Applying new approach methodologies to reduce animal testing under regulatory programmes: a case study of complex (petroleum) substances

NC3R - 11 December 2018 - London

Hans Ketelslegers
Agenda

01 Petroleum refining & substances in a regulatory toxicology context

02 In-vitro developmental toxicity

03 Cat-App: chemical-biological grouping and read across of petroleum substances

04 Developing regulatory acceptance of NAM\(^1\) data: some conclusions from the application on petroleum products

\(^1\) NAM: New Approach Methodologies
Acknowledgements - big thanks to the...
European Petroleum Refiners Association

41 members, representing ~100% of European refining capacity
Petroleum substances: a regulatory (toxicology) challenge

- ~200 Petroleum Substances (PS)
- **Thousands to millions of molecules** (isomers) per PS
- UVCB
  - Unknown or
  - Variable composition,
  - Complex reaction products,
  - Biological materials

https://www.fuelseurope.eu/knowledge/how-refining-works

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Fractionation and Processing of Crude Oil into Petroleum Substances (Complex [reaction] products of Biological origin)

Conversion / upgrading processes
Subsequent distillation steps combined
Hydrodesulfurization / others omitted

Main product types
(usually covering more than one EC number)
- Gasoline
- Distillate fuels
- Residual fuels
- Bitumen
- Others

Distillation
Approximate cut points indicated

Crude oil

Conversion processes
- Solvent extraction, Hydrotreating, Dewaxing
- Reforming
- Hydrocracking
- Catalytic cracking
- Thermal cracking

Conversion products:
- Aromatic extracts
- Lubricants
- Waxes
- Foots oils & Petrolatum
- Asphalt
- Reformate
- Aromatics
- Gas oils
- Residue
- Naphthas
- Gas oils
- Residue
- Alkylate
- Olefins
- Naphthas
- Gas oils
- Residue
- Bitumen
- Residual fuels
- Asphalt
- Lubricants
- Foots oils & Petrolatum

Petroleum Substances are LVGOs:
- Unknown or
- Variable composition,
- Complex reaction products,
- Biological materials
Some aspects of PS UVCBs to keep in mind...

- But neighboring streams overlap: “continuum of PS”
- But variation is limited: “product specifications of PS”
Concawe PS “Categories”, historically based on refining history (1)

1. Low Boiling Point Naphthas (Gasolines)
2. Kerosines
3. Straight-run Gas Oils
4. Cracked Gas Oils
5. Vacuum Gas Oils, Hydrocracked Gas Oils & Distillate Fuels
6. Other Gas Oils
   - Heavy Fuel Oil Components
7. Unrefined / Acid Treated Oils
8. Other Lubricant Base Oils
9. Highly Refined Base Oils
10. Foots Oils
11. Paraffin and Hydrocarbon Waxes
12. Slack Wax
13. Petrolatum
   - Untreated Distillate Aromatic Extracts
   - Treated Distillate Aromatic Extracts
14. Residual Aromatic Extracts
15. Bitumen

In addition CONCAWE has prepared the joint parts of the Registration Dossier for the following stand-alone substances:

- MK1 Diesel fuel (EC number 931-250-7)
- Oxidised Asphalt (EC number 265-196-4)
- Sulfur (EC number 231-722-6)
Concawe PS “Categories”, historically based on refining history (2)

<table>
<thead>
<tr>
<th>Name</th>
<th>EINECS definition</th>
<th>CAS</th>
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<tbody>
<tr>
<td>Asphalt</td>
<td>A very complex combination of high molecular weight organic compounds containing a relatively high proportion of hydrocarbons having carbon numbers predominantly greater than C25 with high carbon-to-hydrogen ratios. It also contains small amounts of various metals such as nickel, iron, or vanadium. It is obtained as the non-volatile residue from distillation of crude oil or by separation as the raffinate from a residual oil in a desphalting or decarbonization process.</td>
<td>8052-42-4</td>
</tr>
<tr>
<td>Residues (petroleum), vacuum</td>
<td>A complex residuum from the vacuum distillation of the residuum from atmospheric distillation of crude oil. It consists of hydrocarbons having carbon numbers predominantly greater than C34 and boiling above approximately 495°C (923°F).</td>
<td>64741-56-6</td>
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<td>Residues (petroleum), hydridesulfurized vacuum</td>
<td>A complex combination of hydrocarbons obtained by treating a vacuum residuum with hydrogen in the presence of a catalyst under conditions primarily to remove organic sulfur compounds. It consists of hydrocarbons having carbon numbers predominantly greater than C34 and boiling approximately above 495°C (923°F).</td>
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<tr>
<td>Residues (petroleum), thermal cracked vacuum</td>
<td>A complex combination of hydrocarbons obtained from the vacuum distillation of the products from a thermal cracking process. It consists predominantly of hydrocarbons having carbon numbers predominantly greater than C34 and boiling above approximately 495°C (923°F).</td>
<td>92062-05-0</td>
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- CAS numbers relate to (last) refining step(s)
- 2010 REACH deadline (>1k tonnes):
  - large numbers of CAS registered (600+) to
  - “secure” company specific refining operations, but
  - CAS within a category are eventually describe the “same” petroleum product! (UVCB nature, i.e., “variable” in composition, but variable within product specifications)

-> in general terms: within one petroleum category, CAS numbers describe different ways of making the same substance
Some aspects of PS UVCBs to keep in mind...

