Development of interim guidance for the inclusion of non-extractable residues (NER) in the risk assessment of chemicals

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EUROPEAN CENTRE FOR ECOTOXICOLOGY AND TOXICOLOGY OF CHEMICALS

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SUMMARY

Decades of discussion have surrounded the definition of the term bound residue and, more recently, non-extractable residues (NER). Significant confusion persists around the irreversibility of the binding and eventual longevity of these residues and the conditions leading to re-release. The question of what is bioavailable and what may become bioavailable over the long-term, commonly referred to as bioaccessible, continues to generate much debate, in particular with respect to how the portion defined as NER should be treated in an environmental risk assessment.

Quantitative information concerning the formation of NER is available for only a small percentage of substances registered on chemical inventories (agricultural chemicals and some pharmaceuticals). The absolute concentration of NER is difficult to define and makes inter-study comparisons difficult, compounded by the use of different extraction techniques, labelling position in the test molecule, and bias originating from standard laboratory testing approaches. It is clear that there is a necessity to establish a number of criteria for standardisation of testing in this domain.

Current regulatory text pertaining to the evaluation of NER is limited to Europe. The definition indicates that the relevant fraction of NER does not include fragments of the parent substance which have been transformed through metabolic pathways leading to natural products, but does not provide guidance on what approaches should be adopted to elucidate NER, nor does it provide guidance on which methods can be used to differentiate between the portion of NER which has been biogenically incorporated.

Traditionally, the regulatory position has been to consider that the entire fraction determined to be NER, whether present as the parent substance, direct metabolite or biogenically incorporated, is bioavailable at any given time with potential toxic effects in the surrounding habitat. This approach does, however, lead to an over-estimation of the potential toxicity and results in a conservative risk assessment since the fraction remaining non-extracted in the matrix, following relatively severe extraction, does not represent a fraction which is bioavailable.

An ECETOC Task Force was formed to assess and critically evaluate the current situation and existing state-of-the-art, to identify where uncertainties lie, and propose interim evaluation procedures for use until further scientifically validated approaches have been developed.

The assessment model presented here is based on a tiered approach, starting with a pre-tier consideration of criteria for waiving the assessment (no emissions to the environment, low annual volume, etc.). Substances not fulfilling waiving then enter the tiered process. Tier 1 considers the physico-chemical parameters of the substance and compares this to a conservative (100% bioavailable) exposure scenario. If the PEC/PNEC ratio is ≥ 1 after application of the relevant safety factor, then there is the possibility to refine the assessment through the use of additional data or via a justifiable amendment to the exposure scenario. Where the PEC/PNEC ratio remains ≥ 1, Tier 2 screening initially considers physico-chemical parameters in combination with the identification of structural alert groups in the substance to assess its potential to form NER. Where potential to form NER exists, adsorption – desorption screening is proposed, before moving on

to ‘soup testing’ of a relevant matrix (soil, sediment) containing NER as exposure medium for a suitable range of organisms.

If the NER demonstrate unacceptable toxicity to biota, further in-depth testing is recommended, as developed in Tier 3, which aims to identify and quantify the overall content and specific toxic elements within the NER fraction.

Recommendations have been made to suggest where further developments of the risk assessment scheme are needed. These include developing structural alerts in Tier 2; validation of an adsorption-desorption screen in Tier 2 and developing ‘soup tests’ in Tier 2 and 3.

Currently no standardised methods are available for either the preparation of exposure medium for performing ‘soup tests’, or for the exposure conditions for the biological phase of such studies. Consequently, the ‘soup test’ proposed serves as a basis for further development, validation, and finally, implementation. Furthermore, the development of suitable guidance on how to interpret the output of these studies will need some refinement.

1. INTRODUCTION

The formation of NER, previously referred to as bound residues, as a dissipation pathway for the 'removal' of chemical substances from the freely available state into a tightly adsorbed state, has been recognised since the 1960s (Bailey and White, 1964; Chiba and Morley, 1968). Quantification of overall NER requires the use of isotopic tracer techniques. Elucidation of chemical moieties constituting the bound fraction currently remains a state-of-the-art practice, requiring a high level of technical infrastructure and in-depth experience.

The operational difficulties related to the definition of NER and the technical challenges related to its quantification may well be responsible for the dearth of clear legislation and lack of technical guidance on this subject matter. On the regulatory front, very few developments have appeared in the official texts for more than two decades. This may be related to the lack of clarity on how NER should be treated in an environmental risk assessment. Views range from consideration of NER as an efficient toxicant removal process, to consideration of NER as a sink, and therefore a future source of toxicants. Despite these polarised views, no new advances have been made over recent years, neither on the science, nor on the legislative front.

Recently, however, significant interest has been generated to re-kindle the debate. This has taken the form of technical workshops:

- European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) – Workshop: Significance of bound residues in environmental risk assessment. 14 – 15 October 2009, Brussels, Belgium (ECETOC, 2010).
- The Food and Environment Research Agency (FERA) – Workshop to assess proposed new technical guidance on how aged sorption studies for pesticides should be conducted, analysed and used in regulatory assessments. 27 – 28 April 2010, York, United Kingdom (UK HSE, 2010).
- UmweltBundesAmt (UBA). Workshop – Nicht-extrahierbare Rückstände – NER. 22 – 23 June 2010, Dessau, Germany (UBA, 2011).

The principal objectives of these workshops centred around:

(i) the development of an operational protocol aimed at standardising the extraction approach with the aim of defining the different levels of bioavailability of residues, and

(ii) the refinement of the 100% bioavailable approach to evaluating the risk of NER to the environment and its ecological impact.

To address these points, ECETOC formed two Task Forces:

a) ECETOC Task Force – Understanding the relationship between extraction technique and bioavailability (ECETOC, 2012) - under the ECETOC science theme "Methods", and

b) ECETOC Task Force – Development of interim guidance for the inclusion of non-extractable residues (NER) in the risk assessment of chemicals – under the ECETOC science theme "Presence of Chemicals in the Environment".

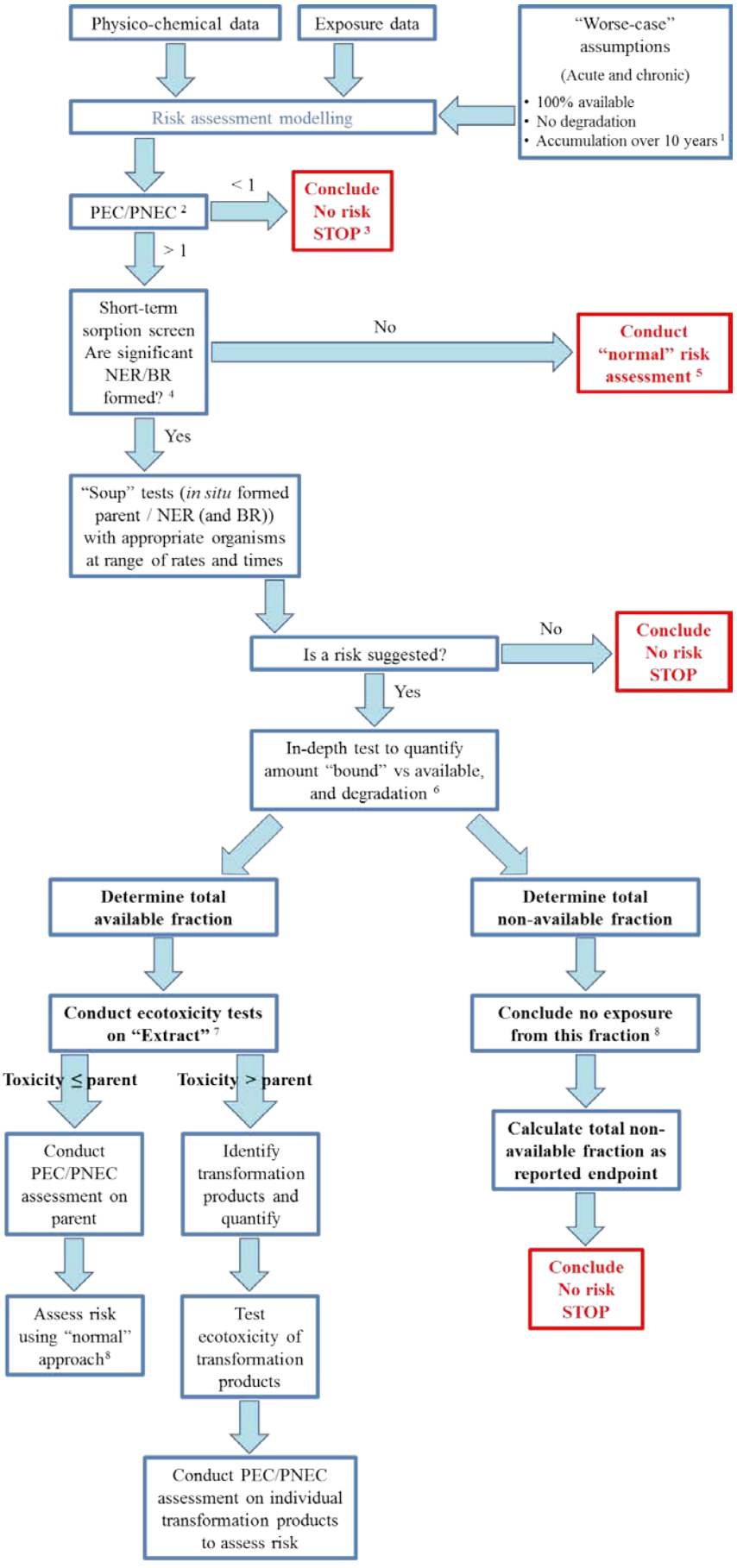
The output from the first Task Force has recently been reported (ECETOC, 2012), and presents an operational framework for the pragmatic fractionation of residues and correlates the extraction step procedure with bioavailability. This current report describes the output from the second Task Force where a Tiered risk assessment approach is presented and supplemented by an intelligent testing strategy as well as an outline of new test set-ups for addressing directly the ecotoxicity of the NER fraction. In conjunction, both Task Forces address and provide a broad reaching development proposal for the majority of the long-standing questions around the ecologically relevant fractions of chemical residues and their bioavailability, and pave the way forward in elucidating the temporal and toxicological significance of NER.

1.1 Terms of Reference

One of the major outputs from the recent ECETOC workshop (ECETOC, 2010) was a framework outlining a possible decision tree (Figure 1) for conducting risk assessment of NER together with key research needs to address the current knowledge gaps. As many of the research needs will take a number of years to deliver, the goal of this Task Force was to develop an interim approach to assessing the impact of NER on current risk assessments for aquatic and terrestrial compartments. The Terms of Reference were:

1. Critically evaluate the proposed risk assessment framework developed following the ECETOC workshop and assess its utility as an interim approach for regulatory assessment of chemicals;
2. Develop suitable guidance and trigger values to enable the decision tree to be used and test the utility of the scheme using suitable case studies;
3. Provide guidance on study design to provide the appropriate quality of data needed for the risk assessment framework to function within a regulatory decision making system.

Figure 1: Risk assessment framework accounting for non-extractable residues as proposed by ECETOC (2010)



Footnotes to the framework

[1] The use of 10 years is assumed as a realistic worst case. Precedent for this is from REACH (ECHA, 2008 in Table R16-10, p 47).

[2] The current agreed safety factors for calculation of all PNEC values should be applied in this assessment.

[3] This is assumed to account for any potential transformation products. A transformation product would need to be significantly (approximately ten times) more ecotoxic than parent or bioaccumulate to present an increased risk.

[4] A screen using realistic environmental matrices should be developed. Methodology, e.g. OECD 106 (2000a) could be modified / developed. Guidance should be developed to define 'significant'.

[5] 'Normal' risk assessment refers to existing regulations, e.g. in the EU, REACH, EMEA, pesticides.

[6] This test should include an agreed framework for extraction methods to relate the behaviour / partition of the chemical to its bioavailability to allow a full assessment.

[7] If extract testing is not practical / desired then you can bypass extract testing and proceed direct to identification and quantification of transformation products to assess risk.

[8] A conclusion of no exposure would be justified on the basis of a robust and agreed extraction framework indicating which fraction was available to organisms. This will need to be supported by suitable data.

1.2 The significance of non-extractable residues in regulatory frameworks

There is general agreement that the formation of NER in soil or sediment can have a significant impact on a chemical's behaviour in the environment and it is therefore important that they should form an integral part of risk assessment of both non-specifically acting chemicals (e.g. narcotic) and chemicals with specific modes of action (e.g. pesticides, pharmaceuticals, biocides and veterinary medicines). Furthermore, it is relevant to reflect upon the aspects of persistence (P/vP), bioaccumulation (B/vB) and toxicity (T) properties of NER given that the mechanisms leading to re-release and the magnitude of re-release events are currently not well understood. However, the significance of NER and exactly how they should be considered in environmental risk and hazard assessments remains the subject of considerable discussion (STEP, 2004; ECETOC, 2010). Barraclough *et al* (2005) describe the regulatory dilemma posed by NER in their review article, entitled, "Bound residues: environmental solution or future problem?" which discusses the contrasting views of the role of bound residue formation and their subsequent toxicity. Depending on the intended uses of the substance, direct or indirect exposure of environmental compartments could occur. Non-extractable residues could either be formed at the site of application or they could be introduced in a compartment together with matrices such as sewage sludge or manure.

Kearney (1976) suggested that "*there are two opposing viewpoints on the question of non-extractable residues formation in soil. It has been seen that non-extractable residues represent a hidden fraction of the original compound or metabolite capable of subsequent release and exertion of long-term biological and ecological effect. A more positive view of the argument is that the bound fraction represents the most effective and safe method of decontamination by rendering the molecule innocuous and allowing slow degradation in the bound state to products that pose no short- or long-term problems. To this day, the contrasting views remain on whether residues bound to solid matrices can be considered an environmentally acceptable route by which potentially harmful chemicals can be processed and immobilised or whether their presence means that the environment is being loaded with chemicals whose future behaviour cannot be predicted*".

Exhaustive extraction methods are commonly used when conducting the assessment of pesticide concentration in soils. Regulatory agencies usually assume that 100% of the amount of a chemical recovered by such techniques is bioavailable (i.e. all of the chemical present is available for degradation or to have potential toxic effects on the biota). However, awareness is now growing among environmental scientists, risk assessors and regulatory agencies that this precautionary approach generally overestimates the exposure concentration by the amount that is not available and therefore over-estimates the level of risk to biota in the environment. It is also well documented that chemicals that are irreversibly bound to solids are less degradable and less toxic than the total residue would predict. It can therefore be argued that current methods for assessing pesticides, which require exhaustive / rigorous extraction procedures to determine the 'non-extractable' or 'bound residue' fractions, do not provide information which is pertinent to evaluating the risks posed by those compounds in the environment. Whilst this position has been recognised by ECPA (2000), and referenced by REACH (2008) and OECD test guidance (2002a,b), there is no agreed guidance on how to determine what is available or not, and how it should be considered in the risk assessment. As a result, it continues to be debated from a scientific and regulatory point of view (Semple and Jones, 2005).

The longer organic compounds reside in the soil / sediment matrix, the less available for uptake by organisms they become, biodegradation kinetics reduce and toxic effects are, generally, buffered (Hatzinger and Alexander, 1995). As they persist, processes occur between the chemical and the matrix that have been termed as 'ageing' and 'sequestration'. The term 'sequestration' refers to the intact chemical that no longer remains available in the matrix, e.g. via physical entrapment in small pores and interstitial cavities which inhibit free movement of the molecule, unless the concerned matrix undergoes some structural change permitting release. The term 'ageing' (Gevao *et al*, 2000) relates to the increasing contact time with the chemical and solid matrix which may allow the association with the solid matrix to become stronger.

The majority of the existing regulatory text for non-extractable and bound residues has focused on active substances, in particular the legislative text for pesticides (91/414/EEC) (EC, 1991) and also more recently for biocides (98/8/EC) (EC, 1998). However, for biocides this has been removed in the revised version of Regulation 528/2012 (EU, 2012). Currently no reference is made to bound or NER in the relevant directives and guidelines for human pharmaceuticals (2001/83/EC and 2004/27/EC) (EU, 2001a; 2004a) or veterinary pharmaceuticals (2001/82/EC and 2004/28/EC) (EU, 2001b; 2004b). A recent draft guideline on determining the fate of veterinary medicinal products in manure by the European Medicines Agency (EMA, 2011) has recognised manure from animals treated with veterinary pharmaceuticals as a source of environmental contamination. This states that non-extractable residues should be considered in the evaluation, but without providing specific guidance. In the case of pesticides, registration requirements and guidance for conducting studies on these compounds has gradually developed over many years. In the European Union (EU) this was first harmonised under Council Directive 91/414/EEC (EC, 1991), which came into force on 26 July 1993. Commission Regulation 1107/2009 (EU, 2011) replaced Directive 91/414/EEC and has applied since 14 June 2011. It will continue to harmonise plant protection products across the EU. Annex II of the directive (on data and information requirements) mentions NER in the determination of pesticide fate and behaviour in soil (7.1.1.1. route of degradation), discussing appropriate studies (soil metabolism) to determine the extent of the formation of NER:

"The investigation of degradation pathways must include all feasible steps to characterise and quantify non-extractable residues formed after 100 days when exceeding 70% of the applied dose of the active substance. The techniques and methodologies applied are best selected on a case-by-case basis."

The biocides Commission Regulation No 528/2012 (EU, 2012) is less specific but makes reference to NER by stating that the extent and nature of bound residues needs to be investigated when understanding fate and behaviour in soil of active substances.

The regulatory texts have tended to focus on the amount of compound that can or cannot be extracted by chemical methods with no clear guidance on an extraction hierarchy or the bioavailability and toxicity of the extracted fraction (Calderbank, 1989; Alexander, 2000). In summary, current regulatory schemes for active substances account for formation of non-extractable or bound residues (ECETOC, 2010), but:

- Rely on an operational definition (non-extractability);
- Do not allow one to conclude on the nature of non-extractable residues;
- Do not quantitatively link occurrence with potential effects; and
- Do not encompass all types of active substances.

To date, interest in NER has mostly focused on European Union legislation (see Table 1). Further reflection on the subject matter has been stimulated in the recent past through national initiatives organised by the Food and Environment Research Agency in the UK (UK HSE, 2010), the German UmweltBundesAmt (UBA, 2011), as well as the expert workshop organised by ECETOC in 2009 to look at the significance of bound residues in environmental risk assessment (ECETOC, 2010).

Table 1: Summary of regulatory requirements for the ERA of NER

Region	Regulation reference	Text relating to NER	Definition of non-extractable residue
EUROPE	COMMISSION REGULATION (EU) No 544/2011 of 10 June 2011, Implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards the data requirements for active substances. Official Journal of the European Union, L 155, Volume 54, 11 June 2011.	Annex - Uniform principles for evaluation and authorisation of plant protection products, as provided for in article 29(6) of Regulation (EC) No 1107/2009, Part I Section 2.5.1.1 - No authorisation shall be granted if the active substance and, where they are of significance from the toxicological, ecotoxicological or environmental point of view, metabolites and breakdown or reaction products, after use of the plant protection product under the proposed conditions of use: - during tests in the field, persist in soil for more than 1 year (i.e. DT_{90} > 1 year and DT_{50} > 3 months), or - during laboratory tests, form non-extractable residues in amounts exceeding 70% of the initial dose after 100 days with a mineralisation rate of less than 5% in 100 days, unless it is scientifically demonstrated that under field conditions there is no accumulation in soil at such levels that unacceptable residues in succeeding crops occur and/or that unacceptable phytotoxic effects on succeeding crops occur and/or that there is an unacceptable impact on the environment.	Non-extractable residues (sometimes referred to as 'bound' or 'non-extracted' residues) in plants and soils are defined as chemical species originating from pesticides used in accordance with good agricultural practice that cannot be extracted by methods which do not significantly change the chemical nature of these residues. These non-extractable residues are not considered to include fragments through metabolic pathways leading to natural products. Annex - Data requirements for active substances, as provided for in article 8(1)(b) of Regulation (EC) No 1107/2009 Section 7.1.1. - Route and rate of degradation. The investigation of degradation pathways must include all feasible steps to characterise and quantify non-extractable residues formed after 100 days when exceeding 70% of the applied dose of the active substance. The techniques and methodologies applied are best selected on a case-by-case basis. A justification must be provided where the compounds involved are not characterised.
EUROPE	DIRECTIVE 98/8/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 February 1998, concerning the placing of biocidal products on the market, Official Journal of the European Community, L 123, 24 April 1998.	ANNEX VI - Common principles for the evaluation of dossiers for biocidal products Section 85 - Where unacceptable contamination of soil is likely to occur, the Member State shall not authorise a biocidal product if the active substance or substance of concern contained in it, after use of the biocidal product: - during tests in the field, persists in soil for more than one year, or - during laboratory tests, forms non-extractable residues in amounts exceeding 70% of the initial dose after 100 days with a mineralisation rate of less than 5% in 100 days, - has unacceptable consequences or effects on non-target organisms, unless it is scientifically demonstrated that under field conditions there is no unacceptable accumulation in soil.	Technical Guidance Document in support of the Directive 98/8/EC concerning the placing of biocidal products on the market – guidance on data requirements for active substances and biocidal products, October, 2000. … bound residues (e.g. in soil or sediment, also referred to as non-extractable or non-extracted residues), are defined as chemical species originating from an active substance after use according to authorisation granted and that cannot be extracted by methods which do not significantly change the chemical nature of these residues. These non-extractable residues are not considered to include fragments of the active substance having been metabolised by soil organisms to natural constituents of humus. Additional information is given in Section 7.2.2.3 - Extent and nature of bound residues [Ann. IIIA, XII.1.4.] - … the nature of

Region	Regulation reference	Text relating to NER	Definition of non-extractable residue
			the bound residues should be characterised as far as possible according to, for example, Schnitzer (1982) or after an acetone/methanol-ultrasonic treatment according to OECD guideline 304A (1981a).
EUROPE	REGULATION (EU) No 528/2012 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 May 2012 concerning the making available on the market and use of biocidal products, L167, 27 June 2012.	Annex II, Section 10.2.7. Extent and nature of bound residues. The determination and characteristics of bound residues is recommended to be combined with a soil simulation study.	
EUROPE	Veterinary pharmaceuticals Commission Directive 2001/82/EC and 2009/53/EC.	No specific mention or guidance on use of NER in the context of ERA.	
EUROPE	Human pharmaceuticals Commission Directive 2001/83/EC and EMEA/CHMP/SWP/4447/00.	No specific mention or guidance on use of NER in the context of ERA.	
EUROPE	European Commission Regulation EC 1907/2006, REACH.	Chapter R.7B – Endpoint Specific Guidance - Knowledge of bound residues and incorporation into biomass also needs to be considered and should be seen as a potential removal pathway.	Chapter R.7B – Endpoint Specific Guidance - The OECD 308 (2002b) Guideline advises as follows: "*Bound residues represent compounds in soil, plant or animal that persists in the matrix in the form of the parent substance or its metabolite(s) after extractions. The extraction method must not substantially change the compounds themselves or the structure of the matrix… In general, the formation of bound residues reduces the bioaccessibility and the bioavailability significantly* [(1)]" [modified from IUPAC, 1984 [(2)]]. Extraction of the sample, often with a suitable organic solvent is generally repeated 3 or 4 times until no further yield is achieved. Typically a range of solvents are used of increasing polarity (e.g. methanol, acetone, acetonitrile and hexane etc.) under ambient conditions. If the entire residual radioactivity cannot be recovered then appropriate solvent may be mixed with weak acids or bases or coupled to ultrasonic extraction. This aims to provide different conditions that may lead to the chemical or metabolite being released back into solution. Finally, the use of strong acids, bases or refluxing could undoubtedly extract the sample more thoroughly but could alter both the compounds of interest and the matrices.

[(1)] DFG, 1998
[(2)] Roberts, 1984

Region	Regulation reference	Text relating to NER	Definition of non-extractable residue
			Such severe extraction techniques are rarely if employed in e.g. routine soil or sediment/water testing. The extraction methods and efficiencies as well as analytical methods and detection limits should always be reported.
UNITED STATES	US EPA/TSCA/OPPTS FIFRA 40 CFR 158	No specific guidance on use of NER in the context of ERA.	
UNITED STATES	US FDA	No specific guidance on use of NER in the context of ERA.	
JAPAN	METI - Chemical Substances Control Law and Act on the Evaluation of Chemical Substances and Regulation of Their Manufacture (Act No. 117).	Japanese CSCL does not consider NER / BR.	
JAPAN	JMAFF	No specific guidance on use of NER in the context of ERA.	

Guidance on how to incorporate information on NER and their bioavailability in environmental risk assessment (and PBT assessment) is lacking. A workshop (ECETOC, 2010) addressed these concerns and identified future regulatory and research needs focussing in particular on:

- The environmental relevance of NER in exposure assessment and limitations of existing biodegradability tests;
- The technical challenges faced in the extraction and categorisation of NER;
- How residue data should be interpreted and analysed, especially data from higher tier biodegradability tests;
- When it can be concluded that NER are not a risk and when they can be considered removed and of no consequence. For risk assessment purposes, can it be said that if a non-extractable pesticide residue in soil is: (a) not bioavailable to plants or soil animals (b) not persistent or (c) not mobile then such residues can be considered insignificant.

In addition there are a number of other uncertainties associated with NER, namely:

- Is a chemical irreversibly bound or is the desorption extremely slow?
- Is the very slowly desorbing fraction the same as NER?
- Can the structure of the matrix change (slowly in time or suddenly) thus making the chemical bioavailable? What are these events, and, what is the significance of the re-mobilisation of NER following such an episode?
- Can the binding capacity of soils or sediments for non-extractables be exhausted?
- How should the formation of bound residues be considered in assessment of persistence?
- Is a quantitative risk assessment possible on a local scale (point source emission) and/or at a regional scale (point source and diffuse source)?
- What are the fundamental differences between a chemical extraction technique and a biological extraction technique?

Clearly, a number of areas need to be addressed if the science underpinning the risk assessment of chemicals which give rise to NER is to be advanced. If the approach to the risk assessment of NER is to be developed, then there is a fundamental need to define more precisely what is meant by ‘bound’ in the context of chemical residues in soil, sediment and biosolids. Regulatory schemes refer to bound residues but without a formally agreed standard procedure for extraction. ECETOC (2012) has proposed a standard framework for extraction methods in an attempt to relate the extractable fractions to bioavailablity and bioaccessibility. Use of such a standard approach should lead to a more consistent interpretation of the data and provide a transparent basis for assessing the potential risk. Understanding the mechanisms of binding and the analytical methods needed to identify them, should assist in the prediction of which chemical-solid-environment combinations may lead to NER.

1.3 Definition of Non-Extractable Residue

'Bound' residues were first mentioned in the literature by Bailey and White (1964) and have subsequently been defined in different ways over the years. A definition of what was considered to be bound residues was published in the US Federal Register (US EPA, 1975) and discussed in more detail by Kaufman (1976). A soil bound residue was defined as "*that un-extractable and chemically unidentifiable pesticide residue remaining in fulvic acid, humic acid and humin fractions after exhaustive sequential extraction with non-polar organic and polar solvents*". Since it is now believed that residues may also bind to clay and clay-humin fractions this definition has since been superseded.

Alternative definitions have been proposed from time to time e.g. by Khan (1982), Klein and Scheunert (1982), Kearney (1982) and by Führ (1987). They are all similar and based on the unextractability of the bound residue using either extraction methods commonly used in residue analysis or methods that do not significantly change the nature of the residues. In all these definitions, unextractable residues which result from the incorporation of $^{14}CO_2$ and small fragments recycled through metabolic pathways leading to natural products, are excluded.

The definition put forward by Roberts (1984), adopted by the International Union of Pure and Applied Chemistry (IUPAC) and generally accepted in the literature is:

"Non-extractable residues (sometimes referred to as 'bound' or 'non-extracted' residues) in plants and soils are defined as chemical species originating from pesticides, used according to good agricultural practice, that are unextracted by methods which do not significantly change the chemical nature of these residues. These non-extractable residues are considered to exclude fragments recycled through metabolic pathways leading to natural products. The extraction method must not substantially change the compounds themselves or the structure of the matrix. The nature of the bond can be clarified in part by matrix-altering extraction methods and sophisticated analytical techniques. To date, for example, covalent ionic and sorptive bonds, as well as entrapments, have been identified in this way. In general, the formation of bound residues reduces the bioaccessibility and the bioavailability significantly."

A subsequent modification widened the scope beyond 'parent material' (pesticides) to explicitly include metabolites:

"Compounds in soils, plants or animals, which persist in the matrix in the form of the parent substance or its metabolite(s) after extraction. The extraction must not substantially change the compounds themselves or the structure of the matrix."

Later modifications included the proviso that bound residues did not include metabolites that were indistinguishable from naturally occurring compounds (Gevao *et al,* 2000). The above definition with this proviso can be applied, not only to soil, plants and animals, but include sediments and biosolids (e.g. sewage sludge and manures). In addition, the definition may be widened beyond pesticides to include specifically acting chemicals (e.g. pesticides, pharmaceuticals, biocides and veterinary medicines) and any other chemical entering the appropriate environmental compartments.

