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Cat-App: New Technologies to Underpin Category Approaches and Read-across in Regulatory Programmes

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Cat-App: New Technologies to Underpin Category Approaches and Read-across in Regulatory Programmes

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1.1. PETROLEUM UVCBs

Petroleum Oil is the largest traded commodity worldwide, either as crude or through refined products (International Energy Agency, 2005). In Europe alone in 2018, around 600 million tonnes of refined petroleum products were manufactured and the refining industry collected some €281 billion of duties for EU economy and generated over €23 billion in added value to local and national EU economies (Concawe data, based on Eurotostat and EU Commission data). The range of petroleum substances (**Figure 1.1.**) manufactured from crude oil is wide, with the most common uses as transportation fuels (diesel, petrol and jet fuel). Other major uses are for energy/heat-raising applications, such as heating oil, and non-energy uses such as the manufacturing of lubricants, plastics, synthetic rubber, fibers and plant protection products. Both local and global economic activity is highly dependent on petroleum products, such that nearly every person in Europe and other developed countries is exposed to these substances through their daily routines. As a consequence, it is essential to ensure the safety of petroleum products to human and environmental health.

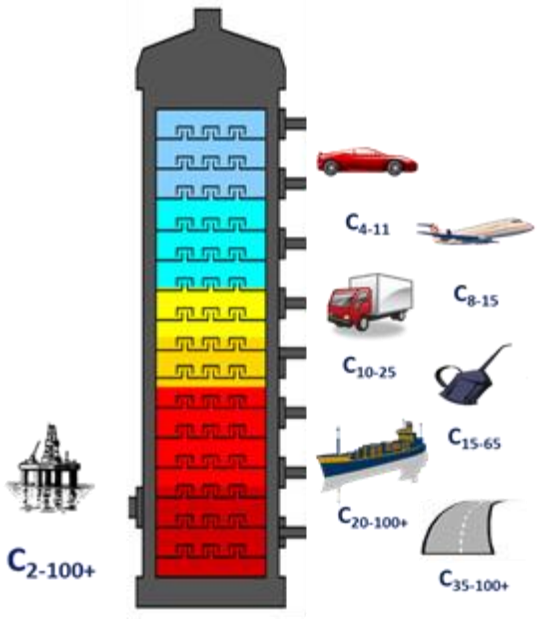

Figure 1.1. **Primary and secondary oil-derived products.** The refinery distillation splits the crude oil into a range of petroleum substances which can be grouped into substance categories, historically based on their manufacturing process and physical-chemical properties. These substances vary in chemical composition, complexity, carbon (C) content, etc. due to the physical-chemical aspects of boiling crude oil, the crude oil source and other factors. Light molecules (low C number) boil off first, and the higher the boiling temperature the heavier the product will be (higher C number) and the more complex it is in terms of number of molecules and isomers (see also **Table 1.1.**). The refinery distillation is most of the time followed by chemical treatment (Reforming, hydrotreating, cracking, …), which process one secondary product at a time and modify its chemical composition. It is also important to notice, in light of grouping and read across assessments, that there is overlap in chemical composition between "adjacent" streams, whereby the heavy end of a low boiling stream will to some extent overlap with the light end of a higher boiling stream. In this way, petroleum substances (PS) form a continuum of substances with significant overlapping chemical composition between CAS numbers within a substance category and between different categories.

Products of petroleum refining are frequently categorized as prototypical UVCB substances (Unknown or Variable composition, Complex reaction products and Biological materials). UVCB substances are one of the most challenging areas in regulatory science because there are few established frameworks for how to evaluate UVCB substances under current chemical regulatory policy and ensure that there is no underestimation of hazard (European Chemicals Agency, 2017). Indeed, the complexity of the chemical composition of petroleum substances (PS), in particular their multi-constituent nature, makes this class of substances a challenge for regulatory assessment under current chemical legislations. The number of individual chemical compounds increases rapidly with carbon number (**Table 1.1.**). The predominant compounds are described by carbon number/boiling point ranges and hydrocarbon types. Generally, carbon number/boiling point ranges are influenced by fractionation whereas hydrocarbon types (n-/i-alkanes, aromatics, olefins etc.) are influenced by chemical processing. To identify the hazards in a correct and practical way, testing is conducted on the whole substances (human health), or on hydrocarbon blocks (environment), rather than on individual constituents or groups of constituents.

Table 1.1. The complexity of the chemical composition of petroleum substances is illustrated here by the increase in number of isomers by boiling point and carbon number range. Numbers are an indication of the potential isomers and used for illustration purposes only.

C number	Boiling point °C (n-alkanes) (*)	Number of isomers (alkanes only!)
3	-42	1
4	-1	2
5	36	3
6	69	5
7	98	9
8	126	18
10	174	75
15	269	4 347
20	343	366 231
25	402	36 777 419
30	450	4 108 221 447
35	490	493 054 243 760
40	525	62 353 826 654 563

There are many UVCB substances on the market (*e.g.*, petroleum products, flavoring agents, fragrances, animal fats and derivatives, vegetable oils and derivatives, natural oils and extractives, biofuels, etc.). However, UVCB substance names and IDs are not adequately specific to permit unambiguous identification. While UVCBs, including petroleum substances, are identified on global chemical inventories with unique Chemical Abstract Services (CAS) numbers and names, they present enormous challenges when evaluating their potential toxicity due to the largely unknown and variable composition of these substances (Clark et al., 2013). For petroleum UVCB substances, the CAS description is a detailed reflection of the refining processes by which the substance is produced, i.e., the 4 CAS for Bitumen all produce a Bitumen substance meeting the specifications of that substance – hence although they are UVCB, the variation between these CAS for that particular

substance is limited to these specifications and a category approach based on manufacturing process is appropriate. Therefore, keeping the 3Rs for toxicology testing in mind (Refinement, Reduction and Replacement; Russell and Burch, 1959), it is not responsible and not needed to test every (petroleum) UVCB CAS number for every endpoint under current regulatory schemes, as this would lead to a high level of unnecessary animal testing while not adding much additional value to the eventual risk management of these substances. Hence, regulators and the industry have a common interest to define a process for (petroleum) UVCB substances to ensure that there is no underestimation of relevant potential human health hazards, while they minimize or eventually replace the use of animals in safety testing to keep regulatory compliance and most importantly to continue a safe production and use of petroleum substances.

1.2. MAMMALIAN TOXICOLOGY OF PETROLEUM SUBSTANCES

Due to the very high production volumes, all Petroleum Substances (PS) were registered in the 2010 REACH[1] deadline (all exceed 1000 tonnes and some exceed 1,000,000 tonnes). Testing proposals to fill the data gaps were submitted where there were missing required *in vivo* animal safety data. One challenge for the regulatory authority (*i.e.*, European Chemicals Agency, ECHA) is the number of registrations from manufacturers of petroleum substances. By the first quarter of 2019, there were 185 PS registered and ~4500 unique registrations. In terms of volume, PS represent ~25% of all chemicals placed on the market in the EU. Because of the large number of registrations and accompanying testing proposals that would potentially be needed to address the data gaps in these registrations, ECHA and the registrants submitting them have a common interest in assuring protection of human health and the environment while being mindful of the animal welfare.

Despite the complexity with petroleum UVCB substances as described earlier, quite a lot is already known on the mammalian toxicology of PS from a wealth of data historically generated in the industry, e.g. under earlier regulatory frameworks such as the dangerous substance directive with the European Chemicals Bureau in the EU and under the High Production Volume Program (HPV) in the USA. In general terms, lower boiling petroleum streams that do not contain polycyclic aromatic hydrocarbons (PAHs) might show lower tier toxicological effects such as mild skin irritation, and in some cases central nervous system effects at higher dose levels. On the other hand, heavier and high boiling PS (HBPS), starting from some of the gas oils and higher boiling, with the potential to contain certain PAH at higher levels have the potential to cause systemic toxicity as well as carcinogenicity and/or reproductive toxicity (Feder and Hertzberg, 2013; McKee et al., 2014; McKee and White, 2014; Roth et al., 2013). This existing toxicological data has been used to fulfil the data requirements in the Concawe REACH dossiers (see next section), and the knowledge of the composition and refining processes coupled with the hazard data forms the basis for developing grouping, read across and testing hypothesis as will be discussed later in the document.

1.3. DATA GAPS, THE NEED FOR NOVEL APPROACH METHODOLOGY DATA IN REGULATORY EVALUATION OF PETROLEUM SUBSTANCES AND CONCAWE REACH STRATEGY FOR HUMAN HEALTH

All PS were registered in the European Union for the 2010 submission deadline under REACH (≥1000 tonnes registration band), comprising more than 4500 individual registrations. A number of these submissions were accompanied by testing proposals

[1] 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

to fill data gaps in specific toxicity endpoints. To minimize the need for testing in vertebrate animals, the majority of data gaps in regulatory submissions and testing proposals of petroleum substances under REACH were addressed by using read-across to similar substances.

Read-across of PS within the REACH framework is typically done by grouping the individual substances into product categories with similar manufacturing processes, physical-chemical properties (including refining history and boiling point/carbon number ranges), and limited analytical chemical information (such as hydrocarbon classes). However, category read-across approaches for (petroleum) UVCBs that are based solely on such broad similarity parameters may not always be considered sufficient and new approaches to facilitate grouping of UVCBs are needed. Under REACH, the similarity principle is mostly build on chemical analytical data (i.e., comparing molecular structures), however, when full chemical characterization of a substance is unattainable, which is the case for most PS, biological data may provide further confidence to prove similarity for read-across and hazard assessment.

Indeed, an authoritative group of European regulators, governmental and academic scientists, concluded in 2014 that "*for substances of complex, and frequently poorly characterized, chemical composition [such as petroleum substances and other UVCB], approaching read across from the perspective of a biological similarity signature would avoid the inherent challenge of using chemical similarity as the main basis of read-across when there is uncertainty and variability in chemical composition*" (Berggren et al., 2015). Thus, expanding the regulatory principle of "read-across" from physical-chemical properties to also include biological information could serve as a much stronger basis for regulatory decisions, both in terms of addressing uncertainties and increasing confidence and transparency.

A proof of concept of the advantages of integrating chemical structure, physical properties and biological data (*in-vivo, in vitro* and *in-silico*), has been presented in a series of publications that laid the ground for this so-called "chemical-biological read-across" approach (Low et al., 2013; Low et al., 2011; Low et al., 2014; Rusyn and Greene, 2018; Rusyn et al., 2012). This approach has been recently endorsed in the US National Academies of Science reports "*Guide on selection of chemical alternatives*" (National Research Council, 2014) and "*Using 21st Century Science to Improve Risk-Related Evaluations*" (National Academies of Sciences Engineering and Medicine, 2017).

It was hypothesized that these principles could help to address the challenges around grouping UVCBs, as will be elaborated on in the next section.

1.3.1. Concawe REACH strategy for human health: grouping, read across hypotheses and Cat-App

If complex substances such as petroleum UVCBs cannot be characterized fully, can biological (mechanistic) information help in describing similarity between substances in order to support grouping of similar substances?

This hypothesis served as a basis for Concawe to initiate the Cat-App[2] project, which aimed to develop a framework based on this chemical-biological grouping by taking advantage of recent innovations in (i) *in vitro* testing, (ii) high-throughput genomics

[2] Cat-App: New Technologies to Underpin **Cat**egory **App**roaches and Read-across in Regulatory Programs

and (iii) integrative data analyses and visualisation into a transparent workflow, for read-across assessment of (complex) UVCB substances in regulatory programmes.

As described above, with the initial REACH registrations in 2010, Concawe utilized the existing toxicological and any relevant data in order to address the required endpoints for human health in the registration dossiers. Data was not available on all registered substances for all endpoints. A data gap analysis was done by Concawe in which no data was available and read-across could not be applied; this identified the need for further experimental data on the reprotoxicity endpoint for some petroleum substance categories. In addition, further data gaps might now be created because both certain read-across assessments as well as some historical data are currently being challenged and might not be accepted by the regulators. As there is a risk that this leads to a large increase in (unnecessary) animal testing, the Cat-App framework was applied to all PS to mitigate this risk by generating data to facilitate chemical-biological read-across which could then inform a new data-gap assessment. Where true gaps exist, i.e., no relevant data are available and read-across cannot be applied, targeted testing would be proposed and performed when the testing strategy is considered relevant –in terms of added value to the risk management process- and accepted by the regulators. (**Figure 1.2.**, see also www.concawe.eu/reach for the Concawe REACH roadmap).

