Concept for a generic information strategy for PBT and vPvB assessement of UVCB substances

Leonards, P.E.G.

published in
Concept for a generic information strategy for PBT and vPvB assessement of UVCB substances
2017

document version
Publisher's PDF, also known as Version of record

IVM Institute for Environmental Studies

Concept for a generic information strategy for PBT and vPvB assessement of UVCB substances

R-16/06

Pim Leonards

Institute for Environmental Studies, VU University

Report: R-16/06
July, 2017

This report was commissioned by: Ministerie I&M

IVM
Institute for Environmental Studies
VU University Amsterdam
De Boelelaan 1087
1081 HV Amsterdam
T+31 20-5989 555
F +31 20-5989 553
E info@ivm.vu.nl

Contents

Table of Contents

1	**Introduction**	**5**
1.1	Background	5
1.2	Outline	6
2	**UVCB assessment approaches**	**7**
3	**Concept generic information strategy for PBT and vPvB assessment**	**11**
3.1	**Constituents approach**	11
3.1.1	Characterisation of constituents in UVCB substances	11
3.1.2	Blocking of constituents	15
3.1.3	Selection of representative structures and *in silico* predictions	18
3.2	**Substance approach**	19
3.2.1	Fractionation of UVCB substance	20
4	**Conclusions and recommendations**	**23**
5	**Acknowledgements**	**24**
6	**References**	**25**

Summary

Within the context of the European REACH (Registration, Evaluation, Authorization, and restriction of Chemicals) Regulation, the SVHC Roadmap aims to identify the highest priority potential substances of very high concern (SVHC) before 2020. With regard to environmental concerns, substances will be assessed for persistency, bioaccumulation, and toxicity (PBT) properties. One group of substances which are more difficult to assess for PBT properties are the UVCBs: substances of unknown or variable composition, complex reaction products or biological materials.

This report aims to set a first step in the process of developing a generic information strategy for the PBT assessment of UVCBs. Two examples of UVCB substances are used in this report to illustrate specific parts of the information strategy: petroleum and coal stream (PetCo) substances and chlorinated paraffines.

The proposed strategy is based on current guidance documents, experience from case studies, and current state of science. The concept follows two lines of evidence: a **constituents approach** and a **substance approach**. The first line is based on the identification of the constituents of the UVCB, followed by grouping (blocks) of constituents with similar properties (e.g. physico-chemical, fate, mode of action), and the selection of representative structures for each block. The representative structures are individually assessed for PBT properties using *in silico* predictions and experimental work. In general, the characterisation of the exact molecular structure of constituents in UVCB substances is challenging or extremely time consuming due to the complexity of the mixtures, even with the latest analytical techniques available. However, for most UVCBs generic structures of the constituents can be generated. This generic structures can be used for blocking constituents with similar properties and to generate representative structures for each block. It is therefore suggested that the generation of generic structures can be used as a first tier assessment of UVCBs. Yet, if information on specific constituents with potential PBT properties are available these should be assessed as well. Predictions of the physico-chemical properties of the representative structures together with (Q)SAR PBT models are then used for the PBT assessment. This information can further guide the experimental work on representative structures and therefore reduce animal testing. Together with the whole substance testing it provides weight-of-evidence on the PBT properties of the UVCB substance.

The second line assesses the UVCB substance as a whole, which can be followed by fractionation of the substance in fractions before experimental testing. With the later concept the main challenges are in the testing methodologies as many UVCB substances are hydrophobic and therefore alternative testing methods are needed.

In this concept there are a number of challenges which are in the field of i) testing methodologies, ii) the applicability domain of the *in silico* models, and iii) each UVCB will have its own specific challenges and therefore a case-by case approach is needed. The current momentum on the development of the strategies for UVCB assessment needs multi-stakeholder involvement from regulators, industry and academia.

1 Introduction

1.1 Background

For the safe use of chemicals in Europe the REACH (Registration, Evaluation, Authorization, and restriction of Chemicals) Regulation is in force. Article 57 of REACH sets the hazard based criteria for substances that may be identified as a substance of very high concern (SVHC). These include substances that are identified as CMRs (carcinogenic, mutagenic or reprotoxic) by CLP, or substances that are persistent, bioaccumulative and toxic (PBT) or very persistent and very bioaccumulative (vPvB). A substance is identified as SVHC once the substance is added to the Candidate list of REACH and a SVHC Roadmap has been developed to screen and assess all most important potential SVHCs by 2020 for further regulatory measures. The EU member states, the European Chemicals Agency (ECHA) and the European Commission execute this Roadmap.

REACH identifies three generic types of substances:

- Mono-constituent substances; where a single constituent makes up for > 80% w/w of the substance composition.
- Multi-constituent substances: where more than one constituent are present in at a percentage between 10 and 80% w/w of the substance composition.
- UVCBs: Substances of unknown or variable composition, complex reaction products or biological materials. In general, such a substance consists of many different constituents, some of which may be unknown.

The present report focusses on the PBT/vPvB assessment of UVCBs. There are approximately 16,000 UVCB substances on the market (Clark et al., 2013). Examples of typical UVCBs are petroleum substances, chlorinated paraffin's (CPs), flavoring agents, fragrances, animal fats and their derivatives, vegetable oils and their derivatives, natural oils and extractives, and biofuels. The composition of a UVCB can be variable or difficult to predict. Often the identity (exact chemical structure) of the constituents in the substance cannot be identified and only generic chemical structures are available. As a consequence, UVCBs are often not described by their chemical content but according to the manufacturing process.

According to REACH a **substance** '*means a chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.*'

In this report "**constituents**" will be used to indicate the chemicals present in the substance.

Some of these substances consist of thousands of constituents. Consequently, the environmental and human risks of UVCB substances are more difficult to assess as this assessment is normally based on the knowledge available for individual constituents.

This report is intended as a first step in the process of developing a generic strategy for the PBT and vPvB assessment of UVCBs and can be used in various working groups that deal with UVCB hazard assessment. Petroleum and coal stream (PetCo) substances

and chlorinated paraffin's are used as two specific case studies to illustrate the strategy proposed. Key questions addressed in this report are:

- What is the state-of-the-art to identify constituents in complex mixtures?
- How to group specific constituents in a UVCB?
- How homogeneous should a constituent group be to be able to perform a PBT/vPvB assessment?
- How can we test groups of constituent for their possible P and B properties?

1.2 Outline

An overview of UVCB assessment approaches is given in chapter 2. This is followed by a proposal for a generic information generation strategy for PBT/vPvB assessment of UVCB substances (chapter 3). Different aspects of the generic information generation strategy are illustrated by case studies to highly the challenges at each step. This report is ended with conclusions and recommendations (chapter 4).

2 UVCB assessment approaches

Worldwide various groups are working on strategies to assess UVCB substances. Examples are the ECHA PBT expert group, US Environmental Protection Agency (US EPA), Environment Canada, and industrial sectors such as the petroleum and personal care industries. Recently, an international workshop was organised by RIFM/ECETOX in Orlando (USA) to address the challenges in risk assessment of multi-component substances and UVCBs.

In this section, brief descriptions of UVCB assessment approaches are given. Note that this report focuses on the information needed to assess PBT/vPvB hazard of UVCBs and not on environmental and human health risk assessment of UVCBs.

ECHA
In Europe PBT/vPvB assessment of substances is required for substances that are manufactured/imported in quantities over than 10 tons per year. A UVCB is a substance that cannot be sufficiently identified by its chemical composition (ECHA). This is because:

- *The number of constituents is relatively large and/or*
- *The composition is, to a significant part, unknown and/or*
- *The variability of composition is relatively large or poorly predictable*

The statement that constituents of a UVCB could not *sufficiently* be identified has considerable consequences for the PBT/vPvB assessment as normally such assessment is based on a single chemical. A different approach is therefore needed to assess the persistency, bioaccumulation and toxicity for multiple constituents. So, ECHA provides guidance on how to perform a PBT and vPvB assessment of multi-constituent and UVCB substances. The most recent update of this guidance is provided in a draft document "Guidance on information requirements and chemical safety assessment Chapter R.11: PBT and vPvB Assessment" (ECHA, 2016). The assessment consists of two phases. In the first phase the constituents in the substance are characterized, and in the second phase information on persistency, bioaccumulation, and toxicity of the constituents is collected.

