Understanding the Relationship between Extraction Technique and Bioavailability

Technical Report No. 117

EUROPEAN CENTRE FOR ECOTOXICOLOGY AND TOXICOLOGY OF CHEMICALS

Understanding the Relationship between Extraction Technique and Bioavailability

Technical Report No. 117

Brussels, May 2013

ISSN-0773-8072-117 (print)

ISSN-2079-1526-117 (online)

ECETOC Technical Report No. 117

European Centre for Ecotoxicology and Toxicology of Chemicals
2 Avenue E. Van Nieuwenhuyse (Bte 8), B-1160 Brussels, Belgium.

Understanding the Relationship between Extraction Technique and Bioavailability

CONTENTS

SUMMARY

It is vitally important in understanding the relationship between extraction techniques and bioavailability that there is a common understanding and agreement on the relevant definitions of so-called 'extractable' and 'bound' residues. Previous attempts have been made to rigorously define these terms, though without relating them in a robust way to the physical and biological processes taking place in the biological compartment of interest. It has therefore been proposed that extraction methods are contextualised in relation to the *in situ* sorption and desorption processes and then, in turn, to bioavailability and bioaccessibility.

Revised definitions of the various sorption processes are provided including an indication of the binding energies for certain attractive forces between chemical moieties and the environmental matrix. ´Bound´ or better covalently bound residues which refer to a defined chemical entity were conceptual differentiated from the irreversibly sorbed residues as a whole. The latter are an unquantifiable mixture of defined chemical entities and natural products into which radiolabelled atoms have been assimilated. This Task Force has developed a model illustrating the key *in situ* processes. A number of different sampling approaches and extraction methods have been proposed for dissolved concentrations, as well as rapidly and slowly desorbed residues. Irreversibly bound residues are extremely difficult to extract without using more severe solvents or conditions which would inevitably alter the properties and structure of the matrix.

The current state of knowledge regarding the nature (and strength) of binding forces involved in the sorption process has been reviewed which assists in the rationale for defining appropriate extraction methods for the various residue pools. The interactions are grouped into three main categories, namely physical and chemical adsorption, covalent binding and sequestration / entrapment. Some chemical moieties with different functional groups or side chains can bind to soil organic matter using mixed modes of action. Predicting the likelihood a chemical will form irreversibly sorbed residues based on structural alerts could be a useful tool in environmental risk assessment. However the subject is a very complex one and our current understanding is insufficient to permit such predictions at present.

Many of the techniques currently employed for extraction of a range of chemicals classes are based on maximising the recovery of a compound and its metabolites from the environmental matrix. By employing organic solvents at elevated temperatures and pressures or even harsher conditions still, it is possible to remove irreversibly sorbed residues, which would not be released under typical environmental conditions. These approaches destroy the organic matrix and result in an overestimation of the releasable fraction of irreversibly sorbed residues. A proposed extraction strategy has been based on extraction and quantitation of the dissolved and rapidly desorbed fraction (for the bioavailable residue) and, in addition, the slowly desorbed fraction (for the bioaccessible fraction). A selection of appropriate extraction solvents and parameters, which represent the best currently available approaches to determine each residue pool, is provided. It is evident these may alter the matrix of interest but nevertheless represent the most robust methodology. When this extraction framework is applied using a considered and rational methodology, it will provide a conservative evaluation of bioaccessible residues. Using an intelligent extraction regime, it is possible to obtain robust laboratory data to assess the bioavailable fraction in an environmental matrix.

With the extraction framework established, further validation is recommended using a series of model compounds. Ideally the selected compounds would cover a range of predicted binding strengths and properties and be representative of various chemical segments. The evaluation would preferably be performed using ^{14}C-labelled products dosed to soils and sediments according to current OECD guidelines. The objective would be to challenge the framework by progressing through the extraction regime using different extractions techniques and conditions, aimed at obtaining the respective dissolved, rapidly desorbed, slowly desorbed and irreversibly sorbed fractions and validating these against appropriate bioassays.

1. INTRODUCTION

1.1 Background and Terms of Reference

An ECETOC workshop "Significance of Bound Residues in Environmental Risk Assessment" was held on 14-15 October 2009 in Brussels. The conclusions (ECETOC, 2010) state that "non-extractable residues are currently characterised by a pragmatic extraction approach by determining whether they are extractable or not". This extraction approach has been historically implemented with various solvents under varying conditions and not necessarily linked to the properties of the chemical, nor the matrix. There is a need to develop a standard framework for extraction methods and to associate the extractable fractions (leachable and NER (non-extractable residues)) with both a level of bioavailability (accessibility) and appropriate test organism(s) for the appropriate environmental compartment. It is recognised that the development of new methods to screen bioavailability of such fractions may be needed to validate this association. This exercise would support a consistent interpretation of the data and provide a transparent basis for assessing the potential risk of NER. It is recommended that an ECETOC Task Force be commissioned to develop a framework for intelligent extraction strategies.

The organising committee for this workshop recommended that a Task Force (TF) should be set up with very specific terms of reference:

- Review current literature describing extraction methods for various chemical groups in different environments and how these different methods correlate with the bioavailability of chemicals in different media.
- Understand the current state of knowledge regarding the mechanisms of binding which may contribute to the rationale for defining the appropriate extraction methods.
- Understand the threshold where extractive techniques transition to 'destructive' methods, resulting in the loss of sample matrix integrity and subsequent ability to characterise the intact chemical
- Propose a framework for an intelligent extraction strategy and make recommendations for future research topics, if necessary.

1.2 Types of chemicals and environmental matrices included in the scope of this work

In initial discussions within the Task Force, it was considered essential to limit the scope (and hence the literature search) to key environmental matrices and to only the major classes of chemicals for which there would be adequate data.

In that respect, 'bound' residues would be limited to those associated with water, soil and sediments, as well as biosolids and sludge from various wastewater treatment plants that could eventually be applied to land. It would exclude 'bound' residues associated with crops, as well as body fluids, such as blood and urine.

Similarly, the classes of chemicals chosen for research were limited to major classes such as agrochemicals, industrial chemicals, pharmaceuticals and petroleum products. Some of these classes of chemicals are discharged to rivers via sewage treatment plants or to land from disposal of sewage sludge. Others, such as agrochemicals can be applied to crops and soil, and ultimately end up as soil residues. Petroleum products may enter the terrestrial environment via spills, land application of sewage sludge or as runoff from roads. Other classes, such as metals and nanomaterials were excluded from the scope of this Task Force.

1.3 Definition of extractable, non-extractable and irreversibly bound residues

'Bound' residues were first mentioned in the literature by Bailey and White (1968). A definition of what was considered to be bound residues was published in the US Federal Register (US EPA, 1975) and discussed in more detail by Kaufman (1976). A soil bound residue was defined as "*that unextractable and chemically unidentifiable pesticide residue remaining in fulvic acid, humic acid and humin fractions after exhaustive sequential extraction with non-polar organic and polar solvents*". Since it is now believed that residues may also bind to clay and clay-humin fractions this definition has since been superseded.

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

The IUPAC (International Union of Pure and Applied Chemistry) Pesticide Commission definition (Kearney, 1982) is as follows. Non-extractable residues (sometimes referred to as 'bound' or 'non-extracted' residues) in plants and soils are defined as chemical species originating from pesticides, used according to good agricultural practice, that are unextracted by methods which do not significantly change the chemical nature of these residues. These non-extractable residues are considered to exclude fragments recycled through metabolic pathways leading to natural products.