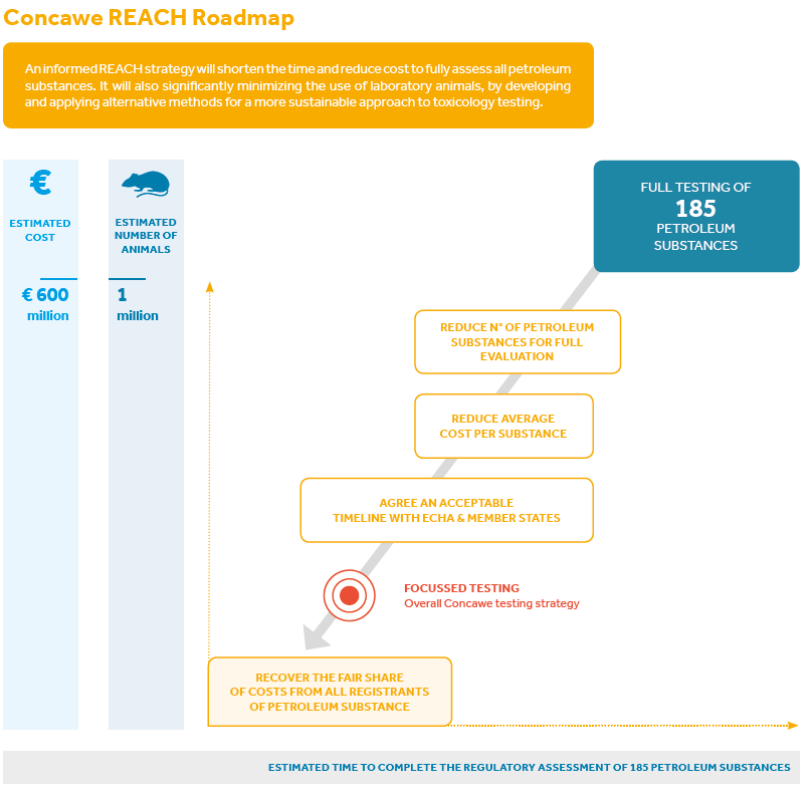

Figure 1.2. **Overview of Concawe REACH roadmap.** An informed testing strategy, for which Cat-App will serve as a basis, will increase the overall efficiency and effectiveness of the toxicological assessments to be conducted under REACH and reduce the overall time to complete the regulatory assessment of the entire PS portfolio. Furthermore, the data generated in this project will increase the understanding on chemical-biological interactions of PS and thereby inform their risk assessment. This Figure is taken from the Concawe REACH roadmap for petroleum substances brochure, which can be found at www.Concawe.eu/REACH

This targeted testing will be performed with the substance predicted to be the most hazardous based on a testing hypothesis for the relevant endpoint. Currently, regulatory focus is on the higher tier toxicological endpoints. For these more complex toxicological endpoints (repeated dose testing, genetic toxicity, carcinogenicity and reprotoxicity), it can be hypothesized based on extensive historical in-vivo data that the observed effects caused by exposure to PS are associated with the level of 3-7 ring PAC[3] in these substances. Based on this read-across hypothesis, a substance with the highest "toxic potential" can be selected for the specific endpoint, and the data generated on this sample will then be conservatively applied to the entire category. Cat-App is expected to provide the basis for the grouping approach that is a prerequisite to this form of read-across and underpin the read-across assessments by providing further biological evidence for the testing hypothesis. The read-across applied here is slightly different from "classical" read-across approaches as these are not applicable to UVCB substances, and might be more similar[4] to the "bridging principle" as referred to in the classification and labelling regulation[5]. This is because the different CAS numbers (see chapter 1.1) are describing slightly different ways of producing a similar substance: for example, all 4 CAS numbers in the Bitumen category contain a wide range of hydrocarbons but are technically the same substance, i.e. Bitumen. Since they have to meet the technical specifications for that substance to be applied and used that way, the variation in chemical composition between these CAS numbers in a specific substance category is limited. In addition, because the physical chemistry of petroleum refining leads to a continuum of substances, there will be significant overlap between different substance categories: the heavy end of a lower boiling refining stream will overlap with the light end of the neighbouring higher boiling refining stream. These are concepts which are of critical importance for a better interpretation of chemical-biological grouping and read-across of PS, and these insights help to facilitate an adapted read-across framework specific to UVCB substances by applying something similar to the bridging principle mentioned earlier.

[3] PAC: polycyclic aromatic compounds. These are polycyclic aromatic hydrocarbons which may include heterocyclics and alkylated molecules. Analytically these are measured by the PAC method 2 (in short PAC2). More information can be found here: https://www.petroleumhpv.org/polycyclic-aromatic-compounds

[4] Similar but not the same, as PS are substances and not mixtures as referred to under CLP

[5] ECHA 2017: Guidance on labelling and packaging in accordance with Regulation (EC) No 1272/2008

2. CAT-APP PROGRAMME OVERVIEW

The Cat-App project was aimed at providing the registrants with an integrative approach to solving the similarity challenges of grouping UVCB substances as presented above. The ***overall objective*** of the project was to develop a framework for category read-across for petroleum UVCB substances, but which should eventually be more widely applicable to other complex (UVCB) substances, using experimental, computational, integrative analysis, and outcome communication elements to meet the regulatory requirements under REACH legislation. It should be noted that the objective of the project is not to provide specific hazard information on the substances that could be used for regulatory purposes. In other words, the intention of Cat-App is not to replace in-vivo testing under current regulations but rather apply the data in order to increase animal testing programs efficiency and effectiveness and thereby facilitate a more informed approach to meet regulatory requirements.

Recent advances in toxicological knowledge and methods enable grouping and read-across between chemical substances for use in research and regulatory domains. The research and development needs for combined prediction approaches that add value by building on and integrating various methodologies into "integrated testing strategies" have been the subject of active debate (Jaworska and Hoffmann, 2010; Piersma et al., 2018). The need for scientifically-valid, generally-accepted, and fit-for-purpose techniques to produce information necessary for a regulatory decision in a standardized but flexible way is currently being met by developing integrated testing strategies for mammalian (Landesmann et al., 2013; Urbisch et al., 2015) and ecological (Lombardo et al., 2014) toxicity. There is a wide range of information that is to be integrated in assessments such as traditional toxicology studies, high throughput *in vitro* tests and quantitative structure activity relationships (QSARs). A number of solutions for integrating such evidence to improve confidence in category groupings has been proposed, especially as it relates to read-across (Rusyn and Greene, 2018). To support such combined approaches, further fundamental work is necessary and the Cat-App programme's research builds on and incorporates current methodologies into cases for "chemical-biological categories" using UVCB substances.

Indeed, the Cat-App programme's research used the latest *in vitro* models to address potential biological targets in humans and mammalian organisms. As explained in the previous chapter, this project was initiated with the aim to generate New Approach Methodologies (NAM) data with a direct application in the Concawe human health strategy under REACH – initially to underpin grouping and read-across approaches. It is expected that such application of NAM data should help to increase the comfort level to progress their regulatory acceptance, which are eventually expected to lead to more targeted and direct alternatives to animal data for rapid batch screening of PS after further development and validation.

2.1. BIOACTIVITY PROFILING AS A NOVEL APPROACH METHODOLOGY FOR GROUPING COMPLEX PETROLEUM SUBSTANCES

Several recent published studies demonstrated the principle of using novel data streams from *in vitro* bioactivity profiling (Grimm et al., 2016) and high-resolution mass spectrometry (Grimm et al., 2017) for grouping complex petroleum UVCBs. Similar approaches have been applied to other categories of UVCBs (Catlin et al., 2018). Collectively, these studies advance the use of novel assessment methods to establish "sufficient similarity" for UVCBs. One of the main challenges facing the petroleum industry with regards to in vitro testing, is how to get lipophilic

substances into an aqueous environment. To overcome this challenge, it was decided to utilize Dimethyl Sulfoxide (DMSO) extracts of petroleum substances. This bioavailability issue was addressed before in the development of screening assays which are applied in the petroleum industry such as the regulatory accepted IP346 and the modified Ames assay (both assays have been recently reviewed by Concawe, and more information can be found at www.concawe.eu). The DMSO extraction procedure used herein (see also **Figure 3.1.**) is designed to concentrate the 'biologically active' fraction, i.e., [3-7 ring] aromatics, of the refinery streams. However, more constituents (i.e., all polar molecules) are extracted which explains that certain refining streams with low to no (3-7 ring) PAH content still have (low levels of) extractable materials; the extracts obtained using this method are used routinely for safety testing (e.g., mutagenicity) and chemical characterization of respective refinery streams. In the remainder of the document, the term **"PS-E"** is used to identify the **Petroleum Substance-DMSO Extracts** when referred to the experimental setting of in vitro testing.

A case study of a comprehensive experimental and computational approach to categorize petroleum UVCBs according to global similarities in their bioactivity, using a suite of *in vitro* models, was presented by Grimm et al. (2016). The study served as a pilot project to Cat-App with the overall goal to determine whether physical-chemical properties and bioactivity from *in vitro* experiments can be used for grouping petroleum UVCBs and assess how such groups correspond to the groupings based on the manufacturing process. Details are described in the peer reviewed paper, in brief: human induced pluripotent stem cell (iPSC)-derived hepatocytes and cardiomyocytes were exposed to DMSO-soluble extracts (as described above) of 21 petroleum substances from five product categories. Concentration-response data from high-content imaging in these cells and physical-chemical properties of the substances (**Figure 2.1.**), as well as targeted high-throughput transcriptomic analysis of the hepatocytes, revealed distinct clusters of petroleum substances. It was found that bioactivity profiling resulted in clustering of petroleum substances in a manner similar to the manufacturing process-based categories. This study demonstrated how novel *in vitro* screening approaches can be effectively utilized in combination with physical-chemical characteristics to group complex substances and enable read-across, which allows for more rapid and scientifically-informed evaluation of their potential health impacts.

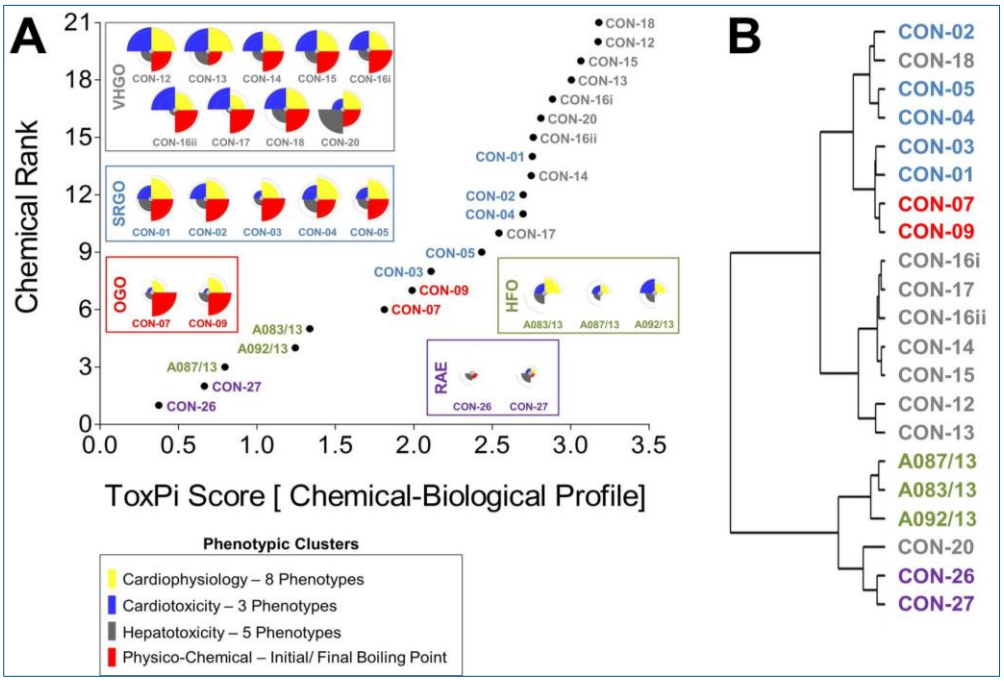

Figure 2.1. **Chemical-biological data-integrative categorization of petroleum UVCBs.** Integration of phys-chem and bioactivity data was performed using ToxPi (A). Four data types comprised 8 cardiophysiology, 3 cardiotoxicity and 5 hepatotoxicity parameters, and 2 phys-chem descriptors (initial and final boiling points). Cluster analysis is shown in panel (B). [Sample coding is explained in Grimm et al. (2016). VHGO = vacuum & hydrotreated gas oils; SRGO = straight run gas oils; OGO = other gas oils; HFO = heavy fuel oils; RAE = residual aromatic extracts]. Image from Grimm et al. (2016).

2.2. CAT-APP GOALS, STRATEGY, APPROACH AND WORKFLOW

The Cat-App project was initiated and funded by Concawe in 2015, with the aim to achieve three goals:

(1) Minimize the need for testing in vertebrate animals under regulatory programmes. One of the aims of REACH is to promote alternative methods to hazard assessment in order to reduce the number of animal testing[6]. REACH provides a number of options for this, including grouping of substances in order to facilitate read-across of data between them – optimizing the use of available toxicology data and of new animal testing needed to address data gaps. The available REACH guidance on this works for relatively simple, mainly mono-constituent substances but is not fit for purpose for complex multi-constituent substances including UVCBs, which is also concluded by ECHA (European Chemicals Agency, 2017). **Cat-App's overall aim** is therefore to develop a framework for grouping UVCB substances that should make this feasible, based on chemical-biological properties combining multiple streams of information comprising petroleum UVCBs substance production type/refining process, physical-chemical properties, chemical analytical profiles, existing (eco-)toxicological data and a comprehensive array of biological responses in a broad spectrum of in vitro systems.