In the characterisation phase, the identity of the main constituents (10 – 80% of the substance) and minor constituents (0.1 – 10% of the substance) present in the UVCB is studied. Information on the **composition** and **identify** of these constituents should be reported as far as possible; if not possible **generic structure** information of the constituents should be provided. For constituents >10% w/w the IUPAC name and CAS number should be provide, and information on typical concentrations and concentrations ranges in the UVCB as well. Note that the identity of all constituents (constituting > 0.1% of the substance) that are relevant for the PBT or vPvB assessment should be provided (Section 4.3.1.1 of the ECHA SID Guidance). However, it should be noted that in case a large number of individual constituents <0.1% are present sufficient data should be provided that these are not PBT/vPvB.

For constituents that could not be identified, as much as possible information on the characterisation should be provided. Often generic information of the constituent is

available, such as boiling point, carbon number, chlorination degree, etc. For example generic information, such as carbon chain length and type of hydrocarbon (alkane, cyclic, aromatic), is available for hydrocarbons in petroleum products. This information can be used to predict persistency, bioaccumulation, and toxicity properties based on experimental data, read across, or (Q)SAR modelling.

After the characterisation phase information on persistency, bioaccumulation, and toxicity properties of both the substance and constituents is collected, predicted or generated by experimental studies. An option for UVCB substances is to group constituents with closely related molecular structures in blocks as has been done for hydrocarbons in petroleum products; the so called hydrocarbon blocking method, see §3.1.2. Read across to the blocks can then be used to collect information on persistency, bioaccumulation, and toxicity. This requires a selection of representative structures for each block.

Care should be taken for testing UVCB substances for persistency, bioaccumulation, and toxicity. Most persistency and bioaccumulation test are not applicable for UVCB testing. For example persistency tests measures sum parameters (e.g. CO_2 evolution or O_2 consumption) and therefore study the whole substance and provide no information on the individual constituents. Ready biodegradation tests should be judged on a case-by-case basis. In a second, tier experimental data or valid (Q)SAR predictions of representative structures of blocks can be generated. For bioaccumulation tests generally the focus is on the accumulation of one single constituent, however, in UVCBs specific constituents can have higher bioaccumulation properties than on average for the whole UVCB. In a first tier K_{ow} or QSARs can be used to predict the bioaccumulation potency of the constituents. If this tier shows potential bioaccumulation, experimental testing of representative structures is required. An important aspect of toxicity testing of UVCBs can be the difference in bioavailability of constituents, which is related to the solubility of the constituents in the media of the toxicity test. For nonpolar compounds that do not dissolve well in toxicity media a different approach should be followed, such as the water accommodated fraction (WAF) approach as used for petroleum derived UVCBs.

In general, UVCB assessment needs a case-by-case approach and the assessment should be based on weight-of-evidence approach. Various types of information can be used for the weight-of-evidence such animal and *in vitro* data, modelling, grouping and use of read-across, (Q)SAR, but also occupational and epidemiological studies. Further expert judgement is required to assess the PBT properties of complex UVCBs.

US EPA

The US EPA follows a similar approach as ECHA for defining chemicals. Two classes as part of the TSCA Chemical Substance Inventory are defined: class 1 and class 2, including UVCBs (USEPA, 2015). Class 1 are single compounds with known chemical structure. Class 2 compounds have unknown or variable compositions, and this class is further divided in multiple compounds with known structures and compounds with no defined molecular formula or with partially defined structures, the UVCBs. The US EPA provides no detailed guidance document on testing strategies for UVCB substances. However, the US EPA provides two health risk assessment guidance documents related to chemical mixtures (USEPA, 1986; USEPA 2000). Both documents

focus on human hazard assessment of chemical mixtures, and the assessment is based on data from three types of mixtures:

- Mixtures of concern
- Toxicologically similar mixtures (same mode of action)
- Mixture of component chemicals (this includes an Interaction Hazard Index)

Other documents related to chemical mixture assessment are the framework for cumulative risk assessment (CRA) (USEPA, 2003), which was further developed in the publication of 2006 (USEPA, 2006).

OECD

The OECD developed a guidance document to group chemicals, which includes a section on UVCBs (OECD, 2007). The OECD considers four key elements to be included, 1) composition data, 2) properties of the constituents, 3) data gap filling using read-across/(Q)SAR, and 4) data gap filling with testing. For the last three key elements representative structures of the substance are needed. As an example, the OECD developed guidance for characterisation of olechemical substances that are useful for hazard assessment (OECD, 2014). In the chemical grouping document of OECD guidance is given for natural complex substances (NCS), and complex inorganic UVCB substances also.

Environment & Climate Change Canada

The risk of substances are assessed under the Canadian Environmental Protection Act (CEPA), for existing substances by "CEPA 1999 and the Assessment of Existing Substances" and for new substances by "CEPA 1999 and the Assessment of New Substances". Environment Canada is responsible for assessing the risks of existing substances. About 23,000 substances are on the existing list and are produced at a quantity of >100 kg per year in Canada. All other substances that are used, imported or manufactured in Canada are covered by the new substance list.

The assessment of substances includes three phases: categorization, screening assessment and in-depth Priority Substances List (PSL) assessment. Multiple lines of evidence are used to assess a substance. For UVCBs empirical data is rarely available for all constituents and therefore only significant constituents of the substances are assessed. In the first phase substance identity is carried out. If the exact structures of the constituents can not be identified representative structures are selected. In the next phase the physical-chemical properties, persistency, bioaccumulation and toxicity data are collected for the constituents or the representative structures. This is followed by a PBT assessment of experimental data and model predictions.

NICNAS, Australia

The Australian Government Department of Health, National Industrial Chemicals Notification and Assessment Scheme (NICNAS) assess the risks of industrial chemicals on occupational exposure, the public health and the environment. For these assessments they use internationally methodologies which are International Programme on Chemical Safety, 1999 and the European Commission, 2003. The risk assessment include hazard identification, hazard assessment, incorporating the dose-response relationship, exposure assessment, and risk characterisation. Assessment are conducted case-by-case and based on a weight-of-evidence. No specific Australian

guidance was found for UVCB substances, however, chemicals assessed can have the label UVCB.

RIFM/ECETOC Workshop

On 2-4 November 2016 in Orlando (USA) an international workshop was organized by RIFM and ECETOX to discuss the development of a strategy to improve the environmental risk assessment of difficult to test multi-component substances. This group consisted of regulatory authorities, research institutes, and industries. The aim of the workshop was to develop appropriate risk assessment and testing methodologies applicable to the assessment of UVCBs. These substances present a challenge to industry and regulatory authorities both in their substance composition, fate and ecotoxicological testing. The results were presented as posters at SETAC Orlando 2016 (Cesnaitis et al., 2016a, 2016b). The posters discussed the risk assessment approaches of UVCBs but also the testing methods of UVCBs (e.g. WAF, biodegradation tests, etc.).

Conclusions

In general, it shows that all UVCB assessment approaches follow a case-by-case and weight-of-evidence procedure. The most detailed approach is provided by ECHA, however, in all approaches limited guidance is provided on testing methods, which is essential to test UVCBs.

3 Concept generic information strategy for PBT and vPvB assessment

The concept of a generic information strategy is based on ideas from guidance documents from the ECHA, US EPA, Environment Canada, and OECD, and practical application of these guidance by industry, and state of the science as will be discussed in this chapter. In all of these documents a number of generic steps are carried out to collect data for the PBT assessment. In general, two lines of evidence are followed, which can simultaneously or in parallel be explored. The first line is **constituents approach** and the second a **substance based** approach (Fig. 1).

A key element in the first constituents approach is the identification of the constituents of the UVCB. This step is followed by grouping (blocking) of constituents, and the selection of representative structures for each block before the PBT assessment is carried out. The first approach consists of the following steps:

- Charaterisation and identification of the constituents in the UVCB substance
- Grouping of constituents (blocking)
- Selection of representative structures of each block
- Data collection, including *in silico* predictions, of PBT/vPvB properties of the representative structures
- Experimental PBT data of representative structures
- PBT assessment of the in silico and experimental data

In the second substance approach the UVCB substance as a whole is tested for PBT characteristics. Additionally, the UVCB substance can be fractionated to reduce the complexity of the substance and to obtain more detailed information on specific fractions of the UVCB.

Both the constituents and substance approach will lead to a final PBT/vPvB assessment. However, the constituents approach will provide more detailed information on the PBT properties of the constituents than the substance approach. An advantage of the substance approach is the ability to study mixture toxicity.

A schematic diagram of the concept with the different steps is shown in Figure 1. Each of the approaches and steps will be discussed in more detail and illustrated with case studies on chlorinated paraffines and petroleum hydrocarbons.