Calderbank (1989) proposed a new direction of the definition by emphasising investigations of the bioavailability of bound residues of pesticides and suggested that a suitable definition for bound residues be related to their equivalence of toxicity with respect to the freely available parent:

"It may be that bound residues in soil could be defined as residues of the intact pesticide or degradation products derived from it that are no longer able to exert their original biological activity to any significant extent and/or which cannot be extracted from the soil by extraction methods which do not degrade the compound unless such methods are able to destroy the soil structure without affecting the compound."

The author goes on to advocate, from a regulatory standpoint, that the removal of pesticide residues to 'ultimate' sinks via formation of bound organic residues, should be recognised as an efficient decontamination process. Calderbank (1989) also points out that the capacity of soils to adsorb these residues, via organic matter breakdown, largely outweighs the potential inputs from pesticide applications.

Führ *et al* (1998) proposed a modified definition of these NER:

"Bound residues represent compounds in soil, plant or animal which persist in the matrix in the form of parent substance or its metabolite(s) after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix. The nature of the bond can be clarified by matrix altering extraction methods and sophisticated analytical techniques. In general the formation of bound residues reduces the bioaccessibility and bioavailability significantly."

A position on decreasing bioavailability and accessibility following formation of NER has been recognised by ECPA (2000), and referenced by REACH (2008), OECD (2002a,b) and US EPA (2008a-h).

Most current definitions addressing bound or NER are focused on the nature of the extraction procedure and its ability to remove a substance from a matrix. These definitions focus on the degree of partitioning between the free and bound fractions but do not always consider the reversibility of any adsorption and how this might change with time. Furthermore, there is little consideration given to the relevance of such extraction procedures for determining bioavailability either for degradation or impact assessment. The following definitions were used in the ECETOC workshop (ECETOC, 2010) to try to address this issue and ensure a common understanding of the terminology.

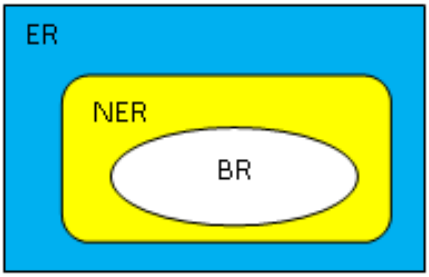
Extractable residue (ER): A residue that is extractable using 'mild' extraction methods. This may include aqueous and cold solvent extraction using methods without excessive added energy. These residues are either freely available, or only weakly adsorbed to the matrix, are considered to be bioavailable and must be considered in any impact / risk assessment.

Non-extractable residue (NER): A residue that is not extractable using 'mild' extraction methods, but extractable under harsher conditions. These conditions may include solvent extraction using methods such as refluxing, microwaves or accelerated solvent extraction (ASE). These residues are strongly associated with the matrix, however they may be potentially reversible, but the partitioning is very much in favour of 'binding' to components of the matrix. Therefore, for risk assessment purposes, this matrix associated fraction is unlikely to be available to indigenous organisms.

Bound residue (BR): A residue that is tightly associated with the solid matrix, often forming covalent (or similar) bonds. These residues usually cannot be released from the matrix or can only be released under extreme conditions where the integrity of the substance and/or matrix is likely to be affected. Such residues are often indistinguishable from the natural organic material e.g. humus in soil. These residues are not available for either degradation or available for indigenous organisms and should not be considered in any impact / risk assessment.

Figure 2 illustrates the above definitions that have been proposed as sub-sets.

Figure 2: Representation of ER, NER and BR (based on Zarfl* et al*, 2009)



ER, NER and BR have been traditionally defined on an operational basis, which is to say that they depend specifically on the methods used to extract the chemical(s). However, the concept of the fraction of chemical that the biological content of a matrix may be exposed, either on an acute or long-term basis, has been proposed as more ecologically relevant end points. The terms of 'bioavailable' and 'bioaccessible' have thus come to the forefront.

Bioavailable (based on Semple *et al*, 2004): Is freely available to cross an organism's cellular membrane from the medium the organism inhabits at a given time i.e. available now (no constraints).

Bioaccessible (based on Semple *et al*, 2004): Is available to cross an organism's cellular membrane from the environment it inhabits, if the organism has access to the chemical. However, the chemical may be either physically removed from the organism or only bio-available after a period of time i.e. available but not within reach from a given place and/or time (constrained or potentially available).

An important implication of the distinction between bioavailable and bioaccessible is that it forces consideration of what is being measured by the biological and chemical techniques over a short-term versus long-term event.

ECETOC (2012) has proposed an extraction regime which relates the extraction solvent and technique to the degree of bioavailability of the residue within the matrix (Figure 3):

Bioavailable fraction - The dissolved fraction and readily desorbable portion extractable with aqueous phase and weak organic solvent mixture is characterised as bioavailable.

Bioaccessible fraction - The slowly desorbed fraction is removed with organic solvent and largely equates to the definition of bioaccessible test substance.

Unavailable fraction - Test substance removed using organic solvent under pressure and/or increased temperature conditions and harsh extraction / digestion techniques are considered representative of irreversibly sorbed non-extractable residue and is categorised as unavailable for biota.

The following regime (Figure 3) has been summarised from the intelligent extraction strategy framework, detailed by ECETOC (2012). The extraction strategy is presented as a "conservative evaluation of bioaccessible residues providing the framework is applied using considered and rational methodology. Through utilising this intelligent extraction regime within a well-designed study, robust laboratory data to assess the bioavailable material in environmental matrices will be measured".

Figure 3: Extraction methodology framework (ECETOC, 2012)

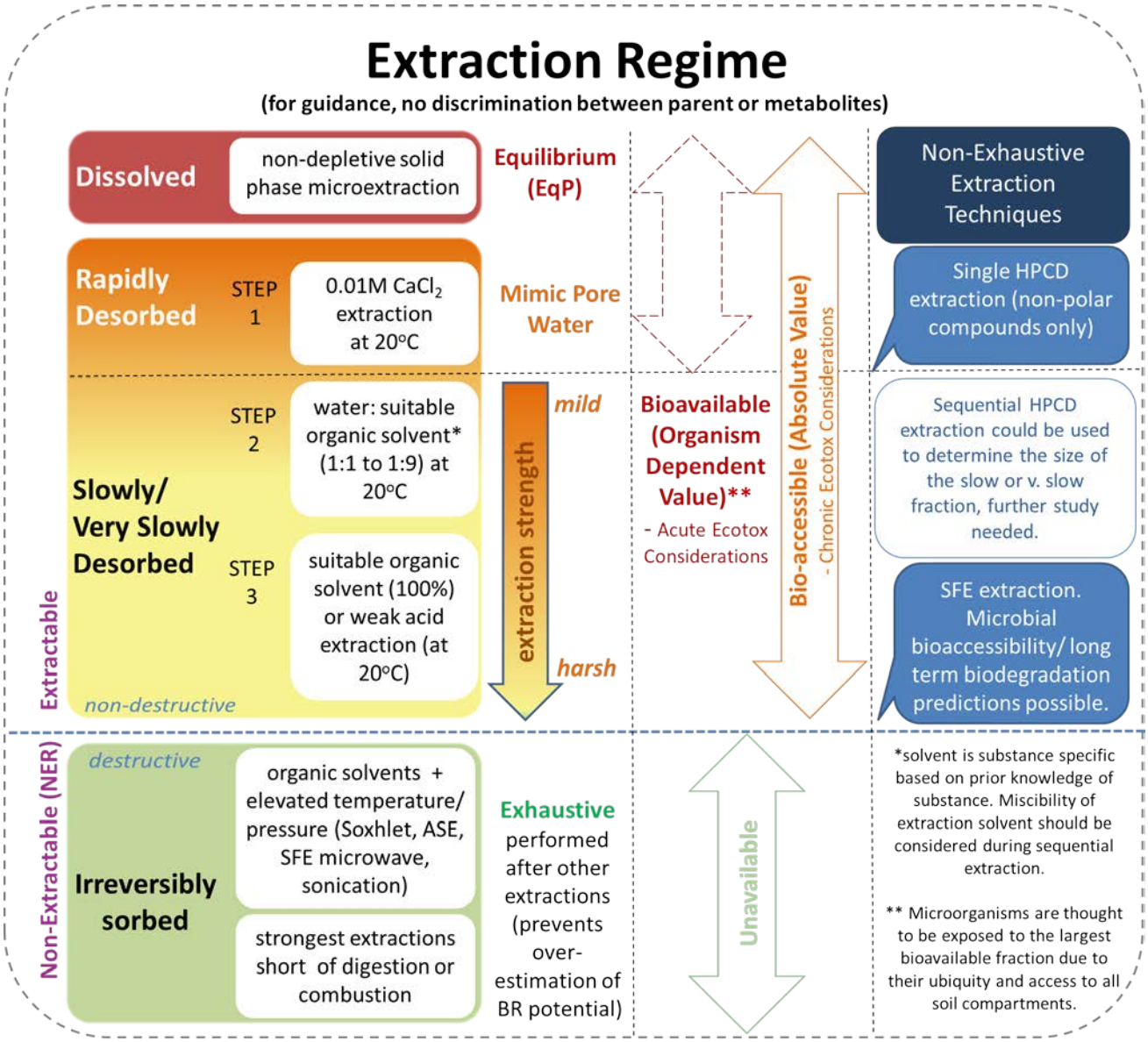


Figure 3 demonstrates the relationship between sequential extraction techniques of increasing severity and bioavailability / bioaccessibility to exposed organisms. It proposes the position of a boundary between accessible and inaccessible material. This is of particular importance in the risk assessment scheme, as it may be used to separate the soil / sediment fractions that are bioavailable / bioaccessible (a distinction between these two fractions has not been made in this report) and the fraction that is considered to contain NER. It allows for the fractions to be assessed separately and conclusions to be made about the risk posed by each one.

It is evident from the literature that many different extraction techniques have been used when considering the issue of residues. However, ECETOC (2012) have drawn recommendations on the practical elements of extractions methods from a review of the literature in order to introduce an element of consistency to their framework. These recommendations may be summarised as follows:

- A solid: extraction solution ratio of 1:5 is preferred, noting that the amount of solid is measured as equivalent dry weight, not wet weight.
- Contact time between solid and solvent should be at least 30 minutes.
- Agitation should be provided by shaking or rolling and not use excessive energy input or destruction of the solid matrix.
- Extraction should take place at ambient temperature and not at an elevated temperature.
- Extraction of the aqueous phase may be performed using solid phase microextraction (SPME) in non-depletive mode.

1.4 Current knowledge on NER and European inventory items

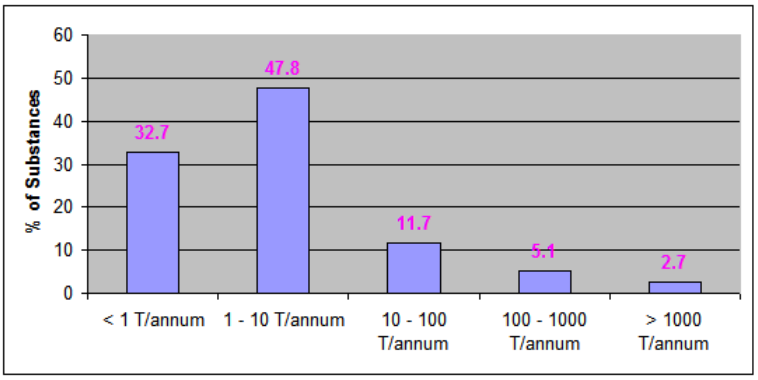
Approximately 110,000 chemicals are registered on the European inventories (EC, 1990; EU, 2002; JRC, 2009). Of these, it is estimated that a full environmental profile, including higher-tier environmental testing, is available for ≤ 1% of inventoried substances. Further, it is probable that a significant portion of the remaining 99% of substances do not possess substantial nor valid experimentally derived information on low-tier environmental screening tests (e.g. ready or inherent biodegradability).

The reality is that a very low number of registered chemicals have a complete environmental dossier. Complete dossiers are in general limited to agricultural chemicals, some industrial chemicals and pharmaceuticals.

In view of the large diversity in quality and quantity of environmental and ecotoxicological information available from one chemical to another, the need to bridge the data gap between those substances having good data sets and those having scarce environmental dossiers, is a challenge. Additionally, there is an economic factor to take into consideration. In effect, only a small number of the 110,000 registered substances fall into the category of high production volume chemicals (HPVCs), which are loosely defined as those substances being produced and/or imported into the European Union at quantities of > 1000 Tonnes

per annum. Figure 4 illustrates the distribution of substances registered in the EU with respect to their tonnage band (JRC, Personal Communication):

Figure 4: Distribution of substances registered in the EU with respect to their tonnage band (JRC, 2013)



In essence, more than 80% of the registered substances are produced and/or imported at volumes of less than 10 Tonnes per annum. This means there are many low volume chemicals to be assessed for their propensity to form NER.

The development of a tiered evaluation approach is, therefore, essential in order to eliminate those substances presenting little, or no, potential to form significant levels of NER during their environmental life-cycle, and target those which present a high potential for adsorption and development of NER.

1.5 Relevant environmental matrices potentially exposed to NER

Emission sources of contaminants can be characterised as either direct or indirect, the former relating to intentional inputs and the latter referring to either accidental contamination, as a downstream result of direct inputs, and/or environmental re-mobilisation of contaminant substances from one point source to another area. Additionally, it is worthwhile to consider the temporal frequency of the emissions to the environment – is it a short-term, acute-type release, or, does it represent a long-term, chronic exposure scenario. As indicated in the Introduction, initial reference to NER was limited to agricultural chemicals, with soil the key environmental compartment. Later, the inclusion of sediment as a matrix of potential interest, impacted either via direct paddy-field application or indirect run-off events to aquatic receiving bodies, was added. However, environmental matrices subject to incorporation of NER also include animal manure, sewage sludge, plant material, and livestock / biotic systems. Table 2 presents the environmental and biological matrices which may be susceptible to contain NER, and describes some of the possible pathways of direct and indirect exposure leading to their contamination.

This report focuses on NER in the soil and sediment compartments, but recognises that other environmental matrices, especially sewage sludge, are also of high importance. It is expected that assessment approaches described herein for soil and sediment will, in the main, also be applicable to sewage sludge.

This Task Force has not considered in detail the category of livestock and biotic systems but will briefly discuss the relevance of NER to bioaccumulation / biomagnification processes.

Table 2: Environmental matrices prone to contamination with NER and potential pathways of direct and indirect contamination

Matrix	Direct Input Source	Indirect Input Source
Sewage Sludge	Industrial on-site STP Household down-the-drain disposal Run-off and flood events	
Soil	Application of agrochemicals Application of fertilisers / nutrients Other soil treatments (e.g. sterilisers) Sewage sludge amendments Industrial classified site Authorised discharge Illegal dumping	Run-off and flood events Irrigation Atmospheric deposition Manure Organic matter breakdown
Sediment	Sewage outfall Aquaculture Oil exploration activities Port / harbour activities Dredging - transport of contaminated sediments Accidental release Illegal dumping	Contaminated water Run-off and fluvial transport Flood events Atmospheric deposition Tidal redeployment of sediment Urbanisation, alteration of water courses
Plants / Vegetation	Spray application Storage conservators, Packing-House products Seed treatment	Uptake from contaminated soil Drift from neighbouring field applications Irrigation Atmospheric deposition
Manure	Contaminated feedstock Agroveterinary products	Trophic accumulation via food-chain
Livestock and Biotic Systems	Contaminated feedstock Agroveterinary products	Trophic accumulation via food-chain

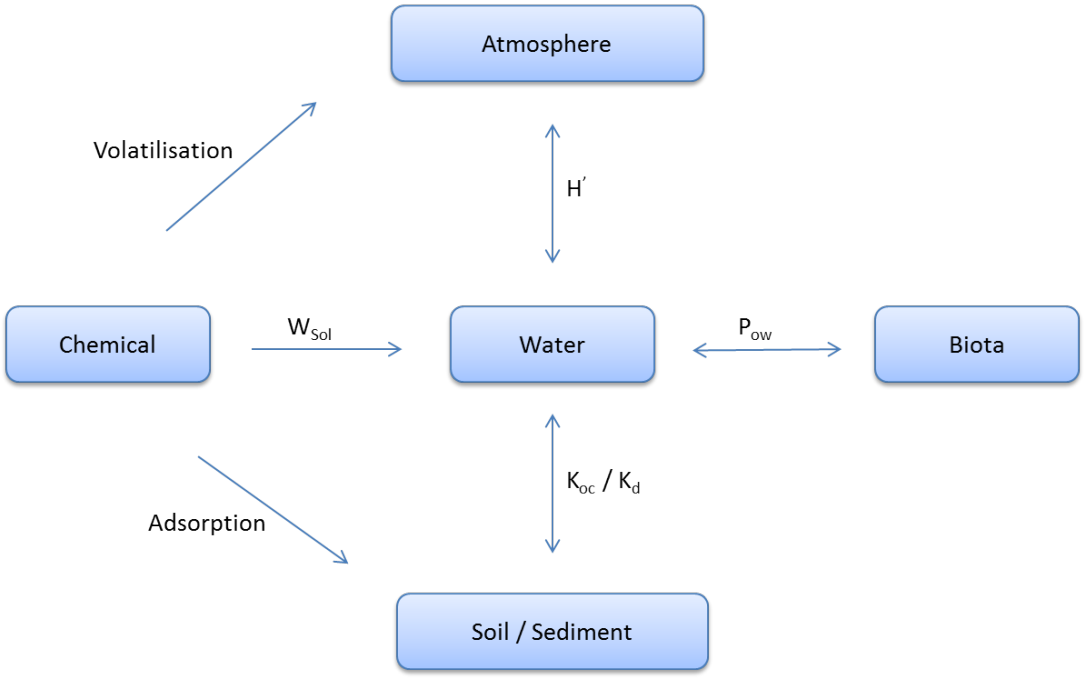
2. PROCEDURES FOR IDENTIFYING THE FORMATION OF NER AND THE POTENTIAL TO FORM NER

Intrinsic characteristics, including physico-chemical parameters and biodegradability help in predicting the environmental behaviour and distribution of chemical substances. The aim of this chapter is to highlight a range of the *in silico* and experimental approaches that may be used to indicate NER formation and provide an overview of the various advantages and disadvantages of each approach.

2.1 Physico-chemical parameter alerts

Physico-chemical properties provide strong indications of potential for partitioning on to solids as they can be directly related to both mobility and persistence of chemicals in the environment (Figure 5). These basic parameters are commonly used by scientists and regulators to describe the fate and behaviour of a chemical in the environment.

Figure 5: Physico-chemical properties and influence on the compartment in which a chemical will reside



2.1.1 Partition coefficient n-octanol/water (P_{ow})

The octanol/water partition coefficient is a key physico-chemical parameter used in the prediction of the environmental fate of chemical substances, notably for the prediction of the potential for bioaccumulation and adsorption to soil and sediment compartments. This particular partition coefficient is the most commonly used, usually expressed as P_{ow} or K_{ow} (octanol/water partition coefficient).

Chemicals which have a high log P_{ow} value (> 3), are more hydrophobic (lipophilic) and more likely to partition into the organic phase in the environment and adsorb on to organic matter (soils / sludge / sediments). A low or negative log P_{ow} value (< 1) indicates that a chemical has a greater affinity for the water phase, and hence, is more hydrophilic and less likely to adsorb on to an organic phase. Chemical substances containing charged ions and polar substituent groups at environmental pH values (e.g. hydroxyl, carboxylic acid, amine, etc.) tend to have lower log D_{ow} (the distribution constant at a given pH) values than substances that are uncharged and lack such polar substituents. However, these ionic/polar substances may sorb to other components of the environmental matrix (MacKay and Vasudevan, 2012). There are no specific trigger values for P_{ow} or D_{ow} reported in regulatory guidance documents which will explicitly determine if a chemical will adsorb and potentially go on to form an NER. Factors such as the biodegradation potential of the chemical and the nature of the solid matrix it is exposed to will all affect NER formation. Trigger values for sediment organism testing derived from log P_{ow} are referenced in REACH guidance (Chapter R7b endpoint specific guidance in REACH, 2008):

"Substances that are potentially capable of depositing on or sorbing to sediments to a significant extent have to be assessed for toxicity to sediment-dwelling organisms. In addition, marine sediment effects assessment is necessary for substances that are known to be persistent in marine waters and may accumulate in sediments over time. In general substances with a K_{oc} < 500 – 1000 L/kg are not likely to be sorbed to sediment (SETAC, 1993). According to this, a log K_{oc} or log K_{ow} of ≥ 3 is used as a trigger value for sediment effects assessment".

This trigger assumes that the substance adsorbs only to the organic fraction of the sediment. This trigger value is not driven by a concern over NER but a more broad concern over the potential risks from substances that tend to partition into soil / sediment. EPI Suite (US EPA, 2011) have provided interpretative guidance on typical substance behaviour for P_{ow} values (Table 3).

Table 3: The interpretive guidance document provided by EPI Suite on estimated physico-chemical values for log P_{ow} (estimated by KOWWIN) suggests the following interpretation of the values

log P_{ow}	Substance behaviour in water
< 1	Highly soluble in water (hydrophilic)
> 4	Not very soluble in water (hydrophobic)
> 8	Not readily bioavailable
> 10	Not bioavailable – difficult to measure experimentally

Log P_{ow} is not an accurate determinant of lipophilicity for ionisable compounds because it only correctly describes the partition coefficient of neutral (uncharged) molecules (Table 3). As detailed in (EU, 2003), for ionising substances the pH dependence of P_{ow} and water solubility should be known and partition coefficients should be corrected according to the pH of the environment.

Generally, soil pH has a minor effect on the sorption of non-ionic molecules; conversely, for ionic compounds the sorption coefficient can be quite sensitive to the pH of the sorbing soil due to differing sorption contributions from ionic and non-ionic species (Franco *et al*, 2009). For surface active substances, it may be

more appropriate to obtain measured distribution coefficient, K_p, values rather than estimations of K_{oc} as these substances tend to concentrate at the interface of the octanol and water layer. Log P_{ow} can be determined either by an appropriate *in silico* estimation method based on the structure of the molecule (QSAR), or by a laboratory test. Extensive guidance is given in Chapter 7A of REACH (2008) for the estimation of QSARs including a comprehensive list of available tools, validation of an estimated QSAR and the various deficiencies for certain chemical classes using QSAR. Several guideline methods exist for the measurement of P_{ow}. Each of these methods has advantages and disadvantages and care is required when selecting which method is most suitable for a particular chemical.

More detailed guidance is provided in Chapter R.7.1.8.5 of REACH (2008) for an integrated testing strategy for octanol/water partition coefficients.

2.1.2 Soil adsorption coefficient (K_{oc}/K_d)

The sorption behaviour of chemical substances in soils, sewage sludge and sediments is described by the K_d, which is the ratio between the concentration of the substance in the solid matrix and the concentration in the aqueous phase at adsorption equilibrium:

$$K_d = \frac{C_{soil}}{C_{aq}} \; or \; \frac{C_{sludge}}{C_{aq}} \; or \; \frac{C_{sediment}}{C_{aq}}$$

where:

C_{soil}	Concentration in the soil	[mg · kg^{-1} dw]
C_{sludge}	Concentration in the sludge	[mg · kg^{-1} dw]
$C_{sediment}$	Concentration in the sediment	[mg · kg^{-1} dw]
C_{aq}	Concentration in the aqueous phase	[mg · L^{-1}]

Values for K_d can vary greatly for the same chemical in different soils or sediments because the organic content has not been considered in the equation. The role of soil organic matter (SOM) is now well studied and understood, where SOM behaves as a non-polar surface and is one of the main sorbents in soils, attracting non-polar organic molecules (Schüürmann *et al*, 2006). The preferred value for determining a soil's ability to adsorb non-polar organic chemicals is K_{oc}, since it considers the organic content of the soil.

$$K_{oc} = \frac{K_d}{F_{oc}}$$

K_{oc} = soil organic carbon / water partition coefficient (L/kg)
F_{oc} = fraction of organic carbon in soil

K_{oc} is the organic carbon normalised partition coefficient. Once K_d is known, using this normalised value, it can be applied to any soil if the soil's organic carbon content is known. For non-polar chemicals, the value of K_{oc} gives a strong indicator of whether or not a chemical is likely to sorb on to soils, sediments or sludges.

A chemical with a high log K_{oc} (> 3.5) value is likely to be adsorbed to soils and sediments and thus, is likely to remain on the soil surface (Table 4). In contrast, a chemical with a low log K_{oc} value (< 1.5) is not likely to be adsorbed to soils and sediments and is likely to leach through these soils and sediments. If not degraded, such mobile chemicals may reach ground and surface waters. The interactions between a chemical – water – soil are in reality more complex as chemicals can also adsorb to alternative sites, such as mineral surfaces (see model test compound DODMAC in Chapter 4) or clay surfaces via ionic interactions. Again, as discussed for P_{ow}, reference to trigger values derived from K_{oc} values are mentioned in guidance for when sediment and terrestrial testing should be considered (Chapter R.7.1.15 in REACH (2008)):

"Substances with a K_{oc} below 500 – 1,000 L/kg are generally unlikely to adsorb to sediment (SETAC, 1993). To avoid extensive testing of chemicals, a log K_{oc} (or log K_{ow}) ≥ 3 can be used as a trigger value for sediment effects assessment. Strong binding behaviour to soil particles (e.g. log K_{ow} > 5, log K_{oc} > 4) might justify immediate long-term soil organism toxicity testing if particular sensitivity and/or persistence is anticipated".

EPI Suite (US EPA, 2011) interpretative guidance (Table 4) is provided for typical sorption behaviour corresponding to K_{oc} value.

Table 4: The interpretive guidance document provided by EPI Suite on estimated physico-chemical values for K_{oc} (estimated by PCKOCWIN) suggests the following interpretation of the values

log K_{oc}	Substance sorption behaviour to soil / sediment
< 1.5	Negligible sorption to soil and sediment, rapid migration potential to groundwater
1.5 – 2.4	Low sorption to soil and sediment, moderate migration potential to groundwater
2.5 – 3.4	Moderate sorption to soil and sediment, slow migration potential to groundwater
3.5 – 4.4	Strong sorption to soil and sediment, negligible to slow migration potential to groundwater
> 4.5	Very strong sorption to soil and sediment, negligible migration potential to groundwater

2.1.3 Water solubility

Water solubility data provides a strong indication on the likely bioavailability of a chemical and if it is likely to be prone to adsorb and potentially form NER. A chemical that is soluble in water will tend to have relatively low adsorption coefficients for soils and sediments. A chemical that is relatively insoluble in water will tend to have higher adsorption coefficients. Table 5 provides further guidance on typical substance properties associated to water solubility.

Table 5: The interpretive guidance document provided by EPI Suite on estimated physico-chemical values suggests the following interpretation of the values for W_{sol}, estimated by WSKOWWIN (US EPA, 2011)

Water solubility (mg/L)	Substance properties in water
< 0.1	Insoluble
> 0.1 – 100	Slightly soluble
> 100 – 1000	Moderate solubility
> 1000 – 10000	Soluble
> 10000	Very soluble

2.1.4 Henry's law constant

Henry's law constant (H') is a measure of the capacity of a substance to undergo exchange from water across the air-water interface into the atmosphere. Table 6 provides further guidance on typical substance properties associated to volatility. H' does not have a direct influence on NER formation nor its prediction.

Table 6: The interpretive guidance document provided by EPI Suite on estimated physico-chemical values suggests the following interpretation of the values for Henry's Law Constant, estimated by HENRYWIN (US EPA, 2011)

Henry's Law Constant		
(atm-m^3/mole)	Pa-m^3/mole	Substance volatility properties in water
> 10^{-1}	> 10133	Very volatile from water
> 10^{-1} – 10^{-3}	101.33 – 10133	Volatile from water
> 10^{-3} – 10^{-5}	1.0133 – 101.33	Moderately volatile from water
> 10^{-5} – 10^{-7}	0.010133 – 1.0133	Slightly volatile from water
> 10^{-7}	< 0.010133	Non volatile

2.1.5 Multimedia modelling

Physico-chemical properties also allow the use of simple fate models to predict the multi-media partitioning of a chemical in the environment (i.e. the likely fate and distribution of a chemical in the environment). Multi-media models such as those developed by Mackay (1991) are often used to evaluate the environmental fate of a variety of chemicals. The concept of fugacity has been widely used to model the concentrations of a substance in different environmental compartments (water, air, soil, sediment, suspended solids and fish). The model estimates the proportion of a compound likely to partition between these compartments, based on a standard release of the chemical into the environment. A sequence of Level I, II and III calculations can be made, which have increasing data requirements each resulting in increasing information about the distribution of the chemical, its susceptibility to transformation and transport and the environmental process and chemical characteristics that most significantly influence chemical fate (Mackay, 1991).

