EXECUTIVE SUMMARY

Reference: PFA.750.011.001

Date: February 2021

Critical literature review of analytical methods applicable to environmental fate studies

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Client	Wood environment and infrastructure solutions UK limited (for ECHA under Framework ECHA/2015/50)
Client's reference	ECHA/2019/236

This report is issued by
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AUTHORISATION RECORD

Conclusions drawn and recommendations made in this report represent the unbiased view of Peter Fisk Associates Limited on the basis of the data presented and obtained.

The name below confirms that this version of the report is complete and final.

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Table of Contents

1. Introduction – background

The evaluation of persistence, bioaccumulation and toxicity (PBT) properties is a mandatory element in the hazard assessment of industrial chemicals and biocides. Determining PBT and very persistent and very bioaccumulative (vPvB) properties, is completed by comparing key properties against the criteria set out in Annex XIII of REACH.

Degradation simulation tests and bioaccumulation tests require that an adequate analytical monitoring procedure is available, in order to understand the fate of the chemical in the test system. The reliability of the results from such studies and their relevance for the purpose of the PBT/vPvB assessment will depend to a great extent on the accuracy, precision and sensitivity of the analytical methods. The use of appropriate, reliable analytical methods in these studies is critical to the outcome and interpretation of the studies, nevertheless it can be practically challenging and costly to apply such methods. There is therefore a need to better define what is technically feasible in terms of quantitative and qualitative analytical monitoring as well as to clarify what information may be regarded as fit for purpose for the PBT/vPvB assessment.

The aim of this project was to perform a thorough, transparent, and critical literature review of analytical methods (and their limitations) applicable to environmental fate studies (e.g. OECD Test Guidelines (TG) 305, 307, 308 and 309). The data gathered were analysed in the context of the (v)P(v)BT assessment under the REACH and the Biocidal Products Regulations. The review also covered the methodologies applied under other EU regulatory regimes.

In doing so, the review addressed the following questions:

- What are the requirements to achieve regulatory compliance under the REACH and Biocidal Products Regulations?

- What are the requirements of recommended technical guidelines?

- What analytical chemistry methodologies are applicable to environmental fate studies?

- What is the impact of the methodological set-up on the relevance and reliability of the information generated from environmental fate studies?

- What are the specific limitations regarding the type of test substance, structural functionality and test substance properties?

- How can current limitations be overcome?

2. Methodology

The scope and the method for the project was defined by an initial review of the requirements for regulatory compliance under EU frameworks[1,2] and associated guidance, in order to consolidate the requirements for the environmental fate studies, to identify key technical challenges, and to focus the subsequent literature search on relevant areas. The test guidelines

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[1] REACH Regulation (EC No 1907/2006), the Biocidal Products Regulation (EC No 528/2012) (BPR).
[2] The Plant Protection Products Regulation (EC No 1107/2009) (PPPR) and the medicinal products regulation (EC No 726/2004).

for persistence and bioaccumulation studies were reviewed and compared, to analyse the requirements of the technical guidelines and to identify areas where an improvement in the regulatory guidance would be beneficial. A wider literature search was then conducted, focussed on the following areas:

- **Current state of the science for environmental fate studies**. This search targeted bioaccumulation and persistence studies conducted according to the relevant OECD guidelines (OECD 305, 307, 308, 309) published in the academic literature and publicly available sources. The focus was on information on analytical methods currently used in such guideline studies and the challenges associated with these analyses.
- **Insights from related areas: challenges and techniques**. This search covered a broader range of literature, and was designed to obtain information on challenges and uncertainties associated with analysis of chemicals in relevant test media (aqueous, sediment, soil, fish) and the approaches used for each stage of the analytical process.
- **Guidelines for validation of analytical methods**. This search targeted information on guidelines for validation of analytical methods that could be applied to environmental fate studies.
- **The use of *in silico* tools** for the prediction of transformation/ degradation products. This was a limited search, with a focus on identifying the extent of the use of such tools in environmental fate studies.

For each of the searches, a series of keyword combinations was developed and used as input in peer-reviewed literature databases and government agency websites. Additionally, a number of further sources (such as relevant textbooks) were reviewed. All obtained literature was subjected to a two-stage screening process (first, screening was based on title and abstract, this was followed by a second screening of the whole paper/report for relevant paper identified in the first screening) and assessed against a set of predetermined inclusion and exclusion criteria to select the relevant studies.