[6] See e.g., article 1, article 13 and article 117(3) of Regulation (EC) No 1907/2006

(2) Practical application of the Cat-App framework in the Concawe REACH strategy as supporting data in the petroleum REACH dossiers, in order to facilitate the completion of data gaps in the endpoint requirements with the already existing *in vivo* toxicological data on petroleum substances.

(3) Inform a broad spectrum of stakeholders globally (commercial entities, regulatory agencies and non-governmental organizations) concerned with public health and wellbeing, to promote (regulatory) use of the Cat-App framework which is expected to eventually have a wide and diverse applicability domain.

2.2.1. Participating organizations

The Cat-App team consisted of the following participating organizations:

Concawe (Brussels, Belgium) 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 REACH product stewardship. The Concawe team consisted of a number toxicologists of member companies. Cat-App project management was led by Hans Ketelslegers, PhD, ERT.

Texas A&M University (College Station, TX, USA) is one of the largest research universities that is ranked in the top 25 public universities in the USA and the top 10 public engineering schools. Research areas at Texas A&M University encompass a wide range of interdisciplinary research resources that include centres, institutes and laboratories dedicated to addressing a wide variety of scientific challenges and opportunities. Texas A&M University's research team was led by Professor Ivan Rusyn MD, PhD who coordinated activities in work packages 1, 2 and 3. His laboratory and staff are well experienced with complex databases and molecular toxicology research. He also worked closely with other principal scientists in this project and has been engaged with the European Commission's Joint Research Centre, European Chemicals Agency, US EPA, California EPA, Texas Commission on Environmental Quality and other regulators.

Public Health England (Didcot, Oxfordshire, United Kingdom) is an executive agency of the UK Department of Health with responsibility for all aspects of public health in England. The Centre for Radiation, Chemical & Environmental Hazards (CRCE) has expertise in toxicology (applied and basic mechanistic understanding) exposomics, transcriptomics, biomathematics, biostatistics and bioinformatics. This expertise is applied in emergency responses, chemical advice, risk assessment, support for expert government committees and peer reviewed research. The leading investigator was Professor Tim Gant, PhD, FBTS, ERT who has extensive experience in transcriptomics and bioinformatics for chemical hazard profiling and mechanistic understanding evidenced by the publication record. He worked in academia and government and has significant experience in the application of toxicology to advance public health.

North Carolina State University (Raleigh, NC, USA) is one of the two flagship research institutions of the University of North Carolina system, with 9 major colleges and schools and over 31,000 students. The University is widely renowned as a trail-blazing national model for research in the public interest and for university-private-government interactions. Of particular relevance are partnerships with the National Institute of Environmental Health Sciences (NIEHS), and the US Environmental Protection Agency (EPA), fostering a dynamic atmosphere

for toxicological research and data from high-throughput screening. The Bioinformatics Research Center (BRC) at NCSU, is an interdisciplinary center devoted to research at the interfaces of quantitative and biological sciences, with strengths in statistical methods applied to toxicological problems. The principal investigator is Professor Fred Wright, PhD who is director of the BRC and a lead investigator on work package 4. Professor Wright is a statistical geneticist with over a decade of experience working with toxicogenomic data.

University of Ulster (Northern Ireland) has an international reputation for excellence and innovation. Its research is characterised by its capacity to shape the future of lives and society through relevant and pioneering research, delivering a range of economic, social or cultural benefit from research of the highest quality. The Northern Ireland Centre for Stratified Medicine (NICSM) at the University of Ulster is a partnership between the Biomedical Science Research Institute, the Clinical Translational Research and Innovation Centre and the Western Health and Social Care Trust. Shu-Dong Zhang, PhD, is a Senior Lecturer in Bioinformatics at NICSM whose research interest include the development of computational, statistical, and bioinformatics methods and their applications in biomedical, pharmacological and toxicological studies. As an expert in gene expression connectivity mapping, Dr. Zhang and his team were responsible for carrying out connectivity mapping related parts of work package 4.

SYNCOM (Ganderkesee, Germany) is a consulting firm with the focus on innovation management. Physicists, Chemists, Engineers and Business managers apply management expertise and know-how on implementation of administrative processes to research and development projects. SYNCOM contributed management and administrative support to the consortium. Additionally, it was responsible for the organisation and steering of the dissemination activities. Klaus Lenz, PhD was the lead of work package 5 and he has been working in the management and administration of industrial R&D projects since 1988.

2.2.2. Cat-App Concept and Approach

The overall ***concept*** of Cat-App was to redefine the regulatory use of a similarity principle through development of the framework based on chemical-biological read-across (Low et al., 2013). Specifically, our ***approach*** was to integrate innovations in (i) *in vitro* testing, (ii) high-throughput genomics, and (iii) integrative data analyses and visualisations into a framework for category read-across of UVCBs. Given its setting and scale, the Cat-App programme has the potential to offer a practical solution to one of the challenging issues in chemical regulation in Europe. The overall programme consisted of five work packages (**Figure 2.2.**). Participating organisation's and their roles in the work programme are also shown in this Figure.

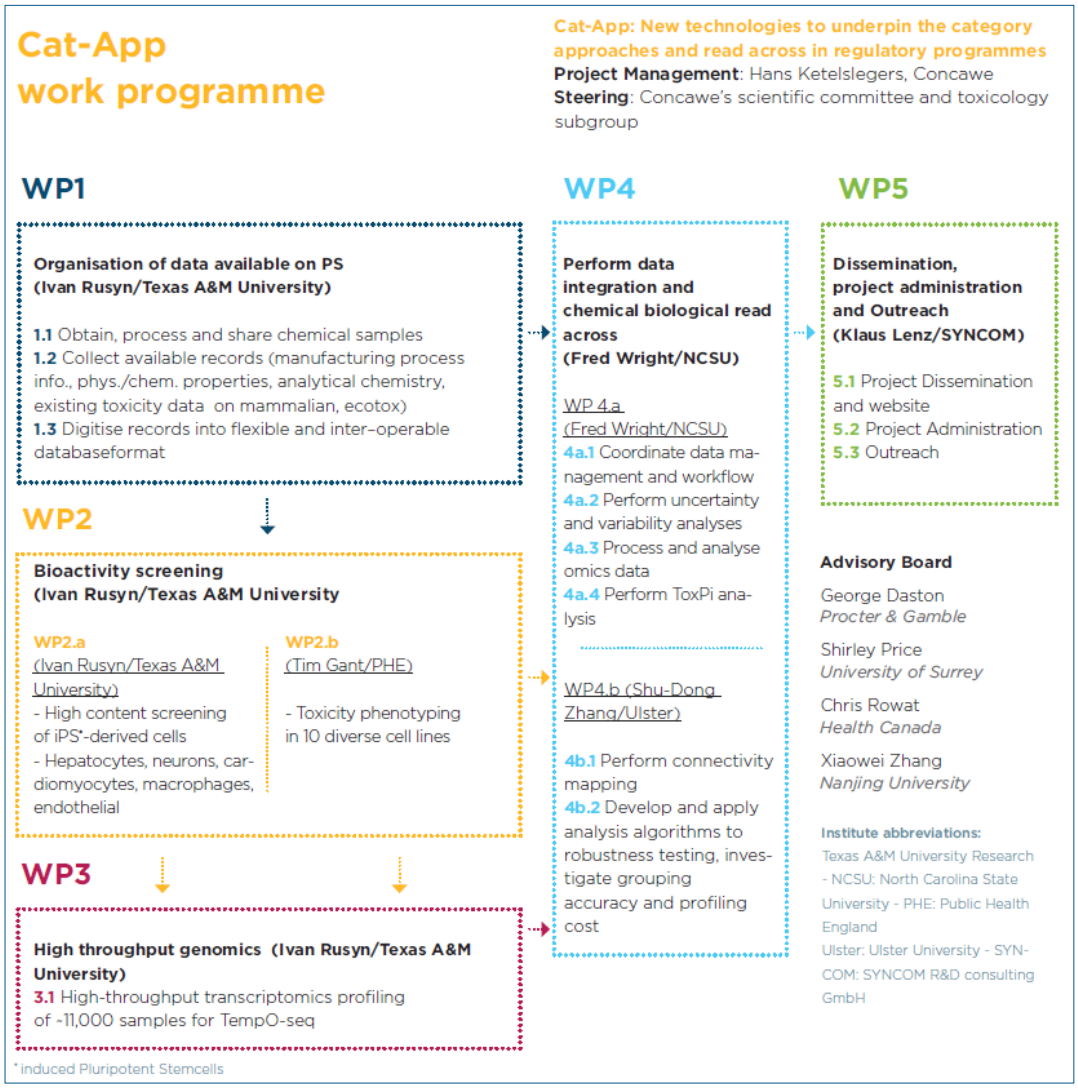

Figure 2.2. Overview of the Cat-App project.

3. BIOACTIVITY PROFILING OF PETROLEUM SUBSTANCES IN HUMAN IN VITRO MODELS

3.1. PREPARING EXTRACTS OF PETROLEUM SUBSTANCES

To facilitate *in vitro* bioactivity profiling experiments, researchers at Texas A&M University obtained, processed and shared with partner Public Health England (PHE) chemical samples (petroleum substance extracts and reference chemicals). First, Concawe coordinated procurement and delivery of all petroleum substances to be tested in Cat-App to Port Royal Research (Beaufort, SC, USA) who performed extraction of petroleum substances into DMSO using American Society for Testing and Materials standard procedure (ASTM International, 2010, E1687-10). The DMSO extraction procedure used herein was designed to concentrate the 'biologically active' fraction (i.e., mostly 3-7 ring polycyclic aromatics, but also other polar constituents) of the refinery streams. The extracts (PS-E, see chapter 2.1) obtained using this method are used routinely for safety testing (e.g., mutagenicity) and chemical characterization of the refinery streams (ASTM International, 2010). The extraction protocol overview is shown in **Figure 3.1.**

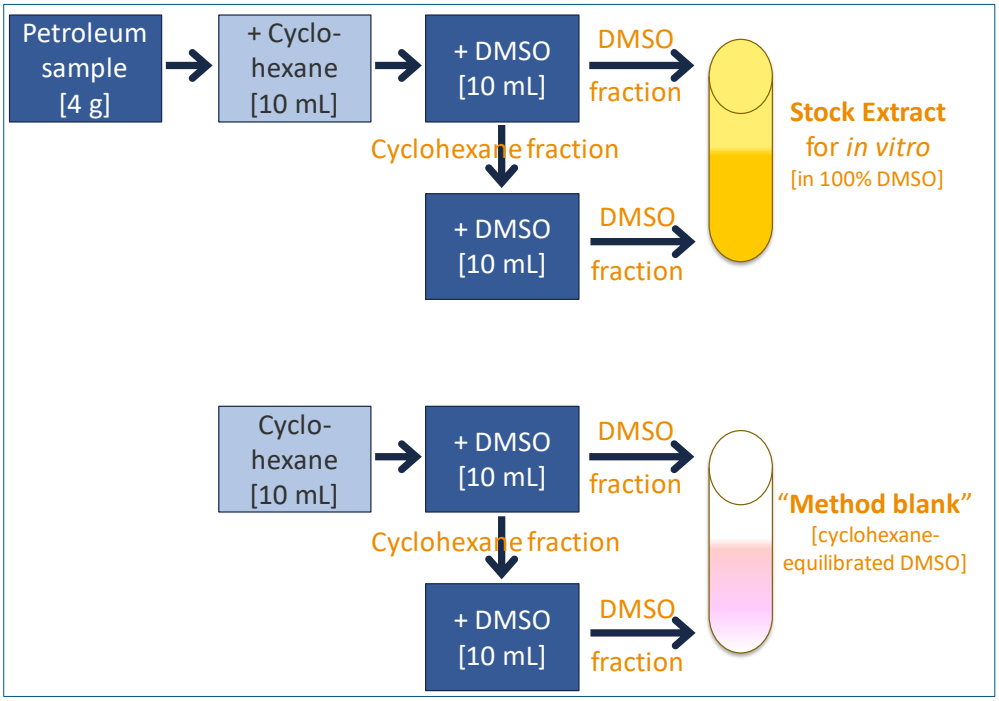

Figure 3.1. **Petroleum substance extraction procedure based on ASTM International (2010) standard method (E1687-10).**

Overall, 141 petroleum substances (which represent the entire continuum of active petroleum substances under REACH) from diverse manufacturing stream categories were used in Cat-App programme. For statistical visualisation purposes, some categories were merged together which leads to the 16 Cat-App specific PS-E categories as shown in **Table 3.1.**

Table 3.1. **Cat-App specific Petroleum substance categories used in this project.** In some cases, closely related substances (PS-E) from different Concawe categories were grouped together solely for statistical and display purposes: HRBO was combined into OLBO (BO), slack waxes and paraffinic waxes were combined (WAX), Bitumens were combined with the single substance Oxidized Asphalt (BIT) and a single MK1 was grouped with Kerosine (KER).

