Illustrative examples with the OECD QSAR Toolbox workflow

Part 2C

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Version	Changes	

Title: Illustrative examples with the OECD QSAR Toolbox workflow (Part 2C)

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1. Acute toxicity

The example illustrates the elements ECHA recommends to take into account when predicting acute toxicity by oral route, in a regulatory context, with the QSAR Toolbox. Please refer to the document "Illustrative examples with the OECD QSAR Toolbox workflow part 1"[1] for an introductory note on the QSAR Toolbox, which provides background information on the software including general considerations on the approach taken.

The example does not focus on the reporting of the results in IUCLID. Endpoint specific guidance can be found in the "Guidance on Information Requirements and Chemical Safety Assessment Chapter R. 7a: Endpoint specific guidance"[2]

In this example, we address the information requirement (regulatory endpoint) for acute toxicity by oral route in Annex VII, 8.5.1 of REACH. The standard tests for this endpoint are the OECD TG 401[3] (EU B.1), 420 (EU B.1 bis), 423 (EU B.1 tris), 425.

The QSAR Toolbox v3.4 does not contain profilers developed specifically for acute oral toxicity. However, it contains the "Cramer" profilers for oral toxicity developed to assign chemicals to different toxicity classes, which can be useful to support the formation of categories for the prediction of acute toxicity, too.

These profilers are accessible in the module "Profiling". The "profilers" are attributes that describe chemical or biological (including endpoint-specific) properties of the substances. The Toolbox calculates them starting from the chemical structure. The two profilers for oral toxicity are called "original" and "extended" toxic hazard classification by Cramer.

The former encodes the original rules established by Cramer[4], while the latter mimics the augmented version implemented in Toxtree[5]. A description of the profilers is accessible in the QSAR Toolbox under Options\Modules\Profilers. The description for toxic hazard classification by Cramer (original) is the following:

Toxic hazard classification by Cramer

"The entered target chemicals are classified in one of three toxic classes:

- *Low (Class I)*

- *Intermediate (Class II)*

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[1] Previous illustrative examples with the OECD QSAR Toolbox workflow can be found on the ECHA website under: http://echa.europa.eu/documents/10162/21655633/illustrative_example_qsar_part1_en.pdf (part 1). Examples on predictions for skin sensitisation and fish short-term toxicity are described at: https://echa.europa.eu/documents/10162/21655633/illustrative_example_qsar_part2_en.pdf (part 2) Examples on prediction of long-term aquatic toxicity to fish are described at: https://echa.europa.eu/documents/10162/21655633/illustrative_example_qsar_part2b_en.pdf (part 2b)
[2] https://echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf
[3] Method deleted from the OECD Guidelines for testing of chemicals and from Annex V to Directive 67/548/EEC. Existing OECD TG 401 (EU B.1) data would normally be acceptable but testing using this deleted method must no longer be performed.
[4] G.M. Cramer and R.A. Ford Estimation of toxic hazard - a decision tree approach. Food and Cosmetics Toxicology, Volume 16, Issue 6, December 1978, Page 255-276.
[5] G. Patlewicz, N.Jeliazkova, R.J. Safford, A.P. Worth, B. Aleksiev. An evaluation of implementation of the Cramer classification scheme in the Toxtree software. SAR and QSAR in Environmental Research. Vol. 19, Nos. 5-6, July-September 2008, Page 495-524.

- *High (Class III)*

Two of the categories are using external files with 440 compounds in "Common component of food" and 107 compounds in "Normal constituents of body", which are borrowed by ToxTree v.2.1.0

The current version of the scheme (2.2) has been modified based on detailed analysis performed with the Munro database (consisting of 613 chemicals) and comparative analysis with Toxtree 2.6.6. The modifications implemented into the schemes are as follows:

- *Implementation of the metabolism node, which removes sulphated and sulphonated groups from the entered chemicals. From this node, the analysis continues with unsulphated and unsulphonated chemicals.*

- *TB will provide two profiling results: for parent and metabolites respectively in the following cases:*

  - *when the chemical undergoes a different type of metabolism such as hydrolysis or unsulphonation/unsulphatation;*

  - *when mixtures are assessed. In this case, TB will provide profiling results for each component of the mixture individually.*

The QSAR Toolbox includes four databases (accessible in the module "Endpoint") that contain experimental data on acute toxicity by oral route[6]:

1. Acute Oral Toxicity database (ChemIDPlus) (10 154 chemicals with10 154 data points; adopted 04/2016).

2. ECHA CHEM (5 205 chemicals with 24 384 data points for acute toxicity; adopted 05/2016).

3. Toxicity Japan MHLW (252 chemicals, 2 914 data points, 3 endpoints; adopted 07/2014).

4. ZEBET database (362 chemicals, 3 867 data points, 2 endpoints; adopted 04/2016).

Keeping in mind that these databases are donated to the Toolbox, the user needs to verify the quality of data used for the prediction, as we will see in the next paragraphs.