The output from these models provides guidance as to which environmental compartment a substance may migrate towards. Modelling output indicating significant partitioning into the soil or sediment compartment provides reasonable evidence that NER formation is possible. However, care is needed in the interpretation

since many of the partition estimates are based on P_{ow} or water solubility predictions, or at best measurement of these physico-chemical properties. As mentioned in Section 2.1.1, P_{ow} focuses on the partitioning to the organic carbon fraction and may not be suitable for use with all chemicals.

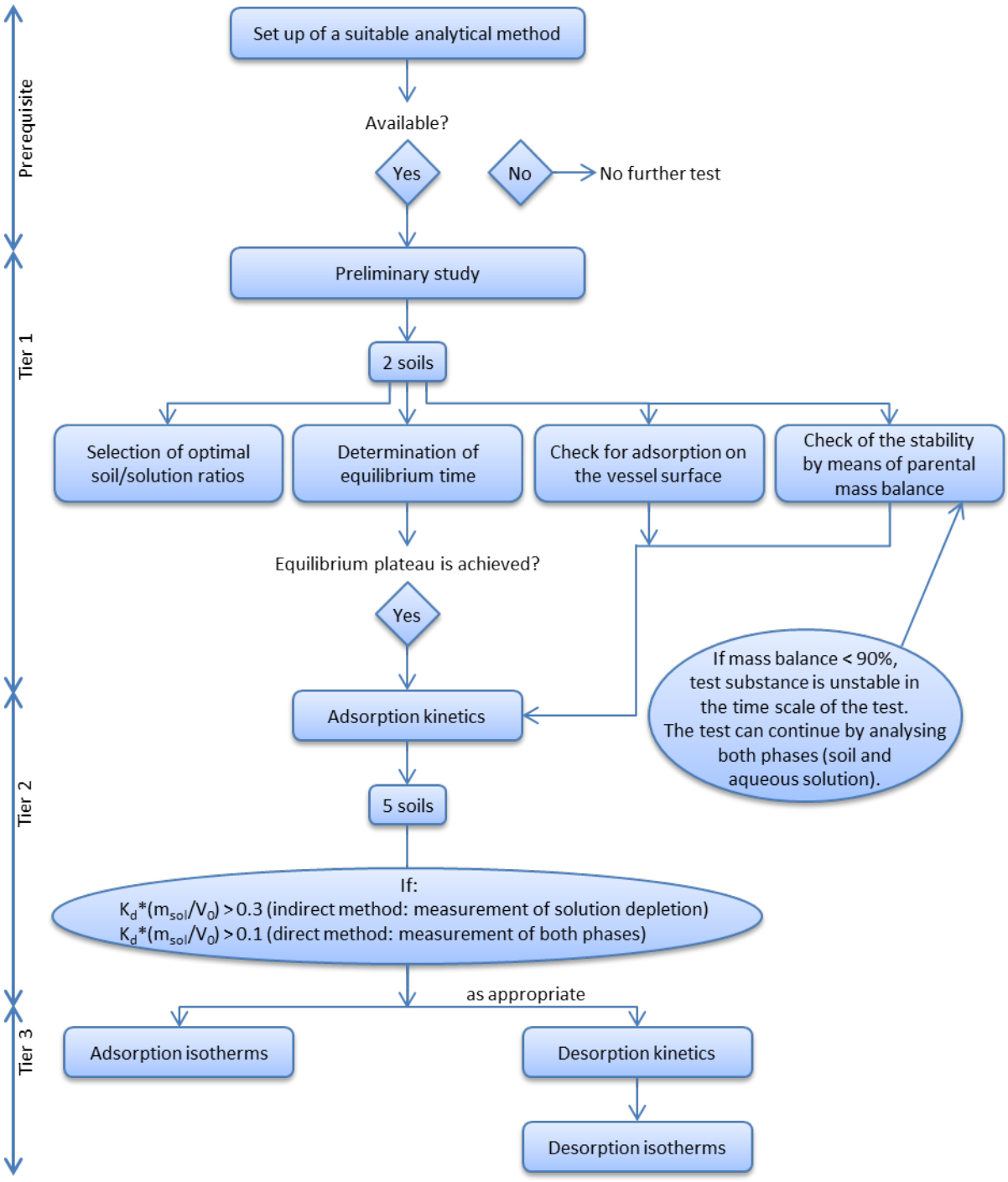
2.2 Adsorption / desorption batch equilibrium experiments

Determining the distribution between solid and aqueous phase compartments is a complex process which depends upon a number of independent factors, the most significant of which are the nature of the adsorbent and the chemical nature of the substance. It is recognised that environmentally relevant parameters and mechanisms beyond the control of standardised laboratory experiments may well have a strong bearing on the distribution and re-distribution of chemical substances. Despite this, batch equilibrium adsorption / desorption studies do provide valuable information with respect to the potential of chemicals to bind to solid matrices, an important element in determining the potential bioavailability of the substance to undergo transformation, for uptake by organisms, potential to leach and potential to form NER. Well defined test guidelines, for example OECD 106 (OECD, 2000a) and OPPTS 835.1230 (US EPA, 2008c) have been developed to study batch equilibrium in soil, and are also applicable to sediments. Another matrix of high importance is sewage sludge, for which several test guidelines exist (ISO 18749:2004 (ISO, 2004); OPPTS 835.1110 (US EPA, 1998a)).

Non-extractable residues can be formed by the parent molecule adsorbing to the solid matrix. However, the formation of NER can often be attributed to a two-step process where the parent molecule undergoes transformation, followed by binding of the metabolite to the solid matrix. To this end, the use of sterile controls may be of interest in attempting to establish the fraction of the adsorbed material which is parent compound and the fraction of the adsorbed material which is formed by transformation products. However, it is not possible to sterilise soil, sediment or biosolids without altering the physical and chemical properties of the matrix (Wolf and Skipper, 1994). Also, the use of chemical sterilisation methods should be used very cautiously since very high concentrations are required to prevent microbial activity and this may affect the observed partitioning (Wick *et al*, 2011). Where possible, sterilisation of these tests should be avoided.

The purpose of the adsorption / desorption test is to obtain sorption values which can be used to predict partitioning under a variety of environmental conditions. Figure 6 describes these tests which generally consist of three tiers:

Figure 6: OECD testing scheme, OECD 106 (OECD, 2000a)



Tier 1: A preliminary study which essentially determines the appropriate test conditions, and also ensure the feasibility of the study by determining the (soil / sediment / sludge) / solution ratio, the equilibration time for adsorption, the amount of test substance adsorbed at equilibrium, the adsorption of the test substance on the surfaces of the test vessels and the stability of the test substance over the test period.

Tier 2: A screening test where the adsorption is studied in a range of different soil / sediment / sludge types by means of adsorption kinetics at a single concentration and determination of distribution coefficients K_d and K_{oc}.

Tier 3: Determination of Freundlich adsorption isotherms to determine the influence of concentration on the extent of adsorption on soils and study of desorption by means of desorption kinetics / Freundlich desorption isotherms.

The originally adopted version of the OECD 106 guideline (OECD, 1981b) was similar but far less intensive. In the 1981 version a preliminary test was performed to ensure the applicability of the method, then a screening test was performed, followed by an advanced test if required. This test is discussed in more detail in the context of NER in Chapter 3.

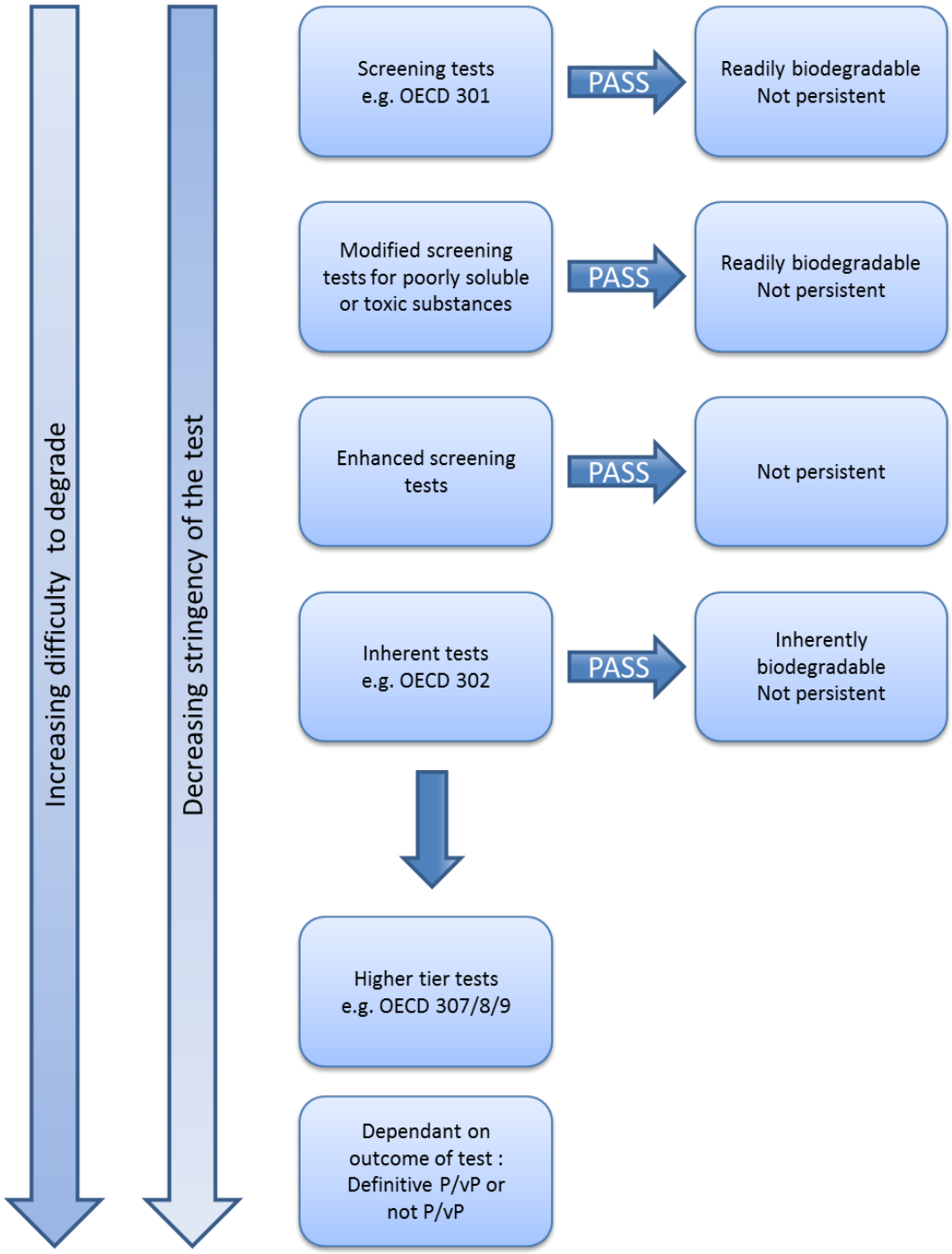
2.3 Biodegradation screening tests

Understanding the intrinsic biodegradation characteristics of a chemical provides essential insights into the fate of the chemical and likely potential to form NER.

Like many screening level approaches for risk assessment of a chemical, models are often first reviewed for predicting biodegradation. Boethling *et al* (2003) validated the BIOWIN models and concluded they were useful for screening-level assessment to predict not-readily biodegradable substances with high accuracy in contrast to confirming ready biodegradability. Other biodegradation models include CATABOL (Jaworska *et al,* 2002) which predicts the extent of biodegradation and also provides information on predicted metabolic pathways and metabolites.

Some ready biodegradation screening tests e.g. 'DOC Die-Away Test' (OECD, 1992a) and 'Modified OECD Test' (OECD, 1992a) and inherent biodegradation screening tests e.g. 'Zahn-Wellens/EMPA' (OECD, 1992b) and 'Modified SCAS' (OECD, 1981c) use DOC (dissolved organic carbon) as the analytical method. Although these are all screening tests primarily concerned with determining the extent of biodegradation of a chemical, they do not differentiate between this and adsorption (unless radio labelled chemicals are used, or inferred from additional tests). Generally, in these test systems if the chemical / organic carbon is removed from the system rapidly (e.g. after 3h) adsorption of the chemical is the assumed mechanism of removal. Comber and Holt (2010) have reported a set of reference chemicals with a known behaviour for use as controls in biodegradation tests which cover a range of profiles from readily biodegradable to persistent and aligned them to a tiered biodegradation testing program (Figure 7) which is typical for determining biodegradation of a chemical.

Figure 7: Hierarchy of screening and higher tier biodegradation tests for determining persistence (Comber and Holt, 2010)



The scheme could be used in the initial phases of screening for NER formation. For example, substances which are classified as persistent, or very persistent, may remain in the environment for a long enough time for them to be incorporated into the matrix and form NER.

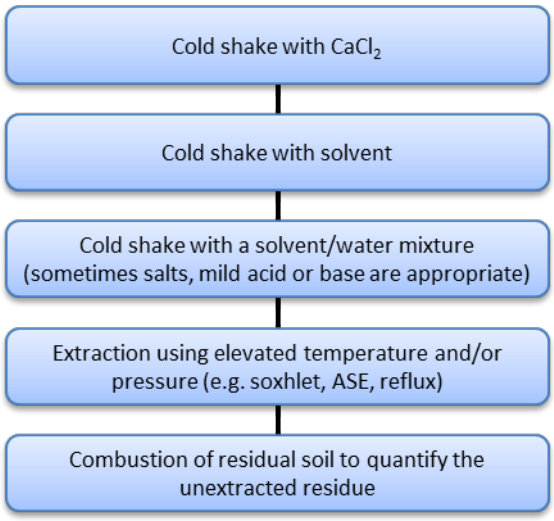
On the other hand, those substances classified as not persistent are less likely to directly form NER as they will be more likely to be transformed or mineralised in a short time period. In limited cases, readily biodegradable chemicals may still have the potential to bind rapidly and form NER.

Even for rapidly mineralised substances, there is a potential to form NER by biogenic mechanisms. Isolation and identification of NER has been a major analytical challenge, and, the interpretation of study results have been almost entirely reported based on total residual radioactivity and do not refer to specific speciation of chemical moieties. A recent study published by Nowak *et al* (2011) studied the nature of NER formed in the fulvic acid and amino acid fractions of soil, amended with farmyard manure on an annual basis. Following addition of ^{13}C-2,4-dichlorophenoxyacetic acid (2,4-D) the soil-manure mixtures were incubated under aerobic conditions for up to 64 days. The incorporation of the ^{13}C-label into living biomass via $^{13}CO_2$ fixation was also studied in parallel. The results of their research indicated that, in the case of 2,4-D, almost all of the bound residue fraction was constituted of biogenically fixed moieties containing natural microbial residues stabilised in the soil organic matter fraction. The authors suggest that the potential risk of bound residues from readily metabolised xenobiotics in soil is highly over-estimated. Differentiating between biogenic NER and chemical binding is analytically very challenging and is not a routine procedure. Therefore, at this time, it is recommended that NER should be considered as a single component in the environmental risk assessment.

2.4 Environmental fate simulation studies

The current test guidelines for determining transformation of a chemical in soil are OECD 307 (OECD, 2002a), OPPTS 835.4100 (US EPA, 2008a) or for the sediment compartment OECD 308 (OECD, 2002b) and OPPTS 835.4300 (US EPA, 2008b). The principles of these tests are that soil or sediment/water samples are treated with the test substance and incubated in the dark in biometer-type flasks or in air flow-through systems under controlled laboratory conditions (at constant temperature). After appropriate time intervals, samples are extracted and analysed for the parent substance and for transformation products. Incubation flasks are removed at appropriate time intervals and the samples extracted with appropriate solvents of different polarity and analysed for the test substance and/or transformation products. Extraction of the sample is generally repeated until an adequate extraction is achieved. Typically, extraction may be performed with increasingly powerful solvents or techniques (Figure 8). The more aggressive extraction methods may remove both extractable residues and NER (ECETOC, 2012). Volatile products are also collected for analysis using appropriate adsorption devices. Using ^{14}C-labelled material, the mineralisation rate of the test substance can be measured by trapping evolved $^{14}CO_2$ and a mass balance established, including the formation of NER.

Figure 8: Typical extraction scheme for determination of residues (ECETOC, 2010)



Currently available test guidelines state applicability to all chemical substances (non-labelled, or, radiolabelled) for which an analytical method with sufficient accuracy and sensitivity is available. They are applicable to slightly volatile, non-volatile, water-soluble or water-insoluble compounds. The tests are not, however, applicable to compounds which are highly volatile from the matrix, although no vapour pressure cut-off has been stated. Whereas non-labelled or labelled test substance can be used to measure the rate of transformation in a matrix, labelled material is required for studying the pathway of transformation, the formation of NER and for establishing a mass balance.

In soil or sediment/water tests, the radioactive test substance is usually measured in the following compartments:

- Volatile fraction of parent, metabolites and CO_2, CH_4 and/or CO_2 in headspace gases.
- Residual CO_2 in water (sediment-water study).
- Parent compound and metabolites in water (sediment-water study, and interstitial soil pore water).
- Parent compound and metabolites in soil / sediment.
- NER in soil / sediment.

Some concerns have been raised regarding the suitability of these biodegradation tests as they can include unrealistically high dose rates. Introduction of test substance may not simulate *in situ* conditions, e.g. chemicals that enter soil on sludge should be dosed on sludge. Likewise veterinary pharmaceuticals that enter soil in manure should be dosed on to manure then added to the soil tests in an appropriate, environmentally realistic proportion; currently this practice is not the norm. Matrix effects are not accounted for, normal processes which occur in the field such as wetting / drying, ploughing / turbation, etc., are not reflected in these simulations. In short, the majority of these environmental 'simulation' studies do not simulate environmental conditions. The design of the current OECD 308 water-sediment test includes a static ratio of approximately 3:1 (v/v) between water and sediment. This ratio shifts equilibrium mass distribution towards the sediment phase, compared to natural conditions, which may result in unrealistically

high levels of NER. Furthermore, incubation unit dimensions (active surface area versus ratio of matrix content) is a recurring theme considered as lending heavy bias to study results.

The nature of the NER have been characterised following fractionation of the organic fraction of soils into humin, humic and fulvic acid portions via the application of sequential strong-acid / strong-base harsh extraction. The impact of such extraction procedures on the integrity of the matrix and residual chemical are, thus, questionable. A range of techniques have been reported to assist with the characterisation of NER. Techniques include solvent extraction, hydrolysis methods, derivatisation of functional groups, model compound investigations, pyrolysis and thermal desorption techniques, immunoassay analysis and spectroscopic techniques. Spectroscopic techniques include Electron Spin Resonance (ESR), Fourier Transform Infra-Red (FTIR), Fluorescence and Nuclear Magnetic Resonance (NMR). The merits of these techniques for determination of organic bound residues are discussed in more detail by Northcott and Jones (2000). Extraction techniques are also reviewed more extensively by ECETOC (2012).

Additional, 'surrogate' information may also be gleaned from both higher tier environmental fate and ecotoxicity studies. Depending upon the particular experimental set-up and, in particular where radiolabelled test material has been used, the portion of the NER in the solid-phase may be determined. Apart from the traditional soil degradation (OECD, 2002a) and sediment-water degradation (OECD, 2002b) studies, which are included in Tier 3 of this evaluation process, an additional insight into adsorption characteristics may be determined from the following test guidelines (see Tables 7, 8, 9), assuming an appropriate test set-up and use of a radioisotopic tracer:

Table 7: Terrestrial and aquatic / sediment fate studies

Reference	Guideline Name	"Surrogate" Information Related to Adsorption
OECD, 2001	OECD 303 – Simulation Test – Aerobic Sewage Treatment: A) Activated Sludge Units, B) Biofilms	Development of sludge K_d and K_{oc} value and NER
US EPA, 1998b	OPPTS 835.3220 – Porous Pot Test	
US EPA, 2008d	OPPTS 835.3240. Simulation Test – Aerobic Sewage Treatment: A. Activated Sludge Units	
US EPA, 2008e	OPPTS 835.3260 Simulation Test – Aerobic Sewage Treatment: B. Biofilms	
OECD, 1981a	OECD 304A – Inherent Biodegradability in Soil	Quantification of NER fraction
US EPA, 1998c	OPPTS 835.3300 – Soil Biodegradation	
OECD, 2004b	OECD 312 – Leaching in Soil Columns	Development of soil K_d and K_{oc} value and NER
US EPA, 2008f	OPPTS 835.1240 – Leaching Studies	
OECD, 2008a	OECD 314 (Parts A through E) – Simulation Test to Assess the Biodegradability of Chemicals Discharged in Wastewater	Development of sludge K_d and K_{oc} value and NER, particularly from 314B and 314C
US EPA, 2008g	OPPTS 835.3280 – Simulation Tests to Assess the Primary and Ultimate Biodegradability of Chemicals Discharged to Wastewater	
US EPA, 2008h	OPPTS 835.2410 – Photo-degradation in Soil	Quantification of NER fraction

Table 8: Terrestrial and aquatic / sediment bioaccumulation studies

Reference	Guideline Name	"Surrogate" Information Related to Adsorption
OECD, 2008b	OECD 315 – Bioaccumulation in Sediment-Dwelling Benthic Oligochaetes	Quantitative analysis of NER in sediment / soil fraction
OECD, 2010a	OECD 317 – Bioaccumulation in Terrestrial Oligochaetes	

Table 9: Terrestrial and aquatic / sediment ecotoxicity studies

Reference	Guideline Name	"Surrogate" Information Related to Adsorption
OECD, 2004c	OECD 218 – Sediment-Water Chironomid Toxicity using Spiked Sediment	Quantitative analysis of NER in sediment fraction
OECD, 2007	OECD 225 – Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment	

This section has summarised many of the existing direct or indirect methods (both *in silico* and experimental) employed for identifying NER formation or potential for NER formation, as well as highlighting the strengths and weaknesses of these various approaches. The following chapter now leads through a proposed framework where many of these methods would be applied in a tiered risk assessment approach, and offers some recommendations of where further research could advance this framework and the science of identifying potential NER formation.

3. TIERED TESTING SCHEME TO ASSESS THE IMPORTANCE OF NER IN ERA

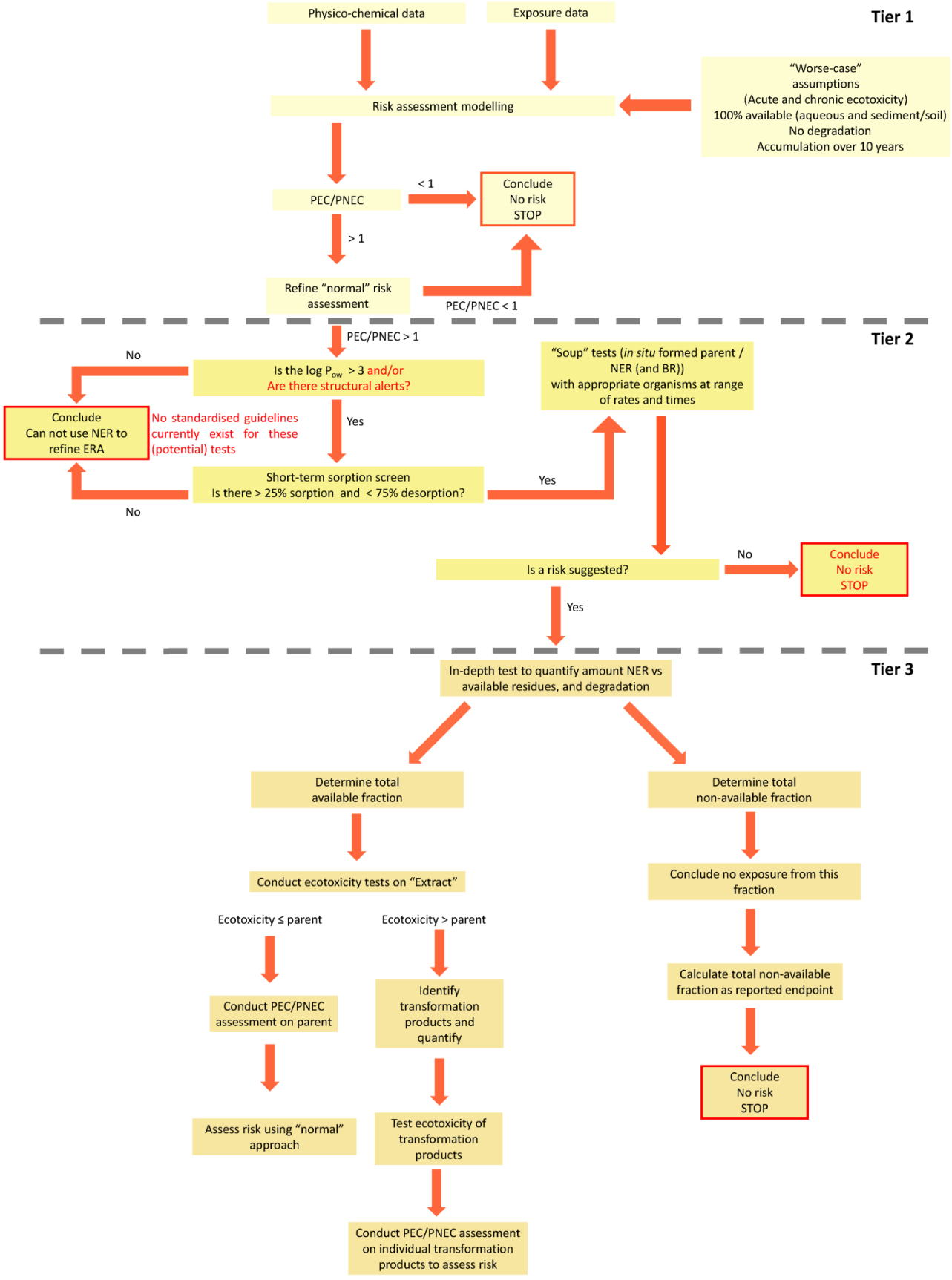
3.1 Introduction to the scheme

The first steps in the risk assessment process for determining chemicals with a potential for forming NER is a screening approach to prioritise chemicals, usually based on hazard and exposure potential. This typically involves collation and evaluation of the relevant physico-chemical data for the chemical and then subsequently to estimate the routes of environmental exposure through use patterns.

This screening approach integrates conservative default assumptions into simple models to compensate for gaps in the data and uncertainties. The assumptions are deliberately designed to be conservative in order to avoid risk decisions based on 'false negatives'. This approach can quickly identify where more refined assessments may be required. The refined assessments are designed to closely simulate a particular exposure scenario and thus require more detailed chemical-, site-, and receptor-specific data and use fewer default assumptions. This approach is less resource intensive (particularly when large numbers of chemicals may be involved) and serves as an efficient means of categorising and prioritising those chemicals that either warrant more tailored and detailed assessments or are of no concern and can be put aside.

Conventional approaches can be taken to discriminate chemicals that present no concern and those that require further work to evaluate whether they are likely to be ecotoxic under proposed conditions of use. The aim is to provide a robust screening method, starting with relatively simple criteria and progressing through steps of increasing complexity, capable of targeting substances of potential risk. The risk assessment scheme is tiered and iterative (Figure 9).