Category	Abbreviation (*)	N of samples in category
Petrolatums	P.LAT	3
Paraffin and Hydrocarbon Waxes/Slack Waxes	WAX (2)	10
Low boiling Point Naphthas (Gasolines)	NAPHTHA	10
Other Lubricant Base Oils/Highly Refined Base Oils	BO (2)	33
Kerosines/MK1 Diesel Fuel	KER (2)	10
Foots Oils	FO	3
Other Gas Oils	OGO	4
Bitumens/Oxidized Asphalt	BIT (2)	5
Residual Aromatic Extracts	RAE	2
Treated Distillate Aromatic Extracts	TDAE	2
Heavy Fuel Oil Components	HFO	27
Unrefined/Acid Treated Oils	UATO	4
Cracked Gas Oils	CGO	8
Vacuum Gas Oils, Hydrocracked Gas Oils & Distillate Fuels	VHGO	10
Straight-Run Gas Oils	SRGO	6
Untreated Distillate Aromatic Extracts	UDAE	4

* The number in brackets represents the number of Concawe categories that were analyzed together in Cat-App, which in total makes 20 categories

3.2. *IN VITRO* BIOACTIVITY SCREENING STUDY DESIGN

Overall, the Cat-App programme used 141 petroleum substance extracts (see Section 3.1) and 20 reference chemicals (see Section 4) that represented the major structural classes of chemistries in petroleum substances: monoaromatics, diaromatics, triaromatics, n-paraffins and, 4-ring aromatics. Reference chemicals were obtained from commercial suppliers and processed to create a dilution series in "Method Blank" (see **Figure 3.1.**, cyclohexane-equilibrated dimethyl sulfoxide). Overall, 4 serial log dilutions of each extract and reference substance were created, aliquoted into 384-well master plates (**Figure 3.2a**) and sealed with aluminium film until use. In addition to the test substances, plates contained a number of quality controls (**Figure 3.2b-e**). Plates were stored at -20°C before use and identical master plates were used at both Texas A&M University and Public Health England.

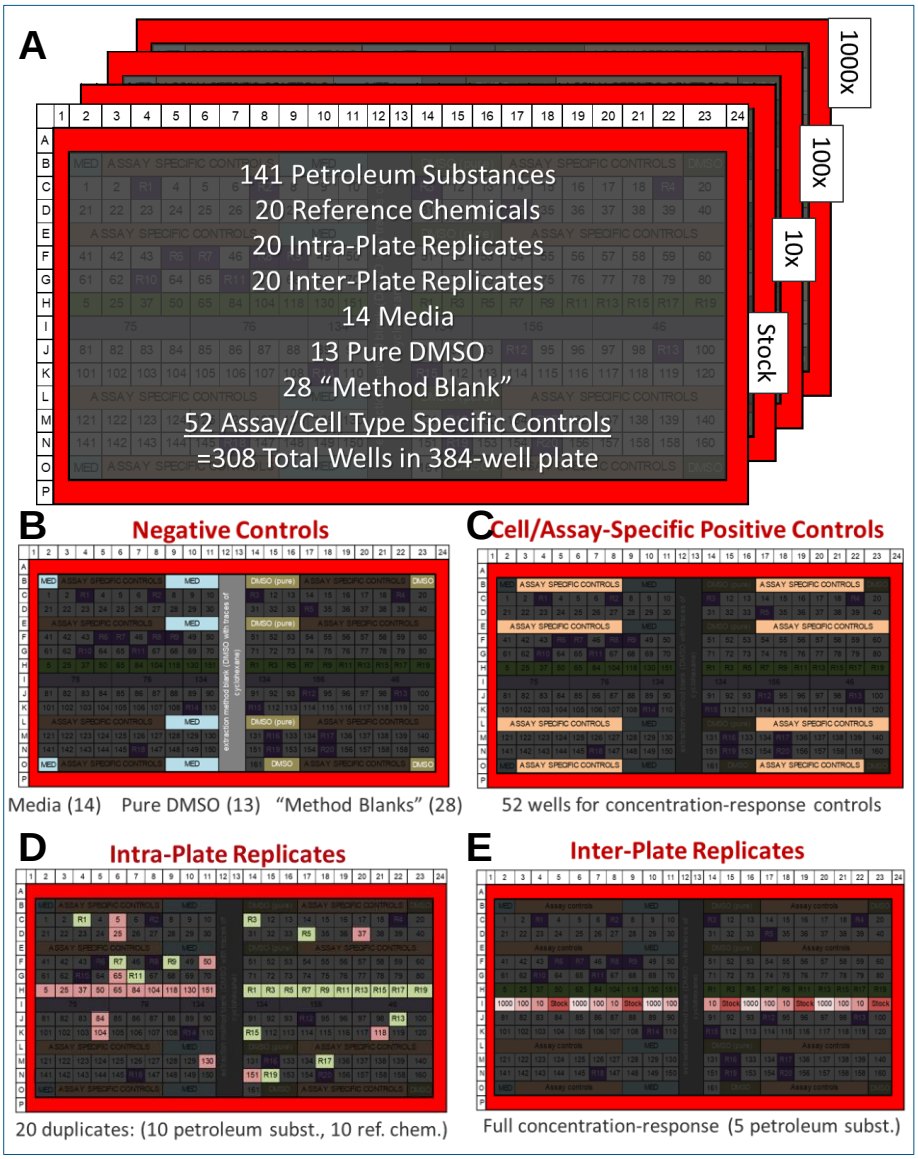

Figure 3.2. **Experimental plate design for Cat-App.**
(A) Overall design of the plate with 308 wells in a 384-well plate utilized for various test substances and controls.
(B-E) Location of various negative, positive, and reproducibility controls is indicated.

3.3. CELL LINE SELECTION

In work package 2, experiments were performed to evaluate bioactivity of 141 PS-E and 20 reference chemicals using five human induced pluripotent stem cell (iPSC)-types (hepatocytes, endothelial cells, neurons, macrophages, and cardiomyocytes), two primary human cell lines (HUVEC and HLMVEC), and eight established (MCF7, A549, HepG2, HepaRG, LN229, HT29, 5H-SY5Y, and A357) human cell lines (**Figure 3.3.**).

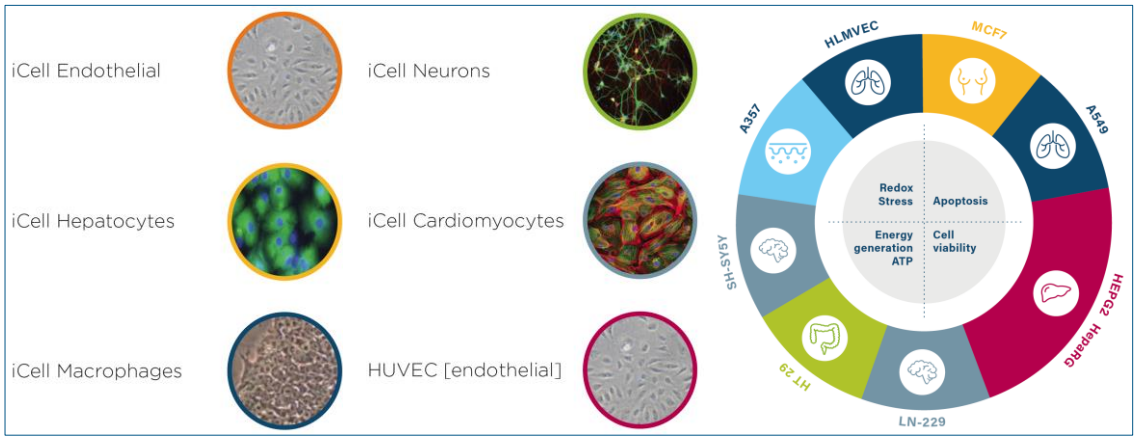

Figure 3.3. **Human cell types used for Cat-App Programme bioactivity profiling.**

Cell type and vendor selections were based on the following considerations. Cells had to be of human origin and represent diverse organs/tissues. We used both "primary" cells, iPSC-derived cells, as well as a number of established cell lines. These *in vitro* models had to be reproducible (i.e., a particular cell/donor can be obtained from a commercial source) and suitable for evaluation of both "functional" and "cytotoxicity" endpoints so that we could assess the specificity of the effects of test compounds. It was considered more important to have a strong screening assay which delivers consistent and reproducible responses regardless of its toxicological functionality rather than having a model with a strong biological relevance, as the aim is to support grouping of substances based on a consistent response and not to predict the toxicological effects of a substance.

Cells were plated in 384-well plates in densities recommended by the supplier, using optimized media supplied by the same company or optimised for density by experimentation for each cell line (House, J.S., et al., 2020). Cells were cultured without treatment for a period of time required to achieve functional capacity. Each cell line was then exposed to vehicle and at least four concentrations (1:10 dilutions) of the DMSO extracts of 141 petroleum substances and 20 reference substances for up to 24 hours. Appropriate negative and positive controls, as well as selected duplicates were included on each plate to assure reproducibility of the results. Multiple plates were seeded and one plate contained all compounds in one concentration. For each concentration, multiple plates were screened. To ensure plate-to-plate reproducibility, select substances were plated in full concentration-response on each plate. Additional plates were included for collecting cell lysates for high-throughput transcriptomic analysis.

3.4. PHENOTYPIC ASSAYS

Bioactivity profiling was conducted using a number of imaging-based and molecular/biochemical assays (**Table 3.2.**).

Table 3.2. **The number of endpoints assayed for each of the cell lines used in Cat-App**

Organ/Tissue	Origin	Cell type name	Number of phenotypes
Skin	Malignant melanoma	A375	3
Lung	Epithelial carcinoma	A549	3
Liver	Cholangiosarcoma	HEPARG	3
Liver	Hepatocellular carcinoma	HEPG2	3
Lung	Microvascular endothelial cells	HLMVEC	4
Gut	Colorectal adenocarcinoma	HT29	4
Brain	Glioblastoma	LN229	4
Breast	Epithelial adenocarcinoma	MCF7	3
Bone marrow	Neuroblastoma	SH-SY5Y	4
Heart	iPSC-derived cardiomyocytes	CM	14
Liver	iPSC-derived hepatocytes	HEP	6
Blood vessel	iPSC-derived endothelial cells	ENDO	9
Blood vessel	Umbilical cord endothelial cells	HUVEC	6
Brain	iPSC-derived neuronal cells	NEUR	4
Blood	iPSC-derived macrophages	MACRO	1
			71

For example, following exposure to PS-E and reference compounds, cell type-specific molecular high-content imaging data such as Ca2+ fluxes, cytotoxicity, mitochondria potential, nuclear morphology, intracellular oxidant production, lipid metabolism changes, formation of vessel or neuronal networks, etc. were collected using appropriate molecular biology reagents and fluorescent dyes, where appropriate.

Assay development and description of many endpoints are included in a number of peer-reviewed publications for several of the cell types used:

- iCell hepatocytes (Grimm et al., 2015; Sirenko et al., 2014a),
- iCell neurons (Sirenko et al., 2014b),
- iCell cardiomyocytes (Sirenko et al., 2013a; Sirenko et al., 2013b; Sirenko et al., 2017),
- iCell endothelial and HUVEC cells (Iwata et al., 2017).

In human established cell lines, cell type-specific high-content data for apoptosis, reactive oxygen species, cell viability and mitochondrial activity was collected using luminescent probe-based assays.

3.5. QUALITY CONTROL METHODOLOGY AND OUTCOMES OF QUALITY ASSESSMENT OF THE BIOACTIVITY DATA

Quality control and uncertainty analysis parts of Cat-App programme utilized various control features for bioactivity assays to evaluate the data and to flag assay and cell line combinations with potentially high signal to noise ratios. First, during data collection phase, several upstream quality control procedures using positive controls (**Figure 3.2c**) were implemented in order to determine that the cells were responding according to expectations in the published assays (see Section 3.4). Second, additional analyses to perform the overall quality control for bioactivity profiling data were based on three criteria: (i) concordance of three types of negative controls (media, pure DMSO, and "method blank" vehicle), (ii) inter-plate replicates, and (iii) intraplate replicates. Only the remaining assays were subject to the QC procedures described here. The quality control procedures were implemented as "flags" for each assay in each cell line so that downstream analyses could be compared in which flagged assays were either included or not included.

There were a number of negative (method) controls (**Figure 3.2b**) that were used as the primary normalization reference within each plate. To assess potential problems with each plate and with unexpected differential behaviour of controls, each negative control well (DMSO or Media) for each assay was first normalized to the method control mean. Excessive variation within or across plates would be potential evidence of undesirable plate effects or differential effects of DMSO and media. Specific details on the quality control parameters are provided in Work Package 4 report.

