Consultancy services to support ECHA in improving the interpretation of Non-Extractable Residues (NER) in degradation assessment

Discussion paper - final report

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Please note that at the date of the publication of this document (June 2018) discussions on the characterisation of NER in relation to the persistence assessment are still on-going and the considerations in this report are based on the current state of the art. Registrants are advised to keep themselves updated with the on-going developments in the field, e.g. via the ECHA website.
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### Glossary

<table>
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<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alkaline extraction of organic matter</strong></td>
<td>A procedure to extract and hydrolyze humic matter for subsequent fractionation into fulvic acids, humic acids and humin.</td>
</tr>
<tr>
<td><strong>ASE</strong></td>
<td>Accelerated Solvent Extraction (extraction with the option of elevated temperature and pressure (cf. PLE)).</td>
</tr>
<tr>
<td><strong>Alkaline extraction and fractionation</strong></td>
<td>See Humic matter</td>
</tr>
<tr>
<td><strong>bioNER</strong></td>
<td>Biogenic NER</td>
</tr>
<tr>
<td><strong>BPR</strong></td>
<td>Biocidal Products Regulation</td>
</tr>
<tr>
<td><strong>Combustion analysis</strong></td>
<td>$^{14}$C-radioactivity associated with solid samples, e.g. soil, sediments etc., are quantified by combustion under defined temperature, oxygen conditions and a metallic catalyst. The evolved radioactive carbon dioxide is trapped in an alkaline solution and subsequently measured by liquid scintillation counting (LSC).</td>
</tr>
<tr>
<td><strong>Degradation</strong></td>
<td>Abiotic and/or biotic degradation processes (in this document mainly referring to late or ultimate degradation by biotic processes, leading to biomass (natural) substances and mineralisation).</td>
</tr>
<tr>
<td><strong>Degradation product</strong></td>
<td>Result of abiotic and/or biotic degradation processes (in this document primarily used for late or ultimate transformation products).</td>
</tr>
<tr>
<td><strong>DT50</strong></td>
<td>Time taken for 50% of substance to disappear from a compartment by dissipation processes</td>
</tr>
<tr>
<td><strong>DegT50</strong></td>
<td>Time taken for 50% of substance to disappear from a compartment due to degradation processes alone</td>
</tr>
<tr>
<td><strong>Dissipation</strong></td>
<td>Disappearance of a chemical in environmental media due to degradation, transport processes and irreversible binding</td>
</tr>
<tr>
<td><strong>Fulvic acids</strong></td>
<td>See Humic matter</td>
</tr>
<tr>
<td><strong>GC-MS</strong></td>
<td>Gas chromatography coupled to mass spectrometry detection</td>
</tr>
<tr>
<td><strong>Half-life</strong></td>
<td>Time taken for 50% of substance to disappear/dissipate from a compartment following single first-order kinetics.</td>
</tr>
<tr>
<td><strong>High temperature catalytic oxidation</strong></td>
<td>See Combustion analysis</td>
</tr>
<tr>
<td><strong>HPLC-MS, HPLC-UV</strong></td>
<td>High performance liquid chromatography coupled to mass spectrometry detection or UV detector, respectively</td>
</tr>
<tr>
<td><strong>Humic acids</strong></td>
<td>See Humic matter</td>
</tr>
<tr>
<td><strong>Humic matter</strong></td>
<td>Humic substances are indicated as a brown to black major component of soil organic matter (SOM), making up 60% to 80% of the SOM. These substances can be divided into three major fractions, i.e., humic acids, fulvic acids and humin, which are defined operationally by the solubility of each fraction under acidic and alkaline conditions. Alkaline extraction of soils or sediments leads to solubilisation of humic and fulvic acids; the remaining insoluble residue contains humin (and inorganic material). After subsequent acidification of the alkaline extract humic acids precipitate, leaving fulvic acids in solution.</td>
</tr>
<tr>
<td><strong>Humin</strong></td>
<td>See Humic matter, fraction remaining insoluble</td>
</tr>
</tbody>
</table>
| **Liquid scintillation counting (LSC)** | A method to quantify radioactivity by mixing a radioactive sample with a liquid scintillator in a solvent and counting the resulting photon emissions. The photons are released from scintillator molecules after excitation by the radioactive material (such as beta particles emission from $^{14}$C-labelled material). Alternatively, a radioactive sample in a
solvent can be passed through a matrix with immobilized scintillators.

**MAE** Microwave Assisted Extraction

**Michaelis Menten kinetics** A model describing the rate of enzymatic reactions by relating the reaction rate and the concentration of a substrate

**Metabolite** Products formed. In biodegradation processes, the term is synonymous with the term biodegradation product; as result of biotransformation within an organism, products are species specific.

**Monod kinetics** An empirical model describing the growth of microorganisms by relating microbial growth rates in an aqueous environment to the concentration of a limiting nutrient or substrate

**MTB** Microbial Turnover to Biomass (model to predict formation of bioNER)

**NER** Non-Extractable Residues

NER type I Sequestered, entrapped NER

NER type II Covalently bound NER

NER type III Biogenic NER (bioNER)

**NMR** Nuclear Magnetic Resonance

**PLE** Pressurised Liquid Extraction at elevated pressure and temperature. This method of extraction is equivalent to the accelerated solvent extraction (ASE).

**PLFA** Phospholipid fatty acids, components of every cell membrane

**Passive sampling** Use of apolar or polar organic polymers to sorb analytes from aqueous solutions, either in the kinetic (uptake) mode or in the equilibrium mode

**PPP** Plant protection products (pesticides)

**Radio-HPLC** High performance liquid chromatography coupled to a radioactivity detector, for instance for profiling $^{14}$C-labelled residues in degradation studies.

**Radio-TLC** Thin layer chromatography and subsequent analysis of radiolabelled residues, e.g. $^{14}$C-residues, on the plate by a radioactivity detector.

**Reflux** A normal refluxing apparatus consists of a heated flask to bring a liquid to the gaseous state and a cooling device above for condensing the gas. A special reflux apparatus is the Soxhlet device, used for extractions of environmental samples. For Soxhlet extractions, a solid sample, often mixed with a drying agent such as sodium sulfate because wet samples may repel lipophilic solvents, is loaded in a porous extraction sleeve made of thick filter paper. The solvent is distilled from a reservoir and is liquified at a cooled condenser from which the hot solvent drips into the sample sleeve. The apparatus is constructed in a way that the solvent is collected and, if the solvent level reaches a drain pipe (a siphon), it is directed back to the sample reservoir where the solvent cycling starts over again. The continuous flux of the hot solvent through the sample allows for very efficient extraction results.

**SFE** Supercritical Fluid Extraction; extraction of mostly a solid matrix, like soil or sediment, by using supercritical fluids as extractants. Supercritical fluids share the properties of liquids (with high dissolving power) and gases (with high diffusion coefficients) above a certain temperature and pressure. Often, carbon dioxide is used as supercritical fluid, sometimes modified by co-solvents like ethanol, because the critical temperature of CO$_2$ (31 °C) and critical pressure (74 bar) are easily achieved.
Silylation is the introduction of a substituted silyl group (R₃Si⁻) to a molecule carrying functional groups with exchangeable protons to a) obtain volatile derivatives of organic chemicals improving detection (e.g. via GC/MS) and subsequent characterisation, and/or b) improve solubility of natural polymers in organic solvents (i.e. polysaccharides and lignin). The latter procedure can be applied to disaggregate humic matter and to release type I (type II NER remain insoluble).

**SOM**
Soil organic matter

**Soxhlet extraction**
Continuous (hours) extraction at elevated temperature (see “Reflux”)

**SPME**
Solid Phase Micro Extraction (passive sampling using thin fibers coated with an apolar or polar organic polymer)

**xenoNER**
NER containing xenobiotic molecules, i.e. the parent substance and transformation products

**TLC**
Thin layer chromatography, for instance two-dimensional (2D-TLC)

**TMAH**
Tetramethylammonium hydroxide, derivatisation agent

**Transformation product**
Includes all transformations; physical, chemical and (a)biotic (in the context of this document often used for early or primary metabolites after transformation of the parent substance).

**UVCB**
Substances of Unknown or Variable composition, Complex reaction products or Biological materials

**Yield**
Microbial biomass yield (biomass formation / substrate consumption)
Executive Summary

The discussion paper is drafted in five chapters, describing the aim of the research, thoroughly reviewing the current scientific state of the art, describing the suggested role of different types of non-extractable residues (NER) in Persistence (P) assessment, suggesting concepts for assessing the role of different types of NERs in the regulatory context of PBT assessment, and research needs to establish a broader base for a better understanding of the underlying mechanisms in NER formation.

Three types of non-extractable residues of generally all chemicals in environmental matrices can be experimentally discriminated (details see Table 2): sequestered and entrapped residues (type I), containing either the parent substance or transformation products or both and having the potential to become released. Release of this type of NER has been observed as exemplified in the main text. Type II NER are those residues that are covalently bound to organic matter in soils or sediments or to biological tissue in organisms and that are considered being strongly bound with very low remobilisation rates like that of humic matter degradation. Type III NER comprises biogenic NER (bioNER) after degradation of the xenobiotic chemical and anabolic formation of natural biocompounds like amino acids and phospholipids, and other biomass compounds.

The Microbial Turnover to Biomass (MTB) model has shown good correlation between the formation of bioNER measured in selected radio-labelled studies and that predicted by the model itself. However, additional research should be performed as the model does not take into account the position of radiolabelling. Depending on the label position, both mineralisation (14CO2 evolution) and formation of NER can differ significantly for the same substance. This is not considered when modelling is performed; therefore, bioNER need to be experimentally quantified.

The proposed extraction sequence to obtain a matrix containing only NER involves three steps: first, readily desorbable residues are extracted thoroughly with aqueous solvents such as a diluted CaCl2 solution. Alternative methods to determine such residues are available and described in the main text. The next step is based on thorough extractions with organic solvent mixtures; care must be taken to choose a suitable solvent depending on the physical-chemical properties of the analyte, its metabolisation status and the sample properties. Finally, the sample is extracted under exhaustive conditions such as elevated temperature and pressure. At this point we define that all adsorbed xenobiotic and related moieties except that of NER should have been released, therefore the amount that has not been recovered is defined as NER. NER can be subsequently differentiated into three NER types by chemical derivatisation of the matrix, i.e. silylation, and by extraction and fractionation of biogenic residues. All extraction steps are described in chapter II.3.1. The extraction scheme was developed for sediment-water systems first and applied also frequently to degradation tests with soils. Principally, the extraction scheme can be applied to all solid or particulate matrices.

bioNER are of no environmental concern and, therefore, can be assessed as such in persistence assessment. Type I NER and type II NER should be considered as potentially remobilisable residues in persistence assessment but the probability of type II release is much lower. For these types the potential of remobilisation needs to be evaluated. The general concept presented is to consider the total amount of NER minus potential bioNER as the amount of xenONER, type I + II. If a clear differentiation of type I and type II is possible, for the calculation of half-life type I NER are considered as not degraded parent substance or transformation product(s). On the contrary, type II NER may generally be considered as (at least temporarily) removed (for exceptions see section II.4.3. Options for the assessment of the potential remobilisation of total NER). However, providing the proof for type II NER is the most critical issue in NER assessment and requires severe additional research. If no characterisation and additional information on NER is available, it is recommended...
to assess the total amount as potentially remobilisable (this is in line with ECHA guidance on PBT assessment (ECHA_2017_R.11)).

I. Purpose and scope
Criteria for the assessment of chemical properties and toxicological and environmental behaviour of industrial chemicals in general, and particularly for biocidal products, plant protection products, and veterinary medicines are summarised in specific European legislations, i.e. regulations (EC_1907_2006), (EC_528_2012), (EC_1107_2009), and guidelines EC 726/2004, 2004/27/EC and 2004/28/EC, respectively. For the assessment of PBT properties under REACH there are guidance documents available (ECHA_2017_R.7b; ECHA_2017_R.7c; ECHA_2017_R.11). These guidance documents provide principles on the assessment of NERs. However, currently there is no detailed description of the extraction techniques to differentiate NER types available. As there is no unified guidance available for the differentiation of different NER types in the general regulatory context (PPP, REACH, ...), here it is suggested an approach based on a thorough review of the current scientific state-of-the-art.

Besides various degradation and transport processes, all chemicals that enter environmental matrices potentially form NER in varying amounts (Barriuso et al., 2008; Kaestner et al., 2014). The regulatory views on NER formation differ considerably, with the two extremes of (i) assuming them as either degraded residues of no environmental concern in the regulation of pesticides (DG_SANCO_2012; FOCUS, 2014), at least if the NER are below or the mineralisation rate above certain threshold values, or (ii) as potentially bioavailable and non-degraded residues (“parent substance”) in the regulation of general industrial chemicals (EC_1907_2006; ECHA_2017_R.7b; ECHA_2017_R.11) if no other information is available. This may be changed if clear indications for ultimate degradation or irreversible immobilisation are available. In other words, NER in the respective matrix are valued either as ‘safe sink’ or as potential ‘hidden hazard’. The present review will argue why the extreme views of NER as degraded vs non-degraded and bioavailable residues have to be reconsidered and why it is necessary to distinguish degradation of chemicals from dissipation, which is only possible by characterizing the underlying mechanisms. A conceptual framework and an analytical toolbox for the characterisation of NER formation as well as potential approaches for the assessment of the NER stability will be provided and a methodology for the general characterisation of NER.

The majority of studies examining NER have been performed in soils; therefore, focus of this review will be on soils. It is reasonable to assume that the principles of binding can be transferred to other matrices like sediments and partly to biological tissues (animals, plants, biomass in sludge etc.). Limitations in the current testing approaches with respect to NER will be discussed and further research needs to address gaps in knowledge are summarised.

II. Scientific state of the art
Chemicals entering the environment undergo various abiotic and biotic turnover processes (Fenner et al., 2013; Gavrilescu, 2005), are taken up by living organisms, leach to the groundwater, and volatilize to the atmosphere, but a part of the chemical will be immobilised as NER (Benoit and Barriuso, 1997). Although these processes have been investigated for decades, formation of NER in soils, sediments and biological tissue (Craven, 2000; Gevao et al., 2003; Mordaunt et al., 2005) are
often considered ‘black box’ (‘still unknown’) in environmental risk assessment of chemicals. Usually, NER were in the past only characterised with respect to the percentage radioactivity associated with the fulvic acid, humic acid and humin compartments (Barraclough et al., 2005; Barriuso et al., 2008; Barriuso et al., 1997; Benoit and Barriuso, 1997; Gevao et al., 2003; Gevao et al., 2000; Loiseau and Barriuso, 2002; Mordaunt et al., 2005). The authors found that contribute to NER formation and analytical characterisation are still only partially understood. These gaps of knowledge have hampered the identification of NER speciation in any complex environmental matrix. Non-covalent binding of organic compounds to environmental matrices occur via multiple mechanisms e.g. electrostatic interactions, hydrogen bonding, van der Waals forces, and hydrophobic interactions (Gevao et al., 2000; Khan and Dupont, 1987; Pignatello, 1989; Senesi, 1992; Wais, 1998); a summary of these binding forces is given in Table 1. With respect to NER, various modes of binding have been postulated and recently also experimentally investigated, as briefly described in the following.

Table 1: Binding interactions of chemicals with environmental matrices that may contribute to the formation of NERs. Binding force data are taken from (Ecetoc_2013_R117).

<table>
<thead>
<tr>
<th>Type I NER: non-covalent binding</th>
<th>Interaction types</th>
<th>Binding force [kJ/mol]</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrostatic</td>
<td></td>
<td>&lt;5 - 350</td>
<td>Electric force between a positively charged pole (ion or dipole) and a corresponding negatively charged pole.</td>
</tr>
<tr>
<td>- Ionic</td>
<td></td>
<td>100 - 350</td>
<td>Ionisable chemicals which exist as cation or anion under environmental conditions bind to corresponding opposite charged matrix components, such as in soil which reveals both a cation and an anion exchanger matrix. Bonding is as strong as covalent binding, but it is reversible.</td>
</tr>
<tr>
<td>- Ion-dipole</td>
<td></td>
<td>50 - 200</td>
<td>Depends on the strength of the dipole of a chemical and the charges of the environmental matrix, e.g. negative charges at clay surfaces or humic matter.</td>
</tr>
<tr>
<td>- Dipole-dipole</td>
<td></td>
<td>5 - 50</td>
<td>Interaction of either a single pair of poles of adjacent molecules or association of one dipole with another one.</td>
</tr>
<tr>
<td>- π-π-stacking</td>
<td></td>
<td>&lt;5 - 50</td>
<td>Interaction of aromatic molecules, one electron rich, the other electron deficient due to the influence of corresponding functional groups.</td>
</tr>
<tr>
<td>- Ligand exchange</td>
<td></td>
<td>50 - 150</td>
<td>For instance in soil, ligands of silicates or metal oxides like hydroxyl groups can be exchanged by ligands (functional groups) of chemicals like catechols, phosphonates, sulfonates, thiols, or carboxylic acids.</td>
</tr>
<tr>
<td>- Charge transfer</td>
<td></td>
<td>5 - 50</td>
<td>Interaction of an electron-rich π-system with an electron-deficient π-system leading to the transfer of an electron and formation of a π-anion and a π-cation. In soil, humic matter can act as electron donor, like in phenols, or electron acceptor, like in quinones, which both can interact with corresponding electron donors or acceptors from environmental pollutants.</td>
</tr>
<tr>
<td>Hydrogen bonding</td>
<td></td>
<td>4 - 120</td>
<td>Attachment of a hydrogen atom to an electronegative atom and subsequent reversible interaction of the formed dipole with another</td>
</tr>
<tr>
<td>Interaction types</td>
<td>Binding force [kJ/mol]</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>electronegative atom. Hydrogen bond donors are for instance hydroxyl or amino groups, typical acceptors are –OH, =O or C=O functional groups.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrophobic</td>
<td>5 - 10</td>
<td>Tendency of polar molecules like water to exclude non-polar molecules, which leads to segregation of water and non-polar substances and causes disruption of the hydrogen bonding network between water molecules. The hydrogen bonds are partially reconstructed by building a water &quot;cage&quot; around the non-polar molecule, leading to significant losses in translational and rotational entropy of these engaged water molecules. The loss of entropy of the encaged water molecules is compensated when several non-polar molecules associate with each other, liberating water molecules from the solvation shells and increasing their overall entropy. In brief, non-polar molecules associate to exclude water and to minimize the surface area in contact with the polar water.</td>
<td></td>
</tr>
<tr>
<td>Van der Waals</td>
<td>0.5 - 5</td>
<td>Sum of attractive or repulsive forces between molecules either between a permanent dipole and a corresponding induced dipole which arises due to polarisation of the electron cloud (Debye force) or between two instantaneously induced dipoles (London dispersion force).</td>
<td></td>
</tr>
<tr>
<td>Type II NER:</td>
<td></td>
<td>Covalent bonding energies are higher than most non-covalent bonds. Some examples for the bond energies [kJ/mol] from (Carruth and Ehrlich, 2002; Petrucci et al., 2007). Thus, covalent bonds are rather stable.</td>
<td></td>
</tr>
<tr>
<td>covalent binding</td>
<td>C-H &gt; 400; C-C, C-N; C-O &gt; 300; C-F &gt; 400; C=C &gt; 600, C=O &gt; 700; C=C &gt; 800</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Some years ago, Barriuso et al. published a comprehensive NER review (which is still general state of knowledge and highly cited) based on registration dossiers of more than 100 pesticides that cover a broad range of physico-chemical properties and structures (Barriuso et al., 2008). The authors found that all pesticides investigated formed NER but in very different quantities from a few percent to more than 90 percent of the applied amounts. The indications from that study apply to all chemicals in general. Although being formed by almost all chemicals that enter soil, sediment and organisms to various extents, NER, are covered only marginally even in recent reviews on the environmental fate of pesticides (Fenner et al., 2013).