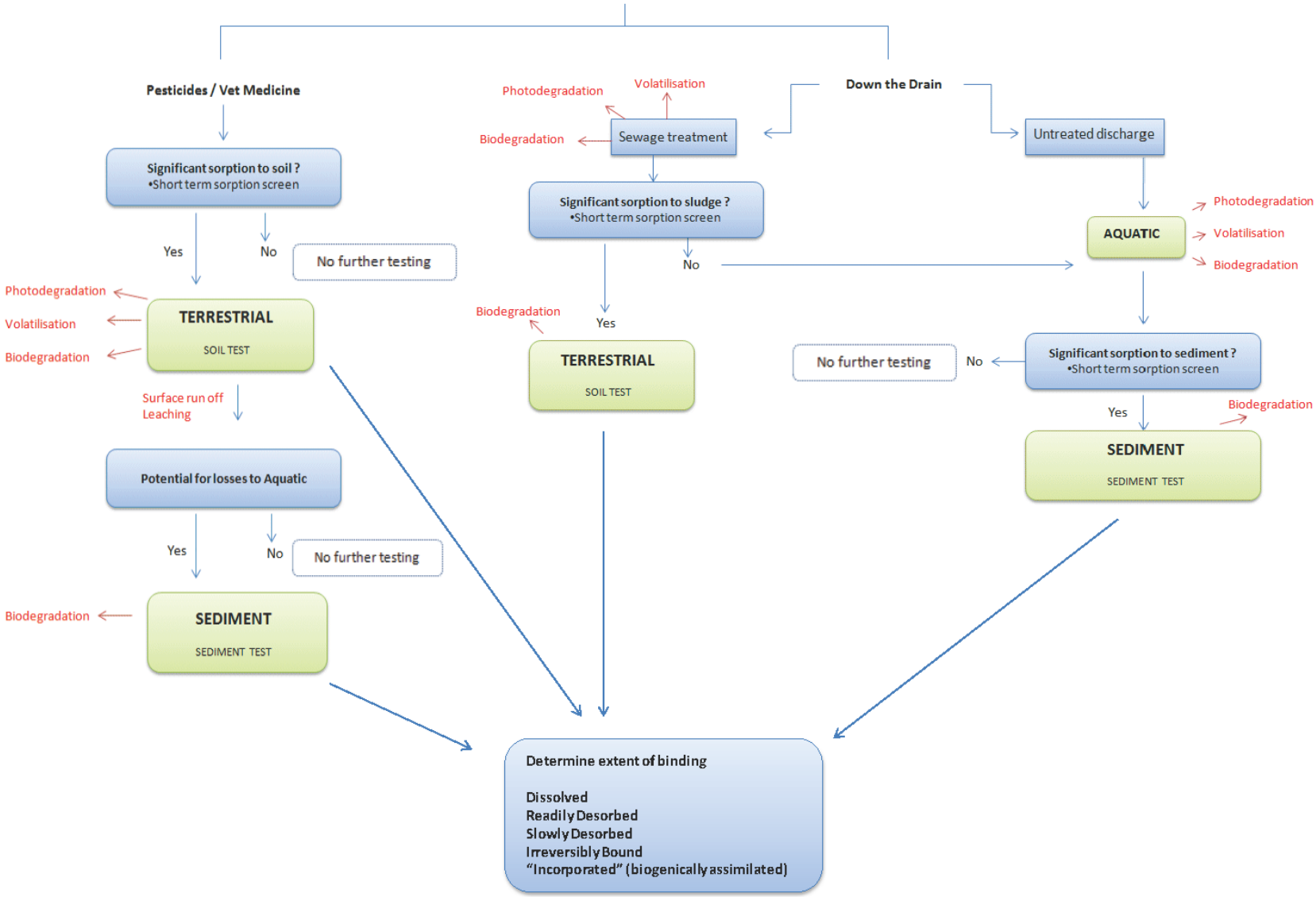
Figure 9: Risk assessment scheme accounting for non-extractable residues



3.2 Potential exposure scenarios and relevant environmental compartments

Consideration of the exposure patterns will determine the relevant compartment testing strategies for determination of the predicted environmental concentration in that compartment (Figure 10) e.g. for a highly water soluble (non sorptive) substance discharged 'down the drain', terrestrial (soil) tests are not appropriate, when the principal environmental exposure for the substance will be via sewage treatment and the aquatic environment. Exposure assessment should also consider previous releases of the chemical to the environment that may give rise to a 'background concentration' as well as sources from natural origins e.g. metals.

Figure 10: Typical exposure scenarios with potential for NER formation and testing strategy



3.3 Waiving criteria

Prior to assessing a chemical in Tier 1 of the framework for NER, an initial screening level assessment should be completed to confirm that a link potentially exists between the chemical forming an NER in an environmental compartment and that biota are likely to be exposed to them. Several criteria may deem that the chemical is of no concern or has no potential for formation of residues and no further risk assessment would be required (for NER formation, other compartments exposed e.g. aquatic, would still need to be completed), some examples may include:

Annual volume

If the substance is produced or used in the EU at < 10 Tonnes per annum, and sufficient data is available to demonstrate that the substance is not PBT or vPvB, or, if the material is intended for research purposes only, then environmental exposure will be limited and NER evaluation would not need to be performed.

Use pattern

If it can be demonstrated that the substance is used in processes where emission to the environment is highly unlikely and that emissions from the production and preparation steps are zero (e.g. from self-contained, closed systems), or, exist in a sequestered non-bioavailable, non-leachable form (e.g. in certain plastics and coatings) then further NER evaluation would not need to be performed.

Substance is an intermediate in a production process

If it can be illustrated that the substance occurs as an intermediate in a confined production process and that no waste residues containing the substance are generated from the procedure, then NER evaluation can be waived.

Physico-chemical data

Highly water soluble chemicals (> 1000 mg/L at 20°C) with log P_{ow} < 1 and log K_{oc} < 1.5 values are highly unlikely to partition on to solids in the environment. Leaching through soils and sediments will be probable pathways in the environment unless the chemical interacts via ionic mechanisms, e.g. via cation exchange. NER would not be expected to be formed and further evaluation would not be necessary.

Biodegradability

If data are available which confirm the chemical is readily biodegradable, it is likely to be of low concern as any NER are likely to be formed via biogenic routes. Persistent residues of the parent compound are also unlikely to be formed, although any potential for hydrophobic metabolites should be considered before waiving of further NER formation assessment.

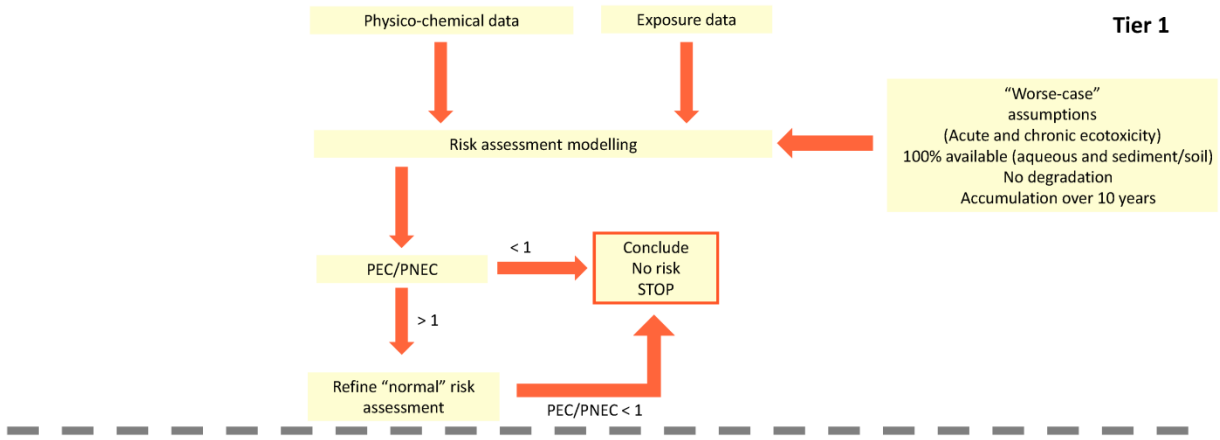
Intrinsic aquatic and terrestrial toxicity

The risk assessment of NER can be waived if the intrinsic toxicity (acute and/or chronic, depending upon the release frequency to the environment of the parent and associated metabolites) has been demonstrated under valid experimental conditions to be low to both aquatic and/or terrestrial life-forms (e.g. EC/LC_{50} > 100 mg/L and > 1,000 mg/kg dry wt soil, or an NOEC > 10 mg/L and 100 mg/kg dry wt soil).

3.4 Tier 1 - Screening assessment assuming 100% bioavailability

The general approach, when generating a screening assessment, for identifying chemicals that pose a potential environmental risk is to assume no formation of NER and follows the principles of standard risk assessment. Initially this involves data collection and validation of the available physical / chemical data, the exposure assessment and any QSAR derived data (Figure 11). This data is then extrapolated to the soil or sediment compartment using equilibrium partitioning and the application of suitable assessment factors to account for the uncertainty of the extrapolation (usually aquatic derived toxicity values).

Figure 11: Introduction to Tier 1 of the NER risk assessment framework



As the first step in the risk assessment process 'worst case' scenario assumptions can be made that 100% of the chemical is available, i.e. there is no reduction in bioavailability and the inherent ecotoxicity could be expressed in proportion to the total exposure concentration. A conservative assumption of the duration of emission to the environment is also necessary to ensure a worst case estimation of PECs for chemicals that might be persistent. As a realistic worst-case exposure scenario, input and potential build-up is assumed for 10 consecutive years. Ten consecutive years of accumulation may not be sufficient for some substances to reach a steady-state situation. To indicate potential problems of persistency in soil, the fraction of the steady-state concentration can be calculated. If the risk characterisation ratio (PEC/PNEC ratio) is < 1 then it can be concluded that there is no further risk and the evaluation process can be curtailed at this point. If the PEC/PNEC ratio is ≥ 1 then potential refinement opportunities of the risk assessment used in the initial screen should be considered.

3.4.1 QSAR modelling

In this initial screen, it is quite probable that the only physico-chemical, ecotoxicity, environmental fate and distribution data for a substance will have been derived from QSARs and models (See Section 2.1). The base set of physico-chemical data (Table 10) should be the minimum data available for use in exposure models and determination of secondary data (partition coefficients) for risk assessment and determining the potential for partitioning.

Table 10: Physico-chemical data base set

Physical – Chemical properties	
Molecular weight	MolW
Octanol-water partition coefficient	P_{ow}
Water solubility	W_{sol}
Vapour pressure	VP
Structure	SMILES notation

The standard of QSAR predictions depend upon the quality and quantity of information used to build the model. REACH guidance documents (REACH, 2008) provide detailed advice on the use of QSAR models as well as the interpretation of their output. Practical guidance also exists with respect to read-across and how chemical 'similarity' is defined for such purposes. To date, QSARs have only been used to predict toxicity to species in the aquatic environment. The QSAR derived aquatic data can be used as input data for the equilibrium partitioning (EqP) method for extrapolation to the soil / sediment environment. Confidence in the derived QSAR must be validated otherwise the recommendation would be to progress directly to generating experimentally derived effects data. QSAR data should only be used for screening purposes to identify chemicals which provide an immediate or delayed concern to soil / sediment communities.

3.4.2 Equilibrium partitioning method

The origins of the equilibrium partitioning method (EqP) were to assess effects on organisms living in the sediment compartment, using aquatic toxicity data and the sediment-water partitioning coefficient. The EqP method makes the assumptions:

- Sediment dwelling organisms and organisms in the aquatic environment are equally sensitive;
- The concentration in the sediment, the interstitial water and organism are in thermodynamic equilibrium;
- Sediment/water partition coefficients can either be measured or derived on the basis of a generic partition method from separately measurable characteristics of the sediment and the properties of the chemical;
- Partitioning is mainly influenced by organic carbon in the solid matrix.

The equilibrium partitioning method may not be suitable for lipophilic chemicals which are likely to adsorb (log P_{ow} > 3) as it only considers uptake via the water phase. Organisms which are exposed primarily through chemicals that are adsorbed to soil particles and taken up by ingestion or contact with the chemical will not be considered, which results in an underestimation in the assessment. This method is not suitable for chemicals with a specific mode of action nor is it suitable for ionic or charged chemicals (at environmental pH values).

The following, based on equilibrium partitioning theory, is applied:

$$PNEC_{sed} = \frac{K_{susp\text{-}water}}{RHO_{susp}} \cdot PNEC_{water} \cdot 1000$$

where:

$PNEC_{water}$	Predicted No Effect Concentration in water	$[mg \cdot L^{-1}]$
RHO_{susp}	Bulk density of wet suspended matter	$[kg \cdot m^{-3}]$
$K_{susp\ water}$	Partition coefficient suspended matter water	$[m^3 \cdot m^{-3}]$
$PNEC_{sed}$	Predicted No Effect Concentration in sediment	$[mg \cdot kg^{-1}]$

The EU (2003) recommends the $PEC_{sed}/PNEC_{sed}$ ratio is increased by a factor of 10 to take uptake via ingestion of sediment into account. If, with this method, a PEC/PNEC ratio ≥ 1 is derived, then tests with benthic organisms using spiked sediment have to be conducted to support a refined risk assessment for the sediment compartment.

The applicability of the equilibrium partitioning method has been evaluated even less for soil than for sediment-dwelling organisms. However, the same general approach is applied to the terrestrial compartment and soil dwelling organisms. To overcome the potential for underestimation when considering lipophilic compounds (log P_{ow} > 5), as described for the sediment compartment the EU (2003) recommends an additional factor of 10 is applied to the final $PEC_{soil}/PNEC_{soil}$ ratio.

$$PNEC_{soil} = \frac{K_{soil\text{-}water}}{RHO_{soil}} \cdot PNEC_{water} \cdot 1000$$

where:

$PNEC_{water}$	Predicted No Effect Concentration in water	$[mg \cdot L^{-1}]$
RHO_{soil}	Bulk density of wet soil	$[kg \cdot m^{-3}]$
$K_{soil\text{-}water}$	Partition coefficient soil-water	$[m^3 \cdot m^{-3}]$
$PNEC_{soil}$	Predicted No Effect Concentration in sediment	$[mg \cdot kg^{-1}]$

As described for sediment PNEC derivation, if with this method a PEC/PNEC ratio ≥ 1 results, then tests with soil dwelling organisms have to be conducted to support a refined risk assessment for the soil compartment.

3.4.3 Assessment factors

Although assessment factors are built on this precautionary approach, they have long been established by authorities across the world and offer a simple and well understood approach to screening risk assessment. They are commonly used from the very early stages of the risk assessment process and are applied to the lowest determined effect concentration that has been measured in laboratory studies or, in some cases derived from QSARs. The scale of the factor which is applied to the assessment is a reflection of the

uncertainty in extrapolating from laboratory toxicity test data (or QSAR), ranging from single species (higher assessment factors applied), to data which is available for a range of trophic levels (lower assessment factors applied). The scaling of the applied factors reflects the confidence in extrapolating the data to the environment i.e. the amount of data available and the nature of it. The assessment factors applied to acute data are higher than those applied to chronic toxicity data. Tables 11 and 12 show examples of the terrestrial assessment factors applied in the US and European risk assessment guidance, respectively.

Table 11: US EPA assessment factors for terrestrial organisms

Available data	Assessment factor
L©C_{50} or QSAR estimate	1000
LIC_{50} or QSAR estimate for minimal three representatives of microbe-mediated processes, earthworms or arthropods and plants	100
NOEC or QSAR estimate	100 or 1000 (based on LIC_{50})
	10 (based on NOEC)
NOEC or QSAR estimate for minimal three representatives of microbe-mediated processes, earthworms or arthropods and plants	10

Table 12: EU assessment factors for soil compartment

Available information	Assessment factor
LIC_{50} short-term toxicity test(s) (e.g. plants, earthworms or microorganisms)	1000
NOEC for one long-term toxicity test (e.g. plants)	100
NOEC for additional long-term toxicity tests of two trophic levels	50
NOEC for additional long-term toxicity tests for three species of three trophic levels	10
Species sensitivity distribution (SSD method)	5-1, to be fully justified on a case-by-case basis
Field data / data of model ecosystems	case-by-case

In general, assessment factors apply an overly conservative approach to ensure the protection of the receiving environment. This is necessary, due to the limited data available and in the case of soil / sediment data, this will often not be bioavailable and end points will have been derived from aquatic toxicity data. In an ideal world, toxicity data would be available for primary producers, consumers and decomposers to assess the risk to terrestrial dwelling organisms.

3.4.4 Calculation of Risk Characterisation Ratio (RCR)

This phase of the evaluation comprises the assimilation and comparison of experimentally- or QSAR-derived ecotoxicity end points (Acute – LC/EC_{50}; Chronic – NOEC) and applies the relevant assessment factor (as discussed based on the quantity and quality of available data) to cover a maximum of eventualities to derive the PNEC. The PNEC is compared to a PEC which has been calculated for a given matrix subject to a specific emission release scenario. The PEC/PNEC ratio is also referred to as the RCR. An RCR value of < 1 indicates that the substance under evaluation is safe for the environment for the use pattern assessed.

An RCR ≥ 1 suggests that either, the environmental emission scenario may need to be refined, and, mitigating practices included in the evaluation, or additional higher tier testing may need to be performed to further clarify the environmental fate of the chemical.

If a worst case scenario has been assumed i.e. that there is no removal of the chemical, the PEC can be refined by following a tiered approach to generate measured data. A biodegradation screen can be performed to confirm whether the chemical is readily biodegradable or not. If the chemical is not readily biodegradable then enhanced or inherent biodegradation tests can be carried out. The highest tier of testing would be a simulation study. The highest level of refinement for estimating environmental concentrations comes from monitoring programmes.

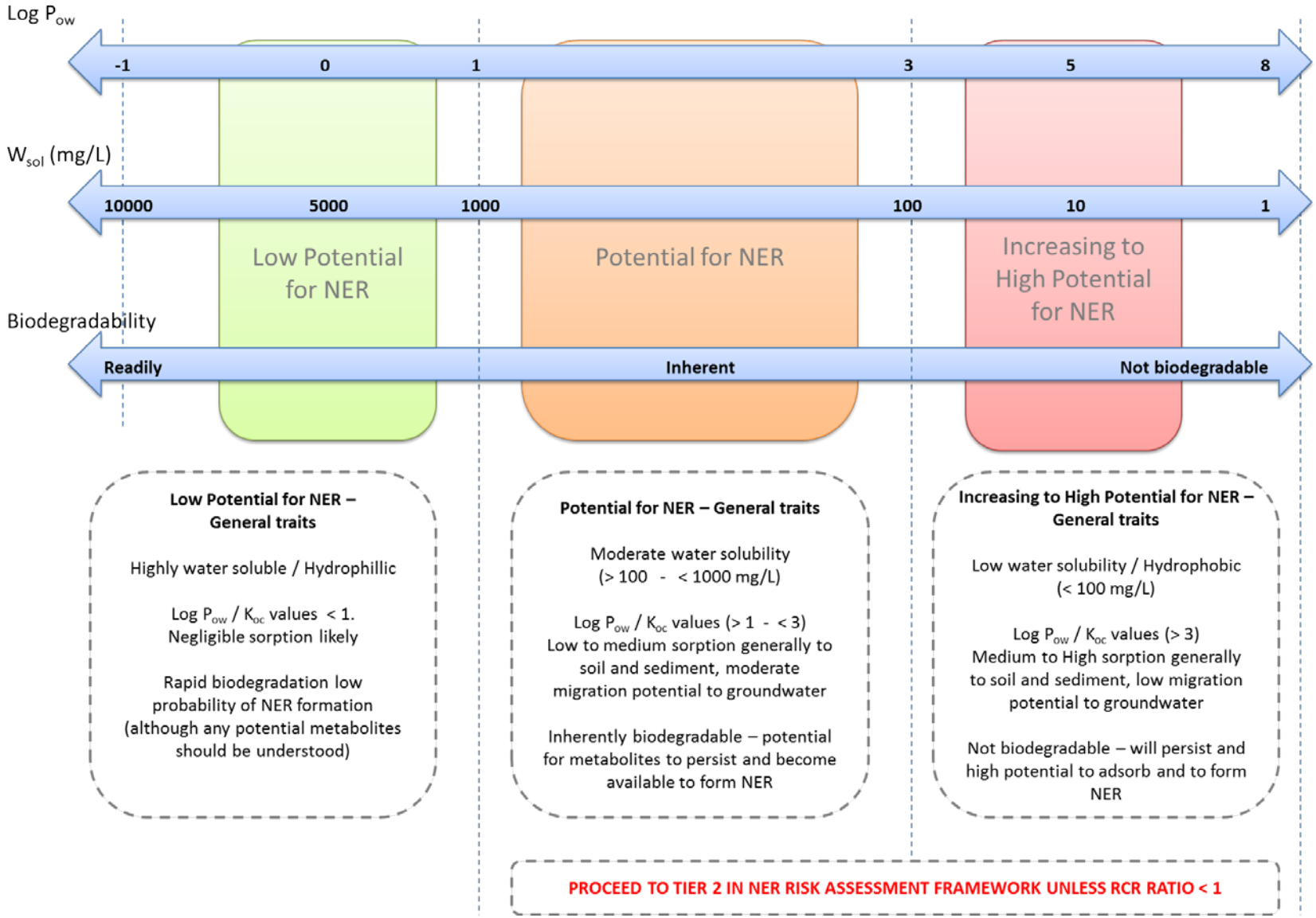
Similarly, the PNEC can be refined by a series of higher tiered experimental testing. If only QSAR data has been used in the assessment, then a base set of aquatic toxicity data will typically be generated. However sediment / terrestrial toxicity tests may be more desirable if the exposure assessment identifies these to be the only compartments that will be exposed to the chemical.

Once the risk assessment, assuming 100% bioavailability, has been completed the outcome would be either that the PEC/PNEC < 1 and no further work would be required, or that the PEC/PNEC ratio remains ≥ 1, in which case the substance should be considered a candidate for Tier 2 of the NER scheme (see section 3.5).

3.5 Tier 2 – Screening for NER

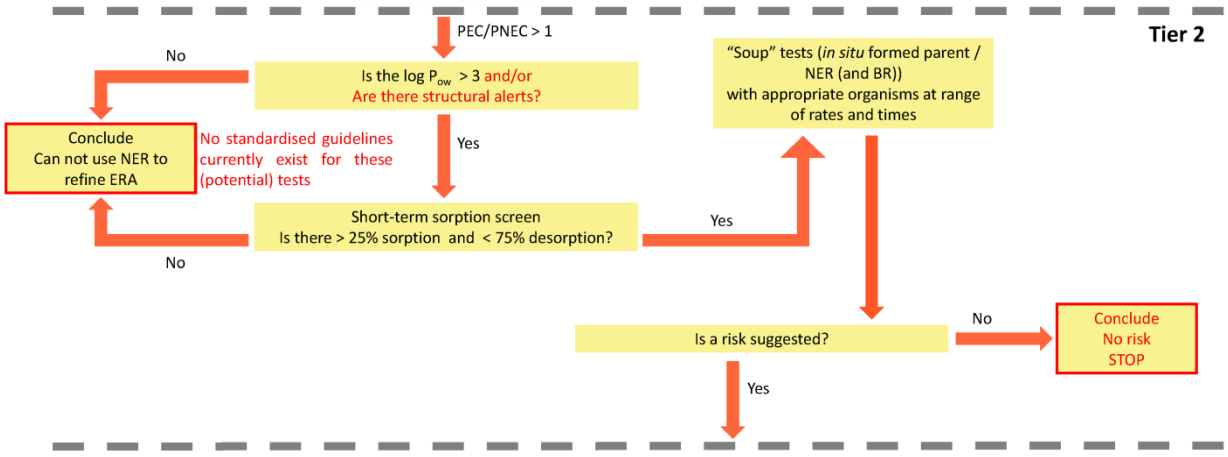
Tier 1 of the proposed framework has assumed initially, a worst case scenario of 100% bioavailability and then discussed the refinement options of the risk assessment if RCR ≥ 1. The potential for NER formation in Tier 2 of the assessment is determined by the chemical's partitioning behaviour and biodegradation profile as summarised in Figure 12. General traits from these properties can help predict the probability of a chemical partitioning to the solid compartment and persisting in that environment to then potentially progress to form NER (Figure 12). However, as discussed, this is not always definitive e.g. some substances may be biodegradable but rapid sorption can then reduce their bioavailability.

Figure 12: Summary of physico-chemical properties and fate profiles and the subsequent potential for NER formation



If the chemical meets the physico-chemical and fate criteria described above for high or moderate potential, then progression to Tier 2 of the proposed NER assessment framework is triggered. Figure 12 provides a qualitative approach for screening chemicals for potential NER formation. In many cases chemicals will have physico-chemical properties and fate profiles which will sit across several of these descriptors. Figure 13 describes Tier 2 of the framework and the proposed screening tests which would offer an intermediate analysis of the chemicals likelihood of NER formation prior to confirmation in Tier 3 studies.

Figure 13: Introduction to the Tier 2 of the NER risk assessment framework



3.5.1 Structural alerts to identify potential NERs

Developing so-called 'structural alerts' to predict which substances are likely to adsorb to sediment and/or soil and which subsequently may form NERs would be very useful. ECETOC (2012) has reviewed the current literature and have concluded that, at present only very rudimentary predictions are feasible, and the reliability is low.

The current approach looks for molecular fragments that are known, or thought, to be related to NER formation. The major difficulty lies within the fact that the matrix (adsorbent) properties also play a key role in this process (e.g. soil organic matter; cation exchange capacity; pH and particle size distribution). The adsorbent properties and the molecule's functional groups lead to different binding forces, often operating simultaneously in the adsorption process within a matrix (MacKay and Vasudevan, 2012). Microbial activity in the adsorbent can also influence the potential for NER formation. Richnow *et al* (1997) reported that the NER formed from parent polyaromatic hydrocarbons were much lower than the NER resulting from the hydroxylated metabolites which are formed by microbial biodegradation. In another example, Nowak *et al* (2011) showed that nearly all NER from 2,4-D were biogenic, i.e. assimilated carbon, forming cellular components. This demonstrates that NER may be formed by incorporation of degradation products, including CO_2, in addition to the binding of chemicals to the soil / sediment structure. This makes predicting NER formation very challenging.

The literature on NER formation has generally focused on plant protection products. Barriuso *et al* (2008) completed a comprehensive review on NER formation in plant protection products using the registration

dossiers submitted for pesticides in the EU. These authors noted that in many cases studies were quite often stopped too early before the final stages of NER formation were complete. In addition, the data reviewed did not distinguish the nature of the NER formed e.g. if it was biogenically incorporated, irreversibly sorbed or slowly desorbed. Another key consideration highlighted in interpreting the data was the dependency of NER formation on the position of the ^{14}C labelling in the chemical structure. The range of NER was generally larger when the ^{14}C labelling was in phenyl, imadazoline and pyrimidine moieties.

Barriuso *et al* (2008) concluded that about 50% of the pesticides reviewed exhibited a low proportion of NER (less than 30% of the initially applied amount) while only 12% of the registered pesticides had a proportion of NER exceeding 70%. Generally, free reactive chemical groups such as aniline and phenol gave rise to a larger proportion of NER. Whereas, the lowest proportion of NER were found for dinitroanilines (< 20%).

More general observations were reported by Bintein and Devillers (1994) who found that the nature of colloidal surfaces of most natural soils were negatively charged and therefore had an affinity for positively charged molecules and a much lower affinity for negatively charged molecules. Generally, anionically charged species have quite low sorption coefficients because they are repelled by the negative net charge of the soil surface, while cationic species are strongly sorbed (Wauchope *et al*, 2002).

Presently, the use of structural alerts to predict NER formation is still very complex and not a definitive approach. Several parameters need to be understood, including the structure of the parent material, the behaviour of the potential metabolites and the behaviour of the adsorbent.

3.5.2 Short-term sorption screen

The current test guidelines for determining sorption include OECD 106 (OECD, 2000a) Adsorption – Desorption using a batch equilibrium test and OPPTS 835.1230 Adsorption – Desorption (Batch Equilibrium) (US EPA, 2008c). These guidelines focus on describing the adsorption-desorption behaviour of a chemical on different soil types (although they both make reference to sediments also).

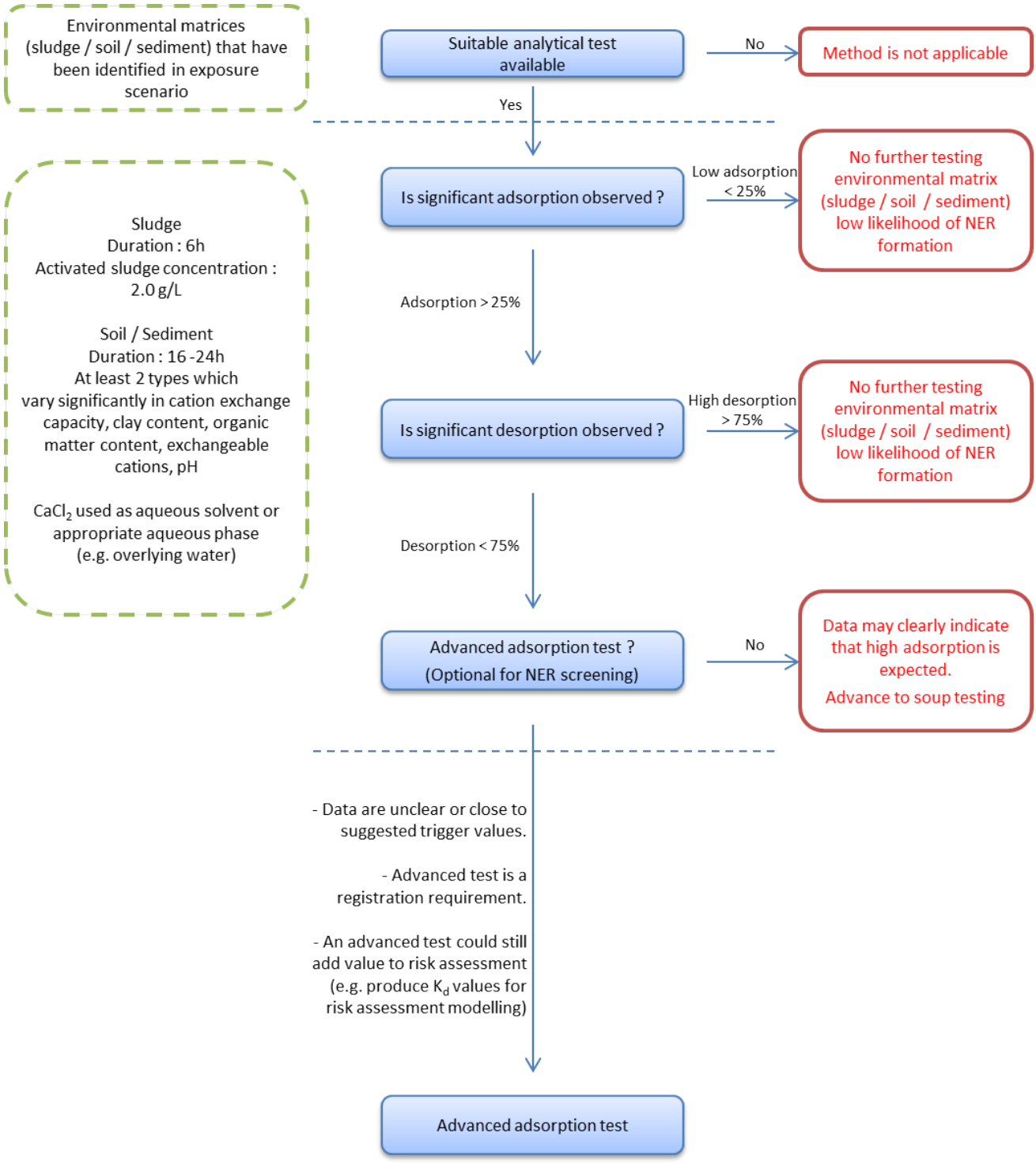
For a Tier 2 screen, these tests are very detailed and there is a need for a simplified procedure (Figure 14). The 1981 version of the OECD 106 guideline (OECD, 1981b) would be suitable to identify if a substance were likely to sorb to soil or sediment. The screen consists of a preliminary test (to ensure the applicability of the method) and a screening test (to assess sorption and desorption characteristics). For the purposes of this tier, only these first 2 tests would be required to indicate if NER formation would be likely. Whilst not required to indicate if NER are likely to be formed, the advanced test (to produce isotherms and K_d values) may optionally be performed. These K_d data could then be used in exposure modelling.