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[6] There is an overlap between the different databases. Hence, the same study may appear in several databases.

2. EXAMPLE 2-ethyltoluene

The example illustrates the workflow for a quantitative hazard assessment of acute toxicity by the oral route for 2-ethyltoluene (CAS number 611-14-3, EC number 210-255-1, SMILES: CCc1ccccc1C) using QSAR Toolbox version 3.4. The endpoint covered in the example is the median lethal dose, i.e. LD_{50}.

2.1 Input

In the "Input" module, the user clicks CAS# under single chemical and enters the CAS number without dashes:

- "611143"

In this example, the structure of the target chemical is introduced in the QSAR Toolbox in the "Input" module using the CAS number. Alternatively, the use of the drawing tool or the SMILES notation returns two chemicals with the same structure but with different CAS numbers.

This is because the Toolbox contains the same structure to represent 2-ethyltoluene (our target substance) and ethyltoluene (a more generic entry (CAS#25550-14-5)). The user can then select the chemical structure according to the relevant CAS number.

In general, a structure can also be selected from a database, inventory, or user list.

2.2 Profiling

The user can select all the available profilers to have an overview of the alerts associated to the structure. In fact, this selection will only affect the computing time but not the successive steps that will lead to the prediction.

The outcome of the endpoint specific profilers toxicity hazard classification by Cramer (original and extended) are:

- Low (Class I)

Figure 1 Profiling results for the target chemical

The QSAR Toolbox provides information to explain profilers, which can be accessed by moving the mouse on the profiling result, clicking the right button and selecting 'Explain'.

In this case, the explanation is that substances classified as Class I are substances with simple chemical structures and for which efficient modes of metabolism exist, suggesting a low order of oral toxicity. The boundaries for each possible profiler result are also described (right click

on the profiling methods and click on 'show boundaries'). This information helps the user to check the reliability of the outcomes of the profilers.

According to the toxicity hazard classification by Cramer profiler, the chemical has low toxicity. The QSAR Toolbox also contains profilers related to repeated dose oral toxicity, protein binding and bioaccumulation that can be useful for supporting the selection of analogues and the predicted results.

As an example, protein binding or bioaccumulation profilers can provide indications on possible toxicity due to bioaccumulation or high reactivity issues. In this example, the target structure does not trigger any protein binding or bioaccumulation alerts.

The outcome from the DART profiler, which indicates the reason for concern for reproductive and developmental toxicity, is not taken into account in this example because is not considered relevant for the prediction of acute toxicity[7].

In this example, the result of the OECD HPV Chemical Categories profiler reports whether the target chemical belongs to one of the chemical categories analysed in the OECD programme. The result for 2-ethyltoluene is the following:

OECD HPV Chemical Categories – C9 Aromatics hydrocarbon solvents

Once the target is found to be part of an OECD HPV chemical category, more information about it can be searched online. The OECD-generated profile (called either the Screening Information Dataset (SIDS), Initial Assessment Profile (SIAP) or the Initial Targeted Assessment Profile (ITAP)) contains brief summaries of SIDS endpoints as well as the major conclusions of the hazard assessment. For this category, the SIAP is available online[8].

The CAS number for the target chemical is not part of the category as such, but "ethyltoluene mixed isomers" (CAS number 25550-14-5) is.

