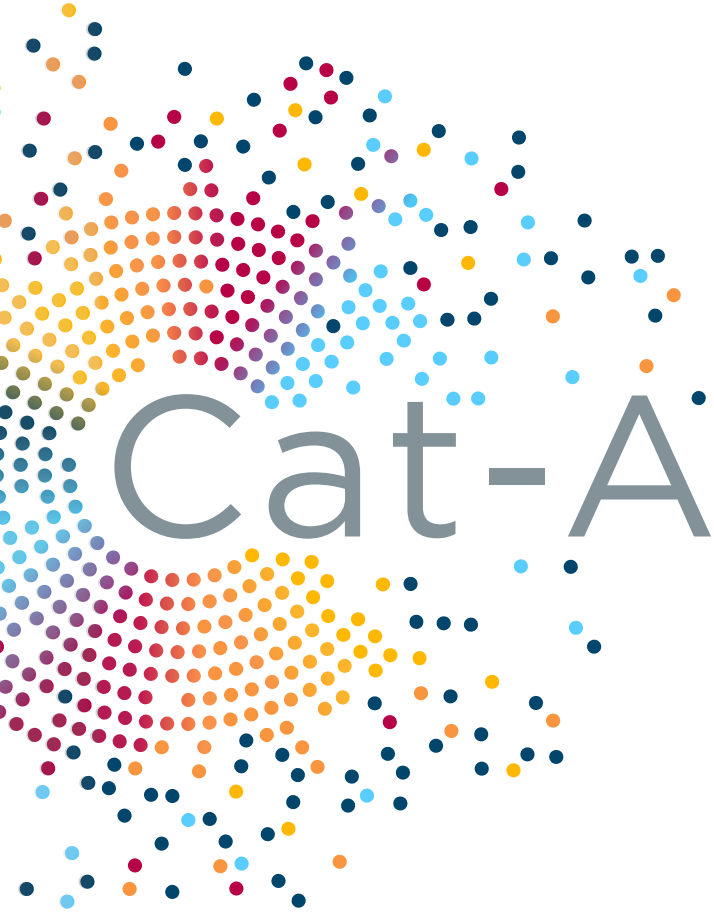


Cat-App

“Aiming to minimize testing in laboratory animals for the regulatory safety evaluation of petroleum substances”

Results 2018

https://www.concawe.eu/cat-app/

Disclaimer: *This brochure is intended for information purposes only to illustrate the concepts, methodologies and thought process around the objectives and results of the Cat-App project. This publication cannot be used as a scientific resource to cite data. The results of the Cat-App project will be published at a later stage in a final project report and peer-reviewed scientific papers. Concawe accepts no responsibility for the use that might be made of the information contained in this brochure.*

Cat-App objectives

Cat-App Project was initiated with the aim to minimize the need for testing in vertebrate animals under regulatory toxicology programmes. Petroleum products are a major challenge for regulatory decision making, due to their chemical complexity of UVCB[1] nature. Under regulatory programmes such as REACH*, this could potentially lead to a large number of (unnecessary) animal testing since the current available alternative strategies do not generally apply to Petroleum UCVB Substances due to this complexity.

Cat-App tries to address this, by developing a novel framework which adds a biological component to the historically used Phys/chem and refining data used for categorizing Petroleum Substances, using state of the art biotechnology tools. This framework will allow to make most optimal use of the already available toxicological information on these PSs by chemical biological read-across, and will help to target additional in-vivo testing in an informed way only where really needed as a last resort, significantly reducing - and avoiding unnecessary animal testing for the registrations of PSs under REACH.

abbreviations:

[1] UVCBs are substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials

*REACH: Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency.