Chemicals which have been identified as being exposed to the terrestrial or sediment environment could be screened using a range of soil / sediment properties (2 different sources). Ideally, with a range which varies significantly in cation exchange capacity, clay content, organic matter content, exchangeable cations, and pH. The initial adsorption screen in these matrices could be monitored over a 16-24 h duration. The screening test would use one test substance concentration (one half of the water solubility limit or 5 mg/L maximum).

For chemicals that will be exposed to sewage treatment then a short-term sludge adsorption screen could be performed using activated sludge from an STP and monitored at e.g. 2.0 g/L of sludge. The test duration could then be performed to cover the typical hydraulic retention time in an STP (~ 6 hours). Care should be taken to ensure that sufficient time to achieve steady state has been allowed, a longer period may be required in some cases (US EPA, 1998a).

If no significant adsorption is measured (< 25%) then the desorption screening test is not required and it is assumed that NER formation is unlikely. If medium to high adsorption is observed (> 25%) the desorption screen would be performed. In the desorption screen, if the substance was readily desorbed (> 75%), then NER are unlikely to be formed. For substances where there is potential to form NER (> 25% sorption, with < 75% desorption) further evaluation would be required. The short-term sorption screen is summarised in Figure 14.

Figure 14: Short-term sorption screen framework

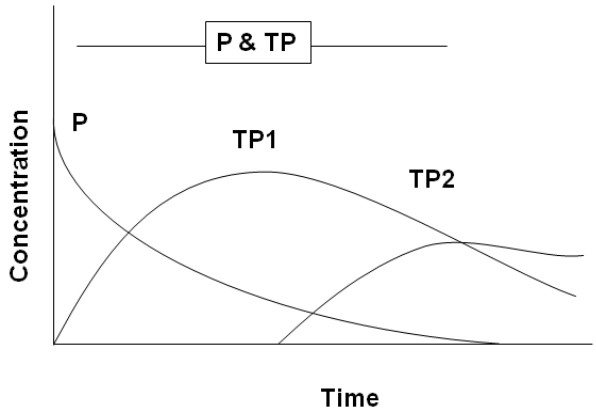


3.5.3 Soup tests

A soup test is an ecotoxicity test (it can also apply to fate testing, e.g. ready biodegradability tests) in which there is insufficient analytical chemistry to ascertain if the added test substance undergoes transformation within the timescale of the test.

Figure 15 illustrates how in soup tests, the lack of analytical chemistry means that it is unknown if any observed ecotoxicity is due to the parent (P) substance, transformation products (TP), or a mixture of P and TP. In a hypothetical example below, only a short-term study (relative to the transformation rate) would address the parent ecotoxicity. Longer-term ecotoxicity tests would address P and TP. The precise timing and proportions of P and TP would be substance and test dependent.

Figure 15: Soup Test



Established aquatic ecotoxicity tests, e.g. OECD test guidelines, aim to maintain the parent concentration in the test, often using flow-through conditions. Even so, some substances are so unstable, e.g. due to hydrolysis, that it is not possible to maintain the parent substance in the test. In these cases, it is normal practice to allow hydrolysis to proceed and test the TPs, and in effect this is a soup test. The static design of sediment and soil ecotoxicity tests means it can be more difficult to maintain the parent test substance concentration, so there is greater potential to conduct a soup test for these ecotoxicity tests. They tend to be longer-term studies (weeks) compared to aquatic acute studies (days). Sediment and soil ecotoxicity tests are frequently carried out based on the nominal concentration, based on quantification of the dosing solution only. Only when extraction and analysis of the P and TP are conducted is it evident if the test was a soup test or not.

Incorporation of ecotoxicity tests during and/or at the end of degradation studies could be used to assess the relative ecotoxicity of the parent and the TPs. Recently, Escher and Fenner (2011) have reviewed the environmental risk assessment of transformation products. These authors focused on biotransformation and (photo)oxidation products and classified existing approaches for transformation product assessment in degradation studies as exposure- or effect-driven. In the exposure-driven approach, transformation products are identified and quantified by chemical analysis followed by effect assessment. This is consistent with the approach reflected in the ECETOC framework (Figure 1) and in Tier 3 of the Risk Assessment Scheme

(Figure 9). In the effect-driven approach, a reaction mixture (soup) undergoes toxicity testing. If a decrease in toxicity compared to that of the parent correlates with a decrease of parent compound concentration, the transformation products are considered to be a lower environmental risk. However, if the observed toxicity increases, or the decrease is not proportional to the parent compound concentration, then the transformation products should be identified, where this is practicable. This concept is used to assess the ecotoxicity of the extracted soil / sediment in Section 3.5.3.1.9 of this report.

When considering the design of the soup test, the parent and/or transformation products must be present for long enough (to attempt to identify them) and at a reasonably constant concentration (to test for ecotoxic effects). It is suggested that they should be present for at least two analytical time points, which for most laboratory environmental fate studies would be a minimum of 14 days. This would also be sufficiently stable to test in many chronic ecotoxicity tests, although longer-term studies e.g. sediment ecotoxicity tests, may be technically challenging if the stability of the transformation products is insufficient for a constant exposure concentration to be achieved.

Where ecotoxicity is explained by the measured concentration of the parent, the risk assessment should be based on the parent chemical. In this case, so long as the test duration is greater than the transformation rate, the transformation products would have a very low ecotoxicity (compared to the parent). No further risk assessment would be necessary as the parent risk assessment will be protective. If significant ecotoxicity were observed, then further investigations would be required as this would indicate the formation of ecotoxic TPs.

Information about safety margins may also be required, depending on the parent/TP PEC/PNEC ratio. In order to develop safety margins it may be possible, depending on factors such as physico-chemical properties and the level of ecotoxicity, to perform tests in an appropriate fate study at the PEC, X10 PEC and/or X100 PEC. However, care must be taken to ensure the fate testing is not conducted at an inhibitory concentration thus preventing biodegradation. If the soup test shows no additional toxicity compared to the control, then these factors could be incorporated into the risk assessment. This approach would require further development and validation before it could be incorporated into the risk assessment scheme.

In the context of addressing NER, in most cases, it is not feasible to fully characterise the NER (made up of bound residues and slowly desorbable residues, which may be extracted using harsh conditions). However, the freely desorbable fraction may be characterised using appropriate analytical methods. Despite the lack of characterisation, soup tests are still very valuable screens since they can show the presence or absence of an adverse effect, without carrying out extensive analytical chemistry. Only in cases where unexplained ecotoxicity was observed should further analysis be considered necessary.

The soup test concept is not a new concept in environmental risk assessment. Examples of where soup testing has been used include:

- Water accommodated fraction (WAF) for testing of oil based products
- Testing of unstable or highly reactive substances
- Testing of mixtures e.g. coal tar and lignosulphonates
- Ecotoxicological effects in soil e.g. plant protection products

- Leachate testing from treated articles e.g. wood and anti-fouling
- Wastewater effluent testing
- Toxicity identification evaluation (TIE)
- Landfill leachates
- Transformation products

In these examples it is the extractable fraction (assumed to be the bioavailable fraction) which is the focus of the ecotoxicity testing. Standardised ecotoxicity tests which could be adapted for the risk assessment of NER include those used in the ecotoxicological effects testing in sediment and in soil. Standardised OECD test protocols for performing these effects assessments have been available for more than 25 years and they have been updated during the intervening period. The test guidelines do not require the detailed analysis that would be needed to quantify the NER, nor the formation of transformation products. In fact, these tests inherently assess the effects of parent and transformation products as well as addressing the bioavailability of the chemicals to the test organism.

Suitable modifications to differentiate between this total residue approach and identifying the bioavailable/non-available fraction could be developed, but currently this is not included in standardised tests. The International Standards Organisation (ISO) issued guidance on the selection and application of methods for the assessment of the bioavailability of contaminants in soil and sediment (ISO, 2008). This guidance also focused on the bioavailable (extractable) fraction and testing their effects.

3.5.3.1 Tier 2 soup test

3.5.3.1.1 Introduction

If a chemical substance demonstrates a significant level of adsorption (> 25%) in the Tier 2 short-term sorption screen (with < 75% desorption), the capacity of the substance to exert toxic effects may be substantially reduced via a reduction in bioavailability (+ bioaccessibility) resulting from these binding / sorption properties. In Tier 2, there is a need to develop a testing framework complementary to the short-term sorption screen, but which screens for ecotoxic effects related to NER.

Currently, no such approaches have been developed and validated for regulatory testing purposes. There may be, however, a means of adapting existing guideline test approaches for such needs.

For simplicity, this phase of testing will be referred to as a Tier 2 soup test. The test is intended to be a relatively simple means of determining if the chemical is likely to produce NER and to examine the potential for the NER to elicit ecotoxic effects within a model system and over an acceptable time-frame.

Bearing in mind the ecological relevance of the end point to be determined in this type of study, it is necessary to consider the bioavailability of the chemical in the exposure matrix to the organism being tested. The extraction methodology framework proposed in Figure 3 suggests that the ‘bioavailable’ fraction (dissolved + rapidly desorbed) of the chemical is immediately available to the organism and, depending upon the biodegradation and/or adsorption kinetics of the chemical, can usually be equated to acute exposure.

However, if the degradation and/or adsorption is slow, then chronic exposure would be more relevant. The slowly and very slowly desorbable fractions are clearly time-dependent and exposure is likely to be observed over a longer duration. The ecologically relevant time period, therefore, means that the Tier 2 soup test should consider chronic effects. However, it is recognised that evidence exists to suggest that re-release of soil NER may be related to some short-term events e.g. wetting and drying cycles (Jablonowski *et al*, 2011), freezing / thawing cycles (Eriksson *et al*, 2001; Zhao *et al*, 2009), physical disturbance of the soil structure (e.g. plant roots, ploughing (Eschenbach *et al*, 1998)). That said, it can also be envisaged that the kinetics of re-adsorption back into the NER state are likely to be rapid.

Tier 2 soup tests would, ideally, be developed and validated for both soil and sediment matrices in order to cover both environmental sinks and various exposure scenarios. As indicated above, no validated soup test procedures exist for either compartment at present. An outline upon which a soup test could be developed for both soil and sediment is proposed in the following section.

3.5.3.1.2 Outline of a soil based Tier 2 soup test

The Tier 2 soil soup test is intended to be a simple screen which aims to detect potential ecotoxic effects exerted by NER. A soil-based test is operationally less complicated to perform than that of a sediment-water soup test, given that:

- Soil can be treated as a single-phase, whereas sediment is a two-phase system comprised of sediment + water;
- The control of ambient test conditions (temperature, moisture content and aerobic conditions) is more straightforward with soil, whereas for sediments changes in oxygen concentration (and redox potential) in sediment and overlying water are difficult to control and ammonium build-up in the sediment of laboratory test systems is often encountered.

This section therefore has focused on a soup test for the soil compartment.

Separate studies, considering three trophic levels (soil microorganisms, earthworms, plants) are proposed. The soup test also adopts a mass balance approach, evaluating both the extractable fraction and the NER. This should give a more complete assessment than focusing on just the extractable fraction.

An outline of the proposed soil soup testing process is given in Figure 16 with details of each stage given below.

3.5.3.1.3 Preparation of aged soil

The conduct of a soil soup test compares the toxicity of a treated and aged soil, containing bioavailable + bioaccessible + NER, with a treated soil containing only the NER, i.e. a soil which has been treated, aged and then suitably extracted using the guidance from ECETOC (2012).

The following approach is proposed as an initial framework for the generation of a 'stock' soil under controlled conditions for subsequent use as an exposure medium and represents a key step in soil soup testing. The use of artificial soil is not recommended since the NER are produced *in situ*, using the naturally occurring microbial and chemical processes present in field collected soils. These may not be active in laboratory formulated artificial soils.

Well characterised (physico-chemical, free- or low-contaminant residue) fresh bulk soil samples would be treated at a suitable concentration and incubated under realistic conditions to allow ageing to develop NER *in situ*. The Task Force suggests that 60 days may be a suitable time, based on the data presented in Ericson *et al* (in press), then extracted using the standard extraction framework (ECETOC, 2012 and Figure 3 in this report) in order to leave the NER portion in the soil. This extracted soil should be treated in a suitable way so as to remove any residual traces of solvent. The structure and viability of the soil may be disrupted during the extraction process, in which case it may be necessary to mix this extracted soil with fresh, viable (previously unexposed) soil (admixed soil). Since the fresh soil will 'dilute' the NER in the admixed soil, higher dosing concentrations (e.g. 10X PEC) may be required to account for this dilution.

The proposed treatments required for a typical terrestrial toxicity study would be:

(i) Controls – Untreated soil incubated as described above (aged for 60 days), with and without added solvent (if used to add the test substance to the treated soils). These controls will then be divided in half. One half would not be subjected to the standard extraction regime and the other would be extracted.

These controls account for the effect of the dosing solvent and the standard extraction regime in the admixed soil. Once experience of the test has been gained it may be possible to simplify the number of control treatments.

(ii) Treated soils – Soil is treated with the test substance (via solvent if necessary) at suitable test concentrations. These are based on multiples of the PEC (at least 10X), but at test concentrations which are sufficiently low to avoid inhibition of the indigenous microorganisms which may inhibit biodegradation. These soils will be aged for 60 days, under controlled conditions, and then treatments will be divided in half. One half of the treated aged soil would be subjected to the standard extraction regime, as depicted in Figure 3, whereas the other half would not undergo extraction.

Once the test treatments (aged and extracted soils) have been prepared they will be admixed with fresh (viable) soil. The ratio of treated soil to fresh soil will need to be determined as this is not a standard guideline procedure. The ratio of the treated soil may need to be low since it will have been essentially sterilised by the extraction process. This ratio will have to be determined in advance, since this will have a bearing on the initial treatment level to be applied to the soil. Depending upon the study requirements, either a single concentration limit test, or a series of admixed soil test concentrations / loading rates could be prepared for the toxicity exposure.

These treatments will account for the difference between NER effects and those from the bioaccessible + bioavailable fractions.

3.5.3.1.4 Toxicity of NER to soil microorganisms

Microorganisms play an important role in the break down and transformation of organic matter in fertile soils, with many species contributing to different aspects of soil fertility. Any long-term interference with these biochemical processes could potentially interfere with nutrient cycling thus altering soil fertility. Transformation of carbon and nitrogen occurs in all fertile soils. Soil microfauna are homogeneously distributed within the soil matrix and exposure and effects are generally more consistent than for macrofauna, which may have a heterogeneous distribution. The small size of soil microorganisms means that they are in 'intimate' contact with the soil and may be capable of directly accessing specific fractions of soil that macrofauna cannot. In this context, soil microorganisms are an ideal trophic level to study for effects of NER.

An adaptation of the existing OECD 216 test guideline (OECD, 2000b) – Soil Microorganisms: Nitrogen Transformation Test – is proposed. This test guideline suggests that if nitrogen transformation is not inhibited, then "it is highly probable that the major carbon degradation pathways are intact and functional". This study typically has a duration of 28 days, can be conducted without specialised equipment and does not require any specific culturing/breeding of the test organism since the guideline uses indigenous soil microbes present in viable soil. Some basic laboratory skills are, nevertheless, required to set-up and conduct this study.

The test soil matrix would be prepared, aged and admixed as indicated above. The soil moisture content would be adjusted to approximately 50% of the maximum water holding capacity. Lucerne grass green meal would be added at an appropriate concentration to supply a source of organic nitrogen. This would be Day 0 of the test. Nitrate concentrations would be analysed on Day 28 of the incubation. Nitrate concentrations monitored are a direct measure of the microbial activity in the soil, statistical analysis of which will determine the significance of effects in the various treatment levels with respect to the control(s).

3.5.3.1.5 Toxicity of NER to earthworms

Earthworms are essential organisms for the re-working of soil organic matter and soil structure. Exposure in soil is via both dermal contact (absorption) and predominantly through ingestion. Earthworms also represent a soil dwelling species which has been widely studied.

This soup test would be an adaptation of the OECD 222 test guideline (OECD, 2004d) – Earthworm Reproduction Test (*Eisenia fetida* / *Eisenia andrei*). Exposure soils will be prepared, aged and admixed as outlined above.

Adult worms are introduced into a range of control or treated admixed soils. Mortality and growth effects on the adult worms are monitored after 28 days of exposure. The adult worms are then removed from the exposure soils, any mortalities noted, the remaining living worms weighed, and any additional observations noted (e.g. lesions). For the reproduction part of the study, the test soil is retained and incubated for a further 28 day period, at the end of which, the soil is carefully sieved and the number of hatched juvenile worms and unhatched cocoons per test level counted. Mortality is the main endpoint from the initial phase

of the study, and fecundity from the latter half of the exposure. Statistical analysis is used to determine any significant effects with respect to the control(s).

3.5.3.1.6 Toxicity of NER to plants

Uptake of pollutants into plant biomass is a major concern, especially in the case of edible species. Various papers have also studied plant growth processes as a potential pathway in liberation of NER from soil via co-solubilisation through secretion of root exudates (White, 2002) and through disturbance of the soil structure as a consequence of root penetration (Eschenbach *et al*, 1998). The addition of a plant species to the Tier 2 soup test is, therefore, considered relevant.

To determine NER effects on plants an adaptation of the OECD 208 test guideline (OECD, 2006a) – Seedling Emergence and Seedling Growth Test – is proposed. This test assesses effects of soil borne pollutants on seedling emergence and early plant growth development. Seeds are introduced into control and treated admixed soils and evaluated for effects following 14 to 21 days, or, at least 50% emergence of seedlings in the control group. Seedling emergence, dry or fresh shoot weight, and, in certain cases shoot height and visible deleterious effects on different parts of the plant are typical endpoints. The choice of plant species should represent suitable taxonomic diversity covering distribution, abundance and specific life-cycle traits. Typically, at least one monocot (cereal) species and 2 dicot species, one of which should be a root crop (e.g. carrot, *D. carota*), the other being a leafy, surface plant type (e.g. lettuce, *L. sativa*) should be considered.

Under the tiered testing scheme, soil soup testing would begin with the soil microorganism toxicity test. In the event where NER was observed to exert toxicity in this study, more advanced, Tier 3, testing would be advocated. In this circumstance, it would not be necessary to perform the earthworm and plant soup test, but to proceed directly to Tier 3 testing. In contrast, if no NER effects were observed in the soil microorganism test, then the Tier 2 soup test with earthworm and plant should be performed to demonstrate no effect over the three trophic levels.

3.5.3.1.7 Sediment soup test

In an analogous way to the approach described in section 3.5.3.1.3, sediment could be prepared for inclusion in suitable sediment ecotoxicity tests. To evaluate biological effects of NER formed in sediments, it is envisaged that the following tests could be modified:

- OECD 225 (OECD, 2007) Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment.
- ISO 10872 (ISO, 2010) Water quality – Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda).
- OECD Draft guideline (2012) *Myriophyllum aquaticum* growth inhibition test in water – sediment system.

These particular organisms have been chosen to represent a range of taxa and exposure pathways. No standardised test methods exist that directly measure the toxicity of sediments to microorganisms, therefore no sediment test that is analogous to the soil nitrogen transformation test is proposed. Tests with *Lumbriculus* would address the issue of uptake and of remobilisation of NER, through sediment ingestion (in an analogous way to earthworms in section 3.5.3.1.5) and exposure to *C. elegans* is mainly through the delicate epidermis but is also through ingestion of fine particle from the sediment (Höss *et al*, 2001). Tests with *Myriophyllum* would assess toxicity to plants. This latter organism is also of interest because *Myriophyllum* can be used in both sediment and water only test systems.

3.5.3.1.8 Interpretation of results

If the Tier 2 soup test shows that there is a significant hazard from the NER, then further testing (Tier 3) would be indicated. If however, no significant hazard were suggested from the NER then this reduction in toxicity should be taken into account in the risk assessment of the NER fraction. However, the bioavailable and bioaccessible fractions still require further evaluation (see Section 3.5.3.1.9). In summary, if the NER fraction was shown to have no significant toxicity, then the PEC would be reduced by the amount of NER. The reduced PEC would then be used to refine the risk assessment.

3.5.3.1.9 Extract ecotoxicity tests

In addition to the Tier 2 soup test to address the NER fraction, it would be necessary to conduct ecotoxicity tests on the extract to complete the mass balance approach to the hazard evaluation. These extracts could be assessed in a similar manner to those described in Section 3.6.3 (Tier 3 testing) using an effect-driven approach. Alternatively, an exposure-driven approach could be used (separating the transformation products, synthesising them and then testing their ecotoxicity) in Tier 3.

Extract preparation

In Tier 2 tests it is assumed that a radiolabelled test substance is not available, but that a specific method of analysis for the parent substance, that is suitable for the extraction matrix, has been developed.

The soil / sediment phase would be extracted using the framework developed by ECETOC (2012). Typically, the make-up of these extracts may include aqueous based extracts e.g. 0.01M $CaCl_2$, water-solvent mixtures, pure organic solvents and mild acidic extracts. Most of these extracts would be expected to be miscible and a combined extract could be produced for ecotoxicity testing. However some manipulation e.g. pH adjustment may be required prior to any attempt to characterise the extract's ecotoxicity. In addition, organic solvents may not be miscible with aqueous extracts e.g. ethyl acetate, hexane etc. In these circumstances some sample manipulation may be necessary, e.g. evaporation of the solvent and re-dissolving the residues in an aqueous based solution. It would be assumed that if the parent substance were recovered from evaporation trials then any transformation products would also be sufficiently non-volatile

to be recovered too. If the substance was volatile, then ecotoxicity testing would be more complicated and suitable methods would need to be developed to introduce the substance / transformation products.

Extract ecotoxicity test

Once the aqueous based extract had been prepared, along with suitable control soil / sediment that had been extracted by the same method, it could then be introduced into the appropriate ecotoxicity test.

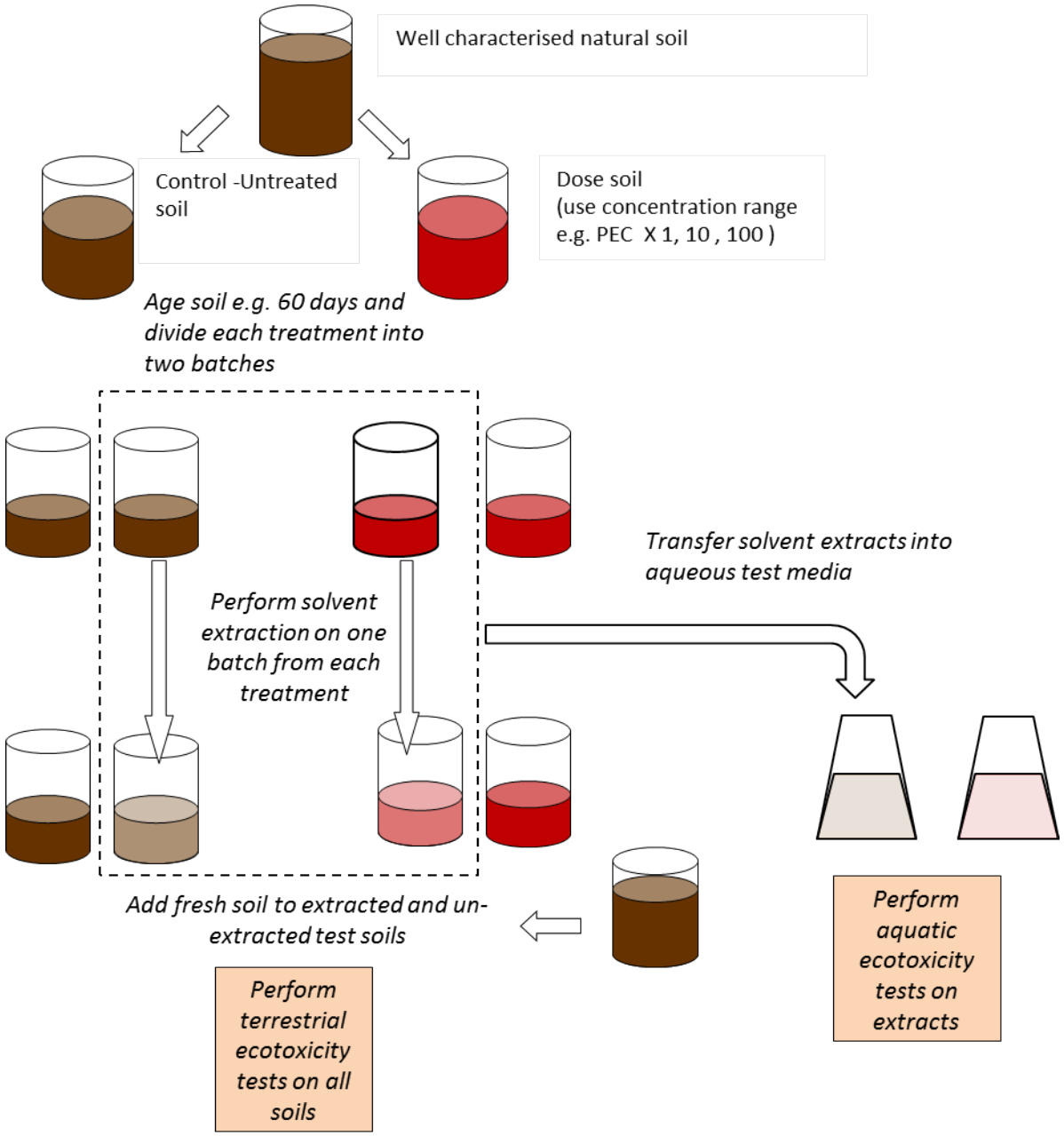
This test would normally focus on a sensitive species from the Tier 1 test results for the parent substance. As the test is a relative test, acute tests could be used. However, longer term tests could be considered but may be prohibitive based on the amount of extract required for these tests.

The extract would also require chemical quantification of the parent substance concentration, so that appropriate dilutions could be made so that some toxicity could be observed. The actual toxicity would be compared to the predicted toxicity from Tier 1 tests on the parent substance. If equal to, or less than, the prediction it suggests that the extract contains parent and/or transformation products that are no more toxic than the parent substance. The risk assessment would then use this extract test data to assign the bioavailable / bioaccessible fraction. This assumes that what is not accounted for in the extract would be in the NER (and be shown to be non-toxic).

If, on the other hand, the ecotoxicity of the extract were greater than predicted from the parent substance data, then further investigations would be required. The data would suggest that a transformation product(s) is(are) more ecotoxic than the parent. In this case either further chemical characterisation would be recommended, or further testing (as described in Tier 3) might be needed to account for the ecotoxicity data.

In an analogous manner, water-sediment systems could be assessed. But, in addition to the ecotoxicity testing of the extracted sediment and the extract, the overlaying water phase would also require ecotoxicity testing and chemical quantification of the parent substance concentration. In this way a ‘mass balance’ approach could be adopted, whereby what is not accounted for in the water and extract would be assumed to be in the NER (and be shown to be non-toxic).