'Chemical species' in this context refers either to the parent material or to derivatives or fragments of it.

'Methods' in this context refers to any procedures, such as solvent extraction and distillation, used to exhaustively remove chemical species from a soil or plant matrix. In each reference to a non-extractable residue, the exhaustive procedure must be given.

If a non-extractable pesticide residue in soil is (a) not bioavailable to plants or soil animals (b) not persistent or (c) not mobile, then such residues can be considered insignificant.

Calderbank (1989) proposed a new direction of the definition by emphasising investigations of the bioavailability of bound residues.

Führ *et al* (1998) proposed a modified definition of these non-extractable residues: "*Bound residues represent compounds in soil, plant or animal which persist in the matrix in the form of parent substance or its metabolite(s) after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix. The nature of the bond can be clarified by matrix altering extraction methods and sophisticated analytical techniques. In general the formation of bound residues reduces the bioaccessibility and bioavailability significantly*".

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

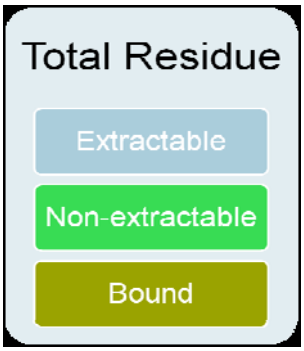
Extractable residue (ER): A residue that is extractable using 'mild' extraction methods. This may include aqueous and cold solvent extraction using methods without excessive added energy. These residues are either freely available, or only weakly adsorbed to the matrix, are considered to be bioavailable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

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

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

ER, NER and BR can be represented by the following figure based on Zarfl *et al* (2009):

Figure 1: ER, NER and BR (Zarfl et al, 2009)



ER, NER and BR are defined on an operational basis, which is to say that they depend specifically on the methods used to extract the chemical(s). In addition, it is only possible to detect BR using methods such as isotopic labelling.

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

Bioaccessible: (based on Semple *et al*, 2004): "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 separated from the organism or only bioavailable after a period of time, i.e. available, but not quite within reach from a given place or at a given time (constrained)". To sum up, bioaccessibility encompasses what is actually bioavailable now plus what is potentially bioavailable. (Semple *et al,* 2004).

Depletion: Removal of a chemical from an environmental compartment. This includes mechanisms such as degradation (including [but not limited to] hydrolysis, photolysis and biodegradation), partitioning and volatilisation.

1.4 The framework model

1.4.1 Sorption and desorption processes in soils and sediments

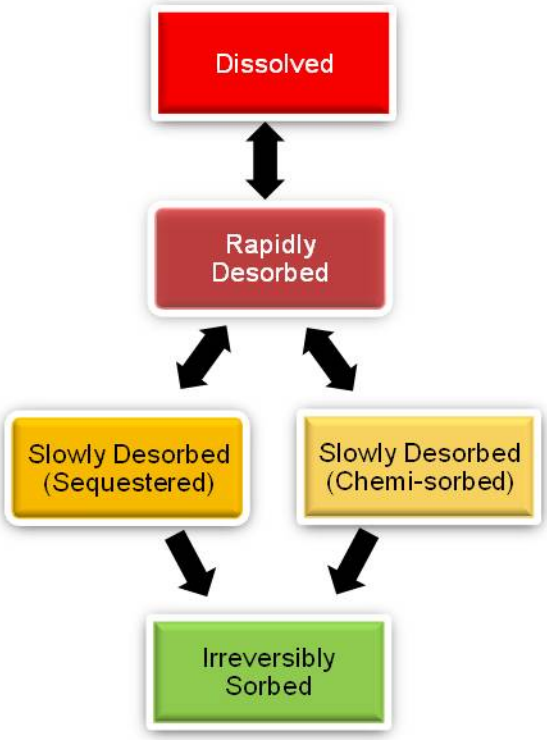
When organic compounds come into contact with soils and sediments they can be taken up by organisms, be degraded by biotic and abiotic mechanisms or transported within the aqueous phase (sediments) or both the aqueous and gaseous phases (soils). All of these processes are controlled to a large extent by the propensity of the compound to sorb to constituents of the solid phase or become physically trapped by them.

The extent to which sorption to soil and sediments takes place is generally described by the soil/water partition coefficient (K_d). Classically, sorption has been conceptualised as a rapid and reversible adsorption process involving partitioning between the compound and organic matter (OM). To reflect this, the K_d value is often normalised by the organic carbon (OC) content of the soil or sediment to give a value termed the K_{oc} (Hamaker and Thompson, 1972).

However, in recent years it has become clear that sorption processes are more complex than this. Depending on the properties of the chemical, other matrix constituents such as clays (Bailey *et al*, 1968; Aharonson and Kafkafi, 1975; Cox *et al*, 1998; Tolls, 2001) and sesquioxides (Leone *et al*, 2002; Kahle and Stamm, 2007) can be important sorbents (Calvet, 1989). Studies conducted over longer timescales have demonstrated that while adsorption is often initially rapid it can also continue to slowly increase over a period of many weeks. This has been suggested to result from the diffusion of the compound into organic matter and intraparticle nanopores (Pignatello and Xing, 1995).

While measurements of adsorption provide an insight into the initial availability of an organic compound, over a longer timescale the rate and extent of desorption of the chemical will be the key determinant of its availability and ultimate fate. The rate that a compound will be desorbed is controlled by the concentration gradient between the solution concentration (influenced by the rate of depletion from the dissolved phase) and the sorbed concentration and any restrictions on desorption as a result of the physical structure of the matrix or the formation of chemical bonds. The extent to which desorption takes place will be determined by the extent of any chemical bonds that may have formed with matrix constituents. The binding mechanisms that can take place are reviewed in Chapter 2 of this report. Such physical and chemical restrictions may serve to delay desorption or to prevent it altogether (irreversible sorption) (Figure 2).

Figure 2: Illustration of various sorption processes between chemical moieties and soils and sediments



In some circumstances the organism(s) of interest can influence the rate and/or extent of desorption. This may be the result of digestion processes or the action of exuded chemicals or enzymes. The significance of such processes is considered in more detail in Sections 1.5 and 1.6.

1.4.2 The relationship between availability, uptake and risk assessment

The definitions of bioavailable and bioaccessible given in Section 1.3 (based on Semple *et al*, 2004) were developed in an ECETOC workshop (ECETOC, 2010). Consideration of these definitions in the context of the sorption processes outlined previously suggests an intuitive overlap between the two.

When considering short term exposure of soil biota it is the 'bioavailable' residue, i.e. that which is immediately available, that is of interest. This equates to the dissolved residue plus the rapidly desorbing fraction.

When assessing longer term exposure the 'bioaccessible' residue is relevant as this represents the quantities that could potentially become available with time. For organisms where exposure is predominantly via the aqueous phase this comprises, the dissolved, rapidly desorbed and slowly desorbed residues. This represents a conservative overestimate of actual uptake as it assumes that the entire 'bioaccessible' residue in a given mass of soil is taken up. Accordingly the 'bioaccessible' residue is appropriate for use in risk assessments.

For some organisms such as earthworms and plants chronic exposure may be influenced by routes of uptake other than via pore water and enhanced desorption. The significance of these processes and their significance when considering uptake is discussed in Section 1.5.