Principal investigators

Concawe is the petroleum industry scientific organisation for environmental, health and safety research relating to the refining of crude oil as well as the distribution and use of petroleum products to benefit the industry itself and the society at large. The scope of Concawe's activities includes research in areas such as fuels quality and emissions, air quality, water quality, occupational health and safety, toxicology and product stewardship.
Hans Ketelslegers, PhD, ERT

Texas A&M University, (Texas A&M University) Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences Texas A&M University. The laboratory and staff are well experienced with complex databases and molecular toxicology research.
Ivan Rusyn, PhD, M.D.

Public Health England (PHE) is an executive agency of the UK Department of Health and has overall responsibility for all aspects of public health in England. The Centre for Radiation, Chemical & Environmental Hazards (CRCE) of PHE provides specialist advice on chemical and radiological issues that affect the UK, inputs into chemical and radiological activities within the EU and internationally as required. The centre carries out an active research program to underpin its advice, and maintain capability and understanding.
Timothy Gant, PhD

Bioinformatics Research Center (BRC) at North Carolina State University is an interdisciplinary center devoted to research at the interfaces of quantitative and biological sciences, with strengths in statistical methods applied to toxicological problems.
Fred Wright, PhD, director BRC

The Northern Ireland Centre for Stratified Medicine (NICSM) at the University of Ulster is a partnership between the Biomedical Science Research Institute (BMSRI), the Clinical Translational Research and Innovation Centre (C-TRIC) and the Western Health and Social Care Trust.
Shu-Dong Zhang, PhD

SYNCOM Research and Development Consulting GmbH is a consulting firm with the focus on innovation management in scientific research and development projects.
Klaus Lenz, PhD

Cat-App work programme

Cat-App: New technologies to underpin the category approaches and read across in regulatory programmes
Project Management: Hans Ketelslegers, Concawe
Steering: Concawe's scientific committee and toxicology subgroup

WP1

Organisation of data available on PS (Ivan Rusyn/Texas A&M University)

1.1 Obtain, process and share chemical samples
1.2 Collect available records (manufacturing process info., phys./chem. properties, analytical chemistry, existing toxicity data on mammalian, ecotox)
1.3 Digitise records into flexible and inter-operable databaseformat

WP2

Bioactivity screening (Ivan Rusyn/Texas A&M University

WP2.a
(Ivan Rusyn/Texas A&M University)
- High content screening of iPS*-derived cells
- Hepatocytes, neurons, cardiomyocytes, macrophages, endothelial

WP2.b
(Tim Gant/PHE)
- Toxicity phenotyping in 10 diverse cell lines

WP3

High throughput genomics (Ivan Rusyn/Texas A&M University)
3.1 High-throughput transcriptomics profiling of ~11,000 samples for TempO-seq

WP4

Perform data integration and chemical biological read across (Fred Wright/NCSU)

WP 4.a (Fred Wright/NCSU)
4a.1 Coordinate data management and workflow
4a.2 Perform uncertainty and variability analyses
4a.3 Process and analyse omics data
4a.4 Perform ToxPi analysis

WP4.b (Shu-Dong Zhang/Ulster)

4b.1 Perform connectivity mapping
4b.2 Develop and apply analysis algorithms to robustness testing, investigate grouping accuracy and profiling cost

WP5

Dissemination, project administration and Outreach (Klaus Lenz/SYNCOM)

5.1 Project Dissemination and website
5.2 Project Administration
5.3 Outreach

Advisory Board

George Daston
Procter & Gamble

Shirley Price
University of Surrey

Chris Rowat
Health Canada

Xiaowei Zhang
Nanjing University

Institute abbreviations:
Texas A&M University Research - NCSU: North Carolina State University - PHE: Public Health England
Ulster: Ulster University - SYNCOM: SYNCOM R&D consulting GmbH

* induced Pluripotent Stemcells

Cat-App work programme: Work package 1

WP1

Organisation of data available on PS (Ivan Rusyn/Texas A&M University)

1.1 Obtain, process and share chemical samples
1.2 Collect available records (manufacturing process info., phys./chem. properties, analytical chemistry, existing toxicity data on mammalian, ecotox)
1.3 Digitise records into flexible and inter-operable databaseformat