With respect to intra-plate replicates, a total of 20 compounds were present in duplicate on each plate, across a range of concentrations (**Figure 3.2d**). Intra-plate replication in each experiment was assessed after a variance stabilization was applied.

With respect to inter-plate replicates, the plate design placed almost all substances at a single dilution per plate. However, 5 substances were placed at a full dilution series for each plate (**Figure 3.2e**), providing an additional source of information on replicability. The QC flag procedure examined whether for each of the dilution-series 'substances normalized values across the plates' vs. 'the dose response values across the separate plates' for the same substance were similar or showed variance.

As a summary of the bioactivity quality control results, we show in **Figure 3.4.** the cell lines and flag outcome across the 15 cell lines. Overall, data from 13 of the 15 cell lines used in the experiments was deemed of acceptable quality for further analyses. Data from 43 assays was used in further data analyses.

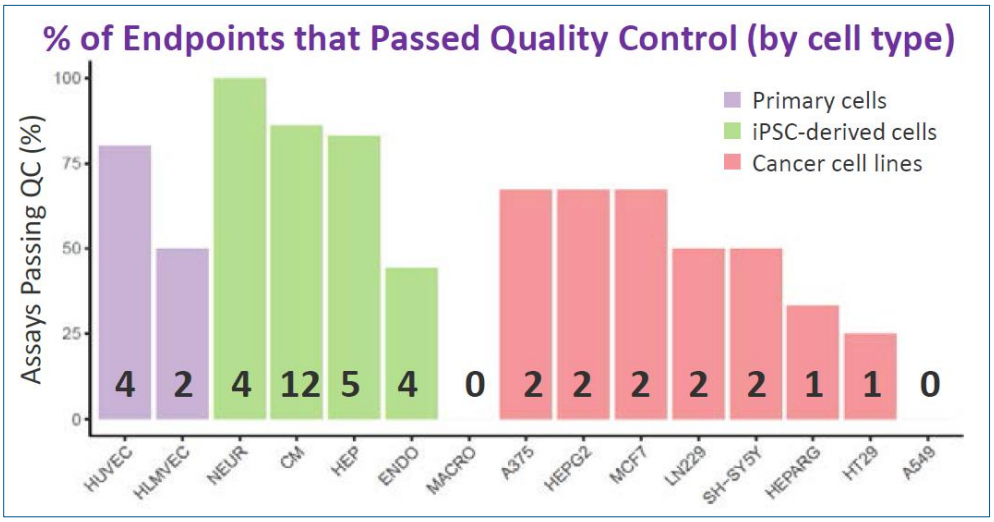

Figure 3.4. **Cell type and assay quality control analysis outcomes.** The bar graph shows the percentage of endpoints passing quality control for each cell type used. The number of endpoints passing QC is shown for each cell type. Abbreviations: NEUR, neurons; CM, cardiomyocytes; HEP, hepatocytes; MACRO, macrophages. Cell types are coloured by their origin as shown in the inset.

3.6. SUMMARY OF THE FINDINGS FROM BIOACTIVITY PROFILING

As a summary across the 43 bioactivity assays/cell lines that passed quality control flags, the ToxPi index was computed (Marvel et al., 2018). Briefly, ToxPi is an analysis and visualization tool that first linearly rescales each assay to the [0,1] interval (0-min, 1=max), and then the user chooses assays to belong to groupings known as "slices." This index was also used in later analyses for comparison with Concawe categories, but here ToxPi was used first as a summary index across all assays. **Figures 3.5a-b** illustrate how assays were grouped into one slice per cell line for overall summaries, with weighting in proportion to the number of assays per cell line. In addition (**Figures 3.5c-d)**, individual cell lines were examined, and assays were grouped into slices according to assay type. This analysis shows that median bioactivity across all cell lines shows a clear gradient among Concawe categories. The PS-E extracts of Unrefined/Acid Treated Oils (UATO) and Untreated Distillate Aromatic Extracts (UDAE) exhibited high degrees of bioactivity, while the PS-E for Waxes (WAX) and petrolatums (P.LAT) but also Base Oils (BO), for example, have low levels. When data were examined for each cell type separately (**Figure 3.5c-d**), additional patterns were discernible. For example, the hepatocytes showed separation into two broad bioactivity regions, whereas the cardiomyocytes showed a gradient of bioactivity among the categories in the bottom half of bioactivity. It is clear that while a gradient of bioactivity exists between the Cat-App categories, there is also appreciable degree of variability in bioactivity within each category. This can be due in part to the combination of Concawe manufacturing categories into Cat-App categories, but also the designation of these substances as UVCB (see also the last paragraph of section 1.3.1), which leads to significant overlap between categories that are not always very similar from a refining perspective (for example, the observed overlap between Naphthas and Base Oils). However, this is also evidence that the use of marker substances to predict bioactivity as an indicator of toxicity is not appropriate.

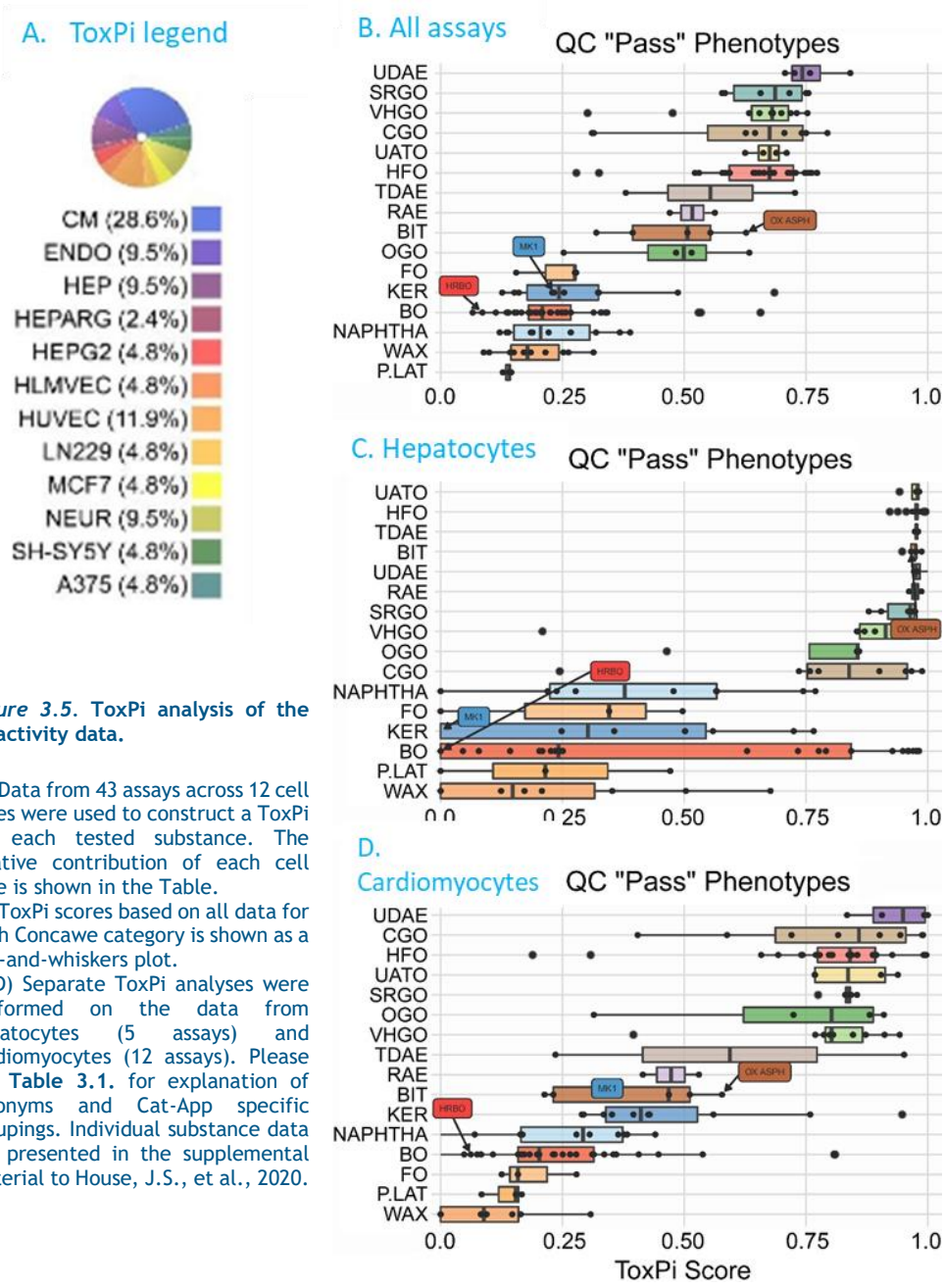

***Figure 3.5*. ToxPi analysis of the bioactivity data.**

(A) Data from 43 assays across 12 cell types were used to construct a ToxPi for each tested substance. The relative contribution of each cell type is shown in the Table.
(B) ToxPi scores based on all data for each Concawe category is shown as a box-and-whiskers plot.
(C-D) Separate ToxPi analyses were performed on the data from hepatocytes (5 assays) and cardiomyocytes (12 assays). Please see **Table 3.1.** for explanation of acronyms and Cat-App specific groupings. Individual substance data are presented in the supplemental material to House, J.S., et al., 2020.

Next, we examined the ToxPi profiles of the individual substances in each Cat-App category. As an example of the differences between categories and similarities within each category, two examples are shown in **Figure 3.6.** ToxPi are shown for 27 PS-E in the heavy fuel oil (HFO) category and 9 PS-E in the Waxes (WAX) category. For the HFO category, most of the PS-E exhibited very similar ToxPi profiles across all cell types indicating an overall similarity in bioactivity (left panel of **Figure 3.6.**). Very different ToxPi profiles were apparent for the WAX PS-E (right panel of **Figure 3.6.**). However, variability among substances in each of the two categories displayed were also apparent, similar to the variability within each category shown in **Figure 3.5b**. For example, two PS-E in the HFO category (top right) were quite different in the effects on cardiomyocytes (blue slice) and other cell types.

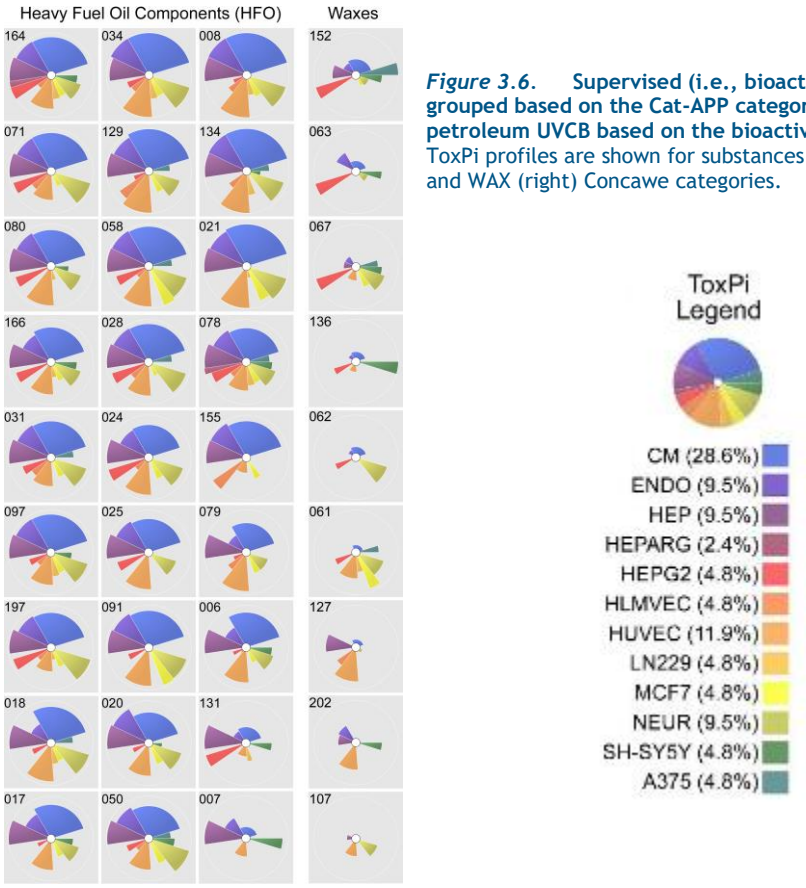

Figure 3.6. **Supervised (i.e., bioactivity data were grouped based on the Cat-APP categories) grouping of petroleum UVCB based on the bioactivity profiling data.** ToxPi profiles are shown for substances in the HFO (left) and WAX (right) Concawe categories.

