exerting non-narcotic modes of action.
Baseline narcosis is generally agreed to represent the minimum aquatic toxicity level of any neutral organic compound, excluding macromolecules that cannot be taken up via passive diffusion through biological membranes. Thus, baseline-narcosis QSARs can be used for predicting the minimum toxicity expected for any neutral organic chemical.

It follows that if the predicted narcosis-level toxicity of a non-narcotic compound is above a relevant regulatory threshold, a respective classification (e.g. dangerous for the environment) would be triggered preliminarily. If this classification is accepted by the user, no further experimental investigation would be needed. However, the user may undertake further action to disprove such a classification. In this latter case, a way forward would be to demonstrate sufficiently rapid detoxification through metabolism, which in turn can be addressed through *in silico* tools (if applicable and sufficiently confident) or experimental investigation.

ECOSAR includes a suite of QSAR models derived (and thus defined) for certain chemical categories such as narcotics and different classes of compounds exerting specific or reactive toxicity. Both the selection of the appropriate class-specific QSAR of ECOSAR and its application proceed automatically, yielding numerical results in terms of LC_{50} or EC_{50} values typically for fish, daphnids and algae covering both acute and chronic exposure times – as far as respective QSARs are available for the chemical class of the target chemical under investigation.

Concerning reactive toxicity caused by electrophilic organics, first local QSARs for Michael acceptors have become available, employing either chemoassay-derived reactivity data (Böhme et al., 2009, 2010) or quantum chemically predicted molecular reactivities (Wondrousch et al., 2010; Schwöbel et al., 2010; Mulliner et al., 2011). The latter, however, require quantum chemical software and respectively coded procedures for calculating the required parameters from three-dimensional molecular structures.

Waiving of tests

In general, for most substances with a log K_{ow} between 1 and 6 a reliable QSAR model for acute baseline toxicity will be available. Thus, in most cases it will be possible to calculate the baseline toxicity of the substance. If the acute effect concentration calculated for baseline toxicity already triggers a regulatory decision (e.g. baseline toxicity <1 mg/L for classification and labelling) this result might be used, but attention should be paid to the fact that the real toxicity of the substance might be much higher due to a more specific mode of action.

In addition, there might be cases where a substance was classified as having a specific mode of action and a valid model for this specific mode of action is available. Although the result of the prediction may not be reliable enough for a definitive risk assessment, it might be possible to base the decision on the results as a worst case.

R.6.1.7.10 Step 7: Overall assessment

In the final step, an overall assessment of the outcome of Steps 1-6 for the chemical and endpoint(s) of interest is made. Expert judgment is required here. There is still relatively little experience with this type of data integration, and further research into the application of decision analysis methods is needed before detailed guidance can be provided. Decision analysis tools based on decision theory might be useful to support this step.

It is recommended to start with a review of the results of the individual steps, also considering the potential need for updating previously collected information. The *Working Matrix* should now be populated with the compound(s) representing the target chemical (which in turn could in principle contain several components with different chemical structures) and all available experimental and non-test data collected or generated during Steps 0-6.

From **Step 1**, all relevant compounds to which the organisms (or tissue) or the environmental compartments of interest are likely to be exposed, should be known. This concerns the parent compound as well as transformation products and metabolites. Waiving of compounds from further treatment according to the criteria of Step 1 should be based on transparent justification and sufficiently reliable (testing or non-testing) information. In case of doubt due to known or expected data or model uncertainties including the issue of model domain mismatch, waiving candidates would need to remain in the evaluation process. Furthermore, in case of insufficient information on the possible occurrence and extent of transformation products, activities to close the respective data gaps are required.

Step 2 generally provided data and information on the assessment-relevant biological activity, concerning ecotoxicological or toxicological effects depending on the endpoint of interest. Expectations on possible modes of action have been derived, and in case of aquatic toxicity a respective expectation concerning narcosis-level vs. excess toxicity has been established.

Step 3 provided an initial assessment, identifying those compounds and endpoints where additional information was required for performing the final assessment.

From **Step 4**, sufficiently similar compounds have been identified to be used as analogues with pertinent experimental information as basis for read-across predictions.

The latter is performed in **Step 5**, which can be applied for both categorical and numerical endpoints – *qualitative read-across* and *quantitative read-across* – and through either averaging sufficiently similar data of reference compounds or through performing a *trend analysis* with reference compounds exerting the same mode of action but different effect levels.

Step 6 provides **QSAR** predictions, which can be used alone or to complement read-across in order to potentially increase the overall confidence in the non-testing method results. In this context, baseline narcosis QSARs are well established and thus considered sufficiently confident, making a discrimination between (expected) narcosis-level and excess toxicity crucial. Moreover, QSARs for predicting acute effects are usually more confident than QSARs for predicting chronic effects, if available at all.

The following list may serve as check list for the information required for Step 7:

1. **Chemical structure**(s) of the compound(s) representing the **target chemical**
2. *If applicable*: Chemical structure(s) of relevant **transformation product**(s) for the environmental compartment(s) of interest
3. *If applicable*: Chemical structure(s) of relevant **metabolite**(s) for the organism of interest
4. *If applicable*: Chemical structure(s) of all **analogue compounds** taken into account
5. **Endpoint**(s) of interest according to the envisaged use pattern of the substance and the associated REACH requirements
6. *If applicable*: **Additional endpoint**(s) of relevance due to information gained during the analysis
7. **Experimental data** concerning relevant physico-chemical and fate-related properties, and relevant ecotoxicological or human toxicological effects, including pertinent information about the data quality
8. *If applicable*: **Waiving** opportunities due to sufficiently limited exposure according to respective guidelines
9. *If applicable*: **Non-test data** for relevant physico-chemical and fate-related properties and for relevant ecotoxicological or human toxicological effects, augmented by pertinent information concerning the respective **model applicability domain**s and expected **levels of confidence**
10. *If applicable*: Adequate **documentation** of the non-test methods used
11. *If applicable*: Remaining data gaps

Inspection of the *Working Matrix* will then indicate one of four different situations concerning the final assessment to be made according to the legal requirement (risk assessment, or classification and labeling):

A) **All experimental data** required for the assessment are **present**, allowing to make the final assessment

B) Initial data gaps (identified in earlier steps of the assessment procedure) could be filled with **sufficiently confident** and documented ***in vitro* or non-testing data**, enabling the final assessment

C) For at least one initial data gap concerning an assessment-relevant endpoint, the **level of confidence** in the respective **non-testing information** is **insufficient**, **equivocal** or known to be too **low**. In this case, exploring additional *in vitro* or non-testing methods is one potential option, possibly going back to Step 2, 4, 5 or 6. If this additional analysis remains unsuccessful, the assessment would require additional experimental investigation.

D) If at least one assessment-relevant information is **without *in vitro* or non-testing method opportunities**, the assessment would require respective experimental investigation.

So far, there is only little regulatory experience in the use of non-testing data. Thus, respective guidance is rather tentative. There is no general scheme to draw conclusions from non-testing data alone or in combination with experimental data available yet. Instead, a thorough case-by-case discussion is required.

A manual of experience is planned to be developed within the EU, which could continuously be updated, revised and improved by a respective procedure to be implemented. This manual will turn practical experience on the validity and acceptance of using QSARs under REACH into a continuously growing REACH QSAR guidance.

3.2 Proposals for R.7b (Conclusions for aquatic pelagic toxicity endpoints including Weight-of-Evidence and Integrated Testing Strategy approaches)

R.7.8.5 Conclusions for aquatic pelagic toxicity

Section R.7.8.3 (information sources) presents an overview of the possibilities to collect available or generate new information of different kinds (*in vivo* testing, *in vitro* testing, non-testing). Section R.7.8.4 provides guidance how the adequacy, i.e. reliability and relevance, of every single piece of information from these different sources can be judged and ranked. Section R.7.8.5 is supposed to guide through the assessment of the toxicity of the substance in cases where the total amount of available information is suitable for regulatory decisions and in cases, where there are data gaps which have to be filled.

The overall purpose of REACH is to provide a high level of protection for man and the environment. To achieve this, the potential hazards associated with chemical substances must be evaluated and to this end, information about the properties of each chemical is needed. At the same time, also according to the REACH regulation, vertebrate animal testing must be restricted to the necessary minimum. Column 1 of REACH Annexes VII–X specifies what is regarded as minimum information requirements. Column 2 of Annexes VII–X as well as Annex XI specify possibilities to modify these requirements. The prerequisite is the availability of other information that is a) equivalent to the results that would be obtained by standard testing and b) adequate for the three regulatory endpoints: Classification and Labelling, PBT assessment and Chemical Safety Assessment. The equivalence and adequacy have to be substantiated by a *Weight-of-Evidence* (WoE) approach, making best use of all existing information.

Figure 4 represents a flow chart which outlines the basic steps of the proposed sequential procedure. This approach uses and modifies the recommendations of Ahlers et al. (2008). Sections 7.8.5.1 – 7.8.5.3 provide detailed information on the different phases of the WoE assessment.

The assessment of a specific ecotoxicological endpoint starts with Phase I (minimum information level), in which at first the structure of the chemical in question is identified (IA), all available substance information is collected (IB) and a preliminary evaluation of uptake and fate (IC) is performed. Within this preliminary evaluation, focus is on the analysis of the physico-chemical properties and the stability of the substance in aquatic systems. Moreover, publicly available sources are searched for *in vivo* data on the endpoint of concern. In this as well as in all following phases, it must be verified whether there are indications that the substance possesses properties of very high concern (SVHC). If this is the case, a separate SVHC assessment is required. If Phase I does not produce sufficient information on the endpoint of concern (e.g. relevant testing results), Phase II (extensive information level) follows in order to search further data on non-testing and read across sources (IIA) as well as *in vitro* (IIB) and *in vivo* (IIC) data.

The compiled information gathered in Phase I and II is assessed by applying Weight-of-Evidence (WoE) approaches. WoE is meant as a qualitative decision making activity aiming at

concluding on the usefulness of available data for covering a required regulatory endpoint. It integrates information from different sources and takes into account various aspects of uncertainty. It requires transparent and comprehensible expert judgement. Therefore, it is essential that all information used, all steps carried out and all conclusions drawn in the evaluation process are fully documented and justified. Besides the gathering of information (see detailed guidance in Sections R.6.1.7 and R.7.8.3), three distinct activities are related to the WoE (IID): 1) the evaluation of the quality of each distinct piece of information, e.g. a test report or a QSAR result (see detailed guidance in Sections R6.1.7 and R.7.8.4), 2) the evaluation of the quality and consistency of results from same data families, e.g. QSAR results obtained from different models and 3) the summary and overall evaluation of the results and evidences with regard to the ecotoxicological endpoint of concern (guidance is given in this section). Guidance on general aspects of a *WoE* approach is provided in Chapter R.4 and in the ECHA Practical Guide No. 2: *How to report weight of evidence* (ECHA, 2010). The latter also provides support on how to integrate a WoE assessment into the IUCLID data set.

Besides qualitative assessments of WoE approaches like *Best Professional Judgement* (BPJ) or *Listing Evidence* or *Causal Criteria*, recent publications also propose quantitative methods in order to achieve a higher degree of objectivity, transparency and repeatability. Quantitative approaches for WoE assessments use weighting/ranking methods (*Scoring*), empirical (*Indexing*) or statistical models (*Quantification*) (Linkov et al., 2009). Examples for such quantitative methods are the *Bayesian* network approach proposed by Jaworska et al. (2010), the Dempster-Shafer theory (Fernández et al., 2009) or the multi-criteria decision analysis (MCDA) (Linkov et al., 2009).

The WoE should allow deciding whether the collected data provide sufficient evidence to cover the respective toxicity endpoint so that further testing is not necessary. If testing cannot be avoided, a test proposal according to the concept of Integrated Testing Strategies (ITS) should be developed in Phase III (IIIA) which ensures that vertebrate animal testing is restricted to the necessary minimum.

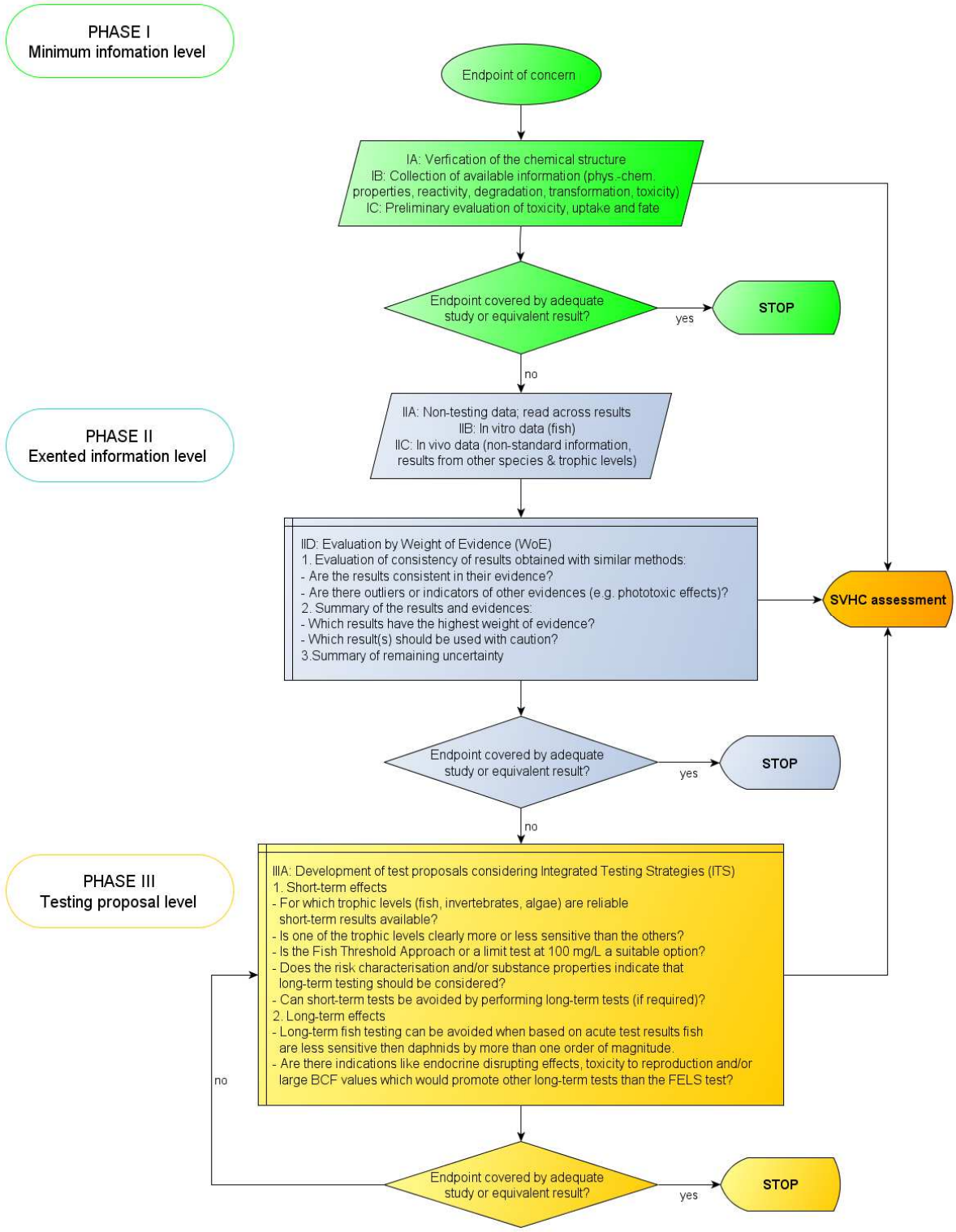


Figure 4. Evaluation of ecotoxicological endpoints using Weight-of-Evidence and Integrated Testing Strategy approaches (⬭: start, ▱: input, ◇: decision, ▭: evaluation, ⬭: output)

R.7.8.5.1 Phase I: Minimum information level

After identification of the endpoint of concern it has to be checked whether information is needed for

(a) Classification & labelling and/or

(b) PBT assessment and/or

(c) Risk assessment, i.e. for derivation of a $PNEC_{aqua}$.

For all three assessments, effect concentrations like LC/EC_{50} or NOEC values need to be identified. However, since for (a) and (b) toxicity threshold levels are the decisive criteria, a range of values or the knowledge that the toxicity value is below or above the threshold level is sufficient to enable a regulatory decision.

In this first phase, it should also be evaluated whether the endpoint can be waived due to exposure and/or regulatory considerations. Following Annex I of the REACH Regulation (*General provisions for assessing substances and preparing Chemical Safety Reports*), the identification of environmental hazards shall be based on all available information, meaning that non-standard information shall also be considered. Therefore, all available substance information should be collected and a preliminary evaluation of this information should be conducted.

The following steps and parameters should be taken into account:

IA: Verification of the structure

This step is essential for the assessment of the mode of action of a substance and for the potential use of non-testing techniques, e.g. QSAR models. In the case of multi-constituent substances (mixtures), it may be necessary to consider two or more structures, if a single representative structure is not considered sufficient (for details see Section R.6.1.7.3).

IB: Collection of available information

Information on the substance of concern should be collected on relevant properties (physico-chemical characteristics, fate, ecotoxicity) from all available sources like databases and estimation tools.

If information on some of these properties is missing, tools like EPISuite or the OECD QSAR Application Toolbox can help to fill these gaps (for details see Section R.6.1.7.3).

IC: Preliminary analysis of uptake, toxicity and fate including identification of possible relevant metabolites/transformation products

A preliminary assessment of expected uptake, toxicity, and fate is performed on the basis of the information collected so far, i.e. an analysis of the chemical structure, physico-chemical properties, degradation pattern, abiotic and biotic reactions involving the parent compound and other information, especially on (eco)toxicity, as available.

At this stage, it is important to evaluate the molecular structure and stability of the substance as well as to identify the relevant metabolites/transformation products. This is essential for the overall hazard assessment of a substance and especially for the evaluation of available *in vivo* results (e.g. for the assessment whether the test concentration was maintained during the test duration in cases where no analytical data are available) as well as for the use of QSAR results (in order to decide if the QSAR models should be used for a metabolite/transformation product rather than for the parent compound). Furthermore, information on the stability of the substance are required when planning studies in order to decide whether exposure should be performed under static, semi-static or flow-through conditions.

Uptake paths and metabolism/transformation mainly depend on the substance properties as well as on the type of organisms (vertebrates, invertebrates, algae) and the developmental stage of the organisms (embryos, larvae or adults). With fish, the major uptake routes for compounds with a log Kow of < 5 will be through the gills and across the skin. The latter is expected to be more significant for embryonic and larval fish than for adults. For compounds with higher log Kow values, uptake via the food chain might be more important than via the water phase (ECETOC Technical Report No. 102, 2007). Further guidance is provided in Section R.6.1.7.4.

If the endpoint cannot be covered by reliable test results, a data search (including a literature search) is recommended. Several databases incorporating ecotoxicological information are publicly available (e.g. the ECOTOX database). Suitable databases are described on pages 92 ff of this guidance document. Suitable literature search machines are available in the internet (e.g. http://www.scirus.com or http://www.ncbi.nlm.nih.gov/pubmed/).

It should be noted that regardless of the evaluation phase and the source of information (standard/non-standard), any indications of properties of very high concern (SVHC) should be followed and verified.

Evaluation of available standard information

If the data search reveals potentially suitable information, the original publication should be verified in order to decide whether the information is reliable and useful for covering the endpoint. Reliability of the data according to Klimisch et al. (1997) should at least be rated with 2 (reliable with restriction). However, less reliable data or data of unknown reliability might be used in a weight of evidence decision (see next phase below).

If potentially useful information has been identified, it must be checked whether the results can freely be used. In case that the data have been published within a regulatory programme, e.g. the OECD HPV chemicals programme, a letter of access might be required for using the information.

How to deal with conflicting data?

As recently published, variability of fish acute toxicity results extracted from databases, e.g. the ECOTOX database, can be very high for the same substance (Hrovat et al., 2009). Besides the fact that for many of the database results a profound documentation of the test conditions was lacking, reported potential reasons were biological (differences in species and life stages) and physical-chemical factors (differences in test temperature, pH and hardness) as well as inter-laboratory and intra-species variability.

When there is more than one set of data on the same species, endpoint, test duration, life stage and testing condition, the greatest evidence is attached to the most reliable and relevant test result. When there is more than one set of data with the same reliability rating, it should be checked whether a specific reason could explain the difference. If no explanation can be found and the results are not more than one order of magnitude apart, they can be averaged by calculating the geometric mean. If results are more than one order of magnitude apart, an average value should not be determined. If the endpoint is crucial for the outcome of the regulatory decision, a repetition of the study may sometimes be the easiest and most efficient solution, especially for non-vertebrate tests. A decision might also be possible on the basis of additional available data, e.g. from studies of a lower reliability rating or from non-testing methods, if these show a distinct tendency in support of a certain result.

What if only secondary data sources are available?

Normally, data from a secondary source will lack several of the criteria required for a sufficient reliability rating and can therefore not be considered for use in regulatory conclusions. An exception can be made when these data have previously been considered under widely accepted/justified programmes which themselves contain adequate review processes for data reliability, e.g. the OECD ICCA/HPV initiative.

Can several data of insufficient reliability provide sufficient information when used in combination?

Some generic guidance on this issue is provided in Chapter R.4. This chapter also mentions the technique of *meta-analysis*, a statistical tool used for analysing the combined data from multiple studies. Such pooling of data may increase the statistical power of certain findings. It requires, however, that the studies from which data are pooled are sufficiently similar with regard to critical parameters of test conditions, set-up, endpoints, reporting etc.

There may be several studies available for the same test substance and the same endpoint, which are deemed to be not fully reliable. However, when used collectively the study results may indicate an effect at approximately the same concentration and time. In these cases there could be justification for using all the studies collectively to conclude on a specific endpoint.

Examples:

- Valid fish toxicity data are only available for a short exposure regime (e.g. 24 h). Tests over 96 h might be available, which cannot be judged as reliable (e.g. because of poor documentation), but which provide information that the main effect occurs within the first 24 h. In this case the 24 h value might be used.
- Toxicity data are available for several time points from a 72 h test. In this case, the time-effect curve may allow extrapolation of the 96 h value.

Do available data allow the derivation of a semi-quantitative result?

This consideration applies in relation to given effect values, for example:

- an LC_{50} value cannot be calculated from an available acute fish test because no mortality was observed, but the tested concentrations are above the EC_{50} value determined for algae or *Daphnia* (retrospective threshold approach).
- an EC/LC_{50} value cannot be derived, because test concentrations were either too high or too low, but it can be stated that the LC_{50} is either above or below a specific regulatory relevant trigger value, such as C&L criteria or the T criterion in PBT assessment.

The summary of the gathered information from the available *in vivo* studies should contain the following:

- Results of standard tests available for all trophic levels?
- Reliable results of non-standard tests available for all trophic levels?
- Reliable results from aggregation of different studies available?
- Reliable half-quantitative results available?
- Description of additional information, the reliability of this information and of its intended use available?

When the data search was not successful, it is recommended to enter the second phase. However, in case that the endpoint of concern does not refer to vertebrates, an applicant might consider skipping Phase II and entering directly into the testing phase (Phase III). With respect to vertebrates and in order to avoid unnecessary testing, it is highly recommended to consider the options of Phase II including WoE.

R.7.8.5.2 Phase II: Extented information level and evaluation by WoE

In this phase, the data search should be expanded to all sorts of information which can be made available for the substance or can be generated in order to help concluding on its toxicity with respect to the endpoint of concern.

Phase II includes consideration of the following issues:

- IIA: Non-testing information and read-across
- IIB: Evaluation of *in vitro* testing data (fish)
- IIC: Evaluation of *in vivo* testing data (other than standard information)
- IID: Weight of evidence assessment of the collected information

IIA: Non-testing information and read-across

The following non-testing (NT) information is useful in order to derive information concerning the substance properties and effects with regard to the endpoint of concern. In Section R6.1.7, it is comprehensively described how to derive and evaluate this information. A short overview is presented in the following.

1. Characterisation of the mode of action (MoA) according to appropriate schemes

The overall assessment of the acute mode of action should take the following questions into account:

- Does the chemical contain structural alerts?
- Is the characterisation using different tools consistent with respect to the mode of action?
- If the results of different classification schemes differ, is there a reasonable explanation?
- Can additional information be derived from the results?

2. Identification and evaluation of possible analogues and read-across

This step includes the following issues:

- Identification of existing or new chemical categories
- Collection of possible analogues
- Evaluation of available experimental data for these analogues with regard to the endpoint of concern

3. Evaluation of QSAR results

This step aims at answering the following questions:

- Are reliable QSAR results available that can be used instead of experimental data, if data gaps are present?
- Can additional information provide a rationale for the waiving of tests?
- Can additional information provide a rationale for the performance of specific additional tests?

IIB: Evaluation of in vitro testing data (fish)

- Are reliable *in vitro* results available?
- Can *in vitro* results provide additional information?

Available *in vitro* tests and their use for regulatory decisions are described in Chapter R.4. At the present (2010), no *in vitro* tests are available that can substitute fish toxicity data. Moreover, no *in vitro* tests are available which cover effects on daphnids or algae. However, *in vitro* data obtained with fish cell lines or fish embryos might be helpful to get further insight into the mode of action of a substance.