More than 200 relevant peer-reviewed studies, guidelines, books, reports and websites were reviewed to provide details of analytical methods applicable to environmental fate studies. The findings were structured to cover the following topics:

- Current practise in guideline bioaccumulation and persistence studies.
- Key aspects to consider prior to conducting biodegradation and bioaccumulation studies.
- Sample handling and extraction.
- Isotopic labelling and quantification of radiolabelled substances.
- Chromatography and stable isotope detection.
- Summary of specific issues for "difficult" substances.

3. Results of the literature search and review

The investigation of the current state of the science for environmental fate studies found relatively few guideline studies in the peer-reviewed literature. Since these studies may not have been conducted in the context of any regulatory framework, some regulatory requirements were not reported (e.g. the identification of transformation and degradation products).

The literature search to gather insights from related areas largely found information from analysis of trace contaminants in the environment rather than from laboratory studies. These

studies nonetheless proved to be a valuable source of information on analytical methods and procedures relevant for environmental fate laboratory studies. Whilst the analytical methods reported in the literature may not, at present, be used in B or P testing they are relevant for the analyses of substances in relevant matrices. The review of these analytical methods provided insights on areas where improvements can be made to the current methods for B and P testing.

General points

Several areas that impact the analytical method development and validity of results were identified:

- *Chemical properties*. Prior to conducting any biodegradation or bioaccumulation study, an understanding of the identity and key physico-chemical properties of the test substance is critical. Water solubility is a fundamental property which determines the feasibility of performing an OECD 305 or 309 study, and also influences key elements of the study design (such as appropriate dosing and sampling methods) and on the analytical methodology (extraction and separation).

- *Metabolites and transformation/degradation products*. An understanding of the transformation products that may be formed during a bioaccumulation or degradation simulation study is also important, especially when concentrations of the transformation products should be evaluated relative to the parent substance. A limited review of available *in silico* tools for the prediction of transformation and degradation products has been completed. These tools may be useful to identify putative transformation products which may guide the development of a suitable analytical method or may indicate whether or not metabolites of concern might be formed. However, their limitations imply that predictions obtained from such tools cannot be used as conclusive evidence and cannot replace the need for experimental identification of metabolites. Experimental identification and quantification of metabolites can be challenging, as reference standards are often not available. In this project, no mention of the use of *in silico* tools for the prediction of transformation/degradation products was found in analysed literature.

- *Artefacts*. The formation of artefacts may be a significant confounding factor affecting the reliability of data generated from environmental fate studies. The significance of artefacts increases as the concentration of test substance in the study decreases. Artefact formation can occur at all stages of the analysis (sampling, extraction and detection). Prevention of artefacts requires careful consideration of the physico-chemical properties of the test substance, and the possible sources of contamination within the test laboratory, extraction solvents and laboratory equipment.

Extraction techniques

The sampling and extraction stages of any study are often reported to have greater error than the separation and detection stages. The efficiency and selectivity of the method for different components, potential artefact formation, and method validity require careful consideration.

Based on the literature identified for this project, extraction techniques from the water phase of bioaccumulation (OECD 305) or degradation (OECD 309) studies included liquid-liquid extraction (LLE), solid-phase extraction (SPE), solid-phase microextraction (SPME) or direct analysis. LLE and SPE were used for a broad range of substances with different properties, as a result of the availability of solid phases with different properties or non-selective character of LLE. SPME was reported to be used for poorly soluble substances, and a study comparing the results from LLE to SPME showed similar results for organochlorine substances.

There are several techniques available for the extraction of substances from solid matrices, The use of Pressurised Liquid Extraction (PLE) and Ultrasound-Assisted Extraction (UAE) may be preferable to the use of solvent (Soxhlet) extraction, as they are faster techniques and

require less solvent. Based on the literature review, PLE and UAE were commonly used as techniques for extracting substances from soil, sediment and fish, and showed satisfactory extraction efficiencies for various substances.

Separation and detection techniques

The selection of separation and detection techniques in a degradation or bioaccumulation study is dependent both on the test substance properties, and whether a radiolabelled, stable isotope labelled, or non-labelled test substance is used. The use of radioactive isotopes allows for detection techniques that are more sensitive compared to detection techniques for stable isotopes, therefore, radiolabelling of the test substance should be considered first when conducting bioaccumulation or degradation studies.

Liquid Scintillation Counting (LSC) is the most common and widely available method for quantifying labelled compounds, in combination with high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) for separation. Using radiolabelled compounds allows for the quantification of the test substance, transformation/degradation products, mineralised and non-extractable residues (NERs). For unlabelled substances quantification can be more difficult as an analytical standard is needed, which are often not available for transformation/degradation products. The quantification of NER and reporting a mass balance are not feasible using unlabelled substances. Using stable-isotope labelled substances, quantification of transformation/degradation products and a mass balance can be calculated using isotope ratio mass spectrometry (IR-MS) analysis. There are examples in the peer-reviewed literature of this technique being successfully applied in soil and sediment degradation studies. Stable-isotope labelling can, however, reduce the cost and technical difficulties associated with radiolabelling.