- But neighboring streams overlap: "continuum of PS"

- But variation is limited: "product specifications of PS"

- Petroleum UVCB can never be fully characterized analytically
Petroleum substances: a regulatory (toxicology) challenge

- 207 Petroleum Substances (PS)
- Thousands to millions of molecules (isomers) per PS
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IUCLID 6 example

*Overview of dossier elements*
REACH dossier - Example of Low Boiling Point Naphthas (Gasolines)

- Example of **ONE** tox study *summarized* in IUCLID and the CSR.
- There are >550 studies (phys/chem, mammalian toxicity, environmental) in total summarized in IUCLID/CSR

The IUCLID stack of papers is a mock-up. The others are real, printed previously for other reasons.

We did not really print the IUCLID file (and don’t recommend anyone to print it...)

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HH endpoints in REACH dossier

- Concawe has completed endpoints with historical data, and some newly generated

- These historical data are currently being challenged by ECHA
  - Exposure route (relevant dermal / inh. [risk based] vs required oral [hazard based])
  - Non-standard guideline / test quality
  - Compositional description test substance

- Concawe will defend historical data...

- ...while acknowledging there are datagaps which need to be addressed to keep dossiers compliant
Current alternative approaches not always applicable to (petroleum) UVCBs

- Learn from other sectors
  - Approaches available for lower tier endpoints (e.g., irritation, acute tox) and well defined chemicals
- However:
  - Real challenge is with higher tier endpoints and complex, multi-constituent substances

From ECHA RAAF report (7 March 2017):
All chemical structures involved need to be considered, grouping of substances on the basis of structural similarity must take account of all constituents, and the predictions within proposed groups must likewise consider the impact of all constituents.

The analysis described in this document confirmed the complexity of read-across approaches for multi-constituent substances and UVCBs.

More work is needed to further develop the RAAF based on the findings described in this document.
The regulatory application\(^1\) of “NAM\(^2\)”

- Number of NAM data submitted to REACH: a handful
- Number of NAM data accepted as “alternatives”: none (?)

To progress the regulatory acceptance of NAM data short term, there is much to gain to start submitting these data in registration dossiers in a weight of evidence approach - focusing on underpinning grouping and read across approaches & supporting hazard data.

\(^1\) From a (petro-)chemicals / REACH perspective; this expressed view is that of the author / presenter of this presentation, and cannot be ascribed to Concawe or its member companies

\(^2\) NAM: New Approach Methodologies
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\(^2\) NAM: New Approach Methodologies
A Concawe approach towards the regulatory application of “NAM”

- Number of NAM data submitted to REACH: a handful
- Number of NAM data accepted as “alternatives”: not a single one

For a more realistic short term application of NAM data, there is much to gain by start submitting these data in registration dossiers in a weight of evidence approach - focusing on underpinning grouping and read across approaches & supporting hazard data.

*In vitro* reprotoxicity hypothesis testing
Concawe PS “Categories”, historically based on refining history

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-> in general terms: within one petroleum category, CAS numbers describe different ways of making the same substance
Concawe PS “Categories”, historically based on refining history

Need for improved analytical chemical descriptors on substance level

Need for improved biological descriptors (e.g., mechanistic data) on substance level

Category or grouping not accepted

...but worst case read-across OK, providing testing hypothesis is proven
3-7 ring PAH hypothesis for Petroleum Substances

Based on historical toxicological data\(^1\) it can be stated that the higher tier mammalian toxicological effects of petroleum substances are associated with the level of 3-7 ring PAH in poorly refined high boiling petroleum substances.

\(^1\) in-vivo toxicological data: see [http://www.petroleumhpv.org/polycyclic-aromatic-compounds](http://www.petroleumhpv.org/polycyclic-aromatic-compounds) for an exhaustive overview of PAC related toxicity of petroleum substances, including public access to relevant papers. Some selected references are Feuston et al., 1994; McKee et al., 1990; McKee et al., 2012; Schreiner et al., 1997; White 2012
Testing hypothesis that 3-7 ring PAH cause developmental toxicity

Battery of in-vitro tests in combination with toxicogenomics...

