Read-across Estimates of Aquatic Toxicity for Selected Fragrances

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Summary — Read-across as a non-animal testing alternative for the generation of risk assessment data can be useful in those cases where quantitative structure–activity relationship (QSAR) models are not available, or are less well developed. This paper provides read-across case studies for the estimation of the aquatic toxicity of five different fragrance substances, and proposes a pragmatic approach for expressing uncertainty in read-across estimates. The aquatic toxicity estimates and their uncertainties are subsequently used to estimate fresh water compartment Predicted No-Effect Concentrations (PNECs), with their two-sided 90% Confidence Intervals (CIs). These PNECs can be used directly in risk assessment. The results of the musk fragrance read-across cases (musk xylene, musk ketone and galaxolide) are compared to experimentally derived PNEC values. The read-across estimates made by using similarity in a hypothesised mechanism of action for (acute) toxicity of musk xylene gave a PNEC of 2μg/L (90% CI 0.0004–13.5μg/L) with the Species Sensitivity Distribution (SSD) approach. This estimated value is 1.8 times above the experimentally-based fresh water PNEC of 1.1μg/L. For musk ketone and galaxolide, the PNEC values based on the SSD approach and employing a toxicity mechanism-based read-across were 2.0 times greater, and 4.9 times below the experimentally derived PNEC values, respectively.

Key words: *aquatic toxicity, andrane, coconut aldehyde, fragrances, galaxolide, musk ketone, musk xylene, PNEC, QSAR, read-across, risk assessment, Species Sensitivity Distribution, SSD, uncertainty.*

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Introduction

The European Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulations advocate the use of non-animal testing methods (1), but guidance is needed on how these methods should be used. The EU FP7 CADASTER (**CA**se Studies on the **D**evelopment and **A**pplication of *In **S**ilico* **T**echniques for **E**nvironmental Hazard and **R**isk Assessment) project aims to provide practical guidance on integrated risk assessment by carrying out full hazard and risk assessments based on non-animal testing data, such as QSARs, for chemicals belonging to four compound classes — (benzo)triazoles, polybrominated diphenyl ethers (PBDEs), perfluorinated substances and fragrances (2). In contrast to the other three CADASTER compound classes, the fragrances group is characterised by its diversity of chemical structures. This class of compounds does not share a single specific chemical functionality, but instead, shares its pattern of use, i.e. all the substances are used as fragrances. Therefore, no specific QSAR models that are applicable to the (whole) class of fragrances are available. Broadly applicable QSAR models, which are considered valid for organic chemicals in general, could be used for the prediction of fragrance toxicity. For instance, the ECOSAR model, developed by the US Environmental Protection Agency (EPA), can be seen as this type of more-generalised model (3).

Another option is to use case-specific read-across to generate estimates of (aquatic) toxicity. Read-across is generally defined as a data gap-filling procedure, in which the property of a substance is considered to be equal to (the average toxicity of) sufficiently-similar and relevant analogue substances, for which experimental data are already available. Read-across as a non-testing method for the generation of toxicity data is explicitly mentioned in Annex XI of the REACH legislation (1). Guidance on how to apply read-across is given in a REACH-specific guidance document (4). Definitions for read-across, similar to the description shown above, are also provided in guidance documents from the EPA (5) and the Organisation for Economic Co-operation and Development (OECD; 6). A general description of the possibilities to use read-across within CADASTER was given in a CADASTER report (7).

Fragrances do possess a number of specific structural functionalities, which are often linked to the type of scent they are producing. These main functionalities comprise esters, aldehydes, nitromusks and polycyclic musks, and alcohols. If the

chemical functionality selected to perform the read-across is similar to the chemical functionality that defines a certain group of fragrances (of a similar scent), then a case study can serve as an example of a read-across procedure that is valid for that group of fragrances. In this paper, five case studies are presented, representing four different fragrance classes for which read-across is used to estimate acute aquatic (fresh water) toxicity. The estimates are generated for three species — a fish, a crustacean (daphnid) and an alga — representing three different trophic levels in the fresh water compartment. These values are subsequently used to derive a Predicted No-Effect Concentration (PNEC) for the fresh water compartment. Two different methodologies for deriving the PNEC are applied: by using assessment factors on the value of the most sensitive species, and by calculating the 95th percentile of a Species Sensitivity Distribution (SSD) through the toxicity estimates. Both are described in the REACH guidance documents on dose–response characterisation for the environment (8). This PNEC value can directly be used for hazard assessment purposes. For risk assessment, the resulting PNEC for the fresh water compartment has to be compared to a Predicted Environmental Concentration (PEC) in the fresh water compartment, as derived by either fate modelling or chemical monitoring. For all the case studies, the results of the read-across procedure are compared to ECOSAR QSAR predictions. The estimated PNECs, based on read-across or QSAR estimates, are compared to the PNECs for fresh water derived from a detailed assessment of all the available experimental data for the three musk fragrance case studies. No comparison with experimental data was performed for the other two case studies.

In order to use read-across estimates in a probabilistic manner in risk assessment, and to permit the assessment of the contribution of read-across to the overall uncertainty in the risk assessment, a concept for the estimation of read-across uncertainty is proposed and worked out in the case studies presented. For the two nitro-musk case studies, this read-across uncertainty is also compared to uncertainty derived from the ECOSAR QSAR by recalculation of the applied QSAR model. For the other three fragrances, the uncertainties in the SSD-derived PNEC, based on QSAR or read-across estimates, are compared.

Methods

QSAR

The ECOSAR (3) program was used to generate QSAR estimates for the different case studies, with log K_{ow} as the (only) input variable for the estimation of aquatic toxicity. In the case studies reported, this value of log K_{ow} is estimated by the KOWWIN™ program (9, 10).

Read-across

The general principles of read-across are described in detail in the REACH, OECD and the North American Free Trade Agreement (NAFTA) guidance documents (4–6). Quantitative read-across is a three-step process:

a) Definition of the basis of similarity used to select analogues. This can be similarity in physicochemical properties, chemical structure, a shared mechanism of action, etc., or a combination of these.

b) A database search for analogues with experimental data for the endpoint of interest.

c) Selection of a (small) number of the most similar substances from step b to read-across the endpoint data of interest.

If only one analogue is selected in step c, the property of the compound of interest is assumed to be similar to the property of the analogue. Hence, when performing a quantitative read-across, the value of the property of interest for the analogue is used as the prediction for the substance of interest. If a number of substances are selected for read-across, different options exist for a quantitative read-across — for example, maximum, minimum or average values of the analogues' property of interest can be taken.