II.1. Bioaccessibility versus bioavailability of organic chemicals
The bioavailability of organic chemicals and their residues in soil and sediment is a growing field of scientific investigation for environmental scientists, although this field was only partially recognised by industries and regulators in environmental sector (Ortega-Calvo et al., 2015). The bioavailability of chemicals in environmental systems is the key factor, which controls their overall fate and effect in soil, in particular their biodegradability and toxicity for biota (Semple et al., 2004; Semple et al.,
Bioavailability is affected by many factors: i) the properties of the compound and the respective soil, ii) aging time in the soil, iii) climate, and iv) organisms of concern and their activity (Katayama et al., 2010). In particular, the type and amount of NER formed will greatly influence the bioavailability of a compound in soil.

Therefore, the assessment of the bioavailability of contaminants and sometimes also of the transformation products in soil is necessary for understanding the potential risks posed by the substance and its NER (Semple et al., 2003). Some authors identified the lack of clarity among environmental scientists regarding the term ‘bioavailability’ and thus proposed the distinction of the two terms bioavailability and bioaccessibility, which is needed for differentiating total concentrations and actual activity (Semple et al., 2004): The bioavailable fraction of a chemical is defined as "that which is freely available to cross an organism's cellular membrane from the medium the organism inhabits at a given time". The bioaccessible fraction is defined as "that which is available to cross an organism's cellular membrane from the environment, if the organism has access to the chemical, however, the chemical may be either physically removed from the organism or become only bioavailable after a period of time." “Bioaccessibility encompasses what is actually bioavailable now and what is potentially bioavailable”. Not only bioavailable compounds present in the water-soluble fraction of soil are available, but also those desorbed from soil over time when a target organism is in direct contact with the soil (Harmesen, 2007). Bioaccessibility includes both the readily available contaminants present in the water-soluble fraction of soil and the contaminants which can become available after desorption from the soil matrix. This includes also the contaminants, which may be released on the long run (slowly reversible sorption/sequestration). The accessibility depends on the desorption conditions (e.g. shaking, temperature, matrix) and desorption time (Reichenberg and Mayer, 2006). Sequestration of contaminants (which includes both strongly sorbed and physically entrapped molecules) within the soil matrix tends to increase with increasing contact time (‘soil aging’) (Johnson et al., 2001; Reid et al., 2000a). A chemical immobilised in SOM or present in the soil solution may however still become bioaccessible when soil conditions change or the matrix is degraded (e.g. increase of pH or SOM content by addition of compost, manure application (Barriuso et al., 1997; Benoit et al., 1996; Kästner and Hofrichter, 2001; Yee et al., 1985). This is particularly relevant for NER derived from veterinary pharmaceuticals in manure or sewage sludge after application to agricultural land.

Besides extraction with dilute salt solutions, e.g. with aqueous CaCl₂ solutions (see section II.4.1), alternative methods to estimate the bioavailability of residues are “mild” supercritical fluid extraction (SFE) with CO₂ as eluent at temperatures below ca. 50 °C (Burgess et al., 2011; Hawthorne et al., 2007; Hawthorne et al., 2012; Katayama et al., 2010), although others reported this technique to also release more tightly bound residues. Anyway, good correlations of SFE extracted residues with bioavailability to different organisms have been reported (Akerblom et al., 2010; Esteve-Turrillas et al., 2005; Hallgren et al., 2006; Sun and Li, 2005). Another method to investigate bioavailable residues is passive sampling such as solid phase microextraction (SPME). This technique only allows quantification of freely dissolved concentrations, which makes it unsuitable for mass balance studies. It does also not take into account that soil organisms like earthworms can additionally be exposed to pollutants bound to soil particles that may be digested in the gut of the worms.

Also extractions with organic polymers like TENAX or XAD4 have been shown as indicators of bioavailability, for instance of PAH to plants (Roncevic et al., 2016) and of pyrethroids to benthic invertebrates (Harwood et al., 2013). In addition, the extraction with cyclodextrin derivatives was shown to predict the uptake of chemicals in earthworms (Gomez-Eyles et al., 2010; Gomez-Eyles et al., 2011; Nelieu et al., 2016; Song et al., 2017) and microbial accessibility (Cuypers et al., 2002;
Papadopoulos et al., 2007; Reid et al., 2000a; Reid et al., 1998; Reid et al., 2000b; Reid et al., 2004; Rhodes et al., 2010; Semple et al., 2007; Stroud et al., 2009). Some scepticism is expressed by the authors whether the cyclodextrin method is suitable as bioavailability indicator because organic chemicals with low aqueous solubility and suitable size or shape will dissolve as inclusion complexes above their inherent solubility, although the above mentioned references proved corresponding correlations.

A model developed by authors of this discussion paper allows a mathematical description of these terms (Kaestner et al., 2014; Rein et al., 2016; Trapp et al., 2010). There, the bioavailable fraction is the chemical activity of the substance in solution, because the chemical activity drives diffusion across cell membranes (Ferguson, 1939; Lewis, 1907; Reichenberg and Mayer, 2006; Trapp, 2000). Bioaccessible is the integral of all substance that is available for uptake into organisms in a certain time period. This includes adsorbed and sequestered molecules that move back into solution when the activity gradient changes, i.e. when the compound is removed from solution by uptake or by degradation. The approach demonstrates that bioavailability and bioaccessibility issues are related to NER type I and are system properties depending on the compound, the soil matrix plus environmental conditions present, the time period under consideration, and the respective organisms (Kaestner et al., 2014).

Various chemical or biological measurements are employed to assess the bioavailability of an organic contaminant and thereby its toxicity for living organisms in soil systems. Chemical measurements involve various ‘harsh’ or ‘mild’ extraction methods of the contaminant from soil (see below), whereas biological ones are based on monitoring the toxic effects of a contaminant taken up by the living target organism (Harmsen, 2007) and on analytical determination of the body burden (Katagi and Ose, 2015; Katagi and Tanaka, 2016). It is generally assumed that the amount of compound recovered by exhaustive ‘harsh’ soil extraction is 100% accessible, when assessing its potential risk for the environment (Alexander, 2000). However, ‘harsh’ methods were considered to be improper for the assessment of the actual bioavailability and thus toxicity of soil associated organic contaminants for the environment (Gevao et al., 2003; Kelsey et al., 1997). Contrary to ‘harsh’ extraction, ‘mild’ extraction and passive sampling methods are useful for the measurement of the bioavailability and bioaccessibility of contaminants in soils in terms of biodegradation or toxicity, since it removes only the contaminant fraction loosely adsorbed to the surface of soil particles (Mordaunt et al., 2005). However, these extraction methods need to be properly designed to mimic uptake by organisms and thus to really reflect the bioavailable fraction.

II.2. Definition and nature of NER
According to the mostly cited IUPAC definition (Roberts, 1984), NER in soils are defined as species originating from chemicals, that remain un-extracted by methods which do not significantly change the chemical nature of these residues. Non-extractable residues are considered to exclude fragments recycled through metabolic pathways leading to natural products. Later a refined definition (Führ et al., 1998) was often accepted: "Bound residues represent compounds in soils, plants, or animals which persist in the matrix in the form of the parent substance or its transformation product(s) after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix.” However, both definitions cause potential for misunderstanding and misinterpretation: they focus on not altering the matrix, which cannot be excluded by many applied methods (see below), and the Führ definition is not considering the formation of biogenic NER. Harder chemical or physical environmental processes such as soil acidification may alter the matrix
and may also change the nature of the xenobiotic and its binding mechanism and strength to the matrix.

The amounts of NER formed in environmental matrices depend on their physicochemical properties. For instance, in soil variations in the clay content, the cation exchange capacity, pH, or organic matter will affect the degradation rates of chemicals as well as NER formation (Guimarães et al., 2018; Nguyen et al., 2018; Zhang et al., 2017). Also the microbiological properties will influence the formation of NER: inoculation of soil with an isoproturon degrading Sphingomonas strain shifted the proportion of biogenic and xenobiotic NER. Mineralisation of isoproturon was enhanced after incubation with the degrader strain and less metabolic products and NER were formed, in which the percentage of bioNER was increased at the expense of xeno-NER (Zhu et al., 2018).

A decade ago it was summarised that no clearly defined soil extraction method exists by which the formation of non-extractable residues can be characterised and quantified (Defra, 2007). In fact, the amount of NER quantified in relation to the total amount of NER in various media like sludge, manure, soil, and sediments depends on the extraction regime, on the matrix, and on the isotope labelling position of the test substance (Ecetoc_2013_R117; ECHA_2017_R.7b; ECHA_2017_R.11). Often, as a first step, aqueous solutions are used to release the rapidly desorbing fraction, for instance in soil mimicking the pore water. The second extraction step includes mixtures of water and organic solvents to mimic the potential uptake by soil organisms; however, this correlation needs experimental validation and would be better tested by comparing passive sampling techniques and organism uptake. Extractions in pure organic solvents like alcohol, acetone or acetonitrile are seen as the last non-destructive stage of the extraction regime (Ecetoc_2013_R117) but are considered ‘harsh’ by others. Using elevated temperature, for instance accelerated solvent extraction (ASE), Soxhlet or microwave extraction, some argue that this might be a destructive technique (Ecetoc_2013_R117), although any extraction method will change the structure of soil components. Anyway, such techniques have been successfully used to characterize and differentiate various NER fractions (see below). This approach will enable the characterisation of the total NER. Changes and destruction of the soil matrix are secondary to obtaining an instantaneous snap shot of the xenobiotic and its transformation product. By using internal and external spikes with the parent moiety any unexpected structural changes of the compound can be identified as resulting from the extraction technique. A unified approach is needed, if NER characterisation and evaluation should be implemented into the PBT and environmental hazard assessment strategies for all chemicals. Such an extraction scheme is proposed in the following.

Since NER have to be quantified by radio isotope labelling (\(^{14}\text{C}\)) of a chemical at the most stable part of the molecule (OECD Guidelines for testing chemicals), the detection can only be related to the labelled atom and not to molecular speciation, and thus the structural identity of NER remains unknown. Dual labelled studies, where the same xenobiotic with the radiolabel at different positions is examined under identical experimental conditions, leads to different results regarding mineralisation, degradation half-lives and NER formation depending on the stability of the labelled molecular moiety. Only by specific spectroscopic techniques after labelling the molecule with suitable stable isotopes, e.g. \(^{13}\text{C}\) or \(^{15}\text{N}\) for corresponding NMR analysis (Berns et al., 2005; Berns et al., 2007; Ertunc et al., 2004; Riefer et al., 2013) or high resolution MS (Girardi et al., 2013; Nowak et al., 2013; Nowak et al., 2008; Nowak et al., 2011; Wang et al., 2016; Wang et al., 2017b; Wang et al., 2017c) or \(^{14}\text{C}\)-labelling combined with LC-MS (Junge et al., 2012) structural features of NER have been elucidated, however, often at elevated concentrations of the test substances.
II.3. Description of different types of NERs

Recently the state of the art regarding NER was reviewed and various types of NER were classified (Kaestner et al., 2014). It was concluded that the total amount of NER is the sum of strongly adsorbed, sequestered or entrapped (type 1) and covalently bound residues (type II) both either derived from the parent substance or from transformation or degradation products; a third type (III) refers to biogenic NER that are derived from biotic degradation (see Figure 1 and Table 2). This degradation results in label transformation to various biomolecules, e.g. amino acids, phospholipids, which has been shown by using stable isotope labelling (13C, 15N) (Girardi et al., 2013; Nowak et al., 2013; Nowak et al., 2011; Wang et al., 2016; Wang et al., 2017b; Wang et al., 2017c). These three NER types are formed by competing processes and discriminating analytical methods have been described (Kaestner et al., 2014) and are presented below in the proposal for an extraction scheme. This concept was based on principal considerations and on the fact that sequestration (a term which includes both strongly sorbed and physically entrapped molecules) in the soil or sediment matrix can be identified using current methods, but the different processes leading to sequestration cannot reliably be separated.

A more sophisticated theoretical concept for separation of adsorbed and entrapped compounds (Eschenbach, 2013) is theoretically valid but cannot be validated by methods because of the complexity of the matrices. In principle, covalent bonds can also be identified using sophisticated chemical cleavage methods (see chapter II.3.2) but this type of NER may be the most critical one for evidencing. As a ‘worst-case scenario’, NER based only on quantitation were often considered to contain dominantly xenobiotic compounds or transformation products with toxicity potential (Loiseau and Barriuso, 2002; Mordaunt et al., 2005). However, methods are available to differentiate various NER types with type III especially of no environmental concern. Therefore, assessment based only on the total amount of NER will overestimate persistence of the substance.

Biogenic residues are of no environmental concern since they are indistinguishable from other biogenic residues and from natural organic matter. Covalently bound NER, in particular if multicovalently bound, are also believed to be of low environmental relevance since such bonds are considered to be rather stable under physiological conditions (Eschenbach et al., 1998; Gulkowska et al., 2013; Haider et al., 2000). Upon a change to acidic or alkaline conditions, to which the residues are sensitive since such bonds are covalently bound. This process is extremely slow as described above with degradation rates of years to decades. Hence, the potential for remobilisation of type I and type II NER needs to be assessed.

An operational approach for NER is proposed related to (Roberts, 1984) considering NER ‘not distinguishable’ from natural organic matter. This may include in practice a certain overlap to the type III NER, because also microbial decay products form organic matter. However, based on the current knowledge type III bioNER can clearly be separated from the xenoNER (type I and II) by identifying the isotope label in the biomarkers. Therefore, the general approach results in the equation: total NER minus bioNER = relevant xenoNER (type I and II); these xenoNER need to be tested for remobilisation potential.

Therefore, ‘exhaustive’ extraction procedures should be used to define the amount of NER. Type I, which can be identified after the silylation procedure, is considered as bioaccessible or becoming bioavailable after release (which is a slow process in the environment). Type II NER is in principle bioaccessible, however only after degradation of soil organic matter, to which the residues are covalently bound. This process is extremely slow as described above with degradation rates of years to decades. Hence, the potential for remobilisation of type I and type II NER needs to be assessed.
Unlike NER types I and II, type III bioNER may become bioavailable since biopolymers and biomass components are degradable even in a particulate state, but these residues are of no environmental concern.

II.4. Methods to extract and identify NER, limitations of the test methods and technical challenges

The immobilisation of pesticides as NER was initially considered desirable (Gevao et al., 2003; Hatzinger and Alexander, 1995; Kelsey et al., 1997; Mordaunt et al., 2005), but since some years it became a concern of scientists and regulatory authorities (Barraclough et al., 2005; ECHA_2017_R.11). Remobilisation and erosion of pesticide-NER may be sources of long-term emission to ground- and surface water which should be investigated. Experimentally difficult also with respect to NER formation in OECD 307 and 308 is the determination of degradation half-lives for highly hydrophobic chemicals with log Kow values above 5 and very low aqueous solubility, e.g., below 10 µg/L. In general, sorption and desorption processes compete with the degradation processes of a chemical, which occur predominantly dissolved in the water phase. Such chemicals are prone to formation of type I NER but may also form type II NER if reactive groups at the structure enable this. If desorption is very slow, the biodegradation is slower than the inherent biodegradability would suggest, i.e., such chemicals are revealing desorption mediated persistence. A recent Cefic project (LRI-ECO32; RWTH Aachen and DTU) aims to differentiate the various processes by comparing the biodegradability in water only systems spiked with microorganisms, capable to degrade the compound, with that in water-sediment systems according to (OECD_308, 2002) and in surface waters (OECD_309, 2004).

In the definition of NER given in (Führ et al., 1998) it was stated that “the extraction method must not substantially change the compounds themselves or the structure of the matrix.” While any changes of the parent substance and of major transformation/ degradation products by the chosen extraction method can be tested by corresponding control experiments, its effect regarding structural changes of the matrix is much more difficult to assess. Soil scientists claim that soil structure is even changed by different moisture contents and the quality of percolating water (Ben-Hur et al., 2009; Emdad et al., 2004). Therefore, any analytical method to extract soil, even by “mild” solutions, will lead to some structural changes. This holds especially for using organic solvent mixtures, both at room temperature and elevated temperatures, when using Soxhlet extraction or Accelerated Solvent Extraction (ASE) or Pressurised Liquid Extraction (PLE). Aqueous solutions for extraction of organic chemicals are only appropriate to test their direct availability for soil organisms, but molecules that are more strongly bound need to be extracted by organic solvents.

The following subsequent extraction procedures are proposed to prepare an environmental matrix (soil, sediment etc.) that only contains NER after extraction of the extractable fractions and subsequent analyses to investigate the nature of NER are described¹ (see Figure 1 and Table 2).

Turnover tests in soil or water plus sediment (OECD_307, 2002; OECD_308, 2002) should also be conducted with sterilised (abiotic) controls in order to gain information about the sorption and

¹ For example, in the uniform principles (Reg EU N 546/2011) it is stated that a pesticide cannot be authorized if in lab studies after 100 d NER is >70 % and mineralization <5 %, unless it is demonstrated that there are no unacceptable effects in succeeding crops and environment. The methodologies in this document may be used to investigate the nature of the NER of these compounds.
abiotic transformation potentials, which is particularly relevant for P/vP assessment and type I NER formation. If abiotically formed NER are much lower in the control than biotically formed NER in the simulation test, this gives a clear indication on NER from degradation products or even from bioNER. If bioNER are actually formed then they can be counted as metabolised substance, similar to CO₂ and can thus be added to the degraded amount. If DT50 values are much lower than real DegT50 values with low amounts of resulting CO₂, there is a strong indication for dissipation with formation of type I and II NER. In this case the DegT50 value should be applied for the P/vP assessment (ECHA_2017_R.11).