...to support the 3-7 ring PAH hypothesis

...justifying the selection of the worst-case representative for testing

...justifying read-across of this test sample to the other group members

...eventually underpinning prenatal developmental toxicity of PS with mechanistic data
mES assay pilot to support devtox-PAH hypothesis

GTL Base Oil (similar results for highly refined base oils):
no aromatics / (3-7 ring) PAH

DAE (extracts):
high (3-7 ring) PAH

Gas Oils (e.g., Diesel):
low to moderate (3-7 ring) PAH

RAE (aromatic extracts) &
heavy fuel oil:
low to high (3-7 ring) PAH

Kamelia et al., Toxicology in Vitro (2017)
mES assay pilot to support devtox-PAH hypothesis

In-vitro results correlate with 3-7 ring PAH content, not 2-3 ring PAH...

In-vitro results correlate with 3-7 ring PAH content, not 2-3 ring PAH...

... and with in-vivo data

Gas Oils (e.g., Diesel): low to moderate (3-7 ring) PAH

RAE (aromatic extracts) & heavy fuel oil: low to high (3-7 ring) PAH
PAH reprotox hypothesis project: putting it all together...

Bioactivity profiling, hierarchical clustering, and chemical ranking using ToxPi GUI 2.0.

ToxPi includes data from - EST (red),
- ZET (blue),
- Calux assays (green),
- PAC2 (yellow)
- (transcriptomics analyses still ongoing, will be included as well)

These data will be integrated into the overall toxicological WoE to support the Concawe intelligent testing strategy
New Technologies to Underpin Category Approaches and Read-across in Regulatory Programmes
Biological activity and high throughput toxicogenomic profiling of human cell models in response to PS exposure

Evaluation of biological response (e.g. phenotypical) changes in stem cells and human cell-lines in response to PS exposure

Unraveling the mechanisms behind the biological responses based on gene expression data

Integrative analysis of all available data to support and visualize groupings for transparent communication of all data
Biological activity profiling of human cell models

Work Package 2a:
Screening of iPSC-derived human cell lines

Figure 1: Phenotypic Profiling of 141 Petroleum Substances and 20 Reference Chemicals

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>iCell Neurons</td>
<td>4</td>
</tr>
<tr>
<td>iCell Cardiomyocytes</td>
<td>14</td>
</tr>
<tr>
<td>HUVEC [endothelial]</td>
<td>5</td>
</tr>
<tr>
<td>iCell Endothelial</td>
<td>9</td>
</tr>
<tr>
<td>iCell Hepatocytes</td>
<td>6</td>
</tr>
<tr>
<td>iCell Macrophages</td>
<td>1</td>
</tr>
<tr>
<td>39 Phenotypes</td>
<td></td>
</tr>
</tbody>
</table>

Work Package 2b:
Screening of human cell lines

Figure 2: Conducted several assays per cell line, leading to ~30 read outs

For quality control purposes and to ensure assay validity, the appropriate control chemicals were used for each assay in all experiments.
Using ToxPi for Grouping Petroleum Substances: Legend

<table>
<thead>
<tr>
<th>Name</th>
<th>Weight</th>
<th># Metrics</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td>4 (7.0%)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>14 (24.6%)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>ENDO</td>
<td>6 (10.5%)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>HEP</td>
<td>7 (12.3%)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>HEPARG</td>
<td>1 (1.8%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HEPG2</td>
<td>2 (3.5%)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HLMVEC</td>
<td>2 (3.5%)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HT29</td>
<td>1 (1.8%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HUVEC</td>
<td>4 (7.0%)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>LN229</td>
<td>2 (3.5%)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MCF7</td>
<td>4 (7.0%)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>NEUR</td>
<td>6 (10.5%)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>4 (7.0%)</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
Example of bioactivity profiling of petroleum substances...

- Heavy Fuel Oils ("unrefined", high 3-7 ring PAH)
- Lubricating Base Oils (more "refined", low to moderate 3-7 ring PAH)
...and ranking of petroleum substances based on their bioactivity profiles using ToxPi (supervised approach)
Correlation between 3-7 ring PAH and bioactivity profiles of PS

ACRONYMS
- UATO: Unrefined / Acid Treated Oils
- AE: Unrefined Distillate, Treated Distillate, Residual Aromatic Extracts
- HFO / PO: Heavy Fuel Oil/ components / Fuels Oils
- CGO / OGG: Cracked Gas Oil / Other Gas Oils
- SRGO: Straight-run Gas Oil
- VGO: Vacuum Gas Oils, hydrocracked Gas Oils & Distillate
- BIT: Bitumen
- KER: Kerosines
- BO: Other Lubricants Base Oils
- WAX: Paraffin and Hydrocarbon wax / Slack Wax
- NAPHTA: Low boiling point Naphthas (Gasoline)
- PLAT: Petroleum
Transcriptomic Data Analysis - Effect of Petroleum Substances

Differentially Expressed Genes [in each of 6 cell types]

Liver performs best!