Some permanent cell lines might express specific characteristics/functions of their source tissue/organ. Their use to characterise more specific modes of action has to be evaluated. Specific modes of action are more likely to be detected with primary cell cultures. For example, primary hepatocytes have been used for studying metabolism, hepatotoxicity, genotoxicity and vitellogenin induction, while isolated gill cells are used for studying the effect on the branchial epithelium. Transfected permanent fish cell lines have been used to detect estrogenic effects of substances.

The fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*), which has been standardized (ISO, 2007) and is routinely used in Germany for whole effluent testing, has also been suggested as a potential alternative to the acute fish test for the testing of chemicals. Recent comparisons of acute fish toxicity and fish embryo toxicity support the potential use of the FET as a replacement for the acute fish toxicity test (Braunbeck & Lammer, 2006; Lammer et al., 2009). However, a systematic comparison of results obtained with both test systems (including a range of substances with different modes of action, a wide range of toxicities in the acute fish test and different physico-chemical properties) is still lacking. As the possible application range and the limitations of the method are not yet clearly defined, use of the fish embryo test should be limited to substances that can reliably be classified as baseline toxicants and that are likely to be able to cross the chorion.

The FET test has also been considered as part of an integrated decision-tree testing strategy for acute environmental toxicity testing which was developed within a REACH project sponsored by the British Department for Environment, Food and Rural Affairs (Defra) (Grindon et al., 2006).

IIC: Evaluation of existing in vivo testing data (other than standard information)

In this step, *in vivo* testing data should be collected and evaluated that were not derived by standard toxicity testing for the endpoint of concern and/or that were obtained with species of other trophic levels.

If the endpoint of concern is e.g. fish acute toxicity, non-standard information on fish toxicity as well as on invertebrate or mammal toxicity data should be considered too. This information is valuable in order to understand the toxicity pattern of the substance of concern as is described in the next step (IID: WoE assessment). Moreover, it might help in order to assess the mode of toxic action.

Guidance on how to evaluate the quality of information from *in vivo* tests is given in the Sections R.7.8.4 and R.7.8.5.1. Guidance on evaluation of modes of action can be found in R.6.1.7.5.

IID: Weight of evidence assessment of the collected information

- Evaluation of the consistency of results from similar methods
- Summary of reliable results and preliminary conclusion on the toxicity of the substance
- Summary of the remaining uncertainty (e.g. due to lack of consistency of data)
- Decision on testing

In this step (IID), all available data from the different sources should be integrated in the assessment in order to understand the toxicity pattern of the substance.

Experimental data (especially standard test results) have the highest priority when conclusions have to be drawn for C&L, PBT assessment and/or PNEC derivation. Non-standard or *in vitro* data as well as non-testing data are important in cases where standard experimental data are missing, not reliable or inconsistent. They are used to verify experimental data and avoid an assessment on the basis of invalid data (e.g. if two acute fish toxicity tests give two different LC_{50} values (e.g. 10 and 100 mg/L) and the chemical of concern is classified as acting by non-polar narcosis with an appropriate QSAR result of LC_{50} = 120 mg/L, more confidence might be given to the 100 mg/L LC_{50} value). Non-testing data can also be considered as additional information, even if experimental data exist. Moreover, they can be used for elaboration of a test design for higher-tier tests or for a decision to perform chronic instead of acute tests (see also next chapter on integrated testing strategies).

At the end, all available information (test data and non-testing information) should be used for a comprehensive conclusion on the endpoint (multi task assessment). This conclusion has to be substantiated and documented. The amount of information necessary to draw such conclusions will definitely be different dependent on the regulatory endpoint. For C&L, in certain cases limit tests may be sufficient as only a decision has to be drawn whether the toxicity is below a certain trigger value, whereas for derivation of the PNEC a quantitative result is required. In the latter case, it is of particular importance to use all available information, as PNEC derivation means to extrapolate from a few monospecies laboratory tests to the maintenance of structure and function of ecosystems. Especially the extrapolation from acute to chronic toxicity is hardly possible. Analysis of a large number of validated data on new and existing chemicals revealed that acute data have only limited predictive value for long-term effects in aquatic ecosystems. The acute/chronic ratio correlates neither with acute toxicity nor with baseline toxicity as modelled through log Kow and no acute/chronic ratio correlation is found across trophic levels, meaning that it is generally not possible to conclude e.g. from *Daphnia* or algal ACR on fish ACR (Ahlers et al., 2006).

In contrast to C+L and PBT assessment which is solely based on intrinsic properties, for PNEC derivation also exposure-based decisions (PEC/PNEC ratio) have to be considered. If, for instance, EC_{50} values for algae and *Daphnia* are available and, in addition, QSAR calculations for fish have been performed and a high PEC/PNEC ratio has been derived, a chronic fish test has to be considered. If the PEC/PNEC ratio is low, no additional data are necessary if not required according to Annex IX and X.

Column 2 of REACH Annexes VII and VIII contains the provision that acute studies do not need to be conducted, if there are mitigating factors indicating that aquatic toxicity is unlikely to occur (for instance if the substance is highly insoluble in water or unlikely to cross biological membranes). However, long-term testing has to be considered, when a substance is poorly water soluble.

There is no scientific basis to define a cut off limit value for solubility below which no toxicity could occur. There may be technical difficulties to perform the test, e.g. the insufficient sensitivity of the analytical method used for the determination of test concentration. Such difficulties and proposed solutions should be clearly documented. For further details see information on testing of difficult substances in Appendix 7.8-1.

Equally, there is no scientific basis to define molecular characteristics that would render a substance unlikely to cross biological membranes.

Thus, no scientifically based cut off criteria for these mitigation factors can be provided at the moment. Nonetheless, it might be possible to decide on a case-by-case basis that acute toxicity to pelagic organisms is unlikely to occur due to very low water solubility and unlikelihood to cross biological membranes. Issues which may be considered in this regard are the indicators used for low likelihood of a high bioaccumulation potential (Chapter R.11). When such indicators are used in the context of triggering derogation from toxicity testing on aquatic organisms, however, a more cautious approach should be used. The reason is that indications of a lack of a high bioaccumulation potential do not necessarily imply the lack of toxicity to aquatic organisms.

In any case, any proposal to deviate from the standard testing requirements in reference to this clause should be carefully justified. For poorly water soluble substances (e.g. water solubility below 1 mg/L or below the detection limit of the analytical method of the test substance), it should be considered to perform a long term test (REACH Annex VII and VIII, 9.1) instead of an acute test bearing in mind any possibilities for waiving (REACH Annex XI).

Further evaluation of the substance properties is required, if in Phase I or II results have been identified which indicate that the substance might be a substance of very high concern (SVHC). SVHC criteria are carcinogenic (C), mutagenic (M), repro-toxic (R), PBT (persistent, bioaccumulative and toxic), vPvB (very persistent and very bioaccumulative) or substances with an equivalent level of concern like endocrine disruptors.

Guidance on evaluation of candidates for SVHC is provided in the REACH *Guidance for the preparation of an Annex XV dossier on the identification of substances of very high concern* and in *Guidance on information requirements and chemical safety assessment,* Chapter R.11 (PBT Assessment). Furthermore, Appendix R.7.8-5 of this Guidance Document deals with the evaluation of potential endocrine disrupting substances.

R.7.8.5.3 IIIA: Development of test proposals considering ITS

Integrated Testing Strategies (ITS) are closely linked to *Weight of Evidence* (WoE) in that the available evidence can help to determine the subsequent testing steps. Results from these subsequent tests may affect the WoE, which leads to a new decision on whether there is any need of further testing. ITS are particularly characterised by flexibility and case specificity. No general ITS can be developed and a case-by-case decision will always be necessary. Guidance on how to develop an individual ITS has to focus on decision making criteria and underlying considerations rather than on ready-to-use procedures.

A general definition of ITS was developed on an EPAA-ECVAM Workshop: '*In the context of safety assessment, an Integrated Testing Strategy is a methodology which increases information for toxicological evaluation from more than one source, thus facilitating decision-making. This should be achieved whilst taking into consideration the principles of the Three Rs (reduction, refinement and replacement)*' (Kinsner-Ovaskainen et al., 2009). From this definition it becomes clear that a key role in ITS is the protection of animals, meaning the avoidance of unnecessary (vertebrate) testing.

A general view, definitions and examples on ITS for different kinds of endpoints can be found in Vermeire et al. (2007). The interaction between ITS and WoE has recently been shown in a comprehensive way using the example of skin irritation classification (Hulzebos & Gerner, 2010).

In the following section, important examples of recent OECD developments on strategies for reducing the number of fish in aquatic toxicity testing are presented.

Threshold approach for toxicity testing in fish (OECD, 2010b)

This approach offers a possibility to significantly reduce the number of fish used in acute fish toxicity testing. It takes into consideration that only the lowest value of the acute toxicity in species of three trophic levels is considered for regulatory purposes.

With the lowest of the two EC_{50} concentrations obtained for algae and *Daphnia* (the Threshold Concentration), a limit test according to OECD TG 203 is conducted, using 7-10 test and 7-10 control fish. In case that no mortality is observed, no further tests are carried out and the acute fish toxicity result (LC_{50}) is reported as *greater than* (>) the Threshold Concentration value. In case that mortality is observed, a full test following OECD TG 203 should be performed.

The same principle could also be applied when instead of juvenile or adult fish, fish embryos or larvae are used for acute toxicity testing.

Rufli and Springer approach to reduce the number of fish in OECD TG 203

Based on an analysis of data from two databases the authors found that using only six fish per concentration LC_{50} estimates are of similar quality as those obtained using seven fish. (Rufli & Springer, 2010; OECD, 2010b). At the time this report was finalised, the OECD discussion about the Rufli and Springer approach was still ongoing. Therefore, no recommendation can be given yet.

OECD Fish Testing Framework

In its Draft Fish Testing Framework document, the OECD has recently published two proposals for fish testing strategies: one for short-term and one for long-term exposure (OECD, 2010b). Although the propsals are not yet finalised both strategies can be recommended for practical use and will be described in short in the following sections.

Similar to the *WoE* approach described in the previous section, the proposed short-term scheme starts with the collection of data on physico-chemical and fate properties in order to allow for deciding on the likeliness of aquatic exposure. The next step foresees gathering of all kinds of toxicity data including QSAR, *in vitro* and *in vivo* results.

For substances, which are not considered to bioaccumulate significantly or which are suspected to be endocrine disrupters (ED), an acute toxicity test is not considered necessary. Long-term testing is suggested in cases where the risk assessment indicates that long-term exposure or long-term/repeated exposure is likely (especially for HPV chemicals).

For substances, for which bioaccumulation potential is expected based on a high log Kow, a high BCF is measured or, prolonged exposure is expected, long-term test is recommended.

For substances with reasonable suspicion of endocrine disruption potential, screening tests following OECD TG 229 or 230 are considered depending on the data requirements deduced from the collected information so far. In some instances direct consideration of long-term toxicity testing, i.e. a fish full-life cycle (FFLC) test, might be justified.

Long-term scheme: If long-term testing is considered necessary and the substance is not suspected to be an ED or a reproductive toxicant, a fish early-life stage (FELS) test is recommended in most cases. If based on the results of this first test, the risk characterisation does not lead to a sufficient margin of safety, life-cycle testing should be considered. Beside the FFLC test, the proposal does also consider the Medaka multi-generation test (MMGT). The decision between the two tests depends inter alia on the BCF and has to be made case by case.

If the evidence is high that the substance is an ED, a fish sexual development (FSD) test is recommended. In the next step an FFLC or MMGT is considered depending on the BCF. If the substance is not suspected to be an ED but is toxic to reproduction, the FSD test can be skipped.

R.7.8.5.4 From integrated testing to integrated assessment

When the *WoE* procedure for evaluation of an ecotoxicological endpoint has been finalised as described above, the amount of validated information may in some cases largely exceed the minimum information requirements of the Annexes of REACH and thus reduce the uncertainties when extrapolating from monospecies laboratory tests to the structure and function of ecosystems. As for PNEC derivation these uncertainties are to be covered by the assessment factors it may be considered to use these factors in a more flexible way according to the altered degree of uncertainty.

Beside the information mentioned above such a *multi-criteria assessment* should also cover:

- The number and representativity of species tested
- The quality of non-standard tests
- The time-dependence of the toxicity
- The steepness of concentration/effect curves

Information from mammalian toxicity is normally not used in standard assessments. Specific guidance on this approach with regard to potential reproductive or developmental toxicity via endocrine modes of action is provided in Appendix 7.8-5

At the end, the derivation of the degree of uncertainty defined in the standard situations and represented by certain assessment factors given by the Section R.10.3 has to be fully substantiated.

4 Documentation of the results of the NT/WoE approach

With the three representative substances nine endpoints were identified in total for which an application of the NT and WoE approach was considered useful. Templates for summarising the results of the approaches have been developed (please see next section). The template for the endpoint 'Endocrine disruption' differs from the template for the acute endpoints, because it was developed on the basis of the proposed 'integrated assessment' described in Figure 7.8-8 of the guideance document R.7b (Appendix R.7.8-5, p. 119).

One example for acute toxicity is presented with the endpoint 'acute fish toxicity of benzanthrone'. The evaluation of the endocrine effects is presented for all three substances in the confidential Annex 3 A.

In section 4.1 the application of the *Weight-of-Evidence* approach as outlined in the REACH guidance document R.7b is described by using this example. Non-testing data were generated following the NT concept as outlined in guidance document R.6. Both non-testing and testing data are integrated in the WoE concept and in the overall evaluation of the substance with respect to the endpoint of concern. The single steps and the results of the evaluation are documented on the following pages. This documentation may serve as a draft template for similar evaluations following the WoE approach.

Regarding appearance in the IUCLID data set (and subsequently in the CSR), the following suggestions are made:

- The collected non-testing and testing data should be entered in the IUCLID section of the endpoint of concern, e.g. 6.1.1 *Short-term toxicity to fish*.
- The results of the single non-testing parameters of the NT approach, e.g. classification schemes, structural alerts or experimental data from analogues could each be entered as an endpoint study record. Certainly, the same applies to QSAR, *in vitro* and *in vivo* results.
- The single records of the same NT/WoE approach should be identified by a certain number or abbreviation in combination with the term *WoE* which should be visible in the title of each record.
- The evaluation of the results of the NT and WoE approach, as documented in the following section, could be entered in the *Discussion* field of the endpoint summary of the endpoint of concern. Alternatively, only the overall evaluation (WoE, point D) and the testing proposal (WoE, point E), if available, is recorded here and the whole document is attached to this section.

4.1 Acute toxicity of benzanthrone to fish

At first sight, the endpoint 'acute fish toxicity' seems adequately covered by a reliable guideline study. However, test data are available that indicate elevated toxicity due to phototoxic effects. Due to the relevance of fish as protected animals and the special mode of action of benzanthrone it was decided to evaluate the acute toxicity of benzanthrone to fish under the aspects of the NT and WoE concepts. Moreover, the comparison to the experimental data was considered helpful in order to rank the results of the non-testing methods.

I NT Approach

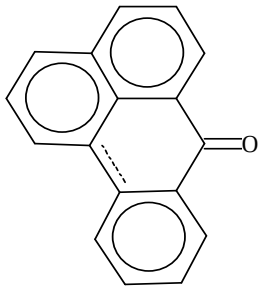
I.1 Step 0: Information collection

To select a representative structure, first the composition of the parent substance has to be characterized. The parent compound hast to be defined, and its chemical structure needs to be obtained. Then available and reliable information for the parent compound should be collected in order to identify data gaps.

Here, benzanthrone is considered as pure compound, i.e. impurities are expected to be of minor relevance. The chemical 2D-structure of benzanthrone has been validated using the QSAR Application Toolbox and the ChemProp database. Furthermore, according to ChemProp there are no different tautomer forms (Thalheim et al., 2010).

Important data of the collected information on benzanthrone are listed in Table 4.

Table 4. General substance information.

Chemical name Benzanthrone	**Structural formula**  **MolWt: 230.27 C17 H10 O1** **000082-05-3 7H-Benz de anthracen-7-one**
CAS No. 82-05-3	
SMILES Code O=C(c(c(c(c1c(ccc2)cc3)c3)ccc4)c4)c12	
Molecular weight (mol/L) 230.27	
Substance group Polycyclic aromatic hydrocarbons (PAH)	
Uses / exposure routes Dyestuff intermediate for anthraquinone-based dyes; use for photosensitization, as charge transport material and in pyrotechnics industry (WIKIPEDIA); Low Production Volume (LPV) chemical (ESIS)	
Classification & labeling / toxicity Not classified in Annex I of Directive 67/548/EEC (ESIS)	

An important issue that needs to be emphasized is the use of benzanthrone as dyestuff intermediate and photosensitizer. This indicates to take into account phototoxicity.

I.2 Step 1: Preliminary analysis of transformation potential, uptake and fate

Transformation potential of the parent compound

At this stage, the environmental transformation processes are considered in order to evaluate whether transformation products have to be taken into account. In Table 5, information about important abiotic transformations (hydrolysis, photolysis) and biotic transformations (microbial degradation, bio-transformation) is listed.

Table 5. Abiotic and biotic transformation processes.

Process	Result	Type of information	Source
Hydrolysis	Not expected	Expert judgment	
Photodegradation in air (DT_{50})	0.6 days	Calculated	EPISUITE v4.00
Biodegradability	No (0%, 4 weeks)	Measured (MITI (I))	NITE (HSDB)
ultimate	Not fast	Calculated	EPISUITE v4.00 (BIOWIN 2)
primary	Weeks … months	Calculated	EPISUITE v4.00 (BIOWIN 3)
MITI	Not fast	Calculated	EPISUITE v4.00 (BIOWIN 6)
Biotransformation half-life in fish	0.4 days	Calculated	EPISUITE v4.00 (BCF/BAF v2.03)

In water, benzanthrone is expected to be stable, i.e. there is no indication of hydrolysis. In air, benzanthrone is expected to be easily degraded by OH radicals (the half-life time at an OH-concentration of $1.5 \cdot 10^6$ cm^{-3} is approximately half a day), but due to a low OH radical concentration in water, the compound is expected to be photolytically stable in the aqueous environment.

Furthermore, a low biodegradation potential is expected for benzanthrone, i.e. no biodegradation has been measured in the MITI (I) test and the estimation methods implemented in EPISUITE also predict a low biodegradation potential.

In contrast to this, a fast biotransformation rate, i.e. a half-life of 0.4 days in fish (10 g wet weight) has been estimated. For this reason, the QSAR Application Toolbox has been used to generate a list of possible metabolites. Both metabolism simulators (liver and microbial) were applied.

From the liver metabolism simulator as the more relevant process (Mekenyan et al., 2004), metabolites based mainly on the following three possible reactions were obtained:

- Oxidising of aromatic rings via monooxygenases (e.g. P450 enzymes), i.e. adding additional OH groups to aromatic rings
- Oxidation of hydroxyl groups
- Reduction of keto groups

The microbial metabolism simulator (Jaworska et al., 2002; Dimitrov et al., 2002, 2004a) generated several metabolites from oxidising steps via dehydrogenases including ring opening mechanisms in further metabolism steps.

A number of these metabolites contain reactive substructures as quinones or Michael-type acceptors. Some potentially reactive metabolites are listed in Table 6.

Table 6. Metabolites with possible reactive modes of action (hydroquinone or quinone substructure) identified by the QSAR Application Toolbox.

Simulator	Structures of metabolites (SMILES code)
Liver metabolism	c12C(=O)c3c(-c4c1c(c(O)cc4)ccc2)ccc(O)c3O
	c12C(=O)c3c(-c4c1c(c(O)cc4)ccc2)cc(O)c(O)c3
	c12C(=O)c3c(-c4c1c(c(O)c(O)c4)ccc2)ccc(O)c3
	c12C(=O)c3c(-c4c1c(c(O)cc4)ccc2)c(O)c(O)cc3
	c12C(=O)c3c(-c4c1c(c(O)c(O)c4)ccc2)cc(O)cc3
	c12C(=O)c3c(-c4c1c(c(O)c(O)c2)c(O)cc4)cccc3
	c12C(=O)c3c(-c4c1c(c(O)c(O)c4)ccc2)cccc3
	c12C(=O)c3c(-c4c1c(ccc4)ccc2)ccc(O)c3O
	C1(=O)C(=O)C2C(=O)c3c4c(C=2C=C1)cccc4ccc3
	c12C(=O)c3c(-c4c1c(ccc4)ccc2)cc(O)c(O)c3
	c12C(=O)C3C(c4c1c(ccc4)ccc2)=CC(=O)C(=O)C=3
	c12C(=O)c3c(-c4c1c(ccc4)ccc2)c(O)c(O)cc3
	C1(=O)C(=O)C2c3c4c(C(=O)C=2C=C1)cccc4ccc3
	c12C(=O)c3c(-c4c1c(c(O)cc2)ccc4)ccc(O)c3O
	c12C(=O)c3c(-c4c1c(c(O)cc2)ccc4)cc(O)c(O)c3
	c12C(=O)c3c(-c4c1c(c(O)c(O)c2)ccc4)ccc(O)c3
	c12C(=O)c3c(-c4c1c(c(O)c(O)c2)ccc4)cccc3
Microbial metabolism	c12C(=O)c3c(-c4c(O)c(O)cc(c14)ccc2)cccc3
	c12C(=O)c3c(-c4c(O)c(O)cc(c14)ccc2)c(O)c(O)cc3
	C(=O)(O)c1c(-c2c(O)c(O)cc3c2c(O)ccc3)c(O)c(O)cc1
	c1(C(c2c(C(=O)O)ccc(O)c2O)=C(O)C(=O)O)c(O)cccc1CO
	C(=O)(O)c1c(C(C(=O)C(=O)O)c2c(O)cccc2CO)c(O)c(O)cc1
	C(=O)(O)c1c(C(C(=O)C(=O)O)c2c(C=O)cccc2O)c(O)c(O)cc1
	C(=O)(O)c1c(C(C(=O)C(=O)O)c2c(C(=O)O)cccc2O)c(O)c(O)cc1
	C(=O)(O)c1c(C(c2c(C(=O)O)cccc2O)C=O)c(O)c(O)cc1
	C(=O)(O)c1c(C(c2c(C(=O)O)cccc2O)C(=O)O)c(O)c(O)cc1
	C(=O)(O)c1c(C(C(C(=O)O)=CC(=O)O)C(=O)O)c(O)ccc1O
	C(=O)(O)C=C

The use of benzanthrone as a dyestuff intermediate and as photosensitizer already indicated potential photo-activity (Step 0). Benzanthrone is a polycyclic fused aromatic compound. This structure also implies possible phototoxicity. Checking the Mekenyan criteria for phototoxicity (Mekenyan et al., 1994) yields a HOMO-LUMO gap (E_{gap}) of 7.43 eV, calculated through the semi-empirical Austin Method 1 (AM1) of MOPAC 2002. This fulfills the Mekenyan criteria for phototoxicity, confirming the expectation. Consequently, this reaction path should be taken into account in the further analysis.

Bioavailability and uptake

The bioavailability can be affected by sorption and volatilization. Relevant physico-chemical properties are listed in Table 7. The sorption of benzanthrone is characterized by the

octanol/water partition coefficient. An experimental value is provided from the EPISUITE database. The estimation via KOWWIN from EPISUITE yields a similar result. The sorption coefficient (K_{oc}) has been taken into account to estimate sorption. The estimation of the water solubility has been carried out to support the validation of ecotoxicity data. The volatility has been evaluated by the Henry's law constant and by the vapour pressure, both estimated via EPISUITE.

Table 7. Properties related to partitioning.

Property / unit	Result	Type of information	Source
Water solubility			
S_w (25°C)	0.78 µM (0.18 mg/L)	Calculated	EPISUITE v4.00
	5 – 10 µM (1.15 – 2.3 mg/L)	Calculated	ChemProp
Partition coefficients			
log K_{ow}	4.81	Experimental	EPISUITE v4.00
	4.73	Calculated	
log K_{oc}	3.78	Calc. (from K_{ow})	EPISUITE v4.00
Volatility: Vapour pressure (VP), Henry's Law Constant (HLC)			
VP [Pa]	2.95E-05	Calc. (Mod. Grain method)	EPISUITE v4.00
HLC [Pa·m³/mol]	6.7E-03 (25°C)	Calc. (Bond est.)	EPISUITE v4.00

Preliminary analysis of transformation, uptake and fate

Benzanthrone only marginally occurs in water due to the rather high log K_{ow} near to 5 and its rather high log K_{oc} of almost 4. The tendency to evaporate is low. Thus, benzanthrone is expected to dissipate into sediments and organic tissues. There is no dissociation in water and, thus, there is no ionization to be expected.

Benzanthrone has a molecular weight of approximately 230, which does not indicate a bulky structure. With the fused aromatic system, benzanthrone has a planar structure. This also does not suggest any size related issues.

Nonetheless, with a maximum BCF in fish of 181 the potential for bioconcentration is supposed to be moderate (Table 8).

Table 8. Bioconcentration (BCF) and bioaccumulation (BAF) of benzanthrone.