Figure 16: Outline of proposed soup test for assessment of NER in soil



3.5.3.2 Bioaccumulation of NER

In principle, NER should not be bioavailable, so bioaccumulation should not occur. However, demonstrating this experimentally by measuring soil or sediment uptake (and potential to accumulate) would be difficult since the availability of radio labelled compound is unlikely. Methods would be restricted to specific analysis both in the soil / sediment NER and the biota into which the uptake is being examined. For the parent compound, this may be feasible but, where there is evidence that TPs are formed, this would not be technically possible at Tier 2. Standardised methods do not currently exist for studying the potential uptake of NER in soil or sediment and the conduct and data interpretation of such tests could be very challenging. For example, differentiating between the uptake via the pore water or from particles in soil / sediments

could be very difficult. In addition to these technical difficulties, it is unclear how these data might be incorporated into the current risk assessment scheme. Therefore, substantial research would be required in order to develop this concept further, prior to it being considered for inclusion into the risk assessment scheme.

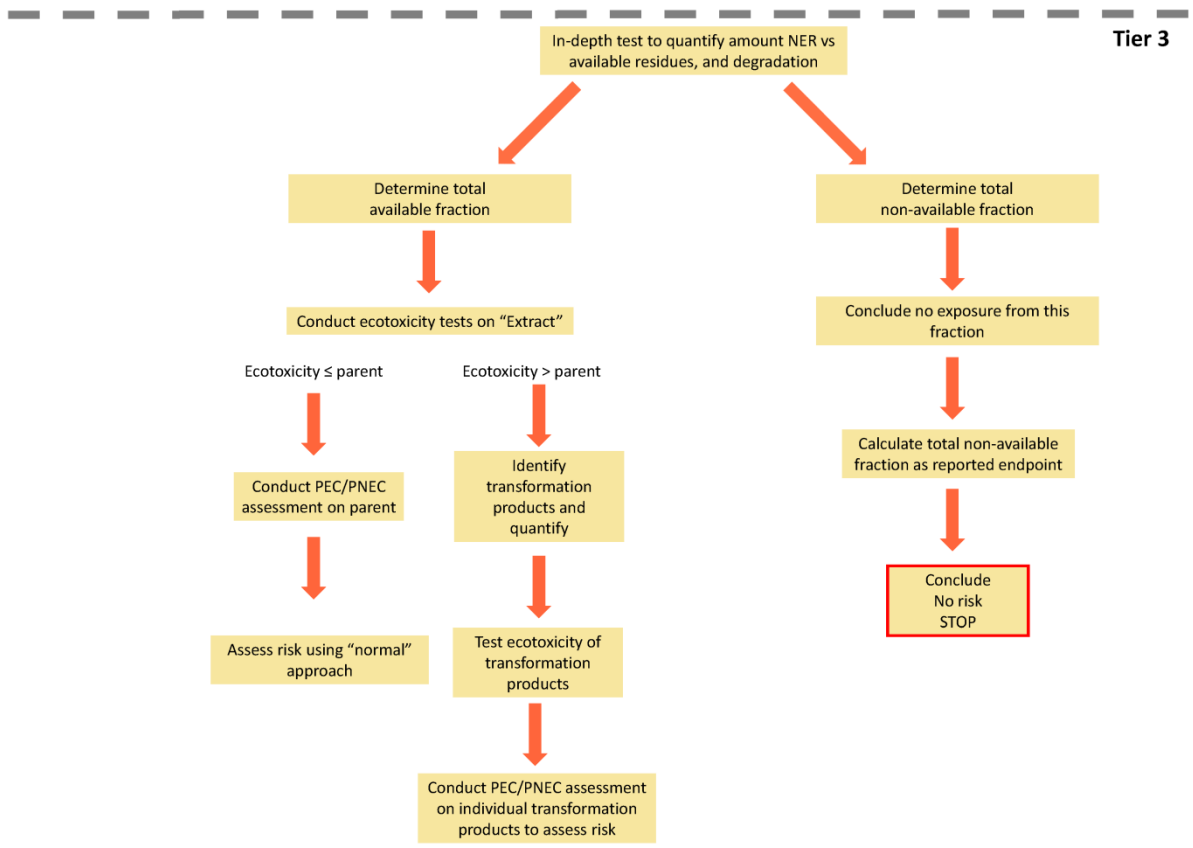
Tier 3 bioaccumulation assessment of NER is discussed in Section 3.6.5.

3.6 Tier 3 in-depth investigations into NER

3.6.1 Introduction to Tier 3

The aim of Tier 3, as depicted in Figure 17, is to provide an in-depth evaluation of NER and how it affects the environmental risk assessment. Substances may have been identified from Tier 2 investigations, or the environmental exposure may directly lead to Tier 3 investigations, e.g. plant protection products.

Figure 17: Introduction to Tier 3 of the NER risk assessment framework



Tier 3 investigations define how much of the originally dosed parent forms NER and characterises the ecotoxicity of the available fraction, within the matrix. This matrix is made up of at least 2 phases, the aqueous phase and the solid phase. Often in nature, and in laboratory test systems, more than 2 phases

co-exist e.g. the formation of colloids or the presence of a dissolved organic matter fraction. This adds to the complexity of the system and the interpretation of the data.

Approaches that have been used to define what is bioavailable / bioaccessible can be grouped into five main categories:

1. Relating the dosed concentration of the parent substance to the ecotoxicity observed in 'intact' matrix in laboratory ecotoxicity tests e.g. OECD OECD 208 (2006a), 216 (2000b), 217 (2000c), 218 (2004c), 219 (2004e), 222 (2004d), 225 (2007), 233 (2010b).
2. Extraction of the parent and/or transformation products using a wide variety of techniques. This has recently been reviewed by ECETOC (2012). The extractable concentrations of parent and/or transformation products are then related to the ecotoxicity observed with 'intact' matrix in laboratory ecotoxicity tests.
3. Relating the measured aqueous phase concentration (either bulk aqueous phase and/or interstitial water) of the parent substance and/or the transformation products with the observed ecotoxicity observed in 'intact' matrix in laboratory ecotoxicity tests.
4. Extraction of the parent and/or transformation products, as described above. The extractable parent and/or transformation products are then isolated, synthesised and tested in aquatic ecotoxicity tests. The observed effects are attributed to the measured concentration of parent and/or transformation product in the extract.
5. The solid matrix, normally soil or sediment, is pre-extracted using a range of methods. The extracted soil / sediment is then blended with varying amounts of fresh soil / sediments. The blended mixture is then used to assess the ecotoxicity and bioavailability of the NER remaining after extraction, e.g. Burgess *et al*, 2011. The approach is the same as for the Tier 2 test; except in Tier 3, supporting analytical chemistry into the identity of the potential transformation products is also included.

In this chapter, we suggest where these approaches may be helpful in understanding the impact of NER on the environmental risk assessment of chemicals.

3.6.2 Soil / sediment simulation studies

Standard OECD guidelines for soil and sediment simulation studies (307 and 308, respectively) have been discussed in section 2.4, as methods for determining the presence of NER. Section 2.4 also discusses potential adaptations to the standard guidelines to introduce greater realism into the test system, for example, by adding the test substance in sludge or manure to soil tests in order to replicate the route of exposure of the test substance. There is an opportunity within the Interim risk assessment to introduce such adaptations within the simulation studies of Tier 3. As has previously been discussed, harsh extraction methods remove fractions beyond those which would normally be bioavailable and bioaccessible. For this reason, soil or sediment from simulation studies should be extracted using the non-destructive methods as described by ECETOC (2012) and which have also been applied to Tier 2 soup tests. The result of this

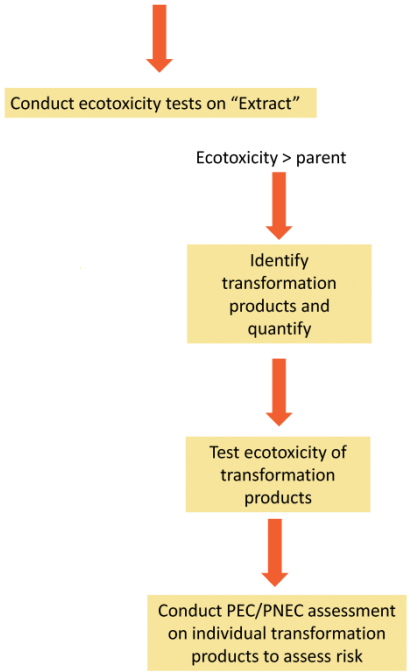
would be to isolate the fraction of media that is considered to be bioavailable / bioaccessible (the extractable fraction) from the fraction that is believed to contain NER (the non-extractable fraction).

The separate extractable and non-extractable fractions would then be analysed for parent compound and transformation products and tested for toxicity with a range of organisms.

3.6.3 Evaluation of the extractable fraction

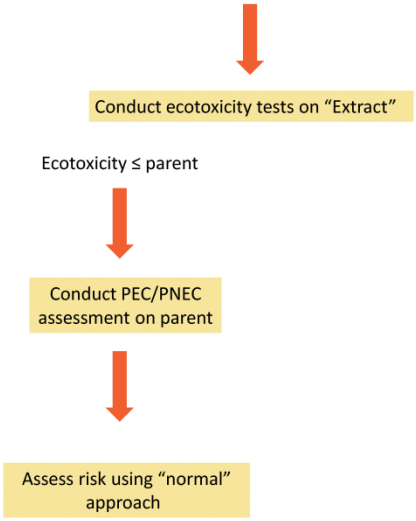
In the context of the Interim risk assessment, extractable residues are thus considered to be test substance and transformation products (TPs, i.e. metabolites, degradation, and reaction products) that are removed from a solid matrix by extraction steps up to, and including, step 3, as shown in Figure 3, Section 1.3. It is assumed that an environmental fate and effect profile has been built up for the parent compound and that this is the initial focus of the risk assessment. However, the same information may not be available for the TPs that are generated during exposure of the parent compound to soil or sediment in a simulation study. TPs may be present in greater amounts than the parent compound, may be more persistent or more mobile and/or more toxic. This issue is currently addressed within the regulatory framework for pesticides (EU, 2009a) which requires effects data for all TPs which account for more than 10% at any time, or more than 5% in at least two sequential measurements, during an environmental fate study. If TPs are considered to be relevant, they must be synthesised and tested separately and risk assessed in the same way as the parent compound, unless sufficient information may be derived from effects studies with similar substances. In the proposed Interim risk assessment scheme, this process is depicted as the middle route of Tier 3 in the flow chart (Figure 18) and has been described as 'an exposure-driven approach' in the literature, which reviews the environmental risk assessment of transformation products (Escher and Fenner, 2011).

Figure 18: Exposure driven assessment



An alternative 'effect-driven approach' is proposed where bioassays are performed on extracts to follow the development of toxicity. This is depicted in the interim risk assessment as the left hand route of Tier 3, as shown in Figure 19.

Figure 19: Effects driven assessment



The effect–driven assessment has been proposed in the Interim risk assessment as a method to circumvent unnecessary synthesis and testing on TPs when they do not pose a significant hazard, over and above that measured with the parent compound. This approach is justifiable on the basis that experience gained from the registration of pesticides shows that degradation products are frequently found to be less toxic than the parent compound. For example, a review of data on the properties and ecotoxicity of pesticides and their transformation products revealed that in 70% of pesticides, transformation products were less or equally toxic as the parent compound (Sinclair and Boxall, 2003). Similarly, an assessment of metabolites arising from the degradation of all major classes of hydrocarbons showed that the metabolites were less toxic, less persistent and less bioaccumulative than the parent molecule (León Paumen *et al*, 2012).

In the first tier of the effect driven approach, ecotoxicity tests are conducted on the extract from simulation studies in order to establish if the toxicity is less than or equal to the level expected from parent compound only. This approach is similar to that described for Tier 2 extract ecotoxicity tests (Section 3.5.3.1.9). If it is, it may be deduced that any transformation product(s) present are not more potent than the parent compound and therefore risk assessment conducted on the parent compound only, is appropriate. If the ecotoxicity is greater than anticipated, it may be concluded that parent compound is transformed into a more hazardous transformation product(s), which would signal the need for greater characterisation and testing to be carried out on the TPs. In practice, this would result in the risk approach being directed back down the middle route of Tier 3 of the scheme (Figure 18).

The success of the effect–driven approach is dependent on the ability be able to test the extract from a simulation study in a suitable bioassay or bioassays. Examples of the use of such bioassays have been

summarised by Escher and Fenner (2011). However, if it is not practical or desirable to test the extract, then the Interim risk assessment process once again proceeds along the middle route (Figure 18).

3.6.4 Evaluation of non-available fraction

The NER is considered to be the fraction of test substance / transformation product that remains in the solid matrix, after the non-destructive extraction techniques have been applied (Figure 3).

The Task Force considers that there is value in conducting biological assays on soil or sediment that has been extracted using non-destructive means following a simulation study. The purpose of conducting biological tests would be to:

- Detect any residual toxicity in the extracted media resulting from exposure to NER / re-mobilised test substance;
- Measure any remobilisation of NER and uptake into test organisms.

It is envisaged that toxicity tests would be similar to those described in Section 3.5.3.1 for Tier 2 soup testing. Test media would be taken from a soil simulation study as described in Section 3.5.3.1.3. For this purpose, the simulation study would need to take the following into account:

- Soil(s) should be fortified with sufficient test substance to provide a concentration that is relevant for risk assessment, multiplied by a factor to allow for dilution with fresh soil for toxicity tests;
- Control soil should be prepared to run in parallel with the simulation study;
- Sufficient volume of soil should be prepared to allow for toxicity tests to be run on the three species. If soil / test substance is limited, it is proposed that the Earthworm Reproduction Test be replaced with OECD 220 (OECD, 2004f) Enchytraeid Reproduction Test.

3.6.5 Relevance of NERs with respect to bioaccumulation

The use of radio labelled compound in a Tier 3 assessment would enable the quantification of NER and to measure uptake of the radioactivity into test organisms. Where suitable separation and identification techniques are used, the radioactivity may be characterised to quantify chemical and/or transformation product uptake. This information would provide valuable information about NER with respect to bioaccumulation. Although generally considered of low bioavailability and limited bioaccessibility, the role of NER on potential bioaccumulation merits discussion. Environmental compartments of concern are the terrestrial, freshwater aquatic and marine compartments, with the principal sinks for NER being soil, freshwater and marine sediments.

The role of biota in both the assimilation and transfer of NER cannot be excluded either, where a portion of a substance bioaccumulated in an organism may, in itself, be non-extractable in the living form, but may be

extractable and/or represent some level of toxic equivalence upon ingestion by another organism in the food chain. A similar phenomenon is likely to exist for terrestrial and freshwater / marine plants which may absorb and then assimilate NER. This assimilation may be via various processes, including gaseous-phase or dissolved-phase uptake, deposition on to leaf surfaces from the particulate or suspended sediment phases. The absorbed chemical then undergoes fixing in the plant as NER, thus, increasing the effective burden in the matrix. Depending upon the edibility of the plant, this may present a potential risk for human and animal via ingestion.

Gevao *et al* (2001) studied the uptake of ^{14}C-residues of Atrazine, Dicamba and Isoproturon by the earthworm *A. longa* exposed over a period of 28 days to freshly spiked soil and soil which had been aged for 100-days, then exhaustively extracted prior to exposure. Uptake of ^{14}C-residues was 2- to 10-times greater in earthworms exposed to freshly fortified soil compared with that of aged soil containing NER only. This suggests that NER are significantly less bioavailable for uptake.

Several authors have considered bioaccumulative uptake of organic substances in earthworms via the dissolved phase by absorption through the skin and compared this to the component accumulated by ingestion (Belfroid *et al*, 1995, Jager *et al*, 2003). Findings suggested that the dietary uptake was, in general, low for the contaminants associated with the solid-phase for substances with a log P_{ow} of < 5, and, that bioaccumulation was relatively well predicted by the equilibrium partitioning calculation approach.

Racke and Lichtenstein (1985) looked at the effects of varying soil microorganism densities on the release of bound residues of [^{14}C]Parathion in soil. Aged soils which had been exhaustively extracted were amended with cow manure and a variety of permutations of other adjuvants, including stimulants and inhibitors of microbial activity. Separate investigations were performed to consider the effects of such amendments on the uptake of radioactivity into oat seeds (*Avena sativa*). Their results indicated that a re-mobilisation of ^{14}C-residue could be attributed to an increase in soil bacteria and fungi which was linked to the addition of cow manure to the aged extracted soil. The magnitude of the release of NER from the soil was greater in the system which had been amended with cow manure compared with extracted soil inoculated with a fresh soil-water component. Mineralisation of the released NER was significantly higher in the manure amended soil incubates. However, uptake of ^{14}C into oat leaves was not enhanced, and, was even suggested to be lower in the manure amended soil. The authors hypothesised that this observation was linked to the lower amount of [^{14}C]NER released as water soluble components in the manure amended permutation (Racke and Lichtenstein, 1985).

The presence of plants has been identified as a trigger factor in the release of aged residues to the soil and uptake to the plant (White, 2002; Zhu *et al*, 2009). However, the mechanisms related to these observations are not currently well understood. The presence of root exudates and plant secretions has been suggested as a potential element leading to the re-mobilisation of aged residues from the soil, rendering it bioavailable for uptake via the plant root system (White, 2002). However, it may be hypothesised that the release of NER and aged residues under cropped conditions results from a combination of effects related to the presence of growing plant biomass, for example macro-disturbance of the soil structure when the plant is planted and micro-disturbance of the soil structure via penetration of the plant root system (Eschenbach *et al*, 1998). A change in hydrological status within the root zone of the soil, especially when irrigation practices are applied, may also affect NER release (Jablonowski *et al*, 2011). Microbial flushes of activity, generated as a

consequence of changes in moisture content, increased aeration and using root exudates as an energy source has been suggested as a cause of NER release (Racke and Lichtenstein, 1985).

Further research has compared the desorption kinetics of phenanthrene and biphenyl from aquatic sediments and the uptake and utilisation by bacteria. It was found that bacteria could act upon sorbed chemical without the necessity of the chemical to undergo initial desorption, and, that the rate of this process was significantly more rapid than the rate of desorption of the chemical from soil (Calvillo and Alexander, 1996; White *et al*, 1997). This observation brings to the forefront an important point when considering the bioaccumulation potential arising from NER – desorption / re-mobilisation kinetics versus the *in vivo* metabolism rate.

Leppänen and Kukkonen (1998) compared the significance of ^{14}C-labelled pyrene uptake via sediment pore water and ingestion of sediment with the sediment oligochaete *Lumbiriculus variegatus* and determined that 61% of the accumulated body burden of pyrene originated from ingested material, the remainder was attributed to dermal absorption. This study did not, however, target the portion of the bioaccumulated fraction related to sediment NER.

The impact of NER on the overall BCF value is likely to be low and, as a process in itself, relatively slow or very slow. Work described above suggests that in soil, the uptake of low- to medium- hydrophobic organic pollutants by the route of ingestion on solid material is low compared to that of the uptake from the dissolved phase, including interstitial pore water.

Organisms which actively re-work and filter soil and sediment are likely to be the most directly impacted, but the overall effect of re-mobilised and ingested NER on the overall body burden will probably depend upon the life-cycle and typical life-span of the organism in question. This does not, however, preclude the effects of the transfer and build-up of the chemical in higher predator species in the food chain.

This topic requires further investigation to look at the importance of NER as an exposure pathway to the overall bioaccumulated fraction. This will require development of guidance on how to set-up and perform relevant exposures under standardised conditions in order to permit comparison between studies.

4. CASE STUDIES

4.1 Introduction

In this chapter, the tiered risk assessment scheme (Figure 9) will be applied to a series of chemical substances of differing chemical class, with a broad range of physico-chemical properties and originating from a diverse portfolio of industrial applications.

The physico-chemical properties, use data, environmental fate parameters and ecotoxicity end points for each of the case study substances is accompanied by text detailing the step-by-step evaluation, according to the framework criteria described in the tiered risk assessment process.

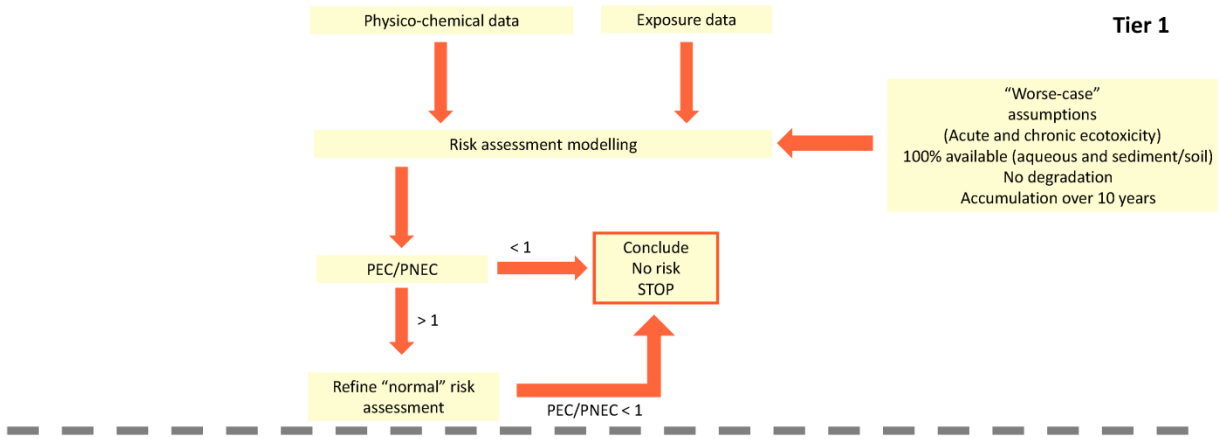
The utility of this approach as a means of screening-out substances of low concern to form NER from further testing is thus demonstrated, and the grey-zones where further in-depth developments are required (e.g. soup tests) highlighted.

4.2 Tier 1 Caffeine

Use pattern / release into the environment

Caffeine is a natural product found in coffee (e.g. 0.9 to 2.6% in green coffee beans), tea, soda beverages, chocolate, and many prescription and over-the-counter drugs, making it the most commonly consumed stimulant. The annual, world-wide, production is 10,000 to 15,000 tons, including 3,000 to 4,000 tons of natural caffeine. Its use in food will be the predominant route of exposure for the environment. Tier 1 of the risk assessment (Figure 20) is based on the discharge to the aquatic environment via sewage treatment.

Figure 20: Tier 1



Exposure assessment

Caffeine has a water solubility of 22 g/L and a log P_{ow} of –0.091 (Table 13). Distribution modelling using Mackay Level 1 indicates that 99.99% of caffeine would be found in the aquatic compartment. The calculated hydrolysis rate is extremely slow, so would not significantly affect the PEC. Concerning biodegradation, there is a 'not valid' study available for caffeine (OECD, 2002c). However, from the structurally analogous theophylline, it can be concluded that caffeine is likely to be readily biodegradable.

No PEC determinations have been published, but measured caffeine concentrations in surface water samples have been reported in the literature. Whilst PEC and MEC (measured environmental concentration) are not equivalent, for the purposes of the assessment in this report, MECs have been used. MECs vary significantly from one location to another. For example, caffeine concentrations for the Ramos River in Brazil were up to 357 µg/L (Ferreira, 2005), whilst the MEC in effluent from a sewage treatment plant was 15 ng/L (Sui *et al*, 2010). Caffeine has been reported to reach concentrations in US streams of 6.0 µg/L, with a median concentration of 0.1 µg/L (Kolpin *et al*, 2002).

Effect assessment

The acute aquatic toxicity (Table 13) has been determined for fish (*Leuciscus idus* LC_{50} (96h) = 87 mg/L), for aquatic invertebrates (*Daphnia magna* EC_{50} (48h) = 182 mg/L) and for algae (*Scenedesmus subspicatus* ErC_{50} (72h), ErC_{10} (72h) > 100 mg/L). Based on these data and an assessment factor of 1000, the PNEC for the aquatic compartment may be calculated as 0.087 mg/L.

Calculation of PEC/PNEC ratios

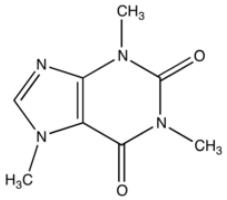
For the purposes of this report, MEC values have been used in place of PEC. The MEC/PNEC was calculated to be < 1, based on reported MECs of 0.1 to 15 ng/L (however, the MEC/PNEC would be > 1 based on the highest reported MEC of 357 µg/L).

Based on the worst case (MEC/PNEC) data, caffeine would be a candidate for refinement of the PEC (based on more exposure data and/or calculation of a PEC), or possibly consideration of screening assessment for the formation of NER. Based on the physico-chemical data (high water solubility and low log P_{ow}) it is not predicted to form significant NER. Structural alerts do not suggest significant NER formation, since there are no phenol or aniline moieties in the molecule.

Conclusion

It is predicted that caffeine is unlikely to form significant NER. Therefore, NER is not considered to have a significant impact on the risk assessment of caffeine.

Table 13: Caffeine case study data

Property	Caffeine
Use	Caffeine is a natural product used in food, beverages and as a pharmaceutical (stimulant)
Main environmental exposure route	Down the drain (via humans it is metabolised in the liver into three primary metabolites: paraxanthine (84%), theobromine (12%), and theophylline (4%))
Chemical name	3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione
SMILES	Cn1cnc2c1c(=O)n(c(=O)n2C)C
Structure	
Structural alert?	No – lacks phenol or aniline moieties
CAS No.	58-08-2
Molecular formula	$C_8H_{10}N_4O_2$
Molar mass	194.19 g/mol
Water solubility	22 g/L at 20°C [1]
Vapour pressure	$4.7 \times e^{-6}$ Pa
pKa	pKa = 10.4 at 40°C [2]
log P_{ow}	-0.091[1]
Distribution modelling using Mackay, Level I (V 2.1)	Predicted that the main target compartment will be water with 99.99% [1]
Fate in STP (partitioning / degradation)	Readily biodegradable. (Read across from theophylline – Guideline: OECD 301A (> 90%) [1])
Fate in freshwater sediment (partitioning / degradation)	No data available
Fate in soil (partitioning / degradation)	K_{oc} = 10 L/kg; log K_{oc} = 1 (EPIWIN calculation) [3] log K_{oc} = -0.0135 (estimated) [1] Expected high mobility in soil
Indication of partitioning to solids?	None
Ecotoxicological Effects	
Aquatic Ecotoxicity	
Acute	Golden Orfe 96h LC_{50} = 87 mg/L *Daphnia magna* 48h EC_{50} = 182 mg/L *Desmodesmus subspicatus* 72h $EC_{50} \geq$ 100 mg/L *Pseudomonas Putida* 17h $EC_{50} \geq$ 3,490 mg/L Activated sludge 3h $EC_{50} \geq$ 1,000 mg/L
Chronic	No data available
Sediment Ecotoxicity	
Chironomus *Lumbriculus* Other	No data available
Terrestrial Ecotoxicity	
Acute	No data available
Chronic	Toxicity to terrestrial plants: *Oryza sativa* Growth inhibition at 0.5 – 10 mM
PEC/PNEC Ratios	
Reported	PNEC = 0.087 mg/L (OECD SIDS); No reported PEC values. Using MEC values, the PEC/MEC ranges from < 1 to > 1.

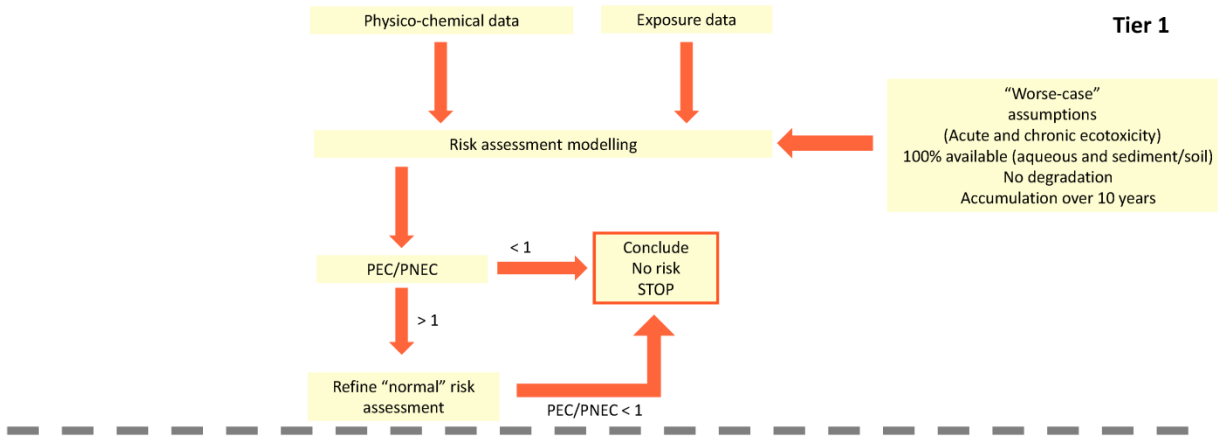
References
[1] OECD, 2002c
[2] Dean, 1985
[3] US EPA, 2011

4.3 Tier 2 DODMAC

Use pattern / release into the environment

DODMAC (dimethyldioctadecylammonium chloride) is a quaternary ammonium compound used in fabric conditioners, shampoos and car washing agents. The use pattern and exposure route into the environment (down the drain) as well as physico-chemical properties (water solubility and log P) do not allow any waiver from risk assessment in the NER risk assessment scheme. The test chemical would progress to Tier 1 of the proposed risk assessment scheme (Figure 21).