Table 2 presents the methodologies that can be used for the identification of the different types of NER.
**Table 2: Types of NER, related properties, and methods for identification**

<table>
<thead>
<tr>
<th>NER types</th>
<th>I(^1)</th>
<th>II(^2)</th>
<th>III(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Properties</td>
<td>sorbed, trapped</td>
<td>covalently bound</td>
<td>label incorporation into biomass</td>
</tr>
<tr>
<td>Formation from</td>
<td>parent substance, + transformation products</td>
<td>parent substance, + transformation products</td>
<td>ultimate degradation and mineralisation</td>
</tr>
<tr>
<td>Evidencing</td>
<td>identification of parent substance + transformation products</td>
<td>identification of cleavage products, (highly difficult)</td>
<td>label in biomarkers</td>
</tr>
<tr>
<td>Formation processes</td>
<td>van der Waals, hydrophobic interactions, etc. (see Table 1)</td>
<td>C-C, C-N, C-O-C, ester bonds (covalent bonds in general)</td>
<td>microbial degradation</td>
</tr>
<tr>
<td>Stability</td>
<td>low to high</td>
<td>high</td>
<td>not relevant</td>
</tr>
<tr>
<td>Release probability of parent substances or transformation/degradation products</td>
<td>low to high</td>
<td>low</td>
<td>not relevant</td>
</tr>
<tr>
<td>Extraction methods for</td>
<td>‘Mild’ to ‘harsh’ for parent substance, transformation products</td>
<td>Cleavage methods for bonds of parent substances and transformation products</td>
<td>Biomarker: PLFA, amino acids, amino sugars</td>
</tr>
<tr>
<td>Analytical method</td>
<td>Typical methods used with radioisotope labelled derivatives include radio-HPLC-UV, radio-TLC, LSC, TopCount, radio-HPLC-MS, oxidative combustion followed by LSC; for additional, structural information, size exclusion chromatography or spectroscopic methods like NMR can be applied. Alternatively to radioisotopes, also stable isotope labelling can be applied with subsequent GC-MS or LC-MS analyses.</td>
<td>GC-MS, HPLC-MS, 2D-thin layer chromatography</td>
<td></td>
</tr>
<tr>
<td>Cleavage methods</td>
<td>Silylation</td>
<td>hydrolysis by KOH (ester-bonds), BBr(_3) (ether bonds), RuO(_4) (C-C bonds), TMAH (Tetramethylammonium hydroxide)</td>
<td>not relevant</td>
</tr>
<tr>
<td>Assessment of remobilisation potentials</td>
<td>for both types of NER: physical treatments: simulation of heavy rain events, hot water extraction, freeze/thaw cycling, grinding, wet/dry cycling chemical treatments: pH changes, long term Tenax extraction, changes in ionic strength, hydrolysis in the presence of Na(^{18})OH or H(_2)(^{18})O biological treatments: application of oxidative and other enzymes with release potential like peroxidases, laccases, and glutathione-S-transferases, treatment with soil feeding organisms</td>
<td>not relevant</td>
<td></td>
</tr>
</tbody>
</table>
The following stepwise approach is proposed for the assessment of extractable residues and the characterisation of different types of NERs in environmental matrices:

**STEP 1**

**Test substance in environmental matrix**

- **Extractions**
  - 1.1 Aqueous solutions → readily desorbable
  - 1.2 Organic solvent - water mixtures → desorbable
  - 1.3 Soxhlet, ASE, PLE, SFE, MAE → slowly desorbable

**Extractable fractions**

**Non-extractable residues**

**STEP 2**

**2.1 Silylation** *  **2.2 Amino acid extraction**

- Released
- Remaining

**Type I NER**  **Type II NER**  **Type III NER**

**MTB approach**

- Large amount of bioNER predicted
- Absence or very low amount of bioNER predicted

**No need for bioNER characterisation**

*Figure 1: Proposed scheme of extraction steps for deriving extractable fractions and investigating NER.*

* The alkaline extraction can be used as an alternative to direct silylation of the NER containing matrix, e.g. soil, each humic matter fraction (fulvic and humic acids and humin) derived by alkaline extraction has to be subsequently silylated to enable the differentiation of type I and type II NER by analysis of parent substances, transformation products and/or biocompounds.

** Amino acid extraction can be additionally performed with the whole sample prior to any extraction. The difference to the amount of bioNER can be considered as the amount of labelled biomolecules that may be extracted in the step 1 procedures.
STEP 1 shows the sequence of three extraction steps: aqueous salt/buffer extraction (bioavailable fraction), solvent/water mixtures (potentially bioaccessible, readily desorbable fraction), and finally exhaustive extraction using ASE/PLE, SFE, MAE to release the extractable fractions (total extractable, remobilisable fraction). This sequence will leave only NER remaining in the matrix, which are defined here as total NER and can be subsequently used to assess and differentiate type I, II, and type III NER. Extractions steps 1.1, 1.2 and 1.3 are recommended extraction steps to consider in order to release the extractable fractions. However, the extraction strategy should be substance tailored and it may be possible that for certain substances not all of these extraction steps are necessary.

In STEP 2 samples must be split into two aliquots or sub-sampled because silylation (step 2.1) and amino acid extraction (step 2.2) are considered to be “destructive” methods and cannot be applied to the same sample sequentially.

Type I and II NER can be differentiated by using silylation (and subsequent analysis for parent substances and transformation products and comparison to the amounts of bioNER formed) of the organic matrix leading to dis-aggregation of humic matter and release of sequestered type I NER, whereas residues covalently bound to the organic matrix or organo-mineral complexes will not be released.

Type III bioNER can be analysed by full hydrolysis of the matrix with subsequent amino acid analysis. The total NER containing matrix may be further analysed with respect to the binding mechanisms using cleavage or matrix disaggregating methods with subsequent suitable analytical techniques like mass spectrometry or NMR spectroscopy. In addition, the remobilisation potential of the total NER (after exhaustive extraction) can be assessed using environmental simulation methods like, acid rain, heavy rain events, drying and rewetting, freeze and thawing, and radical forming enzymes of wood and litter decaying fungi. In any case, however, the speciation always needs to be evaluated for an adequate characterisation of the NER, since these methods do not allow distinguishing the NER types.

A new modelling approach for estimation of the carbon conversion to microbial biomass (MTB approach) has been developed and can now be used to estimate the microbial yield and bioNER formation potential of microbes growing on a specific chemical. This information can help to decide whether additional test for the evaluation of bioNER or sequestered NER type I (and indirectly NER type II) are needed.

II.4.1. Step 1: Determination of extractable fractions (see Figure 1)

1.1 Use of aqueous solutions to determine the amount of residues being easily desorbable.

Aqueous solutions will extract residues that are directly bioavailable for organisms living in the matrix, e.g. soil or sediment; other methods to address the easily desorbable fractions are listed below. A diluted CaCl₂ solution, e.g. 0.01 M, is a suitable solvent for that purpose as the molarity and ionic strength resemble those of the soil pore water (Houba et al., 2000; Peijnenburg et al., 2007). An initial 0.01 M CaCl₂ extraction should be performed at every sampling interval, which is intended to be a surrogate of the pore water in order to determine the ‘easily extractable’ fraction. CaCl₂ extraction has been applied to correlate simazine residue bioavailability. Simazine sorption to soil increased with aging and amounts of simazine extracted by 0.01 M CaCl₂ were clearly correlated to amounts of simazine mineralised by a simazine-mineralizing bacterium (Regitano et al., 2006). Similarly, CaCl₂ extraction mimicked the bioavailable fractions of indaziflam, carbendazim and sulfadiazine in soil (Alonso et al., 2015; Paszko, 2014; Schmidt et al., 2008). Solutions of salts like calcium or sodium nitrate, ammonium acetate or nitrate etc. have also been used for this purpose.
1.2 *Use of organic solvent mixtures to extract thoroughly the matrix.* Available residues should be sequentially extracted at ambient temperature with carefully selected aqueous:organic solvent mixtures (e.g. 50:50 or 20:80 water:acetonitrile; v/v), which at times may be modified with minute amounts (0.1-2.5% v/v) of formic acid, acetic acid and/or ammonia in order to enhance the solubility of the xenobiotic and/or its transformation products. Elevated temperatures are avoided where ever possible for the initial extracts of the samples. Samples will be extracted for prolonged time periods (4 h – 24 h) using physical agitation, e.g., an overhead shaker or a horizontal shaker. Ultra-sonication may enhance the extraction efficiency, but the temperature of the sample should then be monitored. In studies with radiolabeled compounds, sequential extractions should be performed until < 5% of the radioactivity released from the first extraction is obtained. This usually occurs between three and five extractions with one solvent system (Nießner and Schäffer, 2017).

The selection of the proper organic solvents is a critical step. The physico-chemical properties of the analyte, i.e., its volatility, water solubility, the solubility in the organic solvent to be used, the pKa, and the stability, as well as the test matrix properties (such as the moisture and organic matter content of soils and sediments), must be considered (Ecetoc_2013_R117). Properties of some extraction solvents and their relations to properties of analytes are given in Tables 3 and 4.
Table 3: Properties of typical organic solvents and water.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Boiling point (°C)</th>
<th>Snyder polarity index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentane</td>
<td>36</td>
<td>0.0</td>
</tr>
<tr>
<td>Heptane</td>
<td>98</td>
<td>0.1</td>
</tr>
<tr>
<td>Hexane</td>
<td>69</td>
<td>0.1</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>81</td>
<td>0.2</td>
</tr>
<tr>
<td>Toluene</td>
<td>111</td>
<td>2.4</td>
</tr>
<tr>
<td>o-Dichlorobenzene</td>
<td>180</td>
<td>2.7</td>
</tr>
<tr>
<td>Ethyl Ether</td>
<td>35</td>
<td>2.8</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>40</td>
<td>3.1</td>
</tr>
<tr>
<td>n-Butyl Alcohol</td>
<td>118</td>
<td>3.9</td>
</tr>
<tr>
<td>Isopropyl Alcohol</td>
<td>82</td>
<td>3.9</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>66</td>
<td>4</td>
</tr>
<tr>
<td>Chloroform</td>
<td>61</td>
<td>4.1</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>77</td>
<td>4.4</td>
</tr>
<tr>
<td>1,4-Dioxane</td>
<td>101</td>
<td>4.8</td>
</tr>
<tr>
<td>Acetone</td>
<td>56</td>
<td>5.1</td>
</tr>
<tr>
<td>Methanol</td>
<td>65</td>
<td>5.1</td>
</tr>
<tr>
<td>Ethyl Alcohol</td>
<td>78</td>
<td>5.2</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>82</td>
<td>5.8</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>153</td>
<td>6.4</td>
</tr>
<tr>
<td>Water</td>
<td>100</td>
<td>10.2</td>
</tr>
</tbody>
</table>

* Snyder polarity index: Solvent polarity is a function of the dipole moment, proton acceptor or donor properties of chemicals and further properties. The Snyder’s polarity index ranks solvents according to a summation of these properties. The higher the polarity index, the more polar the solvent. Snyder's paper was published in Journal of Chromatography A 92, 223-230 (1974).

Pure organic solvents should be avoided in the first extraction steps because molecules distributed in the interlayers of clay particles in soil may be entrapped by shrinking of the clay when water is removed. Therefore, in the first extraction steps water-miscible organic solvents should be used mixed with small volumes of water, followed by exhaustive extraction pure solvents (or solvent mixtures)2. Extracts should be combined and concentrated prior to radio-profiling for instance via radio-HPLC or radio-TLC.

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2 An important remark: Since transformation products of chemicals usually differ from the parent compound in terms of polarity (most often more polar, sometimes less polar) and chemical reactivity (as well as ecotoxicity), extraction procedures have to be developed during the course of a degradation study. An effective extraction solvent for the parent compound is usually not effective for transformation/degradation products. It is
Table 4: Relative polarity of chemical classes and examples of typical extraction solvents; also mixtures of solvents can be used. The selection is not exclusive and several solvents listed cover a range of chemical classes to be extracted.

<table>
<thead>
<tr>
<th>Relative polarity</th>
<th>Chemical class</th>
<th>Compounds</th>
<th>Typical solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONPOLAR</td>
<td>R-H</td>
<td>Alkanes</td>
<td>Hexane</td>
</tr>
<tr>
<td></td>
<td>Ar-H</td>
<td>Aromatics</td>
<td>Toluene, benzene</td>
</tr>
<tr>
<td></td>
<td>R-O-R</td>
<td>Ethers</td>
<td>Diethyl ether</td>
</tr>
<tr>
<td></td>
<td>R-Hal</td>
<td>Alkyl halides</td>
<td>Tetrachloromethane</td>
</tr>
<tr>
<td></td>
<td>R-COOR</td>
<td>Esters</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td></td>
<td>R-CO-R</td>
<td>Aldehydes, Ketones</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td></td>
<td>R-NH₂</td>
<td>Amines</td>
<td>Acetonitrile, triethylamine*</td>
</tr>
<tr>
<td></td>
<td>R-OH</td>
<td>Alcohols</td>
<td>Methanol, ethanol, acetonitrile</td>
</tr>
<tr>
<td></td>
<td>R-CONHR</td>
<td>Amides</td>
<td>Acetonitrile, dimethylformamide*</td>
</tr>
<tr>
<td></td>
<td>R-COOH</td>
<td>Carboxylic acids</td>
<td>Acetonitrile-water mixtures, diluted acetic acid</td>
</tr>
</tbody>
</table>

* Triethylamine is an ion-pairing reagent and strong base and strips functional groups from the inside of reverse-phase HPLC columns and the surface of silica TLC plates. Dimethylformamide is a viscous solvent with a high boiling point that may also damage the solid phase of chromatographic columns in subsequent analyses. Therefore, both solvents should be used only as modifying additives.

1.3 Exhaustive extraction using Soxhlet, Accelerated Solvent Extraction (ASE) or Pressurised Liquid Extraction (PLE), Supercritical Fluid Extraction (SFE), or Microwave Assisted Extraction (MAE) of the particulate matrix remaining after the described extractions should be applied, in order to release part of the molecules strongly adsorbed to the matrix (EPA, 2014). It has been argued that under such conditions, e.g. high temperature and/or elevated pressure, structural changes of the matrix will occur, but as stated before, any extraction of such matrix will lead to structural changes. It also has to be tested in control experiments whether or not the parent molecule and relevant transformation/degradation products are stable under such extraction conditions. Soxhlet conditions are “not discouraged” in the US-EPA Guidance to determine extractable chemicals from environmental matrices and to achieve a matrix which contains un-extracted pesticide residues (US-EPA, 2014). If feasible, extracts can be analysed by the same methods used for the previous extractions. Recently, a survey with 43 substances and 3 soils showed, that PLE extraction (with methanol/acetone/water (50/25/25%, three cycles with 15 min. each at 100°C) is very efficient (Hogeback and Loffler, 2018, personal communication).

therefore not possible to define optimal extraction conditions at the beginning of the study and to keep this procedure for aged samples; exemptions are of course possible. Both polar and nonpolar solvents or miscible solvent mixtures should be tested for extraction depending on the nature of the residues.
Besides Soxhlet, exhaustive extractions comprise microwave assisted extraction (MAE), ultrasonic, ASE or PLE, and supercritical fluid extraction (SFE) at elevated temperatures and using suitable modifier solvents. For regulatory laboratory testing purposes, Soxhlet is usually the preferred extraction method. ASE and PLE methods are automated and require method development.

The above mentioned methods to release extractable fractions from environmental matrices are described in detail for instance in (Nießner and Schäffer, 2017). The approach of the described extraction steps 1-3 follows in principle that of (Ortega-Calvo et al., 2015) who suggests to use the extraction sequence aqueous solutions, passive sampling extractions (e.g. TENAX), organic solvents at room temperature and at elevated temperature (e.g. ASE), in order to obtain the matrix containing only NER.
Table 5: Some examples of conditions to remove extractable fractions from environmental matrices to obtain non-extractable residues.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Matrix</th>
<th>Solvents and methods to remove extractable fractions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyprodinil</td>
<td>Soil</td>
<td>Methanol</td>
<td>1)</td>
</tr>
<tr>
<td>Simazine</td>
<td>Compost</td>
<td>Methanol:water 9:1 (v:v)</td>
<td>3)</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>Soil</td>
<td>Ethanol:water 9:1 (v:v), Soxhlet</td>
<td>4)</td>
</tr>
<tr>
<td>MCPA</td>
<td>Soil, clay</td>
<td>Methanol; dichloromethane, Soxhlet</td>
<td>5)</td>
</tr>
<tr>
<td>Nonylphenol</td>
<td>Soil, clay</td>
<td>Methanol; dichloromethane, Soxhlet</td>
<td>6)</td>
</tr>
<tr>
<td>Nonylphenol</td>
<td>Soil</td>
<td>Methanol and ethylacetate</td>
<td>7)</td>
</tr>
<tr>
<td>Metalaxyl</td>
<td>Soil, sand, silt, clay</td>
<td>Methanol, Soxhlet</td>
<td>9)</td>
</tr>
<tr>
<td>Clodinafop-propargyl</td>
<td>Sediment</td>
<td>Acetonitrile, Soxhlet</td>
<td>10)</td>
</tr>
<tr>
<td>3,4-Dichloroaniline</td>
<td>Sediment</td>
<td>Methanol</td>
<td>11)</td>
</tr>
<tr>
<td>Isoproturon</td>
<td>Soil</td>
<td>Methanol, ASE</td>
<td>13)</td>
</tr>
<tr>
<td>Tetrabromobisphenol A</td>
<td>Soil</td>
<td>Methanol</td>
<td>14)</td>
</tr>
<tr>
<td>Tetrabromobisphenol A</td>
<td>Soil</td>
<td>Methanol and ethylacetate</td>
<td>15), 16)</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>Soil</td>
<td>Dichloromethane and acetone</td>
<td>17)</td>
</tr>
<tr>
<td>Cypermethrin</td>
<td>Soil</td>
<td>Acetonitrile+water 7+3 (v+v)</td>
<td>18)</td>
</tr>
<tr>
<td>Cypermethrin</td>
<td>Soil</td>
<td>Cyclohexan:Aceton, 1:1 (v:v)</td>
<td>19)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Soil</td>
<td>Ethylacetate, methanol, ammoniumhydroxide, ASE</td>
<td>20)</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>Soil</td>
<td>Methanol, water, HCl, Soxhlet</td>
<td>21)</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>Soil</td>
<td>Acetonitrile, water, MAE</td>
<td>22)</td>
</tr>
<tr>
<td>Carbendazim</td>
<td>Soil</td>
<td>Methanol, Soxhlet</td>
<td>23)</td>
</tr>
<tr>
<td>Isoproturon</td>
<td>Soil</td>
<td>Acetone, Soxhlet</td>
<td>24)</td>
</tr>
<tr>
<td>Metalaxyl</td>
<td>Soil</td>
<td>Acetonitrile, water, ASE</td>
<td>25)</td>
</tr>
</tbody>
</table>

References for Table 5:
1) (Dec et al., 1997a; Dec et al., 1997b; Dec et al., 1997c); 2) (Ertunc et al., 2004); 3) (Berns et al., 2005); 4) (Junge et al., 2011a; Schmidt et al., 2008); 5) (Riefer et al., 2011b); 6) (Riefer et al., 2013); 7) (Shan et al., 2011); 8) (Junge et al., 2012); 9) (Botterweck et al., 2014); 10) (Yuan et al., 2015); 11) (Yuan et al., 2017); 12) (Chen et al., 2017); 13) (Li et al., 2016); 14) (GuJianqiang et al., 2017); 15) (Li et al., 2015); 16) (Liu et al., 2013); 17) (Wang et al., 2017d); 18) (Roberts and Standen, 1981); 19) (Hartnik and Styrishtave, 2008); 20) (Girardi et al., 2011); 21) (Oi, 1999); 22) (Dalkmann et al., 2012); 23) (Lewandowska and Walorczyk, 2010); 24) (Lehr et al., 1996); 25) (Cabrera et al., 2012)
It is obvious from some examples in Table 5, that for the same substance various extraction regimes have been published in order to differentiate extractable fractions and NER. To demonstrate an extreme case (Table 6): the following solvents have been used to differentiate the extractable and non-extractable residues of atrazine in soil. The table shows that extraction conditions cover a broad range and, thus, interpretation of the amount and the environmental relevance of NER is extremely difficult.

Table 6: Extraction conditions to remove extractable fractions from atrazine treated soils to obtain non-extractable fractions. Only examples are presented where ^14C labelled atrazine was used and the amounts of NER were quantified, but many other references on atrazine with even more extraction conditions are available in the literature.