Property	Result	Type of information	Source
BCF	61–181 (Fish)	Experimental	NITE
	180 L/kg ww	Calculated	EPISUITE v4.00
	86 L/kg ww	Calculated (Arnot-Gobas method)	EPISUITE v4.00
BAF	117 L/kg ww (mid trophic)	Calculated (Arnot-Gobas method)	EPISUITE v4.00

Selection of suitable query compounds

The analysis of the degradation processes and rates reveals that significant degradation will only take place in the air compartment. For aquatic toxicity this is less important, so that benzanthrone is considered to be stable in water. For this reason, no transformation products have to be taken into account. However, with respect to the rather high biotransformation rates (short biotransformation half-live), biotransformation products may be important. Further investigations on the relevance of these transformation products would be required here, but were not feasible within the present case study. In extreme cases, additional experimental research would be required.

Here, the further investigation, i.e. Steps 2-6, will focus on the parent compound benzanthrone. Possible metabolites will not be considered separately. However, they will still be taken into account within the parent compound analysis in order to obtain some possible hints on their relevance.

Since phototoxicity has been identified as a particular issue for benzanthrone, its toxicity may differ in dependence of UV radiation. This will be taken into account in the following steps.

I.3 Step 2: Mode-of-action and effect-level classification

In this step, different classification schemes are applied in order to get information on probable modes of action as well as on the effect level. The recommended mode-of-action classification schemes for aquatic toxicity were applied (Table 9).

Table 9. Classification of benzanthrone according to several mode-of-action and effect-level prediction schemes.

Classification Scheme	Result
Verhaar (QSAR Application Toolbox)	Cannot be classified
Verhaar (ChemProp)	Cannot be classified*
Russom (ChemProp)	Class 1 (Nonpolar narcosis)*
Lipnick (ChemProp)	No alert
OASIS toxicity MoA (QSAR Application Toolbox)	Baseline toxicity
Protein binding (QSAR Application Toolbox)	Nucleophilic addition (A_N) at carbonyl group

* Out of the chemical domain (Kühne et al., 2009).

Independently from the applied software it was not possible to classify benzanthrone by the Verhaar scheme (Verhaar et al., 1992). The reason here is the keto group. The Verhaar scheme explicitly defines lists of substructures for each mode of action including non-polar narcosis, but the keto group in benzanthrone does not fulfill any of these structural constraints. Furthermore, the ChemProp analysis (Kühne et al., 2009) reveals that the compound is out of the domain of the Verhaar model.

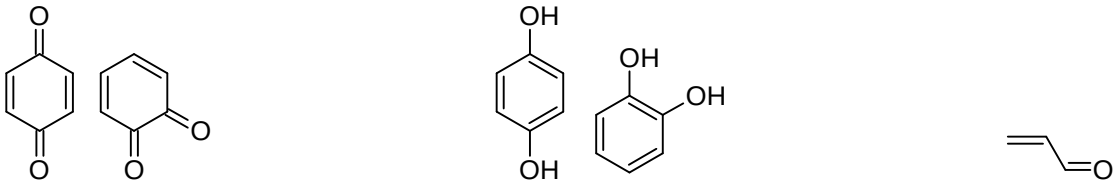
The Russom scheme (Russom et al., 1997) predicts benzanthrone's mode of action to be non-polar narcosis. However, this scheme automatically assigns all compounds without particularly identified substructures to this class. For this reason, consideration of the applicability domain is of particular importance. According to the ChemProp domain analysis, benzanthrone is out of the model domain.

None of the Lipnick rules for excess toxicity (Lipnick, 1991) are fulfilled (ChemProp, no domain information available). The OASIS toxicity MoA predicts benzanthrone to be baseline toxic, while the protein binding profiler identifies a nucleophilic addition to the carbonyl group.

Except the OECD Toolbox prediction regarding protein binding, none of the models indicate excess toxicity or a non-narcosis mode of action. However, this assumption needs to be considered as not very reliable due to the obvious mismatch of the (chemical) applicability domain, resulting from the lack of respective data for similar compounds.

The classification models were not able to confirm the previously identified phototoxic potential of benzanthrone.

Looking at the generated metabolites from Step 1, some reactive groups can be found. Figure 5 shows three structural alerts selected via expert judgment and detected by ChemProp models. Benzoquinones (a) are DNA binding agents, and they are also known to be redox-cycling agents as well as electrophilic reactive Michael-type acceptors. Hydroquinones (b) can be biotransformed into quinones. Michael-type acceptors (c) are known for their protein binding potency. These substructures are well known to trigger unspecific reactive toxicity.



a) Benzoquinone derivative b) Hydroquinone derivative c) Michael-type acceptor

Figure 5. Structural alerts for excess toxicity occurring in metabolites of benzanthrone.

I.4 Step 3: Initial Assessment of transformation routes, uptake, toxicity and fate

In Step 1, benzanthrone has been identified as the parent compound, and its chemical structure has been defined and validated. Preliminary information on benzanthrone has been collected. Benzanthrone is expected to be persistent in water (low biodegrability, no hydrolysis) although it is principally photodegradable. Furthermore benzanthrone has no tendency to evaporate, and probably will be sorbed in soils and sediments. However, despite the rather high log K_{ow} the bioaccumulation potential is relatively low.

Biotransformation is possible, but the relevance of the generated metabolites would need further investigation.

Concerning the likelihood of exposure of aquatic organisms, even though there seems to be a low risk for large scale exposure via the water compartment, there is a considerable risk of sediment contamination.

Classification schemes applied in Step 2 (Russom, Lipnick and the OASIS schemes) as well as the effect-level structural alerts predict benzanthrone to be a baseline narcotic substance. The Verhaar scheme was not applicable at all. However, the reliability of these results is quite low due to the applicability domain mismatch of some models and the suspected domain mismatch for the models without known training sets.

The protein binding screening predicts a possible nucleophile addition at the carbonylic group. At least this hints to a possible toxicity. Together with the low reliability of the other models there is an information gap. This points to the requirement of further investigations.

In Step 2, some potential metabolites have been identified, which contain hydroquinone or quinone substructures known to likely act as redox-cycling agents and as (pro)electrophilic compounds. It is expected that some of the metabolites of benzanthrone reveal significantly higher toxicity than the parent compound. This also needs further investigation.

Benzanthrone has been identified to be potentially phototoxic in Step 1. In consequence, toxicity will depend on the UV radiation. There will be two different toxicity values to take into account in the next steps, including and leaving out phototoxicity.

Summarizing these considerations, the issues to be listed at the end of Step 3 are:

- Molecular structure(s) of the compound(s) of interest to represent the chemical substance under evaluation:

  - Mainly the parent compound, but some metabolites may be of relevance also.

- Endpoint(s) of interest according to the envisaged use pattern of the substance and the associated REACH requirements

  - The endpoint of interest is fish acute toxicity.

- Potentially additional issue(s) of significant relevance due to information gained during the initial analysis

  - Due to phototoxicity, toxicity via UV radiation needs to be considered also.

- Endpoint(s) sufficiently addressed for the final evaluation through the availability of appropriate experimental information

  - There is a reliable experimental value for zebrafish.

- Endpoint(s) in need of further information for their final evaluation according to the REACH requirements

  - There is an experimental value for *Oryzias latipes*, but not reliable (above S_w).

  - There is a non-standard photo-induced toxicity value for fathead minnow.

  - Acute toxicity to fathead minnow without UV radiation is not known.

  - Without UV radiation, narcosis level toxicity is expected but not confirmed.

- In case of evaluation-relevant information gaps: Non-test method(s) options as far as available for potentially addressing the remaining information needs

  - Perform Steps 4-6 to gain further information on fish acute toxicity.

I.5 Step 4: Chemical categories

The aim of grouping approaches is to classify compounds into categories. Three opportunities to determine similar compounds are possible: At best, the compound belongs to an already existing category, and this category contains a sufficient number of compounds and experimental data. Alternatively, new categories are developed *ad hoc*, or structural similarity can be exploited.

In the first grouping step (4a) it is checked whether benzanthrone can be classified into an existing category. The OECD QSAR Application Toolbox suggested two sources with available categories: OECD HPV Chemical Categories and US-EPA New Chemical Categories. Benzanthrone does not belong to any category of the OECD HPV Chemical Categories, but to the neutral organics of the US-EPA New Chemical Categories. However, due to the possible phototoxicity the category neutral organics cannot be applied without additional care. It may only be sufficient for the non-radiation part of the analysis, but is not considered to be useful for this example.

Although several other models to derive analogous compounds are known, these alternatives seem to be “black box” methods. Furthermore, none of these other models are freely available. Therefore, Step 4a did not yield sufficient results.

For Step 4b (newly formed categories), several classification schemes implemented in the QSAR Application Toolbox were used to specify a category (Table 10). They were applied sequentially, i.e. with a relatively broad category. The category was confined and specified in several sequential steps.

Table 10. Newly formed categories using the QSAR Application Toolbox.

Category	**Total number of compounds**	**Number of compounds with fathead minnow 96-h LC_{50} values***
Polycyclic aromatic compounds with additional keto group	614	563
Compounds classified as baseline narcotics by OASIS acute toxicity MoA	198	174
Only compounds without DNA-binding	172	158
Only compounds without estrogen receptor binding (no OH/NH_2 groups)	171	157
Only compounds with keto group enabling nucleophilic addition (A_N)	166	152

* Experimental and estimated values.

First, chemical structures were used to categorize benzanthrone. Fused aromatic systems with ketones were used; the biphenyl functional group was neglected. This group was further confined according to further modes or mechanisms of action.

The final category covers 166 compounds. However, for only three of these compounds experimental fish toxicity data were available. In practice, this would result in the impossibility to use this category for a read-across approach. Nevertheless, for demonstration purposes, estimated fish toxicity values have been included in this example to achieve a sufficient size of the data set.Including the estimated data, the final data set comprises 152 chemicals. Again it needs to be emphasized that this estimated data should not be used for read-across in a real assessment. Obviously to avoid such an inadequate use, the 2.0 edition of the QSAR Application Toolbox does not provide calculated values for that purpose anymore.

Concerning the evaluation of information gained from Step 4, the achieved analogue compounds are comparable in terms of substructures and in the expected modes or mechanisms of action. With regard to the physico-chemical descriptors, at least some key properties of the most similar analogues are in the same range as for the parent compound. The metabolism pathway of analogous compounds could not be compared.

To demonstrate Step 4c (grouping with structural analogues), the ChemProp database was searched for similar compounds based on atom-centered fragments (ACF) similarity. Compounds above the similarity threshold of 0.5 were selected, yielding 15 chemicals as possible reference compounds for read-across (Table 11).

Table 11. Similar compounds based on ACF similarity (Simple 1st order similarity according to Kühne et al., 2009).

Substance	CAS	1st order ACF similarity[1]	Molar mass (g/mol)
Benzophenone	119-61-9	0.875	202
Fluoranthene	206-44-0	0.824	202
Dibenzo(b,d)chrysene-7,12-dione	128-66-5	0.818	154
Biphenyl	92-52-4	0.800	128
Anthracene	120-12-7	0.750	179
Phenanthrene	85-01-8	0.750	166
Naphthalene	91-20-3	0.714	202
Anthraquinone	84-65-1	0.706	154
Acridine	260-94-6	0.688	128
Fluorene	86-73-7	0.645	179
1-Methylacenaphthylene	19345-99-4	0.645	166
Benzo(a)pyrene	50-32-8	0.632	128
1-Hydroxy anthraquinone	129-43-1	0.629	179
2-Aminoanthraquinone	117-79-3	0.629	166
Acenaphthene	83-32-9	0.533	154

[1] Based on the number of common ACFs in both molecules.

I.6 Step 5: Read-across

Read-across means interpolation or extrapolation of values and is based on the identification of similar compounds. Here, similar compounds were obtained from the Steps 4b and 4c.

In Step 0, several database entries for fish toxicity values were collected from the literature and from the QSAR Application Toolbox 2.0 (Table 12).

Table 12. Available fish toxicity data.

	Fish acute toxicity	**Results in µM**	**Results in log [mol/L]**
Experimental values	96-h LC_{50} (BUA 2004)	2.39	-5.62
	50-min LC_{50} (Oris & Giesy, 1987)*	0.215	-6.02
Estimated database value	96-h LC_{50} (QSAR Application Toolbox)	1.30	-5.89

* Also included in QSAR Application Toolbox

The first item (BUA, 2004) is a reliable toxicity value (standard conditions, without any UV-radiation). However, it is measured for zebrafish *Danio rerio* but not for fathead minnow.

The data taken from Oris & Griesy cannot be considered as a normal toxicity value. The goal of their investigation actually was not to determine a somehow normalized LC_{50} for a given radiation. Instead, for a given concentration and radiation the radiation time was varied. In result, a time span is known for which this particular concentrations becomes a LC_{50} value. The QSAR Application Toolbox denotes this value as LT_{50}. In 50 minutes with a certain UV-Vis radiation and a concentration of 0.215 M benzanthrone, 50% of fish larvae died. This indicates an increased toxicity for benzanthrone with UV radiation and confirms the initial assumption.

With regard to the uncertainty of the mode of action, the estimated value from the QSAR Application Toolbox should also be taken with care.

Interspecies considerations

The data base regarding fathead minnow toxicity data is rather poor for compounds with sufficient similarity to benzanthrone. Interspecies correlation may become a possible solution here. To investigate potential interspecies differences between zebrafish and fathead minnow, cross-checking of available toxicity data (QSAR Application Toolbox 1.1) has been carried out (Table 13). Unfortunately the number of compounds with available toxicity values of the two species is also very low, and the small set contains some salts and groups of chemicals without unique structure (e.g., ethoxylated alcohols, $C_{14\text{-}15}$).

At least, in 10 of these 14 cases the toxicities towards these two species do not differ for more than half an order of magnitude, and additionally two are within one order of magnitude. There a two exceptions, N-methyl aniline and N,N-dimethyl aniline. Here, the toxicity towards zebrafish is about 2.5 times higher, resulting in lower LC_{50} values. A possible

explanation is a mode of action yielding excess toxicity to a different amount. Anilines are known for specific effects towards fish.

Table 13. Comparison of the toxicity toward zebrafish and fathead minnow.

Substance	CAS	LC_{50} [µM]	
		Zebrafish	Fathead minnow
1-Pentanol	71-41-0	6.01	4.12
Nitrobenzene	98-95-3	0.91	0.56
Benzonitrile	100-47-0	1.26	0.68
N-Methylaniline	100-61-8	0.0007	0.68
Cyclohexanol	108-93-0	13.80	5.59
Pyridine	110-86-1	6.47	1.21
Hexyl alcohol	111-27-3	1.41	4.10
1-Heptanol	111-70-6	0.54	0.26
N,N-Dimethyl aniline	121-69-7	0.002	0.47
Na Salt of pentachlorophenol	131-52-2	0.004	0.0007
Uranyl acetate	541-09-3	0.01	0.01
Dichlobenil	1194-65-6	0.09	0.04
Na_3 Salt of carboxymethoxy-butanedioic acid	34128-01-3	9.27	8.31
Ethoxylated alcohols, C_{14-15}	68951-67-7	0.004	0.003

With the focus on general fish toxicity and regarding the experimental uncertainty, the interspecies differences can be neglected basically for read-across purposes.

Quantitative read-across with categories

In this step, the toxicity has been estimated using the QSAR Application Toolbox. This software package provides two techniques, read-across and trend analysis. Read-across averages the results of similar compounds. Similarity is obtained by comparing selected properties. The second technique provided by the QSAR Application Toolbox is the more sophisticated trend analysis. It attempts to develop an *ad hoc* model, in which the endpoint is a function of an additional descriptor. For a regression, all analogous compounds are taken into account. The required endpoint for the target compound then is estimated through the application of this specific model.

Here, log K_{ow} or E_{gap} (the E_{gap} or HOMO-LUMO gap is the calculated energy difference between HOMO and LUMO) were applied as read-across descriptors.

As already pointed out, the results of Step 4b have been used for illustration purposes only.

Table 14. Read-across results for LC_{50} by grouping into categories and applying log K_{ow} [1] or E_{gap} as similarity descriptors (QSAR Application Toolbox).

Compound	Number of compounds	Read-across [µM] (mg/L)		Trend analysis [µM] (mg/L)	
		log K_{ow}	E_{gap}	log K_{ow}	E_{gap}

Polycyclic aromatic compounds with additional keto group	563	0.971 (0.224)	0.021 (0.005)	0.730 (0.168)	0.454 (0.105)
Compounds classified as baseline narcotics by OASIS acute toxicity MoA	174	0.849 (0.194)	0.676 (0.156)	1.160 (0.267)	1.400 (0.322)
Only compounds without DNA-binding	158	0.926 (0.213)	0.677 (0.156)	1.140 (0.263)	1.430 (0.329)
Only compounds without estrogen receptor binding (no OH/NH_2 groups)	157	0.926 (0.213)	0.677 (0.156)	1.080 (0.249)	1.050 (0.242)
Only compounds with keto group enabling A_N	152	0.926 (0.213)	0.677 (0.156)	1.110 (0.256)	1.050 (0.242)

[1]Estimated, only for demonstration purposes

Considering the results listed in Table 14, data reduction by classification schemes and profiling does not change the estimated values notably. Only from line one to line two, where the OASIS MOA classification scheme is applied in order to exclude reactive compounds, the estimation results are changing up to factor 3 for E_{gap} as similarity descriptor.

Comparing all estimation results to each other, only the read-across method with E_{gap} as descriptor yields significantly lower LC_{50} estimations than the other methods. This seems to be plausible because phototoxicity is related to the HOMO-LUMO gap.

Detailed examination of individual LC_{50} values used for the toxicity estimation reveals large variations in case of the read-across approaches: There are 1.5 orders of magnitude differences for the approach with log K_{ow} as descriptor and even 3 orders of magnitude for the E_{gap} based approach. These differences do not disappear when narrowing the data set by the several classification steps.

Mainly the results using log K_{ow} are rather close to the experimental value for the zebrafish, thus supposing some confidence. If they were obtained from experimental data instead of calculated values as in case of this demonstration, they could serve as newly gained information in Step 7.

Quantitative read-across with compound selection from similarity

First, similar compounds were selected by ACF similarity (see Step 4c). In the ChemProp database, experimental fathead minnow 96 h LC_{50} values were available for six of the fifteen chemicals (Table 15). As a first estimation, the geometric mean (i.e., the arithmetic mean of logarithmic values) of the toxicities of these compounds was used as toxicity estimation via read-across. With this approach, an LC_{50} of 10.3 µM was achieved. The number of compounds, for which experimental data are available, is rather low and, thus, read-across cannot be recommended. It is shown here for demonstration purposes only.

Closer examination of the analogous compounds identified by ACF similarity reveals that the toxicity of fluoranthene is more than 2 orders of magnitude below the toxicities of the other compounds. Due to this large variation in data, the obtained read-across value may appear unreliable. However, since fluoranthene is one of the most similar chemicals to

benzanthrone (cf. Table 11), there is no justification to omit this value from consideration. Even more, as benzanthrone, fluoranthene is known to be phototoxic to zebrafish

Table 15. Fathead minnow LC_{50} values (ChemProp database) for analogous compounds identified by ACF similarity (ChemProp).

Substance	96-h LC_{50} [µM]	log LC_{50} [mol/L]
Benzophenone	73.2	-4.14
Fluoranthene	0.15	-6.82
Biphenyl	15.1	-4.82
Naphthalene	47.9	-4.31
Acridine	13.8	-4.86
Acenaphthene	11.2	-4.95

Next, the QSAR Application Toolbox (both edition 1.1 and 2.0) was applied. Both techniques, similarity based read-across and trend analysis, were used. In the QSAR Application Toolbox database, data were available for 7 compounds comprising the six chemicals with LC_{50} data from ChemProp and fluorene. The experimental values (Table 16) slightly differ between both versions as well as compared to the ChemProp data. In case of multiple data, the average of them was used for calculation. Again, calculating the geometric mean yields LC_{50} values of 21.5 µM (1.1) and 19.8 (2.0), respectively.

In the default mode of the QSAR Application Toolbox, log K_{ow} (estimated by EPISUITE) is used to pick-up the five most similar compounds (of the 15) for read-across. This yields LC_{50} predictions of 11.0 µM in edition 1.1 and 11.4 µM in edition 2.0.

In addition to the low value for fluoranthene, there is a rather high value for fluorene. Obviously, this increases the average, thus probably increasing the error of this approach. The QSAR Application Toolbox read-across approach excludes naphthalene and acridine, and applys a different algorithm to obtain the result from the individual data. Surprisingly, this works well with keeping in both fluoranthene and fluoren, even though their toxicities are much higher or lower. Obviously, a compensation of both of them occurs.

Table 16. Fathead minnow LC_{50} values (QSAR Application Toolbox) for analogous compounds identified by ACF similarity (ChemProp). LC_{50} in µM, logarithmic values relate to mol/L.

Substance	Edition 1.1		Edition 2.0	
	96-h LC_{50}	log LC_{50}	96-h LC_{50}	log LC_{50}
Benzophenone	81.3	-4.08	75.6	-4.12
Fluoranthene	0.47	-6.33	0.15	-6.82
Biphenyl	12.6	-4.90	16.8	-4.77
Naphthalene	47.9	-4.31	44.3	-4.35
Acridine	12.9	-4.89	13.5	-4.87
Fluorene	602	-3.22	602	-3.22
Acenaphthene	11.2	-4.95	16.5	-4.78

Then, the default parameters for trend analysis based on 96-h LC_{50} (fathead minnow) data were applied. Log K_{ow} (estimated via EPISUITE) was used as descriptor for a linear *ad hoc* model. The toxicity is estimated to be 0.674 µM in edition 1.1 and 1.64 µM in edition 2.0. These results are more than one order of magnitude lower than those from the read-across methods.

The results of all methods used in the Step 4c selection are rather uncertain due to issues discussed already, and thus cannot be taken into account for the risk assessment.

Quantitative read-across without user-defined grouping

In addition to the use of the grouping results from Step 4b and 4c, ChemProp provides some fully automated quantitative read-across models based on atom centered fragments (ACFs) as similarity descriptors. In contrast to the other techniques applies here, no user interaction to define groups or similarities is carried out here. A built-in database with experimental data is used to obtain the results from similar compounds. A model for fathead minnow is available (Schüürmann et al., 2011).

Dealing with the trade-off between a rather broad range of applicability and a reliability that should be as high as possible, the model offers three reliability levels denoted as screening, intermediate and high, differing in similarity thresholds for reference compounds to be taken into account. For plausibility, the implementation additionally checks the results against the estimated water solubility. Furthermore, it tests the applicability domain in terms of the physico-chemical and chemical domain.

Applying the screening levels yields an EC_{50} of 8.2 µM, while the result for the intermediate level is 2.71 µM. At the high level, there are not enough sufficiently similar compounds available, and thus no result is obtained. Since the water solubility (estimated by several models within ChemProp) is about 5–10 µM, the screening level result should be rejected.

However, ChemProp indicates that benzanthrone is outside of the chemical domain of the read-across model (while at least inside of the physico-chemical property domain). Thus, the usefulness of this result is limited.

I.6 Step 6: QSAR

In this step, toxicity estimations using reliable and suitable expert systems should be carried out. The QSAR prediction database does not contain any entry for benzanthrone. Likewise, the QSAR model inventory currently does not provide any model for acute fish toxicity. Other sources for suitable QSARs are required.

There are several models to estimate the baseline toxicity of a compound. Even though no obvious evidence of excess toxicity for benzanthrone (at least except phototoxicity) could be found in the previous steps, results should only be taken as the minimum toxicity.

ChemProp provides two models based on K_{ow} for fathead minnow baseline toxicity (Veith et al., 1983; van Leeuwen et al., 1992) as well as two Abraham type linear solvation-energy relationships (LSER) approaches (Gunatilleka & Poole, 1999; Hoover et al., 2005). The K_{ow}

models yield results similar to the water solubility and thus are equivocal, because the solubility limit cannot be exceeded. The LSER results are near to the experimental value for zebrafish (Table 17). Again, benzanthrone is not within the chemical domain of these models.

Both LSER approaches also provide results for other fish species. This allows for a further comparison to the experimental zebrafish and fathead minnow data in Step 5. The results differ up to 1.5 orders of magnitude. Furthermore, the models suggest fathead minnow is one of the least sensitive species to benzanthrone. In order to assess fish toxicity in general, this needs to be taken into account. The also available K_{ow} model for guppy (Könemann 1981; implemented in ChemProp) yields a result above the water solubility, but again benzanthrone is not in its chemical domain.

ECOSAR v1.0 implemented in EPISUITE v4.00 (Table 18) estimates a fish toxicity (96-h LC_{50}) of 3.07 µM, categorizing benzanthrone as neutral organic with regard to its compound classes.

Furthermore, the QSAR models for fish toxicity implemented in the QSAR Application Toolbox (Table 18) was applied. The models M 2-4 only apply the log K_{ow} (estimated by EPISUITE) as descriptor. They address baseline toxicity and polar narcosis. The largest LC_{50} value (lowest toxicity) is obtained by the model M3 (unpolar narcosis), followed by M2 (combined baseline and polar narcosis), and M4 (polar narcosis). At least for a certain range of K_{ow}, this expected relation directly follows from the coefficients of the equations and thus is no special result for benzanthrone. The QSAR model M1 applies log K_{ow} and E_{lumo} and had been developed to estimate the toxicity of compounds acting via narcosis or unspecific electrophilicity. The estimated LC_{50} value for benzanthrone is lower than from the other models. The QSAR Application Toolbox checks the descriptor domain. Benzanthrone is out of the applicability domain of M1. Since it is inside the domain of the other models, this mismatch can only arise from the E_{lumo} value. All of the models discussed here do not address phototoxicity.