Figure 21: Tier 1



Tier 1

The first step of the Tier 1 risk assessment would be to assume 'worst case' scenarios, that 100% of the chemical is bioavailable and the inherent ecotoxicity could be expressed in proportion to the total exposure concentration. A conservative assumption on the duration of emission to the environment is also necessary to ensure a worst case estimation of PECs for chemicals that might be persistent. QSAR modelling for the derivation of log P_{ow} and water solubility are not applicable to this chemical – DODMAC is a surface-active substance, the log P_{ow} value cannot be used to derive the environmental distribution constants (measured values are required using appropriate methods).

PEC calculation

The DODMAC PECs for the use as fabric softeners, hair conditioners and car washing products are given in Table 14 for the aquatic compartment and Table 15 for the terrestrial compartment (derived from volume data for 1998).

Table 14: Aquatic PECs

Sub compartment	C_{local}	PEC EU region	PEC_{local}
Bulk (water+susp.) [µg/L]	0.40	0.14	0.54
Water phase [µg/L]	0.31	0.11	0.42
Sediment [mg/kg dw]	5.3	1.7	7.0

Table 15: Terrestrial (PECs assuming worst case – all chemical is bioavailable, transferred to sludge and accumulation over 10 years)

Parameter	Value
C_{sludge}	0.24g/kg dw
$PEC_{local\ soil}$	8.12 mg/kg dw

PNEC calculation

Aquatic

The lowest aquatic NOEC in tests with laboratory water of 6 µg/L for *Selenastrum capricornutum*, 3 trophic levels are reported so application factor of 10 applied.

$PNEC_{river\ water}$ *= 6 µg/L / 10 = 0.6 µg/L*

Sediment

In accordance with the EU (2003), the $PNEC_{sed}$ can be estimated from the $PNEC_{water}$ with the equilibrium partitioning method. With a PNEC river water of 0.6 µg/L and a partitioning coefficient of 10,000 L/kg (related to dry weight (dw)), the $PNEC_{sed}$ would be estimated to be 6 mg/kg dw. However, as DODMAC strongly adsorbs to sediments (assumed from structural alerts), according to the EU (2003) an additional factor of 10 should be applied to take uptake via ingestion of sediment into account. Therefore the $PNEC_{sed}$ has to be reduced to 0.6 mg/kg dw.

Terrestrial

$PNEC_{soil}$ = 1,000 mg/kg / 50 = 20 mg/kg

The Tier 1 Risk Characterisation Ratios (PEC/PNEC) are summarised in Table 16.

Table 16: Tier 1: Risk Characterisation Ratios – PEC/PNEC (Emissions via household sewage)

Compartment	PEC/PNEC	RCR
Aquatic	0.54 / 0.6	0.9
Sediment	7.0 / 0.6	11.6
Terrestrial	8.12 / 20	0.4

Degradation

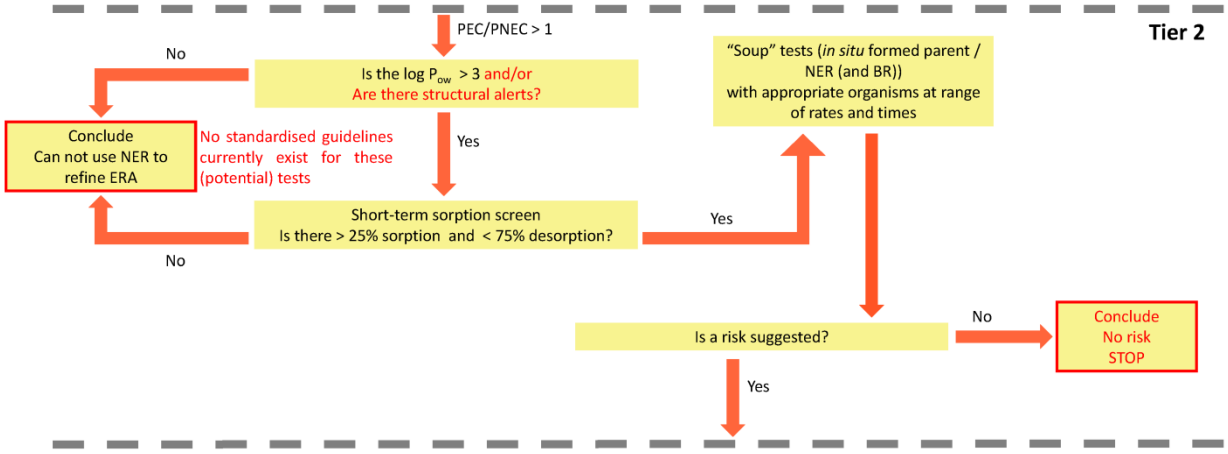
In two low biomass tests with non-adapted inocula, biodegradation of DODMAC was insignificant. No CO_2 production was observed after 28 days in a Sturm test and the biological oxygen demand was 5% after 30 days in a closed bottle test. In a Zahn-Wellens-test (OECD 302B) with industrial activated sewage sludge, DODMAC was eliminated by more than 70% after 3 hours. Elimination reached 92% after 15 days, measured as DOC reduction, a rate of biological degradation could not be determined. In the SCAS test, 80 to 98% of 0.5 mg/L was adsorbed to sludge after 7 days. It was shown in several tests that DODMAC was not readily biodegradable.

Using worst case scenarios and then refinement with biodegradation screening test data, further refinement of the sediment compartment risk assessment is still required (also marginal safety margins achieved in aquatic environment), therefore progress to Tier 2:

Tier 2

Although Tier 2 tests do not currently exist, it is possible to predict the outcome of these tests from the existing Tier 3 data. The first step of Tier 2 (Figure 22) is to consider the log P_{ow} and structural alerts.

Figure 22: Tier 2



NER alerts are triggered by the log P value = 3.8 (measured), very low water solubility and the observations from the inherent biodegradation tests (Zahn-Wellens and SCAS) showing significant adsorption. DODMAC also contains a quaternary ammonium group which is one of the structural alerts for potential NER formation.

Sorption / Distribution

In a test with DODMAC and 3 different sediments, sediment-water partitioning coefficients from 3,833 to 12,489 l/kg dw were analytically determined. The results indicate that the coefficient is more dependent on the nature of the mineral phase than on the organic carbon content. Remobilisation of DODMAC adsorbed onto bentonite was investigated. Activated bentonite (loaded with 34% DODMAC) was treated with water. The substance could not be detected in the water phase. With the detection limit, a distribution coefficient above 10^5 L/kg was calculated. Further investigations demonstrated that DODMAC can be bound very strongly by some minerals, while in others relatively small distribution constants were estimated. Under environmental conditions, the sorption properties of DODMAC probably vary in a wide range depending on the nature of the adsorbent. A value of 10,000 L/kg dw is chosen for both Kp_{sed} and Kp_{soil}. These data suggest that a Tier 2 short-term sorption screen would predict significant sorption, with little desorption. So, DODMAC would potentially form significant NER, and would be a candidate for Tier 2 soup testing.

Aquatic refinement

The lowest aquatic NOEC in tests with laboratory water = 6 µg/L for *Selenastrum capricornutum,* but the lowest NOEC from river water tests (62 µg/L, *Selenastrum capricornutum*) is taken into account in order to calculate the PNEC. This value is supported by other long-term test results with *Microcystis aeruginosa* and *Mysidopsis bahia* for which almost the same values are reported. Refined $PNEC_{river\ water}$ = 62 µg/L / 10 = 6.2 µg/L.

Sediment refinement

For the derivation of the $PNEC_{sed}$, the only available data are from tests where the test organisms were exposed to whole sediment spiked with the test substance. For *Chironomus riparius* a NOEC of 876 mg/kg dw was found. *Lumbriculus variegatus* was less sensitive to adsorbed DODMAC. A NOEC of 5,000 mg/kg dw was found for this sediment ingesting worm. For the nematode *Caenorhabditis elegans* a NOEC of 1,350 mg/kg dw was derived. The NOEC found for the oligochaete *Tubifex tubifex* was 1,515 mg/kg dw (in the same range with the NOECs from the other tests). However, a EC_{10}-value of 550 mg/kg dw could be calculated and that should be used as the basic value for the PNEC derivation. For the derivation of the $PNEC_{sed}$ an assessment factor of 10 is applied to the EC_{10} of 550 mg/kg dw obtained for *Tubifex tubifex*, as long-term tests with species representing three different living and feeding conditions and therefore different exposure pathways are available. The refined $PNEC_{sed}$ = 550 mg/kg dw / 10 = 55 mg/kg dw.

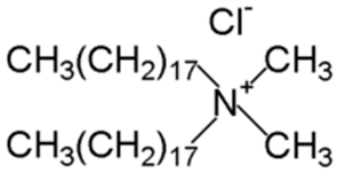
Table 17: Tier 2: Risk Characterisation Ratios – PEC/PNEC (Emissions via household sewage)

Compartment	PEC/PNEC	RCR
Aquatic	0.54 / 0.6	0.9
Sediment	7.0 / 55	0.13
Terrestrial	0.49 / 20	0.025

These data (Table 17) are assumed to reflect the bioavailable concentrations of DODMAC, but expressed as the dosed concentration. In the Tier 2 soup test this would be equivalent to the aqueous / pore water concentration plus the extractable fraction in the sediment. It was not possible to confirm this (nor the lack of effects from the NER fraction) from the existing data (Table 18).

Conclusion: There is at present no need for further information and/or testing or for risk reduction measures beyond those which are being applied as satisfactory RCRs have been established. Progression to Tier 3 of this scheme is not necessary.

Table 18: DODMAC case study data

PROPERTY	DODMAC Dimethyldioctadecylammonium chloride
Use	Fabric softeners / hair conditioner / car washing agents
Main environmental exposure route	Down the drain – sewage
Chemical name	Dimethyldioctadecylammonium chloride
SMILES	CL)II(CCCCCCCCCCCCCCCCCC)CCCCCCCCCCCCCCCCCC
Structure	
Structural alert	Quaternary ammonium N-R_4^+
CAS No.	107-64-2
Molecular formula	C38H80NCl
Molar mass	586.52 g/mol
Water solubility	2.7 mg/L, < 1 mg/L
Vapour pressure	Negligible because of the salt character EPI program an estimated vapour pressure of 10^{-15} Pa
Pka	n/a
log P_{ow}	3.80 (measured)
Distribution modelling using Mackay, Level I (V 2.1)	Air 0% Water 11% Sediment 63% Soil 26%
Fate in STP (partitioning/degradation)	0% after 28 days (OECD 301B) 5% after 30 days (OECD 301D) Zahn-Wellens-test (OECD 302B) 70% after 3 hours, 92% after 15 days, measured as DOC reduction. SCAS test (OECD 302A) 80-98% of 0.5 mg/L DODMAC was adsorbed to the sludge after 7 days. 0% production of $^{14}CO_2$ observed. CAS test 71.2% of 0.01 mg/L DODMAC was adsorbed after 5 days. Based on measurements at different sites of treatment plants estimated the DODMAC fractions being adsorbed onto primary sludge = 31% and onto wasted activated sludge = 24%. Assumed that in all 55% of the used substance is adsorbed and reaches agricultural soils during use of sludge as fertiliser.
Fate in aquatic environment	k_{water} = 0.0047 d^{-1} can be extrapolated for surface water, which would correspond to inherently biodegradable substances (DT_{50} = 150 days).
Fate in soil (partitioning/degradation)	Kp_{soil} 10,000 L/kg dw
Fate in freshwater sediment (partitioning/degradation)	According to the TGD biodegradation in total sediments is assumed to be a factor of 10 lower than in soil: k_{sed} = 1.4. 10^{-4} d^{-1}. Kp_{sed} 10,000 l/kg dw
Indication of partitioning to solids?	Moderate to high

Ecotoxicological Effects	
Aquatic Ecotoxicity	
Acute	Algae: *Daphnia magna*: EC_{50} = 3.1 mg/L (river water) EC_{50} = 0.16 mg/L (laboratory water) Fish: LC_{50} = 1.04 mg/L
Chronic	Algae: NOEC = 0.006 mg/L (laboratory water) NOEC = 0.06 mg/L (river water) *Daphnia magna*: NOEC = 0.38 mg/L Fish: NOEC = 0.05 mg/L
Sediment Ecotoxicity	
Chironomus riparius *Lumbriculus variegates* *Caenorhabditis elegans* *Tubifex tubifex*	NOEC: 876 mg/kg dw NOEC: 5000 mg/kg dw NOEC: 1350 mg/kg dw NOEC: 1515 mg/kg dw EC_{10}: 550 mg/kg dw
Terrestrial Ecotoxicity	
Triticum aestivum	> 1000 mg/kg (14d EC_5)
Linum utisatissimum	> 1000 mg/kg (14d EC_5)
Soil micro-organisms	0% depression of oxygen uptake (28 d)

Reference

All data was obtained from: European Union Risk Assessment Report: Dimethyldioctadecylammonium chloride. Part 1 Environment, Joint Research Centre, EUR 20397, 2002.

4.4 Tier 3 Musk Xylene

Use pattern / release into the environment

Musk xylene (5-tert-butyl-2,4,6-trinitro-m-xylene) is a fragrance ingredient in perfumed products, mainly consumer products (detergents) and cosmetics.

Risk assessment considers emission losses from finished consumer products (detergents, soaps, shampoos, etc.) by the final downstream Private-Use / Household users as 100% down-the-drain disposal.

Annual volume:

Production in the EU ceased in the 1990s and imported volumes were mainly of Chinese origin.

The Research Institute for Fragrance Research (RIFM) and International Fragrance Association (IFRA) compiled the following evolution of importation and use of Musk Xylene in Europe:

1992	174 Tonnes
1995	110 Tonnes
1996	105 Tonnes
1998	86 Tonnes
2000	67 Tonnes

Musk xylene is essentially no longer employed in fragrance compositions in Europe since the recommendation of the IFRA proposal to prohibit the use of Musk xylene in fragrance compositions in 2009 (IFRA, 2009). This recommendation followed a series of assessments indicating the substance to be potentially vPvB (ECHA, 2008). However, the data can be used to assess the potential formation of NER and it's impact on the environmental risk assessment.

Tier 1

The initial assessment (Figure 23), based on QSAR predictions (Table 19) and experimental data (Table 20) suggests that NER could be formed.

Figure 23: Tier 1

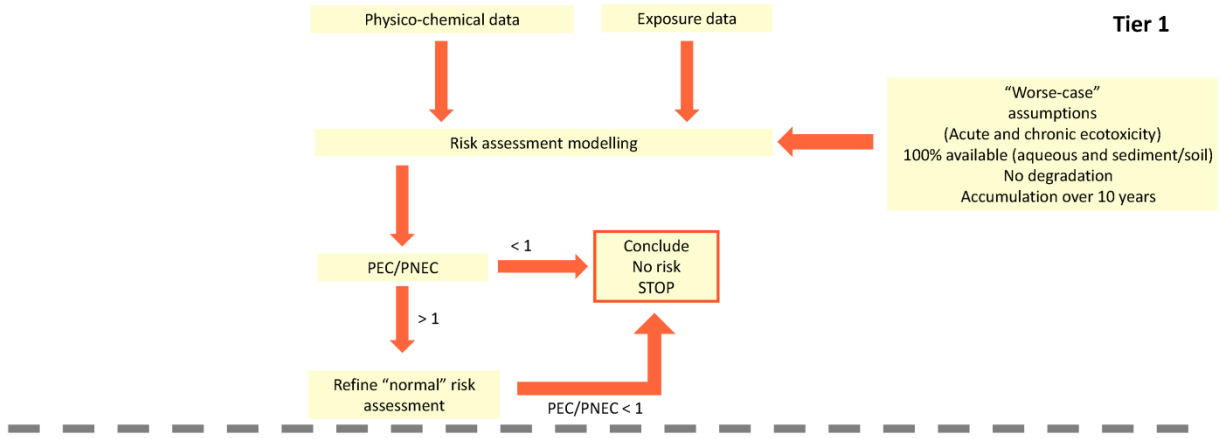


Table 19: QSAR modelling (EPI Suite (v4.10))

log P_{ow}	4.45
Water solubility	0.16 mg/L
Vapour pressure	0.000079 Pa at 25°C

Table 20: Experimentally derived physico-chemical properties

log P_{ow}	4.9
Water solubility	0.15 mg/L
Vapour pressure	Not measured

NER – physico-chemical property alert:
Water solubility < 1 mg/L
log P_{ow} = 4.9 (high probability of partitioning to solid phase)
EPI Suite modelling predicts partitioning to soil / sludge (84.6%) and to sediment (12.3%)

Exposure assessment

Local emissions from private-use – A worse-case scenario is adopted where it is assumed that 100% of the volume modelled (67 Tonnes) is disposed of through STPs as down-the-drain emissions. The 10% rule is applied to derive a Regional consumption quantity (6.7 Tonnes) from the EU-Zone ('Continental') use value. From the Regional value, it is assumed that a fraction of 0.002 is emitted to a main local source. However, the use of musk xylene was significantly higher at the beginning of the 1990s (174 Tonnes per annum). Based on these data, the PEC for the various compartments is summarised in Table 21.

Table 21: Summary of PEC values for different environmental compartments by default values

Scenario	Compartment	PEC	PNEC	PEC/PNEC
Private-use local	Freshwater	0.98 µg/L	1.1 µg/L	0.89
	Freshwater sediment	0.64 mg/kg (dry wt by EqP)	1.38 mg/kg (dry wt)	0.46
	STP water	7.8 µg/L	> 10.7 mg/L	< 0.01
	Atmosphere	4.41×10^{-8} mg/m^3	-	-
	Terrestrial / soil	0.43 mg/kg (dry wt by EqP)	0.26 mg/kg (dry wt)	1.65
	Secondary poisoning–oral, fish	5 mg/kg (wet wt)	1 mg/kg (wet wt)	5
	Secondary poisoning–oral, worm	1.2 mg/kg (wet wt)	1 mg/kg (wet wt)	1.2

PEC/PNEC

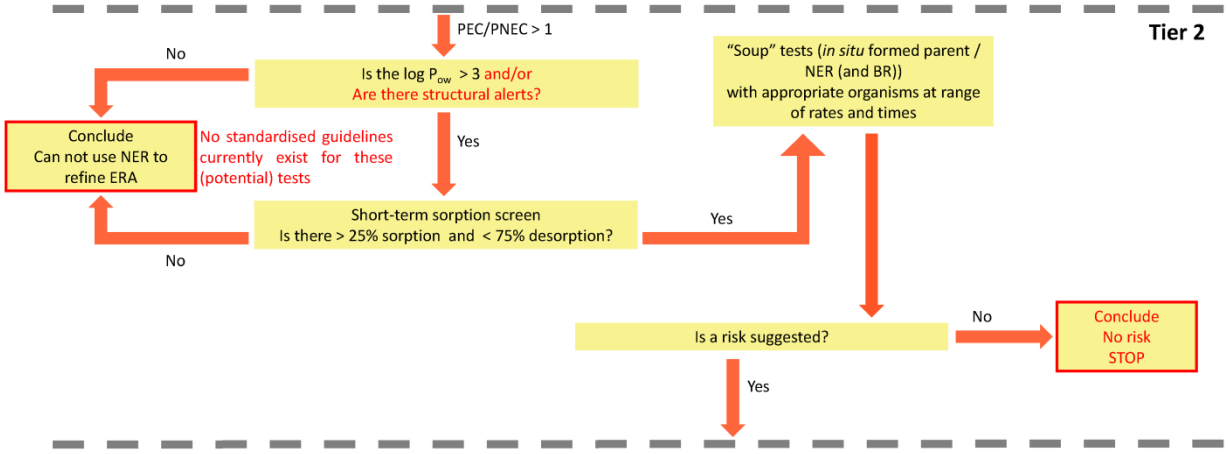
Using the data from Table 21 and Table 22 the PEC/PNEC in soil is 1.65. Since no biodegradation was observed in an OECD 301C test (Table 22), musk xylene would be a candidate for Tier 2 testing.

PEC/PNEC > 1 for terrestrial compartment

Tier 2

Tier 2 (Figure 24) initially considers the log P_{ow} and structural alerts. There are no structural alerts, but the log P_{ow} suggests musk xylene would partition to soil / sediment.

Figure 24: Tier 2



Distribution and adsorption

Using the measured log P_{ow} of 4.9 a solids-water partition coefficient normalised to organic carbon (K_{oc}) of 11,700 L/kg is predicted. A study was conducted by Winkler *et al* (1998) to determine the partition coefficients between water and suspended matter (K_p) in River Elbe water samples. A K_p of 16,300 l/kg was derived. A modified OECD 106 study was undertaken on two marine sediments using ^{14}C-musk xylene, and a K_{oc} value of 15,500 L/kg established.

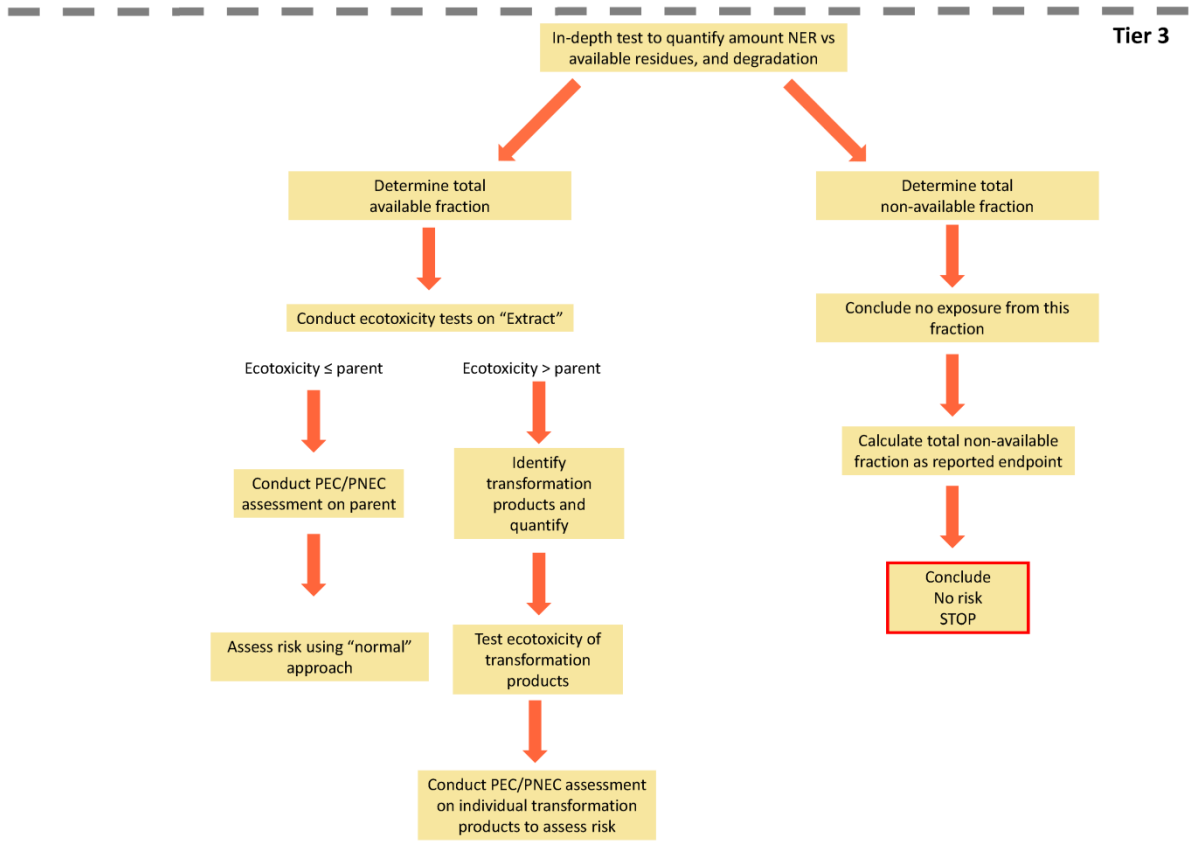
These data suggest that a Tier 2 short-term sorption test would show significant sorption. The scheme would suggest that musk xylene would be a candidate for Tier 2 soup testing.

High log P_{ow} and high experimentally derived K_p and K_{oc}:
= High Potential to form NER
→ Progress to Tier 2 soup testing

Tier 3

Since the Tier 2 soup test does not exist, Tier 3 (Figure 25) data would be the next step.

Figure 25: Tier 3



The modelling and experimentally derived data indicates a high potential for musk xylene to form NER with a potential risk to the sediment and soil compartments.

OECD 308

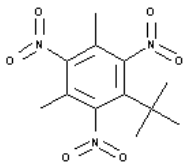
Soup testing has not been reported for musk xylene and its metabolites. However, an OECD 308 study has been performed using ^{14}C-musk xylene on two marine sediment-water systems where 32% NER and 60% NER were formed on the sediment over the 6-month test period, while trapped $^{14}CO_2$ attained only 2 to 8% during the course of the experiment (Table 22).

The results of this study demonstrate that musk xylene would have been a candidate for Tier 2 soup testing and potentially higher-tier studies under Tier 3.

High levels of NER (32 – 60%) determined in an OECD 308 Sediment-Water Study:
= High Potential to form NER confirmed
→ Soup testing in Tier 2 and potential refinement under Tier 3

Conclusion: Musk xylene would have been a candidate for Tier 2 soup testing and potentially further testing under Tier 3 had its use not been restricted within the European market following identification as a potential vPvB substance.

Table 22: Musk xylene case study data

Property	Musk xylene
Use	Fragrance ingredient in perfumed products, mainly consumer products (detergents) and cosmetics
Main environmental exposure route	Down the drain
Chemical name	5-tert-butyl-2,4,6-trinitro-m-xylene
SMILES	N(=O)(=O)c(c(c(N(=O)(=O))c(c1N(=O)(=O))C©©C)C)c1C
Structure	
Structural alert?	O = N = O groups
CAS No.	81-15-2
Molecular formula	$C_{12}H_{15}N_3O_6$
Molar mass	297.3 g/mol
Water solubility	0.15 mg/L [1]
Vapour pressure	0.00003 Pa at 25°C
pKa	Not available
log P_{ow}	4.9 [1]
Distribution modelling using Mackay, Level I (V 2.1)	Indicates that the main target compartment will be soil / sediment 94.6%
Fate in STP (partitioning/degradation)	Not readily biodegradable (OECD 301C) [1] Screening in STP influent vs effluent waters indicates 80pprox.. 95% removal rate [1]

Fate in aquatic environment	$T_{1/2}$ in marine water > 150 days (OECD 309) [1]
Fate in soil (partitioning/degradation)	log K_{oc} = 4.068 l/kg (11'700 l/kg) (predicted) [1] Expected to be of LOW mobility in soil
Fate in sediment (partitioning/degradation)	32 and 60% NER formed in two different marine sediments (OECD 308) [1] K_{oc} between marine-water and sediment = 15,500 l/kg, and K_p (river-water / suspended sediment) of 16,300 l/kg [2]
Indication of partitioning to solids?	Yes (strong adsorption to solids)
Ecotoxicological Effects	
Aquatic Ecotoxicity	
Acute	Algal inhibition: EbC_{50} (72h) > 0.15 mg/L (no effects observed at max. water solubility observed – OECD 201) [2] *Daphnia* immobilisation: EC_{50} (48h) > 0.15 mg/L (no effects observed at max. water solubility observed – OECD 202) [2] Fish Acute: LC_{50} (96h) = 1.2 mg/L [2]
Chronic	Algal inhibition: NOEC > 0.56 mg/L (essentially no effects observed up to max. water solubility) [2] *Daphnia* reproduction: NOEC = 0.056 mg/L [2] Fish: No data available
Sediment Ecotoxicity	
Chironomus	-
Lumbriculus	-
Other	-
Acute	Earthworm: LC_{50} > 50 mg/kg dw, NOEC 50 mg/kg dw (OECD 207) [2]
Terrestrial Ecotoxicity	
Chronic	-
PEC/PNEC Ratios	
Reported	1.65 (terrestrial compartment, private-use scenario)

References

[1] ECHA, 2008
[2] EU, 2005

4.5 Tier 3 Sulfamethoxazole

Use pattern / release into the environment

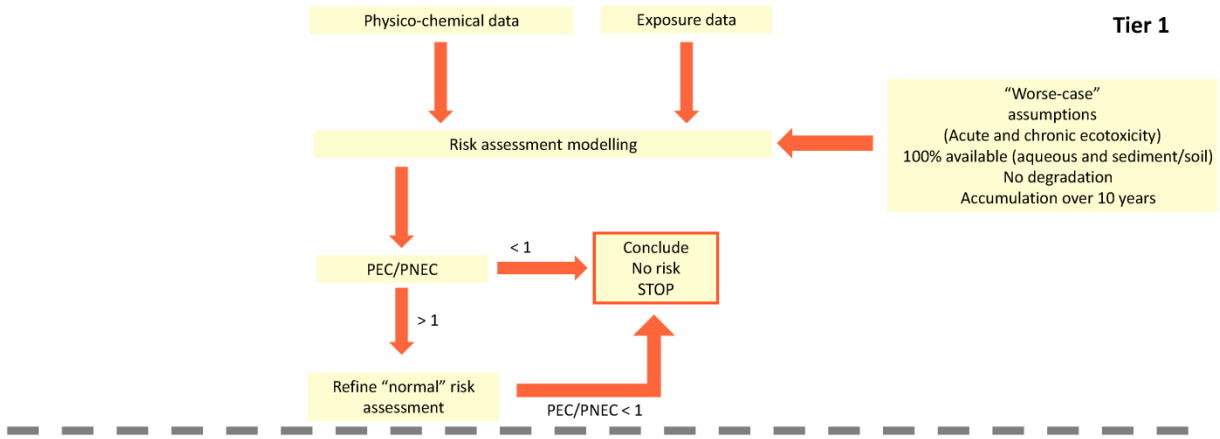
Sulfamethoxazole (4-amino-*N*-(5-methylisoxazol-3-yl)-benzenesulfonamide) is an antibiotic of the sulfonamide class, mainly produced for human use but also has some applications as a veterinary product.