<table>
<thead>
<tr>
<th>Method</th>
<th>Solvents</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temperature, texture size fractionation</td>
<td>Methanol</td>
<td>1), 9)</td>
</tr>
<tr>
<td>Room temp.</td>
<td>CaCl₂, methanol</td>
<td>2)</td>
</tr>
<tr>
<td>Soxhlet</td>
<td>Methanol</td>
<td>3), 10)</td>
</tr>
<tr>
<td>Room temp.</td>
<td>CaCl₂, acetonitrile, methanol, dichloromethane</td>
<td>4)</td>
</tr>
<tr>
<td>SFE</td>
<td>CO₂, methanol as modifier</td>
<td>5), 14)</td>
</tr>
<tr>
<td>Soxhlet</td>
<td>Methanol, dichloromethane</td>
<td>6)</td>
</tr>
<tr>
<td>Room temp.</td>
<td>Methanol</td>
<td>7), 8)</td>
</tr>
<tr>
<td>ASE</td>
<td>Methanol, water</td>
<td>11), 12)</td>
</tr>
<tr>
<td>Room temp.</td>
<td>Methanol, water</td>
<td>13)</td>
</tr>
<tr>
<td>Room temp.</td>
<td>Methanol, dibasic potassium phosphate</td>
<td>15)</td>
</tr>
<tr>
<td>Room temp.</td>
<td>Acetone, water</td>
<td>16)</td>
</tr>
</tbody>
</table>

References for Table 6: 1) (Barriuso et al., 1991); 2) (Houot et al., 1998); 3) (Xie et al., 1997); 4) (Mordaunt et al., 2005); 5) (Kreuzig et al., 2000); 6) (Gevao et al., 2001); 7) (Benoit and Preston, 2000); 8) (Houot et al., 2000); 9) (Loiseau and Barriuso, 2002); 10) (Capriel et al., 1985); 11) (Jablonowski et al., 2009); 12) (Jablonowski et al., 2010); 13) (Hayar et al., 1997); 14) (Khan, 1995); 15) (Zablotowicz et al., 2006); 16) (Dankwardt et al., 1996)

Strongly acidic and alkaline solvents for release of the extractable fraction (with simultaneous partial humic compound extraction) need to be avoided since severe structural changes regarding the inorganic (acids) and organic (alkali) components of the matrix will occur. Strongly acidic/alkaline extracts may be applied as long as they are performed with the knowledge that the matrices and or the xenobiotic may have undergone changes; further sequential extracts of that sample is not advised for structural elucidation of said xenobiotic.

Sub-sampling homogenous samples to perform this type of extract should be treated as routine. If alkaline extractions are performed for releasing humic substances and associated residues, subsequent analyses are needed to further differentiate the xenobiotic residues sequestered or
bound to the humic substances (see explanations below in extraction steps I and II and our comments on this method below). Some authors suggest to extract the slowly desorbable fraction of pollutants in soils by hot or even superheated water (Dadkhah and Akgerman, 2006; Hawthorne et al., 1994; Kronholm et al., 2003; Kuosmanen et al., 2002; Latawiec and Reid, 2010), although others describe that by this method considerable amounts of soil organic matter are extracted as well, which can be seen as a partly destructive technique (Guigue et al., 2014). Nevertheless, these methods also extract biomolecules like proteins and fatty acids and thus the analysis of the speciation is needed for a justified estimation of the remobilisation potential. As mentioned already, structural changes to the matrix will definitely occur under such - and even milder - conditions. Using stronger conditions like Soxhlet or ASE/PLE, it has been shown that the remaining NER in soil may still contain sequestered residues (type I) which by definition have the potential to slowly desorb from the matrix.

Each of the fractions obtained by the above extraction steps 1-3 can be used to analyze the amounts and to identify the structures of the extractable fractions. The residues remaining after the three steps in the matrix are defined as non-extractable residues (NER) but may still include also bioNER (type III). These NER, thus, should be analysed for the different types of NER. Therefore, the characterisation of the bioNER should be included in the next step.

II.4.2. Step 2: Differentiation of NER types (see Figure 1)

2.1 Differentiation and quantification of type I and type II NER by silylation of the matrix: Silylation will replace the exchangeable hydrogen atoms of functional groups in the organic matrix - e.g. carboxylic, hydroxy or amino groups - with trimethylsilyl groups (Haider et al., 2000). The silylation breaks hydrogen bonds between polar functional groups and changes the hydrophilicity of organic matter, resulting in a partial disintegration of the humic substances into smaller fragments, which have been held together in supramolecular aggregates by noncovalent interactions in the original sample. (However, labile compounds and transformation and degradation products may also be destroyed by this method and this needs to be checked for the respective compounds.) If NER are entrapped in the matrix (type I NER candidates), they are released after silylation, while NER formed by covalent binding (type II NER candidates) remain bound to the matrix. Both fractions can only be quantified when radioactively labelled chemicals have been used. However, for the final determination of the type I or II NER extent, the amounts of included bioNER need to be evaluated. In the type I NER containing fraction, this can either be done by quantification of the parent substances and transformation products or the amount of bioNER. The type II NER containing fraction needs to be calculated by the total NER after exhaustive extraction minus total bioNER and identified type I NER. These data can be used as endpoint for quantification of type I and type II NER.

It follows a short description of the silylation procedure, published, e.g., by (Berns et al., 2005) or in a slightly modified form (Wang et al., 2017d): samples of the exhaustively extracted matrix (or, alternatively, of humic matter fractions obtained after alkaline extraction; see below) are dried completely and aliquots are weighed into a Schlenk flask (a reaction vessel typically used in oxygen-sensitive chemistry with a side arm for evacuating and filling with an inert gas). Silylation reaction requires complete exclusion of traces of water and oxygen. Thus, the reaction flasks and the sample are kept under protective gas (argon). All solvents used (such as chloroform, acetone, tetrahydrofuran) are dried before by use of a molecular sieve. The amounts given in the following are exemplary: 30 ml of chloroform, 1.5 g of NaOH plates and 5
ml of trimethylchlorosilane (TMCS) are added to 500 mg dried matrix material and the mixture is stirred for 3 hours. Then, 1.5 g of NaOH plates and 5 ml TMCS are added and the mixture is stirred overnight. The supernatant, i.e. the chloroform fraction, is removed and the matrix is extracted with 10 ml acetone three times by stirring. These supernatants are added to the chloroform fraction. Finally, the matrix is stirred with 30 ml chloroform. All fractions are combined and centrifuged. Volume and amount of radioactivity of the supernatant is determined comprising type I NER residues. The remaining particulate sample is combusted for determining the amount of radioactivity (comprising type II NER).

Silylation is a gentle derivatisation method and has been used for decades in synthetic and analytical chemistry. In the environmental context this pragmatic approach can be applied for quantification of the two NER types I and II, however, it does not provide information about the chemical identity of the NER as long as the released residues are not characterised. Indeed, each fraction derived from these procedures may also contain bioNER. Due to the nature of the silylation method, the analyses should preferably be performed by mass spectroscopic approaches. It cannot be excluded that by silylation some residual type I NER will remain in the matrix, although this seems rather unlikely, but should be investigated by further research, such as repeating the silylation step. If some residual type I NER would remain after this derivatisation method, this would lead to an underestimation of type I NER, which are basically the releasable part, and an overestimation of type II NER.

**Examples for differentiation of type I and type II NER or characterisation of NER are given below:**

The fungicide cyprodinil or its transformation products showing only minor molecular changes were entrapped in the insoluble humin fraction of a soil. Labelling the cyprodinil with $^{13}$C in addition to the radiolabel $^{14}$C allowed to analyse the structure of the residues by NMR spectroscopy after alkaline extraction. In the fulvic acid fraction entrapment of the unchanged parent molecule was unambiguously verified while in the humic acids the independently bound phenyl and pyrimidyl moieties of the cleaved parent were identified (Dec and Bollag, 1997; Dec et al., 1997a).

Similar experiments were performed by (Berns et al., 2005) to investigate the fate of the herbicide simazine in an artificial compost, which was produced from maize and wheat plants grown on sand with $^{15}$N-depleted NH$_4$NO$_3$ as sole nitrogen source. The plants were freeze-dried, ground and mixed with sand for composting. These studies showed formation about 60% NER of the applied amounts of the test substance in soil. Only a small part of the NER was released from soil organic matter by silylation. According to size exclusion chromatography humic acids contained entrapped residues but the humin fraction contained covalently bound residues. Solid-state $^{13}$N-cross-polarisation magic angle spinning NMR of the NER-containing soil indicated that the residues contain simazine transformation products resulting from N-dealkylation and triazine ring destruction. According to quantum chemical calculations to support the interpretation of the NMR data, the NER retaining the hydroxylated triazine ring exist preferentially in the lactam form considering the lactam-lactim tautomeric forms (Berns et al., 2007).

The binding and incorporation of xenobiotics in soil organo-clay complexes has been studied by (Riefer et al., 2011a). They observed that the majority of NER from the phenoxy herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA) is bound covalently to fulvic acids. In other studies, different chemical degradation treatments (potassium hydroxide (KOH), boron tribromide (BBr$_3$), ruthenium tetroxide (RuO$_4$) and thermochemolysis using tetramethylammoniumhydroxide (TMAH)) were used (Riefer et al., 2013; Riefer et al., 2017) to cleave NER linkages between nonylphenol (4-(3,5-dimethylhept-3-yl)phenol) as representative isomer) and soil humic acids isolated from organo-clay complexes (Riefer et al., 2011b; Riefer et al., 2011c). KOH treatment will cleave covalent ester bonds.
formed of xenobiotic residues with organic matter, whereas treatment with boron tribromide, BB\(_3\), a strong Lewis acid, is known to cleave both ester and ether bonds of humic matter. Oxidation by RuO\(_4\) will split the carbon backbone, e.g. at alkyarylomatic and polycyclic aromatic moieties, generating aliphatic carboxylic acids and substituted benzene carboxylic acids.

An even more stringent degradation method is TMAH-thermochemolysis which is used to split humic matter and to characterise different kinds of macromolecular biomolecules and their diagenesis products, e.g., lignin and soil humic acids (Kronimus and Schwarzbauer, 2007; Kronimus et al., 2006). After short incubation times, NER formation from nonylphenol in soil was reported to be mainly due to ester bonding via the aromatic hydroxyl group, but the amount of ester hydrolysed by the chemical treatment decreased over time. The incorporation of nonylphenol into soil organo-clay complexes follows a stereoselective process enriching one of the diastereomers of the studied nonylphenol isomer in the NER in comparison to the diastereomeric ratio of the residues that could be extracted from the soil using non-destructive organic solvents. Subsequent solid state NMR analyses of nonylphenol NER in soil after various incubation times indicated that the aromatic moiety of the parent substance accumulated in the humic acids and reached a plateau of the corresponding integrated NMR signal areas after about six months. However, the exact structure of the xenobiotic residues could not be derived by the NMR analysis (Riefer, 2011; Riefer et al., 2013; Riefer et al., 2011a; Riefer et al., 2011b; Riefer et al., 2011c).

Solid phase NMR and size exclusion chromatography were used to study the NER of the \(^{14}\text{C}\)-labelled antibiotic sulfadiazine (SDZ) in soil which was amended with SDZ-containing pig manure (Junge et al., 2011b). The authors proved that in fulvic acids obtained by alkaline extraction, SDZ non-extractable residues were composed of the parent substance and the hydroxylated transformation product 4-OH-SDZ in about equal proportions. Similar results of parent-containing NER formation were obtained by the same authors in a soil incubation study of the antibiotic difloxacin (Junge et al., 2012). Size exclusion chromatography of soil fulvic acids revealed that the parent substance difloxacin or its transformation products were rapidly integrated into humic matter.

Sequential alkaline extraction of humic matter containing NER of the flame retardant tetrabromobisphenol A (TBBPA) revealed covalent binding via ester bonds as shown by hydrolysis with 1 M NaOH, and via ether bonds following the procedures described by (Martens, 2002) (hydrolysis with 4 M NaOH) (Li et al., 2015; Tong et al., 2016). Residues bound via ester linkages consisted of TBBPA, TBBPA monomethyl ether, and an unknown polar compound. Most of the ester-linked bound residues involved the parent substance TBBPA and the polar compound. Obviously, also type I NER of this substance are formed in soil, because change in redox conditions (anoxic−oxic incubations) released part of the non-extractable residues formed under anoxic conditions. The released residues contained the parent substance and less brominated transformation products (Liu et al., 2013).

After incubation of \(^{14}\text{C}\)-phenanthrene in active soil for 28 days (Wang et al., 2017d), 40% of the initial amount was mineralised and 70.1 ± 1.9% was converted to NER with most of it bound to soil humin\(^3\). Silylation of the humin-bound residues released 45% of these residues, which indicated that they were physically entrapped, whereas the remainder of the residues were chemically bound or biogenic (bioNER were not investigated). By contrast, in sterilised soil, less NER was formed and all of

\(^3\) The recovery was reported to be above 100%, which might be due to experimental errors such as wrong application or aliquot sampling for liquid scintillation counting; in a parallel study with earthworms present in the soil, the overall recovery was 95%.
it was completely released upon silylation, which underlines the essential role of microbial activity in NER formation. It has to be noted, that sterilization was performed by autoclaving, which leads to structural changes of the matrix, and in addition, no attempt was made to verify sterile conditions during the study. Another suitable method for sterilization would have been irradiation with $\gamma$-rays ($^{60}$Co) because it is considered to provide minimal destruction of the soil matrix; however, also effects on biochemical properties of soils have been reported (Blankinship et al., 2014; Gebremikael et al., 2015). Ester-bound phenanthrene transformation products such as 1-hydroxy-2-naphthoic acid, ortho-phthalic acid and 3,4-dihydroxybenzoic acid were analysed by $^{13}$C-CPMAS-NMR and alkaline hydrolysis (Käcker et al., 2002). An estimate of the biomass formation in this study using observed (0.32 gC/gC) or theoretical yield (0.31 gC/gC) gives that 20% of the initially applied $^{14}$C is presumably fixed in biomass (Brock et al., 2017).

Step 2.1 describes the differentiation of type I and type II NER. In order to analyse the amount of type III NER (bioNER), another aliquot of the thoroughly extracted matrix (extraction steps 1.1-1.3, see Figure 1) has to be hydrolysed to release proteinaceous compounds in form of amino acids (see step 2 in Figure 1). However, recent results indicate that also soluble residues from microbial biomass may also be present in the extraction fractions (Nowak, personal communication). That’s why both the non-extracted matrix samples and the extracted ones may be analysed for biomarker (see Figure 1).

Type I NER will slowly desorb from the matrix. The release rate of covalently bound residues (type II) can be assumed to be much lower and presumably similar to degradation rates of soil organic matter (SOM), which is a very slow process. In a review by (Conant et al., 2011) it was assumed that 5-15% of SOM, i.e. the readily degradable, “fresh” organic carbon pool, is decomposed within months to years, 40-50% of SOM, the intermediate pool, within years to decades, and 40-50% of SOM, the stable “old” organic matter, within decades to centuries. This suggests that type II NER in soil are “operationally spoken” irreversibly bound and can be released only in minute amounts and at very slow rates, if at all.

It has been reported that compounds with the potential to form covalently bound residues (type II) – such as anilines – may be released under certain conditions, e.g. microbial mechanisms and physical processes such as changes in pH, temperature (freeze/thaw cycles) and moisture contents (wet/dry cycles), growth of plants; examples of such release studies are summarised for instance in (Ecetoc_2013_R117). However, in these studies no attempt has been made to clarify the differentiation between type I and type II NER and it is reasonable to assume that these compounds formed both types of NER and, presumably, non-extractable type I residues have been released in such studies.

Humic matter fractionation by alkaline extraction: Alternatively to the above described silylation method (extraction 2.1), the ‘classical alkaline extraction of humic matter’ has often been applied. The procedure is described for instance by the International Humic Substances Society (IHSS, www.humic-substances.org) and was modified by numerous scientists in the last decades. NaOH extraction is an extraction method, which destroys the three dimensional structure of the organic matrix in soil and sediment. It resembles a pragmatic approach to extract various organic matter fractions based on their solubility under alkaline and acidic conditions, yielding insoluble humin, humic acids (soluble only under strongly alkaline conditions) and soluble fulvic acids beside other
fractions. The fulvic acid fraction is the fraction of potential concern, since it is assumed to pose the highest potential for leaching into groundwater and, consequently, for co-transport of entrapped and covalently bound pollutants. The alkaline extraction can only be used to quantify the three fractions with respect to the amounts of $^{14}$C radiolabel. These procedures do not differentiate type I, II, and type III NER. Therefore, all three fractions should be subsequently silylated and analysed as described above. Before silylation, fulvic acids have to be lyophilised, and humic acids and humin fractions must be thoroughly dried. The interpretation of the results is difficult, if no parent substances or transformation/degradation products can be identified; this particular result may indicate the formation of degradation products and bioNER. Therefore, the humic matter fractionation by alkaline extraction is not recommended by the authors, because it increases the number of samples that need to be checked for the different NER types. In any case, bioNER will be distributed as well with the humic matter fractionation.

2.2 Quantification of type III NER (bioNER): Both labelling with radioactive ($^{14}$C) (Possberg et al., 2016) and stable isotopes ($^{13}$C, $^{15}$N) (Kaestner et al., 2014) has been successfully applied to quantify the amounts of type III NER using the following extraction method. Basically, the environmental matrix, e.g. soil, is hydrolysed by concentrated (6 M) HCl at 110 °C for 22 hours. The matrix and particularly the proteins are destroyed under such harsh conditions and the hydrolysed extract containing the amino acids is cleaned by passage through a cation exchanger, such as DOWEX. Amino acids will bind to the cation exchanger and after washing can be eluted by ammonia solution. For subsequent gas chromatographical analysis coupled to high resolution mass spectrometry – if stable isotopes like $^{13}$C or $^{15}$N have been used – the amino acids have to be derivatised (Girardi et al., 2011; Nowak et al., 2013; Nowak et al., 2011; Wang et al., 2016; Wang et al., 2017b). In case of $^{14}$C-labelled substances, the cation exchange eluate of the above procedure can directly be analysed by two-dimensional thin layer chromatography. Identification of the extracted amino acid spots on the TLC plate is possible by GC-MS after derivatization, e.g., with ethylchloroformate, and by HPLC-MS (Possberg et al., 2016). Based on amino acid detection of hydrolysed proteins the total living biomass of short term experiments should be calculated by multiplying the amino acid amount by the factor of 2, since the amount of proteins in living microbial biomass is generally around 50% (Madigan et al., 2011). During microbial turnover of microbial biomass, however, the factor decreases and approaches 1.11 for long-term experiments ($\geq$ 120 days) (for details, see Annex 1).

Limitations of the extraction methods, technical challenges. Neither the silylation method to distinguish type I and type II NER nor the method to identify bioNER type III have been standardised for direct inclusion in guidance documents but rather represent methods derived from basic research. Therefore, these methods need to be standardised and to be tested with further chemicals of concern regarding NER formation in order to develop routine methods that could be used for ring tests in order to promote standardisation and validation.

Structural identification of type I and type II residues is a technical challenge and laborious. Stable isotope labelling for subsequent stable isotope NMR ($^{13}$C, $^{15}$N) is possible but has the disadvantage that high concentrations of the test substance have to be used due to the low sensitivity of the NMR method. Mass spectrometric analysis of extracts obtained from fulvic acids and silylated extracts is possible but often hampered by co-extracted matrix components. However, quantification of the various NER types after corresponding labelling is feasible.
II.4.3. Options for the assessment of the potential remobilisation of total NER

Many studies reveal that NER may become released under natural environmental conditions, such as the microbial activity in the rhizosphere of plants or in the digestive tract of animals, but the released residues enter a matrix with degrading activity and may subsequently be transformed or partly mineralized. Release of NER has been observed also by applying artificial conditions that, however, will never (e.g. EDTA addition) or only rarely happen under natural conditions.

Several authors investigated the stability of NER formed during microbial turnover of environmental contaminants such as PAH (Eschenbach et al., 1998) and TNT (Weiss et al., 2004a; Weiss et al., 2004b). NER derived from $^{14}$C-labelled anthracene or the explosive $^{14}$C-2,4,6-trinitrotoluene (TNT) in soil were analysed after simulation of extreme physical, chemical or biological conditions in a systematic manner. These authors used the following treatments for the residue containing soils.

i) **physical** treatment for simulating climatic effects by freezing and thawing or by changing the soil texture via grinding;

ii) **chemical** treatment, the extraction of soil with the metal complexing agent EDTA for estimating the effects of bivalent cations on the aggregation of macromolecular soil organic compounds and the extraction of soil with hot or acidified H$_2$O simulating a millennial rain event and the acid rain impact on the release of NER;

iii) **biological** treatment, the simulation of increased turnover of SOM and the NER by addition of compost or incubation of the soil containing NER with ligninolytic fungi.