For the case studies presented here, the most recent version 3.1 of the freely available OECD QSAR Toolbox (11) software, and the experimental data within this Toolbox, have been used to apply this three-step process. The databases that were selected within the Toolbox to search for experimental aquatic toxicity data are: Aquatic ECETOC, Aquatic Japan MoE, Aquatic OASIS, Aquatic US EPA ECOTOX and the ECHA CHEM databases. Descriptions of these databases are given in the Toolbox program. No literature search to look for additional experimental data was performed. The choice of the similarity used for the selection of the analogues is based on the Toolbox 'Profiling' and 'Category Definition' step; no sub-categorisation of the resulting profile categories was performed. The quantitative read-across for a specific species and toxicity endpoint is then calculated by taking the average of the log-transformed toxicity values (expressed in mol/L) of the five category members which have log K_{ow} values closest to that of the substance of interest.

PNEC estimation by using the Assessment Factor (AF) approach

The derivation of a PNEC for the aquatic (fresh water) compartment from three acute toxicity values representing the three trophic levels — algae, crustacea and fish — is accomplished by taking the lowest of the three estimates for aquatic toxicity and applying an assessment factor of 1000 (8), as given in the following equation:

PNEC = min (L/EC50) / 1000

with L/EC50 being LC50 or EC50 values for at least three different species. Subsequently, the uncertainty of the lowest estimate is also used to estimate the uncertainty in the PNEC value derived, by applying the same assessment factor of 1000 to the 5% and 95% Confidence Limits (CLs). A more elaborate procedure for estimating the uncertainty by taking into account the uncertainty from all three species estimates, is described in another CADASTER contribution, by Golsteijn *et al.* (submitted).

PNEC estimation by using the SSD approach

A PNEC derived from an SSD assumes a log normal distribution of the chronic toxicities of all species in an environmental compartment, and subsequently takes the 5th percentile of this distribution (the so-called Hazardous Concentration 5%, or HC5) as the PNEC. In this case, the PNEC is considered to be protective to 95% of all the species living in the considered compartment. This approach assumes that the true SSD is known, and that it follows a (log) normal distribution. When an SSD is estimated from a sample of toxicity values for different species, the HC5 is estimated by using extrapolation constants (k-values), as proposed by Aldenberg and Jaworska (12). These extrapolation constants take into account the increased uncertainty of the SSD estimation due to the limited sample-size of the species. In this paper, the acute toxicities of five fragrance substances to three species are estimated, by using either read-across or a QSAR. A fixed Acute-to-Chronic Ratio (ACR) of 10 is applied to estimate the chronic toxicity to these species as an acceptable average (13). This is a rough generalisation, as individual ACRs can differ very much from the average values, and might also be related to specific mechanisms of toxicity (13). The three chronic toxicities are subsequently used to estimate the (log normal) SSD. The HC5 based on this SSD is calculated by taking the average of the toxicities and subtracting the standard deviation of the chronic toxicity values multiplied by the extrapolation constant:

log HC5 = average (log L/EC50 values) – k * SD (log L/EC50 values)

where SD is the standard deviation in the log transformed toxicity values, and k is the median (50%) extrapolation constant (12). For a species sample size of three, this k-value is 1.938. Subsequently, the REACH guidance document (8) instructs that an extra safety factor of between one and five should be applied, to account for issues such as low number of species (the recommended minimum is 10 species) and deviations or uncertainty in the log normal distribution of the SSD. As the SSDs for the case studies presented here are based on only three species and use estimated chronic toxicity derived from acute aquatic toxicity, the maximum additional assessment factor of five is applied to the HC5, to arrive at a PNEC estimate:

PNEC = HC5 / 5

Aldenberg and Jaworska (12) also derived extrapolation constants to estimate the 5th and 95th percentiles of the HC5, and these are applied to calculate the 5% and 95% CLs in the SSD-based PNEC. The 5th percentile HC5 k-value for a sample size of three is 7.656, and the 95th percentile HC5 k-value is 0.6391 (12). Therefore, the size of the Confidence Interval (CI) is dependent only on the standard deviation of the toxicity values used to estimate the SSD.

Uncertainty in the estimations

The uncertainty in the QSAR estimates is derived from the linear regression model statistics. By recalculating the ECOSAR QSAR model for dinitrobenzenes, the standard error of the y-estimate can be calculated, and the 5% and 95% CLs based on this standard error. As the ECOSAR regression models for the other classes of fragrances were not recalculated, no CIs for the ECOSAR QSAR estimates can be calculated for the other three fragrances.

A pragmatic approach to the estimation of the uncertainty of an individual read-across estimate has been followed by using the standard deviation of the mean value of the read-across estimate, based on five analogue substances. Log transformed toxicity values, expressed in mol/L, are used to derive the mean. The use of the standard deviation of five analogues in a quantitative read-across seems to give comparable results to uncertainty derived from regression QSAR models. The use of fewer substances (< 5) in the quantitative read-across will possibly lead to an unrealistic estimate of the data uncertainty. The incorporation of more substances (> 5) will, in practice, quickly lead to the taking into account of substances that are not very similar to the

compound of interest. The use of five analogues as the basis of a quantitative read-across is based on experience, and will, in specific cases (very few data, poor analogues, etc.), need to be adjusted.

Selection of fragrances

For the case studies, five different fragrances were selected to represent four different fragrance classes. The chemical structures of the five selected fragrances are given in Figure 1, and their identities and fragrance classes are given in Table 1. The first case study, musk xylene, was selected, because it is a well-known and well-studied fragrance substance. Musk xylene was the most widely used of the fragrances from the nitro-musk category, a type of synthetic musk fragrance that mimics natural musk. It has been used as a perfume fixative in a wide variety of consumer products, and is still used in some cosmetics and fragrances. The use of musk xylene has declined sharply since the mid-1980s, due to safety and environmental concerns. It is a very persistent and very bioaccumulative pollutant in the aquatic environment (vPvB substance), and for these reasons alone, was the first substance to be proposed as a 'substance of very high concern' (SVHC) under the EU REACH regulations (1). Musk ketone is another fragrance from the group of the nitro-musks. Although its structure is very similar to that of musk xylene, one of the nitro groups has been replaced by an acetyl (ethylketone) group. Galaxolide is also considered a musk fragrance, but a polycyclic musk rather than a nitro-musk. It is used in soaps, cosmetics and detergents. Galaxolide possesses a clean, sweet, musky, flowery and woody odour. Coconut aldehyde is, as its name suggests, a coconut fragrance. It is used in the preparation of fruit drink flavours, such as coconut, and also as bitter almond flavour. It is selected as an example of a lactone-type fragrance. Finally, andrane (or 8,9-epoxy cedrane) is one of the few fragrances with an epoxide group. Its olfactory description is that of a precious wood odour, reminiscent of ambergris or sandalwood.