Given the solubility issues with lipophilic PS in in-vitro systems, DMSO extracts were obtained, dilution series prepared and arrayed into 384-well plate format

Additional reference chemicals, positive and negative controls were procured and arrayed onto the chemical plates

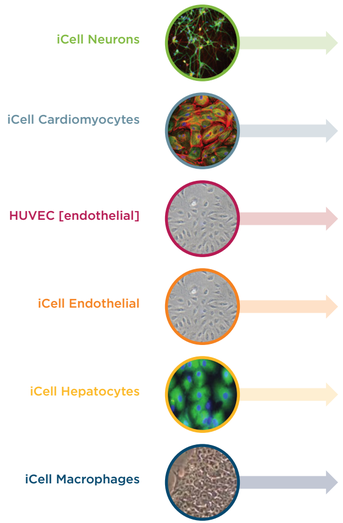
Plates were distributed to participating laboratories for screening

A database of all in vitro bioactivity data was developed for storing, exchanging and visualizing the information

The cell models in WP2a and WP2b were used as tools to measure consistent biological responses of the petroleum substances. These responses were subsequently combined per substance and linked to other data types in WP4, generating global bioactivity profiles to be applied as fingerprints of the Concawe petroleum categories. **The aim with these screening experiments is to add a biological component to the grouping of petroleum substances and not to anchor these responses to any apical hazard endpoint.** Without the necessary additional experimental work for anchoring purposes, these data cannot be used for predictive toxicological applications and this was not the aim of Cat-App.

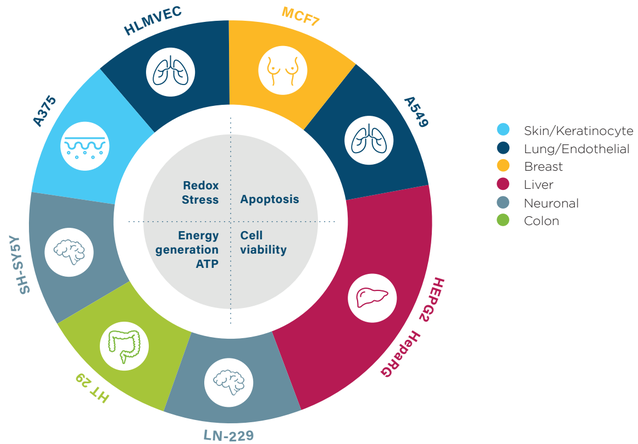
Work Package 2a:
Screening of iPSC-derived human cell lines

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



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

Cat-App work programme: Work package 2a and 2b

WP2

Toxicity screening (Ivan Rusyn/Texas A&M University)

WP2.a
(Ivan Rusyn/Texas A&M University)

- High content screening of iPS-derived cells
- Hepatocytes, neurons, cardiomyocytes, macrophages, endothelial

WP2.b
(Tim Gant/PHE)

- Toxicity phenotyping in 9 diverse cell lines

WP2a:
Screening was performed in **5 human iPS-derived cell types and one primary cell type** representing 5 tissues
Bioactivity assays included measurements of the substance effects on a variety of cell-specific physiological parameters and measures of cell viability

WP2b:
Screening was performed in **9 human cancer cell types** representing 7 tissues
Bioactivity assays included measurements of the substance effects on basic cell functions and viability

Work Package 3: **high throughput transcriptomics profiling**

WP3

High throughput genomics (Ivan Rusyn/Texas A&M University)

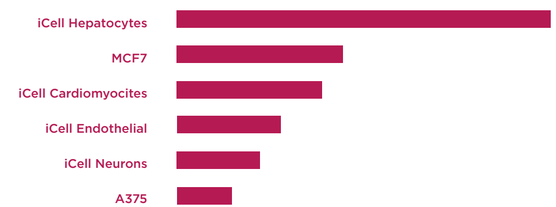
3.1 High-throughput transcriptomics profiling of 13,500 samples for TempO-seq