Collectively the dissolved, rapidly desorbed, slowly desorbed and irreversibly sorbed residues constitute the 'total parent residue'. Use of this value in risk assessment will overestimate the exposure of biota, as the irreversibly sorbed fraction is neither bioavailable nor bioaccessible on a timescale relevant to soil and sediment dwelling organisms.

1.4.3 The relationship between sorption / desorption and chemical extraction methods

Much of the available information on the extractability of chemical residues from soils and sediments is derived from studies designed to determine their rate and route of degradation. Within these, the most robust data sets are derived from studies that utilise radioisotopes to enable all of the applied chemical mass to be accounted for. In such studies the following terms have gained widespread usage:

- Extractable residues
- Non-extracted residues
- Bound residues

These are defined on an operational basis, which is to say that their magnitude and composition depend specifically on the methods used to extract the chemical(s) and degradates. As outlined in Section 1.3, a number of authors including ECETOC have tried to rigorously define these terms. Despite this, they still cannot be directly related in a robust way to the physical (sorption/desorption) and biological (bioavailability / bioaccessibility) processes taking place in the biological compartment of interest *in situ*. Depending on the choice of solvent system the extractable residue will generally be a gross overestimate of the bioavailable residue and may over or under estimate the bioaccessible residue. The Task Force therefore

proposes that extraction methods are considered on the basis of how they relate to *in situ* sorption and desorption processes and hence to bioavailability and bioaccessibility.

An important point to note is that extractable and non-extractable residues refer specifically to a defined chemical entity, which may be the parent chemical or a degradate. However, because the bound residue is quantified by a destructive measurement of the residue remaining in the soil or sediment after extraction, its composition remains unknown. For clarity the Task Force proposes that a clear conceptual differentiation is made between irreversibly sorbed residues (which refer to a defined chemical entity) and 'bound residues' (which are an unquantifiable mixture of defined chemical entities and natural products into which radiolabelled atoms have been assimilated).

A further weakness of most definitions of non-extractable and bound residues is that they state that the extractant used should not alter the matrix of interest. In reality, however, most extractants alter the matrix to some extent (Hayes *et al*, 1975). Pragmatically the Task Force proposes that the suitability of extraction methods should be based on their ability to measure the relevant residue fraction(s) (dissolved, rapidly desorbed and slowly desorbed). The impact on the matrix should only be taken into account when investigating the nature or behaviour of the irreversibly sorbed and assimilated residues when changes to the matrix may be a relevant consideration.

Finally, the dynamic nature of these processes must also be considered. It is well established that sorption tends to increase with time, although in some circumstances perturbations to the environmental compartment of interest can result in enhanced desorption (these are reviewed in Section 3.2). The model (Figures 3a and 3b) which builds on the approaches of Reichenberg and Mayer (2006), Semple *et al* (2007) and Frische *et al* (2003) illustrates the key *in situ* processes.

Figure 3a: Framework model describing the fate processes for chemical moieties in soils and sediments

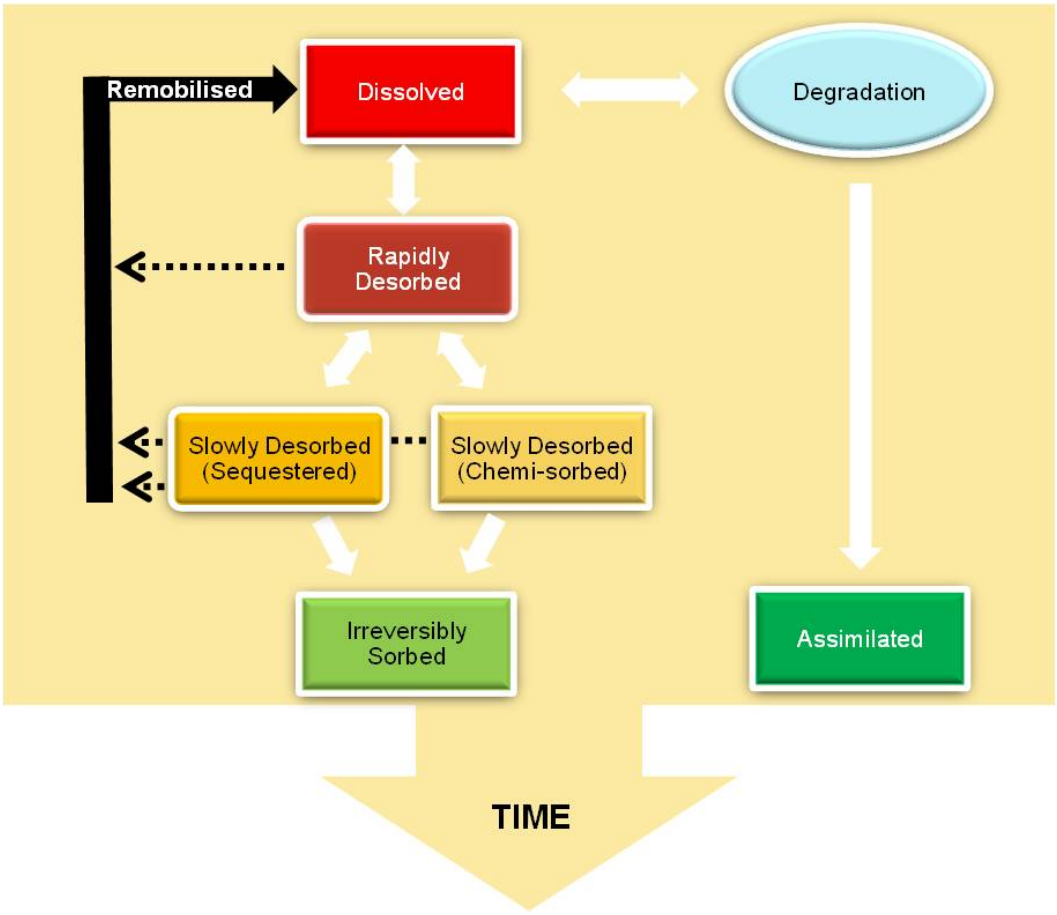
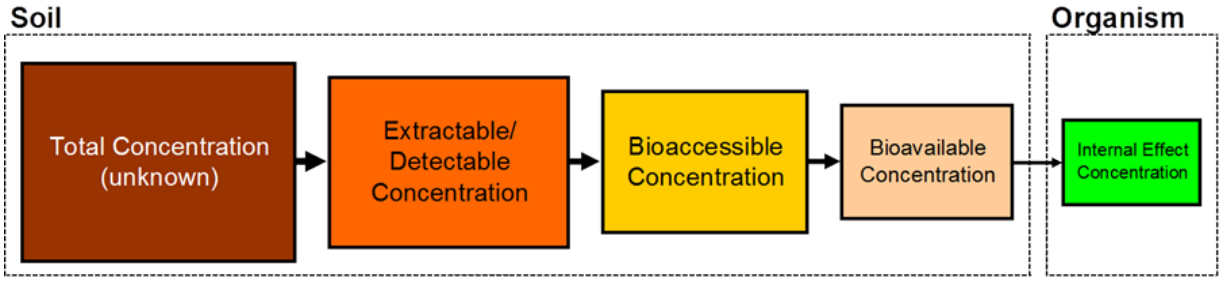


Figure 3b: A representative model illustrating the relative fraction sizes of a chemical in soil (adapted from Frische* et al*, 2003)



The next step is to establish clear conceptual boundaries for each *in situ* residue fraction and then evaluate how they can be robustly quantified. This is a fundamentally different approach to that used in most radiolabelled degradation studies where the historical emphasis has been on extracting as much as possible of the total radioactive residue then retrospectively trying to assess the biological and environmental significance of those residues. This had led to the current unsatisfactory approach of equating 'extraction strength' to 'availability' with little reference to how what is extracted relates to the processes taking place in the compartment of interest in the field.