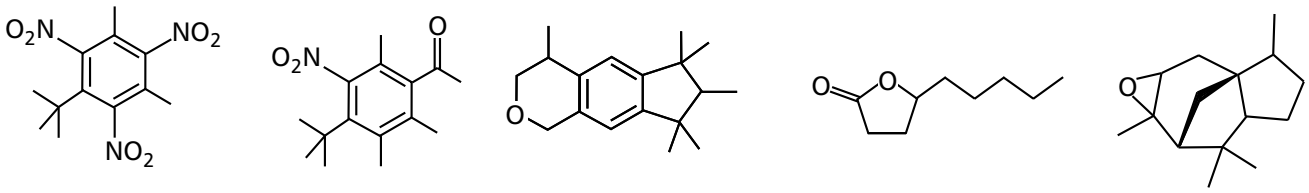
Results

Aquatic toxicity estimates for musk xylene

ECOSAR (QSAR) results

The ECOSAR QSAR model selects the sub-model for dinitrobenzenes, when making predictions for musk xylene. The predictions from the dinitrobenzene sub-model indicate that this substance is more toxic than expected on the basis of a narcosis-type toxicity mechanism. The results of the QSAR estimates from the ECOSAR QSAR for musk xylene (3) are given in Table 2.

Figure 1: Chemical structures for the five fragrance substances selected for the case studies



Musk xylene Musk ketone Galaxolide Coconut aldehyde Andrane

Table 1: The five fragrances selected for the read-across case studies

Name	Chemical name	CAS No.	Fragrance class
Musk xylene	5-tert-butyl-2,4,6-trinitro-*m*-xylene	81-15-2	nitro-musk
Musk ketone	4′-tert-butyl-2′,6′-dimethyl-3′,5′-dinitro-acetophenone	81-14-1	nitro-musk
Galaxolide	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-γ-2 benzopyran (HHCB)	122-05-5	polycyclic musk
Coconut aldehyde	gamma-nonalactone	104-61-0	lactone
Andrane	8,9-epoxycedrane	13567-39-0	epoxide

Table 2: Acute aquatic toxicity estimates for the five fragrance substances

	Musk xylene	Musk ketone	Galaxolide	Coconut aldehyde	Andrane
Fish, 96-hour LC50, mortality					
ECOSAR	0.23 (0.0009–59.6)	0.28 (0.0010–73.4)	0.036[d]	19.2[d]	1.28[d]
Read across[a]	0.052 (0.0019–1.39)		2.38 (0.011–510.6)		
Read-across[b]	0.324 (0.0071–14.8)	0.321 (0.0071–14.6)	0.19 (0.0017–22.2)	3.56 (0.71–17.8)	0.023 (0.00003–15.5)
***Daphnia*, 48-hour EC50, immobilisation**					
ECOSAR	1.34[c]	1.65[c]	0.038[d]	39.1[d]	1.51[d]
Read-across[a]	10.7 (0.301–383.3)		0.14 (0.0002–83.5)		
Read-across[b]	3.07 (0.124–76.2)	3.04 (0.122–75.5)	1.13 (0.129–9.85)	99.5 (7.40–1336.9)	0.15 (0.10–0.22)
Alga, 96-hour E_rC50, growth rate					
ECOSAR	0.27 (0.092–0.80)	0.29 (0.099–0.852)	0.114[d]	16.5[d]	1.26[d]
Read-across[a]	3.57 (0.117–109.2)		3.86 (0.037–401.1)		
Read-across[b]	5.31 (0.281–100.3)	5.26 (0.278–99.3)	0.18 (0.0015–20.1)	0.063 (0.0002–20.6)	0.058[e]

5% and 95% confidence limits are given in brackets.

[a]*Read-across based on chemical similarity, by using the structure of dinitrobenzene as scaffold for analogue selection for musk xylene, and by using only neutral organic structures for galaxolide.*
[b]*Read-across based on hypothesised mechanism of toxicity, by using nitrenium ion formation as analogue selection criterion for musk xylene and musk ketone, quinoid precursor for galaxolide, direct acylation by acetates for coconut aldehyde, and epoxide radical formation for andrane.*
[c]*No two-sided 90% CI could be calculated as the QSAR regression model is based on a single experimental observation.*
[d]*Only the polynitrobenzene sub-model from ECOSAR (applicable to musk xylene and musk ketone) was recalculated, and a two-sided 90% CI of the QSAR prediction is calculated. No CI is calculated for the QSAR predictions for galaxolide, coconut aldehyde and andrane.*
[e]*Read-across for* Pseudokirchniella subcapitata *is based on a single epoxide analogue, by using a reported algae E_rC50 at 72 hours. No CI can be calculated.*

It should be noted that these QSAR estimates reveal a number of drawbacks that are specific to the ECOSAR model and this case study. These drawbacks provide a good general idea of when read-across might be more suitable as a method for filling data gaps. These QSAR predictions have some weak points, which are discussed below. The acute toxicity QSAR for fish is based on a limited data set of 14 dinitrobenzenes, and the regression coefficient (R^2) of the relationship with log K_{ow} is only 0.27. This translates in the relatively large standard error of the y-estimate (sy) of 1.21 log units for musk xylene. The fact that the quality of the QSAR is not very high is, at least partly, reflected in the increased uncertainty. However, the QSAR also uses toxicity data from various fish species. This makes the QSAR estimate less appropriate for use in an SSD approach for estimation of the environmental PNEC, where single-species toxicity estimates are preferred.

Table 3: Read-across of fish (*O. mykiss*) toxicity for musk xylene

	LC50, 96 hour, mortality		*Oncorhynchus mykiss* (n = 20 / 62 values)		
	MW	log K_{ow}	mg/L	–log(mol/L)	SD
Musk xylene	297.10	4.45	0.0519	6.76	0.71
Analogues used for read-across:					
Pendimethalin	281.14	4.82	0.7900	5.55	
Binapacryl	322.12	4.49	0.0500	6.81	
Fluazinam	463.95	4.02	0.0630	6.87	
Fluchloralin	355.05	5.07	0.0230	7.25	
Dinobuton	326.11	3.94	0.0140	7.37	

The structural scaffold of dinitrobenzene was used to search analogues. The five analogues with log K_{ow} values closest to musk xylene were selected.