~35 MILLION

gene expression data points collected on more than **11,000 samples** in 6 cell models (most responsive cell models from high content in-vitro screening (WP2a and 2b) were selected for transcriptomics, see page table on page 14)

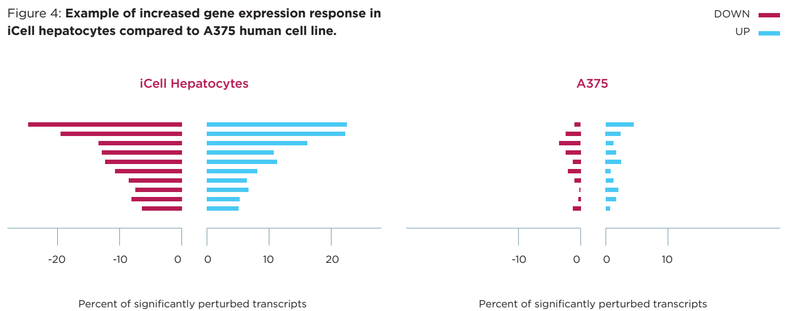
Transcriptomic Data Analysis Effect of petroleum substances

Figure 3: **Performance of cell models in terms of gene expression changes as percentage of the total.**



iCell Hepatocytes constantly showed most significant gene expression changes in response to extracts of petroleum substances (left panel), and strongest effects were observed in the heavier petroleum substances with higher PAH content (lower panel).

Figure 4: **Example of increased gene expression response in iCell hepatocytes compared to A375 human cell line.**



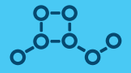
Cat-App in numbers

141 SUBSTANCES

&20 REFERENCE CHEMICALS

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

~340,000 DATA POINTS FROM THE PHENOTYPIC PROFILING OF PETROLEUM SUBSTANCES IN 15 CELL LINES



4 DIFFERENT PHYSICAL CHEMICAL CHARACTERIZATIONS ON ALL 141 SUBSTANCES

3-4 ASSAYS CONDUCTED ON HUMAN CELL LINES

~35,000,000

GENES EXPRESSION DATA POINTS ON MORE THAN 11,000 SAMPLES IN 6 CELL LINES

TRANSCRIPTOMICS MEASUREMENTS, PERFORMED ON

4 iPS*-derived cells

2 human cell line

5 iPS* Cell Lines

8 Human Cancer Cell Lines

2 Human Primary Cell Lines

THIS WORK WAS PREPARE BY THE CAT-APP TEAM (AS SHOWN IN THE CAT-APP WORK PROGRAMME) AND THE TOXICOLOGY SUBGROUP OF CONCAWE.

* iPSC = Included Pluripotent Stem Cells

Work Package 4:
integrative data analysis and chemical-biological grouping

WP4

Perform data integration and chemical biological read across (Fred Wright/NCSU)

WP 4.a
(Fred Wright/NCSU)
4a.1 Coordinate data management and workflow
4a.2 Perform uncertainty and variability analyses
4a.3 Process and analyse omics data
4a.4 Perform ToxPi analysis

WP4.b (Shu-Dong Zhang/Ulster)

4b.1 Perform connectivity mapping
4b.2 Develop and apply an-alysis algorithms to robustness testing, investigate grouping accuracy and profiling cost

This table show the number of assays that were done on the respective cell models, and how well these models performed.

The six models with highest correlation coefficients were taken forward for high throughput transcriptomics profiling.