Table 17. Baseline toxicity estimated in ChemProp.

Model	LC_{50} (µM)	Log LC_{50} [mol/L]
Log K_{ow} models		
Veith et al., 1983	11.2	-4.92
van Leeuwen et al., 1992	8.23	-5.08
LSER model: Gunatilleka & Poole, 1999		
Fathead minnow (*Pimephales promelas*)	3.16	-5.50
Guppy (*Poecilia reticulata*)	0.71	-6.15
Golden orfe (*Leuciscus idus melanotus*)	0.13	-6.90
LSER model: Hoover et al., 2005		
Fathead minnow (*P. promelas*)	3.49	-5.46
Guppy (*P. reticulata*)	0.70	-6.15
Golden orfe (*L. idus melanotus*)	0.10	-6.99
Bluegill (*Lepomis macrochirus*)	2.18	-5.66
Goldfish (*Carassius auratus*)	0.95	-6.02
48 h Medaka high-eyes (*O. latipes*)	1.26	-5.90
96 h Medaka high-eyes (*O. latipes*)	0.11	-6.95

Table 18. Toxicity estimates by EPISUITE and QSAR Application Toolbox models with default parameters.

Model	Acute fish toxicity LC_{50} [µM]	Acute fish toxicity LC_{50} (log [mol/L])
Baseline (ECOSAR)	3.07	-5.51
M1 (narcosis +electrophile)	1.84	-5.74
M2 (narcosis)	2.70	-5.57
M3 (non-polar narcosis)	3.94	-5.40
M4 (polar narcosis)	2.65	-5.58

Analysis of the log K_{ow} models

Most of the QSAR models applied here employ log K_{ow}. The results of both ChemProp models are rather similar, as well as the four QSAR Toolbox models. The ECOSAR result is insight the range of the Toolbox models, but the ChemProp results are much higher, indicating lower toxicity, and in particular exceeding the water solubility.

The underlying equations are listed in Table 19. Obviously, ECOSAR and M3 are very similar to the van Leeuwen model. This suggests that the observed differences between the ChemProp models (including van Leeuwen) and ECOSAR and the QSAR Application Toolbox results (including M3) may be mainly caused by different log K_{ow} values.

Table 19. Model equations of the K_{ow} models for fathead minnow 96 h LC_{50}.

Model	log K_{ow} (exp.) EPISUITE	log K_{ow} (calc.) EPISUITE	log K_{ow} (calc.) ChemProp

	4.81	**4.727**	**4.323**
	LC_{50} models in ChemProp		
Veith	8.25	8.55	11.19
van Leeuwen	3.17	3.73	8.23
	LC_{50} model in EPISUITE		
ECOSAR	2.60	3.07	6.97
	LC_{50} models in QSAR Application Toolbox		
M1 (narcosis +electrophile)	1.65	1.84	3.14
M2 (narcosis)	2.31	2.70	5.73
M3 (non-polar narcosis)	3.34	3.94	8.78
M4 (polar narcosis)	2.30	2.64	5.18

Table 20. Estimation results applying different log K_{ow} values (results in µM).

Model	**log K_{ow} (exp.) EPISUITE: 4.81**	**log K_{ow} (calc.) EPISUITE: 4.727**	**log K_{ow} (calc.) ChemProp: 4.323**
Veith	8.25	8.55	11.19
van Leeuwen	3.17	3.73	8.23
ECOSAR	2.60	3.07	6.97
M1 (narcosis +electrophile)	1.65	1.84	3.14
M2 (narcosis)	2.31	2.70	5.73
M3 (non-polar narcosis)	3.34	3.94	8.78
M4 (polar narcosis)	2.30	2.64	5.18

The models included in the QSAR Application Toolbox as well as the ECOSAR model use a log K_{ow} estimated by KOWWIN from EPISUITE. ChemProp performs a model selection based on compound classes, for benzanthrone it applies a fragment model (Marrero & Gani, 2002). In Table 20, the predictions of the discussed models applying the different log K_{ow} values (including the experimental value from the EPISUITE database) as input data are shown. The Veith model remains above the solubility limit in all cases. The van Leeuwen model result indeed now agrees to ECOSAR and M3 with the K_{ow} values taken from EPISUITE.

When applying other K_{ow} models known to be reliable, the result of the log K_{ow} prediction is 4.67±0.27 (ACD/LogP, Advanced Chemistry Development, Inc. 2009) and even 5.07 (SPARC). Taking the van Leeuwen model, this will yield LC_{50} values of 4.17 (ACD, regarding the uncertainty, 2.3 … 7.7) and 1.9 (SPARC). Due to the logarithmic natures of the K_{ow} prediction models as well as of the K_{ow}-LC_{50} relationship, the rather small uncertainty obtained from ACD already yields values from below the EPISUITE model almost up to the estimation based on the Marrero & Gani K_{ow} prediction.

The results demonstrate the importance to validate not only the results, but also the model input parameters. In practice, the best known data should be used. Usually, experimental values should be preferred.

I.7 Step 7: Overall assessment

In this step, all available testing and non-testing information is combined and evaluated. This starts with reviewing Steps 1-6.

Step 1. Benzanthrone has been predicted to be stable in water. With regard to partitioning, sorption into soil, sediment and organic tissues is likely. Bioaccumulation is expected to be moderate. Furthermore, phototoxicity is suspected. Probably, there is a rapid biotransformation. Metabolites were identified, but their relevance could not be specified.

Step 2. Classification schemes predict narcosis as mode of action with the exception of binding to proteins via a carbonyl group (A_N mechanism). The application of effect level structural alerts yields the expectation of narcosis effect level. However, the model results due to applicability domain mismatches the model results should be considered with care. Some metabolites possess reactive substructures (benzoquinones, hydroquinones and Michel-type acceptors).

Step 3. Phototoxicity is expected for benzanthrone and thus identified as additional important parameter for further investigations. The relevance of toxic metabolites also needs to be considered, but was not addressed in this case study.

Step 4. No predefined category could be applied. New categories were formed and similar compounds were identified. Both approaches provide analogous compounds.

Step 5. Several read-across methods were applied. However, since the number of available data was very poor, calculated data were used for demonstration purposes. In a real assessment, no valid results would have been obtained here. Alternatively, an automated read-across model could be applied, but benzanthrone was out of the model domain and thus the result is not reliable.

Step 6. The baseline toxicity towards fish was estimated by several models. Interspecies considerations indicate differences mostly within one order of magnitude, but it turned out that fathead minnow is probably one of the least affected fish species. Thus, focusing on fathead minnow toxicity would underestimate the fish toxicity in general. Comparison to the few experimental data does not reveal larger disagreemets. Phototoxicity could not be addressed.

Using the checklist developed for Step 7, the results for acute fish toxicity of benzanthrone can be summarized as follows:

1. Chemical structure(s) of the compound(s) representing the target chemical: This has been done correctly. There is a clearly defined structure, and there are no ambiguities as e.g. tautomers.

2. Chemical structure(s) of relevant transformation product(s) for the environmental compartment(s) of interest: No significant transformation products have been identified.

3. Chemical structure(s) of relevant metabolite(s) for the organism of interest: There is a remarkable biotransformation potential. Metabolites with possible relevance for toxicity were identified, but the probability of their occurrence needs to be further explored.

4. Chemical structure(s) of all analogue compounds taken into account: Analogue compounds have been identified, but generally there were only few experimental data available for them.

5. Endpoint(s) of interest according to the envisaged use pattern of the substance and the associated REACH requirements: This example focuses on aquatic toxicity. Thus, acute fish toxicity has been addressed.

6. Additional parameter(s) of relevance due to information gained during the analysis: Phototoxicity needs to be considered.

7. Experimental data concerning relevant physico-chemical and fate-related properties, and relevant ecotoxicological or human toxicological effects, including pertinent information about the data quality: Only very few experimental values were available.

8. Waiving opportunities due to sufficiently limited exposure according to respective guidelines: Even though the partitioning properties indicate limited exposure in water, this is not sufficient for waiving.

9. Non-testing data for relevant physico-chemical and fate-related properties as well as for relevant ecotoxicological or toxicological effects, augmented by pertinent information concerning the respective model applicability domains and expected levels of confidence: Physico-chemical and fate-related properties basically could be estimated with sufficient confidence. Toxicity estimations were possible, but less reliable.

10. Adequate documentation of the non-testing methods used: Cf. Steps 1, 5 and 6.

11. Remaining data gaps: There is only limited confidence on the acute fish toxicity, and a lack of information on phototoxicity. The relevance of metabolites with potential excess toxicity needs to be explored.

II WoE approach

II.1 Phase I: Minimum information level

The results of the initial assessment following Steps 0-3 of the NT approach are summarized below:

A) Verification of the structure

The structure of benzanthrone was verified.

B) Collection of available information

and

C) Preliminary analysis of toxicity, uptake and fate including identification of possible relevant metabolites

The evaluation of the physico-chemical and fate properties reveals that benzanthrone is expected to be stable in water, but can adsorb to sediment and suspended particles. The evaporation potential is low. Based on measured data, the bioaccumulation potential in fish is moderate.

Exposure of fish via the water phase is expected to be the main route, but oral exposure is also likely. Biotransformation in fish is estimated to be fast (0.4 days), but the relevance of identified potential metabolites is not assessed further. It is, however, expected that some of the metabolites are more toxic than the parent compound.

The following classification schemes were applied in order to predict benzanthrone's mode of action: Verhaar (OECD Toolbox, ChemProp), Russom, Lipnick (both in Chemprop), OASIS toxicity MoA and the Protein binding scheme (both in the OECD Toolbox). The Verhaar scheme was not applicable to benzanthrone. The Russom scheme identified benzanthrone as baseline toxic, but the result was not considered further due to application domain problems.

The Lipnick scheme did not indicate excess toxicity. Except for the OECD Toolbox protein binding profiler, which predicted a nucleophilic addition at the carbonylic group, benzanthrone was classified as baseline narcotic by the OASIS toxicity MoA and a ChemProp structural alert model for fish toxicity. However, in the latter model benzanthrone was again out of the applicability domain. Due to these shortcomings, resulting from a lack of respective data for similar compounds, the classification scheme results should be considered with care.

The classification models were also not able to confirm the previously identified phototoxic potential of benzanthrone. However, based on its use as dyestuff intermediate and photosensitizer, phototoxicity of benzanthrone can be expected, and the compound was indeed identified to fulfill the Mekenyan criteria for phototoxicity. Therefore, this possible property will be taken into account in the further analyses.

Although the water solubility of benzanthrone is low (0.18 mg/L at 25°C), exposure based waiving is not an option, since the substance might still be available for fish in concentrations which may induce toxic effects.

Note: The preliminary assessment of available *in vivo* data as reported in the BUA report No. 254 (2004) indicated that a reliable result on fish acute toxicity derived according to OECD TG 203 could be used for covering this endpoint. Therefore, the evaluation of this endpoint

could have been terminated at this point. However, in order gain experience with the NT and WoE approaches and since benzanthrone is suspected to have unconsidered effects (phototoxicity), the evaluation is continued. *In vivo* results are listed and evaluated in Phase IIC.

II.2 Phase II: Extented information level incl. evaluation by WoE

IA) Evaluation of available non-testing data and read-across

Grouping and read-across

In Steps 4 and 5 of the NT approach, a search for categories and analogous compounds of benzanthrone was carried out. Existing categories were not found (OECD Toolbox), but new categories could be formed. Grouping methods implemented in the OECD QSAR Application Toolbox and the ChemProp software identified analogue compounds, which are comparable in terms of substructures or the expected mode of action.

With the OECD Toolbox the most specified group (*compounds with keto group enabling nucleophilic addition*) existed of 166 compounds, but only for three of them experimental data are available. With the ChemProp software analogous compounds were selected by ACF similarity. Fifteen possible reference compounds were selected which were above the similarity threshold of 0.5. Experimental results on acute fish toxicity are available for six of these compounds.

Due to the poor experimental database of the category formed by the OECD Toolbox, the prediction results of the two read-across models (read-across and trend analysis) were predominantly based on calculated values and are not considered further in the evaluation. The structural analogues identified by ACF were also checked for experimental data in the OECD Toolbox. Although one additional result could be obtained, the database is again too small for reliable use for read-across.

The last method applied, a fully automated read-across model implemented in ChemProp, was not useful because benzanthrone was not in the applicability domain of the model. Moreover, the LC_{50} results of all the available methods showed large variation with no reliable trend. Therefore, the results of the different read-across methods are not considered further in the WoE assessment.

QSAR results (NT Step 6)

QSAR results were gathered with the OECD Application Toolbox, the ChemProp software and with ECOSAR (v1.0). As benzanthrone was not in the applicability domain of the models implemented in ChemProp, these results are not considered further.

With ECOSAR, benzanthrone was classified as neutral organic and an LC_{50} of 0.71 mg/L (3.07 µM) was estimated, indicating baseline toxicity.

Results of the Toolbox models M2 (narcosis), M3 (non-polar narcosis) and M4 (polar narcosis), for which benzanthrone is within the applicability domain, predict LC_{50} values between 0.61 mg/L (2.65 µM) and 0.91 mg/L (3.94 µM). None of the models is capable of predicting photoxicity.

IIB) Evaluation of existing in vitro data

There are no data from *in vitro* methods available which could reflect acute fish toxicity.

IIC) Evaluation of existing in vivo data

In total, three experimental results on acute fish toxicity of benzanthrone are available. The corresponding LC_{50} values differ widely as shown in Table 21.

Table 21. Available *in vivo* results on fish acute toxicity of benzanthrone.

Reference	Guideline	Species	Result	Remark
BUA report No. 251 (2004) (BASF AG, 1992)	OECD TG 203	Zebrafish (*Danio rerio*)	96 h LC_{50} = 0.55 mg/L (2.4 µM)	GLP, without chemical analysis
BUA report No. 251 (2004)	Japanese Industrial Standard JIS K 0102-1986-71	Medaka (*Oryzias latipes*)	48-h LC_{50} > 100 mg/L (> 434 µM)	No data on GLP and chemical analysis, effect concentration far above water solubility of benzanthrone
Oris and Giesy (1987)	No guideline test	Fathead minnow (*Pimephales promelas*)	0.83 h LC_{50} = 0.05 mg/L (0.2 µM); LT_{50} = 0.83 h	Photo-induced toxicity; with chemical analysis

The study on *Danio rerio* (BASF AG, 1992 cited in BUA, 2004) was conducted under GLP conditions following an internationally harmonised guideline (OECD 203). Although no analytical monitoring was conducted the study can be considered as of sufficient reliability. Regarding its physical-chemical properties, benzanthrone is expected to be stable in water for the duration of a 96-h study.

Normally, this study would be sufficient to fulfil the REACH requirements for fish acute toxicity. However, as possible phototoxity of benzanthrone is suspected, which would not be detected in a standard OECD 203 study, further investigation of this endpoint is required. The *D. rerio* study (BASF AG, 1992 cited in BUA, 2004) will therefore only be considered as helpful additional result.

The second study mentioned in the BUA report was conducted with *Oryzias latipes* following a Japanese guideline. No data on GLP conditions and analytical monitoring are available. Besides the fact that the duration of the study (48 hours) is too short to fulfil the standard requirements as outlined in the REACH guidance document R.7b, the result appears to be not valid given that the effect concentration (> 100 mg/L) is far above the calculated water solubility limit of 0.18 mg/L. Moreover, it is by several orders of magnitude higher than the other available results.

The third study was performed with *Pimephales promelas* (Oris & Giesy, 1987) and was not conducted under guideline conditions. The study was designed to investigate the effects of UV radiation on the toxicity of benzanthrone. Larvae were first exposed for 24 h to a solution of benzanthrone (nominal: 0.0316 mg/L, measured: 0.0495 mg/L) in the absence of solar UV radiation. Test solutions were then replaced and larvae were placed under a laboratory system light bank simulating natural sunlight. Light was filtered to eliminate >99% of the radiation of

wavelengths below 315 nm. Solar UV radiation intensities were monitored: UV-B (290-336 nm) was 20 µW/cm^2, UV-A (336-400 nm) was 95 µW/cm^2. The test solutions were renewed at 12 h intervals. Larvae were fed brine shrimp once daily prior to changing test solutions. Benzanthrone concentrations were measured at 0 and 12 h. The median lethal time (LT_{50}) was determined. Mortality of the controls was less than 5% in all tests. Benzanthrone showed an acute photo-induced toxicity against *P. promelas* larvae. With an LT_{50} of 0.83 hours (0.83 h LC_{50} = 0.05 mg/L), benzanthrone had the lowest median lethal time of the 12 tested PAHs, i.e. benzanthrone had the greatest absorption-specific photo-induced toxicity.

Similar results were observed with invertebrates (*Daphnia magna*) by Newstedt and Giesy (1987) using comparable experimental conditions.

In conclusion, a guideline study of good reliability is available, but there is strong indication that photo-induced toxicity is relevant for benzanthrone.

IID) Overall evaluation by WoE

Summary of the results and evidences

Within the present project, environmental fate and exposure were only evaluated to a very limited extent and no predicted environmental concentrations were derived. In the present case study, it was assumed that no waiving due to exposure considerations is possible. Although the ESIS website lists benzanthrone as LPV (Low Production Volume) chemical, a tonnage band of more than 100 t/a is assumed in this evaluation. Hence, the REACH requirements following Annex VII - IX would have to be taken into account.

Benzanthrone is a PAH with low water solubility. However, its solubility is sufficient to be relevant for consideration of acute and chronic effects. Based on the available information it is expected to be relatively stable in water. The high log K_{OW} (4.81) indicates potential for adsorption to soils, sediments and organic tissues. Uptake via food is possible, but due to the relatively low BCF (< 200) is regarded to be of minor relevance. The predominant uptake path in fish would be through the gills. Biotransformation of the substance in the organisms is expected to be rapid. Metabolites may be of higher toxicity than the parent compound, but were not investigated further in this case study.

Most of the applicable classification schemes predict benzanthrone as baseline narcotic substance, but benzanthrone was outside the applicability domain of most of the models. However, benzanthrone fulfilled the Mekenyan criteria for phototoxicity.

Results from the different read-across methods as implemented in the ChemProp software as well as in the OECD Toolbox are not considered further due to an insufficient number of experimental results. In addition, benzanthrone was not in the applicability domain of some of the models.

Results of the available QSAR methods, having benzanthrone within their applicability domain, estimate the fish acute toxicity within a similar range. With ECOSAR, benzanthrone is predicted to be baseline toxic with an LC_{50} of 0.71 mg/L (3.07 µM). With the OECD Toolbox, Results of the M2, M3 and M4 model were similar with LC_{50} values between 0.61 mg/L (2.65 µM) and 0.91 mg/L (3.94 µM).

The available *in vivo* data indicate that the toxicity of benzanthrone to aquatic organisms is increased by photo-induction. Using intensive UV radiation, a 0.83-h LC_{50} of 0.05 mg/L (0.22 µM) was observed in fathead minnows. Without UV radiation, the LC_{50} is clearly higher as

can be deduced from a standard toxicity test with zebrafish (96-h LC_{50} = 0.55 mg/L = 2.39 µM). One result (medaka 48-h LC_{50} > 100 mg/L) was considered as not valid.

Evaluation without consideration of the in vivo results

Assuming that no *in vivo* results are available, the following decision on testing has to be based only on the non-testing information and on data from analogous compounds.

In the case of benzanthrone, the used classification schemes and read-across methods did not yield reliable results, mainly because of applicability domain problems and a lack of experimental data of analogous compounds. With one software program, a potential for phototoxicity was identified. None of the available QSAR methods considers this type of toxic action.

Within the present project, it could not be investigated whether any metabolites are relevant for acute fish toxicity and should be considered further.

The results for baseline toxicity (ECOSAR) and toxicity due to the different narcosis types (narcosis, non-ploar narcosis, polar narcosis) do not differ remarkably. The lowest LC_{50} value is 0.61 mg/L (2.65 µM) for polar narcosis (OECD Toolbox, M4).

The collected non-testing information is not sufficient to draw a substantiated conclusion on acute fish toxicity. With respect to classification and labelling, the LC_{50} can be expected below 1 mg/L as a worst case. Due to this fact and considering the lack of ready biodegradability as well as the high log K_{OW}, benzanthrone would have to be classified as ‘R50/53’ (DPD/DSD) and/or ‘Acute category 1 / Chronic category 1’ (GHS), respectively.

With respect to the risk assessment, no PNEC can be deduced from the available information. Therefore, further testing would be required. Due to the low water solubility and taking the REACH requirements following Annex IX into account, long-term testing should be considered. The potential for photoxicity has to be further investigated.

Evaluation with consideration of the in vivo results

The endpoint appears to be adequately covered with the test result from a GLP guideline study following OECD TG 203. The 96-h LC_{50} as determined with the zebrafish *Danio rerio* is 0.55 mg/L, leading to the same classification as mentioned above.

However, based on the Mekenyan criteria and *in vivo* data (Oris & Giesy, 1987) there is a strong indication that under certain conditions higher toxicity is observed, which is due to phototoxic effects. The result of a non-guideline study leads to a 0.83-h LC_{50} of 0.05 mg/L (Oris & Giesy, 1987). This result would not influence the classification according to DPD/DSD or GHS, but would result in a much lower PNEC. Moreover, the aspect of phototoxicity might need to be considered in case long-term testing is required.

As mentioned above, phototoxicity was observed in a non-guideline study performed in the laboratory under very specific test conditions. The authors (Oris & Giesy, 1987) reported that their method reflected natural sunlight conditions. This should be verified. If natural sunlight conditions can induce phototoxicity of benzanthrone, the results of the available phototoxicity study can be used as basis for regulatory purposes and risk assessment.

In Table 22, the UV radiation conditions as reported in the two biological studies (Oris & Giesy, 1987; Newstedt & Giesy, 1987) are compared to average worst-case values (at noon in

sunny southern regions). As can be deduced from the data, UV-B radiation in the two studies was similar to the worst-case values as measured in India. However, UV-A radiation under natural conditions is much lower than in the two biological tests. Therefore, further investigations are required to elucidate whether natural sunlight is able to induce the phototoxic effects of benzanthrone.

Table 22. UV radiation conditions in the biological studies compared to natural worst-case conditions

Study subject	UV radiation	Remark	Reference
Fathead minnow (*Pimephales promelas*): acute toxicity	UV-A (336-400 nm): 95 µW/cm^2 UV-B (290-336 nm): 20 µW/cm^2	Simulated sunlight; light was filtered to eliminate >99% of the radiation wavelengths below 315 nm	Oris & Giesy, 1987
Waterflea (*Daphnia magna*): acute toxicity	UV-A: 120 µW/cm2 UV-B: 25 µW/cm^2	See above	Newstedt & Giesy, 1987
Optimal utilisation of UV rays for phototherapy	UV-A: 4.7-6.6 µW/cm2 UV-B: 19.5-40.2 µW/cm^2	Average values from one year measurements between 12:00 and 13:15 in Coimbatore, India	Balasaraswathy et al., 2002

Since no data on algae growth inhibition are available, further testing is required, especially because the exposure of algae under standard test conditions includes permanent and strong illumination. Therefore, a high toxicity of benzanthrone to algae is expected. In addition, long-term testing needs to be considered. A testing strategy has to be developed.

IIIA) Test proposal considering ITS

Taking into account the available results on fish, the endpoint appears to be adequately covered. QSAR data support the 96-h LC_{50} of 0.55 mg/L. In addition, experimental data provide evidence that due to photo-induced effects an enhanced toxicity has to be expected.

Such phototoxicity data are also available for daphnids (5.4 h-EC_{50} = 0.035 mg/L). Fish and daphnids reveal similar sensitivities to photo-activated benzanthrone. Since both studies were not performed according to standard procedures and the exposure periods were clearly shorter than would normally be required, a direct use of the results in a risk assessment cannot be recommended. Moreover, it needs to be verified whether under natural sunlight conditions a phototoxic effect of benzanthrone to aquatic organisms has to be expected. Once this has been confirmed all following studies have to consider these conditions.

As already mentionend, the available results are sufficient for classification and labelling.

Under normal circumstances, i.e. if the substance of interest would not be suspected to reveal phototoxic effects, acute testing of fish and invertebrates would not be necessary because long-term testing has to be performed anyway. However, due to the findings and reasons mentioned above, in this case it might be wise to first assess the acute toxicity of benzanthrone in standard toxicity tests, but under natural sunlight conditions. The latter

conditions need to be defined considering worst case conditions (in Europe). Testing should start with invertebrates in order to gather experience with the illumination system. Fish should be tested in a second study. Beside that the effects of phototoxicity can be assessed within these studies, the comparison of the sensitivity of invertebrates and fish will help to decide whether long-term testing can be avoided with one or the other trophic level.

The following test strategy is proposed:

1) Perform the following studies:

- Acute toxicity to invertebrates under natural sunlight conditions
- Acute toxicity to fish under natural sunlight conditions

2) Decide on whether phototoxicity has to be considered and perform the following studies:

- Algae growth inhibition study
- Activated sludge respiration inhibition test

3) If based on the acute test results, one group of organisms (fish or invertebrates) are likely to be more sensitive than the other by more than one order of magnitude, long-term testing should be performed with the more sensitive group (considering specific UV light intensities, if phototoxicity can be expected under natural conditions). If both organism groups show similar sensitivity, long-term testing should be conducted with both groups starting with invertebrates.