3.1 Constituents approach

3.1.1 Characterisation of constituents in UVCB substances

There are a number of issues with the PBT assessment of UVCBs. One major issue is the chemical complexity of the UVCB substance and consequently the challenges in the determination of the composition and identify of constituents in the mixture. In general, not all constituents can be identified and often only generic structures can be

determined without exact chemical structure. Other criteria to be included in the characterisation of the UVCB are the concentration range and typical concentrations.

Still, identification is a key factor in the assessment of UVCBs. In this section the state-of-the-art on analytical techniques that are helpful in the identification process are briefly discussed. The feasibility of these techniques, if they are commercially available or only accessible at the research level are discussed as well.

The ECHA guidance document declares that spectral (ultra-violet, infra-red, nuclear magnetic resonance or mass spectrum) and analytical (high performance liquid chromatogram (LC), and/or gas chromatogram (GC)) data of the UVCB constituents should be provided. The current analytical techniques are able to identify a broad range of chemical structures using a combination of separation techniques (chromatography) with spectral detection techniques. However, note that a standardized method is not available for UVCB characterisation and a large effort and expertise is needed to develop methodologies.

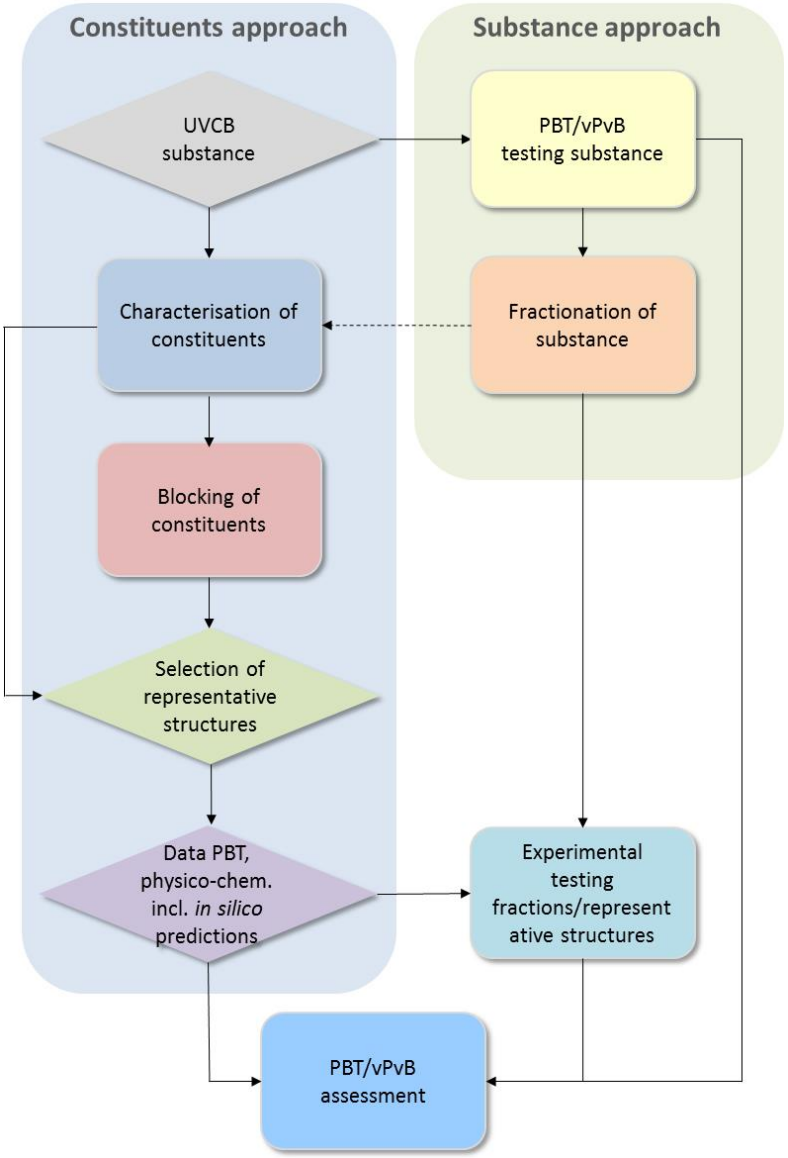

Figure 1: Conceptual framework to PBT/vPvB assessment of UVCB substances.

In the last decade significant developments in analytical techniques for the identification of organic chemicals especially in the field of mass spectrometry occurred. These techniques enhanced the fields of research on identification of unknown substances (non-target screening) in the indoor and outdoor environment, but also in the field of unknown endogenous substances in organisms and plants. Most progress has been made in the field of high resolution mass spectrometry (HRMS) which can be used for the structure elucidation of compounds. Modern HRMS instruments, such as Time-of-flight (TOFMS) and Orbitrap MS, are powerful and sensitive techniques to determine accurately the mass of organic molecules which in turn can be used to predict the elemental composition. In combination with LC or GC and sophisticated software data mining of unknowns become efficient and very powerful. These techniques are commercially available and used in many laboratories worldwide.

For very complex mixtures such as petroleum hydrocarbons or chlorinated paraffin's comprehensive two dimensional chromatrography techniques are used to increase the separation power. In the petroleum industry two dimensional gas chromatography (GCxGC) is applied (Bloomberg et al., 2004; Forbes et al., 2006). Nowadays, for more polar and thermal label compounds two dimensional liquid chromatography can be used (LCxLC) (e.g. Ouyung et al., 2015 and 2016). The two dimensional techniques are able to separate thousands of compounds especially in combination with mass spectrometry. The advantage of GCxGC or LCxLC for the characterisation of UVCBs when using the correct analytical settings is to generate structured chromatograms where generic structures are displaced as roof tiles. An example for chlorinated paraffines showing groups of chlorination for the C_{10} alkane is shown in Figure 2. These analytical approaches could be of help to find generic representative structures.

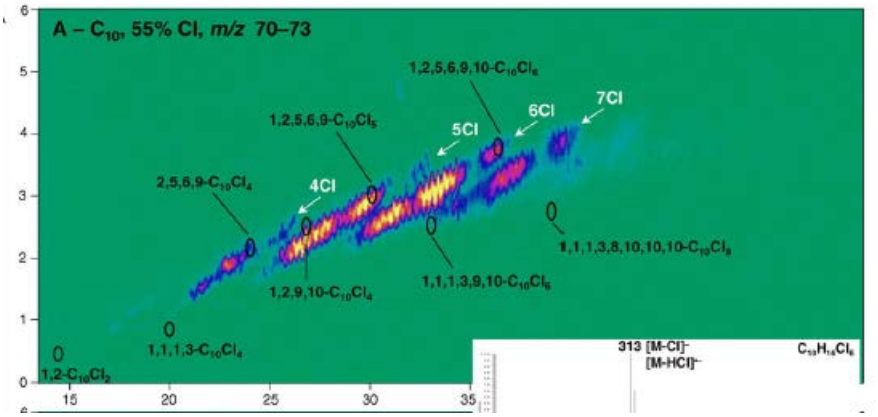

Figure 2: GCxGC-ECD chromatogram of chlorinated C_{10} 55% Cl (picture from Korytar et al., 2005).

Case study chlorinated paraffines: characterisation of constituents

In this section a case study is discussed to show the challenges with the characterisation of chlorinated paraffins (CPs). It will also show the state-of-the-art analytical capabilities to identify constituents in CP substances.

CPs are complex mixtures consisting of thousands of congeners with varying carbon length and chlorine atoms, and therefore a UVCB. CPs are widely used in various applications such as lubricants in metal-cutting industry, flame retardants, plasticizers, rubbers, paints, sealants (Muir et al., 2000). The general formula for CPs is $C_nH_{2n+2-z}Cl_z$. Commercial CPs mixtures are divided by their chain length in 3 groups, the short-chain CPs (SCCPs) (C_8-C_{13}), median-chain CPs (MCCPs) (C_{14}-C_{17}) and long-chain CPs (LCCPs) (>C_{18}) with a chlorination degree that varies from 30-75% (De Boer at al., 2015). CPs are high production volume chemicals with a global production of more than 1000 ktons $year^{-1}$ in 2009 (van Mourik et al., 2015). Evidence show that SCCPs are persistent, have potential to bioaccumulate (Zeng et al., 2011; Stevens et al., 2003), toxic to aquatic organisms (Shaw at al., 2010; El-Sayed et al., 2010) and occur in remote areas (Van Mourik et al., 2016) and therefore the SCCPs has been listed under the Stockholm Convention as Persisted Organic Pollutants (POPs). The European framework directive included the SCCPs on the list to be monitored in water. Relevant data on the potential hazard, fate and the levels of MCCPs and LCCPs in the environment are scarce to facilitate international regulations. One of the main reasons for the lack of data on CPs is the limitation regarding sufficient analytical methods and suitable quantification standards.