The QSAR for the daphnid is based on only one experimental observation and a fixed value representing the toxicity at the solubility limit. Therefore, the regression is defined by two single points, with a perfect fit and without any uncertainty.

The QSAR for the alga uses three experimental observations (plus the fixed value representing toxicity at the solubility limit), and the fit of the QSAR model is good ($R^2 = 0.86$). This leads to a relatively small prediction uncertainty, expressed in the sy value of only 0.23 log units. It is questionable whether this is an adequate estimate of the prediction uncertainty, as the model fit of these three observations is probably not representative of the variability in algal toxicity data.

Read-across by using the ECOSAR class 'dinitrobenzenes'

For the read-across procedure, the OECD QSAR Toolbox was applied, which permits the simultaneous search of various databases for relevant experimental data, and allows the user to vary the similarity parameters that are used to select the read-across analogues. First, the same structural boundaries as applied by ECOSAR (polynitrobenzene functionality), are used to search for analogues and to perform read-across by using those analogues that have the most similar log K_{ow} values to that of musk xylene. In Table 3, the fish toxicity read-across value (average) and the standard deviation for musk xylene are given, together with the data of the analogues used for the read-across. This read-across is performed for one specific species of fish, *Oncorhynchus mykiss*, for which 62 measured values of the 96-hour LC50 were available, for 20 different substances. Information on the read-across and the analogues used for read-across for this and all other substances and endpoints are detailed in the supplementary information, which is available online. It is also possible to perform read-across by using all of the available measured values of 96-hour LC50 for any fish species. This makes the results more comparable to the ECOSAR QSAR prediction, but less suitable for use in an SSD approach to estimate the PNEC. The read-across with all the experimental fish toxicity values gives a value of 0.066mg/L for musk xylene, with the two-sided 90% CI from 0.0025 to 1.75mg/L. This result is almost equal to the *O. mykiss* toxicity read-across (0.052mg/L). The same five analogues are selected as the most similar to musk xylene, and the (average) fish toxicities reported differed very little from the species-specific toxicities for *O. mykiss*. The CI is also similar — the standard deviation of the log transformed toxicity values is 0.71 for all the fish, as well as for the *O. mykiss* read-across result. The detailed read-across data are given in the online supplementary information.

Similarly, read-across estimates can be generated for a single crustacean species, *Daphnia magna*, and a single algal species, *Pseudokirchneriella subcapitata*. The number of acute toxicity data available for these single species read-across estimates is smaller than for the fish read-across, resulting in the selection of different substances for use in the read-across. The results of the read-across of daphnid and algal toxicity for musk xylene are shown in Table 2. Detailed information on the specific read-across case, with information on the analogues used to derive the read-across value for toxicity and the standard deviation, can be found in the online supplementary information.

The log K_{ow} range spanned by the analogues used for the daphnid and the alga is much wider, with the lowest value for 1,3-dinitrobenzene of around 1.6. However, the structural feature (polynitrobenzene in this case) is what largely determines the aquatic toxicity, rather than the log K_{ow}. This is shown by the higher toxicity of trinitrotoluene (LC50 = 0.57mg/L) when compared to trifluralin (LC50 = 1.5mg/L), despite the fact that the K_{ow} of trifluralin is a factor of 2000 higher than the K_{ow} of trinitrotoluene. In addition, the standard deviation of the algal read-across value is very similar to that of the fish read-across estimate, despite the wider log K_{ow} range spanned by the analogues.

Read-across by using a hypothesised mechanism of action for toxicity

Read-across is highly dependent on the parameter of similarity that is chosen by the user to define the set of analogues. In this case, in order to compare the results of the read-across procedure with the results from an existing and available QSAR model (ECOSAR), we used the same definition of similarity as used by that model (i.e. the substances should all have a dinitrobenzene functionality present in their structure). If possible, however, the basis for read-across should be formed by a mechanistic hypothesis. The OECD Toolbox allows for such a similarity hypothesis by using the profiling functionality. When analysing the profile of musk xylene, it seems that this substance has a specific reactivity, which might be the cause of its excess toxicity. The hypothesised mechanism of toxic action is nitrenium ion formation, and subsequent organic radical formation that causes toxicity through radical damage.

Although this type of reactivity is linked to the presence of (aromatic) nitro-groups, it gives a broader definition of the type of structures that are thought to be able to exert the same type of toxicity as musk xylene, but also specifies structural

requirements in addition to the presence of a nitroaromatic sub-structure. When this similarity definition (substances able to form nitrenium ions) is applied, a different set of analogues for read-across is extracted. The detailed results for the read-across based on this hypothesis for the toxic mechanism of action are given in the online supplementary information. A summary of the results of the read-across are shown in Table 2.

The absolute value (0.32mg/L) of the mechanistic read-across is almost an order of magnitude higher than the read-across by using the ECOSAR classification as its similarity basis (0.052mg/L). The standard deviation is only slightly larger (0.83 log units *versus* 0.71 log units, respectively). The basis for the read-across is very different, as only one analogue, fluchloralin, is used in both read-across procedures.

Similarly, for the read-across of acute toxicity for *D. magna*, three different read-across analogues are selected when employing the nitrenium ion formation hypothesis, leading to a read-across estimate which is a factor of three lower than that based on the ECOSAR classification. For the algal species (*P. subcapitata*), only one different analogue is selected, and the read-across estimate is subsequently more-or-less the same (5.3mg/L *versus* 3.6mg/L). The results for *Daphnia* and the alga, together with the previous results of the QSAR and read-across estimates for fish toxicity, are shown in Table 2. The values are given with their 5% and 95% CLs, based on the standard error (QSAR) and standard deviation (read-across) of the estimates.