**Examples of the potential remobilisation of total NER are given below:**

Neither physical nor biological treatments caused a mobilisation of $^{14}$C-anthracene and $^{14}$C-TNT NER in soils. A release of approximately 15% $^{14}$C from NER in soil containing residues from anthracene and TNT was observed after addition of the complexing agent EDTA, however no degradation products were characterised (Eschenbach et al., 1998). Also, the simulation of acid rain and hot water extraction mobilised $^{14}$C-TNT residues in soil of about 30 to 50%. The NER released by these mobilisation treatments were analysed for the presence of the parent substances and their known main transformation products. Neither anthracene nor TNT or transformation products were found to be released from SOM after EDTA addition, acid rain or hot water treatment, which indicates that the NER in these experiments were either not composed of sequestered parent substances or that they were not released by the methods applied. In addition, the identification of some labelled amino acids in residues from labelled phenanthrene and TNT indicated that the NER derived from the microbial turnover in compost or straw-amended soils may be partly of biogenic origin (Richnow et al., 2000; Weiss et al., 2004a). For TNT 11% of the NER were identified as potential bioNER and later it was shown that steam or hot water extractions released considerable amounts of bioNER (Weiss et al., 2004a). The absence of the relevant toxic components after mobilisation shows that the parent substances were transformed into different compounds during the residue formation process. Overall data demonstrate, that NER formed during microbial degradation of TNT or anthracene were very stable on the long run. In addition, the stability of NER is generally depending on their age, as shown in soil experiments with 15-days-old NER and 90-days-old NER derived from 2,4-D. The addition of fresh soil to “young” NER induced their mineralisation, whereas the aged NER were stable and turned over at rates similar to SOM (Lerch et al., 2009).
Soil treated with $^{14}$C-parathion contained after 7 weeks of incubation 27% of the applied radiocarbon as NER. After mixing the NER containing soil with fresh soil for 4 weeks 26% of NER was mineralised and 5% became extractable with the parent substance as main component. This indicates that soil microorganisms play an important part in the release and metabolism of NER. Introducing ‘fresh’ matrix like compost or soil will have both disadvantages and advantages, such as sample dilution, changes in microbial population, potentially increased availability of the extractable fraction, and reduction in potential ongoing toxic affect to biomass associated with a static test system. Promotion of microbial activity by addition of easily degradable substrates (cow dung) increased the release of NER (Racke and Lichtenstein, 1985). Plant root exudates can lead to the remobilisation of aged residues as shown by (White, 2002) with DDE (dichlorodiphenyldichloroethylene) residues and its uptake in squash, pumpkin, cucumber, melon; the presence of DDE residues in the plants was thereby observed. Plant root exudates can lead to the remobilisation of aged residues as shown by (White, 2002) with DDE (dichlorodiphenyl dichloroethylene) residues and its uptake in squash, pumpkin, cucumber, melon; the presence of DDE residues in the plants was analytically verified. However, in this study, no attempt was made to determine the amount of NER in the soils. NER from chlorophenols can also be released by microbial activity as shown by $^{14}$C-labelled 2,4.-dichlorophenol coupled to natural and synthetic humic acids; release rates after 12 weeks incubation were below 10% of the total NER (Dec et al., 1990). $^{14}$C-atrazine formed high NER in soil, i.e. more than 50% of the applied amount. After incubation with *Pseudomonas* species 30-35% of the NER amount was released. The parent substance and its hydroxy- and mono-dealkylated analogues were released into the incubation mixture and were subsequently metabolised by the two species involving dechlorination and dealkylation (Khan and Behki, 1990).

Also physical manipulations may lead to the release of NER, although not directly proven. Balesdent et al. (2000) found that tillage and dry-wet cycling increases the decomposition of soil organic matter and it can be speculated that pollutants forming NER type I may be released subsequently under such conditions. A further argumentation would be that drying of soil leads to shrinking of soil organic matter and sequestered residues may get more tightly bound or their diffusion rate decreased. Similarly, soil wetting will lead to expansion of soil organic matter which may facilitate the diffusion of type I NER pollutants from the matrix (Lennartz and Louchart, 2007). A similar effect of drying-wetting of soil was shown for other pesticides (Jablonskowski et al., 2012). However, others investigated that dry-wet cycling may lead to either increased or decreased decomposition of soil organic matter, and thus the correlation of this environmental condition with NER type I release may be related to the fate of soil organic matter in general (Fierer and Schimel, 2002). Similarly inconclusive, freeze-thaw cycles may lead to both increased and decreased release of NER depending on the soil type and (probably, but not investigated) on the NER type (Weiss et al., 2004b; Zhao et al., 2009; Zhao et al., 2013).

Few investigations have been reported on investigations whether the pH will affect the formation and release of NER. pH changes are very relevant as micro-organisms such as fungi and bacteria release acidic and alkaline compounds as metabolic products. Indirectly, Li et al. (2016) concluded from soil incubation studies of isoproturon that different amounts of NER were formed in presence of different bacterial communities. Since pH shifts in soil will also change microorganism communities, this will also affect the formation of NER. However, the authors did not investigate the release of NER under different pH values. Yee et al. (1985) found that about 25% of NER of prometryne were released by adding buffer at pH 4 and 8 of soil and 2 weeks incubation. The afterwards extractable radioactivity contained the parent substance and transformation products. The release was explained by structural changes of the humic matter when changing the pH, which was actually observed by other soil scientists (Chen and Schnitzer, 1976).

Growth of plants in NER containing soil can promote some remobilisation of the NER label by either shift in pH in the root zone, solubilizing exudates or increased microbial activity in the rhizosphere.
Methyl-parathion forms about 32% NER in soil after 46 days of incubation; extractions to yield NER comprised several steps with various solvents like acetone, methanol, chloroform, isopropyl alcohol. After extraction, either earthworms or plants were inserted into the NER containing soil and in both cases some NER became extractable (yielding about 5% lower amounts of NER) and some part of the released residues were taken up in the worms and the plants (Fuhremann and Lichtenstein, 1978). Similar results were obtained regarding the uptake of NER of dinitroaniline herbicides in soil by plants (Helling and Krivonak, 1978), whereas sulfadiazine NER in soil were only taken up to a low extent by earthworms and plants (Heise et al., 2006). Low uptake was observed for cypermethrin NER in wheat plants up to a level of about 0.3 ppm (Roberts and Standen, 1981). Root exudates were shown to release parts of bound PAH in soil (Gao et al., 2017).

The fate of plant NER in organisms has been studied by several groups by administering isolated plant material containing only non-extractable residues after thorough extraction. If radiolabelled substances were used, the radioactivity present in urine, bile, carcass, and organs is considered as bioavailable, whereas elimination in feces is taken as evidence of no release. Many examples show that in fact parts of NER become bioavailable after ingestion (Schmidt, 1999). As an example, Sandermann et al. (1992) showed that NER of 3,4-dichloroaniline in wheat - mainly associated to lignin - becomes in part (up to ca. 20% of NER) bioavailable when fed to rats and lambs. The herbicide propanil is taken up in plants and forms several transformation products. Parts of the residues can be extracted from the plant material, but after exhaustive extraction parts of the residues remain as NER. When the extracted plant material is submerged with water – to simulate flooding at river banks – and becomes degraded, the non-extractable residues are in part released to the water phase containing beside others the toxic transformation product 3,4-dichloroaniline (Chen et al., 2017).

Richnow et al. (1994) proved covalent ester binding of acidic PAH transformation products in soils and sediments by alkaline hydrolysis with $^{18}$O labelled NaOH. After ester cleavage, the presence of $^{18}$O in the released carboxylic acid is a clear evidence of covalent linkage. However, in addition to covalent binding, part of the same transformation products was also strongly sorbed to the soil and sediment and the ratio of both binding types could be estimated by comparing the amounts of $^{18}$O labelled carboxylic acids and those containing no $^{18}$O label. Similarly, 2,2-bis(chlorophenyl)acetic acid, a carboxylic acid transformation product of DDT as well as metalaxyl acid are covalently bound to humic matter in sediments by esterification as shown by Na$^{18}$OH hydrolysis (Kalathoor et al., 2015).

Solid phase extractions, e.g. using TENAX (a 2,6-diphenylene-oxide polymer), have been used to differentiate to assess bioaccessible or bioavailable concentrations and non-extractable residues of pollutants in particulate media. (van Noort et al., 2014) concluded that contaminant fractions desorbed from sediment to TENAX after a few hours can be used as a proxy for bioavailable concentrations.

Oxidative enzymes of wood decaying fungi favor the formation of NER. (Botterweck et al., 2014) showed, that the addition of laccase to soil incubated with metalaxyl promotes NER formation. When added to sterilised soil, NER formation was twice as high as in the sterilised soil alone and both ester and ether bound linkages of the main transformation products metalaxyl acid with humic matter were formed. In fact, laccase addition to soil can be applied as remediation technology due to irreversible, covalent binding of pollutants to soil organic matter (Ahn et al., 2002). Such mechanism was confirmed also early with other oxidative enzymes (peroxidase, tyrosinase) (Dec and Bollag, 2000). To our knowledge, only few studies have been performed to investigate whether such enzyme activities may also lead to remobilisation of non-extractable residues of chemicals (Eschenbach et al.,
but the release from PAH and TNT amounted only to a few percent of the NER. The use of enzymes to hydrolyze NER of xenobiotics in soil or plants as type I and type II NER may also release residues bound to biomolecules such as cellulose, starch, proteins, lipids, sugars, lignin, as well as type III NER, i.e., the interpretation of release rates in terms of the various NER types may be difficult.

II.5. Methods for predicting NER formation and Microbial Turnover to Biomass (MTB) approach

Chemicals that are easily biodegradable show high mineralisation rates and are thus prone to formation of biogenic NER, whereas those which are poorly biodegradable (persistent) with low mineralisation rates will mainly form type I and type II NER (Kaestner et al., 2014). Based on this assumption, the data set of (Barriuso et al., 2008) can thus be divided in two classes of chemicals with respect to NER formation, one forming xenobiotic (type I and II), the other forming biogenic NER (type III) as sketched in Figure 2. With respect to the formation of type I and type II NER, the prediction just from substructures of chemicals is still difficult due to the complex influences of environmental conditions like the soil water content and temperature as well as of the experimental setup, for instance the extraction conditions. However, it is possible to define structural moieties that have an increasing or decreasing effect on NER formation, but molecules may contain both competing types of substructures which render predictions ambiguous. NER related bulk molecular properties of compounds (hydrogen bonding, basicity, polarity, polarizability, and size/mass) and also certain functional groups that increase (carbamate, phenol, carboxyl, nitro) or decrease (imine, nitrile) the potential for NER formation have been defined (Kühne, 2017; Trapp et al., 2018). However, for a predictive model with acceptable uncertainty experimental data are still too limited for verification.

Figure 2: Modelling and experimental approaches to differentiate the three NER types whose amounts depend both on the properties of the test substance and the environmental conditions.

Microbial Turnover to Biomass (MTB) approach. A few years ago, a clear correlation between the amount of CO$_2$ formed during degradation of the xenobiotic and biomass growth has been found (Loos et al., 2012), but the inverse model used could have been improved by taking the differentiation of the three types of NER into account. A simple relation has been recently established between released CO$_2$ (as indicator of microbial activity and mineralisation), biomass yield, and biogenic NER formation. This relation can be used as a screening tool or indicator for bioNER development. The estimation of bioNER formation with the MTB method is a two-step
process: first, the theoretical growth yield is estimated from thermodynamics and molecule structure. Second, the yield together with the information about CO₂ production (determined experimentally in a biodegradation test) is used to calculate the microbial biomass growth. If the experiment is long-term, then also the biomass turnover in the microbial food chain is considered. The sum of living and dead biomass plus organic matter originating from this dead biomass contributes to bioNER (Trapp et al., 2018). The MTB method is described in Annex 1, including limitations and uncertainties, followed by two calculation examples.

The MTB tool can be used in the P assessment as a screening tool for the estimation of the likeliness of type III versus type I and II (xenoNER). The real formation of bioNER should then be tested.

Microbial growth and degradation of the test chemicals lead to the incorporation of labelled carbon into the microbial mass, resulting in biogenic NER without any environmental relevance. The modelling approach was applied in order to estimate the formation of microbial biomass and the microbial growth yield (Brock et al., 2017; Trapp et al., 2018). The MTB approach needs minimum input data, all of them are readily available. Recently, the microbial growth yields of 40 organic chemicals of environmental concern (including 31 pesticides) were estimated. The results were compared to experimental values and the results of other methods for yield estimation that are available in the literature. The MTB method performed best for the tested chemical and PPPs. Moreover, with the theoretical biomass yield and using the released CO₂ as a measure for microbial activity a range for the formation of biogenic NER could be predicted. For the majority of the PPPs, a considerable fraction of the NER was estimated to be biogenic.

The MTB yield method is quite new (Trapp et al., 2018). It has been tested versus available data, but experience is still limited and can thus only be an indicator. The MTB yield estimate has shown the best performance for the yield prediction of xenobiotics like plant protection products but still had a mean average error (in comparison to experimental data) of 49% with both overestimation and underestimation (Brock et al., 2017); the high deviation is due to failure in few cases, and reasons for the failure could be identified (and are listed in Annex 1). There are few measured yields for xenobiotics available for comparison to the estimates, and this data has also considerable uncertainty. Validation with ¹³C-studies showed good agreement to measured growth yields of 2,4-D and ibuprofen (Trapp et al., 2018) and much higher bioNER formation was found than type I and II NER; more of such data will be helpful and are currently under production.

The calculated bioNER from MTB yields can be recommended as a screening approach but not as definitive proof for bioNER formation; experimental methods to verify bioNER formation exist and need to be performed. Once sufficient (positive) experimental data have been gained it may be possible to rely on the calculated bioNER alone.

III. Concepts for assessing the role of NER in the regulatory context

III.1. Different types of NER in P assessment

Currently there are contrasting positions with respect to NER in the general European legislation on chemicals and their annexes, REACH (EC_1907_2006), on biocides (EC_528_2012), and on plant protection products (EC_1107_2009), and the related technical guidelines (Brusseau et al., 1991), (ECHA_2017_R.7b), (ECHA_2017_R.11). For the REACH legislation, when simulation tests are requested, NER are mentioned and suggested to be determined but the extraction methods are not
specified. For the PBT assessment under REACH, and for biocides and plant protection products (PPP) the characterisation of NER is requested but without standardised guidance. For PPP, the databases and experience are more advanced in comparison to the general chemicals and that’s why the references in the scientific part were dominantly derived from pesticide fate assessment. Here, focus is the PBT assessment for general assessment of chemicals based on this scientific background.

For the past decade NER were mentioned in the ECHA guidance documents (ECHA_2016_R.7a; ECHA_2017_R.7b; ECHA_2017_R.7c; ECHA_2017_R.11) and several related reports but only limited details for the assessment of NER were available. This finally resulted in series of workshops (Ecetoc_2009_WR17), (Ecetoc_2012_WR24), (Ecetoc_2015_R29), (UBA_2010), statements and a preliminary interim guideline (Ecetoc_2013_R118) for general NER consideration, which are sometimes highly contradictory in the assessment of the relevance of NER. The impact of NER was always discussed for degradation experiments according to OECD 307 and 308, but how to deal with NER in PBT assessment was further specified in the recent ECHA guidance.

Although the potential release of NER was discussed on many workshops (Ecetoc_2009_WR17), (Ecetoc_2012_WR24), (UBA_2010), the working document on NER dealing with the ‘Evidence Needed to Identify POP, PBT and vPvB Properties for Pesticides’ (DG_SANCO_2012) stated: “Unextractable residues should be excluded from further assessment. They can be considered as degradation loss and not bioavailable and therefore unable to exert toxicity”. For the last couple of years, this approach was applied by industry and most approval authorities for pesticides without taking into account the consequence of underestimation of the potential impact of NER, which are well known from the scientific literature (Kaestner et al., 2014). However, the guidance committed that “Future Guidance might foresee taking into account ‘adsorbed unextractable residues’, which could be mobilised in the long term and become relevant for further assessment”.

These different viewpoints on NER may cause confusion and are for example directly influencing the re-approval strategies of PPP products. Based on the strategies in PBT assessment many PPP, in particular the older ones, would be regarded as persistent and thus may become not approvable, if re-approval decisions have to be taken now. Vice versa the concept of general degradation loss (DG_SANCO_2012) is certainly not conservative and not scientifically justified, since NER can clearly be divided into xenoNER with hazard potential and surely non-relevant bioNER.

Contrary to the previous considerations (SANCO), the recent ECHA Guidances (ECHA_2017_R.7b), (ECHA_2017_R.7c), and (ECHA_2017_R.11) for PBT assessment are considering NER as derived from parent substances or transformation and degradation products and being bioavailable, if no other data are showing evidence for degradation, for example the decrease of NER similarly to the mineralisation, and non-remobilisable properties of the NER. In other words, this means a ‘hidden hazard’ in case of default information. The idea behind is that these compounds may be remobilised on the long run by processes like freezing and thawing, drying or rewetting, other changes of the physical or chemical conditions (pH, temperature, water contents, etc.; see table 2), and by microbial degradation of soil organic matter (SOM) in future. In addition, changes of the exposure conditions, for example for NER derived from veterinary pharmaceuticals in manure and following application of manure on agricultural land may also lead to a mobilisation potential (see chapter II.3).

More detailed differentiation of NER types is nowadays possible and should be considered in the PBT assessment (see Table 2). So, if the likeliness for the formation of biogenic NER is very high (presumably derived from the in-silico assessment with MTB and the confirming mineralisation in the fate assessment), the consideration of NER to be derived from parent substances and primary transformation/ degradation products is not justified and here the analysis of bioNER is suggested.
For the remaining NER of type I (for type II only if there are indications for remobilisation potential), a common approach of remobilisation assessment methods is suggested (see Table 2 and chapter II.4.3). A guide value for remobilisation of NER may be suggested: only if no or very low amounts of residues are released (for soil organic matter, < 2 % C/a), we consider this as no remobilisation. The turnover of 2% C per year is the average respiration of a living soil including soil organic matter under aerobic condition (Conant et al., 2011; Keiluweit et al., 2017). Remobilisation experiments are an operational approach, and the absence of remobilisation is no conclusive evidence for covalent binding. Note that the turnover of organic matter applied with manure or sewage sludge is fast may also lead to a release of the label. At this point a clear weight of evidence approach is needed and such an approach will be provided in the next chapter.

III.2. Concept for characterisation of NER

Based on our scientific analysis of the NER formation and the screening of the available related documents it can be stated that characterisation of NER can be embedded in the general PBT assessment of chemicals in REACH and the Biocidal Products Regulation (BPR). This has to be related to the differentiation of various types of NER. The approach follows the suggested classification of type I (strongly sorbed, trapped and sequestered parent substances or transformation and degradation products, type II (covalently bound transformation and degradation products), and type III (biogenic NER) as defined in chapter II (see Table 2). It is proposed to generally consider unknown total NER as remobilisable parent or transformation products, if no additional information is available. If clear indications for bioNER and also for covalently bound type II NER are available (unless there exist indications for a remobilisation potential) these NER can be considered as “safe sink” with no remobilisation potential in terms of transformation/degradation products. Single bonds like ether linkages of type II NER are also assumed to be rather stable but this type of bonding needs to be validated in the assessment procedures of the potential remobilisation (see Table 2). However, providing the proof for type II NER is the most critical issue in NER characterisation and requires severe additional research.