Concerning acute toxicity, the following information is reported: *"Acute toxicity studies (oral, dermal and inhalation routes of exposure) have been conducted in rats using various solvent products containing predominantly mixed C9 aromatic hydrocarbons (CAS RN 64742-95-6). Inhalation LC50's range from 6 000 to 10 000 mg/m3 for C9 aromatic naphtha and 18 000 to 24 000 mg/m3 for 1,2,4 and 1,3,5-TMB, respectively. A rat oral LD50 reported for 1,2,4-TMB is 5 grams/kg bw and a rat dermal LD50 for the C9 aromatic naphtha is >4 ml/kg bw. These data indicate that C9 aromatic solvents show that LD50/LC50 values are greater than the limit doses for acute toxicity studies established under OECD test guidelines."*

All the profiling results suggest that the target chemical may have low acute oral toxicity. This information can be further confirmed when filling the data gap for this endpoint.

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[7] It is worth mentioning that one attempt to make a prediction using the DART profiler in the sub-categorisation process also led to similar results in terms of predicted LD50. However, some of the analogues containing ethyl substituents were excluded from the selection of analogues, reducing the confidence in the predicted value.

[8] http://webnet.oecd.org/hpv/ui/handler.axd?id=a0bd2c68-c19d-4044-9095-6685d36510c6

2.3 Endpoint

In the "Endpoint" module, the user retrieves experimental data for the target chemical from the selected databases. As mentioned before, the Toolbox contains four databases with LD_{50} data, but the user may want to select all human health hazards databases to retrieve all available toxicological information on the target.

To retrieve the experimental data that may be in one or more of the QSAR Toolbox databases, click "Endpoint" in the top menu, select the relevant databases and click "Gather". The retrieval can be further limited to endpoints (in this case for example to "acute toxicity" by selecting "choose…" in the pop-up window: "Read data?"). The next pop-up window is reporting repeated values originating from different sources. It is recommended to choose the "Select one" option.

In this example, no data for acute toxicity are found for the target.
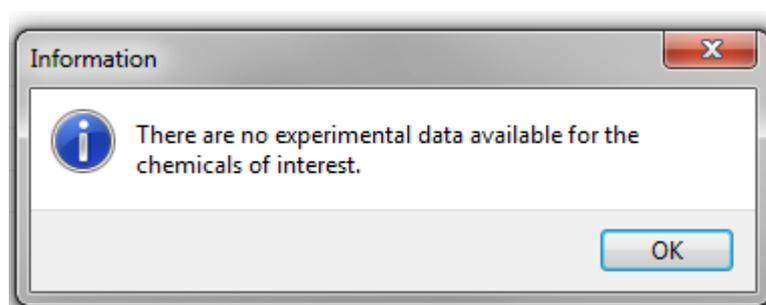
Figure 2 No data found for the target

2.4 Category definition

In the "Category Definition" module, the user chooses the profiler(s) for the analogue selection.

To ensure chemical similarity among analogues, the recommendation is to start with a "chemical" profiler. In the "Grouping methods" window, select "Organic functional groups (nested)", and click on "Define".

Only the chemicals found in the selected databases will be taken into account.

The target is profiled as "Alkyl arenes" and "overlapping groups" by the organic functional groups (nested) profiler, therefore only the analogues with the same functionalities will be retrieved from the selected databases. In this case, only alkyl arenes with at least two substituents (identified as "overlapping groups" by the Toolbox) will be selected.

Afterwards select "Strict"[9] at the bottom of the pop-up window.
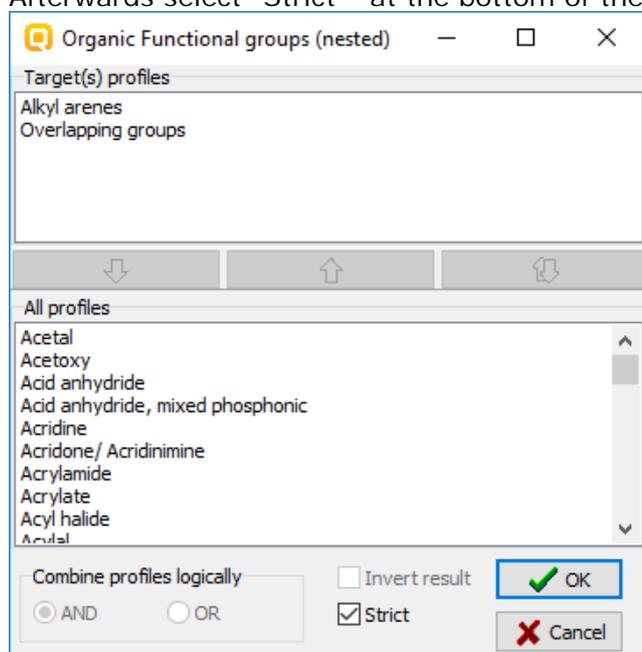
Figure 3 Functional groups to be included in the initial category

It is recommended to start the selection of analogues with a "structural" profiler rather than an endpoint specific one because, according to REACH, the final selection has to include chemically similar analogues.