It is extremely challenging to identify the constituents in CP mixtures and also to quantify CPs. Until today separation of hundreds to thousands of CP congeners can be achieved using multi-dimensional separation techniques, see later, however the exact structure configuration of the chlorine atoms cannot be achieved only generic structures based on the carbon number and number of chlorine atoms. For example all C_{10} isomers with 7 chlorines can be separated and grouped (e.g. Korytar et al. 2005; Xia et al., 2014; Bogdal et al. 2015).

Another challenge in CP analysis is the quantification of CPs, which is highly important for persistency and bioaccumulation tests. Currently, only semi-quantitative analyse is possible due to a lack of proper analytical standards. The analytical standards available do not match the chlorine configuration of the constituents present in the technical CP mixtures. Commercial standards contain chlorine atoms at the end positions of the carbon chain while the CPs present in the technical mixtures mainly have chlorines in the middle of the chain. For many years commercial companies try to synthesize individual CPs with chlorine atoms in the mid position but this has not been successful yet. The importance of these standards is reflected in the large differences in detector responses between CP congeners. Analytical standards should therefore match the chlorine substitution of the technical mixtures.

Quantification nowadays is done with commercial available technical CP mixtures. However, only limited number of analytical CP mixtures with different chain length and chlorine content are commercially available. In contrast, over the last 10 years globally more than 200 CP formulations have been used in various application (Zeng et al., 2005). Each of these formulation contain CPs with different carbon length and chlorination degree. Also during weathering or biotransformation the carbon length and chlorination degree of the technical CP mixtures may changes. Therefore is it rather challenging to find commercial technical CP mixtures that are suitable for all CPs in environmental samples. For example a differences in the CP chlorine content between mixture and sample can result in a variation of > 100% (Van der Veen et al. 2015) when the most often used technique, electron capture negative ionization mass spectrometry(ECNI-MS), is used (Van Mourik et al., 2015). This technique shows an

increased response factor of CP congeners with increase chlorine content, and compensation for this increase is need, i.e. good match with analytical CP mixtures. A promising compensation technique was developed (Reth et al., 2005) whereby no chlorine matching pattern is needed between the standards and sample, however still suitable standards are needed to quantification and improve the quality of the reported data (Van Mourik et al., 2015). The results of a first interlaboratory study showed that the variation in the analysis of a standard solution variety between 22-56% (van der Veen et al., 2012). If real life samples (fish extract) were analysed the variation increased to 137% and the results felt in two groups with had difference of about 10-fold in concentration. This variation is probably due to the different types of analytical techniques used to quantify CPs.

Over the years many different analyse techniques have been developed for the quantification of CPs. Gas chromatography (GC) coupled with high resolution MS (HRMS) (Tomy wet al., 1997) and low resolution MS (LRMS) (Reth et al., 2005) operating in the ECNI mode are the most common techniques used for routinely quantification SCCPs. A disadvantage of using ECNI-MS is that it is sensitive for SCCPs but sensitivity decreases for MCCPs, and LCCPs are almost undetectable. Also lower chlorinated paraffins ($<Cl_5$) are hardly detectable with ECNI-MS (Zenack et al., 2005). Lower chlorinated paraffins are detectable with electron capture detection (ECD) especially in combination with GC x GC (Korytar et al., 2005; Xia et al., 2014). GCxGC-ECD is a powerful tool to separate CP isomers and this technique is also able to detect lower chlorinated CPs (Cl_2-Cl_4). Still it has not been employed by many laboratories which may be because of the relative complicated optimization and time consuming quantification (Van Mourik et al., 2015). GC x GC has also been successfully used in combination with ECNI- time of flight (TOF)-MS by Korytar et al. (2005). With GC x GC-ECD and GC x GC-ECNI-TOFMS SCCPs, MCCPs and LCCPs could be analysed in one single run. However, with GC x GC-ECNI-TOFHRMS lower chlorinated paraffins ($<Cl_5$) are still hardly detectable.

Recently Bogdal et al. (2015) developed a promising analytical method using liquid chromatography (LC) coupled to atmospheric pressure chemical ionization (APCI) – TOFHRMS. Without analytical column the extract was directly injected into the qTOF-HRMS followed by quantification of CPs by mathematical deconvolution using technical CP mixtures. The advantage is that SCCPs, MCCPs and LCCPs can be quantified in one single run. This analytical method seems to be a promising alternative for the GC-ECNI-HRMS method as also lower chlorinated paraffins (Cl_2-Cl_4) could be detected.

3.1.2 Blocking of constituents

To reduce the complexity of an UVCB substance CONCAWE introduced a grouping of constituents approach for petroleum hydrocarbons, the Hydrocarbon Block Method (CONCAWE, 1996). This approach was used to perform an environmental risk assessment of petroleum substances. The hydrocarbon block method was accepted by the European Commission and implemented in the EU TGD for risk assessment (EC, 2003). The underlying principle of the hydrocarbon block method is grouping of constituents with similar physico-chemical and fate properties. This grouping should result in blocks of constituents with similar fate and hazard properties. Beside this blocking approach two other approaches are possible. Blocking based on groups of closely related chemicals or similar mode of action.

To be able to perform blocking analytical characterisation data consisting of known structures and generic structures of the constituents is of high importance. For most UVCB substances generic structures of constituents can be generated.

For example, the generic structures of petroleum substances are based on hydrocarbon types. Blocks are made on these generic structures in combination with properties such as chain length, degree of branching, or boiling point (Table 1). Other options to block constituents could be polar and non-polar functional groups in relationship to chain length (e.g. surfactants), chain length in combination with the presence and position of heteroatoms for instance chlorine (e.g. chlorinated paraffins), presence and number of aromatic rings, and number and position of different functional groups.

Table 1: Example of hydrocarbon blocking scheme. n-P: normal paraffin; i-P: iso paraffin; MoN: monoNaphthenic s; DiN: diNaphthenic s; PolyN: poly-Naphthenic s; MoAr: Mono-aromatics; DiAr: di-aromatics; DiAr: di-aromatics; PolyAr: polyaromatics; NMAr: Naphthenic mono-aromatics; NDiAr: Naphthenic di-aromatics.

Block	Carbon no.	n-P	i-P	MoN	DiN	PolyN	MoAr	DiAr	PolyAr	NMAr	NDiAr
1	4										
2	5										
3	6										
4	7										
5	8										
6	9										
7	10										
Etc.	Etc.										

Independent how groups or blocks are generated a scientific justification of the blocking scheme is needed. Additionally, technical issues related to the block scheme can be part of the justification. For instance, not all petroleum hydrocarbons in the analysis of petroleum products are well separated from each other and therefore can be grouped in a single carbon block. For the analysis of petroleum hydrocarbon analysis GCxGC is used. This is a very power technique to characterize in detail the hydrocarbon mixtures. Individual constituents can be separated based on carbon number and chemical functionality. However, some hydrocarbon blocks can overlap. Therefore, the aliphatic hydrocarbons are separated from the aromatic hydrocarbons before GCxGC analysis. This makes quantification possible for a large group of hydrocarbon blocks. Still, constituents in some blocks are more difficult to identify and separate from other blocks. One of these are the poly-naphthenics. Only limited information on the separation of poly-naphthenics (e.g. steranes, triterpanes, and triaromatic steranes) is available (Frysinger and Gaines 2010). Which is partly due to limited number of analytical standards available. These standards show that overlap can occur in constituents between poly-naphthenic blocks, e.g. some C_{21} poly-naphthenics elute in the retention area of C_{20} poly-naphthenics (CONCAWE, 2010). For the PBT assessment the C_{20} and C_{21} poly-naphthenics can therefore be grouped in one block.