The boundary between the dissolved and sorbed phases is conceptually obvious and hence little justification of the suitability of the proposed analytical methods to differentiate this boundary (beyond their analytical robustness) is required.

The rapidly desorbed phase can be robustly delineated by consideration of the short duration (< 1 week) over which the exchange needs to take place to be relevant when considering acute exposure.

Distinguishing between the slowly desorbed fraction and the irreversibly sorbed fraction is also a question of timescale rather than a clear cut boundary. As this also represents the point at which residues become unavailable to organisms of interest then the appropriate timescale over which desorption takes place must be on a comparable scale to the lifespan of such organisms. Use of an appropriate bioassay would enable the boundary of the slowly desorbed residue to be determined. For this purpose the use of soil microbes that mineralise chemicals of interest are extremely useful.

A comprehensive review of the influence of microbial chemotaxis and transport across the outer membrane has on bioaccessibility is beyond the scope of this report and the reader is directed to the work of Harms and Wick (2004), Pandey and Jain (2002) and Parales *et al* (2008). However, it is appropriate to outline a rationale to support the contention that the mass of chemical that can be mineralised by a specific degrading microbe represents the best available measure of the bioaccessible residue.

Under natural conditions soil bacteria have optimised their shape, physiology and behaviour to improve their ability to utilise suitable substrates. Bacteria are among the smallest organisms in soil being several micrometers in length and approximately 1 μm^3 in volume (Wood, 1995). The small size, shape and motility of bacteria enable them to move towards food in soil, a process termed chemotaxis. Predation and size can however, result in the exclusion of bacteria from 30-50% of the pores in soil (Postma and van Veem, 1990; Harms and Bosna, 1997). This natural limit on bioaccessibility can be circumvented in bacteria bioassays by the use of artificially high cell densities facilitating both more rapid results and more extensive degradation than under normal conditions. Bacteria are commonly differentiated into gram-positive or gram-negative on the basis of the cell wall reaction with a violet dye (or 'Gram' stain). The outer membrane of gram-negative bacteria facilitates the uptake of hydrophilic chemicals via water filled pores called porins. This membrane presents a barrier to hydrophobic compounds that have to be taken up via specific channels or transport mechanisms. Such hydrophobic compounds can then diffuse through the cytoplasmic membrane. A number of active transport mechanisms exist that facilitate the transport of hydrophilic compound and aromatic acids (Bitton, 2011; Parales *et al*, 2008). Whilst by no means fully understood it is clear that such uptake mechanisms facilitate the uptake of xenobiotics with a wide range of polarities. In the case of specific degrading bacteria the ability of the organisms to then effectively metabolise the substrate results in a depletion at the cell surface and hence a steep concentration gradient between the solid and liquid phases which will result in higher substrate transfer (desorption) rates. Other adaptive strategies to optimise substrate availability such as biofilm formation and the release of biosurfactants also exist (Johnsen *et al*, 2005) but it is not clear if they are relevant to the cultures used as bioassays. On this basis it is reasonable to assume that such microbes mineralise the total bioaccessible (total reversibly sorbed) residue and therefore provide an appropriate benchmark for the performance of the solvent extraction schemes. Furthermore, the quantities extracted or mineralised can be compared directly as the total soil volume can be considered as having been exposed in each case, which would not be the case if larger organisms such as

earthworms were used. A number of studies have used this approach and the findings are summarised below.

Bacterial species capable of mineralising phenanthrene (*Pseudomonas* sp. strain R and *Pseudomonas putida*), 4-nitrophenol (bacterium WS-5) and triazine herbicides (*Pseudomonas* sp. strain ADP) have been used to assess how well different solvent extraction approaches estimate bioaccessibility. In the case of phenanthrene, extraction using butanol, both with and without agitation, and extraction with acetonitrile / water (1:1) without agitation, removed more compound than was mineralised. In contrast, more polar solvents such as ethanol and methanol mixtures with water all removed less phenanthrene than was mineralised (Hatzinger and Alexander, 1995; Kelsey *et al*, 1997). A similar approach for phenanthrene using hot and superheated water was used by Latawiec *et al* (2008). Extractions at 40 and 80°C underestimated mineralisation, extraction at 160°C removed similar amounts as were mineralised and extraction at 180 and 200°C overestimated mineralisation. Similar results were also observed for 4-nitrophenol with butanol extraction removing significantly more of the compound than was mineralised (Hatzinger and Alexander, 1995).

Kelsey *et al* (1997) also compared the extraction of the triazine herbicide atrazine to the quantity mineralised. A range of solvents covering a wide range of polarities were used with and without the use of agitation. In all cases, extraction without agitation removed less of the compound than was mineralised. Conversely, extraction with agitation using methanol/water (1:1) or methanol alone extracted more atrazine than was mineralised. Recovery of atrazine by extraction also declined at a slower rate over time than uptake by earthworms. An alternative approach was used by Barriuso *et al* (2004) to investigate atrazine availability. After ageing in two soils for up to 8 weeks samples were either; not extracted, extracted with 0.01 M $CaCl_2$ (shaking for 16 hours) or extracted with 0.01 M $CaCl_2$ followed by aqueous methanol (80:20 v/v, with shaking for 4 hours). After the relevant extraction procedure samples were inoculated with *Pseudomonas* sp. strain ADP. In both soil types more atrazine was mineralised than was extracted by 0.01M $CaCl_2$ but slightly less than was extracted by 0.01M $CaCl_2$ followed by aqueous methanol. This method was subsequently used with simazine (Regitano *et al*, 2006), another triazine herbicide that is mineralised by *Pseudomonas* (ADP). As with atrazine the entire $CaCl_2$ extractable residue was mineralised as was most (but not all) of the solvent extractable residue. With time, the aqueous methanol available residue increased in the ADP inoculated soils in contrast to the non-ADP treated samples in which the methanol extractable residue continued to decline. This suggests that the residue available to ADP was declining with time and that aqueous methanol was removing more of the residue with time than was bioaccessible to ADP.

The number of compounds that can be studied in this way is currently limited by the availability of suitable microbial species that can mineralise the test compound. For the compounds tested however, the data indicate that extraction with solvents compatible with the properties of the compound of interest generally remove a greater proportion of the residue than is bioaccessible to the microorganism. This suggests that the bioaccessible fraction of a residue will, in general, be solubilised by short extractions using a suitable cold solvent and some form of agitation. Furthermore, these data suggest that degradation studies designed to determine the rate of degradation of a chemical should not include residues (of the parent compound) that are released using more exhaustive extraction regimes as these are not bioaccessible in the soil. Their inclusion in the rate calculation will therefore result in apparent degradation rates that are slower than in reality.

The suitability of available extraction methodologies for quantifying each fraction of the residue is reviewed in Chapter 2.

1.5 The contribution of uptake via digestion

Exposure to chemicals can occur directly via passive uptake through the skin from interstitial water and free chemical products present in the soil organic matter (Lord *et al*, 1980). For some organisms such as earthworms uptake can also take place through intestinal ingestion. Several papers have studied uptake of chemicals when earthworms are burrowing or feeding (e.g. Belfroid *et al*, 1994, 1995; van Leeuwen and Hermens, 1995; Leppänen and Kukkonen, 1998; Jager *et al*, 2003).