One of the possible hypotheses that explains the variability in bioactivity within each category is the variability in 3-7 ring PAC content of each substance (see PAH hypothesis for PS, section 1.3.1). To examine whether 3-7 ring PAC content might be associated with overall bioactivity, the relationship between bioactivity of the substances and the 3-7 ring PAC content in each substance was evaluated. It was hypothesized that the variability in bioactivity within each category is related to the variability in 3-7 ring PAC content of each substance. Therefore, the bioactivity, expressed as a cumulative ToxPi score for each substance, was compared for each UVCB to the 3-7 ring PAC content expressed as a proportion of DMSO-extractable PAHs. Specifically, 3-7 ring PAC content score was calculated by taking the sum of aromatic ring content (for 3 through 7 ring -containing constituents) times the percent total weight of DMSO-extractable polycyclic aromatic compounds (PAC) determined by PAC-2 Method as described by Gray et al., 2013.

Consistent with the hypothesis, the overall fit for the ToxPi scores based on the bioactivity data from all 13 cell types (**Figure 3.7.**) showed a strong positive correlation (Spearman rho=0.89) with the PAH 3-7 ring content of each substance.

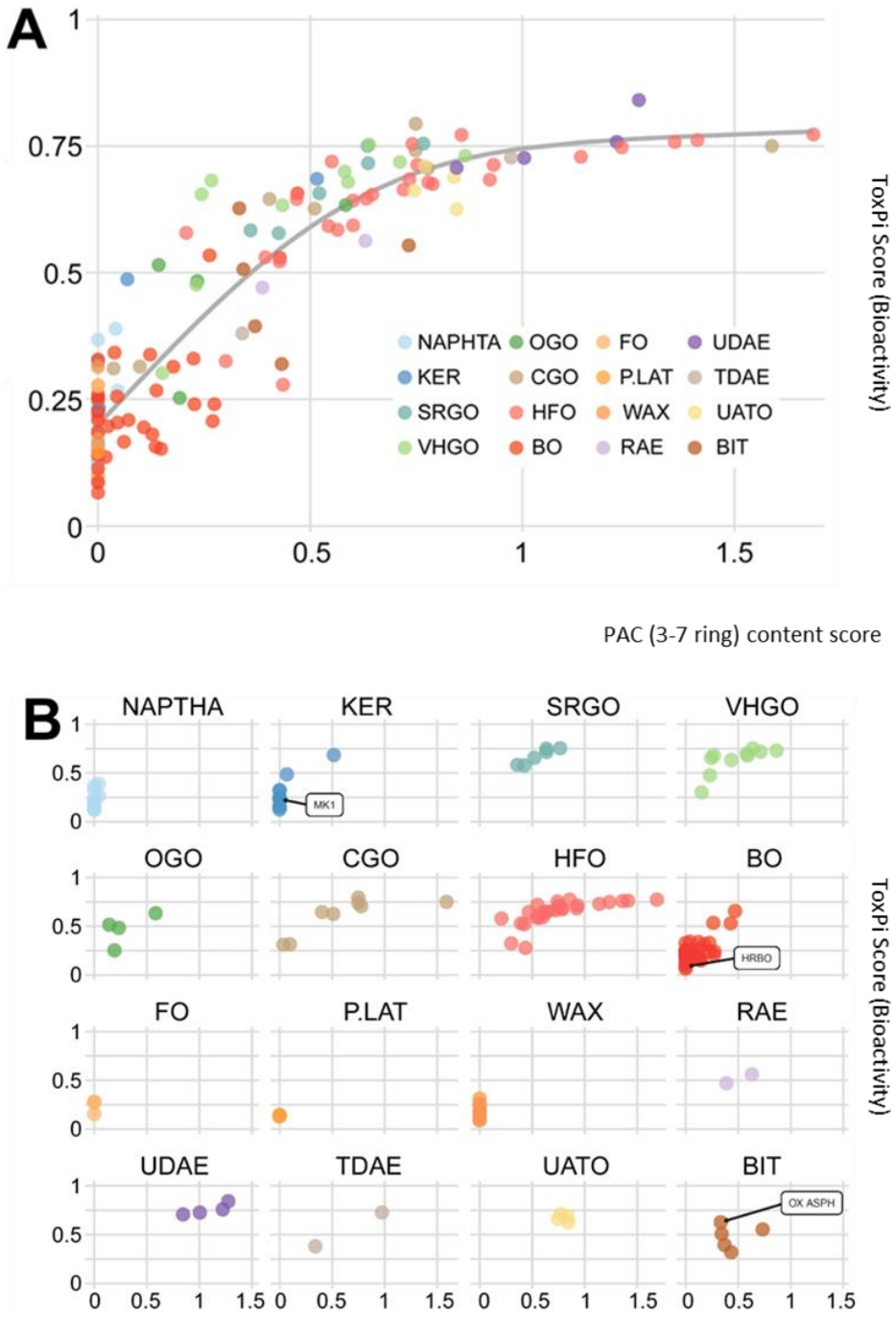

Figure 3.7. **Relationships between bioactivity-based ToxPi scores of PS-E and PAH 3-7 ring content score of the petroleum UVCB used in Cat-App.** The image on the left (A) shows the overall correlation plot with all substances included. X-axis is PAH 3-7 ring content score that was calculated by taking the sum of aromatic ring content (for 3 through 7 ring –containing constituents) times the percent total weight of DMSO-extractable polycyclic aromatic compounds (PAC) determined by PAC-2 Method. Y-axis is the cumulative ToxPi score of each substance based on the bioactivity in 13 cell lines. Each substance is marked by a colour that corresponds to Concawe Cat-App specific categories. The images on the right (B) show the same information but each plot contains the substances for a Cat-App specific category. Note: for statistical visualisation reasons the Concawe categories were merged into 16 classes shown here by 16 colors. Subcategories are noted in the right panel: MK1 (in KER), HRBO (in BO) and Oxidized Asphalt (in BIT). For further explanation on Cat-App specific acronyms and groupings refer to **Table 3.1**. See supplemental material of House, J.S., et al., 2020 for cell-specific correlations.

The right panels of the **Figure 3.7.** show examples of the individual Concawe categories. For those categories with relatively higher 3-7 ring PAC content, strong trends in the categories are observed showing increased bioactivity correlated to increased 3-7 ring PAC content. In contrast, for categories with low to negligible 3-7 ring PAC content these trends were not observed. Overall, the results presented in this Figure corroborates the known relationship between the content of PACs, especially of 3-7 ring type, in the petroleum refining products with their potential health hazard (see section 1.3.1).

We also note that the ToxPi bioactivity should not be interpreted as a quantitative indicator of the health hazard potential of a substance because ToxPi scores are based on a relative comparison of the cumulative effects of the substances included in the analysis. ToxPi are informative only in the context of a particular dataset. ToxPi profiles of the individual substances (**Figures 3.6.** and **3.7.**) aid in visualizing the patterns in the effects of each substance on in vitro cell-based models. We show that there is similarity in the patterns of effects by the substances in a category, while there are appreciable difference between categories. In addition, trends in the total ToxPi scores and their correlation with PAH 3-7 ring content are helpful as further supporting data, integrated with other data types (e.g., analytical, *in vivo*, other *in vitro*), in the selection of the most representative substances from each category for further testing. This application in an overall integrative testing strategy maximises the efficient use of animals needed for toxicological assessments of petroleum UVCBs and reduce the overall time to complete the regulatory assessment of petroleum substances (**Figure 1.2.**).

4. BIOACTIVITY PROFILING OF KNOWN CONSTITUENTS FOR GROUPING OF PETROLEUM SUBSTANCES

A number of reference compounds were selected for testing in parallel with PS-E, to investigate if these could add additional value to the assessment and grouping of PS. The reference compounds were selected from those that are frequently used as "marker molecules" for complex petroleum-derived substances in analytical chemistry methods, such as GCxGC-FID (ASTM International, 2011), and for which a strong *in vitro* database already exists. Specifically, we selected 20 "reference substances" (**Figure 4.1.**) that are representative of the major known chemical structural classes in petroleum substances: n-paraffins, mono-, di-, tri- and higher order aromatics. These substances also have been used in ToxCast screening (Judson et al., 2009; Williams et al., 2017), as well as many of them have *in vivo* toxicity data, and thus other information is available on them for potential comparison. The 20 reference chemicals were included in all screening among petroleum substance extracts and other controls (see section 3.2 and **Figure 3.2a**).

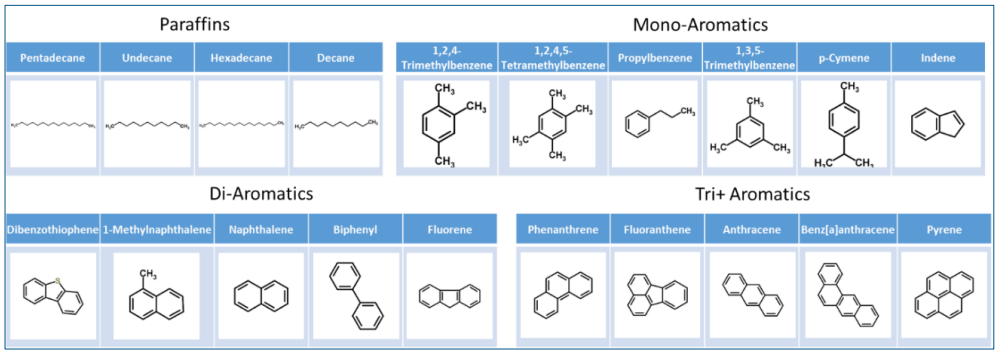

Figure 4.1. **Reference compounds used in Cat-App.**

We tested whether bioactivity profiles of the PS-E correlate with those of individual reference compounds, which might be informative in a further weight of evidence approach. We found that few strong correlations exist among petroleum UVCBs and the reference chemicals for all categories, which indicates that read-across from further safety data on those reference chemicals to the whole UVCB is of limited relevance. This also further supports the hypothesis that data on the PS-E is based on the complex interactions of the chemistry and are not driven by one constituent in the UVCB.

It was considered that these data are outside the current scope of the report, hence do not add further value and will therefore not be described in more detail here. The relevant data, as well as all data generated under Cat-App, will be published in a secured online Cat-App database (more details on accessibility and other relevant information will be published on www.concawe.eu/cat-app).

5. MECHANISTIC UNDERPINNING OF THE BIOACTIVITY-BASED GROUPING OF PETROLEUM SUBSTANCES THROUGH HIGH-THROUGHPUT TRANSCRIPTOMIC ANALYSIS

The transcriptomic profiling of ~3,000 transcripts was conducted on 6 cell types (**Figure 5.1.**) using the TempOSeq technology (Grimm et al., 2016; Yeakley et al., 2017). Cell types were selected from the original 15 based on several criteria: (i) cells with assays passing QC for bioactivity; (ii) cells that represent a diverse set of tissues and/or organs, and (iii) priority was given to human iPS- cells. Overall, 4 iPSC and 2 cancer cell lines were selected for transcriptomic analysis. To enable accurate quantification of transcriptional concentration and to evaluate differential expression analysis and perform assessment of transcriptional dose-response, a comprehensive pipeline was developed in Cat-App (House et al., 2017).

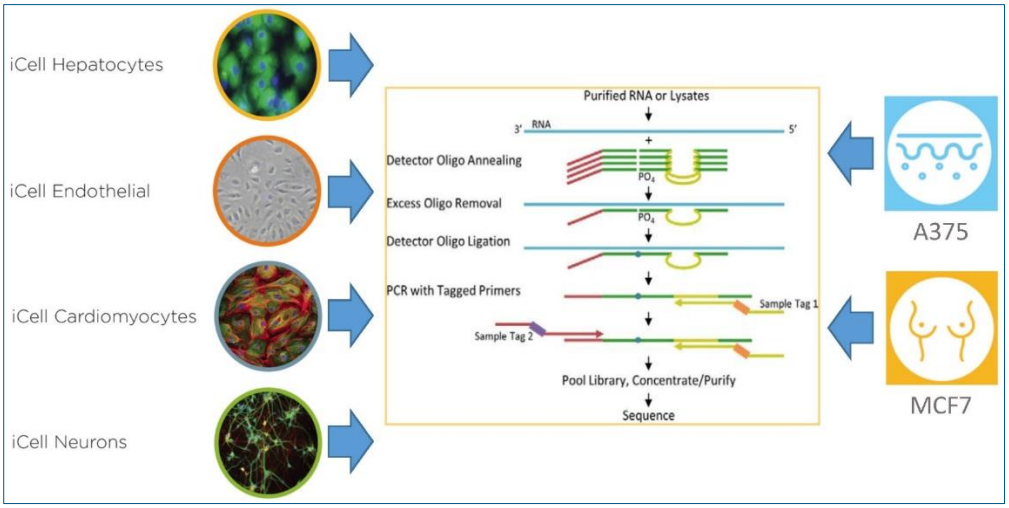

Figure 5.1. **Transcriptomic analysis of the biological effects of UVCBs and reference chemicals was conducted on 6 cell types as indicated in the figure.** The method used for these experiments is detailed in (Grimm et al., 2016; Yeakley et al., 2017) and the data processing pipeline in (House et al., 2017).