In the following steps of the prediction workflow, a sub(categorisation) based on mechanistic information is possible. At the end of the prediction workflow, once the final group of analogues is identified, this similarity assessment is further confirmed by applying expert

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[9] During the definition of the category, it is possible to select whether the analogues have to comply strictly with the functional groups/alerts of the target or if they can also contain additional functionalities/alerts. In the first instance, it is recommended to tick the "strict" option to select only close analogues of the target, excluding compounds with additional functionalities.

judgement (as also shown later in this example).

After the selection of the starting profiler, all the chemicals found in the selected databases and having experimental data are displayed in the Toolbox matrix and become part of the starting category.

In this example, ChemIDPlus, ECHA CHEM, Toxicity Japan MHLW and ZEBET databases are selected.

After gathering the analogues, a pop-up window asks which data to read. Only acute toxicity data are selected.

A group of 43 analogues is identified (42 and the target). If the Toolbox identifies repeated values across databases, a pop-up window appears showing the repeated values. The recommendation is to click "Select one" and then "OK" to remove the repeated values.

Figure 4 Repeated values

Acute toxicity data are not available for all analogues. There are 135 acute toxicity data points from 40 of these analogues. 39 data points for 89 chemicals refer to LD$_{50}$, which is the target endpoint.

At this point, a further sub-categorisation is needed to refine the selection of analogues and data points. The sub-categorisation will be performed in the data-gap filling module, where the chemicals removed in each step are visually highlighted in the plot, helping the user to follow and understand the sub-categorisation process.

2.5 Data-gap filling

In the "Data Gap Filling" module, the user chooses:

- the endpoint to predict (LD$_{50}$)

- the type of approach for filling the data-gap (read-across)

- the sub-categorisation strategy (described below)

The Toolbox offers three different methods for data-gap filling: read-across, trend analysis and (Q)SAR models. In this case, read-across is the preferred option given the availability of data for some analogues structurally very close to the target. To select the endpoint, the user needs to click in the cell of the data matrix corresponding to the target endpoint and chemical (in blue in the figure below).

Figure 5 Selection of the target endpoint

Click read-across in the top-left corner of the screen and "Apply". When clicking on "Apply" to start the prediction process, a pop-up window of the QSAR Toolbox appears checking for possible data inconsistencies. The window offers the possibility to filter the data according to the experimental details and to choose the scale/unit for data-gap filling.

Filtering according to the experimental conditions is possible also later in the process, therefore in this step we do not limit the selection. Anyway, it is not possible to use all data points due to the inconsistency among the units (mg/kg and mL/kg bw). The most appropriate unit to select for oral toxicity is mg/kg and this leads to the exclusion of 14 data points.
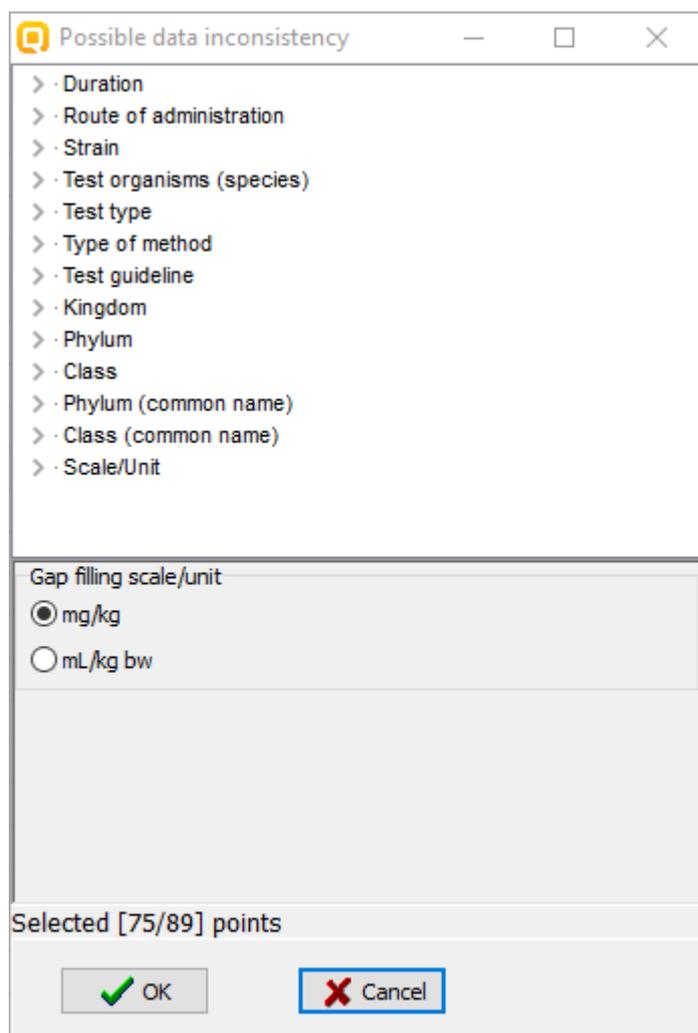
Figure 6 Data inconsistency pop-up window