The NER risk assessment is based on its use as a human pharmaceutical where release to the environment is from human excretion (no metabolism assumed) which is discharged to surface water via sewage treatment.

Tier 1

Tier 1 of the risk assessment (Figure 26) is based on information from an assessment of European Medicines Agency (EMEA) guidelines on ERA of human medicines (Grung *et al*, 2008).

Figure 26: Tier 1



Exposure assessment

Phase I of the EMEA guidelines calculates the PEC in the freshwater aquatic compartment. Estimation of the PEC is based on the maximum daily dose of sulfamethoxazole and default parameters using the following formula:

$$PEC_{surface\ water}\ (mg/L) = \frac{DOSE_{smx} \times F_{pen}}{WASTEW_{inhab} \times DILUTION}$$

where:

$PEC_{surface\ water}$ = Predicted environmental concentration for surface water
$DOSE_{smx}$ = Maximum daily dose of sulfamethoxazole consumed per inhabitant
F_{pen} = Market penetration factor of active ingredient (0.01)

$WASTEW_{inhab}$ = Volume of wastewater generated per inhabitant (200 L $inhab^{-1}day^{-1}$)
DILUTION = Dilution of effluent in recipient (10 x)
Values in parentheses are defaults.

The maximum daily dose of sulfamethoxazole is 2,400 mg which, when substituted into the formula, gives a $PEC_{surface\ water}$ of 12 µg/L. This is greater than the EMEA action limit for further investigation of 0.01 µg/L, which triggers a Phase II Tier A, fate and effects assessment of the pharmaceutical.

Effects assessment

The EMEA Phase II Tier A proposes three standard chronic toxicity tests, from which NOECs may be determined. An assessment factor (AF) is then applied to the lowest NOEC to derive a PNEC. The size of the AF depends on the number of trophic levels represented in the chronic tests. An AF of 50 was set for sulfamethoxazole as chronic test data (Table 23) were available for algae and a crustacean (i.e. two trophic levels). The lowest NOEC recorded for sulfamethoxazole was 5.9 µg/L, which was determined in a 96h algal growth inhibition study with the cyanobacteria *Synechococcus leopolensis.* The $PNEC_{surface\ water}$ for sulfamethoxazole therefore becomes 5.9/50 = 0.118 µg/L.

Calculation of PEC/PNEC ratios

Based on the unrefined, predicted values described above, the PEC/PNEC ratio for sulfamethoxazole is 12/0.118 = 102.

Refinement of the PEC/PNEC ratio

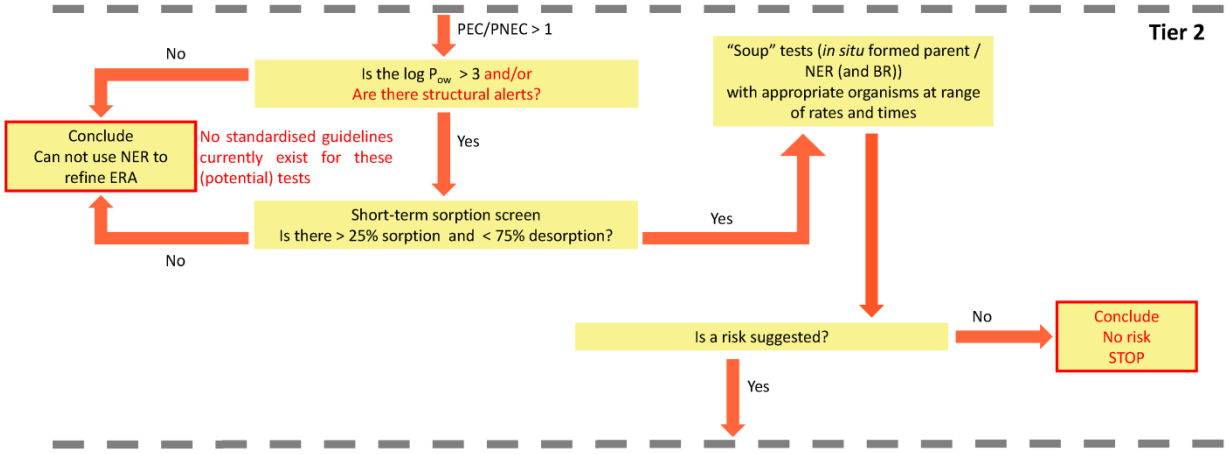
The initial PEC was based on the assumption that 100% of the administered pharmaceutical was available in the receiving water. In order to refine the PEC for sulfamethoxazole, removal through adsorption and degradation in an STP is taken into account. The reduction through adsorption to suspended solids was calculated to be only 0.1% and sulfamethoxazole is not easily degraded by microbes (only 4% biodegradation was measured in a 28 day closed bottle test, Table 22. Consequently, the impact of the STP had little effect on the PEC i.e. the refined PEC was 11.4 µg/L, giving a PEC/PNEC ratio of 97, which means that further risk assessment is required.

Tier 1 assessment concludes PEC/PNEC > 1
→ Tier 2

Tier 2

The initial phase of Tier 2 (Figure 27) assesses the potential for NER formation from log P_{ow} data and structural alerts.

Figure 27: Tier 2



Evaluation of log P_{ow} and structural alerts

Sulfamethoxazole has a measured log P_{ow} of 0.89, which would suggest that it would not strongly adsorb to solid matrices. However, it has been proposed that certain chemical structures may be involved in active binding mechanisms and that the presence of these structures in a substance could be used as an alert to the possibility of NER formation. Among the functional groups that have been implicated as being potential structural alerts, are aniline and phenol groups. An aniline group is found within sulfamethoxazole, therefore, according to the interim risk assessment, its presence would trigger further investigation into the formation of NER by conducting a short-term sorption screen.

NER – Structural Alert:
Aniline moiety
→ Short-term sorption screen

Short-term adsorption screen

A detailed adsorption / desorption study was carried out (Table 23) with sulfamethoxazole in two soils types, high and low organic content soils, according to OECD guidelines. For high organic (7.1% OC) soil, it was found that the amount of sulfamethoxazole adsorbed and desorbed was 60% and 27%, respectively. For soil with a low organic content (0.37% OC), the amount adsorbed and desorbed was 10% and 84%, respectively. According to the interim risk assessment, the results obtained with the high organic content soil would trigger the continuation of assessment of NER, as adsorption was greater than 25% and desorption less than

75%, if these data were reflected in the Tier 2 short-term sorption test. The same did not hold true for low carbon content soils. However, Tier 2 soup testing would be recommended to evaluate this further.

> 25% adsorption and < 75% desorption in short-term sorption screen with high OC soil
→ Soup testing

Soup testing

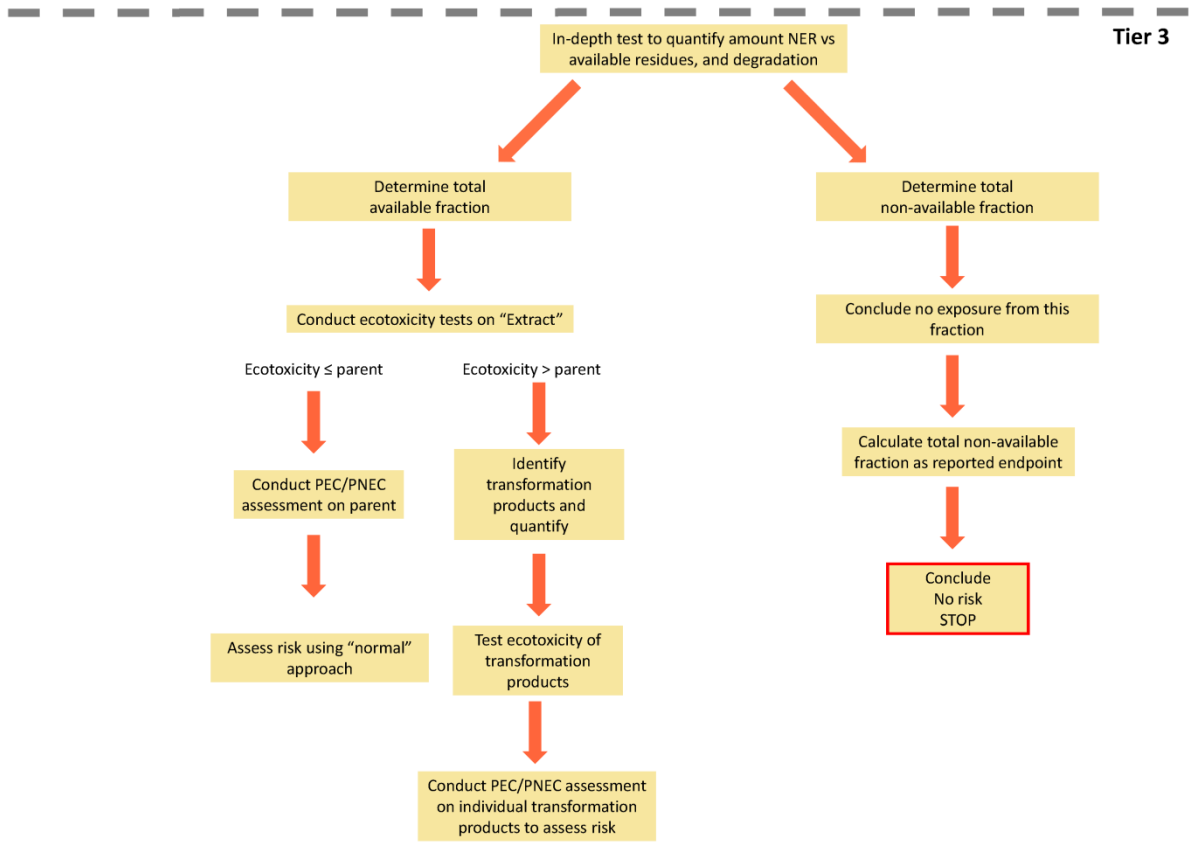
No information could be found for sulfamethoxazole, or any related sulfonamide antibiotic, that has been tested in soup type test system. The interim risk assessment therefore proceeds to Tier 3, in depth testing, to quantify NER formation and degradation.

No information on soup testing reported
→ Tier 3

Tier 3

The first step of Tier 3 (Figure 28) would focus on a soil or sediment-water test to quantify the NER. Typically, these tests are based on the OECD 307 (OECD, 2002a) or OECD 308 (OECD, 2002b) test guidelines, respectively.

Figure 28: Tier 3

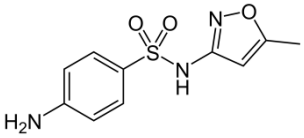


The formation of NER in soil was studied using [^{14}C]sulfamethoxazole, with and without the addition of a test slurry (liquid bovine manure). Non-extractable residue formation was > 70% of the applied radioactivity (extractable with ethyl acetate, Table 23). Evidence that this was a non-extractable fraction comes from the same study with the structural analogue of sulfamethoxazole – [^{14}C]sulfadiazine. After sequential extractions with different solvents (ethyl acetate → methanol / hydrochloric acid → chlorotrimethylsilane) the amount of sulfadiazine that remained bound was still 74% of that applied.

The remobilisation of non-extractable [^{14}C]sulfamethoxazole residues from ethyl acetate extracted soil samples, was monitored using the earthworm, *Lumbricus terrestris*. After 14 days, the burrowing activities of the earthworms had completely re-moulded the test soils. However, > 90% [^{14}C]sulfamethoxazole remained as NER. The amount of radioactivity taken up by the worms was 0.3 ± 0.2% and 1 ± 0.5% in soils, with and without the addition of slurry, respectively.

Conclusion: Sulfamethoxazole has been shown to form NER in soil and there is evidence to suggest this fraction has low bioavailability to earthworms. The affinity of the molecule for solid matrices would suggest that, if applied in a soup test system, NER would be formed and toxicity would not be observed in extracted soil.

Table 23: Sulfamethoxazole case study data

Property	Sulfamethoxazole
Use	Sulfamide antibiotic used mainly as human pharmaceutical but also used for veterinary purposes
Main environmental exposure route	Effluent from municipal sewage treatment
Chemical name	4-amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide
SMILES	O=S(=O)(Nc1noc(c1)C)c2ccc(N)cc2
Structure	
CAS No.	723-46-6
Molecular formula	$C_{10}H_{11}N_3O_3S$
Molar mass	253.279 g/mol
Water solubility	0.29 mg/L [1]
Vapour pressure	1.3 e^{-7} mgHg
pKa	1.8 and 6
log P_{ow}	0.89
Distribution modelling using Mackay, Level I (V 2.1)	
Fate in STP (partitioning/degradation)	Sulfamethoxazole is not readily biodegradable. 4% degradation measured in closed bottle test after 28 days [2]. Modelling (EPI SUITE v4), predicts STP removal of 1.88%; 98.12% will be discharged in effluent.
Fate in aquatic environment	Data not available
Fate in soil (partitioning/degradation)	K_{oc} 530 L/kg (high organic soil) K_{oc} 62.2 L/kg (low organic soil) [3]
Fate in freshwater sediment (partitioning/degradation)	Data not available
Indication of partitioning to solids?	Non-extractable (ethyl acetate extraction) residues amounted to 93% versus 5% extractable after 14 days from soil amended with slurry; 85% and 11% respectively from un-amended test soil. Nature of NERs not investigated [4].

Ecotoxicological effects	
Aquatic ecotoxicity	
Acute	*V. fischeri* (Microtox) 15 min EC_{50} = 344 mg/L *D. magna* 48h EC_{50} = 174 mg/L *O. Latipes* 96h LC_{50} ≥ 100 mg/L [5]
Chronic	Algal inhibition (*Synechococcus leopolensis*): NOEC = 0.0059 mg/L Invertebrate reproduction (*C. dubai*): NOEC = 0.25 mg/L Fish (*D. rerio* ELS): NOEC > 8 mg/L [6]
Sediment ecotoxicity	
	Data not available
Terrestrial ecotoxicity	
Acute	*O. sativa* (Rice) $EC_{50(germination)}$ = 8 mg/L Soil respiration inhibition 2 day EC_{10} = 7 mg/kg [7]
Chronic	Data not available

References

[1] Plumb, 2002
[2] Alexy *et al*, 2004
[3] Drillia *et al*, 2005
[4] Heise *et al*, 2006
[5] Kim *et al*, 2007
[6] Ferrari *et al*, 2004
[7] Liu *et al*, 2009

5. DISCUSSION AND CONCLUSIONS

One of the major outputs from an ECETOC workshop in 2009 to investigate the significance of bound residues in environmental risk assessment was a framework outlining a possible decision tree for conducting risk assessment of NER (Figure 1). ECETOC subsequently established a Task Force whose goal was to:

1. Critically evaluate the framework developed following the ECETOC workshop and assess its utility as an interim approach for regulatory assessment of chemicals.

Having critiqued the framework the Task Force have concluded that the framework developed from the workshop provided a sound approach to addressing the issue of risk assessment of NER. The developed scheme presented in this report (the risk assessment scheme) has made very few major changes to the framework. The main developments have been:

- Presenting the framework as a series of Tiers, to help clarify each step of the process.
- In Tier 2, the introduction of using structural alerts to screen for ionisable / polar substances with potential to form NER, despite having a low log P_{ow}.
- Developing the principle of the Tier 2 soup test and extending it to address some aspects of Tier 3 (more in depth tests). Tiers 2 and 3 have been developed to evaluate the potential risk posed by NER.

2. Develop suitable guidance and trigger values to enable the decision tree to be used and test the utility of the framework using suitable case studies.

Guidance and trigger values have been developed where this has been feasible. However, some aspects will still require further validation, for example, the proposed Tier 2 soup test methodology and the applicability of the extraction framework (ECETOC, 2012). The trigger values, such as those selected for adsorption / desorption, the structural alerts and the choice of exposure concentrations in relation to environmental exposures, have been based on existing test guidelines or published literature. However, existing risk assessment methodology could not be applied in the context of assessing NER.

The utility of the scheme, for use in risk assessment, has been demonstrated using case studies, such as DODMAC, musk xylene and sulfamethoxazole. These substances would be suitable test substances for further investigation of the utility of the scheme. It is expected that risk assessment of these substances using Tier 2 studies would have demonstrated that:

a. A quantifiable amount of each substance would form NER, and
b. the fraction containing NER would not be ecotoxic.

Caffeine was included as it was predicted not to form NER and was used to demonstrate how this type of substance could be eliminated from NER risk assessment in the first tier of the scheme.

3. Identify current knowledge gaps and provide guidance on study design to provide the appropriate quality of data needed for the risk assessment framework to function within a regulatory decision making system.

The Task Force has identified where the scheme was incomplete and has suggested areas that would warrant further work. The Tier 2 soup tests provide a useful intermediate step between the overly conservative Tier 1 risk assessment and the technically demanding Tier 3 tests. This is currently missing in substance risk assessment methodology. However, the Task Force has drawn from other regulatory arenas and published literature and applied their principles to the risk assessment of NER.

Soup tests have been designed to be sufficiently rigorous (including aging processes, using adequate control treatments and the selection of the appropriate test species), and the Task Force has adopted a 'mass balance' approach assessing the risk of the extract as well as extracted fractions. These soup tests facilitate evaluating the toxicity of NER for the purpose of risk assessment without requiring extensive analysis, where this is unnecessary.

The first chapter of this report describes the background literature on how NER have been dealt with in a regulatory context. In the context of this report, the task force has discussed the scope of its remit and focused on the soil and sediment-water compartments. Other compartments, such as sewage sludge, manure, plants and livestock, whilst being important, have not been investigated to the same extent, and validated testing methodologies for these matrices are less broad-reaching. It is, however, expected that the principles developed for soil and sediment could be applied to these other environmental compartments.

Chapter 2 highlights a range of *in silico* and experimental approaches to identify chemicals which may be susceptible to adsorption, the first step in forming NER, and provide an overview of the various advantages and disadvantages of each approach. Overall, except for non-polar substances, the use of physico-chemical properties such as log P_{ow}, water solubility and the Henry's Law constant do not adequately predict the formation of NER. Hence, there is a need for more predictive tools and these are described in Chapter 3.

In chapter 3, the framework has been split into 3 Tiers:

Tier 1 – Risk assessment assuming 100% bioavailability (a conservative (worse case) risk assessment). If the PEC/PNEC ratio is greater than 1, then it is possible to perform a refinement step using justifiable data. If the PEC/PNEC ratio remains ≥ 1, the substance enters Tier 2 evaluation. However, if no risk is suggested (PEC/PNEC is < 1) then no further evaluation would be required. At this stage of the risk assessment it is assumed that NER are not formed. Therefore, no additional testing or alteration to the risk assessment is warranted.

Tier 2 – NER screening risk assessment. Initially, this tier addresses the hydrophobicity (log P_{ow}) and looks for structural alerts to suggest if NER formation is likely. Whilst the science of predicting NER from structural alerts is not well developed some basic rules of thumb have emerged from the literature, e.g. phenol and aniline moieties do seem to lead to higher levels of NER, as does the presence of cationic moieties. However, there are suggestions that other moieties may also be linked to NER formation. To develop these rules, the Task Force suggests that more research would be necessary.

If the predictions suggest NER may be formed, then a screen to confirm / refute them would be helpful. The Task Force has suggested that a screen based on a previous version of the OECD 106 test would be suitable (Section 3.5.2). It uses a modest number of soils and gives an indication of adsorption, whilst not being too

onerous. This guideline also suggests when significant adsorption / desorption occurs within the test. The triggers (25% adsorption with 75% desorption) have been adopted within the scheme.

The final stage of Tier 2 (if the substance fulfils the previous requirements for further evaluation) assesses the hazard of NER by conducting ecotoxicity testing. Several options both in soil and in water-sediment systems were considered before concluding that initially a microbial test in soil would be the most suitable candidate test in this tier. Microbes were selected as they perform an important role in soil fertility, are homogeneously distributed and their small size means they are 'intimately' in contact with the soil. This means they are likely to be exposed to NER during the ecotoxicity test. In order to conclude on the risk assessment of NER at this stage, it is suggested that chronic ecotoxicity tests at three trophic levels would be required. An outline on how to perform such studies is detailed in Chapter 3. The reality is that tried and tested methods do not exist for regulatory purposes. These tests would need to be developed beyond the concept developed by the Task Force. They would need to be validated with reference substances which have suitable properties and have been extensively studied in regard to NER risk assessment. If concerns were still evident after the Tier 2 screens, then further in depth evaluations (Tier 3) may be required. If, however, no risk was suggested then further testing would not be required.

Tier 3 – In depth NER risk assessment. In this final tier, the amount of extractable and non-extractable residue would be characterised using appropriate environmental fate guidelines (e.g. OECD 307 or OECD 308) and utilising the extraction framework developed by ECETOC (2012). Once degradation processes had been allowed to transform the substance and NER had been formed, the NER would be considered to be non-available. To demonstrate any environmental risk, testing similar to that outlined for Tier 2 (Section 3.5.3.1) may be required. The extractable fraction may be evaluated by conventional approaches of separation and identification of the transformation products. This would be followed by the synthesis of these substances and subsequent ecotoxicity testing to indicate which, if any, were of ecological concern. An alternative approach could be developed to use a more targeted (effects-driven) approach to identify if there were a need for more detailed evaluation. These methods have not been formalised into substance risk assessment guidelines, so this would require further work before a standardised approach could be adopted.

In an attempt to use the scheme, Chapter 4 describes how it has been used to examine selected substances in a series of case studies. Four existing substances, for which differing levels of environmental data were available in the literature, were selected. Since the testing described in Tier 2 does not currently exist, no substances stopped at this stage. Caffeine would not be expected to form NER, so would not progress beyond Tier 1. However, DODMAC, musk xylene and sulfamethoxazole would progress to Tier 3. If the in-depth study data for DODMAC, musk xylene and sulfamethoxazole were reflected in Tier 2 screens, it is likely that these substances could have avoided progressing to Tier 3. More case studies, or preferably new substances, should be evaluated using the scheme. This would identify where further refinement of the scheme may be needed.

In this final chapter (Chapter 5) some recommendations have been made for future improvements and suggested potential future research activities.

Future recommendations

The use of the scheme with new examples, ideally with limited existing data would be helpful. This would test the scheme with no pre-conceived ideas of the outcome.

Tier 2 screening for structural alerts

Whilst some literature suggests certain moieties may be associated with high levels of NER, more data using standard approaches (and more standardised extraction methods) is needed to develop more robust predictions. To use these rules, it is suggested that more research would be necessary. For example, details of the extraction method could be investigated and similar extraction methods grouped together. Other variables, such as soil and sediment characteristics, could also be grouped. Standardisation might then make it practical to identify trends within existing pesticide data (e.g. Barriuso *et al*, 2008). In addition, other classes of chemicals (e.g. human pharmaceuticals) are now being examined in soil and water-sediment tests, such as OECD 307 and OECD 308, where the formation of NER is observed (Ericson *et al*, In press). Pooling these diverse datasets will be a challenge but may also aid in identifying new structural alerts for NER formation.

Tier 2 screening for adsorption and desorption

Whilst the proposed approach is based on a screening phase of a superseded test guideline, the approach needs to be developed for freshwater sediments and some validation carried out to ensure the data are robust. Developing the use of desorption data in risk assessment of NER is also recommended to characterise the rate/extent of release of these residues.

Tier 2 soup tests

The report describes an approach which focused on soil, but this requires some minor development and validation. However, a suitable test using a sediment-water system would need research, development and validation before it can be recommended for use in this risk assessment scheme. It is recommended that the substances used in the case studies (Chapter 4) could be used as a validation set for such work.

Tier 3 testing methods for ecotoxicity evaluation of extracted residues

Currently there are no standard methods, except for isolation and testing of each transformation product. An approach that tests the whole extract and applies an effect-driven philosophy is recommended. In addition, further guidance is required in the use of such data in a risk assessment. For example, the selection of appropriate test species and how many trophic levels has not been defined. Also, relating the exposure concentration to the observed effects in extracts (with uncharacterised transformation products) would need to be considered.

Tier 3 testing to demonstrate that the NER is of low risk

This is challenging as some agreement on what tests would be required and how many different organisms / trophic levels / conditions would need to be developed and agreed in a regulatory scheme. Whilst, some approaches which may be appropriate are suggested, any further development would require agreement with the regulatory community to ensure acceptance in a regulatory context.

Conclusions

The framework developed after the 2009 ECETOC workshop (ECETOC, 2010) is a suitable approach with which to develop the risk assessment of NER.

The Task Force has critiqued the elements of the framework, and where possible, it has suggested triggers and further guidance.

The scheme now includes the extraction framework developed by a parallel ECETOC Task Force which addressed the relationship between extraction technique and bioavailability (ECETOC, 2012). The extraction framework describes an approach which can be used to identify the portion of extractable and NER using a range of appropriate extraction methods. Tier 2 of the scheme has been developed further as a screening tier and has some elements which are also applicable to the Tier 3. For example, the Task Force has addressed concerns about the potential risk of the NER remaining after suitable extraction of the extractable residues (the bioavailable and bioaccessible fractions). This has been challenging since there is no regulatory guidance and the published literature tends to focus on assessing the extractable residues.

In principle, if NER are not extractable, then they cannot be available to elicit an effect on the biota. However, some concerns remain about the validity of this principle. Some suggestions on how this could be addressed have been made. Whilst this Task Force has developed the risk assessment scheme, knowledge gaps remain where further work is required and these have been highlighted in the report.

ABBREVIATIONS

AF	Assessment factor
ASE	Accelerated solvent extraction
B	Bioaccumulation
BCF	Bioconcentration factor
BR	Bound residue
DOC	Dissolved organic carbon
DODMAC	Dioctyl dimethyl ammonium chloride
D_{ow}	Distribution constant at a given pH
$DT_{50/90}$	Disappearance Time of 50/90% of the substance
dw	Dry weight
EC	European Commission
EC_{50}	Effective Concentration, 50%; median effective concentration
ELS	Early life stage
EMEA	European Medicines Agency
EqP	Equilibrium partitioning
ER	Extractable residue
ERCs	Environmental release categories
ESIG	European Solvents Industry Group
ESR	Electron spin resonance
EU	European Union
FERA	The Food and Environment Research Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FTIR	Fourier transform infra-red
H’	Henry’s Law Constant
HPCD	Hydroxypropyl-beta-cyclodextrin
HPVC	High Production Volume Chemical
IFRA	International Fragrance Association
ISO	International Standards Organisation
IUPAC	International Union of Pure and Applied Chemistry
K_d	Solid/liquid partition coefficient
K_{oc}	Organic carbon normalised solid/liquid partition coefficient
K_{ow}	Octanol-water partition coefficient
K_p	Partition coefficient
LC_{50}	Lethal Concentration, 50%; median lethal concentration

MEC	Measured environmental concentrations
NER	Non-extractable residue
NMR	Nuclear magnetic resonance
NOEC	No observed effect concentration
OC	Organic content
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
P	Persistence
PBT	Persistent, bioaccumulative, toxic
PEC	Predicted environmental concentration
pH	Potential of Hydrogen
PNEC	Predicted no effect concentration
P_{ow}	Partition coefficient n-octanol/water
QSAR	Quantitative structure activity relationship
RCR	Risk characterisation ratio
REACH	Registration, evaluation, authorisation and restriction of chemicals
RIFM	The Research Institute for Fragrance Research
SCAS	Semi-continuous activated sludge
SFE	Supercritical fluid extraction
SMILES	Simplified molecular input line entry system
SOM	Soil organic matter
SPME	Solid phase micro-extraction
STP	Standard Temperature and Pressure
T	Toxicity
TP	Transformation product
TSCA	Toxic Substances Control Act
UBA	UmweltBundesAmt
US EPA	United States Environmental Protection Agency
vPvB	Very persistent, very bioaccumulative
W_{sol}	Water solubility

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