In total, over 10,000 samples were processed for TempO-seq, resulting in over 35 million data points. The quality control procedures were performed at the plate level, ensuring sufficient read counts, as well as identification of suspect samples due to low correlation with remaining control samples. After passing earlier quality control steps, fold change of the highest concentration tested as compared to method blank was calculated. Probes with false discovery rate *q*-values <0.10 for each cell line were then used to calculate transcriptional point of departure, as detailed in House et al. (2017).

The overall transcriptomic assessment of the number of differentially expressed genes supports the view that iPSC hepatocytes are most responsive to exposure with hydrocarbon-containing complex substances, probably because they are more metabolically-competent than other cell types studied (**Figure 5.2.**). In addition, PS-E with the highest PAH 3-7 ring content have elicited the most pronounced effects on gene expression in hepatocytes and other cell types.

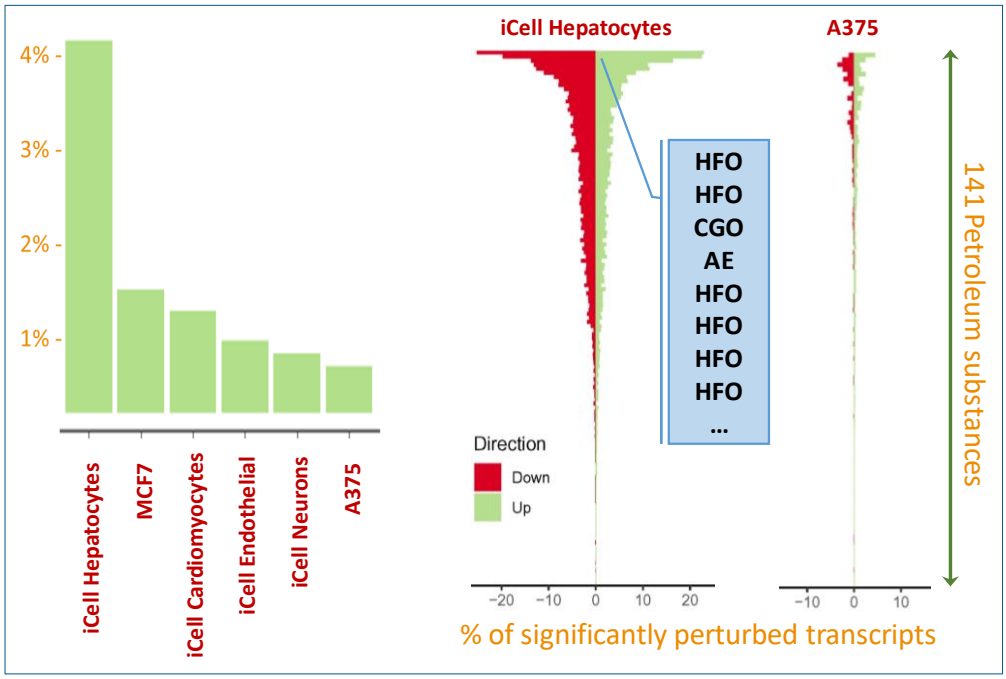

Figure 5.2. **Transcriptional effects of DMSO extracts of petroleum UVCBs on gene expression in 6 cell types.**
Left, the fraction of transcripts affected by all substances. For each cell type, ~3,000 transcripts were evaluated across all 141 substances. For example, 4% represents the proportion of differentially expressed genes in the iCell hepatocytes. Right, substance and cell type-specific effects of the petroleum UVCBs are shown. iCell hepatocytes and A375 cells, representing the cell types that had the most and least pronounced UVCB-induced transcriptional effects, are shown as examples where substances are ranked by the total number of transcripts significantly affected by treatment. Colours represent the directionality of change. The top 8 substances (indicated by their Concawe category) are shown in the insert for hepatocytes.

Next, we determined whether the degree of an effect on gene expression can be used to group petroleum UVCBs. When transcriptomic data from all cell types was combined, there was little evidence of group-specific effects (**Figure 5.3.**). However, iCell hepatocyte-only data showed much more pronounced separation among categories, with Residual Aromatic Extracts (RAE) showing the greatest and waxes (WAX) the least overall effect on gene expression. Again, strong separation between the substances that contain higher levels of 3-7 ring PAC on one end and higher refined substances with lower PAC levels on the other end of the continuum.

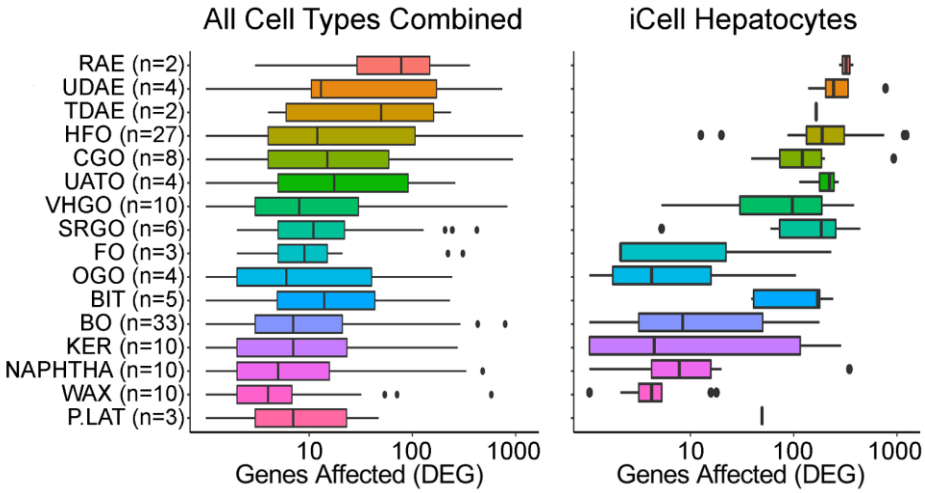

Figure 5.3. **Transcriptional activity for Cat-App specific categories.** (See **Table 3.1.** for further explanation on acronyms for Cat-App specific categories). Left, the number of differentially expressed genes in all 6 cell types based on all DMSO extract data for each Concawe category is shown as a box-and-whiskers plot. Right, data for hepatocytes only are shown.

We also tested whether transcriptional activity of the PS-E of the individual petroleum UVCBs was correlated with the PAH 3-7 ring content of the PS. PAH 3-7 ring content score was calculated by taking the sum of aromatic ring content (for 3 through 7 ring structures) times the percent weight from DMSO extract data. **Figure 5.4.** (left) shows that Spearman correlations of differential gene expression in each cell type or all cell types with 3-7 ring PAH content varied widely with hepatocytes exhibiting the highest correlation. In order to ascertain how closely the aromatic ring content is associated with the ToxPi bioactivity from all *in vitro* bioactivity data and transcriptomics, we conducted PCA. **Figure 5.4.** (right) shows strong correlation (Spearman 0.75) between the first principal component (PC1) and the ToxPi score for *in vitro* bioactivity and transcriptomics.

Table 5.1. **Correlation (Spearman and Pearson) between gene expression (number of differentially expressed genes) and PAH content (level of 3-7 ring PAH) of UVCB substances.** Correlations and p-values for each cell type and all cells combined are shown.

Cell type	Spearman correlation	p-value	Pearson correlation	p-value
A375	0.24	0.005	0.13	0.12
iPSC CM	0.11	0.2	0.51	10^{-11}
iPSC ENDO	0.18	0.04	0.2	0.02
iPSC HEP	0.75	10^{-26}	0.76	10^{-26}
MCF7	0.20	0.02	-0.02	0.8
iPSC NEUR	0.09	0.3	0.29	10^{-4}
All Cells	0.62	10^{-16}	0.7	10^{-22}

6. PREDICTIVE INTEGRATIVE ANALYSIS OF THE BIOACTIVITY, HIGH-THROUGHPUT TRANSCRIPTOMIC AND ANALYTICAL CHEMISTRY DATA FOR GROUPING OF PETROLEUM SUBSTANCES

One relevant research question of interest is whether the extensive data compiled and generated for Cat-App enables the generation of supervised predictive models for Concawe categories. The term 'supervised' denotes that we use the existing categories to train a model and then apply a "leave one out" approach to predict in which category the specific substance belongs based on its chemical and / or biological profile.

As a comprehensive summary of available data, we included data in three major types. The analytical data (A) consisted of the 3-7 ring PAH data in %wt DMSO extractables as explained earlier (2-7 ring PAC content), with the aromatic ring percentages and total weight, GCxGC data with specific compound constituency (naphthalene, anthanthrene, benzo[e]pyrene, etc…) as well as additional PAH ring content, and SIMDIS data with initial boiling points, final boiling points and 5% increments. Any missing data were mean-imputed. Initial exploratory analysis suggested that higher prediction accuracy could be achieved with vetting of the analytical data, and so the final analytical predictive data used consisted of priority, per1-per 7, relative PAH 3-7 and PAH 4-7 content, and final boiling point. The Bioactivity data (B) consisted of all the phenotypic assays that passed QC (**Figure 3.4.**). These are a matrix of the dose point of departure values for each of 141 substances across the 43 assays. Finally, Expression data (E) using TempO-seq fold-changes (max dose vs. controls) were utilized from hepatocyte gene expression on ~2800 genes (**Figure 5.3.**).

As the number of potential features vastly outnumbered the number of UVCB substances, a penalized machine learning approach (the PAMR method implemented in R) was used for classification, with leave-one-out cross validation. The leave-one-out approach ensures that the classification accuracy is realistic, because each of the 141 UVCBs is held out in succession and not used in training the model. Another way to describe the estimated accuracy is the predicted accuracy if an entirely new UVCB were to be classified. As some of the 21 original Concawe substance categories for the 141 UVCBs had too few members for this statistical classification approach, analyses were performed using the 16-category version of Concawe categories (**Table 3.1.**) used for the colour scheme shown in section 5.

Figure 6.1. shows statistical classification accuracy for analytical data (accuracy 43%) and bioactivity data (accuracy 38%). The combination of analytical and bioactivity data gave a higher accuracy (45%). The categories are ordered by mean PAH 3-7 relative content, and it is apparent that several of the category errors are in fact assignments to categories of similar PAH 3-7 content (shown in light green). Assignments to more "distant" categories are shown in yellow and red, highlighting substances that merit further scrutiny. We note that random assignment of UVCBs would give a much lower accuracy (as low as ~20%, depending on the random category assignment approach used). One salient feature of classification using bioactivity data alone is that predictions tend to concentrate on the two largest categories, base oils and heavy fuel oils (**Figure 6.1.**, middle panel). The combination of analytical and biological data (**Figure 6.1.**, right panel) provides not only the highest accuracy, but the spread of classifications across the categories is better explainable in terms of the 3-7 ring PAC content of the substances related to their observed biological activity.

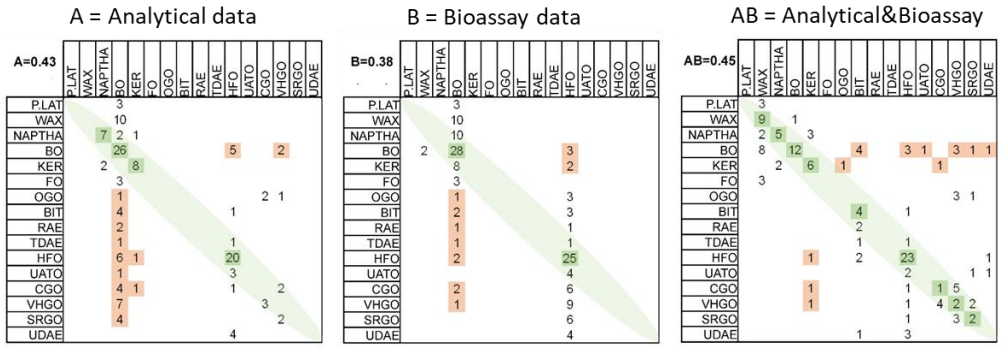

Figure 6.1. **The results of leave-one-out classification analysis (by PAM-R) using analytical and bioactivity data alone and in combination.**

Analyses including the expression data alone show reduced accuracy (31%, **Figure 6.2.**, left). One possible explanation for this result is the so-called "curse of dimensionality," i.e. the number of features (genes) that are examined is too large for effective classification. Indeed, when expression data are added in combination with analytical and bioactivity data (**Figure 6.2.**, right), the accuracy (39%) is still poorer than for analytical+bioactivity as shown in **Figure 6.1**.