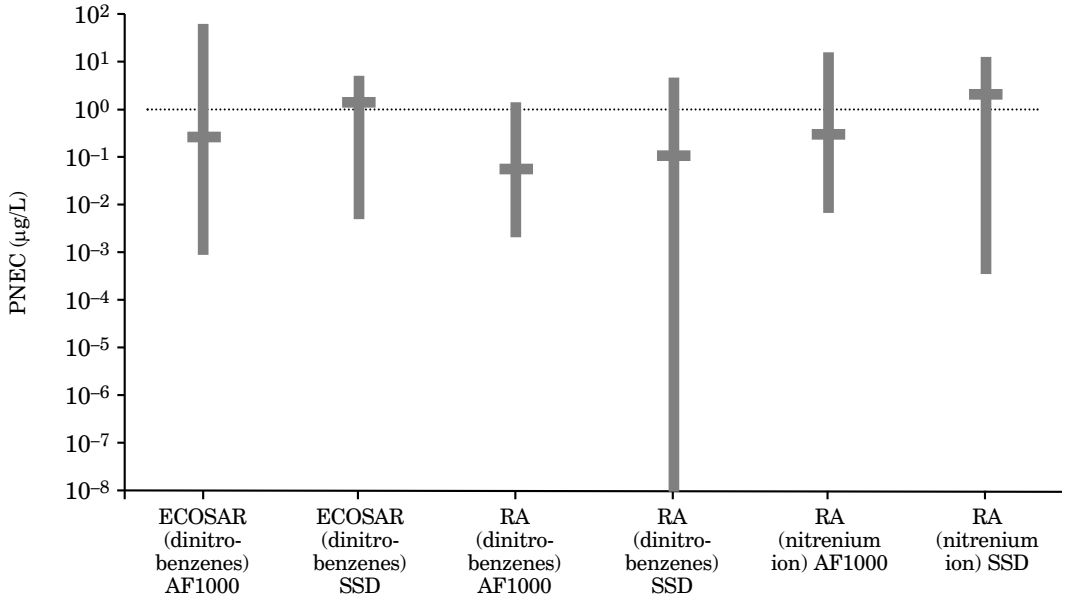
PNEC derivation from QSAR and read-across estimates

Two approaches can be followed to derive a PNEC from these acute toxicity values, namely, a direct estimate of the PNEC by using assessment factors (Assessment Factor [AF] approach), or a Species Sensitivity Distribution (SSD) approach. The PNEC estimates for musk xylene are given in Table 4. The value for the PNEC derived in the EU Risk Assessment Report (EU RAR) for musk xylene (14), based on a proper evaluation of all the experimental data (including measurements of chronic aquatic toxicity) was 1.1µg/L. A graphic representation of the PNEC estimates and their uncertainties, together with the EU RAR-derived values, is given in Figure 2.

Aquatic toxicity estimates for musk ketone

Musk ketone is structurally very similar to musk xylene, and both are examples of the fragrance class of nitro-musks. This substance is also consid-

Figure 2: Lower (5%), median and upper (95%) percentiles of the fresh water PNEC estimates for musk xylene



The EU RAR-derived fresh water PNEC of 1.1 µg/L is shown as a dotted line in this graph. See the text for explanation of the names of the different PNEC estimation procedures.

ered to be a nitrenium ion-former, according to its OECD Toolbox profile. The major difference between musk ketone and musk xylene is the log K_{ow}, which is 4.31 for the former compared to 4.45 for the latter. The QSAR and read-across results for musk ketone are given in Table 2.

The small differences in the absolute values of the ECOSAR prediction when compared to the predictions for musk xylene are due to the different log K_{ow} value used for the predictions for musk ketone. In addition, the minor variations in the read-across estimates are due to the slightly different molecular weights of musk ketone and musk xylene. The read-across results expressed in mol/L are identical to those for musk xylene (see online supplementary information). The same drawbacks, as noted for musk xylene, apply to the results of the ECOSAR QSAR for musk ketone.

The PNEC estimates based on these toxicity estimates, by using both the assessment factor method and the SSD estimation procedure, are summarised in Table 4. The PNEC estimates for musk ketone are all around 1μg/L, which is expected, based on the value derived for musk xylene. The EU RAR-derived fresh water PNEC value for musk ketone is 1.0μg/L (15). The AF approach seems to yield more-conservative PNEC estimates, as both SSD-derived values are above the AF-derived values, and above the EU RAR experimentally-derived PNEC. There is no trend in the CI, as both the largest and the smallest CIs are for the SSD-derived PNEC. A graphical representation of the PNEC estimates and the two-sided 90% CIs is shown in Figure 3, together with the EU RAR-derived PNEC value of 1.0μg/L.

Aquatic toxicity estimates for galaxolide

The profiling of galaxolide in the OECD QSAR Toolbox does not indicate specific structural features that are linked to specific toxicity mechanisms, or unspecific reactivity that might lead to increased toxicity. The substance is profiled as a neutral organic substance. The ECOSAR QSAR model also applies the sub-models for neutral organic substances to galaxolide. A read-across based on neutral organic substances from the OECD QSAR Toolbox should therefore give results comparable to the ECOSAR QSAR predictions for galaxolide. As the neutral organics sub-model from ECOSAR is not recalculated, no CIs of the QSAR prediction are calculated. The results of the QSAR and read-across estimations, assuming galaxolide is a neutral organic substance, are summarised in Table 2.

The parent substance, galaxolide, is a neutral organic substance. However, its oxygen-containing aliphatic ring can be metabolised into a quinoid-like structure (a phenol-like structure with the potential to isomerise into a quinone). In the OECD QSAR Toolbox Profile, this is indicated in the functional groups profile as 'quinoid precursor'. As quinone-like structures are known to have

Table 4: Fresh water PNEC estimates for five fragrance substances

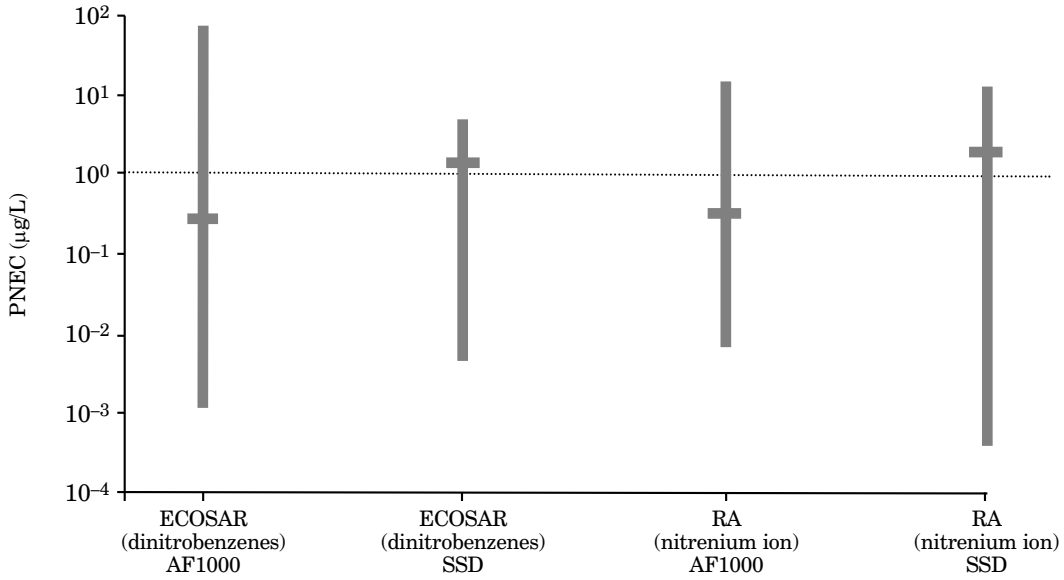
Based on:	Musk xylene	Musk ketone	Galaxolide	Coconut aldehyde	Andrane
ECOSAR (AF)	0.23 (0.0009–59.6)	0.28 (0.0011–72.3)	0.036[a]	16.53[a]	1.26[a]
ECOSAR (SSD)	1.33 (0.0051–4.69)	1.43 (0.0043–5.34)	0.31 (0.0074–0.71)	190.0 (13.7–345.2)	22.1 (12.4–25.2)
Read-across 1 (AF)	0.052 (0.0019–1.39)		0.14 (0.0002–83.5)		
Read-across 1 (SSD)	0.107 (1.1×10^{-8}–4.16)		0.68 (0.00002–6.94)		
Read-across 2 (AF)	0.324 (0.0071–14.8)	0.321 (0.0071–14.6)	0.18 (0.0015–20.1)	0.063 (0.00020–20.6)	0.023 (0.00003–15.5)
Read-across 2 (SSD)	1.97 (0.0004–13.5)	1.95 (0.00004–13.4)	0.89 (0.0023–3.47)	0.045 (3.2×10^{-1}–5.35)	0.193 (0.00094–0.646)