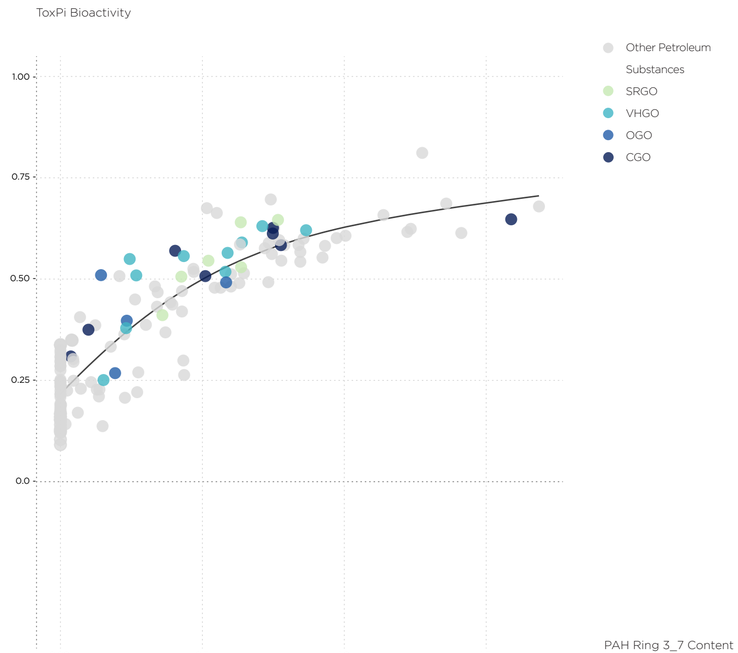
*Row height is proportional to the number of assays performed on the respective models

CELL TYPE	SPEARMAN CORRELATION	ASSAYS*	TRANSCRIPTOMICS?
ALL ASSAYS	0.88	77	
A375	0.08	5	Yes
A549	0.06	3	
CM	0.83	16	Yes
ENDO	0.80	11	Yes
HEP	0.81	8	Yes
HEPARG	0.32	3	
HEPG2	0.27	2	
HLMVEC	0.21	4	
HT29	0.21	4	
HUVEC	0.74	5	
LN229	0.27	4	
MACRO	0.72	1	
MCF7	0.37	5	Yes
NEUR	0.71	6	Yes

Figure 5**: Using ToxPi to rank substances in the chemical-biological space, global continuum of petroleum substances**
(see explanatory text on page 15)

Figure 6: **Using ToxPi to rank substances in the chemical-biological space, per Concawe substance category (supervised approach)**
(see explanatory text on page 15)

Figure 7: **To illustrate the continuum of petroleum substances, the 4 Concawe Gas Oil categories are highlighted here as an example of overlapping «neighboring» petroleum streams, adding support to inter-category read across assessments**



Work Package 4:
integrative data analysis and chemical-biological grouping (all assays)

Petroleum substances were scored based on their bioactivity profiles following the ToxPi methodology (ref needed) and subsequently ranked in the chemical-biological space. The chemical space is represented by 3-7 ring PAH data obtained with the PAC2 methodology[1].

A strong positive correlation (rhos=0.88) was observed between 3-7 ring PAH and bioactivity profiles of the petroleum substances, showing that petroleum substances which are mostly of aliphatic nature (waxes, petrolatums, certain base oils) have lower bioactivity, whereas the more heavy petroleum substances with higher PAH content have higher bioactivity (figures 5 and 6). This is a pattern which is generally known in terms of the mammalian toxicological hazards of petroleum substances build further confidence in the methodological approach and add to the quality control of the data.

Overall this provides additional supporting evidence to the hypothesis that the main bioactive constituents in the heavier petroleum substances are 3-7 ring PAH, depending on their bioavailability, a hypothesis on which grouping and read across assessments can be build to help target eventual toxicology testing of these substances in animals only where really needed (figure 7).

[1] PAC-2 method as summarized e.g., in Gray, et al., (2012). Assessing the Mammalian Toxicity of High-Boiling Petroleum Substances under the Rubric of the HPV Program Regulatory Toxicology and Pharmacology. For more information see http://www.petroleumhpv.org/polycyclic-aromatic-compounds.