Following these steps, the first plot is generated. It shows one LD$_{50}$ data point per chemical on the Y-axis[10], expressed as log $1/LD_{50}$ in mol/kg, and the octanol-water partition coefficient (log Kow) calculated from EPIWIN (KOWWIN ver. 1.67) on the X-axis.

Please note that the active descriptor on the X-axis can be changed at any time. In read-across predictions with the Toolbox, the selection of the active descriptor is not used in the calculation of the predicted value and therefore is not as important as it is in the trend analysis predictions, where the active descriptor is included in the equation to predict the toxicity.

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[10] By default, the Toolbox aggregates multiple values for each chemical and shows the average value, however, this option can be changed to show all available values, see later in the text.
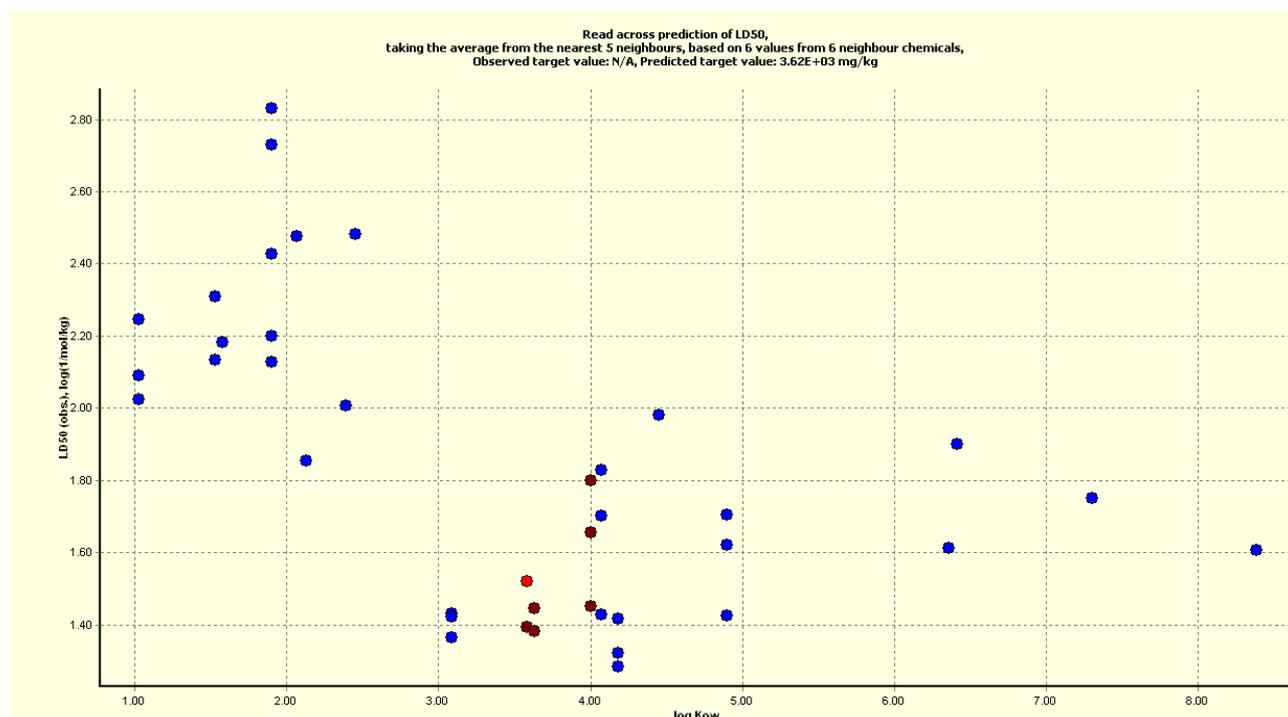
Figure 7 Starting plot