Percent of significantly perturbed transcripts

PS with high 3-7 ring PAH induce highest responses!

Direction
- Red: Down
- Green: Up

141 Petroleum substances
Hepatocyte Gene Expression: Group-Specific “Signatures”? 

Highest expressed genes are all involved in (PAH) metabolism related biological pathways
3-7 ring PAH hypothesis for Petroleum Substances

The percentage weight of 3-7 ring PAHs in the UVCB is the most active contributor to the bioactivity observed.
Chemical-biological grouping and read across; Cat-App data to support HH REACH testing

For further testing in animal studies, based on these data a sample can be selected which is expected to be most responsive in that in-vivo study, applied as a representative worst case* for that group of substances.

*The worst case means worst case test sample for the in-vivo test, not because it is worst case in terms of bioactivity as these data cannot be interpreted as correlation to eventual toxicological effects.
141 substances & 20 reference chemicals

39 phenotypes measured on iCells (some unique to the cell line in question)

\(~340,000\)

4 different physical chemical characterizations on all 141 substances

3-4 assays conducted on human cell lines

\(~35,000,000\)

4 IPS\(^*\)-derived cells

2 human cell line

5 IPS\(^*\) Cell Lines

8 Human Cancer Cell Lines

2 Human Primary Cell Lines

Data points from the phenotypic profiling of petroleum substances in 15 cell lines

Genes expression data points on more than 11,000 samples in 6 cell lines

This work was prepared by the CAT-APP team (as shown in the CAT-APP work programme) and the toxicology subgroup of CONCAWE.

* IPS = Included Pluripotent Stem Cells
Cat-App: conclusions from work to date to take forward for future efforts

- Cat-App data add a biological component to the grouping of Petroleum Substances
- This is expected to address some of the regulatory challenges on application of read across for our complex substances
- The data also add further mechanistic support for our testing hypotheses
- Which all together should help to significantly reduce high animal consuming REACH testing

Next Phase:
- **Obtaining acceptance** of these data as part Concawe HH REACH strategy
- **Further work** will be recommended in the final Cat-App project report, based on project results and discussion with the regulators

Overview of informed and tiered intelligent testing strategy

Target animal testing where needed as a last resort, based on a worst case approach rather than targeting all substances

Basis for testing strategy:
- Historical (in-vivo) data
- Mechanistic data
- Available human data
- Cat-App

Mutagenicity / Genetox including screening assays

“optimized OECD 422s”:
- OECD 474 (in-vivo micronucleus) will be added, as well as PAC biomarker analysis in urine
- OECD 411 added to this study will inform the repdose endpoint
- the OECD 421 aspects of the text will serve as a DRF for the reprotox studies

Carc WoE, including screening assays

Repeated Dose toxicity (build into OECD 422s)

Reprotoxicity (PNDT & EOGRTS) (OECD 422s inform as screening studies and used as DRFs here) in-vitro screening battery

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Some thoughts on the regulatory application NAM data

Based on the experience with Concawe REACH work...

- REACH is generally set up to assess substances on a case by case basis, with an emphasis on the need for new in-vivo data.
- More “holistic” type of thinking is needed to avoid unnecessary animal testing.
- This would progress the regulatory acceptance of alternative data...
- ...and can be achieved in steps, starting by including them in informed and targeted testing strategies (“R-eduction”)
- But can only be achieved by breaking the silo’s between academia, industry and regulators (e.g. PetCo²)

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² ECHA Petroleum and Coal substances working group: https://echa.europa.eu/petco-working-group

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Some thoughts on the regulatory application NAM data

Based on the experience with Concawe REACH work...

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Risk of getting lost in an endless detour?

Taking “preliminary” regulatory action (i.e., “tick the box” strategy rather than “result driven”) would again ignore the possibility to make a significant step towards application of NAM data in view of “the 3Rs” in a regulatory context.
How Concawe efforts fit into the “3Rs*” on animal testing in regulatory toxicology

- **Refinement**
  - ITS, more efficient appl. of OECD guideline studies

- **Reduction**
  - In-vitro screening, WoE to support grouping & read across, worst case approaches

- **Replacement (eventually)**

For a more realistic short term application of NAM data, there is much to gain to start submitting and accepting these data in registration dossiers in a collaborative effort between academia, industry and regulators – initially focusing on underpinning grouping and read across approaches & supporting hazard data.

Thank you for your attention

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