It is difficult to assess the quantitative contribution and significance of each route of uptake and accumulation of residues in earthworms but a number of models have been developed.

One model was developed based on the equilibrium partitioning (EqP) theory applied to a passive process (Connell and Markwell, 1990; Van Gestel and Ma, 1988, 1990). The organisms take up chemicals through the skin from the freely dissolved phase in the pore water. Using adsorption coefficient data, it is possible to predict the residues in the organisms.

More recent models have integrated the other route of uptake (ingestion). Belfroid *et al* (1995) was one of the first to develop a model which incorporated both routes of exposure into the accumulation of residues. This model is based not only on the uptake from interstitial water by passive diffusion through the skin membrane but also on the soil ingestion. Results showed that the contribution of dietary uptake was small (< 10%), except for very hydrophobic compounds with log K_{ow} values > 5. The estimation of body residues in these circumstances could be calculated based on the equilibration partitioning model alone, thus resulting in a small underestimation compared to the total body burden in the earthworm.

Jager (2004) also describes these two routes of uptake in earthworms with hexachlorobenzene using a similar model. Results show that the predicted body residues are no more than 20% higher than the estimate based on EqP. The author confirms that body residues are not expected to exceed the EqP estimate by more than 50% for other chemicals or different earthworm species. The deviation is related to the digestive efficiency of the earthworms. The same author (Jager, 2003) has also experimentally described the routes of exposure and their quantitative contribution. In order to do this, he ligatured the anterior end of worms with tissue adhesive to prevent them feeding. Results confirmed the fact that the contribution of the gut route increased with increasing hydrophobicity of chemical. Moreover, the final body residues did not exceed EqP predictions by more than 25%, with the conclusion that specific modelling of the gut compartments is not really necessary and such assessments could therefore rely on just EqP.

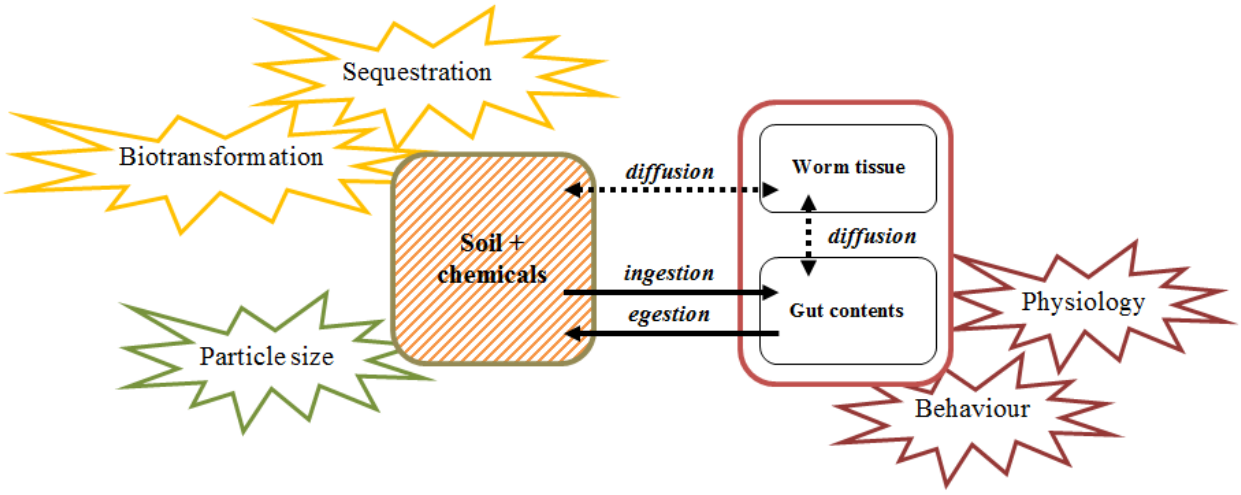
Other experiments have specifically studied the incorporation of bound residues into earthworms, where the extractable portion in the soil matrix was previously removed. For example, incorporation of bound residues of atrazine, dicamba and isoproturon into earthworm tissues has been observed (Gevao *et al*, 2001) as well as for methyl [^{14}C] parathion (Fuhremann and Lichtenstein, 1978). Both researchers observed that the level of tissue residues was higher in experiments with soil containing freshly spiked pesticides compared

with aged soil containing non-extractable residues. No explanation was provided by the researchers on how bound residues can become biologically available and be taken up by earthworms.

Leppänen and Kukkonen (1998) studied the dietary route of high hydrophobic chemicals in earthworms. They compared the significance of pore water and ingested sediment as accumulation routes of sediment bound pyrene in *Lumbriculus variegatus*. Their results have shown that the ingestion of the hydrophobic chemical pyrene was the dominant route of uptake by earthworms (approx. 61%). It leads to a high underestimation from the EqP when assessing body residues, confirming that the contribution of dietary uptake is dominant only for hydrophobic compounds.

However, the accumulation of chemical in earthworms can be influenced by many other parameters and make the assessment of the earthworm body burden very difficult to predict (e.g. Fuhremann and Lichtenstein, 1978; Ebert, 1992; Leppänen and Kukkonen, 1998; Belfroid *et al*, 1996). Belfroid *et al* (1996) have shown that earthworm behaviour, the size of the organisms, the density of the population, their physical avoidance of highly contaminated sites and burrowing behaviour influenced the uptake. The difference in behaviour was also observed in the study of Leppänen and Kukkonen (1998) where the rate of uptake differed between worm groups (previously grouped by size or age), relating to different starting time of soil ingestion. Ebert (1992) has detected remarkable differences in the accumulation of chemicals in two earthworm species. Moreover, other elements such as the sequestration process, biotransformation of chemicals and soil particle size can also influence the body burden and result in deviation from the actual levels of residues accumulated in organisms (e.g. Belfroid *et al*, 1996; Jager, 2003). As an example, the bioavailability of a chemical decreases with increasing contact time with soil. Residues are assimilated less in earthworms when the soil is aged than in freshly spiked soil (e.g. Kelsey *et al*, 1997; Tang *et al*, 1999; Morrison *et al*, 2000) and therefore the model could overestimate the chemical concentration in earthworms in aged soils.

Figure 4: Scheme representing the routes of uptake by earthworms and the influencing parameters (modified from Jager, 2004)



In conclusion, the uptake of chemicals by earthworms can occur through two routes; ingestion of soil and passive diffusion through the skin. For less hydrophobic compounds (log K_{ow} <5) the major route of accumulation will be uptake by passive diffusion, whereas ingestion becomes a more relevant route of uptake for very hydrophobic compounds (log K_{ow} >5). The relationship between increased uptake via ingestion with increasing lipophilicity suggests that differences in partitioning behaviour as a result of differences in the environment in the gut and dermis are key, not enhanced release due to the digestion process. Appropriate solvent selection when dealing with very non-polar compounds should still therefore still facilitate extraction of the residue that is bioaccessible to earthworms in circumstances where uptake via digestion is significant. As well as the hydrophobicity of the chemical, other factors, such as the organism itself, its behaviour, the nature of the soil and the ageing of the chemical can influence the uptake. The uptake and bioaccumulation of chemicals by earthworms, which is an interactive process between soil/sediment, water and the organism itself, has been studied by various researchers (e.g. Oliver, 1984; Beyer, 1996; Kelsey *et al*, 1997; Kelsey and Alexander, 1997; Tang *et al*, 1999, 2002; Morrison *et al*, 2000). Such behavioural influences on the extent of uptake combined with the physical constraints on contact with a given volume of soil mean that earthworms are a poor choice for bioassays designed to determine the bioaccessible residue in soil.