The above block approach is based on physico-chemical and fate properties and less on similarity in hazard characteristics (mode of action) of constituents. Hazard characteristics are more related to functional groups or the position of groups/atoms

on molecules. Therefore, an alternative block approach, not used so far, could be based on hazard properties (mode of action) of the constituents. In this case *a priori* hazard property information of individual constituents is needed which requires the exact structure identify of the constituents. It also requires, for a specific mode of action, the relative equivalent potencies (REP) of these constituents. Experimental or (Q)SARs generated REP values in combination with information on the occurrence of the constituents in the UVCB substance can finally result in specific mode of action based blocks, generating a toxicity profile of the substance. The feasibility of this approach depends on i) the exact structures of the constituents, ii) experimental data on the mode of action, or iii) suitable (Q)SARs for the modes of action. This shows already the challenges in achieving such approach as for most UVCBs individual constituent data is not available. One option could be to use a marker constituent if the toxicity of an UVCB is driven by this marker (OECD, 2007).
To provide a feeling of the hazard based block approach an example is given for polychlorinated biphenyls (PCBs), a substance with potentially 209 different constituents. The position of chlorine atoms of PCBs is strongly linked to the mode of action. Substituents with chlorines at the non-ortho and mono-ortho position show dioxin-like toxicity, while di-ortho congeners does not. REP values for all non- and mono-ortho congeners are available and therefore a dioxin-like toxicity block can be formed. If blocking would have been based on the carbon number in combination with the chlorination degree dioxin-like CBs would have been separated over different blocks that included constituents without dioxin-like toxicity. The feasibility of a hazard based blocking is possibly an approach for the future when (Q)SARs for hazard assessment are further established. Especially the data generated by the US EPA ToxCast program and the (Q)SAR toolbox of OECD could be of great interest. ToxCast has screening information over 9,000 chemicals and information from more than 1,000 high-throughput assay endpoints. The OECD toolbox includes (Q)SAR models and read-across to predict physico-chemical properties, environmental fate and transport, ecotoxicological hazards, and human health hazards.

One other aspect of the block approach not yet discussed is the resolution of the blocks. In general care should be taken in the resolution of the blocks. For petroleum hydrocarbons the smallest block size is the single carbon number and the carbon type, as shown in Table 1. To assess the uncertainty and variation of the blocking scheme an uncertainty analysis was carried out for petroleum hydrocarbons by van de Meent (2008). This study investigated the environmental fate factors and human intake fractions for exposure and risk calculations using the hydrocarbon block method. Fate and human intake factors were based on QSAR predictions. One of the factors studied was the block resolution on the outcomes of the Risk Coefficient Ratio (RCR) (PEC/PNEC ratio). Two types of block resolution for the carbon number/boiling point range were tested. Low resolution consisting of two chemical classes (aliphatic or aromatic) and seven boiling points. The high resolution scenario used twelve chemical classes (comparable to Table 1) and thirteen boiling classes. The results showed that the uncertainty factors of the block means for fate and intake fractions were not significantly different between the low and high resolution scenarios. This means that a higher resolution did not give significantly improved risk calculations than the low resolution scenario. Van de Meent concluded that the hydrocarbon block scheme was close to the point of minimum estimation error and was recommended to be applied for the risk assessment of petroleum substances.

For PBT assessment principally it is advisable to set-up a block scheme with the highest resolution possible as this will improve the assessment by enhancing the which blocks (constituents) that fulfil the PBT criteria. If blocks are too wide similarities in fate or bioaccumulation properties will disappear and constituents in a block may be wrongly classified. This can be shown by plotting the $LogK_{ow}$ values of for example mono-aromatics, di-aromatics, naphthenic mono-aromatics, and naphthenic di-aromatics (Fig. 3). $LogK_{ow}$ values are from the CONCAWE Library. This figure shows that for each class with increasing carbon number, and consequently an increasing complexity of the molecular structures, the K_{ow} values increase. It also shows that overlap exists in K_{ow} between blocks. If some blocks would have been combined to one block, for instance C_{16} to C_{18} di-aromatics, the average K_{ow} would have been increased with 0.5 units, and therefore may be misclassifying some constituents. It is therefore recommended to group the constituents in individual carbon number groups.

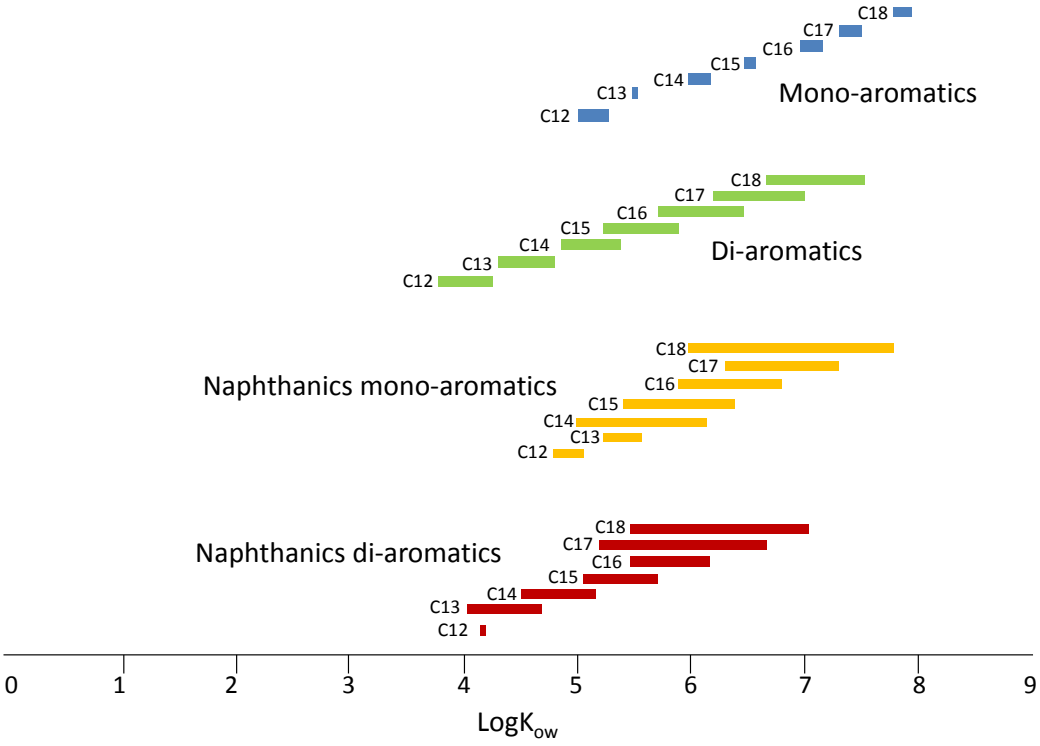

Figure 3: Variation of predicted $LogK_{ow}$ (CONCAWE library) of hydrocarbons (mono-aromatics, di-aromatics, naphthenic mono-aromatics, and naphthenic di-aromatics). Each line represents a hydrocarbon block.

3.1.3 Selection of representative structures and *in silico* predictions

Selection of representative structures for each block is the next step before PBT properties are collected, predicted, or experimentally determined. Selection can be based on analytical data availability, production process/sources, literature, by expert judgement, or by *in silico* predictions. For petroleum hydrocarbons representative structures were based on basic structure information (Quann, 1998) and over 1500 structures (C_4-C_{41}) were described in the CONCAWE hydrocarbon library. This library is also used in the PetroTox model to predict the toxicity of hydrocarbons. This library did not contain hetero-hydrocarbons (N and O) except sulfur (Rorije et al., 2012).

Therefore, CONCAWE performed recently a study on the occurrence of nitrogen, oxygen and sulfur containing heterocyclics in petroleum products (CONCAWE, 2016). Two dimensional GC in combination with element selective detectors was used (Bloomberg et al. 2002, 2004). Eleven different sulphur and nitrogen classes of hydrocarbons were identified in petroleum products. No O-containing constituents were found (<0.1%), whereas the mean percentage occurrence of S-heterocyclics was 0.58%, and total N-heterocyclics only 0.02%. Representative structures of the S-heterocyclic hydrocarbons should be added to the CONCAWE library so that they can be assessed for PBT.

Another approach to generate representative structures is computational methods. This approach has been described by Dimitrov et al. (2015) and uses the generic structures of the UVCB. The system was specially developed for UVCB substances and is a systematic approach to generate structures of constituents of the UVCB. The methodology is not only able to generate constituents but also to select representative structures (this can be user controlled). The model uses Generic Simplified Molecular-Input Line-Entry System (G Smiles) and Generic Graph (GH Graph) formats. The generated constituents data can be used as input to other models.

If representative structures have been generated the first tier would be to predict physico-chemical properties and use these in (Q)SAR models to predict the fate, toxicity and bioaccumulation models such as the OECD Toolbox. An important aspect is to check if the (Q)SAR model domains fit the constituents domains. Based on the (Q)SAR predictions and physico-chemical properties representative structures could be selected for further experimental studies. This approach will reduce animal testing. The experimental studies form a second line of evidence in the PBT assessment.