PNEC estimates are in μg/L. 5% and 95% confidence limits are given in brackets.

The PNECs are based on the acute aquatic toxicity estimates from ECOSAR or read-across, given in Table 2. Two methods for extrapolation are applied: by using the Assessment Factor (AF) approach or the Species Sensitivity Distribution (SSD) approach.

[a]*Only the polynitrobenzene ECOSAR sub-model (applicable to musk xylene and musk ketone) was recalculated, to calculate a two-sided 90% CI of the QSAR prediction. No CI are calculated for QSAR predictions for galaxolide, coconut aldehyde and Andrane.*

Figure 3: Lower (5%), median and upper (95%) percentiles of the fresh water compartment PNEC estimates for musk ketone



The EU RAR-derived fresh water PNEC of 1.0 µg/L is shown as a dotted line in this graph. See the text for explanation of the names of the different PNEC estimation procedures.

increased toxicity compared to the narcosis-type toxicity of neutral organic substances, a read-across is performed by using the mechanistic hypothesis of quinone-like toxicity after metabolism. The results for this read-across are also given in Table 2.

The high log K_{ow} of galaxolide, as used by the ECOSAR QSAR models (log K_{ow} = 6.26), leads to estimates of high toxicity (i.e. low LC50 values). The log K_{ow} of galaxolide is so high that bioavailability might determine the observed toxicity. This could be an explanation for the relatively large difference between the QSAR estimates and the read-across estimate from neutral organic substances. The read-across is based on the experimental toxicities of substances with equally high log K_{ow} values, which do not show the high toxicities predicted by the ECOSAR QSAR. The mechanistically-based read-across, involving the use of the quinoid precursor structure to select analogues, leads to lower LC50/EC50 values for the fish and alga toxicity but, surprisingly, a higher EC50 estimate for *Daphnia*.

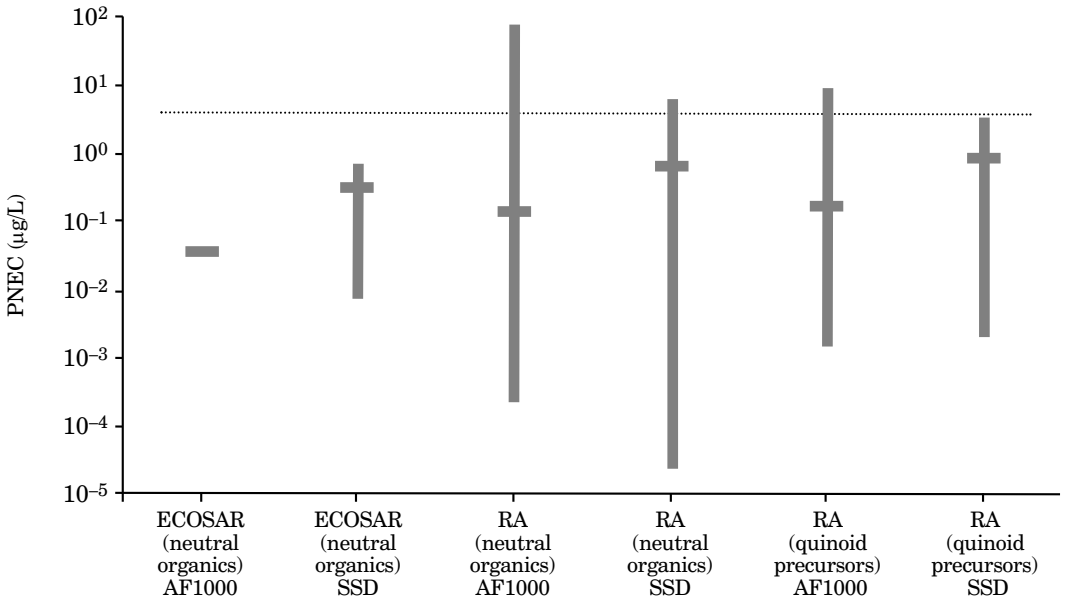
The PNEC estimates based on these toxicity estimates, by using both the direct assessment factor method and the SSD estimation procedure, are given in Table 4. A graphical representation of the PNEC estimates and their two-sided 90% CIs is shown in Figure 4. With the exception of the PNEC based on the lowest ECOSAR estimate, and applying an AF of 1000, all of the estimated PNECs are in the range of 0.1–1µg/L. A fresh water PNEC was derived from experimental (chronic) toxicity data in the EU RAR for galaxolide (16), giving a PNEC for the fresh water compartment of 4.4µg/L. This value is also included in Figure 4.

Aquatic toxicity estimates for coconut aldehyde

The ECOSAR model selects the category of esters for the prediction of the aquatic toxicity for coconut aldehyde. No CI was calculated for the ECOSAR prediction, as the esters sub-model was not recalculated. Profiling coconut aldehyde with the OECD QSAR Toolbox yields an indication that this substance might be capable of direct acylation involving a leaving group through the presence of an acetate functionality (or a precursor). The results from the ECOSAR ester sub-model and from read-across by using the acetate functionality, are given in Table 2.