Figure 8a: **Using ToxPi Grouping Petroleum Substances: Supervised**

Heavy Fuel Oils

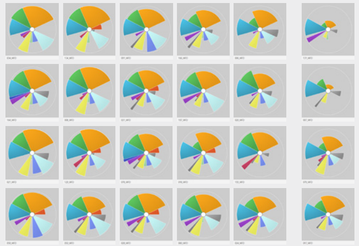
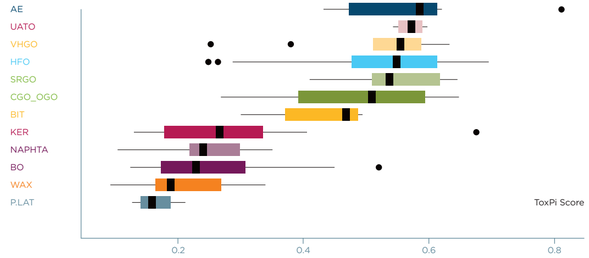


Figure 8b

Libricating Base Oils

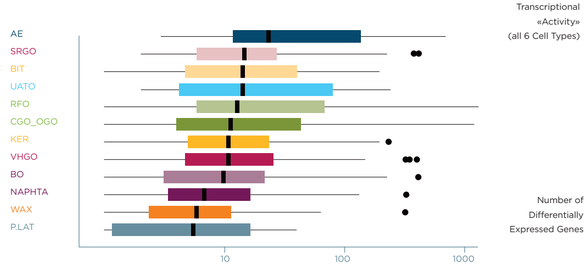
Figure 9: **Combinated data for 15 cell types**

ToxPi's, scored per substance as explained earlier, were grouped per Concawe category. This example shows a clear different pattern between Heavy Fuel Oils (**figure 8a**), which are heavy unrefined petroleum substances, and Lubricant Base Oils (**figure 8b**) which are generally more refined. This scoring was done for all Concawe categories and subsequently ranked based on their bioactivity profile, resulting in a positive trend from the lighter and higher refined heavy substances at the bottom left to the lesser refined heavy substances at the top right (**figure 9**). These data show that bioactivity profiles are to some extend different between Concawe categories, but there is significant overlap as well which is expected given the fact that these substances represent a continuum based on their refining history.



The number of differentially expressed genes was calculated per petroleum substance, which were subsequently grouped based on the current Concawe categories (supervised approach). A positive trend going from lighter and more refined heavier substances (least response) to heavier less refined substances (highest response) was observed again, similar to what was observed with the bioactivity data presented in **figure 9**, with significant overlap in gene expression profiles between the different categories. The top 20 genes that were most significantly affected, in particular by the heavier unrefined petroleum substances, were mostly involved in (PAH) metabolism such as the Cytochrome P450 enzyme family.

Figure 10: **Transcriptomic Data Analysis – Effect of Petroleum Substances**



ACRONYMS

UATO: Unrefined / Acid Treatered Oils

AE: Untreated Distillate, Treated Distillate, Residual Aromatic Extracts

HFO - FO: Heavy Fuel Oil components / Foots Oils

CGO - OGO: Cracked Gas Oil / Other Gas Oils

SRGO: Straight-run Gas Oil

VHGO: Vacuum Gas Oils, Hydrocracked Gas Oils & Distillate

BIT: Bitumen

KER: Kerosines

BO: Other lubricants Base Oils

WAX: Parafin and Hydricarbon wax / Slack Wax

NAPHTA: Low Boiling point Naphtas (Gasolines)