3.2 Substance approach

The second approach to assess PBT properties of an UVCB is testing of the whole substance, which can be followed with the fractionation of the substances. The fractions can subsequently be tested on PBT properties. One of the issues with testing UVCBs as a substance is the challenge in testing methods. Many UVCBs have a hydrophobic character and are therefore difficult to dissolved in the exposure medium. Often the water accommodated fraction (WAF) approach is used to dissolved UVCBs compounds in water for aquatic toxicity testing (OECD, 2000). The WAF procedure for petroleum products have been described by Girling (1989), Tadokoro et al. (1991) and CONCAWE (1992). The WAF is only used for substances that are not fully soluble in water. Briefly, the method consists of the addition of the test substance to water followed with mixing for an extensive period and a settling phase. Only the fraction that is dissolved is removed and used for testing. The formation of dispersions and emulsions should be avoided. The mixing duration and energy are important parameters and will influence the composition and particle size. For each loading in a toxicity test a separate WAF should be made as the dissolved concentration is dependent on the loading; constituents which are less soluble may not change with different loadings but the loading of more soluble constituents will. The loading can be used for dose-response relationships which is similar as the nominal concentration used in toxicity test. Determination of the concentrations of the constituents in the WAF would be a more appropriate way to determine a dose-response relationship, but is more difficult to establish for UVCBs. The WAF method has a number of advantages

such as i) inclusion of mixture toxicity effects, ii) takes into account differences in water solubility of multi-constituents mixtures, and iii) the procedure can be standardized. The method also has a number of disadvantages. It is a challenge to maintain stable water concentrations and consequently keep the composition proportional to the start conditions. Hydrophobic constituents will bioaccumulate more than less hydrophobic constituents in the test organism which will change the composition pattern in the water, and therefore depletion of specific constituents can occur.

An alternative approach to the WAF is passive dosing (e.g. Smith et al., 2010, Brown et al., 2001, Kiparissis et al., 2003, Mayer and Holmstrup, 2008 and Turcotte et al., 2011). The principle is based on the partitioning of a compound between a solvent and a reservoir material, this is often polydimethylsiloxane (PDMS) and this reservoir is then is used to load the test system. The loaded compound is partitioning between the loaded PDMS into the test medium and keeps the concentration of exposure constant during the test despite losses due to sorption, bioaccumulation, biotransformation etc. This system has been used for many applications such as polycyclic aromatic hydrocarbon exposure and toxicity analysis of various systems (e.g. Smith et al., 2013; Vergauwen et al., 2015; Oostingh et al., 2015; Stibany et al., 2017). There is only one study that used passive dosing for an UVCB (Heger et al., 2016). This study used passive dosing for *in vitro* toxicity (cytotoxicity and AhR activitiy) testing of refinery products (gasoline, diesel and biodiesel) and compared the method with the WAF approach(Heger et al., 2016). This study concluded that the test concentrations were within water solubility. Another advantage of passive dosing was that concentration of hydrocarbons where more stable during the test period. However, the analysis method to determine the hydrocarbon concentrations needs further attention. Another point not discussed is that UVCB constituents can have a wide range of K_{ow} values, depletion of the loaded PDMS material for the more water soluble constituents could therefore occur and needs further attention. Passive dosing is certainly an interesting option but has mainly been used for single compound exposure. Further research should focus on passive dosing of complex mixtures including UVCB substances with a wide range of physio-chemical properties. Passive dosing should also study in more detail the composition in the loading system and toxicity medium.

3.2.1 Fractionation of UVCB substance

Already in the 1970's group type fractionation of petroleum substances was performed by HPLC (e.g. Suatoni and Garber, 1976). They were only able to separate a limited number of hydrocarbons groups. Today, the separation power of LC columns is much improved and a larger number of hydrocarbons groups can be separated. Still, it is impossible to have the same resolution of separation as in the characterisation phase as LC or GC separation is complemented with the power of mass spectrometry for the identification of constituents.

Examples of analytical techniques to fractionate petroleum substances are LC or GC chromatography but also distillation is an option for more volatile constituents. These techniques are usually used in a low resolution option and only a limited number of fractions are collected. For petroleum hydrocarbons often open column chromatography or HPLC is used and less than 10 fractions are generally collected

(e.g. Mao et al., 2008; Sutton et al., 2005; Vrabie et al., 2012). Fractionation of petroleum hydrocarbons is based on hydrophobicity and aliphatic, aromatic, polar or resin fractions are collected to reduce the complexity of the mixture before detailed hydrocarbon characterisation is performed by LC-MS, GC-MS or GCxGC-ToFMS. In some studies fractionation was used to get more insight in the constituents that can bioaccumulate or cause the toxicity (e.g. Jonker et al., 2016; Vrabie et al., 2012). In these studies four fractions (saturates, aromatics, resins, asphaltenes) of crude and refined oils spiked sediment were tested for the potency of bioaccumulation using an oligochaete worm. The internally accumulated compounds were extracted and tested with *in vitro* assays (aryl hydrocarbon receptor (AhR) agonist) (Vrabie et al., 2012). The most important fractions were further fractionated to reveal more information on the constituents that had the potential of persistency, bioaccumulate, and caused the toxicity. A two-step fractionation procedure was also performed by Radovic and co-workers (2014) of North Sea crude oil and residual heavy fuel oil. An open column fractionation (aliphatics, aromatics and polars) was used as a first step followed by a HPLC fractionation of only the aromatics in 15 fractions. Fractions were tested with *in vitro* assays for AhR agonist and androgen receptor (AR) antagonist. These studies showed that fractionation of a substance provides further insight in the hazard profile of petroleum constituents. Linking the observed effects to specific constituents is still difficult due to the complex mixture of fractions. The relevance of these studies for the risk assessment needs further consideration but it provides further evidence on the complex issue of mixture toxicity.

Today, high-resolution fractionation is possible to further unravel the complexity of UVCB substances using ultra high performance liquid chromatography (UPLC) columns (Booij et al., 2014; Jonker et al., 2015). Fractions are collected from the UPLC column in 96 wells, which shows the increased resolution of fractionation (fractions of less than 10 seconds are collected). The 96 well plate can further be used for toxicity testing using a cell seeding approach. The feasibility of this fraction technique in combination with toxicity testing (algae or *in vitro* assays) has been shown for environmental samples recently (Booij et al., 2014; Jonker et al., 2015).

The latest developments shows that even two dimensional LC (LCxLC) fractionation is feasible (Ouyang et al., 2016; Brown et al., 2016). The feasibility of this technique has been shown for fractionation of effluent water from a waste water treatment plant in combination with an acetylcholinesterase inhibitor assay.

Another option to fractionate petroleum substances and isolate constituent is preparative GC where fractions from the GC outlet are collected by trapping (Sutton et al., 2005); only a restricted number of fractions can be collected). In this study preparative LC fractionation was preceded before preparative GC. The latest developments shows that high resolution fractionation by GC is also possible (Pieke et al., 2013). This technique is able to collect fractions of the complete GC chromatogram, and is currently only available at the research level. Fractions of a few seconds are collected in 96 well plates, before bioassay testing. The feasibility of this methods has been shown for environmental samples using an *in vitro* AhR assay (Pieke et al., 2013).

To show the feasibility of fractionation methods for PBT testing an estimation of the amount of constituents collected after LC or GC fractionation can be made. We assume the following situation. An UVCB substance is fractionated in 100 fractions and the constituents are equally divided over the fractions. A concentration of 1 g/l is injected

with HPLC and fractionated. A normal volume to inject for a HPLC column is 10 µl which is equivalent to 10 µg per injection, resulting in 0.1 µg of constituents in each fraction. If the fractionation is repeated 100 times, which is possible due to the high repeatability of the current LC systems, a total of 10 µg per fraction is collected. The 10 µg is than available for persistency, bioaccumulation or toxicity testing.

4 Conclusions and recommendations

In general, a UVCB assessment is done a case-by-case basis as it requires more sophisticated analytical techniques and testing protocols.

The proposed concept of a generic information strategy for PBT assessment of UVCB substances is based on two lines: a **constituents approach** and a **substance approach**. The constituents approach requires:

- Characterisation of the UVCB
- Blocking of constituents with similar properties
- Selection of representative structures for each block
- Collection, prediction and/or experimental data of PBT properties of the representative structures

For most UVCB substances generic structures of the constituents can be generated. It is suggested that these generic structures can be used as a first tier assessment. The generic structures can be used for blocking constituents with similar properties and to select representative structures for each block. The predicted PBT data of these representative structure can function as a first tier to steer the experimental studies of the PBT assessment.

In the substance approach the substance is tested as a whole which sets a number of methodological challenges such as solubility issues as many UVCB has a hydrophobic character. One option is to fractionate the substance in fractions before experimental P, B or T testing.

There are an number of challenges in the UVCB assessment:

- A key aspect is related to the methodologies used to test the substances.
- Another aspect is the uncertainties in model predictions of constituent data.
- An third aspect is the data interpretation at the regulatory site as the assessment is based on a case-by-case study and weights of evidence approach.