1.6 The contribution of root exudates to uptake by plants

In most circumstances, risk assessments involving the uptake of chemicals by plants are not concerned with the effects on the plants, but instead on the potential effects on other organisms as a result of consumption of residues in the plants. In the case of plant protection products, determination of the quantity and nature of residues taken up by plants following soil treatment is a regulatory requirement in many parts of the world, thus direct measurements are available for use in risk assessments. For risk assessments on produce grown on land which is potentially contaminated with other chemicals, then the extent of chemical uptake by plants may have to be estimated from other study types, or from the analysis of samples from the site. To facilitate such extrapolations it is important to know how chemical residues in soil are taken up by plants.

Comprehensive reviews of chemical uptake by plants are available (Collins *et al*, 2006; Hellstrom, 2004). These indicate that there are two principal pathways for the entry of chemicals into plants from soil: uptake by roots from the soil solution and via shoots as a result of volatilisation. For the purposes of this report, the focus will be on uptake from soil solution. For most chemicals, uptake from the soil solution is passive and is mediated by partitioning of the chemical into soil organic matter. As outlined elsewhere in this report, this is probably an over-simplification. However, for the purposes of risk assessment, this is a conservative approach as the total residue is considered to be potentially available. The robustness of this hypothesis was tested against a number of data sets by Boesten (1993). For plants, it was found that approximately ten studies on uptake qualitatively supported this hypothesis. A mixed picture was apparent with quantitative tests, where, in the three available studies, the soil concentrations were higher, lower and approximately equal to that expected. In all three cases the concentration in the liquid phase was calculated from adsorption data rather than being directly measured, and this was highlighted as a potential reason for the discrepancies. An important caveat is that in many cases the system of interest is not at equilibrium either as a result of degradation or increasing sorption with time (Ronday *et al*, 1997). It is therefore important to ensure that the measurements made are relevant to the risk assessment scenario under consideration.

In the context of the framework model, uptake by plants can therefore be estimated from the dissolved concentration using equilibrium partitioning theory. Given the relatively lengthy duration of exposure of plants, a conservative method for the extraction of the total plant available residue would include the dissolved, plus both the rapidly desorbed and slowly desorbed fractions.

A number of species of plants are known to produce root exudates that can modify both the physical and biological environment in the soil around the plant root. These changes may, in some cases, result in enhanced desorption of residues from the solid phase. Plants of the *Cucurbita* genera were shown by White (2001 and 2002) to reduce the soil concentration of *p,p'*-DDE around the plants, and to take up more of the compound from soil than other species. A similar effect was observed with corn (*Zea mays*) root exudates by Yoshitomi and Shann (2001). In this case, the mineralisation of ^{14}C-pyrene was stimulated to a similar extent in the rhizosphere of growing plants, and by the addition of corn root exudates to soils. White also demonstrated that organic acids typically found in root exudates (citric, oxalic, malonic etc.) increased the desorption of *p,p'*-DDE from soil, leading the author to propose that the enhanced uptake was as a result of increased desorption resulting from the action of the root exudates. Malic and citric acid were also found to enhance desorption of the herbicide isoproturon from a Eutric gleysol, but desorption was reduced in the case of a Hap udic cambisol (Ding *et al*, 2011). This suggests that the influence of root exudates depends on the mechanisms of sorption involved. Conversely, Zhu *et al* (2009) demonstrated phenanthrene desorption was only enhanced when both root exudates and an intact microbial community was present. In the presence of NaN_3, enhanced desorption was not observed with oxalate, citrate or corn root exudates.

The uptake by plants of residues from soil after exhaustive extraction of the parent compound has been studied for a number of compounds including cyprodinil (Dec *et al*, 1997), monolinuron (Suss and Grampp, 1973), dinitroaniline herbicides (Helling and Krivonak, 1978), prometryn (Khan, 1980) and trifluralin (Mostafa *et al*, 1982). In most cases, some uptake of the remaining residue into plants was observed, which accounted for 0 to *ca*. 5% of the initially applied amount. It is difficult to contextualise the significance of these findings because: 1) the extraction methods were generally designed to remove the parent compound, hence residues of degradates may have been left in the soils; 2) the disturbance of the soil prior to planting, and, in some cases, the inclusion of additional matrix materials may have resulted in the repartitioning of sequestered residues.

In conclusion, the available literature indicates that in most circumstances the residue available for plant uptake comprises the dissolved and rapidly desorbed fraction. In a few cases, plants may enhance desorption of chemicals from soil and it may be appropriate to take account of the slowly desorbed fraction when preparing risk assessments.

2. MECHANISMS OF INTERACTION BETWEEN CHEMICAL MOIETIES AND ENVIRONMENTAL MATRICES, ASSOCIATED BINDING FORCES AND POSSIBLE MECHANISMS OF RELEASE

2.1 Binding forces, interactions and mechanisms

The current state of knowledge regarding the mechanisms of binding and their binding forces has been reviewed. The aim of this exercise was to contribute to a rationale for defining appropriate extraction methods for the various dissolved, rapidly desorbed, slowly desorbed and irreversibly sorbed residue fractions.

2.1.1 General processes and interactions

After a chemical is released into the environment its behaviour is mainly governed by two processes:

1. degradation/transformation,
2. sorption.

The degradation / transformation itself is often influenced or directed by sorption processes. For example, strong sorption of a compound to the soil or sediment matrix will most probably result in reduced transformation of that compound due to its reduced bioavailability.

The sorption strength of a chemical compound and its corresponding interaction with environmental matrices will strongly impact on the environmental behaviour of the compound itself. A detailed knowledge of the type and mechanism of sorption or rather the binding mechanism of a chemical to the environmental matrix, e.g. soil, sewage sludge, etc. may contribute to the elucidation of its behaviour in the environment. In addition, the knowledge of the binding mechanism of a chemical may also contribute to the choice of an appropriate extraction method of this chemical from different environmental matrices.