For non-labelled substances a range of chromatographic techniques can be selected, however, LC or GC are typically used. Commonly these LC or GC based systems are connected to mass spectrometers, although other detectors can be used. Mass spectrometers have the advantage that they are selective and sensitive and can be used in a targeted as well as non-targeted (screening) approach. The examples in the literature showed that parent substances and transformation or degradation products were predominantly analysed by targeted techniques such as MS/MS, however, in some cases non-target detectors (high resolution MS) are being used for the identification of degradation products. The use of soft ionisation and high resolution MS techniques could be further investigated in the identification of transformation/degradation products as information on the accurate masses of the transformation/degradation products can be obtained.

Analytical method validation

Whether an analytical method used in fate studies is validated and thereby generating reliable and robust data is key for interpreting the outcome of these studies. According to OECD guidelines 305, 307, 308 and 309, information on recovery, repeatability and sensitivity should be provided. In the fate studies assessed in the literature review, information on limit of detection (LOD), recovery or repeatability was not always provided. When provided, recoveries were generally within the range stated in the OECD guidelines and LODs were generally below the minimum required values stated in the guidelines regardless of the extraction, separation and detection technique used. Information on the calibration curve, accuracy and precision of the analytical method and the stability of the analyte in a given matrix (under set storage conditions) were generally not reported, and yet are key information to assess the validity of the analytical

method.

‘Difficult’ substances

Some substance types present particular challenges in environmental fate studies, both to select an appropriate study design and to develop a reliable analytical methodology. The substances that have the potential to cause technical challenges in performing bioaccumulation and degradation studies are similar to those defined as ‘difficult substances’ in OECD GD 23[3]. Where a substance or transformation product possesses such ‘difficult’ properties, the impact of these properties must be considered at all stages of the study. In some cases, the feasibility of the study itself may be defined by these ‘difficult’ properties.

Combinations of difficult properties may present particular challenges for study design and analytical methodology. Some of the key issues identified include:

- *Poor solubility*. Very poorly soluble hydrophobic substances present particular challenges in bioaccumulation and surface-water simulation studies. Passive dosing is a method of maintaining freely dissolved concentrations of hydrophobic organic chemicals (Log K_{ow} > 5) that are poorly soluble, and therefore has the potential to be used to achieve stable aqueous concentrations for a bioaccumulation study via aqueous exposure. Passive dosing is mentioned in both OECD 23 and OECD 264[4], and is becoming more widely employed in bioaccumulation testing. Losses from sorption of the analytes to sample containers and extraction material is also a potential problem with poorly soluble hydrophobic substances, and measures to prevent this should be taken, for example avoiding the use of glassware and using polytetrafluoroethylene (PTFE), borosilicate (i.e. Pyrex®) glassware. For very poorly soluble substances, the sensitivity of the analytical method required to quantitatively monitor the test substance in solution may be a significant challenge, e.g. in an OECD 309 study, analytical determination of 90% dissipation of the test substance from the system may not be feasible if the solubility of the test substance is too close to the LOQ of the analytical method (and quantification of degradation/transformation products may also not be feasible).

- *High volatility*. For highly volatile substances, biodegradation and bioaccumulation studies may require significant adaptations of the test system to prevent loss of the test substance from the system. Consideration of these adaptations is beyond the scope of this project. In the context of the analytical methodology, losses of volatile test compounds and transformation products can occur at all stages of sample handling and extraction, however there are fairly well-established methods to handle this type of compound (e.g. US EPA Methods 8270E, 8260 D).

- *UVCBs.* At present, there is very little practical consideration in the test guidelines or regulatory guidance for conducting environmental fate studies with a complex substance such as UVCB or multi-constituent substances. The available test guidelines assume that a highly pure test substance is used and do not acknowledge the technical challenges of selecting and synthesising/isolating individual constituents (and any attempt at radiolabelling these constituents) or in isolating a fraction of the UVCB/multiconstituent substance for testing. Considerations for UVCB/multiconstituent substances include appropriate extraction and separation techniques for constituents with significantly different physico-chemical properties, and feasibility of method validation, given the potential lack of availability of reference standards.