4) Derive the PNEC and conduct the exposure and risk assessment for pelagic and benthic organisms. If risks are identified for sediment organisms, further testing, e.g. on sediment-dwellers should be considered.

5) Either the NOEC from fish or invertebrate long-term studies can be used for PBT and risk assessment.

5 Conclusions

5.1 Guidance Documents: Practicability and suggested improvements

Within this project, the guidance documents R.6 (Chapter 6.7.1 'NT approach'), R.10 (Chapter 10.2.2.2) and R.7b (mainly Chapter 7.8.5 'WoE approach' and Appendix R.7.8-5 'Evaluation of endocrine effects') were reviewed, applied and commented. Shortcomings identified during their application were documented and suggestions for improvements were developed for some important chapters. In total, more than 120 points were commented. Many of these were related to editorial and technical aspects, e.g. misleading cross references, but some restructuring of the sections is also considered necessary. Specific comments were made on those points where information was not precise enough or lacking.

The main target within the project was the development of improvements of the NT and WoE approach.

5.1.1 R.6: Non-testing approach

Step 0 ('Collecting information') is well practicable. A remark about the reliability of data and a suggestion to consider special models for mixtures, if available, should be added.

Step 1 ('Preliminary analysis') is also practicable, although metabolism should be more in the focus due to potential hazards caused by metabolites. A useful addition would be a list with examples of properties and chemical groups of concern. Unfortunately, not much non-commercial software is available.

Step 2 & Step 3: Although the steps 'Application of classification models' and 'Structural alerts' are well practicable it is not useful to separate these steps. Classical classification schemes and structural alerts are both approaches to estimate modes of action. The guidance document suggests that classical classification models are needed to derive structural alerts. However, structural alerts can also be identified with other methods.

Step 4: This step is logical and practicable, but it should be renamed to 'Initial assessment of transformation routes, uptake, toxicity and fate'.

Step 5: Although several aspects mentioned in the step 'Read-across' are practicable, the step is not well structured. It should be separated into two steps, grouping and read-across. In addition, some minor revisions of the grouping step are suggested. For instance, if a compound is found in an existing group it is necessary to check the suitability of this group. In the similarity approach, no methods are indicated to determine similarity directly from the chemical structure, i.e. using structural alerts or atom centred fragments (ACF). The 'Read-across' step can be reduced to the part of applying read-across estimation methods.

Step 6: The 'QSAR' step is well applicable, although the possible limitations of QSAR methods could be elaborated further.

Step 7 ('Final Assessment') does not need much change. However, the limitations of the named methods should be added.

In all steps, considering the applicability domains of the employed models is essential.

5.1.2 R.7b: Weight-of-evidence approach

In general, this section (R.7.8-5) considers all of the necessary steps needed to be able to perform an endpoint evaluation by weight of evidence (WoE). However, from a practical point of view it should be restructured at some points in order to lead the applicant in a more helpful way through the necessary steps. Furthermore, some additional information as well as updates on the state of the art regarding useful methods should be provided.

In the current version of the WoE approach, the single steps on data collection, i.e. compilation of physico-chemical properties, *in silico* methods, grouping and read-across as well as the collection of results from *in vitro* and *in vivo* methods are arranged in a successive way. However, it is suggested to rearrange these steps so that it becomes obvious that the information derived from these different sources can be collected independently. In a quantitative evaluation, it might be useful to weight the different data with ranks. However, the presented WoE approach is mainly a qualitative assessment, for which expert judgement on each evidence and an overall evaluation is needed. The problems which can come up during this evaluation are discussed in section 5.2.

The WoE concept should be re-structured from the 'successive-step-wise' approach to a more practical approach, which is divided into three evaluation phases: collection and preliminary evaluation of available information (Phase I: Minimum information level), (2) an extended data search and evaluation including WoE (Phase II: Extended information level) and – optionally – (3) developing of test proposals considering integrated testing strategies (ITS) (Phase III: Testing proposal level). The first phase should consider the collection of all kinds of available substance information by internal and external data search in order to perform an intial characterisation of the substance properties with respect to uptake, fate and toxicity. Before entering the second phase, some general issues should be checked. For instance, with respect to invertebrates and algae it might be more useful to directly perform a study instead of conducting a complex WoE procedure, which might be more costly and time-consuming and leads to a higher uncertainty with regard to the respective endpoint. In cases where in this or in a following phase indicators have been identified that the compound might be a candidate for a substance of very high concern (SVHC), a separate SVHC assessmentis required. Phase II follows in order to extend the search on non-testing and read across data as well as on *in vitro* and *in vivo* data. Here, non-standard information and experimental results from other species and trophic levels are collected. The compiled information gathered in Phase I and II is assessed by weight of evidence. For this purpose, the reliability of the single results is rated and the consistency of results obtained with similar methods is evaluated. An overall conclusion is drawn based on the results with the highest weight of evidence and considering the remaining uncertainty.

In cases where the endpoint of concern cannot be covered adequately by the information collected so far, a testing proposal has to be developed in Phase III. The proposal should consider all possible options in order to avoid unneccessary vertebrate testing. In the existing guidance document, the fish threshold approach is the only example for an integrated testing strategy (ITS) that helps reducing the number of fish used in aquatic toxicity testing. Meanwhile, some more ITS with respect to aquatic toxicity have been developed. Recently, the OECD has published proposals for fish testing strategies with respect to short-term and long-term toxicity. These proposals are included in the ITS section of the revised WoE approach.

5.1.3 R.7b: Evaluation of the endocrine disrupting potential

In Appendix R7.8-5, all steps are considered that are necessary to evaluate whether a substance has endocrine disrupting potential. Figure R.7.8-8 provides a very useful scheme on how the assessment procedure should be performed. It would be very helpful to move this figure and the text on pages 115 – 118 from the end of the section to its beginning, so that an overview of the whole assessment is given before single steps and, then, tests are presented.

For some of the steps, additional guidance is required to more effectively instruct the user of the guidance documents and to avoid that relevant available information is not considered in the assessment:

More guidance should be provided on how information derived from mammalian screening assays for endocrine activity and other human health endpoints from repeated-dose toxicity, carcinogenicity and reproductive toxicity studies should be evaluated with regard to the endocrine disrupting potential in aquatic organisms. Brief information on those tests / endpoints that provide relevant information would, for example, be extremely helpful. With regard to this issue, the outcome of the research project FKZ 206 67 448/05 performed for the UBA (‘Entwicklung struktur- und risikobasierter Methoden zur Identifizierung von Chemikalien mit Verdacht auf endokrine Wirkungen zur Priorisierung für das Zulassungsverfahren unter REACH’) and the draft ‘Guidance document on the assessment of chemicals for endocrine disruption’ (OECD, 2010c) appear to be very useful. In addition, cross references to the relevant sections dealing with the evaluation of human health endpoints should be included in Appendix R7.8-5.

Although *in silico* and, especially, *in vitro* screening data are likely to represent the majority of the available data on possible endocrine disrupting potential, guidance on evaluation of these data is rather limited. This is most important for substances, for which only *in silico* and / or *in vitro* data and / or mammalian toxicity data are available. Based on this information, the registrant has to evaluate, if there is concern of potential endocrine mode of action using all available information (including environmental fate and exposure). While the results from tests with aquatic vertebrates allow for a comparison of effect concentrations with predicted (or measured) environmental concentrations, this is not possible for the above-mentioned data. Additional guidance is needed to aid the registrant in evaluating if there is concern. The draft ‘Guidance document on the assessment of chemicals for endocrine disruption’ (OECD, 2010c) provides some input regarding this issue.

In addition, the following general issues deserve some further attention:

Metabolisation in humans and /or animals and transformation in the environment may lead to an increased endocrine activity. At present, metabolites / transformation products are not mentioned in Appendix R7.8-5. Should possible metabolites, which are e.g. identified using the OECD Toolbox and which are predicted to have a high endocrine activity, be included in the assessment? Some further guidance on this issue would be very helpful.

In some cases, endocrine effects are only observed at substance concentrations that are in the range of or only slightly below concentrations causing general toxic effects. It is known that endocrine endpoints as e.g. vitellogenin levels in female fish can also be affected by general toxicity and non-endocrine toxic modes of action such as hepatotoxicity (see e.g. OECD, 2009a). This issue should be mentioned in Appendix R7.8-5.

As the development of test methods for endocrine effects has proceeded significantly in the last few years, some updates on the state of the art regarding useful methods should be provided as indicated in Annex 1 to this report.

5.2 Evaluation of the selected substances: Results and general remarks

The guidance documents were applied to the selected substances with respect to specific endpoints in aquatic toxicity (acute toxicity and endocrine disrupting potential). The NT and WoE approach were applied to acute toxicity endpoints in order to evaluate whether these endpoints can be waived due to available non-testing information. In the following passages, some remarks are presented on the application of the NT and WoE approach in general and on the usability of non-testing methods in particular.

5.2.1 Non-testing methods

Classification models and structural alerts are independent approaches enabling prediction of probable modes of toxic action. Some modes of action like phototoxicity cannot be identified based on chemical structure alone, but need to be identified using several descriptors. More attention should be paid to metabolites due to their potential to reveal a higher toxicity than the parent compound.

Read across can be applied in case of suitable analogous compounds. The most difficult and most important part is the correct definition of analogous compounds. The criteria for suitable similarity are still not clearly defined. Especially in case of a small number of analogous compounds, where the choice of one unsuitable compound may change the prediction results completely, the application of additional methods is necessary. To check consistency of the prediction results it is recommended to apply more than one similarity descriptor and more than one method for read-across.

For a given endpoint, predicted toxicity values should not differ by more than one order of magnitude. Higher differences may either indicate an unsuitable prediction method, unsuitable analogous compounds or an insufficient number of analogues or different modes of action, which are not covered by the applied non-testing methods.

If no experimental data for the query compound are available, the experimental values for some of the analogous compounds could be used to increase the correctness of the prediction results or at least to get an idea of possible prediction errors.

5.2.2 Acute toxicity to fish

Regarding the acute toxicity to fish (and other aquatic organisms), benzanthrone is certainly a special case. This is mainly due to the potential of expressing phototoxic effects. Most of the applied non-testing methods do not consider these kinds of effects. Therefore, the results have to be used with caution. However, it could not be clarified within this project whether phototoxicity would play an important role under natural sunlight conditions. If this is not the case, some of the non-testing results could be used with higher confidence within the NT and WoE approach.

The fact that benzanthrone was outside the applicability domain of many of the non-testing models already indicates that the substance might be an outlier. This refers in particular to the

classification schemes and the predicted mode of action as well as to some of the read-across and QSAR models. Anyhow, one software package (MOPAC2002) demonstrated that benzanthrone fulfilled the Mekenyan criteria for phototoxicity.

The available QSAR methods having benzanthrone within their applicability domain estimate the fish acute toxicity within a similar range. The predicted LC_{50} values coming from QSAR and read-across models integrated in ECOSAR, ChemProp and the OECD Toolbox were in good agreement with the measured LC_{50} (0.55 mg/L) from the standard toxicity test (OECD 203). In contrast, measured LC_{50} values for analoguous compounds identified by ACF (ChemProp method) were predominantly higher than 0.55 mg/L. None of these models considers phototoxicity, which was identified for benzanthrone in experimental studies, leading to a clearly higher toxicity (0.83-h LC_{50} = 0.05 mg/L).

Concluding on the evaluation of this endpoint it can be stated that with or without consideration of the *in vivo* data, the result with respect to classification and labelling would be identical. However, covering this endpoint with non-testing data only, e.g. for PNEC derivation, cannot be recommended, especially when considering the possible phototoxicity.

In the present case, REACH requirements for a tonnage band of more than 100 t/a were assumed. In such a case, long-term testing has to be considered too. Since REACH foresees the possibility of long-term testing instead of short-term testing, especially for substances with poor water solubility, acute studies for fish and invertebrates could normally be waived. However, in the special case of benzanthrone it is recommended to first check whether phototoxic effects are to be expected under natural sunlight conditions. This could for example be done in guideline studies on acute toxicity to fish and invertebrates (*Daphnia*), which are performed under natural UV radiation conditions.

Following this, the application of the non-testing methods could not result in the avoidance of *in vivo* tests. However, a chronic fish test might be avoided in case it can be shown that in acute tests daphnids are more sensitive than fish by more than one order of magnitude.

Overall, the most critical points in the application of non-testing methods are the knowledge of the reliability and applicability of the single methods as well as the interpretation of their results. Therefore, it is essential that the developed methods are transparent and well documented. When using and interpreting non-testing methods, it has become evident that for each parameter (e.g. mode of action, grouping, QSAR) the application of different tools and models is essential. Only the overall assessment of the data gives enough evidence whether a non-testing result can reliably be used in a WoE approach.

In the case of benzanthrone it has also been shown that *in vivo* results which at a first glance would be rated as reliable might lead to an underestimation of the substance toxicity. Hence, this underlines the requirement of collection and evaluation of all available data, as it is stated as a starting point in the evaluation of a substance.

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Annexes

Annex 1: Comments on REACH Guidance Documents R.6, R.7b and R.10

Annex 2: Literature data on acute toxicity of 2,4,6-tribromophenol, benzanthrone and benzophenone-2

The following annexes are not intended for publication and have, therefore, not been included in the present copy of the report:

Annex 3A: Evaluation of the endocrine disrupting potential of 2,4,6-tribromophenol, benzanthrone and benzophenone-2

Annex 3B: Study report '*In vitro* assessment of the endocrine activity of benzanthrone and 2,4,6-tribromophenol'

Annex 3C: Study report '2,4,6-Tribromophenol: a short-term fish screening assay for endocrine effects'

Final Report

on

UBA R&D Project

Review and Enhancement of New Risk Assessment Concepts under REACH

(Überprüfung und Weiterentwicklung neuer Konzepte zur Risikobewertung unter REACH)

Annex 1:

Comments on REACH Guidance Documents R.6, R.7b and R.10

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April 2011

CONTENTS

1 Comments on QSAR chapters

1.1 Comments on R.6 (Chapter 6.7.1: NT approach)

Reference	Content	Comment	Recommendation
Step 0			
R.6.7.1.3, p. 34, 1st para, line 3 f.	The purity/impurity profile might be useful at a later stage to explain discrepancies between experimental and non-testing data.	The guidance document points to the fact that the composition of a chemical including its possible impurities (purity / impurity profile) enables to explain discrepancies between experimental and non-testing data. It is suitable to decide whether to use a model for a single compound or to model multi-constituent substances. Thresholds to distinguish have not been defined yet.	Addition of a hint that the purity/ impurity profile is useful for model selection (for a proposal see report, R.6.1.7.3 Step 0).
R.6.7.1.3, p. 34, 1st para, line 4 ff.	In the case of multi-constituent substances (mix-tures), it may be necessary to model two or more structures, if a single repre-sentative structure is not considered sufficient.	In case of multi-constituent substances it may not only be necessary to model several compounds. It is also possible that simply modelling all components separately yields false results. Interactions between these compounds have to be considered.	Addition of a hint that special methods of prediction of mixture toxicity may be needed due to the fact effects of a mixture may differ from the sum of effects of its components (for a proposal see report, R.6.1.7.3 Step 0).
R.6.7.1.3, p. 34, 3rd para	Collect available information for the parent compound	A useful addition of the guidance document would be a list of the required basic physico-chemical properties of the compounds.	Add examples or a cross link (for a proposal see report, R.6.1.7.3 Step 0, list of relevant properties).
R.6.7.1.3, p. 34, 3rd para	Collect available information for the parent compound	In addition to the previous comment, an important issue has not been considered: A check whether data are reliable should be added, especially if different data sources are used.	Add a paragraph dealing with data reliability.
Step 1			
R.6.7.1.4, p. 36, 3rd para, last two lines	What chemical reactivity (what type(s) of reactions) is expected for the parent compound	Possible metabolites should also be checked in the same way as the parent (at least in case of expected biotransformation). For example, the possible metabolites of benzanthrone have a much higher hazard potential than the parent compound.	Add a recom-mendation that possible metabolites should also be treated like the parent compound (for a proposal see report, R.6.1.7.3 Step 1).
General Remark on Steps 2 & 3			
R.6.7.1.5, p. 36; R.6.7.1.6, p. 36 f.	Structure of NT approach	Step 2 & 3 should be combined to one step, e.g. called “MOA Analysis”.	The section should be revised: MOA analysis (including classifi-cation schemes by Verhaar, Cramer, Russom etc. and structural alerts as

Reference	Content	Comment	Recommendation
			independent approaches) (for a proposal see report, R.6.1.7.5 Step 2).
Step 2			
R.6.7.1.5, p. 36	Cramer et al., 1978; Verhaar et al., 1995	The proposed MoA classification schemes may not be suitable for all substances, e.g. the Cramer scheme predicts the hydroquinone and catechol derivatives to have low toxicity, while in fact they have the highest toxicity in the fish assay. Besides that, the Verhaar scheme is not able to classify approximately half of the selected compounds.	The application domain or at least an indication of the model limitations should be added (for a proposal see report, R.6.1.7.5 Step 2).
R.6.7.1.5, p. 36		The difference between the MoA and the potential for toxicity has to be pointed out. The problem with application domains of classification schemes and QSAR models has to be mentioned.	Add a paragraph to point out the difference between these two issues incl. the information where to put the main focus (for a proposal see report, R.6.1.7.5 Step 2).
Step 3			
R.6.7.1.6, p. 36 f.	Several commercial software programs are available for analysing structural alerts	Unfortunately only commercial software is mentioned for determination of structural alerts. This may be an obstacle in the application of the guidance document. In case that such software is not available, it is impossible to generate structural alerts except via expert judgement.	Available free software or alternative approaches for developing structural alerts should be added (for a proposal see report, R.6.1.7.5 Step 2).
Step 4			
R.6.7.1.7, p. 37, last two paragraphs	This evaluation step should also help to define the hazard and risk assessment strategy that is further supported by applying the subsequent steps.	In this step the results of the first steps are summarized. Next to the mentioned first evaluation of potential hazards of the target compounds, all applied methods should be evaluated with regard to their suitability.	Possible pitfalls should be mentioned at this point (for a proposal see report, R.6.1.7.6 Step 3).
General Remark Steps 5 & 6			
R.6.7.1.8, p. 37 ff.; R.6.7.1.9, p. 39f.	Structure of NT approach	The structure of Step 5 is not ideal. It is proposed that this Step is structured into 'grouping of chemicals' (using the two basic methods shown under step 5a and step 5b) and 'filling of data gaps'.	Steps 5 and 6 should be completely restructured: Step 5: Grouping and search for analogous compounds; Step 6: Data Gap Filling by Read-Across and QSAR. . (for a proposal see report, R.6.1.7.7-9

Reference	Content	Comment	Recommendation
			Step 4-6).
Step 5			
R.6.7.1.8, p. 38, 2nd para	Step 5b. Similarity assessment	In case that a chemical cannot be associated to an existing compound class, some possible methods of similarity assessment are mentioned. Other approaches to define similarity of chemicals can be built on classification models based on MoAs or using ACFs. A first approach can be performed with the OECD Toolbox, the second with ChemProp.	Add further similarity categories: ACFs and classification models based on MoAs (for a proposal see report, R.6.1.7.7,8 Step 4,5).
R.6.7.1.8, p. 39, 2nd para	Collect information for analogues and update working matrix	Several problems need to be mentioned, when it comes to filling data gaps: Which endpoints are available? Is it possible to combine data from different endpoints or even different species, if the data base is poor? Which confidence do we have in the data? Discrepancies can appear because of uncertainties and different MoA (e.g. phototoxicity).	Add a paragraph how to deal with large discrepancies between endpoint values (for a proposal see report, R.6.1.7.8 Step 5).
R.6.7.1.8, p. 39, 3rd para	Perform read-across	Which descriptor(s) should be used for similarity? Is there any correlation and inter-correlation between selected descriptors?	Add suggestions which descriptors can be used and how to deal with (inter)correlation problems (for a proposal see report, R.6.1.7.8 Step 5).
R.6.7.1.8, p. 39, 3rd para	Perform read-across	In this subsection, a statement on possible problems and the large estimating uncertainties is missing.	Add a remark considering the large estimating uncertainties (for a proposal see report, R.6.1.7.8 Step 5).
Step 6			
R.6.7.1.9, p. 40, 1st para, line 4	Relevant (Q)SAR models	In this subsection, a very important problem is not addressed, i.e. the problem with the application domain of classification schemes and QSAR models. If a compound is out of the application domain all results have to be considered with great caution.	Such a statement should be added (for a proposal see report, R.6.1.7.9 Step 6).
Step 7			
R.6.7.1.10, p. 40, 4th para f.	In the final step, expert judgement is used to reach an overall assessment of the outcome of Steps 1-6 for the chemical and endpoint(s) of interest.	There is only little experience with this overall assessment step. Next to what is mentioned in this subsection, possible uncertainties and pitfalls (e.g. not very reliable data, relevant modes of action that are not considered and incorrect application domains) have to be considered here for all applied models.	Add limitations in the use of QSAR models. . (for a proposal see report, R.6.1.7.10 Step 7).

1.2 Comments on R.10 (Chapter 10.2.2.2)

Reference	Content	Comment	Recommendation
Part: Schemes for the prediction of the mode of action/structural class of a compound			
10.2.2.2, p. 12, 1st para, line 6	Excess toxicity definition	The additional comment in brackets is unclear.	Change to "reactive or specific acute modes of action"
10.2.2.2, p. 12, 2nd para, line 2	Suggested models	Verhaar and Russom have a limited application domain.	Information on these limitations should be added (in appendix).
Part: Qualitative information from structural alerts			
10.2.2.2, p. 13, para 1, line 2	Structural alert models	Lipnick, von der Ohe et al. only cover some modes of action.	Information about the modes of action that are included and examples for modes of action that are not included should be added.
Part: QSAR Predictions from expert systems			
10.2.2.2, p. 14, 1st para, line 3	Expert systems	ChemProp (Osiris Version) and the OECD Toolbox do not appear in the appendix of expert systems.	The missing software should be added in table R10-20

2 Comments on R.7b (Chapters R.7.8.1-7.8.5 and Appendix R.7.8-5)

Commenting of Chapter R.7b is presented in two parts, the first giving the editorial, technical and specific comments and the second with more general and structural comments.

2.1 Editorial, technical and specific comments

2.1.1 Chapters R.7.8.1-7.8.5

Reference	Content	Comment	Recommendation
R.7.8.1, p. 9, 1st para, last line	Introduction to aquatic pelagic toxicity	While extrapolation of aquatic toxicity to marine conditions and even sediment toxicity is possible, extrapolation to terrestrial organisms is not possible	Delete 'and soil'.
R.7.8.1, p. 10, last para and last but one para	Ref. to other sections	The guidance for the evaluation of sediment toxicity is not provided in a separate document, but in the same document in Section R.7.8.11. The reference to the ED section is App. R.7.8-5.	Change cross ref. accordingly.
R.7.8.1.2, p. 10, 4th para, last line	Cross ref. to Section R.7.8.11	Ref. not valid	Ref. should read App. R.7.8-5.
R.7.8.1.2, p. 10, 5th para, last line	Cross ref. to Section R.7.8.11	Ref. not valid	Ref. should read App. R.7.8-5.
R.7.8.2, p. 11	Information requirements for aquatic pelagic toxicity at different tonnage levels	The Annex VII-X requirements are summarised very briefly. It would be desirable to list and explain the requirements including escape clauses in detail in this section. Besides that, the triple repetition of reference to mitigating factors is confusing.	To be checked and revised if deemed necessary.
R.7.8.2, p. 11, 2nd para	Mitigating factors indicating that aquatic toxicity is unlikely to occur	No explanation or definition is given here regarding 'highly insoluble in water' and 'unlikely to cross biological membranes'. On page 40/41 and App. R.7.8-1 the term 'Highly insoluble' is explained in more detail.	The mitigation factors should be explained in more detail in this section or it should be referred to a section where more information is found.
R.7.8.2, p. 11, 3rd para	'Short-term testing on invertebrates does not need to be conducted if adequate information on environmental classification and labelling is available.'	No explanation or definition is given here regarding the term 'adequate'.	What is meant with 'adequate information' should be explained in more detail in this section or a reference should be made to a section where detailed information is found (e.g. R7.8.5.1).
R.7.8.2, p. 11, 4th para	Cross ref. to Section R.7.8.7	Ref. not valid	Correct ref. App. R.7.8-1?
R.7.8.3.1, p. 12	Data on aquatic pelagic toxicity - *In vitro* data	The status on validation of the *in vitro* methods should be updated. A reference to section R.7.8.4.1 (Testing data) is	A list of validated methods to be integrated; a reference

Reference	Content	Comment	Recommendation
		missing.	should be included.
R.7.8.3.1, p. 13, 2nd para	Cross ref. to Section R.7.8.11	Ref. not valid	Ref. should read App. R.7.8-5.
R.7.8.3.1, p. 13, 4th para	Cross ref. to Section R.7.8.8	Ref. not valid	Ref. should read App. R.7.8-2.
R.7.8.3.1, p. 13, 6th para	Cross ref. to Section R.7.8.8	Ref. not valid	Ref. should read App. R.7.8-2.
R.7.8.3.1, p. 13, 7th para	Cross ref. to Section R.7.8.8	Ref. not valid	Ref. should read App. R.7.8-2.
R.7.8.3.1, p. 13, 8th para	Cross ref. to Section R.7.8.8	Ref. not valid	Ref. should read App. R.7.8-2.
R.7.8.3.1, p. 14, 2nd para	Cross ref. to Section R.7.8.8	Ref. not valid	Ref. should read App. R.7.8-2.
R.7.8.4, p. 16, 1st para	Cross ref. to Section R.7.8.7	Ref. not valid	Correct ref. App. R.7.8-1?
R.7.8.4, p. 16, 2nd para	Cross ref. to Section R.7.8.7	Ref. not valid	Correct ref. App. R.7.8-1?
R.7.8.4, p. 16, 6th para	WoE, ref. to R.4.4	The WoE for aquatic pelagic toxicity is described in Section R.7.8.5; a reference to R.7.8.5 is missing.	Ref. should be included.
R.7.8.4.1, p. 17-30	Data on pelagic toxicity	This subsection is very large and the level of (unnumbered) headers, though formatted in different fonts, remains unclear.	Introduce further indenture levels and subsection numbers.
R.7.8.4.1, p. 19, last but one para	Cross ref. to Section R.7.8.7	Ref. not valid	Correct ref. App. R.7.8-1?
R.7.8.4.1, p. 21, 2nd para	Cross ref. to Section R.7.8.7	Ref. not valid	Correct ref. App. R.7.8-1.
R.7.8.4.1, p. 21, last para	Cross ref. to Section R.7.8.7	Ref. not valid	Correct ref. App. R.7.8-1.
R.7.8.4.1, p.23ff	Guidance on specific test types for freshwater species	This section appears to be not clearly arranged. The main acute aquatic endpoints (fish, *Daphnia*, algae) should appear with clear headers and should also be separated clearly from each other. For each endpoint, a list or table of parameters to be considered would be useful including factors, which dis-qualify a study result.	The section should be structured more clearly (please see recommendations in comment column).
R.7.8.4.1, p. 23 ff., 1st para	Guidance on specific test types for freshwater species	The introduction refers to the 'evaluation of data from non-standard ecotoxicity tests'. However, this subsection explicitly addresses standard OECD tests. It is not completely clear, what the purpose of this subsection is.	The introduction should make clear that varied conditions are set into relation to standard testing.