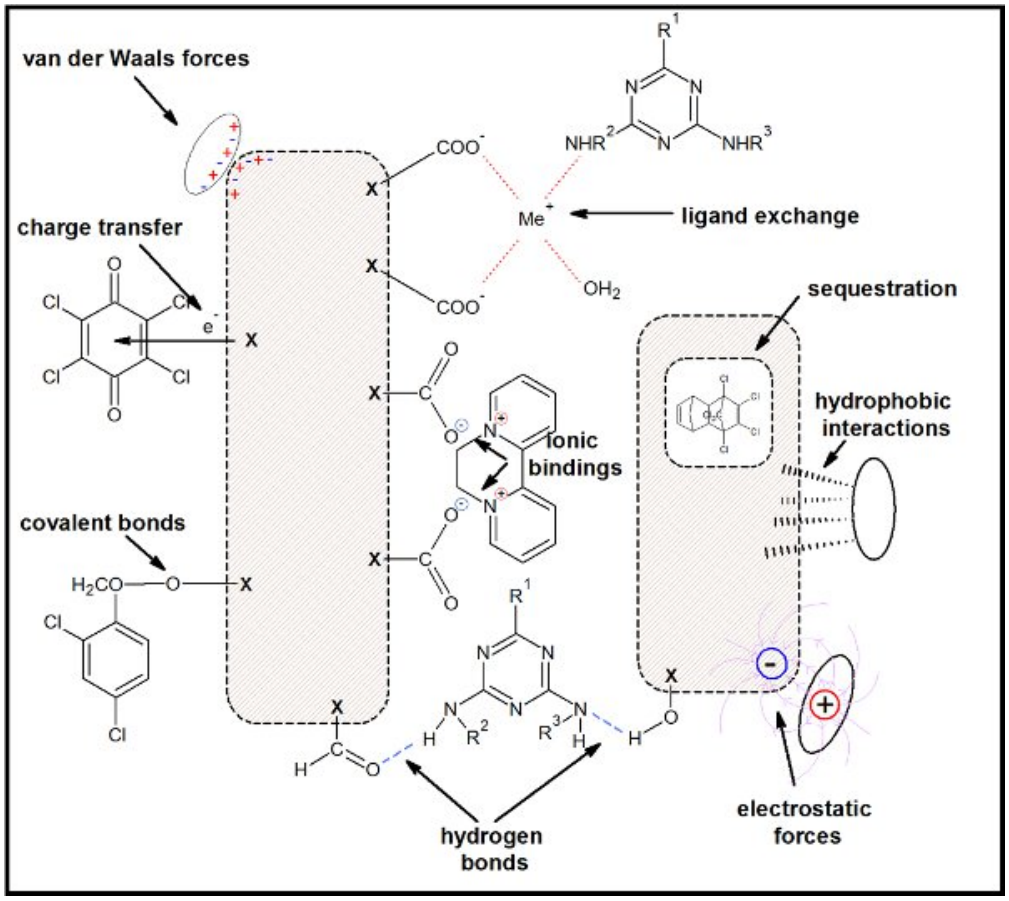
In the following sections the main mechanisms of interactions between certain chemical moieties in pesticides, pharmaceuticals, industrial chemicals and petroleum hydrocarbons are described and typical binding energies detailed. Sorption itself comprises different types of physical and chemical interactions (Von Oepen *et al*, 1991).

The retention mechanisms involved in sorption may be explained by several different processes. In some cases the sorption interaction is dominated by one type of interaction alone. However, in most cases different types of interaction appear simultaneously, e.g. involving interactions between moieties or specific groups of such chemicals (and/or their metabolites) and compartments of the environmental matrix, e.g. clay fraction, organic matter, etc. These interactions can be grouped into three main categories of interaction:

1. physical and chemical adsorption,
2. covalent bonding,
3. sequestration / entrapment.

An illustration of the main mechanisms of interaction between functional groups in compounds and the organic matter in soil and sediment is shown in Figure 5. A summary of the various bond types and their typical bond strengths is shown in Table 1.

Figure 5. Scheme representing certain types of interaction between chemical moieties and soil organic matter (modified from Senesi (1992), Andreux (1997) and Klaus et al (1998))



A number of authors have reviewed the nature of binding forces involved in the adsorption process (Khan, 1982; Calderbank, 1989; von Oepen *et al*, 1991; Senesi, 1992; Andreux, 1997; Lerch *et al*, 1997; Klaus *et al*, 1998; Stegmann *et al*, 2001). From these it is possible to establish the current state of knowledge about typical interactions between chemicals and environmental matrices and the formation of non-extractable residues.

2.1.2 Physical and chemical adsorption

2.1.2.1 Van der Waals forces (0.5 - 5 KJ/mol)

These interactions are very weak and arise from the polarisation of the electron cloud by the proximity of an adjacent nucleus, resulting in a weak electrostatic interaction. They are non-directional and lead to macroscopic effects only if combined in large numbers. Such intermolecular interactions are the basis for the formation of cell walls, micelles, vesicles or membranes. Billions of amphiphilic molecules interact weakly in these systems to form macroscopic structures. The individual interactions are weak (0.5 - 5 KJ/mol) and depend on the contact area (nm^2) and distance between molecules.

Van der Waals interactions may be divided into dispersion (London) and exchange-repulsion terms. The dispersion interaction is an attractive force resulting from the interaction of fluctuating multi-poles in adjacent molecules. The exchange-repulsion interaction involves an overlap of the wave functions of electrons between molecules, which results in an attractive force. If they occupy the same space at the same time, then repulsion occurs.

Interactions are small and less significant for small compounds however they become more important for larger molecules as the individual forces are additive. These forces may play a major role in adsorption processes of large non-polar compounds to suitable (hydrophobic) sites in soil matrix compartments. This type of binding is fully reversible.

2.1.2.2 Hydrophobic interactions (5 - 10 KJ/mol)

Hydrophobic interactions result when non-polar molecules are within a polar solvent e.g. H_2O. Non-polar molecules group together to exclude water (i.e. hydrophobic). By doing so, they minimise the surface area in contact with the polar solvent.

In large molecules, such as proteins, the hydrophobic (non-polar) parts of the molecule will tend to turn towards the inside, while the polar parts will tend to turn towards the surface of the molecule in the corresponding polar solvent.

2.1.2.3 Hydrogen bonds (4 - 120 KJ/mol)

A hydrogen bond may be regarded as a special kind of dipole-dipole interaction. The hydrogen atom is attached to an electronegative atom (leading to a dipole) and interacts with another electronegative atom. Hydrogen bonds are directional and therefore of special importance in molecular recognition. The lengths, strengths and orientation of hydrogen bonds vary over a wide range. In the solid state a single hydrogen bond can be sufficient to determine the structure; in solution or gas phase it may influence the aggregate structure. Typical hydrogen bond donors are: –OH, -NH, -(CO)NH but also C-H is a possible donor in special cases. Typical bond acceptors are: -OH, -O-, =N-, C=O.

This type of binding is reversible and a typical hydrogen bond between water and a hydronium ion has a binding energy of approximately 14 KJ/mol (Markovitch and Agmon, 2007). Recent investigations into the ^{13}C-CPMAS-NMR technique have given evidence of H-bonds being involved in the binding of 2-aminobenzothiazole to natural organic matter in addition to covalent amide bonds as shown by Berns *et al* (2005).

2.1.2.4 Electrostatic bonds (0 - 350 KJ/mol)

This is the attraction due to the electric force of a positively charged pole, e.g. an ionised molecule or dipole and the corresponding negatively charged pole or dipole. The different types of electrostatic binding interactions are listed below.

Ionic interactions (100 – 350 KJ/mol)

This is the most powerful electrostatic binding interaction. If a xenobiotic exists in a cationic or anionic form, it can bind to soil or other matrices at negatively or positively charged sites e.g. as a result of cation exchange, which has been reported for paraquat and diquat (Hayes and Himes, 1986). Ionic bonding is comparable in strength to covalent bonding with bond energies of 100 – 350 KJ/mol. However, it can be reversed.

Ion-dipole interactions (50 – 200 KJ/mol)

The strength of an ion-dipole interaction depends on the charge of the soil surface, the strength of the dipole of the sorbate and on the orientation of the dipoles in space. The interaction of an ion, such as Na^+, with a polar molecule, such as water, is an example of an ion-dipole interaction. The bond strength is in the range of 50 – 200 KJ/mol.

Dipole-dipole interactions (5 – 50 KJ/mol)

Typical bond strengths are in the range 5 – 50 KJ/mol. Alignment of one dipole with another can result in significant attractive forces. The interaction can be between a single pair of poles on adjacent molecules (type I) or alignment of one dipole with another (type II). An example of a type II interaction is an alignment of two opposing carbonyl compounds creating a bond with strength of around 20 KJ/mol (equivalent to a strong hydrogen bond).