The plot shows that most of the analogues selected with the functional groups profiler have relatively low toxicity. Only a few analogues (in the top left of the plot) show higher toxicity, with LD_{50} values of around 2 log units (expressed as 1/mol/kg, corresponding to ~500 mg/kg).

These chemicals may either be genuine outliers of the category (and, in this case, the prediction has to take into account these values) or have some structural or mechanistic differences from the other analogues not caught by the organic functional groups profiler. A further refinement of the category based on reactivity, kinetic properties and experimental conditions can help to make the category uniform.

To better visualise the next steps, it is advised to click on "Calculation options" and change "Data usage" from "average" to "all". By doing this, each data point will be shown in the plot and not only one (average) value per substance as per default option.

In the "Select/filter data" option, the user applies additional filters for analogue selection.

For this example, the starting set of 39 analogues and 89 data points was sub-categorised as follows (note that sub-categorisation removes all data points associated to the substances filtered out, opposite to the filtering by test conditions used later that removes single data points):

1. Subcategorise -> Toxicity hazard classification by Cramer (original)[11]: 21 analogues left

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[11] The profiler 'Toxicity hazard classification by Cramer (extended)' would remove, in this case, some analogues that are structurally very close to the target. These analogues are identified as benzene analogues and assigned to the high toxicity category. However, the experimental acute LD50 of the analogues show toxicity values in line with the other category members for this endpoint. The Cramer (original) profiler is therefore preferred for this example.

This sub-categorisation step removes high and intermediate toxicity analogues clearly different from the target due to the presence of nitrogen and long chain substituents. The remaining analogues all have "low" toxicity according to this profiler.

2. Subcategorise -> Bioaccumulation – metabolism alerts: 13 analogues left

This profiler has been developed for fish bioaccumulation. However, we note that this profiler identifies all analogues with isopropryl and terbutyl fragments, which are not present in the target; while the remaining analogues are more similar to the target and share the same metabolic profile. In this situation, where many data points and similar structures are available, a strict refinement in the selection of analogues is advisable. In other cases where only a smaller number of analogues might be available, such strict sub-categorisation might not be possible.

At this point, 54 values from 13 analogues are left. Note that the next filters according to the test conditions remove data points and not analogues as such (at this step, the analogue is removed only if all data points associated to it are filtered out).

3. Filter points by test conditions -> Route of administration - dermal (removes four data points and one chemical, leaving 12 analogues)

Given that the endpoint to predict is oral LD50, results obtained from the dermal exposure route are not relevant.

A visual inspection of the category members shows consistency from a chemical point of view. Consistent low toxicity and basic metabolic/bioaccumulative properties were taken into account by the sub-categorisation step.

The only remaining issue pending is that of data quality for the analogues' data. The user is recommended to critically check each of the data points considered for the prediction. For the purpose of this example, an automatic quality check was done by applying additional filters to data points.

4. Filter points by test conditions -> Reliability - 1 (19 points left)

Data with reliability 1 are more reliable than data with reliability 2.

5. Filter points by test conditions -> Purpose flag – key study (18 points retained)

Data flagged as key study may be more relevant than supporting studies.

After all the filters mentioned above, seven analogues (six sources and the target) and 18 data points are left. By default, the Toolbox uses the average value among the five closest analogues to calculate the predicted result (the option can be changed in the "calculation options").