Reference	Content	Comment	Recommendation
R.7.8.4.1, p. 23, Algae tests	Guidance on evaluation of EC_{50} values from algae tests	It may be the case that EC_{50} values are available, e.g. from literature (without raw data), without indication whether these refer to growth rate or biomass integral or other parameters such as cell density. There is no guidance given in this chapter how to deal with this case (see also R.7.8.5.3). Note that in its GHS Guidance Document (ECHA-09-G-02-EN, 2009), ECHA states on page 411 that in those cases classification should be based on the lowest EC_{50} available.	The described case should be handled here and a recommendation should be given whether these results may fully be used or only as part of a WoE.
R.7.8.4.1, p. 26, 2nd para	Cross ref. to Section R.7.8.8	Ref. not valid	Correct ref.?
R.7.8.4.1, p. 26, 2nd para	Cross ref. to Section R.7.8.7	Ref. not valid	Correct ref. App. R.7.8-1.
R.7.8.4.1, p. 26, 4th para	Cross ref. to Section R.7.8.7	Ref. not valid	Correct ref. App. R.7.8-1.
R.7.8.4.1, p. 27, last para	Non-testing data on aquatic pelagic toxicity (QSARs)	This subsection has the same level as the 'Testing data on aquatic pelagic toxicity' (p. 17), but gets lost because of the same format as the specific test guidelines (cf. p. 23-25).	Introduce further subsection numbers.
R.7.8.4.1, p. 29, 5th para	Cross ref. to Section R.7.8.3	The reference can be skipped since no significant information on 'Structural alerts' can be deduced from this section.	Ref. R.7.8.3 should be deleted; instead ref. to R.6.1.7.6.
R.7.8.4.1, p. 29, 4th para	Cross ref. to Section R10.2.2.2	Ref. not valid	Correct ref. Table R.10-16 (p. 55).
R.7.8.4.2, p. 30, 8th para	Remaining uncertainty	The sentence 'The more chronic … the remaining ??? is less.' lacks the word 'uncertainty' at the end.	Edit.
R.7.8.4.3, p. 30 f.	Exposure consid-erations for aquatic pelagic toxicity requirements	Exposure considerations have already been briefly addressed in the introduction to Section R.7.8.4 (p. 16) and the main issues of this section are effects.	It is proposed to move the whole subsection R.7.8. 4.3 (p. 30 f.) to the end of p. 16 (and to remove the header).
R.7.8.5, p. 31-42	Conclusions for aquatic pelagic toxicity and ITS	The 'Weight of Evidence' (WoE) approach is elaborated over more than 10 pages without a single header numbering. The first indenture level (R.7.8.5.***1***) refers to 'Concluding on suitability for Classification and Labelling'	Revise the structure of this section (*for a proposal see ch. 3.2 of the main report*).
R.7.8.5, p. 31-42	Conclusions for aquatic pelagic toxicity and ITS	The 'Weight of Evidence' (WoE) approach is structured in successive steps for collection and evaluation of non-testing and testing information. It is suggested to rearrange these steps so that it becomes obvious that the information derived from these different sources can be collected independently from each other.	Revise the structure of this section (*for a proposal see ch. 3.2 of the main report*).
R.7.8.5, p. 33, Fig. R.7.8-2	Suggestion for a *Weight of Evidence*	The flow-chart does not clarify the difference between the general steps (to	Redesign the flow-chart (*for a proposal*

Reference	Content	Comment	Recommendation
	approach	be performed once) and those steps, which have to be repeated for each endpoint. The iteration with the ITS is not represented in the chart.	*see main report, ch. 3.2, Figure 4*)
R.7.8.5, p. 33	Step 1	A list of relevant phys.-chem.- properties is missing. Additionally, tools for generating phys.-chem. and fate data should be indicated like EPISuite or the OECD Toolbox.	Add a list of relevant physico-chemical properties (*for a proposal see main report, ch. 3.1, R.6.1.7.3*).
R.7.8.5, p. 34, 1st para	Term 'expected uptake'	There is no description what is meant in detail, what kinds of uptake routes are possible and how this can be assessed (the guidance in R.6.1.7.4 appears to be not sufficient).	A clear description of this term should be included (*main report: ch. 3.2, R.7.8.5.1, subsect. IC*).
R.7.8.5, p. 34, 2nd para	Term 'relevant metabolites'	There is no description what is meant with 'relevant metabolite' and how this can be assessed (the guidance in R.6.1.7.4 appears to be not sufficient).	A definition of a 'relevant' metabolite is necessary as well as information how to deal with it under REACH. A description how to identify relevant metabolites should be included here and/or in other sections.
R.7.8.5, p. 34	Step 2	Programs and tools for generating results on structural alerts and mode of action should be indicated, e.g. the OECD Toolbox and ChemProp.	Add the respective information or a cross reference to section 6 (main report: c*h 3.2, R.7.8.5.2: ref. to section 6 added*).
R.7.8.5, p. 36	Step 3	Programs and tools for generating results on grouping and analogue substances should be indicated, e.g. the OECD Toolbox, ChemProp.	Add the respective information or a cross references, e.g. to section 6 (main report: c*h 3.2, R.7.8.5.2: ref. to section 6 added*).
R.7.8.5, p. 36, 3rd para	Cross ref. to Section R.7.8.4	This cross reference is made to a chapter where similar unspecific information is given. In turn this chapter refers to Section 6.2.	The reference can be deleted since a reference to other relevant GD sections is already made in this paragraph.
R.7.8.5, p. 36, 6th para	How to deal with conflicting *in vivo* data?	It might be desirable to discuss the potential problems in more detail: • Is 'most relevant' connected to 'most sensitive' (= worst case)? • How to deal with conflicting results from different species, e.g. various fish species? • How to deal with results from studies without analytical monitoring? • How to deal with results from studies that were conducted according to	To be checked and revised/amended if deemed necessary.

Reference	Content	Comment	Recommendation
		outdated guidelines?	
R.7.8.5, p. 38	Step 4a	Programs and tools for generating QSAR results should be indicated, e.g. the OECD Toolbox, ChemProp, EPISuite.	Add the respective information or a cross reference to section 6 (main report: c*h 3.2, R.7.8.5.2: ref. to section 6 added*).
R.7.8.5, p. 38, last but one para	Reliable QSAR results	Beside that this section might need an update, two major questions arise: • How is an analogue defined? • What is a close analogue? Or the other way round: • What makes an analogue to be not 'close' anymore?	This appears to be more an issue of Section 6, but in the context of this chapter those questions should also be answered briefly here or a cross reference to Section 6 should be included (main report: c*h 3.2, R.7.8.5.2: ref. to section 6 added*).
R.7.8.5, p. 39 f.	Step 5: Overall assessment	Besides a qualitative evaluation (expert judgement) quantitative assessments of the weight of evidence, e.g. like Bayesian networks (Jaworska et al., 2010) or the Dempster-Shafer theory (Fernández et al., 2009) are also possible.	Add information (main report: information is added to c*h 3.2, R.7.8.5*)
R.7.8.5, p. 39 f.	Step 5: Overall assessment	Meanwhile there is practical guidance from ECHA available how to include WoE results into the IUCLID (ECHA, 2010). This should be mentioned here.	Add information (main report: information is added to c*h 3.2, R.7.8.5*)
R.7.8.5, p. 39, 3rd para	Use of *in vitro* tests for regulatory decision (ref. to R.3 and R.4)	The link to Section R.3 appears to be not helpful since there is no essential information on this topic to be found.	The reference should be deleted.
R.7.8.5, p. 40, 2nd para	Step 5:	The last para of Step 5 is hardly understandable.	Rephrase and elaborate (for a proposal see main report, ch. 3.2, R.7.8.5.2, subsect. IID).
R.7.8.5, p. 40, 4th para	Cross ref. to Section R.7.8.7	Ref. not valid	Correct ref. App. R.7.8-1.
R.7.8.5, p. 40 f.	Step 6: Intrinsic physico-chemical properties	The 2nd para of this sub-section indicates that there is no 'cut-off limit value for solubility below which no toxicity could occur'. The 4th para of this sub-section suggest such a value of 1 mg/L for moving from acute to chronic testing.	The legitimation of the threshold value should be explained in more detail.
R.7.8.5, p. 40 f.	Step 6: Threshold approach for tox-icity testing in fish	An OECD Guidance Document on the threshold approach for acute fish toxicity testing" has been finalised (OECD, 2010a).	Amend this subsection accordingly (main report: information is added to c*h 3.2, R.7.8.5.3*).
R.7.8.5, p. 40 f.	Intelligent Testing Strategies (ITS)	Some more ITS with respect to aquatic toxicity testing have been developed and	Amend this subsection accordingly (main

Reference	Content	Comment	Recommendation
		published. Reasonable proposals like those suggested by OECD (2010d) should be included in the ITS section of the revised WoE approach.	report: ch. 3.2, R.7.8.5.3).
R.7.8.5, p. 42, 1st para	Cross ref. to Section R.7.8.11 (Information from mammalian toxicity)	Ref. not valid; Moreover, the promised 'specific guidance' cannot be found in the ED section. The cross reference found there (page 112) to 'Section 6 of this appendix' cannot be allocated.	Ref. should read App. R.7.8-5 (main report: ch. 3.2, R.7.8.5.4); the other ref. should be newly defined.
R.7.8.5.2, p. 46	Concluding on suitability for PBT/vPvB assessment	The header, or at least the first introductory sentence should make clear that this section is dealing exclusively with the T-criterion of the PBT triple. Consequently, mentioning 'vPvB' is obsolete.	Revise.
R.7.8.5.3, p. 49, point 3.	Prediction of relative species sensitivity	The methods for prediction of relative species sensitivity are poorly described and/or not properly referenced.	Amend explanations and/or references; add bullet points to the list.

2.1.2 Appendix R.7.8-5 'Assessment of available information on endocrine and other related effects'

Reference	Content	Comment	Recommendation
App. R.7.8-5, p. 102 ff.	Header numbering	Indenture levels would be helpful in this Appendix	Add.
App. R.7.8-5, p. 102, 1st para, lines 6-7	'and none of the screening and testing methods discussed has been fully validated or approved as OECD Test Guideline'	Should be updated (see also specific comments below).	The sentence should e.g. read 'and only some of the screening and testing methods discussed have been fully validated or approved as OECD Test Guideline'.

Reference	Content	Comment	Recommendation
App. R.7.8-5, p. 102, 1st para, lines 8-9	'Relevant information... may also be derived from... mammalian screening assays for endocrine activity and other human health endpoints from repeated-dose toxicity, carcinogenicity and reproductive toxicity studies'	In Appendix R7.8-5, little guidance is provided on how information derived from mammalian screening assays for endocrine activity and other human health endpoints from repeated-dose toxicity, carcinogenicity and reproductive toxicity studies should be evaluated.	Further information on those tests that can provide relevant information should be provided. Cross refs to the relevant sections dealing with evaluation of human health endpoints should be added. With regard to this issue, the outcome of the research project FKZ 206 67 448/05 performed for the UBA and the draft guidance document on the assessment of chemicals for endocrine disruption (OECD, 2010c) are certainly useful.
App. R.7.8-5, p. 102, last para, 1st point in the bulleted list	'information indicating potential endocrine activity in aquatic organisms (from human health endpoints...)'	In Appendix R7.8-5, little guidance is provided on how information from human health endpoints should be evaluated with regard to potential endocrine activity (see also previous comment).	See previous comment.
App. R.7.8-5, p. 103, 2nd para	Objective of the guidance	The term 'serious adverse effects' is not specified and not properly distinguished from 'adverse effects', which is used at other places of the Appendix.	The terms should be defined.
App. R.7.8-5, p. 103, 2nd para	Objective of the guidance	The proper reference is 'Article 57 f' instead of 56 f.	The reference should be corrected
App. R.7.8-5, p. 103, 1st para of section 'Non-testing data', lines 2-3	'explained in the main part of this guidance document'	Ref. too unspecific	Ref. to R.6.1 and R.10.
App. R.7.8-5, p. 103-104, para 'Non-testing data'	Information and its sources on non-testing data	Information and given references might need an update.	To be checked and updated if considered necessary.
App. R.7.8-5, p. 104-109	Testing data	The whole sub-section 'Testing data', including *in vitro* screening data and *in vivo* screening and testing data is more a compilation of assays and tests rather than guidance and a scheme for decision making. There is a considerable amount of redundancy with p. 110-112.	The section should be restructured and redundant parts should be removed.

Reference	Content	Comment	Recommendation
App. R.7.8-5, p. 104, last para, lines 1-2	'At present, validated *in vitro* assays and internationally accepted Test Guidelines for regulatory purposes are not yet available.'	Should be updated. An OECD test guideline for a 'Stably transfected human estrogen receptor-α transcriptional activation assay for detection of estrogenic agonist-activity of chemicals' (test guideline 455; OECD, 2009a) is available.	Inclusion of validated test methods.
App. R.7.8-5, p. 105, 4th para, line 1	Information on prevalidation of two receptor binding assays	Specific information on both assays is missing.	Information on the names of both tests and, if possible, references should be added.
App. R.7.8-5, p. 105, 4th para, line 4	Information on an assay based on the androgen receptor from rat prostate cytosol	More specific information would be helpful.	Information on the exact name of the test and, if possible, a reference should be added.
App. R.7.8-5, p. 105, 4th para, lines 5-6	Information on an assay based on the estrogen receptor from rat uterine cytosol	More specific information would be helpful.	Information on the name of the test (rat uterine cytosolic (RUC) estrogen receptor (ER)-competitive binding assay) should be included.
App. R.7.8-5, p. 105, para 5, last sentence	'has been evaluated in the Japanese Report in peer review at the OECD'	A reference for the mentioned report is missing. In addition, the information should be updated. Were several reporter gene assays validated or is only one assay, the stably transfected transcriptional activation (TA) assay to detect estrogenic activity (as stated in the following para, lines 1-2) meant?	Should be checked and updated, if required. A reference for the Japanese report should be added.
App. R.7.8-5, p. 105, lines 3-4	'Prevalidation of four transcriptional activation assays for ER and AR (anti)agonists detection'	More specific information would be helpful.	Information on the exact names of the four tests and, if possible, references should be added.
App. R.7.8-5, p. 106, 1st para	Information on vitellogenin assays with primary hepatocytes	More specific information would be helpful.	It should be specified which tests are validated. Key references should be indicated.
App. R.7.8-5, p. 106, 2nd para, line 5	Reference to OECD draft detailed review paper on steroidogenesis (2002)	Should the reference be replaced by the final detailed review paper on steroidogenesis screening assays and endocrine disruptors (EPA contract No 68-W-01-023)?	Should be checked and updated, if required.
App. R.7.8-5, p. 106, 2nd para, last line	Reference to OECD draft detailed review paper on aromatase (2002)	Should the reference be replaced by the final detailed review paper on aromatase (EPA contract No 68-W-01-023)?	Should be checked and updated, if required.

Reference	Content	Comment	Recommendation
App. R.7.8-5, p. 106, 3rd para, line 2	Information on an assay based on the H295 human adrenocortical carcinoma cell line	More specific information would be helpful.	The name of the test (H295R steroido-genesis assay) and a reference should be indicated.
App. R.7.8-5, p. 106, 3rd para, lines 3-4	Information on prevalidation studies on human recombinant aro-matase	More specific information would be helpful.	The name(s) of the test(s) and, if possible, references should be indicated.
App. R.7.8-5, p. 106, 4th para	Information on ECVAM website	This sentence should not be placed in the section on steroidogenesis assays.	Move sentence to the end of the introductory section on '*In vitro* screening data' (p. 195, 2nd para).
App. R.7.8-5, p. 106, 6th para, line 1	'At present, there are no validated *in vivo* screening assays for the identification of substances with potential endocrine activity...'	Should be updated (see also specific comments below).	Several guidelines are available / have been updated by now. The sentence should be adapted accordingly.
App. R.7.8-5, p. 107, 2nd para	Information on the 21-day fish screening assay	This test guideline has recently been finalised (test guideline 230, OECD 2009b). In addition, a second test guideline for a fish screening test for endocrine effects, a short term re-production assay, is now available (test guideline 229, OECD 2009c). This test should also be mentioned in the section on 'Screening assays'.	The section should be updated accordingly.
App. R.7.8-5, p. 107, 3rd para	Information on the fish sexual development test	A revised draft of this test guideline has been published recently (OECD, 2010b).	The section should be updated accordingly.
App. R.7.8-5, p. 107, 4th para	Information on the fathead minnow reproduction test	The guideline for this test has been finalised (test guideline 229, OECD 2009c, see previous comment) in a slightly modified form, allowing the use of three fish species, fathead minnow, medaka and zebrafish. Please note that this test is now considered as a screening test and not as a confirmatory test. A draft guidance document on fish gonadal histopathology is now available (OECD 2009d).	The section should be updated accordingly. In addition, it should be moved to the previous sub-chapter 'Screening Assays'.
App. R.7.8-5, p. 107, last para	Information on fish full life cycle and two-generation tests	A detailed review paper on fish life-cycle tests is now available (OECD 2008a).	This reference should be included.

Reference	Content	Comment	Recommendation
App. R.7.8-5, p. 108, 2nd para	Information on the amphibian meta-morphosis assay	The guideline for this test has been finalised (test guideline 231, OECD 2009e).	The section should be updated accordingly.
App. R.7.8-5, p. 108, 4th para	Information on an enhanced test guideline 211, *Daphnia magna* reproduction test	Revision of test guideline 211, '*Daphnia magna* reproduction test' has been completed (OECD 2008b). An annex has been added to describe procedures for identification of neonate sex.	The section should be updated accordingly.
App. R.7.8-5, p. 109, para 'Non-testing data'	Evaluation of QSAR results (general information)		To be checked and updated if considered necessary.
App. R.7.8-5, p. 109, 3rd para, line 3	Reference to the TGD	Is the technical guidance document on risk assessment of new notified substances, existing substances and biocidal products (EC, 2003) meant?	A clear reference should be added.
App. R.7.8-5, p. 109, 3rd para, line 3	Reference to the 'main text on aquatic toxicity'	Ref. too unspecific	Ref. to R.6.1 and R.10.
App. R.7.8-5, p. 109, 3rd para, lines 4-5	Reference to the 'general introduc-tion'	Ref. too unspecific	The reference should be specified.
App. R.7.8-5, p. 109, last 2 paragraphs	Non-testing data	These general statements contribute little to evaluation of endocrine effects.	Revise accordingly.
App. R.7.8-5, p. 110, 1st para	Information on evaluation of *in vitro* screening data	*In vitro* screening data are likely to represent most of the available data on possible endocrine activity of the substances to be evaluated. However, guidance on evaluation of *in vitro* data is at present rather limited.	Additional guidance should be provided.
App. R.7.8-5, p. 110, 1st para, line 2	Reference to the TGD	Is the technical guidance document on risk assessment of new notified substances, existing substances and biocidal products (EC, 2003) meant?	A clear reference should be added.
App. R.7.8-5, p. 110, 1st para, line 2	Reference to the 'main text on aquatic toxicity'	Ref. too unspecific	Ref. to R.7.8.4.1.

Reference	Content	Comment	Recommendation
App. R.7.8-5, p. 110, 1st para, lines 2-4	'...data from mammalian systems may also provide information of relevance to aquatic organisms'	In Appendix R7.8-5, no guidance is provided on how information from mammalian systems should be evaluated.	As mentioned above, guidance should be provided how information from toxicological tests should be evaluated, and cross refs to the relevant documents on evaluation of human health endpoints should be added.
App. R.7.8-5, p. 110, 2nd para, lines 1-2	Reference to the 'general parts of this guidance document'	Ref. too unspecific	Ref. to R.7.8.4.1.
App. R.7.8-5, p. 110, 4th para	'21-Day Fish Screening Assay, draft TG proposal'	This test guideline has recently been finalised (test guideline 230, OECD 2009b).	Both reference and text should be updated accordingly.
App. R.7.8-5, p. 111, 2nd para	Information on evaluation of the 'Fathead minnow reproduction test'	This test guideline has recently been finalised in a slightly modified form, allowing the use of three fish species, fathead minnow, medaka and zebrafish (test guideline 229, OECD 2009c).	The section should be updated accordingly.
App. R.7.8-5, p. 111, 2nd para, last sentence	'Guidance documents are in preparation in the US and the OECD to assist pathologists...'	A draft guidance document on fish gonadal histopathology is available (OECD, 2009d).	The reference to the draft guidance document and, if possible, a reference to the US guidance document should be added.
App. R.7.8-5, p. 111, 4th para	'21-Day Amphibian Metamorphosis Assay, draft TG proposal'	As mentioned above, the guideline for this test has been finalised (OECD 2009e).	The section should be updated accordingly.
App. R.7.8-5, p. 112, 2nd para	'...rather than identifying any specific endocrine mode of action... (except for the proposed enhancement to the existing *Daphnia* reproduction test)'	In the revised test guideline 211, '*Daphnia magna* reproduction test' (OECD 2008b), an annex has been added describing procedures for identification of neonate sex. As is detailed in the following paragraph (lines 3-7), sex ratio in *Daphnia* is no endpoint that is specific to an endocrine mode of action.	The last part of the section '(except for the proposed enhancement to the existing *Daphnia* reproduction test)' should be omitted.
App. R.7.8-5, p. 112, last two lines of the 2nd para and 3rd para	Ref. to the 'proposed enhancement to the existing *Daphnia* reproduction test'	As mentioned above, revision of test guideline 211, '*Daphnia magna* reproduction test' has been completed (OECD 2008b).	The section should be updated accordingly.