P.LAT: Petrolatum

Cat-App:
Conclusions from work to date to take forward for future efforts

- In-vitro bioactivity profiles of petroleum substances (PS) show a strong positive correlation with their (3-7 ring) PAH content
- PS can be ranked in the chemical-biological space (low -> high PAH content and bioactivity) - representing the continuum of petroleum substances on the upper right end of the space
- This ranking in agreement with the PAH hypothesis for PS which states that certain specific toxicological effects observed in heavier PS are associated with the level of 3-7 ring PAH in these substances, and adds another line of data to the overall weight of evidence that is being built holistically across the continuum of PS to further support this hypothesis
- By themselves, individual bioactivity data and gene expression profiles show a trend across the spectrum of PS, with large intra-category variation and inter-category overlap.
- The selected assays are limited in their ability to isolate petroleum categories fully...
- ...but the data help to further build the holistic picture that PS constitute a continuum of substances, in which certain overlapping groups can be identified and read-across hypothesis built
- Cat-App will be applied as part of an intelligent testing strategy with the goal to reduce animal testing by leveraging the data from the continuum of PS, and target animal testing only as a last resort
- Cat-App is the largest to date “case study” that was aimed at testing whether and how in vitro bioactivity [including transcriptomics] can be used to support grouping of UVCBs
- Ambitious goals of Cat-App (in terms of the type and number of substances, cell types, phenotypes and data analysis questions) have been met with respect to both scientific output and timelines
- Further work is needed to fully unlock the potential of Cat-App

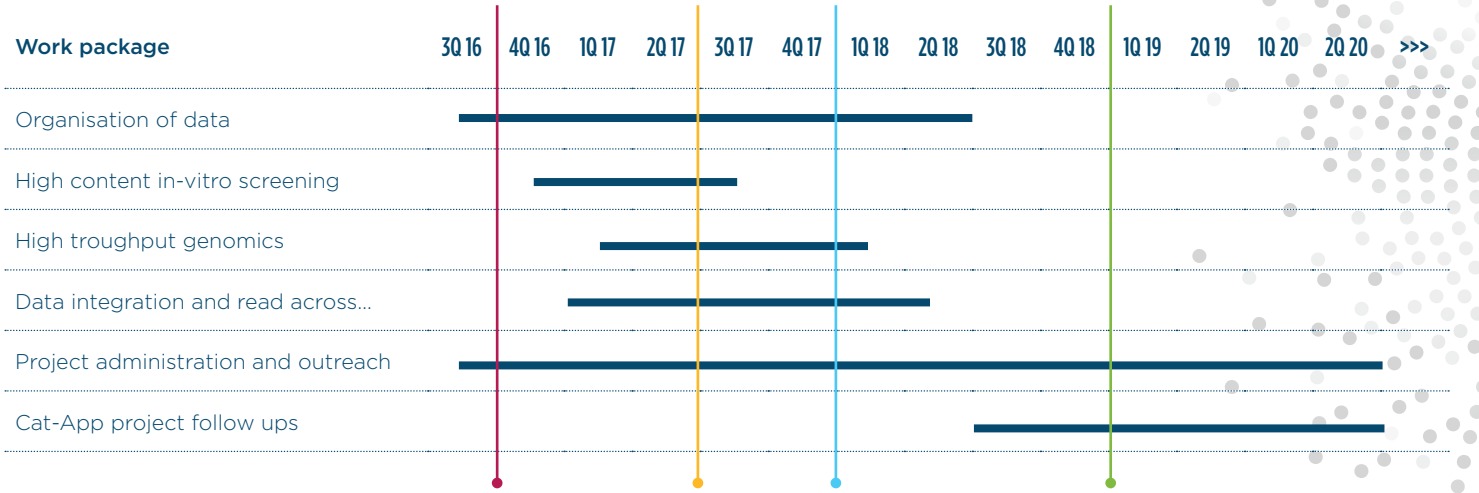
Timeline

MS1: All petroleum substances available as DMSO extracts for testing in in vitro assays

MS2: Quality control report of preliminary in vitro analyses

MS3: Initial workflow for Chemical-Biological Read-across and ToxPi visualisation available

Cat-App final project report



More to come on www.concawe.eu ...

Concawe
Boulevard du Souverain 165,
1160 Brussels, Belgium
Hans.Ketelslegers@concawe.eu