To improve the differentiation of NER the described methodology is suggested for incorporation in the PBT assessment (ECHA_2017_R.11), since the differentiation will directly determine the evaluation of the P criterion. Also implementing more initial in silico approaches is suggested in accordance with current ECHA guidance documents (ECHA_2017_R.7b; ECHA_2017_R.7c; ECHA_2017_R.11). Besides the principal assessment of chemical properties and the potential biodegradability with physical and chemical parameter alerts, bioavailability and structural alerts, as well as matrix alerts need to be considered, for example for the assessment of chemicals in manure with subsequent matrix changes. In addition, the MTB approach (Trapp et al., 2018) is suggested to be used for the general estimation of the biomass yield and the bioNER formation potential in order to obtain information before setting required OECD degradation or fate simulation tests. For example, indications for bioNER can be cross-validated by assessing CO₂ formation in degradation experiments or the bioNER just analytically confirmed. Vice versa, dominantly NER type I or II forming pesticides can be identified, which may not need further evaluation of bioNER, since all NER are considered to be remobilisable. This approach is very important for not wasting time with inappropriate testing of a certain chemical without a specific NER focus and is also helpful for the interpretation of results derived from these tests. The general concept of the MTB application is to consider the total NER minus potential bioNER as the amount of type I + II NER (see Figure 2). Based on this relation, additional tests (step 2; see Table 2 and chapter II.3) for distinction of NER types I and II can be performed in order to adapt the characterisation strategy to the potential outcome.
Type I is considered to include remobilisation potential whereas type II is considered to be irreversibly bound (unless there are indications for the opposite). The MTB yield estimation can also be applied in order to gain information about P indication – very low biomass yields are an indicator for persistence – and this can be verified with the related amount of CO₂ formed. Further tests could assess the remobilisation potential as suggested above, if no conclusion of the P assessment can be retrieved without these data.

The differentiation of type I and II NER (counted as non-degraded substance) (see step 2.1) does not need to be performed, when taking into account the mineralisation and experimental bioNER (step 2.2) allow to conclude, that the substance and its transformation products are not P.

III.3. How to deal with complex mixtures and natural compounds?
The ECHA considers mixtures and substances of unknown or variable composition, complex reaction products or biological materials (UVCBs) also for the P assessment (ECHA_2017_R.11 Section R11.4.2.2). These complex mixtures are approached by constituent profiling, identification of known constituents, or block profiling (functional blocks), if no known constituent can be identified. Others may be impurities or additives in the respective formulation. However, the proposed approach for NER characterisation in a narrow sense can only be applied for `known constituents´ with a defined CAS number, because the approach is generally based on the application of isotope labelled compounds. In a broader sense, it may also be applied to mixtures and substances of unknown composition as long as known compounds or constituent blocks, or the compounds of most critical concern can be identified.

If the constituents of a complex mixture are known, approaching either as mono- or multi-constituent substance should be considered. If the most critical constituent can be identified, the general suggested approach of NER characterisation is valid. In that case the actual fate and turnover can be evaluated based on the behaviour of the respective constituents (or of a set of certain constituents, block profiles, etc.) with PBT potential in the respective mixture, if the mixture can be supplied with the labelled constituents of interest. If feasible, the fate and turnover of the compounds in the mixture should always be compared to the behaviour of the pure compounds in order to evaluate additional effects caused by the mixture.

If only the `whole substance approach´ is applicable without supply of any labelled compound, the NER cannot really be quantified. In fact, the sequential extraction-differentiation approach (see fig. 1) can be applied but only with high analytical efforts. However, the experimental analysis of bioNER is not possible, only the potential release based on the silylation method or other remobilisation methods.

The MTB approach (see Annex 1) can be applied to each constituent separately, or a block of constituents, if a common chemical structure can be identified. Then a lumped biomass yield can be calculated. If the physical and chemical properties of the mixtures are highly different from the known constituents, for example for specific compounds in non-aqueous-phase-liquids (NAPLs), the fate and distribution behaviour can be affected (Kaestner et al., 2014; Rein et al., 2016). NAPLs may cause mass transfer problems and result in not fully developed biomass yields.

In addition, if the number of constituents is relatively large and/or the composition is to a significant part unknown and/or the variability of composition is relatively large or poorly predictable, the validity of the suggested approach may be limited. In any case the chemical composition of UVCB
substances to be assessed require information on source and manufacturing such as: identity of starting materials/source (and ratio), reaction steps/mechanisms, plant operating parameters (e.g. temperatures/pressures), solvents/reagents used, details of any clean-up/purification steps. This will enable identifying the potential functional constituents or profiles with P potential. In particular for highly variable mixtures, the turnover and fate assessment may be performed with average concentrations of the main constituents or those of particular interest.

IV. Recommendations for further research

Limitations of the approach. Biodegradation and also NER formation depends on the properties of the compounds, the concentrations present, the microbes in the test systems and their pre-exposure to the compounds, their growth behavior and the general conditions of the respective test. In addition, the total amount of NER in the experiment always depends on the efficiency of the extraction method. Hence, there are multiple causes of diverging results even for one compound in one test system and this is actually a very difficult problem and cannot be solved with general considerations. Adding an additional analytical parameter like NER makes the situation even more complex. Therefore, only a weight of evidence evaluation of each test result is possible and needs to be taken with care and expertise.

Type I NER (sequestered residues). The following investigations should be performed with soils or sediments which have been thoroughly extracted after incubation with a relevant chemical and, thus, contain NER only. The release of parent substances, transformation/ degradation products, and transformation products forming type I NER should be systematically studied, also with respect to the question whether this release is associated to the degradation rate of natural soil organic matter. When the embedding matrix, i.e. the humic material, is depleted by degradation, then the thermodynamic potential (the active concentration) of the pollutant is increased and partitioning into the pore water will be increased. Further research is proposed on the correlation of bioavailability and ecotoxicology and the potential to form type I NER: chemicals which have been shown to form this type of NER should be specifically investigated for their bioavailability using analytical tools like passive sampling and by exposure to soil and sediment dwelling organisms. It has been proposed that a part of the reversibly immobilised residues of NER can be released by TENAX extraction; as shown in soil incubated with trinitrotoluene the ecotoxicity after TENAX treatment diminished because the potentially bioavailable fraction was removed (Harmsen et al., 2017).

Type II NER (covalently bound residues). Additional specific limitation may occur from the fact the currently no general method is available for the characterisation of type II NER (covalently bound NER). This causes uncertainties since this type of NER can only indirectly be assessed by subtracting bioNER and type I NER from the total amount of NER. As long as no data are available for type II NER they have to be considered similar to type I NER.

It is speculated that covalent binding to humic matter is a rather stable immobilisation mechanism but research is needed to study this hypothesis under various environmental conditions, such as shifts in pH or in redox conditions or by addition of hydrolyzing enzymes. Such investigations may be performed in cases where type II NER has been unambiguously demonstrated. Cleaving of covalent ester-bonds by use for instance of labelled water (H₂¹⁸O) or sodium hydroxide (Na¹⁸OH) can clearly be elucidated as shown for the transformation/degradation products of DDT and metalaxyl which were bound by ester-linkage to humic matter (Kalathoor et al., 2015). Also, specific investigations should
be performed to clarify release mechanisms of xenobiotics covalently bound to humic matter fractions in soils and sediments (fulvic acids, humic acids). Such studies have been performed with model humic substances (Bollag, 1991; Bollag et al., 1998) but should be extended with humic matter obtained from natural resources.

**Type III NER (biogenic residues).** With respect to biogenic NER a model to predict the formation potential has been described (Brock et al., 2017; Trapp et al., 2018). This approach is new and needs to be confirmed by investigation of more chemical substances. Also, the effect of repeated applications of a chemical to soil (as in case of spray series of pesticides) on the formation of type III NER should be further investigated: adaptation of soil microorganisms and accelerated degradation has been described (Jablonowski et al., 2010; Krutz et al., 2010; Snigowski et al., 2012) but so far not with respect to NER formation. Principally, such effects can be considered by the MTB method.

The MTB potential yield estimation method is, like other yield estimation methods, based on thermodynamics and stoichiometry, combined with elementary microbiology knowledge. The bioNER estimation from the MTB is based on elementary principles, and the method is a promising tool for the interpretation of degradation tests. Some limitations are listed in the Annex 1, but the main issue here is that there are few data for testing and validation of this method available. It is therefore recommended to use the calculated bioNER from MTB yields for now as an indicator but not as definitive proof for bioNER formation. Once sufficient (positive) experience has been gained it may be possible to rely on the calculated bioNER alone. Of particular research interest are:

- Microbial yield and label assimilation (depending on the label position) during starvation degradation and co-metabolism as well as the turnover of proteins or biomass (factor f in the equations). Microbial biomass turnover is in particular rapid in activated sludge systems.
- MTB yield estimation for incomplete mineralisation (endproduct is a stable transformation product and not CO₂).
- Application of the new MTB method to more experimental results, preferably from OECD tests relevant for P assessment.

**General issues.** There is still a set of issues that remain to be evaluated for setting guidelines on NER characterisation that were in part already raised at the NER workshop of the German Environmental Agency (UBA_2010, 2010):

- There is urgent need for standardisation of the NER extraction methods and this may be performed by an annex to the OECD tests 307 and 308 without starting a separate standardisation procedure. Regular ringtests should be performed to validate the proposed silylation technique for distinction of type I and type II NER as well as the hydrolysis method for determining bioNER. The efficiency of the silylation method to release all type I NER should be tested, e.g., by repeating silylation of the matrix.
- There is also need for standardisation of the NER remobilisation assessment methods, which are also less sufficiently described in the literature.
- The relationship between extraction technique and bioavailability/bioaccessibility: there are currently several methods available to assess the actual potential bioavailability or bioaccessibility (Ecetoc_2013_R117), however, the release and the accessibility on the long run or under changes of the environmental conditions is still an open question.
- Improve differentiation and quantification of sequestered, covalently bound and biogenic NER types of environmentally relevant chemicals and study the potential correlation of the
bioavailability with potential ecotoxicity of NER, especially of those chemicals forming significant amounts of type I NER. Study the applicability of the passive sampling methods in this context.

- The chemical mechanisms underlying NER formation: the understanding of the potential chemical and biochemical mechanisms is not the actual problem that needs to be solved in legislation and guidance; the dominating problem is the fact that beside the determination of bioNER no mechanistic methods are available that can distinguish between sorbed/sequestered (type I) and covalently bound (type II) residues. This can so far only be achieved on qualitative and quantitative terms by chemical derivatisation (e.g. silylation), but a mechanistic understanding of the two processes is missing. Only if released transformation/degradation products and transformation products can unambiguously be related to an ester bond by hydrolysis in the presence of $^{18}$O- or $^2$H-labelled H$_2$O evidence for a covalent binding mode can be provided (see chapter II).

- A special case of NER may be the conjugates of pharmaceuticals in NER assessment, in particular if entering the environment by activated sludge or manure. Of course, soluble conjugates of metabolites or parent compounds linked to low molecular compounds (phase II metabolites) are no NER in sensu stricto but they may also be associated to particulate matter in environmental systems. The remobilisation potential of such compounds clearly needs to be tested.

- Special consideration has to be given to the class of poorly water soluble substances with log Kow values above about 6, which have a high tendency to adsorb to particulate matter like soil or sediment. These compounds quickly partition from the aqueous phase to pores of the humic matter becoming strongly sorbed or sequestered (type I NER). Even if the inherent biodegradability is high they become rather persistent in the sequestered state if desorption rates are very slow. Persistence is clearly influenced by partitioning to particulate matrices as has been shown by many examples: even biodegradable substances like proteins persist after immobilisation to a solid matrix.

- Investigation of the temperature dependence on the formation of total NER and on the amounts of the three NER types is missing.

- Concepts to describe the competing kinetics of adsorption, sequestration and biodegradation kinetics are available, and at least one model is available that can, with reasonable input data, estimate simultaneous formation of type I NER and type III bioNER, namely the unified model for sorption and biodegradation (Kaestner et al., 2014; Trapp et al., 2018). However, few studies have been performed where detailed experimental data has been used for comparison to simulation results, and more research is helpful for confirmation and verification of model concept and output.

- Similarly, mathematical tools that simulate the outcome of OECD tests, e.g., (OECD_307, 2002; OECD_308, 2002; OECD_309, 2004), would be useful both for interpretation and confirmation of the test results. The development and test of such models is recommended. As pointed out, a distinction of the types of NER of chemicals in soils and sediments is possible and corresponding analytical tools are at hand. This means, the proof of concept has been established but unfortunately so far only with a limited number of chemical substances.

- The release of non-extractable residues by environmentally relevant conditions such as changes in ionic strength, changes in pH, freeze-thaw cycles, wet-dry cycles has to be further investigated with a set of chemicals covering various functional groups.

- Systematic evaluation of new approval dossiers after implementation of the present NER strategy is suggested.
• There seems still discussion about the hazard from NER II. Risk assessment is not the issue of this paper, but of course, the risk characterisation of the NER fractions in terms of identifying the speciation of type II NER can also impact the methods for their characterisation.
Annexes
Annex 1. Brief description of the MTB method including limitations

Microbial Turnover to Biomass (MTB) approach. The MTB method uses the relation between released CO$_2$ (as indicator of microbial activity and mineralisation), biomass yield, and biogenic NER formation. The estimation of bioNER formation with the MTB method is a two-step process: first, the theoretical growth yield is estimated from thermodynamics and molecule structure. Second, the yield together with information about the CO$_2$ production (e.g. determined experimentally in a biodegradation test) is used to calculate the microbial biomass growth. If the experiment is long-term, then also the biomass turnover in the microbial food chain is considered. The sum of living and dead biomass plus organic matter originating from this dead biomass contributes to bioNER (Trapp et al. 2018).

Step 1: Microbial yields

Biomass yield is defined as the biomass $X$ (in g biomass, or in g C) formed from the utilised substrate $S$ (in g substrate, or in g C):

$$Y = \frac{dX}{dS}$$

Yield estimation. A new method named “MTB” (microbial turnover to biomass) was specifically designed to be applicable for yield estimates for degradation of xenobiotics in general and in particular also for degradation of pesticides. The MTB method is based on Gibbs free energy, Nernst equation, and microbial available electrons (Brock et al., 2017; Trapp et al., 2018). The estimated yield, together with the principle equations above, allows predicting the potential of a chemical to form type III NER from the CO$_2$-evolution during degradation tests. The method is thus also very important for the characterisation of the other types of NER. According to the relation total NER – bioNER = type I and II NER, the fraction of xenoNER can be estimated from the quantified total NER and the estimated bioNER potential (see also Figure 2).

The MTB potential yield estimation method is, like other yield estimation methods (McCarty, 2007) based on thermodynamics and stoichiometry, combined with elementary microbiology. The MTB calculation method gives the potential growth yield of microbes metabolizing a single substrate. It is valid for pure strains or mixed strain cultures. It is valid for pure substrate degradation as well as for starvation degradation (growth on multiple substrates, often falsely termed co-degradation), if the sole labelled substrate is the target compound. In these cases, catabolism and anabolism occur together, which means that CO$_2$ is produced while biomass is formed. Yield estimates can be done for oxygen as terminal electron acceptor (aerobic degradation), but also with nitrate or sulphate (anaerobe degradation). Due to thermodynamic reasons, the yield is highest with oxygen and lowest with sulphate.

Step 2: Calculation of bioNER (type III NER) from microbial yields and CO$_2$-formation

For complete aerobic mineralisation of substrates by microbes, all labelled carbon by definition either turns into CO$_2$ or into biomass, and their fractions are determined by the yield. This relation can be used to calculate the maximum amount of bioNER that can form during mineralisation of carbon labelled substrate, and which is the biomass formed during growth on the substrate. The formation of CO$_2$ during degradation tests is hereby used as indicator for the fraction of substrate that has been mineralised. The bioNER estimation gives a range of potential bioNER formed from the
substrate. The higher value (bioNER is microbial biomass X) relates to when the growth of the microbes on the substrate is just finished. In case of longer experiments, in particular with high initial substrate concentrations and subsequently high degrader biomass after the growth period (typically 2-4 weeks in soil experiments), some of the carbon fixed within biomass will be turned over to new biomass (with release of CO₂) in the microbial food web or ends up in soil organic matter (SOM) after decay (Miltner et al., 2012). Due to this turnover, CO₂ increases and bioNER decreases with time. The lower value of the bioNER prediction (soil organic matter SOM and remaining biomass X) relates thus to longer experiments (120 days in OECD 309).

For substrate utilised for mineralisation, the carbon of the substrate forms microbial biomass and CO₂. Thus, if the unit g C is used, the relation holds that

\[ \text{total X = Y S and} \]
\[ \text{total CO}_2 = (1 - Y) S \]

The ratio of X (bioNER) and CO₂ is thus

\[ \frac{X_{\text{bioNER}}}{CO_2} = \frac{Y}{(1 - Y)} \] after the initial growth phase (1-3 weeks)

From this ratio, the amount of fresh biomass formed during mineralisation can be calculated. Labeled C fixed in biomass X counts to bioNER, so this relation gives the upper amount of C turning into bioNER. During longer experiments (>100 to 200 days), microbes decay and are digested in the microbial food chain. This forms non-living soil organic matter SOM, but also new microbial biomass and more CO₂. In the long run, about 40% of the labelled bulk carbon in biomass X (mainly the protein fraction) turned into SOM, 10% remained within living biomass, the sum of both is fraction \( f = 0.5 \), while \( 1 - f = 0.5 \) or 50% of the carbon forms CO₂ (Miltner et al., 2012), formalised:

\[ \frac{[\text{bioNER}]}{[CO_2]} = \frac{f \times Y}{(1 - Y) + (1 - f) \times Y} \times [CO_2] \] for long-term experiments (≥ 120 days)

(all units here in g C).

The factor \( f \) is independent from the original substrate, it solely depends on how the microbial biomass that was formed by growth on the labeled substrate (and that is thus labeled itself) is turned over in the microbial food chain. The values given (i.e., the factor \( f = 0.5 \)) are purely empirical and were derived by tracking the fate of 13C-labelled bacterial cells in soil (Miltner et al. 2012).

The relation between bioNER, CO₂ and yield is based on basic principles of microbiology and can be used to interpret experimental results from degradation studies. Estimated yields (microbial biomass formed per g of substrate) can be applied for calculating the turnover of a substrate to microbial biomass.

As can be seen from the equations, the bioNER prediction with this method is closely related to the production of CO₂ in the degradation test. If the CO₂-development is low (for example, below a threshold value of < 5%), the bioNER formation potential is negligible.
Protein measurement. \( X \) in the equations above stands for the whole biomass (g C) formed from the utilised substrate \( S \) (g C). However, in section “II.3.2. Differentiation of NER types” section II is described how bioNER in the protein fraction (determined by hydrolysed amino acids) can be determined by amino acid extraction. Typically, half of the dry biomass is proteins (Tchobanoglous et al., 2002). Thus, the labelled carbon in the protein fraction \( P \) is half of that in living biomass, i.e. \( P = 0.5 \times X \) (Madigan et al., 2011), and therefore

\[
\frac{[P]}{[CO_2]} = \frac{\frac{1}{2} Y}{(1 - Y)}
\]

after the initial growth phase (1-3 weeks)

During biomass turnover, fatty acids and sugars are decomposed quickly, while the protein fraction is more persistent (in particular when adsorbed to soil or sediment). The factor \( f_P \) of labelled C in proteins remaining in the protein fraction during microbial turnover is about 0.9 (Miltner et al., 2009). Still, about 50\% (1 – \( f \)) of living biomass is mineralised to \( CO_2 \) and adds to the total \( CO_2 \) formation. For long-term experiments thus holds for the labelled C in the protein or amino acid fraction:

\[
[P_{\rightarrow CO_2}] = \frac{f_P \times \frac{1}{2} Y}{(1 - Y) + (1 - f) \times Y} \times [CO_2]
\]

Limitations and uncertainties of the estimation:

Yield: In case of incomplete degradation with accumulation of metabolic products, a yield estimation is still possible, if the products are known and quantified. Examples for incomplete degradation are fermentation processes, or the formation of stable transformation products (e.g., AMPA from glyphosate). The yield for complete degradation with mineralisation and biomass formation is always higher than that of incomplete reactions. The Biocatalysis and Biodegradation Database BBD at ETH Zurich may help judge whether incomplete biodegradation with accumulation of transformation products is likely (BBD_database).