Reference	Content	Comment	Recommendation
App. R.7.8-5, p. 112, section on 'Mammalian toxicity data'	Information on evaluation of mammalian toxicity data	In Appendix R7.8-5, little guidance is provided on how mammalian toxicity data should be evaluated with regard to potential endocrine effects in aquatic vertebrate species. Some information is given on p. 116-117, but more specific guidance would be desirable.	As mentioned above, information on the tests, which can provide relevant information, should be provided, and the specific sections of the chapter on 'Human health assessment', where guidance on evaluation of the relevant data is given, should be indicated.
App. R.7.8-5, p. 112, 8th para	Ref. to 'section 6 of this Appendix'	Ref. not valid	Include correct ref.
App. R.7.8-5, p. 112 f., bottom/top	Relevance of endocrine activity for classification	The concept of creating a 'safety net' for substances, which do not fall under the 'core set of criteria', is not properly explained or referenced to the REACH Regulation.	Explain and/or make a reference to the Regulation.
App. R.7.8-5, p. 114, 5th para, lines 4-5	'available information on a accordance with the principles outlined in the previous sections'	Copy and paste error	This part of the sentence should be omitted.
App. R.7.8-5, p. 114, last para	Suitability in relation Art. 57 (f)	The regulatory circumstances, under which a CA may request non-standard data are not properly specified.	Explain and/or make a reference to the Regulation.
App. R.7.8-5, p. 115, 2nd para	Suitability in relation Art. 57 (f)	The meaning of this paragraph is not clear.	Delete or revise.
App. R.7.8-5, p. 115, 5th para	Integrated assessment of potential endocrine activity	The sentence 'This section … requirements of REACH' is dispensable.	Delete or revise.
App. R.7.8-5, p. 116, 2nd para	Ref. to 'sections 3 and 4'	Ref. not valid	Ref. to p. 103-104
App. R.7.8-5, p. 116-117, section on 'Information from mammalian toxicity data'	Information on evaluation of mammalian toxicity data	As mentioned above guidance on how mammalian toxicity data should be evaluated with regard to potential endocrine effects in aquatic vertebrate species is very limited, and more specific guidance would be desirable.	Instead of only listing the relevant endpoints, a table should be included that gives an overview of the relevant tests, the corresponding endpoints, and the specific sections of the chapter on 'Human health assessment', where guidance on evaluation of these tests is given. As indicated above, the outcome of the research project FKZ 206 67 448/05 performed for the

Reference	Content	Comment	Recommendation
			UBA and the draft guidance document on the assessment of chemicals for endocrine disruption (OECD, 2010c) contain very useful information.
App. R.7.8-5, p. 117, 3rd para	Weight of evidence	The relation of the WoE approach here (in the context of 'Integrated Chemical Safety Assessment') to the WoE approach in R.7.8.5 (p. 31 ff.) is not clear.	Revise.
App. R.7.8-5, p. 117, 3rd para, line 4	Ref to 'section 4'	Ref. not valid	Include correct ref.
App. R.7.8-5, p. 117, 4th para, lines 3-6	'Endocrine-specific assays are... under development and validation...'	As detailed above, some test guidelines have been finalised in the meantime.	The sentence / the whole section should be updated accordingly.
Figure R7.8-8, part 1	'Preliminary indication of potential endocrine activity in aquatic organisms'	In the second colomn of the figure, *in vitro* screening assays for thyroid activity should be included.	*In vitro* tests for thyroid activity should be mentioned.
Figure R7.8-8, part 2	'Indication of specific endocrine modes of action in intact aquatic organisms'	What is defined as 'strong concern'?	Further guidance is required.

2.2 General and structural comments

2.2.1 Chapters R.7.8.1-7.8.5

Reference: *R.7.8*

Content: *Endpoint specific guidance*

Comment:

The objective of the project was to test the usefulness of the 'Guidance on information requirements and chemical safety assessment' (GD – Guidance Document), in particular Chapter R.7b (Endpoint specific guidance), by applying it to several substances. It was expected that the Guidance Document provides a step-by-step procedure to collect and assess the required data. In fact, the introduction to the endpoint specific guidance (Chapter R.7a, p. 13, 3rd para) states that the 'guidance for each specified endpoint has been developed as a stand-alone report addressing …'. However, this is not the case in Chapter R.7b. Additional information on the pathway of performing the chemical safety assessment (CSA) and creating the chemical safety report (CSR) is necessary, in order to understand the structure of Chapter R.7b. Some essential hints are given in the following:

The context of Chapter R.7b is presented in the pathfinder figure on page 4. However, in order to understand this figure, it is necessary to understand Part A of the GD (Introduction to the GD) where this figure is more deeply elaborated in figures and text. The outcome of the CSA is implemented in the Chemical Safety Report (CSR), which is structured according to the data requirements of the REACH-Regulation (Annexes VII to XI). The four steps to fulfil the information requirements are set out in Annex VI of the Regulation and elaborated in Part A of the GD (p. 9f.), as well as in Part B.2.1 of the guidance on Hazard Assessment (p. 10 ff.). This already indicates that essential information is scattered throughout the entire GD.

The main point about data gathering is that to start with, 'all' available information should be collected (cf. in particular Part A, p. 19 last para) and not only data required in accordance with standard test guidelines. Once the relevance of available data has been assessed (possibly applying the weight-of-evidence approach), the data may be compared with the requirements of the Regulation (the outcome is reported in the CSR). Only thereafter, if data gaps remain, the registrant may decide on appropriate testing to fill the gaps. With other words, the introduction to Chapter R.7b lacks reference to the intended result, i.e. the CSR. The registrant, who starts with the endpoint specific guidance, is likely to be confused unless he already possesses a complete overview on the entire CSA process. With this process in mind, it becomes understandable why Chapter R.7b is a comprehensive compilation of test methods and strategies, but hardly contains advice for decision making. Further explanation on the structure of guidance in Chapter R.7b is given in the introduction to Chapter R.7a.

Recommendations:

The 'endpoint specific guidance', should be redesigned to be more a practical manual to fill in the CSR. Besides this a clearer reference to the underlying process description would be helpful.

Reference: *R.7.8.1 – R.7.8.5*

Content: *Aquatic pelagic toxicity*

Comment:

The target of the endpoint specific guidance is the 'understanding of the toxic profile of the substance …' (Section R.7.8.4; 1st para; p. 15). However, it is not defined in any part of the Guidance Document what this 'toxic profile' includes and which format it is supposed to have. It may be suspected that the 'toxic profile' conforms to the Chemical Safety Report (CSR), as conceptualised in the Appendix to Part F. At two other occasions, R.7b refers to a 'toxicity pattern' of the substance (Section R.7.8.5; Step 5; p. 39; and R.7.8.5.4; Overall conclusion; p. 52), which might be similar to the 'toxic profile'. On page 40 (Section R.7.8.5) a 'comprehensive conclusion on the endpoint (multi task assessment) … has to be substantiated and described in the text'. The mentioning of a 'text' indicates that the user is supposed to write some kind of a dossier for the substance (i.e. should this be integrated in the CSR?).

The singular endpoint referenced in the phrase above (R.7.8.5 p. 40) refers to the information requirements as outlined in the Annexes VII-XI of the REACh-Regulation. However, the description of these Annexes in Section R.7.8.2 (p. 11f.) is not helpful, since the user of the guidance does not see which test endpoints are required based on the tonnage band. The information on the requirements has to be extracted from the Regulation itself. Some explanation to the endpoints is placed in Section R.7.8.4.1 (p. 23 ff.), but this section is introduced as 'evaluation of data from non-standard ecotoxicity tests', while it actually also contains the standard tests required in the Annexes. For the user of the guidance it is difficult to identify, which tests are actually required and which not.

Consequently, it does not become immediately obvious in the guidance that the *Weight of Evidence* approach is not applied to create an 'understanding of the toxic profile of the substance', but merely to assess the relevance of available data for a single endpoint. The individual steps of the WoE need to be repeated for each endpoint. Within the CSR template (Appendix to Part F) is does not become completely clear, whether the WoE approach is part of the 'Data waiving' or the 'Discussion' of an endpoint section (e.g. 'short-term toxicity to fish').

Currently, the guidance document gives explanations on the background of requirements and evaluations of aquatic toxicity. The current structure: R.7.8.3 'information sources', R.7.8.4 'evaluation', R.7.8.5 'conclusion' contains partly redundant information. So far, the only structured guidance, which specifies how a 'toxic profile' should be compiled and presented, is available in the CSR template. Such guidance should then include examples for the endpoint evaluations and the WoE presentations.

Recommendations:

In principle, the last paragraph of the comment should be turned into actions. Please see the main report, chapter 3.2 for a proposal of a revised section R.7.8.5 and chapter 4.1 for a proposal on how to document an NT/WoE evaluation of a specific endpoint.

Reference: *R.7.8.3 – R.7.8.4*

Content: *Information on aquatic pelagic toxicity and its sources / Evaluation of available information on aquatic pelagic toxicity*

Comment:

The structure of these two subsections is unclear and the information presented is not easily transferred into guidance for discussing the actually available or missing data within the CSA. The description of the sections in the introduction to Chapter R.7a (p. 13, 4th para) already contains a large amount of redundancy. Section R.7.8.3 contains only one subsection R.7.8.3.1 with a header (Data on aquatic pelagic toxicity – Testing data on aquatic pelagic toxicity), that is also repeated in subsection R.7.8.4.1. Subsection R.7.8.3.1 contains a listing of testing approaches divided into 'in-vitro', 'in-vivo – single species', 'in-vivo – multiple species' tests, QSARs, and grouping approaches; this would have suggested a subsection numbering there. The listing remains relatively superficial and would better fit as introductory paragraphs to the following Section R.7.8.4, where general remarks are repeated anyway.

Referencing back to Section R.7.8.2, where the data requirements in the Annexes of the REACH-Regulation are addressed, it should be reminded that the Regulation is very specific on the tests to be presented (Column 1), with some derogations mentioned in Column 2. Therefore, a listing of tests as in Sections R.7.8.3 and R.7.8.4 cannot be a basis for 'deciding on the aquatic pelagic tests to perform' (cf. R.7.8.3, p. 16, last 2 paras, 'Other considerations'). Only if the regularly required test is not available, the registrant has the opportunity to propose other tests in the context of 'weight of evidence' (WoE) and the 'intelligent testing strategy' (ITS). This step-by-step process is not made explicit in the Guidance. The ITS is mentioned once at the very beginning of the Guidance (Section R.7.8.1.2) and then again at the end (Section R.7.8.5). At this stage (R.7.8.4, p. 16), it remains unclear why and how a registrant should 'decide' on a certain test.

Furthermore, if a study has been considered a 'key study' of good quality in the IUCLID, a further WoE is not necessary. Therefore, the justification of presenting the information on required key data at this place of the Guidance is unclear.

Besides that, if 'exposure considerations' are already addressed in Section R.7.8.3 (p. 16, last 2 paras, 'Other considerations'), it would be helpful to specify them as it is done in Subsection R.7.8.4.3 (p.30).

Recommendations:

The structure of Sections R.7.8.3 and R.7.8.4 should be revised by merging the (test-level specific) text from Section R.7.8.3 to R.7.8.4. Thus, redundancies are removed and the presentation of information should be more in a way of a manual for preparing a CSR (with view to the ITS of the following Section R.7.8.5), rather than a comprehensive list of test methods. Subsection R.7.8.4.3 (p. 30) should be merged with the introduction to Section R.7.8.4 (p. 16).

Reference: *R.7.8.4.1, p. 19-22*

Content: *Data on aquatic pelagic toxicity / CHECKLIST*

Comment:

This checklist for assessment of *in vivo* (single species) testing is quite detailed on one hand, but does not specify the test conditions precisely on the other hand. The checklist may be helpful for the assessment of non-standard tests, older tests and imperfect study reports. However, for tests, which are performed to agreed test guidelines under GLP, the test conditions, as mentioned in the checklist, are precisely determined with good cause. Besides that, standard tests are addressed in the following subsection 'GUIDANCE OF SPECIFIC TEST TYPES …'.

Recommendations:

The purpose of the subsection 'CHECKLIST' is not clear and should be specified in an introductory sentence. It should be checked whether all relevant issues are covered in the list.

Reference: *R.7.8.5*

Content: *Conclusions for aquatic pelagic toxicity and integrated testing strategy (ITS): Weight-of-evidence (WoE) approach*

Comment:

In general and from a theoretical point of view, the WoE approach appears to be a useful tool for possible filling of data gaps. As it is stated in several text passages, expert knowledge is needed and case-by-case decisions have to be made.

With respect to the standard endpoints in aquatic ecotoxicology, which are fish and *Daphnia* acute toxicity as well as algae growth inhibition, a more specific guidance for these three endpoints would be desirable. Under Step 6 the 'Threshold approach for toxicity testing in fish' is explained. Since the WoE is also related to ITS, it would be adequate to also list the existing circumstances under which testing, e.g. of fish acute toxicity, can be omitted in this or in a referenced chapter. In several text passages in R7.b, e.g. App. 7.8.5.3, it is explained that fish do not need to be tested when it is likely that invertebrates or algae are at least a factor of 10 more sensitive than fish. However, how to determine this without testing? Here is again a strong interaction between the chapters which becomes evident after reading all relevant sections.

Recommendations:

The structure of the guidance document should be revised in general in order to give clear advice on single endpoints (including the WoE). Please see the main report, chapter 3.2 for a proposal of a revised section R.7.8.5.

2.2.2 Appendix R.7.8-5 'Assessment of available information on endocrine and other related effects'

Reference: *Appendix R7.8-5*

Content: *Structure of the Appendix*

Comment:

In Appendix R7.8-5, all steps are considered that are necessary to evaluate whether a substance has endocrine disrupting potential. Figure R.7.8-8 provides a very useful scheme on how the assessment procedure should be performed. However, some restructuring of Appendix R7.8-5 is suggested as outlined below.

Recommendations:

It would be very helpful to move Figure R.7.8-8 and the text on pages 115 – 118 from the end of the section to its beginning, so that an overview of the whole assessment is given before the single steps and, then, the different tests are presented.

Reference: *Appendix R7.8-5*

Content: *Metabolites / transformation products*

Comment:

Metabolisation in humans / animals and transformation in the environment may lead to an increased endocrine activity. At present, metabolites / transformation products are not mentioned in Appendix R7.8-5. Should possible metabolites that are, for example, identified using the OECD Toolbox and that are predicted to have a high endocrine activity be included in the assessment?

Recommendations:

Some further guidance on this issue would be very helpful.

Reference: *Appendix R7.8-5*

Content: *Endocrine effects at high substance concentrations*

Comment:

For some substances, endocrine effects are only observed at substance concentrations that are in the range of or only slightly below concentrations causing general toxic effects. However, endocrine endpoints as, for example, vitellogenin levels in female fish can also be affected by general toxicity and hepatotoxicity (see e.g. OECD, 2009b).

Recommendations:

Some further guidance on the evaluation of endocrine effects, which are only observed at substance concentrations in the range of or slightly below concentrations causing general toxic effects, would be very helpful.

Reference: *Figure R7.8-8, part 1*

Content: 'Preliminary indication of potential endocrine activity in aquatic organisms'

Comment:

Apart from the information that 'all available information, including environmental fate and exposure' should be considered, little guidance is available on how 'concern of potential endocrine mode of action' should be determined based on molecular structure, mammalian toxicity data and *in vitro* screening data. While the results from fish tests (parts 2 and 3 of figure R7.8-8) allow for a comparison of effect concentrations with predicted or measured environmental concentrations, this is not possible for the above-mentioned data on which the 'preliminary indication of potential endocrine activity in aquatic organisms' is based. QSAR data, *in vitro* screening data and mammalian toxicity data only provide information on relative activity (e.g. compared to a positive control).

Guidance is lacking on how the registrant should proceed, if only such preliminary information on potential endocrine activity is available. What is defined as 'strong concern'? How should potential concern be evaluated based on these data taking 'environmental fate and exposure' into account? May further testing be waived in case that exposure of the aquatic environment is demonstrated to be negligible? In which cases are further tests required? Calabrese et al. (1997), which is cited in R7.8-8, provides a methodology for a relative ranking of substances with potential endocrine (estrogenic) activity. This approach is helpful for substances for which there is conflicting information, and also for prioritising substances for further testing. However, it does not provide an indication on the threshold of concern required to trigger further testing. Should *in vitro* tests be performed in all cases where structural alerts indicate a potential for endocrine effects? Should (additional) *in vitro* tests be performed in cases where available information from QSARs or *in vitro* tests is conflicting? Should an *in vivo* screening test be performed in all cases where *in vitro* tests indicate an endocrine effect, even if this effect is very weak, or should such an *in vivo* test only be performed in case that the effect exceeds a certain threshold (which is certainly not easy to define)? The draft 'Guidance document on the assessment of chemicals for endocrine disruption' (OECD, 2010c) provides some information regarding this issue.

Recommendations:

Further guidance is required regarding this issue.

3 References

Calabrese E.J., Baldwin, L.A., Kostecki, P.T., Potter, T.L. (1997). A toxicologically based weight-of-evidence methodology for the relative ranking of chemicals of endocrine disruption potential. Regul. Toxicol. Pharmacol. 26, 36-40.

ECHA (2009). Guidance on the Application of the CLP Criteria. Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures. ECHA-09-G-02-EN, 25-Aug-2009.

ECHA (2010).Practical guide 2: How to report weight of evidence. ECHA-10-B-05-EN. ISBN-13: 978-92-9217-028-8, ISSN: 1831-6727, 24-Mar-2010.

Fernández A, Rallo R, Giralt F (2009). Uncertainty reduction in environmental data with conflicting information. Environ. Sci. Technol. 43: 5001-5006.

Jaworska J, Gabbert S, Aldenberg T (2010). Towards optimization of chemical testing under REACH: A Bayesian network approach to Integrated Testing Strategies. Regul. Toxicol. Pharmacol. 57: 157-167.

Laws, S.C., Yavanhxay, S., Cooper, R.L., Eldridge, J.C. (2006). Nature of the binding interaction for 50 structurally diverse chemicals with rat estrogen receptors. Toxicol. Sci. 94, 46-56.

OECD (2008a). Detailed review paper on fish life-cycle tests. Series on testing and assessment, No. 95. OECD, Paris.

OECD (2008b). *Daphnia magna* reproduction assay. OECD guideline for testing of chemicals, No. 211. OECD, Paris.

OECD (2009a). Stably transfected human estrogen receptor-α transcriptional activation assay for detection of estrogenic agonist-activity of chemicals. OECD guideline for testing of chemicals, No. 455. OECD, Paris.

OECD (2009b).21-Day fish assay: a short-term screening test for oestrogenic and androgenic activity, and aromatase inhibition. OECD guideline for testing of chemicals, No. 230. OECD, Paris.

OECD (2009c). Short-term reproduction test. OECD guideline for testing of chemicals, No. 229. OECD, Paris.

OECD (2009d). Draft Guidance Document on Fish Gonadal Histopathology. OECD Environmental Health and Safety Publications. Series on Testing and Assessment. OECD, Paris.

OECD (2009e). The amphibian metamorphosis assay. OECD guideline for testing of chemicals, No. 231. OECD, Paris.

OECD (2010a). Short guidance on the threshold approach for acute fish toxicity. OECD Environmental Health and Safety Publications. Series on Testing and Assessment No. 126. ENV/JM/MONO(2010)17. OECD, Paris.

OECD (2010b). Draft proposal. Fish sexual development test. Version of 21 December 2010.

OECD (2010c). Guidance document on the assessment of chemicals for endocrine disruption. Draft, Version 9 (17 November 2010).

OECD (2010d). Draft fish testing framework document. Available at http://www.oecd.org/document/12/0,3343,en_2649_34377_1898188_1_1_1_1,00.html.

Final Report

on

UBA R&D Project

Review and Enhancement of New Risk Assessment Concepts under REACH

(Überprüfung und Weiterentwicklung neuer Konzepte zur Risikobewertung unter REACH)

Annex 2:

Literature data on acute toxicity of 2,4,6-tribromophenol, benzanthrone and benzophenone-2 to aquatic organisms

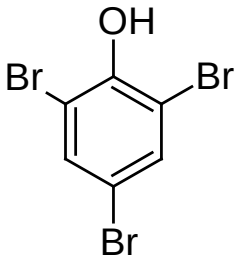
FKZ 3708 65 407

April 2011

CONTENTS

1 2,4,6-Tribromophenol

1.1 Substance data

Name: 2,4,6-Tribromophenol	**Structural formula**:  	**Candidate for category (1/2/3):** 1
CAS No.: 118-79-6		
SMILES Code: Oc(c(cc(c1)Br)Br)c1Br		**Substance group**: Brominated phenol
Uses / exposure routes: Brominated flame retardant, antiseptic, germicide, wood preservative, intermediate for PCP and for production of poly(dibromophenylene oxide), a flame retardant (HSDB)		**Production volume / producers**: HPV; Eurobrom (ESIS)
Classification & labelling: Not classified in the Annex I of Directive 67/548/EEC		**Database hits**: ESIS, HSDB, OECD, NITE, ECOTOX, Scorecard

1.2 Main characteristics (phys.-chem. / fate)

Endpoint	Value	Measured / calculated	Source / Reference
Molecular weight	330.8	Calc.	EPISuite v4.00
Water solubility (mg/L)	70 (15°C) 50 (25°C;) 10 (25°C)	Meas. (-) Meas. (OECD 105) Meas. (OECD 112)	EPISuite v4.00 (Yalkowsky & Dannenfelser, 1992); OECD SIDS, 2005
p*Ka*	ca. 6.2	Calc.	SPARC
log K_{ow}	3.7 3.89	Meas. (OECD 117) Meas. (OECD 107)	OECD SIDS, 2005
log D_{ow}	ca. 3.3	Calc.	logD calc.
HLC (Pa*m³/mol)	3.59E-03 (25°C)	Calc. (Bond method)	EPISuite v4.00
Hydrolysis	No hydrolysis	Meas.	OECD SIDS, 2005
Ready biodegradability	Biodegradable (49% BOD/ThOD)	Meas. (MITI (I))	NITE / OECD SIDS, 2005
BCF	513	Meas.	OECD SIDS, 2005
Indirect photolysis (OH rate constant)	0.4749E-12 cm³ /molecule*sec (22.5 d half-life – 12h light)	Calc.	EPISuite v4.00

Biotransformation	0.5 d (half-life – 10 g fish, 15°C)	Calc.	EPISuite v4.00
ΔH_f	-9.66 kJ/mol	Calc.	MOPAC, 2002
E_{gap}	8.88 eV	Calc.	MOPAC, 2002

1.3 Ecotoxicity

1.3.1 Fish acute toxicity

Substance data

Substance	**LC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
2,4,6-Tribromophenol	96-h LC_{50} = 1.1 mg/L (3.3 µM)	OECD 203 (with GLP and chem. analysis)	OECD SIDS, 2005	Exp. (*Cyprinus carpio*)
Original reference:	DSBG/Bromine compounds Ltd. (1998). 96-hour acute toxicity study in carp with 2,4,6-tribromophenol (FR-613), (STATIC). 14 August 1998.			
Evaluation:	Original reference not available, but documentation in OECD SIDS sufficient for evaluation. A range finder and two separate studies were performed. Results were similar. Critical endpoint.			
Reliability:	2 (valid with restrictions as indicated in the OECD SIDS)			

Substance	**LC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
2,4,6-Tribromophenol	96-h LC_{50} = 1.5 mg/L (4.5 µM)	OECD 203 (with GLP and chem. analysis)	NITE / OECD SIDS, 2005	Exp. (*Oryzias latipes*)
Original reference:	Environmental Agency of Japan (2000). Ecotoxicity testing report, Test Number 10034. Acute toxicity to himedaka (*Oryzias latipes*). Unpublished report, Japan Food Research Laboratories.			
Evaluation:	Original reference not available, but documentation in OECD SIDS sufficient for evaluation.			
Reliability:	1 (valid without restriction as indicated in the OECD SIDS)			

Substance	**LC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
2,4,6-Tribromophenol	96-h LC_{50} = 6.5 / 6.8 mg/L (19,7 µM / 20.6 µM)	APHA (1971)	OECD SIDS, 2005	Exp. (*Pimephelas promelas*)
Original reference:	Phipps G.L., Holcombe G.W., Fiandt J.T. (1981). Acute toxicity of phenol and substituted phenols to the fathead minnow. Bull. Environ. Contam. Toxicol. 26: 585-593.			
Evaluation:	Documentation in OECD SIDS sufficient for evaluation.			
Reliability:	2 (valid with restrictions as indicated in the OECD SIDS)			

Substance	LC_{50} (incl. test duration)	Guideline	Source / Reference	Remarks (exp./calc./species)
2,4,6-Tribromophenol	96-h LC_{50} = 6.25 mg/L (18.9 µM)	No guideline test	Broderius et al., 1995	Exp. (*P. promelas*)
Full reference:	Broderius S.J., Kahl M.D., Hoglund M.D. (1995). Use of joint toxic response to define the primary mode of toxic action for diverse industrial organic chemicals. Environ. Toxicol. Chem.14: 1591-1605.			
Evaluation:	Acute toxicity of a range of organic chemicals (including 2,4,6-tribromophenol) was determined in a 96 h flow-through test with 26- to 34-d-old fathead minnows. Purity of the test substances was at least 95%. Fish were exposed at 25°C to 4 or 5 toxicant concentrations and a control with two replicates for each test. Toxicant concentrations were measured daily. All tests were performed without using solvents. LC_{50}-values were calculated using the trimmed Spearman-Karber method. For 2,4,6-tribromophenol, an 96 h LC_{50} of 6.25 mg/L was derived.			
Reliability:	2 (valid with restrictions as indicated in the OECD SIDS)			

Substance	LC_{50} (incl. test duration)	Guideline	Source / Reference	Remarks (exp./calc./species)
2,4,6-Tribromophenol	48-h LC_{50} = 4.4 mg/L (13.3 µM)	DIN (2001)	Kammann et al., 2006	Exp. (*D. rerio*, embryos)
Full reference:	Kammann U., Vosbach M., Wosniok W. (2006). Toxic effects of brominated indoles and phenols on zebrafish embryos. Arch. Environ. Contam. Toxicol. 51: 97-102.			
Evaluation:	The zebrafish embryo test was carried out according to DIN (2001). Fertilized eggs were exposed for 48 h in 24-well plates (5 eggs in 1 ml test solution per well). Test substances were dissolved in DMSO (final concentration of DMSO in test solutions: 1%). The tests were replicated twice. They included a solvent control, but no control without solvent. Copper (as copper sulfate dehydrate; 0.5 mg/L Cu) and 3,4-dichloroaniline (3.7 mg/L) were used as positive controls. LC_{50}- and EC_{50}-values were derived by iterative maximum likelihood estimation. The selected concentrations of Cu and 3,4-dichloroaniline led to ca. 50-60% lethal effects. For 2,4,6-tribromophenol, an LC_{50} of 4.4 mg/L was derived. EC_{50}-values for the endpoints lack of pigmentation, spinal deformations and yolk sac edema were 5.7, 3.9 and 3.1 mg/L. Nonpolar narcosis is suggested as major mode of action of the studied bromophenols.			
Reliability:	Scientifically acceptable, but no chemical analysis and no control without solvent. Test method has not yet been validated / accepted as alternative to the acute fish test for the testing of chemicals.			