The QSAR estimates for the three aquatic species are close to each other, whereas the read-across estimates show a difference of a factor of 1500 between the toxicity estimate for the alga and that for *Daphnia*. This leads to significant differences in the derived fresh water PNEC estimates based on the QSAR or the read-across toxicity esti-

Figure 4: Lower (5%), median and upper (95%) percentiles of the fresh water compartment PNEC estimates for galaxolide



The EU RAR-derived fresh water PNEC of 4.4 µg/L is shown as a dotted line in this graph. See the text for explanation of the names of the different PNEC estimation procedures.

mates. The values for the derived PNEC values and their two-sided 90% CIs are shown in Table 4. The small differences between the three QSAR estimates for aquatic toxicity consequently lead to a relatively high value of PNEC (close to the actual acute toxicity values). This is due to the small standard deviation in the QSAR toxicity estimates, which also leads to a small CI of the SSD-estimated PNEC. On the other hand, the large difference between the read-across values for the alga and *Daphnia* give a large standard deviation, and subsequently a relatively low PNEC estimate (far from the acute toxicity values) and a very large CI. The PNEC values for coconut aldehyde and their two-sided 90% CIs are graphically represented in Figure 5.

Aquatic toxicity estimates for andrane

The ECOSAR model selects the epoxides submodel when predicting aquatic toxicity for andrane. Profiling andrane in the OECD QSAR Toolbox also indicates that this functionality (epoxide) could be the basis of the aquatic toxicity mechanism, due to unspecific reactivity toward proteins through epoxide ring opening. The QSAR model and read-across are therefore based on the same assumption, and use the same functionality to select analogues. The results of the aquatic toxicity predictions are given in Table 2. The read-across estimates are a factor of 10 (for *Daphnia*) to 55 (for the fish) below the ECOSAR estimates. The fish toxicity QSAR from ECOSAR is based on six epoxides substances. However, only two substances have a log K_{ow} > 1.6, with values of 3.2 (estimated) and 3.3 (measured). Andrane has a log K_{ow} (estimated) of 4.35. In the read-across procedure, the selected epoxide analogues have log K_{ow} values of 4.56 to 6.43, with the substance having the highest value, 9,10-epoxy stearic acid, showing the lowest toxicity of the five read-across analogues.

The read-across for *Daphnia* is based on five read-across analogues with reported toxicity values close to each other, and with K_{ow} values similar to those of andrane. The two-sided 90% CI is therefore very small, with the 5% CL at 0.10mg/L and the 95% CL at 0.22mg/L. The QSAR for *Daphnia* toxicity is based on four substances with the highest log K_{ow} of 3.3, more than a log unit below the log K_{ow} of andrane.

Read-across for 96-hour E_rC50 (i.e. the concentration inducing 50% inhibition of the growth rate) for the alga was not possible, as only one analogue with experimental data for this endpoint could be located in the OECD Toolbox, i.e. 2,3-epoxypropanol. This substance has a log K_{ow} of –1.09 and a measured toxicity of 261mg/L, but, based on

the log K_{ow} and its small molecular structure, it is not considered a valid analogue for read-across to andrane.

When evaluating 72-hour values for algal toxicity, four epoxide substances with experimental data were found. However, only one had a sufficiently similar log K_{ow} to that of andrane. The read-across estimate of 0.58mg/L for andrane is therefore based on this single analogue, with a log K_{ow} of 4.22, and toxicity to the alga of 0.09mg/L. A CI cannot be calculated for the algal toxicity estimate.

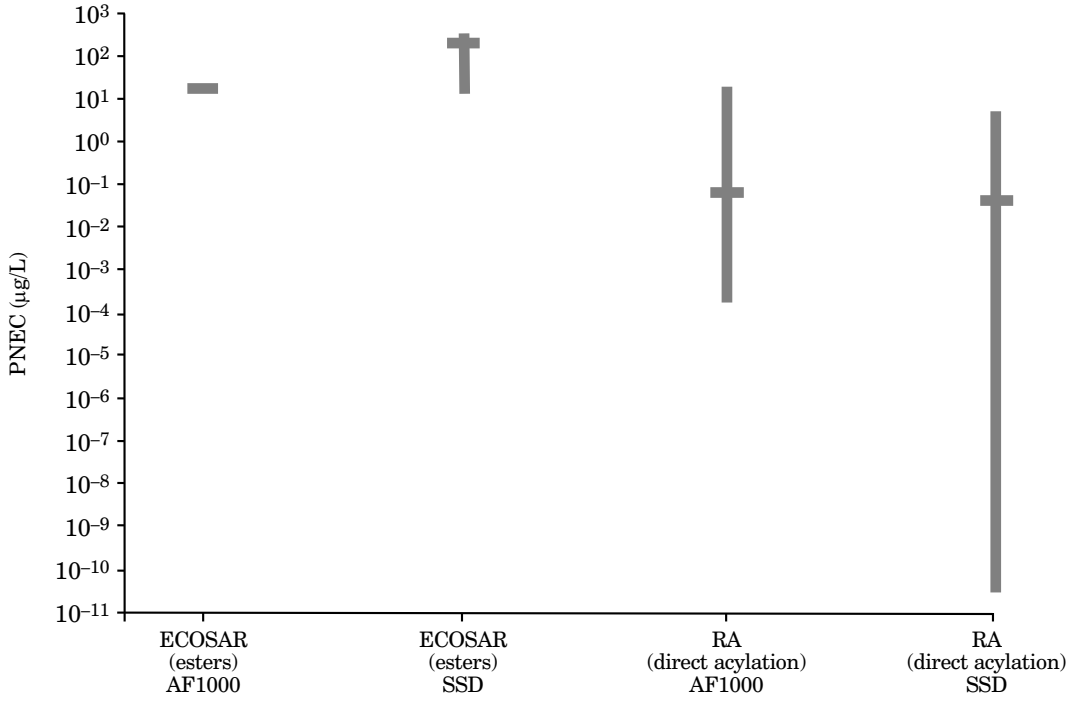
The QSAR estimates for the three aquatic species are close to each other, as was the case for the coconut aldehyde. In the SSD approach, this leads to relatively high PNEC estimates with small CIs. The values for the derived PNEC values and the two-sided 90% CIs are given in Table 4. The PNEC values for andrane, and their two-sided 90% CIs, are graphically represented in Figure 6.

Discussion and Conclusions

In general, the PNECs based on ECOSAR (QSAR) and read-across predictions of aquatic toxicity are within one order of magnitude. When the read-across procedure is based on (roughly) the same underlying toxicity data as the QSAR, this is to be expected. The uncertainty in the read-across estimate, as proposed in this paper, should then also be roughly similar to the uncertainty of the QSAR model estimate, as both methods use the variability of the underlying data to define uncertainty. The practical approach presented here, to use the standard deviation of the five closest analogues used for read-across, gives acceptable results.