Based on the literature review, identification and quantification of any transformation/degradation products has not been attempted in studies with UVCB/multi-

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[3] OECD 23; Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures
[4] OECD 264; Guidance Document on Aspects of OECD TG 305 on Fish Bioaccumulation

constituent substances. GC-GC methods are recommended in the REACH guidance for identification of constituents, however, availability, cost and inter-laboratory reproducibility are problematic. Fractionation of the sample by liquid chromatography (LC) or possibly thin layer chromatography (TLC), followed by a separate analysis of the fractions using an appropriate detector, such as high-resolution mass spectroscopy (MS) or nuclear magnetic resonance (NMR) could also be considered.

4. Conclusions and recommendations

Whilst the OECD test guidelines (OECD 305, 307, 308, 309) allow the use of radiolabelled or non-labelled test substance, radiolabelling is the ideal technique for test substances used in bioaccumulation and degradation studies and is recommended in the regulatory guidance. However, it can be extremely expensive and requires expertise to perform radiolabelled synthesis, and in some cases the synthesis of appropriately radiolabelled test material may not be possible. The guidelines do not differentiate between radioisotopes and stable isotopes, and the mass balance is only discussed in radiolabelled terms. Since it is partly possible to determine NERs with stable isotope labelling, there is the potential for greater use of stable-isotope labelled test substances which are generally cheaper and may present fewer technical difficulties than the use of radiolabelled substances.

When using a non-labelled test substance, any mass-balance requirement cannot be met. Analytical methods for identification and quantification of the test substance itself are usually well developed, however identification of transformation/degradation products is challenging and quantification of these products even more difficult. It may be feasible to make some attempt to quantify any transformation/degradation products, however this is likely to depend on how chemically distinct the products are from organic substances naturally occurring in the matrix. Precise quantification of non-labelled transformation/degradation products is unlikely to be possible without the identification and subsequent synthesis of these products as analytical standards for calibration. Where possible, a well-defined and well-characterised test substance should be used in testing.

The importance of the availability of reliable measured water solubility data prior to planning any proposed bioaccumulation or degradation study should be emphasised, since it is a critical and fundamental property which needs to be taken into account at all stages of the study.

Separation and detection techniques are available for the majority of substance types. For some substances, such as ionising and very polar substances, techniques such as derivatisation or non-standard chromatography column types may be required.

The current limit of quantification (LOQ) of analytical instrumentation should not be generally be considered a limiting factor for environmental fate studies. The use of appropriate sample clean-up and extraction have the potential to improve the reliability of test results without the same cost and availability issues as use of new chromatography/detection techniques.

Based on the analyses of the literature and the conclusions drawn, the following recommendations are also made:

Use and applicability of extraction techniques:

- Regardless of the extraction technique used, issues such as efficiency and selectivity of the method for different components, potential for artefact formation, and method validity require careful consideration.
- Although LLE is still a commonly used extraction technique for extractions from water, SPE is faster and requires less solvent than LLE and can be applied to a wide range of chemicals due to the availability of different solid phases, and its use should be encouraged where applicable.
- The use of microextraction techniques should be investigated for the analysis of water samples. For this technique no solvents are needed, and it has a wide applicability due to availability of different fibres.
- For solid phases, PLE or UAE are recommended as they are fast extraction techniques that require small amounts of extraction solvent. These methods are automated or can be operated in larger batches.

Identification and quantification of transformation /degradation products and closing the mass-balance:

- The determination of mass-balance in studies using stable-isotope labelled compounds should be encouraged. The use of stable isotopes in combination with IR-MS should be explored in environmental fate studies as an alternative to the use of radiolabelled substances, where radiolabelling is not possible.
- Mass spectrometry is a powerful method for identification and/or quantification. Increased use could be made of soft ionisation, tandem MS/MS and high-resolution MS (HRMS), particularly for degradation products. HRMS can allow for exact mass determination of the parent substance and degradation products, and can be used with both non-labelled and radiolabelled compounds.

Information required to demonstrate method validation:

- Information on quality assurance and quality control of the analytical method should always be provided as this information is key to interpret the study results.
- The validation parameters should include information on the calibration curve, accuracy, precision, recovery, sensitivity and stability.

In addition, the following opportunities for further research have been identified:

- Whilst techniques for separation and quantification of radiolabelled test compounds are well established, the use of fractionation techniques followed by further analysis could be developed. Such second-dimension analysis may reduce the LOD and allow identification of transformation/degradation products where direct identification is difficult.
- Further exploration of the use of passive sampling techniques, both in aqueous media and in soil/sediment matrices, would be beneficial particularly for testing poorly soluble substances.