Analogues data

Not necessary as the endpoint is covered.

1.3.2 Invertebrates acute toxicity

Substance data

Substance	EC_{50} (incl. test duration)	Guideline	Source / Reference	Remarks (exp./calc./species)
2,4,6-Tribromophenol	48-h EC_{50} = 0.26 mg/L (0.8 µM)	OECD 202 (with GLP and chem. analysis)	OECD SIDS, 2005	Exp. (*Daphnia magna*)
Original reference:	DSBG/Bromine Compounds Ltd. (1998). Acute toxicity study in *Daphnia magna* with 2,4,6-tribromophenol (FR-613), (static). 4 August 1998.			
Evaluation:	Original reference not available, but documentation in OECD SIDS sufficient for evaluation. Critical endpoint.			
Reliability:	2 (valid with restrictions as indicated in the OECD SIDS)			

Substance	EC_{50} (incl. test duration)	Guideline	Source / Reference	Remarks (exp./calc./species)
2,4,6-Tribromophenol	48-h EC_{50} = 2.2 mg/L (6.7 µM)	OECD 202 (with GLP and chem. analysis)	OECD SIDS, 2005	Exp. (*D. magna*)
Original reference:	Environmental Agency of Japan (2000). Ecotoxicity testing report, test number 10032. Acute toxicity to daphnid (*Daphnia magna*). Unpublished report, Japan Food Research Laboratories.			
Evaluation:	Original reference not available, but documentation in OECD SIDS sufficient for evaluation.			
Reliability:	1 (valid without restriction as indicated in the OECD SIDS)			

Substance	EC_{50} (incl. test duration)	Guideline	Source / Reference	Remarks (exp./calc./species)
2,4,6-Tribromophenol	48-h EC_{50} = 1.31 mg/L (4.0 µM)	No data (no chemical analysis)	OECD SIDS, 2005	Exp. (*D. magna*)
Original reference:	Kopperman H.L., Carlson R.M., Caple R. (1974). Aqueous chlorination and ozonation studies. I. Structure-toxicity correlations of phenolic compounds to *Daphnia magna*. Chem.-Biol. Interact. 9: 245-251.			
Evaluation:	Documentation in OECD SIDS sufficient for evaluation.			
Reliability:	4 (not assignable as indicated in the OECD SIDS)			

Analogues data

Not necessary as the endpoint is covered.

1.3.3 Algae toxicity

Substance data

Substance	EC_{50} (incl. test duration)	Guideline	Source / Reference	Remarks (exp./calc./species)
2,4,6-Tribromophenol	72-h ErC_{50} = 1.6 mg/L (4.8 µM); NOErC = 1 mg/L (3.0 µM)	OECD 201 (with GLP and chem. analysis)	OECD SIDS, 2005 / NITE	Exp. (*Selenastrum capricornutum*)
Original reference:	Environmental Agency of Japan (2000). Ecotoxicity testing report. Test Number 10031, growth inhibition test to algae (*Selenastrum capricornutum*). Unpublished report, Japan Food Research Laboratories.			
Evaluation:	Original reference not available, but documentation in OECD SIDS sufficient for evaluation; critical endpoint.			
Reliability:	1 (valid without restriction as indicated in the OECD SIDS)			

Substance	EC_{50} (incl. test duration)	Guideline	Source / Reference	Remarks (exp./calc./species)
2,4,6-Tribromophenol	72-h ErC_{50} = 0.4 mg/L (1.2 µM); NOErC = 0.1 mg/L (0.3 µM)	OECD 201 (with GLP and chem. analysis)	OECD SIDS, 2005	Exp. (*Selenastrum capricornutum*)
Original reference:	DSBG/Bromine Compounds Ltd. (1998). Fresh water algal growth inhibition test with 2,4,6-tribromophenol. FR-613. 4 August 1998.			
Evaluation:	Original reference not available, but documentation in OECD SIDS sufficient for evaluation. Due to shortcomings in the analytical data and/or their documentation the study was downgraded.			
Reliability:	4 (not assignable as indicated in the OECD SIDS)			

Analogues data

Not necessary as the endpoint is covered.

1.3.4 Other ecotoxicity data (e.g. *Daphnia* or fish long-term)

Daphnia long-term toxicity

Substance	NOEC (incl. test duration)	Guideline	Source / Reference	Remarks (exp./calc./species)

2,4,6-Tribromophenol	21-d NOEC = 0.1 mg/L (0.3 µM)	OECD 211 (with GLP and chem. analysis)	NITE / OECD SIDS, 2005	Exp. (*D. magna*)
Original reference:	Environmental Agency of Japan (2000). Ecotoxicity testing report. Test Number 10033. Reproduction test to *Daphnia magna*. Unpublished report, Japan Food Research Laboratories.			
Evaluation:	Original reference not available, but documentation in OECD SIDS sufficient for evaluation; critical endpoint.			
Reliability:	1 (valid without restriction as indicated in the OECD SIDS)			

Fish embryo and larvae toxicity

Substance	**NOEC (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
2,4,6-Tribromophenol	LOEC = 0.22 mg/L (0.7 µM) (NOEC not indicated)	no guideline test; not clear if LOEC based on nominal or measured concentration	Neilson et al., 1990	Exp. (*Danio rerio*)
Original reference:	Neilson A.H., Allard A.-S., Fischer S., Malmberg M., Viktor T. (1990). Incorporation of a subacute test with zebra fish into a hierarchical system for evaluating the effect of toxicants in the aquatic environment. Ecotoxicol. Environ. Saf. 20: 82-97.			
Evaluation:	Effects of 2,4,6-tribromophenol on zebrafish embryos and larvae were evaluated according to the method described by Dave et al. (Environ. Toxicol. Chem. 6, 61-71): Exposure was started 2 to 4 hours after spawning. Embryos and larvae were exposed at 26°C. Test solutions were renewed daily and the number of dead embryos and larvae was recorded. No food was provided and the test was terminated when at least 90% of the larvae at all concentrations had died. 'Median effective times' for hatch and survival were determined for each test concentration and the controls. From these 'median effective times', the LOEC was derived. Toxicity of 2,4,6-tribromophenol was studied at pH 6.2, 7.2 and 8.2 using 2-(N-morpholino)ethane-sulfonic acid, 3-(N-morpholino)propanesulfonic acid and piperazine-N,N'-bis(2-hydroxypropanesulfonic acid), respectively, as buffers. Concentrations of 2,4,6-tribromophenol were determined by gas chromatography. Measured concentrations of 2,4,6-tribromophenol were on average 85% of nominal concentrations. No further detail on the results of the chemical analysis is provided, i.e. it is not clear, whether recoveries in the different experiments / at different pH differed. LOEC-values of 0.10 mg/L (pH 6.2), 0.22 mg/L (pH 7.2) and 0.80 mg/L (pH 8.2) were derived for *D. rerio*. Neither NOEC-values nor the spacing factor between tested substance concentrations are indicated.			
Reliability:	3 (not reliable): no guideline test, little information on experimental methodology and test results (e.g. performance of controls), little information on results of chemical analysis, use of buffers (which might have influenced the test result), test method is			

	questionable (exposure of larvae without feeding until starvation).

2 Benzanthrone

2.1 Substance data

Name Benzanthrone	**Structural formula**	**Candidate for category (1/2/3):** 2
CAS No. 82-05-3		
SMILES Code O=C(c(c(c(c1c(ccc2)cc3)c3)ccc4)c4)c12		**Substance group** PAH
Uses / exposure routes Dyestuff intermediate for anthraquinone-based dyes; use for photosensitization, charge transport material and in pyrotechnics industry.		**Production volume / producers:** LPV (e.g. Zeneca, ACNA (ESIS); BASF AG: < 500 t/a (BUA report)
NOTE: Following the BUA report, production at BASF/Germany was terminated in May 2003		
Classification & labelling Not listed (and not in priority list as foreseen under EEC 793/93)		**Database hits:** EPIWIN, WIKI (en), HSDB, MITI, ECOTOX

2.2 Main characteristics (phys.-chem. / fate)

Endpoint	**Value**	**Measured / calculated**	**Source / Reference**
Molecular weight	230.27	Calc.	EPISuite v4.00
Water solubility (mg/L)	0.18 (25°C) (0.78µM)	Calc.	EPISuite v4.00
p*K*a	--	Calc.	SPARC
log K_{OW}	4.81	Meas.	EPISuite v4.00
log D_{OW}	--		
HLC (Pa*m³/mol)	6.7E-03 (25°C)	Calc. (Bond est.)	EPISuite v4.00
Hydrolysis	Not expected	Expert judgem.	
Ready biodegradability	No (0%, 4 weeks)	Meas. (MITI (I))	MITI (HSDB)
BCF	61 – 181 (fish)	Meas.	MITI
Indirect photolysis (OH rate constant)	18.00E-12 cm³ /molecule*sec (1-8 d half-life – 12h light)	Calc.	EPISuite v4.00
Biotransformation	0.39 days (Half-life – 10 g fish, 15°C)	Calc.	EPISuite v4.00
ΔH_f	+169.32 kJ/mol	Calc.	MOPAC, 2002

E_{gap}	7.43 eV	Calc.	MOPAC, 2002

2.3 Ecotoxicity

2.3.1 Fish acute toxicity

Substance data

Substance	**LC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
Benzanthrone	96 h LC_{50} = 0.55 mg/L (2.4 µM)	OECD TG 203 (with GLP, without chem. analysis)	BUA report No. 251, 2005	Exp. (*Danio rerio*)
Original reference:	BASF AG 1992. Report on the study of the acute toxicity; zebra fish (*Brachydanio rerio* Ham. U. Buch.). Project No. 17F0688/905157. Unpublished report, 22 July 1992 (as cited in BUA report)			
Evaluation:	96-hour static test under GLP without analytical monitoring; purity of test substance: 99%; NOEC = 0.1 mg/L; LC_{100} > 2.15 mg/L (information from IUCLID as included in BUA report).			
Reliability:	1 (valid without restriction as indicated in the BUA report)			

Substance	**LC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
Benzanthrone	48 h LC_{50} > 100 mg/L (> 434 µM)	Japanese Industrial Standard JIS K 0102-1986-71	BUA report No. 251, 2005	Exp. (*Oryzias latipes*)
Original reference:	Chemicals Inspection & Testing Institute Japan (ed.) (1992). Data of existing chemicals based on the CSCL Japan. October 1992.			
Evaluation:	Original reference not available. Test result based on nominal substance concentration.			
Reliability:	3 (not reliable). In the BUA report, a reliabilty of '1' was assigned. However, given that the effect concentration (> 100 mg/L) is far above the calculated water solubility limit of 0.18 mg/L and that the test duration only was 48 h, the test was downgraded.			

Substance	**LC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
Benzanthrone	0.83 h LC_{50} = 0.05 mg/L (0.2 µM); LT50 = 0.83 h	No guideline test	Oris and Giesy (1987)	Exp. (*P. promelas*)
Original reference:	Oris J.T., Giesy J.P. Jr. (1987). The photo-induced toxicity of polycyclic aromatic hydrocarbons to larvae of the fathead minnow (*Pimephales promelas*). Chemosphere 16: 1395-1404.			

Evaluation:	Photo-induced toxicity of benzanthrone and 11 other polycyclic aromatic hydrocarbons (PAHs) to larvae of *P. promelas* was studies. Larvae (7 d post-hatch) were exposed in 300 ml glass dishes (20 to 25 larvae per dish) containing 150 ml of test solution or control water with two replicates per treatment. Larvae were first exposed for 24 h to a solution of benzanthrone (nominal: 0.0316 mg/L, measured: 0.0495 mg/L) in the absence solar UV radiation. Test solutions were then replaced and larvae were placed under a laboratory system light bank simulating natural sunlight. Light was filtered to eliminate >99% of the radiation of wavelengths below 315 nm. Solar UV radiation intensities were monitored: UV-B (290-336 nm) was 20 µW/cm^2, UV-A (336-400 nm) was 95 µW/cm^2. Solutions were changed at 12 h intervals. Larvae were fed brine shrimp once daily prior to changing test solutions. Benzanthrone concentrations were measured at 0 and 12 h. The median lethal time (LT_{50}) for the twelve PAHs in fish was determined. Mortality of the controls was less than 5% in all tests. None of the tested PAHs exhibited toxicity during the first 12 h of exposure in the dark. Benzanthrone showed an acute photo-induced toxicity against *P. promelas* larvae. With an LT_{50} of 0.83 hours, benzanthrone had the lowest median lethal time of the 12 tested PAHs, i.e. benzanthrone had the greatest absorption-specific photo-induced toxicity.
Reliability:	2 (valid with restrictions – scientifically acceptable as indicated in BUA report)

Substance	**LC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
Benzanthrone	96-h LC_{50} = 0.7 mg/L (3.0 µM)	-	EPISuite v4.00	Calculated (class: neutral organics)
Reference:	-			
Evaluation:	-			
Reliability:	4 (not assignable – Documentation of QSAR validation insufficient for assessment; not in line with OECD recommendations)			

Analogues data

Substance	**CAS No.**	**MW (g/mol)**	**96-h LC_{50} [mg/L] ***	**96-h LC_{50} [µM]**	**Remarks (exp./calc./species)**
Fluoranthene	206-44-0	202.3	0.031	0.15	mutagen
Benzophenone	119-61-9	202.3	14.8	73.2	nonmutagen
Naphthalene	91-20-3	128.2	6.14	47.9	
Acenaphthene	83-32-9	154.2	1.73	11.2	nonmutagen
Biphenyl	92-52-4	154.2	2.33	15.1	nonmutagen
Acridine	260-94-6	179.2	2.47	13.8	mutagen

* Measured data from UFZ database (converted from log values to µM and mg/L).

2.3.2 Invertebrates acute toxicity

Substance data

Substance	**EC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
Benzanthrone	5.4 h EC_{50} = 0.035 mg/L (0.015 µM); LT_{50} = 0.224 d	No guideline test; chem. analysis	Newsted & Giesy, 1987	Exp. (*Daphnia* sp.)
Reference:	Newsted J.L., Giesy J.P. (1987). Predictive models for photoinduced acute toxicity of polycyclic aromatic hydrocarbons to *Daphnia magna*, Strauss (Cladocera, Crustacea). Environ. Toxicol. Chem. 6: 445-461.			
Evaluation:	The test consisted of 3 groups of 20 daphnids, each in 200 mL of aqueous test substance solution. The nominal test concentration was 34.3 µg/L. During the first 24 h of the exposure, the daphnids were kept in the test solution under laboratory conditions, i.e., 16 h lightness, 8 h darkness, no UV light. During the following 24 h of exposure, the daphnids were subjected to simulated sunlight, i.e. UV radiation for 12 h. The test solution was renewed after the transfer of the daphnids from the laboratory to the simulated sunlight conditions, and again at the end of the simulated sunlight exposure. Within the first part of the exposure, i.e., 24 h under laboratory conditions, no mortality was observed. Mortality occurred during the second part of the exposure, when daphnids were subjected to simulated sunlight (UV radiation). The measured test concentration of benzanthrone was 35.1 µg/L. The measured concentration of benzanthrone in daphnids was 79 nM/g wet weight. The actual median lethal time for benzanthrone was LT_{50} = 232 min. The LC_{50} given as nominal value was deduced from the LT_{50} value (based on BUA, 2004).			
Reliability:	2 (valid with restrictions – scientifically acceptable as indicated in BUA report)			

Substance	**EC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
Benzanthrone	48-h EC_{50} = 0.6 mg/L (2.6 µM)	-	EPISuite v4.00	Calculated (class: neutral organics)
Reference:	-			
Evaluation:	-			
Reliability:	4 (not assignable – Documentation of QSAR validation insufficient for assessment; not in line with OECD recommendations)			

Analogues data

Substance	CAS No.	MW (g/mol)	48- h EC_{50} [mg/L] *	48- h EC_{50} [µM]	Remarks (exp./calc./ species)
Fluoranthene	206-44-0	202.3	0.11	0.54	mutagen
Benzophenone	119-61-9	202.3	7.6	37.6	nonmutagen
Fluorene	86-73-7	166.2	0.427	2.6	nonmutagen
Anthraquinone	84-65-1	208.2	10.0	48.0	mutagen
Anthracene	120-12-7	178.2	0.427	2.4	mutagen
Phenanthrene	85-01-8	178.2	0.778	4.4	mutagen
Naphthalene	91-20-3	128.2	9.72	75.8	
Acenaphthene	83-32-9	154.2	2.33	15.1	nonmutagen
Biphenyl	92-52-4	154.2	3.37	21.9	nonmutagen
Acridine	260-94-6	179.2	2.77	15.5	mutagen

* Measured data from UFZ database (converted from log values to µM and mg/L).

2.3.3 Algae toxicity

Substance data

Substance	EC_{50} (incl. test duration)	Guideline	Source / Reference	Remarks (exp./calc./species)
Benzanthrone	96-h EC_{50} = 0.9 mg/L (3.9 µM); NOEC = 0.56 mg/L (2.4 µM)	-	EPISuite v4.00	Calculated (class: neutral organics)
Reference:	-			
Evaluation:	-			
Reliability:	4 (not assignable – Documentation of QSAR validation insufficient for assessment; not in line with OECD recommendations)			

Substance	EC_{50} (incl. test duration)	Guideline	Source / Reference	Remarks (exp./calc./species)
Benzanthrone	Carcinogenic in algae	-	BUA report No. 251 (2005)	Exp. (*Porphyra tenera*)
Original reference:	Ishio S et al. (1971). In: Adv. Water Pollut. Res. Proc. Int. Conf., 5th 1970. Ed. SH Jenkins, 2, III-18/1-III-18/8.			
Evaluation:	Young leaves of *P. tenera*, a marine red alga, were exposed to benzanthrone at a concentration of 0.2 ppm (emulsion in Tween 20; in order to avoid incidence of cancer due to an excess of Tween 20, the concentration of this solvent was 4 ppm) for 40 days. The exposure to benzanthrone resulted in changes indicative of a cancerous disease, and confirmed the carcinogenic potential of benzanthrone on *P. tenera* (based on BUA, 2005).			

Reliability:	2 (valid with restrictions – scientifically acceptable as indicated in BUA report)

Analogues data

Substance	**CAS No.**	**MW (g/mol)**	**72/96-h EC_{50} [mg/L] ***	**72/96-h EC_{50} [µM]**	**Remarks (exp./calc./species)**
Phenanthrene	85-01-8	178.2	0.408	2.3	mutagen
Acenaphthene	83-32-9	154.2	0.522	3.4	nonmutagen
Acridine	260-94-6	179.22	0.636	3.6	mutagen
Benzo(a)pyrene	50-32-8	252.32	0.015	0.06	mutagen, high carcinogenicity

* Measured data from UFZ database (converted from log values to µM and mg/L).

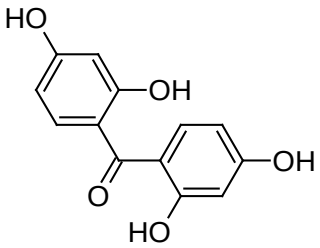
2.3.4 Other ecotoxicity data (e.g. fish long-term)

Substance data

No data.

3 Benzophenone-2

3.1 Substance data

Name: Benzophenone-2; 2,2'4,4'-tetrahydroxy-benzophenone	**Structural formula**: 	**Candidate for category (1/2/3):** 3
CAS No.: 131-55-5		**Possible endocrine mechanism (estrogen / androgen):** Estrogen
SMILES Code: O=C(c(c(O)cc(O)c1)c1)c(c(O)cc(O)c2)c2		**Substance group:** Benzophenones
Uses / exposure routes: UV filter (Kant. Lab. Basel, CH)		**Production volume / producers**: LPV; e.g. BASF (ESIS)
Classification & labelling: Xn; R22; R36/37/38		**Database hits**: ESIS

3.2 Main characteristics (phys.-chem. / fate)

Endpoint	Value	Measured / calculated	Source / Reference
Molecular weight	246.22	Calc.	EPISuite v4.00
Water solubility (mg/L)	399 (25°C) (1.62 mM)	Calc.	EPISuite v4.00
p*K*a	pKa 1 = ca. 7.5 pKa 2 = ca. 8.5 pKa 3 = ca. 11.1; pKa 4 = ca. 13.6	Calc.	SPARC
log K_{ow}	2.78	Calc.	EPISuite v4.00
log D_{ow}	ca. 2.7	Calc.	logD estimation
HLC (Pa*m³/mol)	3.66E-011 (25°C)	Calc. (Bond est.)	EPISuite v4.00
Hydrolysis	Not expected	Expert judgem.	
Ready biodegradability	No	Calc.	EPISuite v4.00
BCF	8.2	Calc.	EPISuite v4.00
Indirect Photolysis (OH rate constant)	200.56E-12 cm³ /molecule*sec (0.05 d half-life – 12h light)	Calc.	EPISuite v4.00
Biotransformation	0.002 d (half-life – 10 g fish 15°C)	Calc.	EPISuite v4.00

ΔH_f	-673.12 kJ/mol	Calc.	MOPAC,2002
E_{gap}	8.61 eV	Calc.	MOPAC, 2002

3.3 Ecotoxicity

3.3.1 Fish acute toxicity

Substance data

No experimental data available.

Substance	**LC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
Benzophenone-2	96-h LC_{50} = 8.1 mg/L (32.9 µM)	-	EPISuite v4.00	Calculated (class: phenols, poly)
Reference:	-			
Evaluation:	-			
Reliability:	4 (not assignable – documentation of QSAR validation insufficient for assessment; not in line with OECD recommendations)			

Substance	**LC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
Benzophenone-2	96-h LC_{50} = 41.5 mg/L (169 µM)	-	EPISuite v4.00	Calculated (class: neutral organics)
Reference:	-			
Evaluation:	-			
Reliability:	4 (not assignable – documentation of QSAR validation insufficient for assessment (not in line with OECD recommendations)			

Analogues data

Substance	**CAS No.**	**MW (g/mol)**	**96-h LC_{50} [mg/L] ***	**96-h LC_{50} [µM]**	**Remarks (exp./calc./species))**
Methyl-2,4-dihydroxybenzoate	2150-47-2	168.15	45.3	269	Exp.
4,4'-Oxybisphenol	1965-09-9	202.21	5.83	28.8	Exp.

* Measured data from UFZ database (converted from log values to µM and mg/L).

3.3.2 Invertebrates acute toxicity

Substance data

No experimental data available.

Substance	**EC_{50} (incl. test**	**Guideline**	**Source / Reference**	**Remarks**

	duration)			**(exp./calc./species)**
Benzophenone-2	48-h EC_{50} = 26.0 mg/L (106 µM)	-	EPISuite v4.00	Calculated (class: phenols, poly)
Reference:	-			
Evaluation:	-			
Reliability:	4 (not assignable – documentation of QSAR validation insufficient for assessment; not in line with OECD recommendations)			

Substance	**EC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
Benzophenone-2	48-h EC_{50} = 26.3 mg/L (107 µM)	-	EPISuite v4.00	Calculated (class: neutral organics)
Reference:	-			
Evaluation:	-			
Reliability:	4 (not assignable – documentation of QSAR validation insufficient for assessment; not in line with OECD recommendations)			

Analogues data

Substance	**CAS No.**	**MW (g/mol)**	**48-h EC_{50} [mg/L] ***	**48-h EC_{50} [µM]**	**Remarks (exp./calc./species)**
Bisphenol A	80-05-7	228.29	9.1	39.9	Exp. (nonmutagen, no carcinogenicity)
2,5-Dihydroxy-benzaldehyde	1194-98-5	138.12	20.9	151	Exp.

* Measured data from UFZ database (converted from log values to µM and mg/L).

3.3.3 Algae toxicity

Substance data

No experimental data available.

Substance	**EC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
Benzophenone-2	96-h EC_{50} = 1.8 mg/L (7.3 µM); NOEC = 0.4 mg/L (1.6 µM)	-	EPISuite v4.00	Calculated (class: phenols, poly)
Reference:	-			
Evaluation:	-			

Reliability:	4 (not assignable – documentation of QSAR validation insufficient for assessment; not in line with OECD recommendations)

Substance	**EC_{50} (incl. test duration)**	**Guideline**	**Source / Reference**	**Remarks (exp./calc./species)**
Benzophenone-2	96-h EC_{50} = 15.6 mg/L (63.4 µM); NOEC = 6.5 mg/L (26.4 µM)	-	EPISuite v4.00	Calculated (class: neutral organics)
Reference:	-			
Evaluation:	-			
Reliability:	4 (not assignable – documentation of QSAR validation insufficient for assessment; not in line with OECD recommendations)			

Analogues data

Substance	**CAS No.**	**MW (g/mol)**	**72/96-h EC_{50} [mg/L] ***	**72/96-h EC_{50} [µM]**	**Remarks (exp./calc./species)**
4,4'-Isopropylidene-diphenol	80-05-7	228.29	2.87	12.6	Exp. (nonmutagen, no carcinogenicity)

* Measured data from UFZ database (converted from log values to µM and mg/L).