To use all of the seven analogues selected, the user needs to change the "Prediction approach options". Confidence range can also be shown by changing the "visual options". In this case, the prediction is an LD_{50} = 4 870 mg/kg bw (4 160 to 5 690 taking into account 95% confidence interval). Another (conservative) approach is to read-across from the highest toxicity value (i.e. from analogue CAS 105-05-5, 1,4-diethylbenzene, LD_{50} > 2 000 mg/kg bw). Regardless of the approach chosen, the prediction would be above the highest classification threshold relevant for classification (2 000 mg/kg bw).
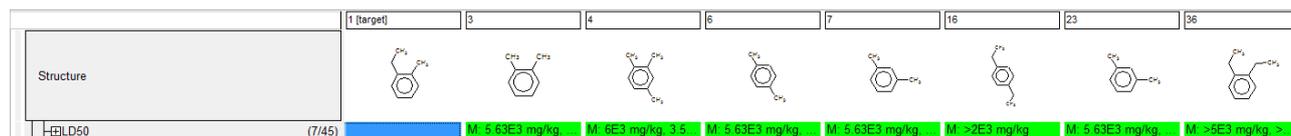
Figure 8 analogues and their respective LD50 values
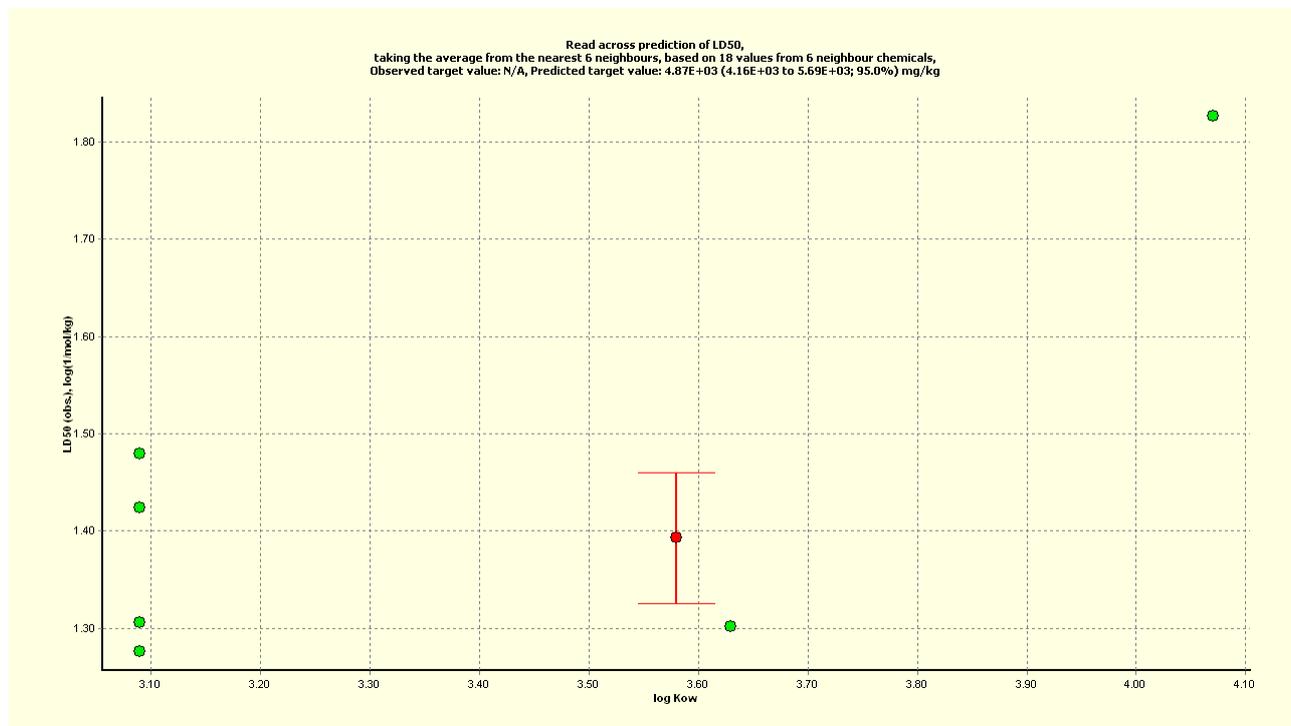
Figure 9 Final prediction plot

At this point, the analysis is concluded and can be entered in the data matrix by clicking on "Accept prediction".

2.6 Report and category summary

Once the prediction is accepted for reporting, the "report" module of the QSAR Toolbox gives the possibility to semi-automatically generate a standalone file to report the result with a pre-defined template, providing information on the modelling procedure (applicability domain, information about the group members, etc.).

To generate a report for the prediction, the user has to open in the module "Report" the Predictions in the window "Available data to report" by double clicking, selecting the specific prediction, and then clicking the button "Create".