Yield estimates are not valid (because yield is null) for non-growth supporting co-metabolism and for use of the target substrate as electron acceptor. In these two cases, the substrate is depleted, but microbes do not or only slowly grow on it, and thus \( CO_2 \) development is none or small. Then, also biomass growth and subsequent bioNER formation will be none or small and indicate type I or II NER. This will be predicted by the MTB approach.

In mixed cultures (natural inoculum) and environmental samples (OECD_307, 2002; OECD_308, 2002; OECD_309, 2004), complete mineralisation to \( CO_2 \), incomplete mineralisation with formation of transformation products and co-metabolism without microbial growth may occur simultaneously. It is therefore likely that the estimated potential yield is the upper limit of growth yield in such tests.

bioNER: Deviations from the predicted range of bioNER may occur

- in all cases in which the MTB yield estimate does not apply (see above), among them: the degradation is incomplete, i.e., transformation/degradation products accumulate (this can be considered in the assessment if the nature and amount of transformation products is known); the
degradation is (partly or fully) anaerobic; methane (CH$_4$) is formed instead of CO$_2$ (again, this can be considered if known); if nitrate or sulphate are the electron acceptors, then the yield is lower.

- if there is significant storage of carbon within carbonates or microbes, e.g., in form of carbohydrates or poly-$\beta$-hydroxy acids. In this case, the release of CO$_2$ by mineralisation is delayed. The equations for the bioNER assume, however, immediate release of CO$_2$. In consequence, the true bioNER may be higher than predicted from CO$_2$ and yields, in particular if refixation of labelled CO$_2$ may occur (Miltner et al., 2005).

- the natural inoculum does not contain microbes with enzymes for efficient and complete mineralisation of the substrate and the potential yield is not reached.

- the substrate is toxic to microbes or inhibits enzyme reactions (McCarty, 2007; Miltner et al., 2012; Rein et al., 2007).

- the $^{13}$C- or $^{14}$C labels present in carboxylic moieties or other highly oxidised positions of the parent substance can be lost as CO$_2$ even without any further transformation of the compound. In this case, mineralisation of the entire molecule is generally overestimated. The MTB can also be used to calculate yields and bioNER from incomplete reactions. The only constraint is that the products and the educts, and their Gibbs energies, are known.

**BioNER formation under non-growth conditions**

BioNER is also formed under non-growth conditions. The reason is that substrate can also be used for maintenance. Maintenance is here defined as the replacement of microbial biomass, either within a living cell (i.e., new proteins and reserve substances are formed), or by replacement of decaying cells with new cells.

The growth of microbes is described by the Monod equation, plus a term for decay of microbes:

$$\frac{dX}{dt} = \frac{\mu_{max} a}{K_M + a} \times X - b \times X$$

where $dX/dt$ is the change of microbial biomass with time (g microbial biomass dw per day), $\mu_{max}$ is the maximum growth rate (d$^{-1}$) and $b$ is the decay rate of microbes (death rate, d$^{-1}$). $K_M$ (g m$^{-3}$) is the chemical activity at which the substrate consumption rate is half of its maximum (half saturation constant), and $a$ is the chemical activity of the substrate (equivalent to the concentration of substrate truly dissolved in water) (g m$^{-3}$).

The first term is of the equation forms the growth rate, which depends on the chemical activity (or the concentration) of the substance. For small substrate concentrations, the growth is slower than the decay or death of the microbes. This is known as "non-growth conditions" because no growth of microbes is observed. Still, there is some growth: only, it is smaller than the death of the microbes, resulting in a negative net growth (or slow decay) of the microbial population. Thus, even under these non-growth conditions, there is still microbial biomass formed (as replacement for the decaying biomass), and thus also bioNER is formed. The figure A 1.1 shows the difference between pure decay (X_nonlabeled), the growth on labeled substrate (X_labeled) and the resulting net growth of the microbial population total X (which is still in decay, but slower than decay without maintenance, shown as X_unlabeled). It follows that even under non-growth or maintenance conditions (i.e. growth slower than decay), the yield equation and the bioNER equations are valid and can be used.
Figure A 1.1. $X_{\text{nonlabeled}}$ is pure decay of microbes with decay rate $k_{\text{death}} = 0.05 \text{ d}^{-1}$; $X_{\text{labeled}}$: growth of new microbes with growth rate 0.01 d$^{-1}$ on labeled substrate that replace the initial (unlabeled) microbes; total $X$: net growth (i.e. slower decay).
Annex 2: Fictive application example for the application of the suggested methods

This fictive example was added because there is not yet a real example data set for the application of the new procedures. The data represent data obtained during the registration process, as outlined in ECHA guidance document Chapter R.11. The example shows how to interpret the results in case the substance is rapidly mineralised and high amount of bioNER is formed.

(Fictive) Experimental Results

Fictive experimental results from a degradation test in soil are shown in Table 1 and Figure 1. At the end of the test, day 56, only 1.5% of the initially applied 14C-label is extractable, 50.75% are found in CO2 and 47.76% in the non-extractable fraction (NER).

Table 1. Fictive results of a degradation test, values given as % of initially applied 14C-label.

<table>
<thead>
<tr>
<th>time</th>
<th>extractable</th>
<th>mineralised</th>
<th>total NER</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>83.58</td>
<td>5.97</td>
<td>10.45</td>
</tr>
<tr>
<td>14</td>
<td>64.18</td>
<td>11.94</td>
<td>23.88</td>
</tr>
<tr>
<td>28</td>
<td>11.94</td>
<td>43.28</td>
<td>44.78</td>
</tr>
<tr>
<td>56</td>
<td>1.49</td>
<td>50.75</td>
<td>47.76</td>
</tr>
</tbody>
</table>

Figure 1. Plot of fictive results of a degradation test.
The OECD test guidelines recommend to fit a log-linear plot to the data in the exponential decay phase. This gives a slope of \(-0.0726\) d\(^{-1}\) or a half-life DT50 of 9.5 days for the fit to the extractable labeled C (\(R^2 = 96\%\)). The slope is \(-0.0139\) d\(^{-1}\) or half-life DegT50 of 49.9 days for the fit to the non-mineralised fraction (100% minus %CO\(_2\), \(R^2 = 89.8\%\)) (Figure 2). If the test was an OECD 309 (fresh water), the criterion for P is a half-life higher than 40 days. In this case, the substance would be considered not persistent if only half-life of extractable was considered. On the contrary, it would be considered persistent if only mineralisation was taken into account. The guidelines R.11 state: "Tests should report the degradation rate (or degradation half-life) in each medium determined through mineralisation, e.g. volatile \(^{14}\)C-CO\(_2\), and/or direct substance analysis".

Next it is introduced how to use the proposed approach and how to interpret the results.

**Experimental procedure to determine NER**

**Step 1: Determination of extractable residues**

Step 1.1 Extraction with water

Step 1.2 Extraction with pentane-water

Step 1.3 Accelerated solvent extraction (micro-wave-assisted extraction)

overall, 1.5% extractable (see Table 1)

**Calculation of bioNER with MTB**

The measured CO\(_2\) (% of initial \(^{14}\)C-label) is 50.75% percent. The MTB calculation indicates 21% formation of bioNER. Conclusion: formation of bioNER is likely, analysis for bioNER recommended.
Step 2: Differentiation of NER types

Figure 2 shows the result of the extraction.

Step 2.2 Amino acid extraction

Step 2.2 amino acid extraction: The determination of amino acids (AA) gives 21% of labeled C in AA. This means there is maximally 42% of label in bioNER (factor 2). Having 42% label in bioNER and 48% in total NER means that up to 87.5% of NER is bioNER.

The minimum factor is 1.1 (Annex 1); 1.1 times 21% is 23% of initially added labeled C as minimum of label in bioNER (48% of total NER is then bioNER) (that value that is close to the MTB estimate, compare also Trapp et al. 2018).

Step 2.1 Silylation

31% of label is found in the silylation extract, while 17% remain in the matrix. By assuming an equal distribution of bioNER between the two fractions obtained by silylation (an assumption which should be tested in future research), we can calculate the absolute amount of NER I and NER II. Of the 31% label in the extract, a fraction of 0.48 to 0.875 is bioNER, i.e. the amount of NER I ranges from 4% to 16%. Similar, of the 17% remaining in the matrix, between 2% and 9% represent NER II (Figure 2).
**Figure 2.** Determination of NER fractions.

*Note to Figure 2: The amino acid conversion factor to biomass varies between 2 and 1.1 (cf. Annex 1). In this Figure, only the calculations based on factor 2 have been presented.*

**Possible complications**

Some problems may occur:

i) bioNER may also be in the extractable fraction

Solution: make an additional analysis of the complete sample (including aqueous phase and extractable phase). In the present example, ≤ 1.5% of $^{14}$C is in the extractable fraction (a negligible amount).

ii) the fraction of bioNER in the silylation extracts may not be constant. Above we assumed that the fraction of bioNER is the same in the extract of the silylation, and in the remaining fraction. This allowed us to calculate type I NER and type II NER. In fact, we have yet no proof for this.
Solution: analyse the extract (of the silylation) for parent compound and early metabolites, if possible.

iii) The factor 2 to convert from amino acids to bioNER is valid for living microbes. If the microbial degrader population decays, the necromass is consumed in the microbial food chain. the most stable fraction of the biomass is proteins, and thus their fraction increases. This is the reason why the MTB method calculates a range of result (see Annex 1. Brief description of the MTB method including limitations).

Solution: The same range was used here, for the conversion of amino acids into bioNER. The fraction of labeled C in the amino acid fraction of the biomass increases from about 0.5 to 0.9 during the turnover (Miltner et al., 2009; Trapp et al. 2018; Annex 1), i.e. the factor varies from 2 to 1.1. Both factors were applied above. The true result will in most cases be between these ranges, and the closer to factor 1.1 the longer the growth phase of the microbes has passed. For the present example, bioNER is still the largest fraction of NER, even with the low factor 1.1, and the decision remains unchanged.

Use of the result for the interpretation of data

Our suggestion is to count $^{14}$C in bioNER to the mineralised fraction. For all t, the %metabolised (mineralised and biomass) can be calculated, and Table 1 can be expanded to Table 2. The data are plotted in Figure 3, a log-plot to derive loss rates is shown in Figure 4.

DT50 and DegT50 values can be derived from the log-plot. DegT50 is fitted to the sum of CO$_2$ and bioNER (Figure 4).

\[ t1/2 \text{ extractable } = \frac{\ln(2)}{0.0726} = 9.55 \text{ d} \]
\[ t1/2 \text{ non-metabolised } = \frac{\ln(2)}{0.0466} = 14.87 \text{ d} \]
\[ t1/2 \text{ non-mineralised } = \frac{\ln(2)}{0.0139} = 49.87 \text{ d} \]
\[ t1/2 \text{ xenoNER } = \frac{\ln(2)}{0.0107} = 64.78 \text{ d} \]

While the fit to the CO$_2$-data gives a half-time of 50 d, the fit to the sum of CO$_2$ and bioNER-data gives a half-life of only 15 d. With this low half-life, the compound is no longer suspect of being persistent.

Note: The half-lives above have been calculated only based on the conversion factor of 2 between amino acid and biomass.

Summary

This example shows that the substance would be considered not persistent if only the half-life of extractable label was considered. On the contrary, it would be considered persistent if only mineralisation was taken into account (as described in guidelines R.11). If both mineralisation and
bioNER are taken into account, then the substance will not be considered persistent anymore. Finally, the half-life of XenoNER suggests that this fraction is persistent. Its disappearance will depend on the rate of remobilisation (with subsequent degradation) of this fraction.

**Figure 3.** Mass balance with characterisation of NER type I, II and III.

**Figure 4.** Determination of rates for DT50 and DegT50 by a log-plot. "Non-mineralised" is 100% - (CO₂ + bioNER).
Table 2. Fictive results of a degradation test (%$^{14}$C-label), with fraction of NER added. "Metabolised" is sum of label CO$_2$ and bioNER.

<table>
<thead>
<tr>
<th>time</th>
<th>extractable</th>
<th>mineralised</th>
<th>xenoNER</th>
<th>NER I</th>
<th>NER II</th>
<th>bioNER</th>
<th>metabolised</th>
<th>total NER</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>83.6</td>
<td>6.0</td>
<td>10.4</td>
<td>8.4</td>
<td>2.0</td>
<td>15.0</td>
<td>21.0</td>
<td>25.4</td>
</tr>
<tr>
<td>14</td>
<td>64.2</td>
<td>11.9</td>
<td>9.0</td>
<td>7.0</td>
<td>2.0</td>
<td>14.9</td>
<td>26.9</td>
<td>23.9</td>
</tr>
<tr>
<td>28</td>
<td>11.9</td>
<td>43.3</td>
<td>9.0</td>
<td>7.0</td>
<td>2.0</td>
<td>35.8</td>
<td>79.1</td>
<td>44.8</td>
</tr>
<tr>
<td>56</td>
<td>1.5</td>
<td>50.7</td>
<td>6.0</td>
<td>4.0</td>
<td>2.0</td>
<td>41.8</td>
<td>92.5</td>
<td>47.8</td>
</tr>
</tbody>
</table>
Annex 3. Reassessment of compounds using the suggested approach

**Annex 3.1 Triclosan estimation of bioNER formation with MTB method**

**a. Purpose**

The existing data base is re-evaluated using additionally the MTB method for bioNER estimation.

**b. Available data**

Triclosan (TCS) is well investigated by many scientific studies. The data used for the estimation of bioNER formation are shown in Table A 3.1.

**Table A 3.1. Data of Triclosan used for the estimation of NER formation.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Unit</th>
<th>Comment</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>log D</td>
<td>5.21</td>
<td>log (L/L)</td>
<td>≤ pH 7</td>
<td>ACD/i-Lab</td>
</tr>
<tr>
<td>pKa</td>
<td>7.8; 8.8</td>
<td></td>
<td></td>
<td>ACD/i-Lab, two methods</td>
</tr>
<tr>
<td>Koc</td>
<td>8380</td>
<td>L/kg</td>
<td>≤ pH 7, estimated</td>
<td>(Franco and Trapp, 2008)</td>
</tr>
<tr>
<td>Water solubility</td>
<td>4.6</td>
<td>mg/l</td>
<td>no pH given, neutral species</td>
<td>ACD/i-Lab</td>
</tr>
<tr>
<td>Chemical formula</td>
<td>C₁₂H₇Cl₃O₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molar mass</td>
<td>289.54</td>
<td>g/mol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gibbs free energy</td>
<td>+86</td>
<td>kJ/mol</td>
<td>at standard conditions, pH 7 and I 0.01 M</td>
<td><a href="http://equilibrator.weizmann.ac.il/">http://equilibrator.weizmann.ac.il/</a></td>
</tr>
</tbody>
</table>

**c. Yield estimation**

The theoretical yield (g biomass formation dry weight per g substrate mineralised) is calculated using the MTB method (Brock et al., 2017).

Reaction: C₁₂H₇Cl₃O₂ +12 O₂ --> 12 CO₂ + 3 HCl + 2 H₂O

ΔG° of reaction partners (kJ/mol):

<table>
<thead>
<tr>
<th>Substance</th>
<th>ΔG° (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>-394</td>
</tr>
<tr>
<td>O₂</td>
<td>0 (elementary)</td>
</tr>
<tr>
<td>H₂O</td>
<td>-237.13</td>
</tr>
<tr>
<td>HCl</td>
<td>-131.23</td>
</tr>
</tbody>
</table>
The calculated yield for aerobic complete mineralisation is 0.231 g microbial biomass dw per g substrate, or 0.246 g C per g C. The calculation in all steps follows the description in (Brock et al., 2017; Trapp et al., 2018). It was done in Excel and is shown in Annex 4 step by step.

Interpretation: A yield of 0.231 g microbial biomass dw per g substrate is principally not limiting biodegradation. Similar values were obtained for phenanthrene (0.21 g/g Adam et al. 2016) or 2,4-D (0.28 g/g, Trapp et al. 2017), which were not recalcitrant to biodegradation in these studies.

d. Calculation of bioNER

bioNER is both living biomass X, dead necromass, and soil organic matter formed by this (labeled) dead biomass. X and SOM can be calculated from CO₂-formation and the yield Y using the equations:

\[
\frac{X}{[CO_2]} = \frac{Y}{(1-Y)} \quad \text{Eq. 1}
\]

\[
\frac{\text{bioNER}}{[CO_2]} = \frac{f \times Y}{(1-Y)+(1-f)\times Y} \quad \text{Eq. 2}
\]

where X is living biomass (at the end of the growth stage, without decay), CO₂ is the carbon dioxide released, bioNER is the sum of living and dead biomass and SOM at the end of a long-term experiment, and Y is the yield (all units in g carbon label).

e. Interpretation of biodegradation tests using the equations for bioNER formation and the yield estimated with MTBStudy 1 (Federle et al., 2002) cited in the SEV report an Activated Sludge (AS) test with 2.5 g AS per liter of water, test duration 72 and 50 days. This resulted in 78.4 to 90.1% CO₂ formation, and 2.2 to 6.8% in biomass (this is part of bioNER). 1.5 to 4.4% of the applied radioactivity was found adsorbed to AS. The recovery was 94% and 95%.

The calculated bioNER formation (with eq. 2 for long-term tests) is 11% to 13%, i.e. an overestimation of the measured values. A value of 6.8% is achieved when a degradation of AS to CO₂ with a rate of 0.025 per day is assumed (i.e., a half-life of 28 days for the AS in the system; this value can be confirmed when respiration is measured; typical half-lives for respiration of AS are about 10 days). The adsorption of radioactivity to AS was 1.5 to 4.4% and is consistent with the high Koc of triclosan (8380 L/kg, Table X).

Study 2, Simulation tests (water and sediments). Sediment to water volume ratio 1:3.3; dosage 0.109 mg/l, up to 104 days of incubation. ^14C in CO₂ was 21.4 and 29.1%; in sediment was 69.2 to 74.2%, aerobic.

It is stated in the report on the resulting NER: “In sediment, the amount of non-extractable radioactivity steadily increased during incubation. At the end of incubation, means of 32.4% and
33.0% of the applied radioactivity remained unextracted from river and pond sediments, respectively. Further harsh extractions using acidic conditions under reflux extracted a maximum of 3.5% of the applied radioactivity from the sediment on day 104.” Calculated bioNER for this study with eq. 2 (long-term) is 3.0 and 4.1%, i.e. about 10% of the total NER. Thus, about 90% of the NER is either fraction I (sequestered and entrapped) of NER II (covalently bound). Given the high Koc of triclosan, the formation of NER I due to sequestration and entrapment is likely.

Study 3, Biodegradation in soil

The study was made in accordance with OECD guideline 307. 14C-TCS was applied with 0.2 mg/kg soil using three soils. The study operated over 124 days, and the overall recovery was 97.5%. At the end of the study (day 124), extractables represented between 13% and 17% of applied for the corresponding soils. Soxhlet extraction released an individual amount of up to 6% of applied radioactivity. A small fraction of the NER was extracted using acidic harsh extraction under reflux (max. 2-3% of applied). Methyl-triclosan (M3) was present.

14CO2 reached maximum levels of 14%, 16%, 12% and 5% in soils on day 124. The total formation of NER (bound residues) was 57 to 71%.

The calculation bioNER formation with the highest CO2-development (16%) is 5.23% with equation 1 and 2.25% with equation 2. This means that less than 10% of the total NER is likely to be bioNER. Most of the NER is present either as NER I or as NER II. Given the high Koc of triclosan, the formation of type I NER is likely.