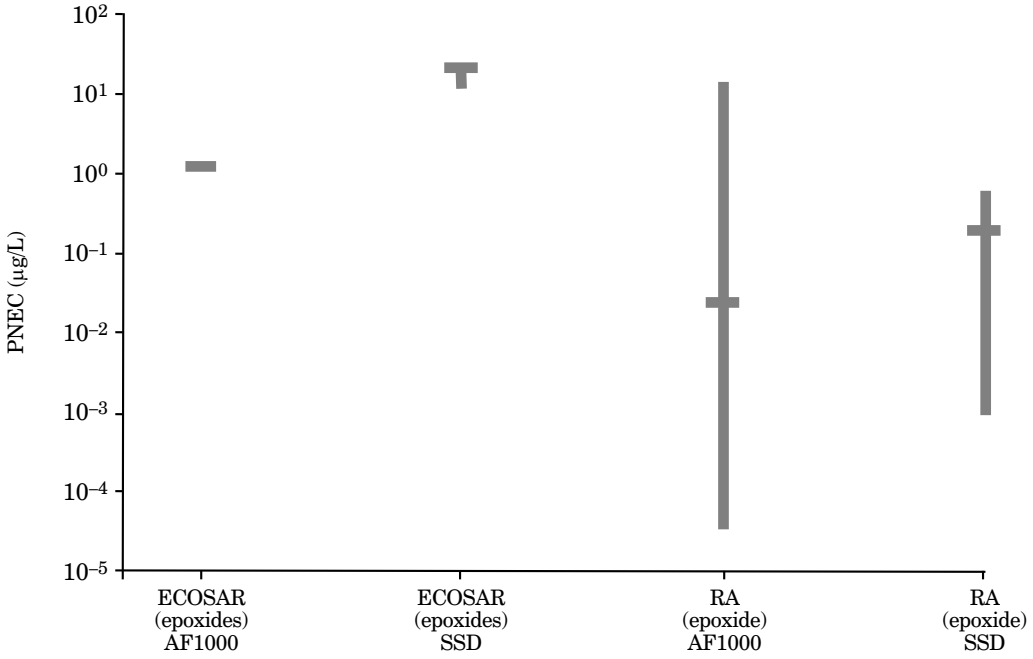
In those cases where the QSAR model is based on a very small training data set, both the QSAR estimate and the uncertainty calculated from the (regression) QSAR model can be misleading. Very small training data sets were observed for *Daphnia* (n = 1) and the alga (n = 3) QSAR models applied to the nitro-musks, and *Daphnia* (n = 4) and alga (n = 3) models for epoxides applied to andrane. The read-across estimates for the nitro-musks were based on much broader data sets, and the CI of the read-across estimate is therefore thought to be more representative of the uncertainty in the toxicity estimate. The read-across for andrane was similarly limited in the number of observations as the ECOSAR QSAR. The selection of more relevant analogue(s) in the read-across

Figure 5: Lower (5%), median and upper (95%) percentiles of the fresh water compartment PNEC estimates for coconut aldehyde



See the text for explanation of the names of the different PNEC estimation procedures.

Figure 6: Lower (5%), median and upper (95%) of the fresh water compartment PNEC estimates for andrane



See the text for explanation of the names of the different PNEC estimation procedures.

procedure for andrane, compared to extrapolation from the QSAR training data sets, therefore gives more confidence in the read-across values for toxicity.

The PNEC estimates from both the AF and the SSD approach are considered conservative estimates, which, at least, should not lead to the overestimation of a PNEC based on experimental, chronic data. However, the estimated values are relatively close to the actual PNECs derived in the EU RARs. This can be attributed (in part) to the fact that the k-values, as proposed by Aldenberg and Jaworska (12), only very weakly reflect the increased uncertainty, due to the low number of species used to estimate the SSD. Within the CADASTER project, recommendations for new extrapolation constants have been worked out (17). These yield more-or-less similar results when the number of species for an SSD are considered, as recommended in the REACH guidance (10–15 species; 8), but give more conservative estimates of the PNEC for the (more uncertain) SSD approach, by using low numbers of species (as in the case studies presented here).

The SSD procedure for PNEC estimation has the advantage that estimating uncertainty in the PNEC value is independent of the uncertainty of the individual data points used to construct the SSD. In a separate paper, Aldenberg and Rorije (17) argue that the uncertainty of the individual data points can be neglected, especially for SSDs based on small numbers of species. A drawback to this SSD approach is that, in those cases where the estimates are very close together, the PNEC estimate will be relatively high and will have a small confidence interval. This is shown for the ECOSAR-based SSD cases of coconut aldehyde and andrane. For these two case studies, the ECOSAR QSAR estimates for the three trophic levels (algae, crustacea and fish) were very close to each other. This leads to relatively high PNEC estimates with very small CIs intervals. The read-across procedure subsequently gave very different estimates. It seems that the database of the ECOSAR sub-models for these specific cases is not sufficiently descriptive of the fragrance substances of interest. Another advantage of the read-across procedure over the ECOSAR QSAR estimates is the possibility of generating read-across values for different species of fish, daphnids and/or algae. This will increase the reliability of the SSD estimate. This was not performed for the case studies in this paper. However, the databases contain sufficient experimental data to make different-species read-across possible for these selected fragrances.

For the three case studies of musk fragrances, the estimated PNEC values were in good agreement with PNEC values based on experimental

data and derived in an international risk assessment framework. However, based on the agreement with the experimentally derived values, no conclusion can be drawn on the superiority of one estimation method over another.

In general, it seems good practice to compare a QSAR estimate of toxicity with an independently derived read-across estimate. The latter forces the user to evaluate the data on which the analogues are selected. Such an evaluation is also possible for QSARs, provided that the training data set for the QSAR is publicly available, and includes substance identities. The case studies presented here show that such a comparison points the user toward additional existing data, shows the possible data selection that is performed to derive the QSAR model, and helps to define an operational hypothesis about a mechanism of toxicity. Such an evaluation will greatly enhance the acceptability of theoretically-derived toxicity predictions for use in hazard or risk assessment in a regulatory context.

Online Supplementary Information

The supplementary electronic material includes a worksheet with all read-across and QSAR estimated toxicity values as given in Table 2, the toxicity and structure data on the substances selected as analogues for the different case studies of read-across, and the derivation of the PNEC values and the 5% and 95% confidence limits as given in Table 4.

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