Alternatively, the user can generate a report directly from the data matrix by hovering over the prediction and clicking the right mouse button. Selecting "Report" from the pop-up menu will generate a report, and selecting "IUCLID" will produce and attach a report directly to a IUCLID template file.

The report also contains manually editable fields that the user should complete to justify the procedure followed (i.e. category definition, hypothesis, and description). Here, we summarise some of the considerations needed for the assessment of the category and the prediction.

They are based on good practices and expert judgement and are not hard-coded in the QSAR Toolbox as such. To support and justify the category hypothesis, it is highly recommended to manually produce and include a data matrix (not shown in this example) reporting physico-chemical, ADME and other toxicological properties of the category members.

a. *Category definition*

A category of eight analogues (a target and seven source chemicals) is defined for the purpose of data-gap filling of acute toxicity by the oral route (as LD50). The analogues are shown in Figure 8. In this example, considerations on impurities are omitted. For REACH substances, impurities need to be analysed and discussed.

b. *Hypothesis for grouping chemicals*

The working hypothesis is that the selected analogues can be grouped together for the prediction of acute toxicity by the oral route. The hypothesis is based on the profilers' outcome, which supports the assumption that the analogues are structurally related and share similar reactivity and metabolic profiles.

c. *Category description*

- The category members are di- and tri-substituted (methyl or ethyl) C8-C10 toluenes. The category members do not present any additional functional groups. For each analogue, at least one LD_{50} for acute toxicity by the oral route is available. The LD_{50} values are expressed in mg/kg bw.

- The covered hydrophobicity range in terms of log K_{ow} is between 3 and 4.1 (based on calculated log Kow values available in the QSAR Toolbox). The experimental log K_{ow} values, also available in the Toolbox, are close to the calculated ones. The log K_{ow} of the target chemical is in the middle of this range, at around 3.5. Solubility covered by the analogues is from 30 to 160 mg/L. (based on experimental solubility values available in the QSAR Toolbox). The experimental water solubility for the target is 74.6 mg/L. The value fits in the range defined by the analogues. The molecular weight of the target is 120 Da and fits into the range defined by the analogues (from 106 to 134 Da).

d. Strategy used (data-gap filling method)

Predicted value (LD_{50}) = 4 870 mg/kg bw (4 160 to 5 690; 95%)
Quantitative read-across calculated as average of the category members.
The LD_{50} is presented in log (1/LD_{50} in mol/kg).
Number of data points (N) = 18

2.7 Additional weight of evidence considerations

There is a screening information dataset (SIDS) initial assessment profile (SIAP) prepared by US/ICCA (International Council of Chemical Associations) in the context of OECD Cooperative Chemicals Assessment Programme (CoCAM) for the category: "C9 Aromatic Hydrocarbon Solvents Category".

The category includes the chemical "Benzene, ethylmethyl (ethyltoluene mixed isomers)" (CAS 25550-14-5), which is a mixture of isomers also containing the target chemical. For acute toxicity, the report concludes "C9 aromatic solvents show that LD50/LC50 values are greater than limit doses for acute toxicity studies established under OECD test guidelines". This line of evidence supports the conclusion of low toxicity from read-across.

2.8 Conclusions

The read-across prediction obtained with the QSAR Toolbox indicates low acute toxicity by the oral route (4 850 mg/kg bw). A more conservative approach within the same category would predict a toxicity above 2 000 mg/kg bw. The selection of analogues used for the prediction is supported by the profiler's outcome, which indicates chemical, endpoint specific reactivity and metabolic similarity among the analogues.

The Cramer profiler suggests low toxicity for all category members. The quality of the data used for the prediction has been automatically taken into account by considering only data points from key studies with a Klimisch score of 1. Please note that for REACH registration purposes, a more exhaustive manual analysis of data quality and permission to refer to the data is expected.

The SIAP for the chemical category "C9 Aromatic Hydrocarbon Solvents Category", which includes a mixture of isomers containing the target chemical, indicates an acute toxicity greater than limit doses for acute toxicity studies established under OECD test guidelines (5 000 mg/kg bw).

Taking all the presented evidence into account in a weight-of-evidence approach, an LD50 for acute toxicity by oral route > 2 000 mg/kg bw is predicted for 2-ethyltoluene (CAS 611-14-3, EC 210-255-1), for the purpose of this illustrative example.