A key challenge of UVCB substance assessment is related to the methods used to test persistency, toxicity and bioaccumulation. Many UVCBs are difficult to dissolve in water or cover a very broad range of polarities (from polar to non-polar). Novel testing strategies should be developed.

Models are used to predict the physico-chemical, fate, bioaccumulation and toxicity properties of the constituents but how well do the model fit the application domains? The model should be fit for purpose, and the uncertainties in predictions and variation between models should be further evaluated. Extension of the models for specific mode of action is required.

To gain the momentum of the assessment of UVCBs it is highly important to involve multi-stakeholders (regulators, industry and academia) in the process of the concept development and testing strategies as they can provide different angles to the process. It is recommended to discuss and align the framework between regulatory bodies (e.g. ECHA, US EPA, Environment Canada, etc.).

5 Acknowledgements

I would like to acknowledge Fleur van Broekhuizen, Emiel Rorije, Pim Wassenaar, Eric Verbruggen, and Joop de Knecht from RIVM for the discussions on the UVCB PBT assessment. Dan Salvito (RIFM) for the discussions on the UVCB PBT assessment concept.

6 References

Blomberg, J. et al. 2002. Gas chromatographic methods for oil analysis. J Chrom. A 972, 2, 137-173.

Blomberg, J. et al. 2004. Comprehensive two-dimensional gas chromatography coupled with fast sulphur-chemiluminescence detection: implications of detector electronics. J Chrom. A 1050, 1, 77-84.

Bogdal, C., Alsberg, T., Diefenbacher, P.S., MacLeod, M., Berger, U., 2015. Fast quantification of chlorinated paraffins in environmental samples by direct injection high-resolution mass spectrometry with pattern deconvolution Anal. Chem., 87, 2852–2860.

Booij, P., Vethaak, A.D., Leonards, P.E.G., Sjollema, S., Kool, J., Voogt, P. de & Lamoree, M.H. 2014. Identification of Photosynthesis Inhibitors of Pelagic Marine Algae Using 96-Well Plate Microfractionation for Enhanced Throughput in Effect-Directed Analysis. Environ. Sci. Technol., 48(14), 8003-8011.

Brown. T.A., Jacksona, B.A., Bythellb, B.J., Stensona, A.C. 2016. Benefits of multidimensional fractionation for the study andcharacterization of natural organic matter. J. Chrom. A, 1470 (2016) 84–96.

Cesnaitis, R., de Knecht, J., Eisenreich, K., Embry, M., Fernandez, M., León-Paumen, M., Jenner, K., Lyon, D., Dan Salvito. D., 2016a. Developing a strategy to improve the ERA of difficult to test multi-component substances Part 1: Background. Natural Complex Substances (NCS)

Cesnaitis, R., de Knecht, J., Eisenreich, K., Embry, M., Fernandez, M., León-Paumen, M., Jenner, K., Lyon, D., Dan Salvito. D., 2016b. Developing a strategy to improve the ERA of difficult to test multi-component substances Part 2: Workshop Output. Natural Complex Substances (NCS)

Chen, M.Y., Luo, X.J., Zhang, X.L., He, M.J., Chen, S.J., Mai, B.X., 2011. Chlorinated paraffins in sediments from the Pearl River Delta, South China: spatial and temporal distributions and implication for processes Environ. Sci. Technol., 45, 9936–9943.

Clark, C.R, McKee, R.H., Freeman, J.J., Swick, D., Mahagaokar, S., Pigram, G., Linda G. Roberts, L.D. Smulders, C.J., W. Beatty, P.W. 2013. A GHS-consistent approach to health hazard classification of petroleum substances, a class of UVCB substances. Regulatory Toxic. and Pharm. 67, 409–420.

CONCAWE. 1992. Ecotoxicological testing of petroleum products: test methodology. CONCAWE report 92/56.

CONCAWE. 1996.environmental risk assessment of petroleum substances: the hydrocarbon block method. CONCAWE report no. 96/52.

CONCAWE, 2010. CONCAWE effluent speciation project. CONCAWE report no. 3/10.

De Boer, J., El-Sayed Ali, T., Fiedler, H, Legler, J., Muir, D.C.G., Nikiforov, V.A., Tomy, G.T., Tsunemi, K., 2010. Chlorinated paraffins. The handbook of Environ Chemistry, vol 10, Springer, Berlin, Heidelberg.

Dimitrov S.D., Georgieva, D.G., Pavlov, T.S., Karakolev, Y.H., Karamertzanis P.G., Rasenberg, M., Mekenyan, O.G. 2015. UVCB substances: methodology for structural description and application to fate and hazard assessment. Environ Toxicol Chem. 34(11):2450-62.

EC. 2003. Technical Guidance Document on Risk Assessment in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances, Directive 98/8/EC of the European Parliament and of the Council

concerning the placing of biocidal products on the market. European Commission Joint Research Centre, Ispra, Italy, EUR 20418 EN/1.
ECHA, 2012. Draft Guidance on information requirements and chemical safety assessment Chapter R.11: PBT and vPvB Assessment. ECHA reference: ECHA-12-G-24-EN.
El-Sayed, T., Legler, J., 2010. Overview of the Mammalian and Environmental Toxicity of Chlorinated Paraffins. in: de Boer, J. (Ed.). Chlorinated Paraffins, pp. 135-154.
ESWI, 2011. ESWI Study on Waste Related Issues of Newly Listed POPs and Candidate POPs. Consortium ESWI (Bipro, Umweltbundesamt and Enviroplan) for the European Commission.
Frysinger, G.S. and Gaines R.B. 2001. Separation and identification of petroleum biomarkers by comprehensive two-dimensional gas chromatography. J. Separation Sci. 24, 2, 87–96
Forbes et al. 2006. Application of comprehensive two-dimensional as chromatography (GCxGC) for the detailed compositional analysis of gas oils and kerosines. Poster presentation, SETAC Europe, Annual Meeting, The Hague.
Girling, A.E. 1989. Preparation of aqueous media for aquatic toxicity testing of oils and oil-based products: a review of the published literature. Chemosphere, 19(10/11), 1635-1641.
Gramatica, P. 2007. Principles of QSAR models validation: internal and external. QSAR Comb. Sci. 26, 2007, No. 5, 694 – 701.
Heger, S.,Bluhm, K., Brendt, J., Mayer, P., Anders, N., Schäffer, A., Seiler, TB., Hollert, H. 2016. Microscale In Vitro Assays for the Investigation of Neutral Red Retention and Ethoxyresorufin-O-Deethylase of Biofuels and Fossil Fuels. Plos One, sept, 1-24.
Houde, M., Muir, D.C.G., Tomy, G.T., Whittle, D.M., Teixeira, C., Moore, S. 2008. Bioaccumulation and trophic magnification of short-and median-chain chlorinated paraffins in food webs from lake Ontario and Lake Michigan. Environ. Sci. Technol. 42, 3893-3899.
Howard P., Meylan W., Aronson D., Stewart S., Parkerton T., Comber M. 2006. Prediction of Environmental Fate and Transport Properties in Support of the Hydrocarbon Block Approach to Risk Assessment, Society of Environmental Toxicology & Chemistry, 8-11 May 2006, The Hague, The Netherlands.
Jonker, W., Lamoree, M.H., Houtman, C.J., Hamers, T., Somsen, G,.W., Kool, J. 2015. Rapid activity-directed screening of estrogens by parallel coupling of liquid chromatography with a functional gene reporter assay and mass spectrometry. J. Chrom. A., 1406, 165–174.
Jonker, M.T.O., Candido, A., Vrabie, C.M., Scarlett, A.G., Rowland, S.J. 2016.Synergistic androgenic effect of a petroleum product caused by the joint action of at least three different types of compounds. Chemosphere, 144, 1142-1147.
King D.J., Lyne R.L., Girling A., Peterson D.R., Stephenson R., Short D. 1996. Environmental risk assessment of petroleum substances: the hydrocarbon block method. Concawe Report no. 96/52. Brussels, Belgium.
Korytar, P., Parera, J., Leonards, P.E.G., Santos, F.J., De Boer, J., Brinkman, U.A.T., 2005. Characterization of polychlorinated n-alkanes using comprehensive two-dimensional gas chromatography-electron-capture negative ionization time of flight mass spectrometry. J. Chromatogr. A. 1086, 71-82
Mao, D., Van De Weghe, H. Diels, L., De Brucker, N., Lookman, R., Vanermen, G. 2007. High-performance liquid chromatography fractionation using a silver-modified column followed by two-dimensional comprehensive gas chromatography for detailed group-type characterization of oils and oil pollutions. J. Chrom. A, 1179 (2008) 33–40

Muir, D.C.G., Stern, G.A., Tomy, G.T., 2000. Chlorinated Parraffins Chapter 8, vol 3, Spinger, Berlin, Heidelberg.