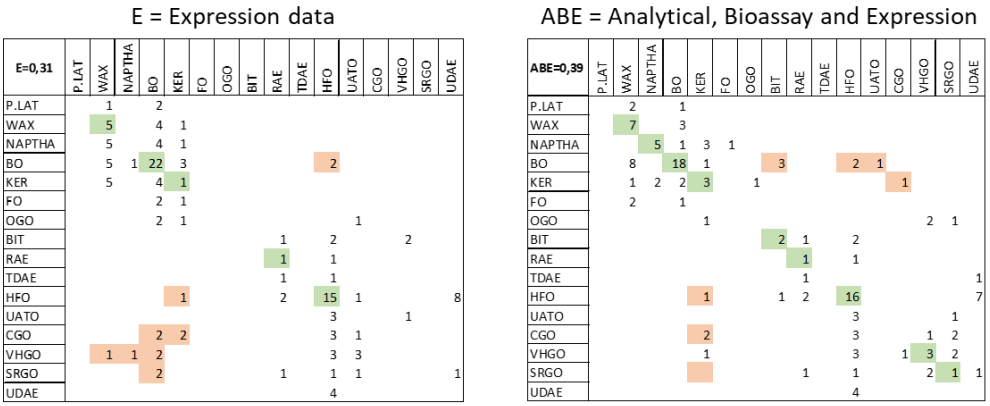

Figure 6.2. **The results of leave-one-out classification analysis using expression alone (left panel) and all three data types (analytical+bioactivity+expression, right panel).**

These analyses suggest that the incorporation of both bioactivity and analytical data allow for more accurate categorization of substances. More targeted approaches that focus on categorization of petroleum UVCBs that have little to no PAH ring composition may be instructive to help differentiate the substances for which little biological activity was observed here, and is therefore expected to possibly increase accuracy overall. Careful follow-up analyses can focus on data features that are characteristic of each substance, and further examination of "misclassified" substances.

7. DISCUSSION

The Cat-App is a first-of-its-kind project that attempted to determine whether new approach methodologies that are based on *in vitro* bioactivity and gene expression profiling can be used to ascertain similarities among complex petroleum UVCB substances. Because of the chemical composition complexity and expected variability in composition of the petroleum UVCB within and among the manufacturing classes, it was questioned to what extent the manufacturing stream/phys-chem properties would be sufficient to support "similarity" of complex UVCBs. Therefore, Cat-App aimed to establish a biological fingerprint that could be used in conjunction with the other inherent (phys/chem) properties to serve as a basis for grouping these substances. The ultimate goal was to incorporate the biological component as an additional piece of information to help underpin the exisiting grouping exercise thereby increasing the potential to reduce animal testing under regulatory programmes. It is recognized that grouping materials with variable and complex identities can be difficult in light of traditional evaluation tools, and adding the biological component adds an additional component for consideration.

The Cat-App project demonstrated that bioactivity profiling of complex UVCBs is a feasible path towards characterization of "sufficient similarity" for complex substances based on NAM data. It is the largest to date "case study" that was aimed at testing whether and how *in vitro* bioactivity and gene expression data can be used to inform grouping of UVCBs. Ambitious goals of Cat-App, in terms of the number of substances, cell types, endpoints and data analysis questions, have been met with respect to both scientific output and timelines. This project demonstrates how a focused 2-year programme can yield both the novel knowledge and informative data that are immediately applicable to decision-making.

One of the main findings of Cat-App is that there is a clear relationship between *in vitro* bioactivity profiles and the PS-E of the petroleum UVCB (i.e., DMSO extract as explained in chapters 2 and 3 of this document). By using the PS-E of the substance, it is recognized that this does not represent the full substance, however as the role of Cat-App is not for hazard identification, the use of a normalized control helps to eliminate questions over what is available or testing, as it provides a consistent matrix to test in the biological endpoints, thereby allowing direct comparison of the results both within and across groups and categories. It is well established that some manufacturing categories of petroleum UVCB, such as HFO for example, contain substances that can vary widely in terms of their 3-7 ring PAC content. Indeed, we observed both the overall strong correlation between bioactivity and the categories of UVCBs (for example, HFO – which have overall much higher PAC content than e.g. waxes - in general show high bioactivity whereas waxes show low bioactivity), but also significant variation within some PS categories (HFO have a large range from low to high PAC containing substances which is reflected in the spread of bioactivity observed in this category). This trend was enforced by the fact that the biological data separate out the two foots oils from the HFO group of substances when they were intially grouped together for statistical visualisation purposes (e.g., **Figure 3.5. & 3.7.**), which is well explainable as Foots Oils are much closer to Waxes from a refining perspective. More specifically, the 3-7 ring PAC content of these PS-E correlated strongly with bioactivity. Petroleum substances can be ranked in the chemical-biological space (low → high 3-7 ring PAC content and bioactivity), representing the continuum of petroleum substances. This ranking is in agreement with the PAC hypothesis for petroleum substances which states that certain specific toxicological effects observed in heavier (average molecular weight) substances are associated with the level of 3-7 ring PAC in these substances. Thus, Cat-App adds an important additional mechanistic information to the overall weight of evidence

that is being built holistically across the continuum of petroleum substances to further support this hypothesis.

It is also evident that existing Concawe categories contain substances with significant inter- and intra-category overlap as expected based on the phys-chem characteristics of PS. Indeed, petroleum substances are a continuum in terms of their chemical composition with "adjacent" streams overlapping: to some extent, there will be overlap between the heavy end of a low boiling stream and the light end of the adjecent higher boiling stream (see also **Figure 1.1.**). Despite these expected similarities, the bioactivity data are able to clearly distinguish among the substances that should not be placed into the same category based on their refining properties and/or product specifications. For example Heavy Fuel Oils (HFOs) are clearly different from Foots Oils, and although these were initially "grouped" together for statistical purposes, it is interesting to see that they are identified as different from HFOs which is correct from a refining perspective.The same was observed for other substances categories, where for example highly refined base oils were less bioactive then the other LBOs -with which they were grouped for this project (**Figure 3.5. & 3.7.**).

Another goal of Cat-App was to determine what new approach methodologies and models are most informative in terms of decision-making for complex petroleum UVCB substances. We found that data derived from assays of biological activity in iPS cell-derived models was more informative in terms of their ability to group substances and ascertain trends in bioactivity. We posit that this finding is most likely the product of the retained organotypicity of these cell types as compared to established cancer cell lines. In addition, in this study, data from iPSC-derived hepatocytes was most informative for separating the substances in terms of their overall bioactivity trends, consistent with the ability of these cells to metabolise PAH-containing substances to reactive intermediates. Also, assays based on iPSC-derived cardiomyocytes were able to provide some separation between the UVCB categories with substances that have low to negligible PAC content, which poses the question whether there may be different types of molecules other than PAC playing a role in the observed biological response, which could be interesting follow-up work to investigate: whether generating and adding data to the intergrative analysis on the biological response of the non-PAC part of the UVCB can further improve the overall grouping. Nevertheless, these categories are chemically very different ranging from low-boiling naphtha to high-boiling bitumen.

Gene expression data generated in this project are massive in terms of the number of substances and cell types analyzed, more than 35,000,000 data points were obtained. These data provide further support to the observations from in vitro bioactivity profiling and offer additional mechanistic insights. We found that liver cells proved to be most informative, most signifciantly expressed genes were all involved in biotransformation/PAH metabolism related pathways. In addition, the PS-E that triggered most noticeable gene expression responses were the ones with the highest PAH content. This is further mechanistic support to the PAH hypothesis for petroleum substances, as it confirms mechanistic pathways for 3-7 ring PAHs via interaction with receptors in hepatocytes to alter gene expression.

We tested whether multi-dimensional *in vitro* bioactivity, gene expression, or analytical characterization data on petroleum UVCBs can be used to classify each substance into a particular Concawe category. We found that each of these data streams alone has significant power to predict what category or overall group the substance may belong to; however, mis-classification was observed relatively frequently as well. Importantly, combinations of these data, a so-called chemical-biological read-across, was most powerful in eliminating mis-classifications. These

data offer strong support for the need of various data streams to increase confidence in grouping of complex UVCBs.

Overall, the data do support the use of the current Concawe categories which are based on refining history, and add further biological insights to this grouping in terms of chemical-biological trends across the continuum of PS which is important in view of generating read-across hypotheses.

In parallel to petroleum UVCBs, we tested whether some of the reference ("R20") chemicals, molecules representing the common classes of hydrocarbons found in petroleum refining products, could be used as exemplar molecules to evaluate the full UVCB. With limited exception, correlations between these R20 compounds and UCVBs on biological responses were relatively weak, indicating that single chemicals were usually insufficient to replicate the diversity of compounds in a UVCB on phenotypic and transcriptomic cellular responses. The noted exceptions, i.e., limited instances where indeed similarity in biological responses were observed, seem to be for when the UCVB contained particularly high amounts of PAH and the R20 chemical was a PAH, further supporting the PAH hypothesis. In conclusion, the data from Cat-App indicate that individual reference chemicals are not sufficient to replicate the complexity and diversity of the petroleum refining-derived UCVBs.

8. CONCLUSION

Cat-App data add an important and informative biological component to the chemical-biological trends observed in both the overall continuum (i.e., the hydrocarbon space) of PS as well as within PS categories. An interesting observation from these data is that they were able to discriminate specific substances from categories they were initially forced into: for statistical purposes, foots oils were initially merged with heavy fuel oils, which are completely different from a refining perspective, and the biological data were able to pick these out. Similarly, highly refined base oils are different from lubricating base oils and again based on the chemical-biological data these were assigned differently. These data therefore have the potential to underpin the overall grouping of these substances by showing chemical-biological trends, an aspect that can address the challenge from regulators that grouping PS UVCBs on their phys/chem data only (the historical approach taken in Concawe) is not sufficient.

The next step is to then apply this further in supporting read-across assesments and testing strategies to avoid unecessary (duplicate) animal testing. Althought the Cat-App data provide further insights and supporting information for the PAH hypothesis that is the basis for several higher tier toxicological effects observed with PS, these data are not intended to predict toxicity and therefore their application on this aspect is limited to hypothesis building. Starting from chemical biological grouping of PS, an approach could be to look at the endpoint under consideration more specifically and integrate further mechanistic insights from other -more targeted- in vitro work ongoing at Concawe, as well as specific historical in vivo data relevant to the specific endpoint. Such an integrated analysis would help select the most representative samples for further testing, as well as further supporting read-across of historical data.

This can be done in an integrated testing strategy as described earlier in this report to address human health endpoint requirements under REACH, in a holistic approach which looks at the continuum, the full hydrocarbon space, of PS. Besides avoiding unnecessary animal testing, this approach should also help to reduce time to complete the regulatory assessment of 185 PS overall (see **Figure 1.2.**). Therefore, Cat-App data will be integrated into the Concawe product dossiers in REACH submissions as supporting WoE information at the heart of an intelligent testing strategy. Further work that could be considered following the outcomes of this project is to investigate the observed variation in response with the DMSO extact of PS that have neglegible levels of 3-7 ring PAH, to investigate other possibilities of dosing PS to *in vitro* systems instead of using a DMSO extract (e.g., passive dosing options as applied in assays used for ecotoxicological assessments), application of the best performing models to samples under current regulatory investigation to provide additional biological and mechanistic support for those experimental and read-across assessments, as well as to address the variation within a CAS number (one of the challenges with UVCBs) and other areas.

The practical approach taken with Cat-App describes a direct regulatory application of New Approach Methodology (NAM) data, to support grouping (and subsequently read-across) assessments. The framework presented petroleum substances as a case study, but its concept is more generally applicable and therefore expected to help further progress the regulatory acceptance of these types of new biological data - initially as supporting information, but on the longer term with the aim to help develop alternatives to animal testing.

9. GLOSSARY

AE	Aromatic Extracts
BO	Base Oils
BRC	Bioinformatics Research Center
C	Carbon
CAS	Chemical Abstract Services
CGO	Cracked Gas Oils
CRCE	Centre for Radiation, Chemical & Environmental Hazards
DMSO	Dimethyl Sulfoxide
ECHA	European Chemicals Agency
EPA	Environmental Protection Agency
EU	European Union
GCxGC	Comprehensive Two-Dimensional Gas Chromatography
HBPS	Heavier and High Boiling PS
HFO	Heavy Fuel Oil(s)
HPV	High Production Volume Program
HRBO	Highly Refined Base Oils
iPSC	induced Pluripotent Stem Cell
LBO	Lubricating Base Oils
NAM	New Approach Methodologies
NCSU	North Carolina State University
NICSM	Northern Ireland Centre for Stratified Medicine
NIEHS	National Institute of Environmental Health Sciences
OGO	Other Gas Oils
OLBO	Other Lubricating Base Oils
PAC	Poly-cyclic Aromatic Compounds
PAH	Polycyclic Aromatic Hydrocarbons
PCA	Principal Components Analysis
PHE	Public Health England
PS	Petroleum Substance(s)
PS-E	Petroleum Substance-DMSO Extracts
QC procedures	Quality Control procedures
QSARs	Quantitative Structure Activity Relationships
RAE	Residual Aromatic Extracts
REACH	Registration, Evaluation and Authorisation of Chemicals
SRGO	Straight-Run Gas Oils
tDAE	treated Distillate Aromatic Extracts
UATO	Unrefined/Acid Treated Oils
uDAE	untreated Distillate Aromatic Extracts
UVCBs	Unknown or Variable composition, Complex reaction products and Biological materials
VHGO	Vacuum Gas Oils, Hydrocracked Gas Oils and Distillate Fuels
WoE	Weight-of-Evidence

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