The small fraction of the bound residues that was extracted using acidic harsh extraction under reflux (max. 2-3% of applied) may correspond to the amino-acid fraction (i.e. bioNER), and this would also be in accordance with the calculated bioNER, but without further characterisation (see also chapter II.3) this remains uncertain.

A second study was conducted according to U.S. FDA Technical Assistance Document (section 3.12). The degradation of 14C-TCS in three soils with an application rate of 0.2 mg/kg dry soil was studied (Colgate-Palmolive Company, 1994b, cited in the SEV report). NER in soils ranged from 12.6% to 80.5%, average NER was 56.3%, 28.4% and 54.4%. The mean cumulative radiolabeled 14CO2 evolution was 20.1%, 11.9% and 13.6% after 64 days of testing.

Calculated bioNER for these values are 1.6% to 2.8% with eq. 2 and 3.9 to 6.4% with eq. 1. This means, as before, bioNER do account for less than 10% of the total NER and thus do not change the conclusions derived from the previous tests.

f. Conclusions

The formation of CO2 in all tests indicates that triclosan can be mineralised by microbial enzymes. The highest CO2-evolution was found in the study with the smallest particulate matter content (activated sludge, 2.5 g/L). The stronger triclosan could adsorb in the tests, the smaller was the evolution of CO2 and the higher was the formation of NER. Due to the very high and rapid adsorption, TCS in biodegradation tests with sediments or soils will rapidly be bound to the matrix. This favors sequestration, and thus the formation of NER I (and perhaps also NER II). Calculation of the theoretical microbial yield with the MTB method and the calculation of bioNER formation showed that the majority of NER was not bioNER and thus principally partly remobilisable. Summarised, the
high Koc of triclosan limits the bioavailability, limits thus biodegradation and favors NER I formation, hence leads to recalcitrance of the substance in the simulation tests.

Annex 3.2 77PD estimation of bioNER formation with MTB method

a. Purpose

The formation of bioNER of 77PD is estimated.

b. Available data

Little information on 77PD (N,N'-Bis(1,4-dimethylpentyl)-p-phenylenediamin, CAS No. 3081-14-9) is published in the scientific literature. The physical-chemical properties given in this summary are log Kow 6.3 and log Koc mean is 4.76 (range 4.53 to 5.11). The chemical dissociates at pKa 7.5.

c. Yield estimation

The estimation of bioNER formation is based on chemical formula and chemical structure (Figure 3). The chemical formula is C\textsubscript{20}H\textsubscript{36}N\textsubscript{2} and the molar mass is M 304.58 g/mol (source: http://www.chemnet.com/ChinaSuppliers/52319/Antioxidant-77PD--1966460.html). The Gibbs energy formation is unknown and was set to zero (this gives an underestimate of the yield of a few % when \(\Delta G\) is positive, and a few % overestimate if it is negative, Brock et al. 2017). 77PD is a compound consisting mostly of carbon and hydrogen (20 C and 36 H). The oxidation status of the carbon in the molecule is -1.5, which means that the aerobe oxidation of the substance can yield a lot (in total 110) electrons. Of the 36 hydrogen atoms, 31 are directly connected to carbon, and thus 62 of the electrons produced by carbon oxidation can be used by microbial catabolism (56% of the Gibbs energy). Subsequently, -77PD is principally a good substrate for aerobic microbial metabolism, and the microbial yield is estimated using the MTB method to 0.70 g microbial biomass dry weight per g substrate, or 0.472 g C / g C. Note that the numbers are both uncertain due to missing Gibbs energy, and unknown reaction (both nitrate or ammonia may be formed, depending on whether the microbes use or not the nitrogen in their metabolism). However, the error of the yield estimate is typically less than 10% if the Gibbs energy of the substrate is unknown (Brock et al. 2017).

For these types of compounds the considerations of the sterical hindrance in the MTB approach need to be mentioned again. In particular, the multi-methyl substitution of alkyl chains are increasing the resistance against biodegradation with increasing amount of substituents. For example, pristane and phytane are known to be relatively stable in crude oils and the ratio of alkanes to pristane and phytane is thus an indicator for the extent of degradation of crude oils in the reservoirs. Therefore, the potential yield estimated by MTB needs to be particularly assessed in experimental approaches.

In addition, the very high log Kow and log Koc is expected to strongly reduce the bioavailability of the molecule in all degradation tests with organic phase (activated sludge, sediments or soil), because microbial enzymes typically only attack dissolved molecules. Another obstacle to degradation is that branched molecules have steric limitation of degradation due to high activation energy. The methyl groups attached to the alkane structure of 77PD may slow down or inhibit the microbial degradation (Figure A 3.1). This is not considered in the yield estimation (which is solely based on the nutritional
value, and the thermodynamic energy content of a molecule). The MTB yield estimate excel sheet is shown in Annex 4.

![Chemical structure of 77PD](http://www.chemnet.com/ChinaSuppliers/52319/Antioxidant-77PD--1966460.html)

**Figure A 3.1. Chemical structure of 77PD (source: http://www.chemnet.com/ChinaSuppliers/52319/Antioxidant-77PD--1966460.html)**

d. **Calculation of bioNER**

bioNER is both living biomass $X$, dead necromass, and soil organic matter formed by this (labeled) dead biomass. $X$ and SOM can be calculated from $\text{CO}_2$-formation and the yield $Y$ using the equations:

$$\frac{[X]}{[\text{CO}_2]} = \frac{Y}{(1-Y)}$$  
Eq. 1

$$\frac{\text{bioNER}}{[\text{CO}_2]} = \frac{f \times Y}{(1-Y)+(1-f) \times Y}$$  
Eq. 2

where $X$ is living biomass (at the end of the growth stage, without decay), $\text{CO}_2$ is the carbon dioxide released, bioNER is the sum of living and dead biomass and SOM at the end of a long-term experiment, and $Y$ is the yield (all units in g carbon label).

e. **Interpretation of biodegradation tests and bioNER formation**

**Screening tests**

In a modified MITI test (OECD Guideline 301C), 12% of 77PD were degraded, based on $\text{O}_2$-consumption. Using eq. 1 it can be calculated that another 11% of the 77PD-carbon was assimilated into biomass.

In an inherent biodegradability study comparable to OECD Guideline 301B (Registration dossier (Study report, 1979), the parent substance 77PD showed 50% degradation measured as $\text{CO}_2$.
evolution after 35 days. It was concluded that consequently, the parent compound should be considered as “not inherently biodegradable”.

**Biodegradation in soil**

An OECD 307 soil simulation test is available with the analogous substance 7PPD. 7PPD has a molar mass of 282 g/mol, the structure is shown in Figure A 3.2. Four different soils were tested. A considerable amount of CO₂ formation was observed (which may result from ultimate biodegradation of parent compound or hydrolysis products). Table A 3.2 shows the mass distribution of total non-extractables (NER) and CO₂ after 0, 1, 3, 7, 15, 28 and 56 days. Less than 10% CO₂ evolved, and this corresponds to 9% biomass (part of bioNER, eq. 1) and 3% organic matter (eq. 2). The table also shows the calculated amounts of NER I and NER II. The numbers change only slightly, bioNER (X biomass, eq. 1) is about 11% of NER at the end of the experiment.

In a second test under anaerobic conditions, less than 1% CO₂ was formed, which underlines that under such conditions 77PD is very persistent in soil.

**Table A 3.2. Mass distribution of 7PPD in the OECD 307 soil simulation test.**

<table>
<thead>
<tr>
<th></th>
<th>0 d</th>
<th>1 d</th>
<th>3 d</th>
<th>7 d</th>
<th>15 d</th>
<th>28 d</th>
<th>56 d</th>
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<tbody>
<tr>
<td>Total non-extractables</td>
<td>26.9</td>
<td>49.9</td>
<td>60.4</td>
<td>77.6</td>
<td>77.1</td>
<td>81.9</td>
<td>76.4</td>
</tr>
<tr>
<td>CO₂</td>
<td>0</td>
<td>1.2</td>
<td>4.2</td>
<td>3.5</td>
<td>8</td>
<td>9.7</td>
<td>8.1</td>
</tr>
<tr>
<td>biomass X (eq. 1)</td>
<td>0.0</td>
<td>1.1</td>
<td>3.8</td>
<td>3.1</td>
<td>7.2</td>
<td>8.7</td>
<td>7.2</td>
</tr>
<tr>
<td>calculated bioNER (eq. 2)</td>
<td>0.0</td>
<td>0.4</td>
<td>1.3</td>
<td>1.1</td>
<td>2.5</td>
<td>3.0</td>
<td>2.5</td>
</tr>
<tr>
<td>NER I + II - X</td>
<td>26.9</td>
<td>48.8</td>
<td>56.6</td>
<td>74.5</td>
<td>69.9</td>
<td>73.2</td>
<td>69.2</td>
</tr>
<tr>
<td>NER I + II - bioNER</td>
<td>26.9</td>
<td>49.5</td>
<td>59.1</td>
<td>76.5</td>
<td>74.6</td>
<td>78.9</td>
<td>73.9</td>
</tr>
</tbody>
</table>

Figure A 3.2. Chemical structure of 7PPD. Source: http://www.eastman.com/Products/Pages/ProductHome.aspx?Product=71093129&list=products

**River water, 6PPD**

For 77PD no simulation tests in water or sediment are available. A simulation test in water is however available for the analogue substance 6PPD (CAS No. 793-24-8, Figure A 3.3). It has a molar mass of 268.4 g/mol and the sum formula C₁₈H₂₄N₂. In the simulation tests with Mississippi river water, 96% disappeared, in deionised water 88%, indicating rapid abiotic degradation.
f. Conclusions

Based on the MTB yield estimation, 77PD is a very good substrate for aerobic biodegradation, with yields of 0.7 g biomass per g substrate, or 0.47 gC per gC. The very high log Kow and log Koc indicate, however, quite limited bioavailability in soils and sediments. The screening test according to OECD Guideline 301B shows 50% CO\textsubscript{2}-evolution; the theoretical yield indicates formation of 45% biomass. Together, this corresponds to almost complete (95%) metabolism, if the test compound was the sole source of carbon in the test. No tests in water, soil or sediment are available. Simulation tests with related substances show some CO\textsubscript{2}-evolution, but the major fate of the substance is formation of NER. According to the MTB estimation, only up to 10% of this NER is irreversibly fixed bioNER, most NER is NER I (sequestered and entrapped) or NER II (covalently bound). This result is plausible, given the high Koc of 77PD.

Figure A 3.3. Chemical structure of 6PPD.
Source: http://www.chemicalland21.com/specialtychem/finechem/6PPD.htm
Annex 4. Yield calculation for example chemicals

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
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<td><strong>Appendix 1. Tricosan Yield calculation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enter name</td>
<td>Tricosan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enter structure</td>
<td>C12H27O3S2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No of C</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enter molar mass</td>
<td>289.54 (g/mol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enter reaction (in names)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1 + S2 = P1 + P2 + P3</td>
<td>C24H54O6S2 + 12 O2 → 12 CO2 + 3 H2O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>enter deltaGO-values</td>
<td>usually negative</td>
<td>n times</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate S 1</td>
<td>86</td>
<td>1 kJ/mol</td>
<td></td>
<td></td>
<td>86 Tricosan</td>
</tr>
<tr>
<td>Substrate S 2</td>
<td>0</td>
<td>6 kJ/mol</td>
<td></td>
<td></td>
<td>0 oxygen</td>
</tr>
<tr>
<td>Product P1</td>
<td>-384</td>
<td>12 kJ/mol</td>
<td></td>
<td></td>
<td>-4728 CO2</td>
</tr>
<tr>
<td>Product P2</td>
<td>-237.13</td>
<td>2 kJ/mol</td>
<td></td>
<td></td>
<td>-474.26 H2O</td>
</tr>
<tr>
<td>Product P3</td>
<td>-131.23</td>
<td>3 kJ/mol</td>
<td></td>
<td></td>
<td>-393.69 HCl</td>
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<td>dGO net energy</td>
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<td></td>
<td></td>
<td>-5681.95 kJ/mol</td>
</tr>
<tr>
<td>pH release</td>
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<td></td>
<td></td>
<td></td>
<td>F Faraday constant 96.5</td>
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<tr>
<td>energy for pH shift 0 to 7</td>
<td>-39.67 kJ/mol</td>
<td></td>
<td></td>
<td></td>
<td>n Elektronen 48</td>
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<tr>
<td>correction for delta G</td>
<td><strong>-5681.95</strong> kJ/mol</td>
<td></td>
<td></td>
<td></td>
<td>electrical potential e Volt 1.23</td>
</tr>
<tr>
<td>Calculation of Y anabolic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction of C in dry cell</td>
<td>0.88 gC/gcell</td>
<td></td>
<td></td>
<td></td>
<td>number of OH bonds 6</td>
</tr>
<tr>
<td>molar mass M of C</td>
<td>12 g/mol</td>
<td></td>
<td></td>
<td></td>
<td>CHP2 = ribose electrons available 12</td>
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<td>Y anabol</td>
<td><strong>271.7</strong> g dry cell per mol</td>
<td></td>
<td></td>
<td></td>
<td>fraction energy available for b 0.25</td>
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<td>Calculation of Y catabolic</td>
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<td></td>
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<td></td>
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<tr>
<td>deltaG0 of ATP</td>
<td>-80 kJ/mol ATP</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ratio be dG reaction / dG ATP</td>
<td><strong>71.02</strong> mol ATP per mol substrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y ATP</td>
<td>5 g dry cell per mol ATP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y cota (thermodynamics)</td>
<td>355.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y cota (biota)</td>
<td><strong>88.8</strong> g dry cell per mol</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1/Y anabol</td>
<td>0.004</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>1/Y total</td>
<td>0.015 mol/g</td>
<td></td>
<td></td>
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<tr>
<td>Y total</td>
<td>56.9 g/mol</td>
<td></td>
<td></td>
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<tr>
<td>Ytotal unit g biomass / g Substr</td>
<td><strong>0.231 g/g</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>correction factor</td>
<td>1.066 unit conversion from g biomass dry g substrate to g C1 g C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ytotal in the unit g C / g C</td>
<td><strong>0.2463 g C/g C</strong></td>
<td></td>
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</tr>
</tbody>
</table>
### Appendix 2: Microbial yield of 77PD

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<th>Enter name</th>
<th>77PD</th>
<th>number of C atoms</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enter structure</td>
<td>C20H8NO2</td>
<td>number of H atoms</td>
<td>36</td>
</tr>
<tr>
<td>Enter molar mass</td>
<td>204.58 (g/mol)</td>
<td>number of O atoms</td>
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</tr>
<tr>
<td>Enter reaction (in names)</td>
<td>S1 + S2 = P1 + P2 + P3</td>
<td>number of N atoms</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>20H2O + 18H2O + 2NH3 + 2NO3</td>
<td>number of P atoms</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>number of S atoms</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>number of Cl atoms</td>
<td>0</td>
</tr>
</tbody>
</table>

**enter deltaG0-values usually negative in times**

| Substrate S1 | 0 | 1 kJ/mol | 0 | 77PD, unknown |
| Substrate S2 | 0 | 6 kJ/mol | 0 | oxygen |
| Product P1   | -394 | 20 kJ/mol | -7880 | CO2 |
| Product P2   | -237.13 | 15 kJ/mol | -3556.95 | H2O |
| Product P3   | -26.5 | 2 kJ/mol | -53 | NH3 |

**dG0 net energy**

| dG0 net energy | -11489.95 kJ/mol | -11489.95 kJ/mol |

**H+ release**

| H+ release | 0 mol |

**energy for pH shift 0 to 7**

| -39.87 kJ/mol |

**correction for delta G**

| -11489.95 kJ/mol | electrical potential E Volt | 1.08 |

**Calculation of Y anabolic**

| Fraction of C in dry cell | 0.58 gC/gcell | number of CH bonds | 31 |
| molar mass M of C | 12 g/mol | CH4 = nido electrons available | 62 |
| Y anabol | 452.8 g dry cell per mol | fraction energy available for b | 0.564 |

**Calculation of Y catabolic**

| deltaG0 of ATP | -80 kJ/mol ATP |
| ratio be dG reaction / dG ATP | 143.62 mol ATP per mol substrate |
| Y ATP | 5 g dry cell per mol ATP |
| Y cata (thermodynamics) | 718.1 g dry cell per mol |
| Y cata (biota) | 404.8 g dry cell per mol |
| 1/Y anabol | 0.002 mol/g |
| 1/catalbol | 0.002 mol/g |
| 1/Y total | 0.005 mol/g |
| Y total | 213.7 g/mol |
| Ytotal g biomass /g Subs | 0.702 g/g |
| correction factor | 0.673 |
| Ytotal in the unit g C / g C | 0.4720 g C/g C |
References


Benoit P, Barriuso E. Fate of 14C-ring-labeled 2,4-D, 2,4-dichlorophenol and 4-chlorophenol during straw composting. Biology and Fertility of Soils 1997; 25: 53-59.


Brock AL, Kästner M, Trapp S. Microbial growth yield estimates from thermodynamics and its importance for degradation of pesticides and formation of biogenic non-extractable residues. SAR and QSAR in Environmental Research 2017; (submitted).


Ecetoc_2015_R29. Workshop Report 29: Defining the role of chemical activity in environmental risk assessment within the context of mode of action: Practical guidance and advice, Snowbird, USA.


EPA. Guidance for addressing unextracted pesticide residues in laboratory studies. 2014.


Gomez-Eyles JL, Collins CD, Hodson ME. Using deuterated PAH amendments to validate chemical extraction methods to predict PAH bioavailability in soils. Environmental Pollution 2011; 159: 918-923.


Houba VJG, Temminghoff EJM, Gaikhorst GA, van Vark W. Soil analysis procedures using 0.01 M calcium chloride as extraction reagent. Communications in Soil Science and Plant Analysis 2000; 31: 1299-1396.


Junge T, Classen N, Schaffer A, Schmidt B. Fate of the veterinary antibiotic C:14-difloxacin in soil including simultaneous amendment of pig manure with the focus on non-extractable residues. Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes 2012; 47: 858-868.


Kästner M, Nowak KM, Miltner A, Schaffer A. (Multiple) Isotope probing approaches to trace the fate of environmental chemicals and the formation of non-extractable 'bound' residues. Current Opinion in Biotechnology 2016; 41: 73-82.


Khan SU, Behki RM. Effects of Pseudomonas species on the release of bound C-14 residues from soil treated with C-14 atrazine. Journal of Agricultural and Food Chemistry 1990; 38: 2090-2093.


Latawiec AE, Reid BJ. Sequential extraction of polycyclic aromatic hydrocarbons using subcritical water. Chemosphere 2010; 78: 1042-1048.


Lerch TZ, Dignac MF, Nunan N, Barriuso E, Mariotti A. Ageing processes and soil microbial community effects on the biodegradation of soil 13C-2,4-D nonextractable residues. Environmental Pollution 2009; 157: 2985-2993.


Mordaunt CJ, Gevao B, Jones KC, Semple KT. Formation of non-extractable pesticide residues: observations on compound differences, measurement and regulatory issues. Environmental Pollution 2005; 133: 25-34.


REACH_Annex_VI. Harmonised classification and labelling for certain hazardous substances.


Reid BJ, Stokes JD, Jones KC, Semple KT. Nonexhaustive cyclodextrin-based extraction technique for the evaluation of PAH bioavailability. Environmental Science & Technology 2000b; 34: 3174-3179.


Sun HW, Li JG. Availability of pyrene in unaged and aged soils to earthworm uptake, butanol extraction and SFE. Water Air and Soil Pollution 2005; 166: 353-365.


Yuan Y, Zhang P, Schaffer A, Schmidt B. 3,4-Dichloroaniline revisited: A study on the fate of the priority pollutant in a sediment-water system derived from a rice growing region in Italy. Science of the Total Environment 2017; 574: 1012-1020.