Mutlu, E., Warren, S.H., Matthews, P.P., Schmid, J.E., Kooter, I.M., Linak, W.P., M. Gilmour, I., DeMarini, D.M. 2015. National Health and E Health effects of soy-biodiesel emissions: bioassay-directed fractionation for mutagenicity. Inhalation Toxicology, 27 (11), 597-612

OECD. 2007. OECD Series on Testing and Assessment Guidance on Grouping of Chemicals. OECD report ENV/JM/MONO(2007)28.

OECD. 2000. Guidance document on aquatic toxicity testing of difficult substances and mixtures. OECD report ENV/JM/MONO(2000)6.

OECD. 2014. OECD guidance for characterizing oleochemicals substancers for assessment purpose. OECD report ENV/JM/MONO(2014)6.

Oostingh, G.J., Smith, K.E.C., Tischler, U., Radauer-Preiml, I., Mayer, P. 2015. Differential immunomodulatory responses to nine polycyclic aromatic hydrocarbons applied by passive dosing. Toxicol. In vitro, 29 (2), 345-351.

Ouyang, X., Leonards, P.E.G., Tousova, Z., Slobodnik, J., de Boer, J., Lamoreet, M.H. 2016. Rapid Screening of Acetylcholinesterase Inhibitors by Effect-Directed Analysis Using LC x LC Fractionation, a High Throughput in Vitro Assay, and Parallel Identification by Time of Flight Mass Spectrometry. Anal. Chem. 88 (4), 2353-2360.

Peterson, D.R. 1994. Calculating the aquatic toxicity of hydrocarbon mixtures. Chemosphere 29, 2493-2506.

Pieke, E., Heus, F.A.H., Kamstra, J., Mladic, M., Velzen, M. van, Kamminga, D., Lamoree, M.H., Hamers, T., Leonards, P.E.G., Niessen, W.M.A. & Kool, J. 2013. High-resolution fractionation after gas chromatography for Effect Directed Analysis. Anal. Chem., 85 (17), 8204-8211.

Radovic, J.R., Thomas, K.V., Parastar, H., Diez, S., Tauler, R., Bayona, J.M., Josep M. 2014. Chemometrics-Assisted Effect-Directed Analysis of Crude and Refined Oil Using Comprehensive Two-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry. Environ. Sci. Techol. 48 (5),3074-3083.

Reth, M., Zencak, Z., Oehme, M., 2005. New quantification procedure for the analysis of chlorinated paraffins using electron capture negative ionization mass spectrometry J. Chromatogr. A, 1081, 225-231.

Shaw, S.D., Blum, A., Weber, R., Kannan, K., Rich, D., Lucas, D., Koshland, C.P., Dobraca, D., Hanson, S., Birnbaum, L.S., 2010. Halogenated flame retardants: Do the fire safety benefits justify the risks? Rev. Environ. Health 25, 261-305.

Smith, K.E.C., Dom, N., Blust, R., Mayer, P. 2013. Controlling and maintaining exposure of hydrophobic organic compounds in aquatic toxicity tests by passive dosing. Aqua. Toxicol., 144, 341-341.

Stevens, J.L., Northcott, G.L., Stern, G.A., Tomy, G.T., Jones, K.C., 2003. PAHs, PCBs, PCNs, organochlorine pesticides, synthetic musks, and polychlorinated n-alkanes in UK sewage sludge: survey results and implications. Environ. Sci. Technol., 37, 462–467.

Stibany, F., Schmidt, S.N., Schaffer, A. Mayer, P. 2017. Aquatic toxicity testing of liquid hydrophobic chemicals - Passive dosing exactly at the saturation limit. Chemosphere, 167, 551-558.

Suatoni, J.C. and Garber, H.R. 1976. Hydrocarbon Group-Type Analysis of Petroleum Fractions [b.p. 190°-360°C] by High Performance Liquid Chromatography. J Chromatogr Sci (1976) 14 (11): 546-548.

Sutton, P.A., Lewis, C.A., Rowland, S.J.. 2005. Isolation of individual hydrocarbons from the unresolved complex hydrocarbon mixture of a biodegraded crude oil using preparative capillary gas chromatography. Organ. Chem. 36, 963-970.

Tadokoro, H., Maeda, M., Kawashima, Y., Kitano, M., Hwang, D. and Yoshida, T. 1991. Aquatic toxicity testing for multicomponent compounds with special reference to preparation of test solutions. Ecotox. Environ. Safety., 21, 57-67.

Tomy, G.T., Stern, G.A., Muir, D.C.G., Fisk, A.T., Cymbalisty, C.D., Westmore, J.B., 1997. Quantifying C10–C13 polychloroalkanes in environmental samples by high-resolution gas chromatography/electron capture negative ion high-resolution mass spectrometry. Anal. Chem., 69, 2762–2771.

USEPA, 1986. Guidelines for the Health Risk Assessment of Chemical Mixtures (EPA/630/R-98/002).

USEPA, 2000. Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures (EPA/630/R-00/002 - August 2000).

USEPA, 2003. Framework for Cumulative Risk Assessment - EPA/630/P-2/001F (Washington DC).

USEPA, 2006c. Considerations for Developing Alternative Health Risk Assessment Approaches for Addressing Multiple Chemicals, Exposures and Effects - EPA/600/R-06/013A (Washington DC).

USEPA, 2015. Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials (UVCB Substance) on the TSCA Inventory. 2015-5 report:.

van der Veen, I., Cofino, W., Crum, S., De Boer, J., 2012. Interlaboratory study on the analysis of chlorinated paraffins in environmental matrices, phase 1. IVM Institute for Environmental Studies, Amsterdam. QUASIMEME Report W-12/11.

Van Mourik, L,M., Leonards, P.E.G., Gaus, C., De Boer, J., 2015. Recent development in capabilities for the analysing chlorinated paraffins in environmental matrices: A review. Chemosphere, 136, 259-272.

Van Mourik, L.M., Gaus, C., Leonards, P.E.G., de Boer, J., 2016. Chlorinated paraffins in the environment: A review on their production, fate, levels and trends between 2010 and 2015. Chemosphere. 155, 2016, 415–428.

Van de Meent, D. 2008. Environmental fate factors and human intake fractions for exposure and risk calculation of petroleum products with the hydrocarbon block method. Netherlands Center for Environmental Modelling, report Environmental Science no. 322.

Vergauwen, L, Schmidt, S.N., Stinckens, E., Maho, W., Blust, R., Mayer, P., Covaci, A., Knapen, D. 2015. A high throughput passive dosing format for the Fish Embryo Acute Toxicity test. Chemosphere, 139, 9-17.

Vrabie, C.M., Sinnige,T.L., Murk,A.J., Jonker, M.,T.O. 2012. Effect-Directed Assessment of the Bioaccumulation Potential and Chemical Nature of Ah Receptor Agonists in Crude and Refined Oils. Environ. Sci. Technol. 46, 1572-1580.

Wang, Y., Li, J., Cheng, Z., Li, Q., Pan, X., Zhang, R., Liu, D., Luo, C., Liu, X., Katsoyiannis, A., Zhang, G., 2013. Short- and medium-chain chlorinated paraffins in air and soil of subtropical terrestrial environment in the Pearl River Delta, South China: distribution, composition, atmospheric deposition fluxes, and environmental fate Environ. Sci. Technol., 47, 2679–2687.

Xia, D., Gao, L., Zhu, S., Zheng, M., 2014. Separation and screening of short-chain chlorinated paraffins in environmental samples using comprehensive two-dimensional gas chromatography with micro electron capture detection. Anal. Bioanal. Chem., 406, 7561–7570.

Zencak, z., Borgen, A., Reth, M., Oehme, M., 2005 Evaluation of four mass spectrometric methods for the gas chromatographic analysis of polychlorinated n-alkanes J. Chromatogr. A, 1067, 295-301.

Zeng, L., Temporal trends and pattern changes of short- and medium-chain chlorinated paraffins in marine mammals from the South China Sea over the past decade.

Zeng, L., Wang, T., Wang, P., Liu, Q., Han, S., Yuan, B., Zhu, N., Wang, Y., Jiang, G., 2011. Distribution and trophic transfer of short-chain chlorinated paraffins in an aquatic ecosystem receiving effluents from a sewage treatment plant. Environ. Sci. Technol., 45, 5529–5535.