Cation - π interactions (5 - 80 KJ/mol)

Many transition metal cations (such as Fe^{2+}, Pt^{2+} or Ag^+) form complexes with π-systems. These interactions are strong and can be considered to be covalent in nature, because of the bonding situation involving the π-orbital of the unsaturated ligand and the d-orbitals of the metal ions. However alkali and earth alkali metal cations show much weaker interactions to π-systems and these are clearly non-covalent.

π - π Stacking (0 - 50 KJ/mol)

This is a weak electrostatic interaction, typically between aromatic rings with one binding partner more electron-rich, the other more electron-poor. There are two possible orientations observed in this interaction, either a 'face to face' orientation or an 'edge to face' orientation.

Ligand exchange (50 - 150 KJ/mol)

Ligand-exchange processes of ions such as Mg(II), Ni(II), Ca(II), Na(I) are very fast (up to 10^9/sec) whereas the ligand-exchange process of Pt(II), Pt(IV), Ru(II), Os(II), Ir(II) and Cr(III) are very slow and may take hours or even days at ambient temperature. Ligand-exchange mechanisms are detailed elsewhere (Van Eldik and Hubbard, 2006). There is a wide range of metal-ligand bond strengths (50 - 150 KJ/mol).

Charge transfer (5 - 50 KJ/mol)

These are a special case of π-stacking or ion-ion interaction. Here an electron-rich π-system interacts with an electron-poor π-system and transfers an electron (sometimes with the help of light). This leads to a π-cation and a π-anion with strong attractive electrostatic forces. Typical bond strengths are in the range 5-50 KJ/mol.

The existence of charge transfer complexes has been demonstrated with residues capable of being ionised, via electron donor-acceptor mechanisms. Structures from humic substances have the capacity to act as electron donors (e.g. diphenol) or acceptors (e.g. quinone) and interact with corresponding electron acceptors or donors from contaminants. As a result there may be the formation of free radicals (quinone/semiquinone type) in humic substances.

2.1.3 Covalent binding

Most chemists are fully conversant with classical covalent chemical bonds, such as C-H, C-C, O-H and N-H. These single bonds usually have strengths of some 250 - 500 KJ/mol (König, 2011). Double bonds as in C=O and C=N and triple bonds as in dinitrogen (N≡N) have bond strengths up to 500 and 800 KJ/mol, respectively. Covalent binding involves the chemical reaction of a chemical, or its degradation product, with a natural organic molecule in the matrix, e.g. soil. The consequence of such a linkage is the loss of the chemical identity of the contaminant.

Covalent bonds are chemically formed between the chemical residues and the matrix, e.g. soil organic matter. They can involve ester-, peptide-, ether, C-C and C-N bonds, depending on the functional groups of the compounds or fragments which are formed during the microbial degradation. The compounds without functional groups could receive the introduction of a reactive group by microbial or chemical degradation. Carbonyl, quinine and carboxyl groups associated with different humic substances can result in covalent binding and hence NER. This type of interaction is persistent as the covalently bound residues become an integral part of the humic substances. Within the framework model, covalently bound residues would fall into the category of 'irreversibly sorbed', to be liberated only with extreme harsh methods which would destroy the soil matrix, e.g. digestive extraction methods.

Dec *et al* (1997) utilised ^{13}C-NMR-spectroscopic methods to characterise the nature of binding of NERs of cyprodinil. For improvement of the NMR spectra they used ^{13}C depleted humic material and a special silylation procedure. The binding of anilazine and its main metabolite dihydroxy-anilazine to aquatic humic substances has also been studied by Klaus *et al* (1998) using ^{13}C-NMR spectroscopy. Similarly, Witte *et al* (1998) examined the binding of ^{13}C-labeled 2-aminobenzothiazoles to humic acids using ^{13}C NMR experiments (1998). ^{19}F NMR spectroscopy was used to show that the 2,6-diamino product of trifluralin formed covalent bonds with fulvic acids (Strynar *et al*, 2004). Using ^{13}C-CPMAS-NMR spectroscopy Käcker *et al* (2002) proved that hydroxylated metabolites of phenanthrene were bound covalently via carboxyl groups to humic acids. Additional structural assignments indicated the presence of an ether bond between hydroxylated metabolites of phenanthrene and humic substances. In all these publications the proof of the covalent bonding was done using artificial model substances as equivalent for humic substances or special conditioned humic substances or very harsh extraction methods destroying the matrix.

Bollag (1992) emphasised the reaction and binding mechanisms that occur via oxidative coupling processes e.g. dichlorophenol with syringic acid. Oxidative coupling is mediated by a number of abiotic and biological catalysts including microbial enzymes. These depend on the structure of the intermediate radicals which occur.

As shown in the above studies, covalently bound residues or part of them could only be extracted with harsh, matrix-destroying, extraction methods. Even under such harsh extraction methods, some covalent bonds were shown to persist using NMR experiments. It is evident that those covalent bonds between a chemical compound and the matrix will not be broken up under environmentally relevant conditions. Therefore under environmentally relevant conditions a release of the original parent compound is most unlikely.

Table 1: A summary of the various bond types and their typical bond strengths

Types of bond (Intermolecular)	Type	Examples of compound	References
Van der Waals	Short-range induced-dipolar attractions	Common to all molecules, stronger for some (e.g. chlorobenzenes)	König, 2011 Szecsody and Bales, 1991
Hydrophobic	Partitioning of non-polar compounds from aqueous phase into SOM	PAHs, aromatic hydrocarbons, pesticides	Israelchvili, 1992 Dickinson, 1997 von Oepen *et al*, 1991
Sequestration	Physical entrapment in cavities of matrix, e.g. soil micropores	1,2-dibromoethane (EDB) in soil micropores; organocations in silicate clays	König, 2011 Steinberg *et al*, 1987
Hydrogen	Special kind of dipole-dipole force that occurs when an H atom is bonded to very electronegative atoms, such as O, F or N. The H-O, H-N and H-F bonds are very polar	H-bonds between humic substances and s-triazines or 2,4-D. Also sorption to clay minerals	Israelchvili, 1992 Dickinson, 1997 König, 2011 Pignatello, 1989 Hamaker and Thompson, 1972
Ligand exchange	Interaction between cations or anions with organic or inorganic soil components	Non-specific sorption involves electrostatic interactions, whereas specific ionic sorption refers to +ve or –ve charged sites of the soil	Van Eldig and Hubbard, 2006 von Oepen *et al*, 1991
Charge transfer	These interactions result from the formation of a donor-acceptor complex between an electron-donor molecule and an electron-acceptor molecule	Two classes of complexes – electron rich π-donors (aromatics, alkynes) - ion pair donors (alcohols, amines) Likely mechanism for sorption to humins	König, 2011 von Oepen *et al*, 1991
Ionic bonding	Ionic compounds result from the combination of two elements of widely different electronegativities	Ionic compounds (e.g. sodium chloride) consist of extended, highly organised aggregates in which +ve and –ve ions alternate	König, 2011
Covalent bonds	Strong chemical bond	Organic compounds consist mainly of covalent bonds	König, 2011

2.1.4 Sequestration / entrapment

One possible mechanism for the interaction of organic compounds with soil involves partitioning to pores within the soil matrix. In particular, the sorption capacity of micropores is extremely high because molecule sorption in micropores follows a mechanism of pore filling of the adsorption space rather than surface coverage (Cheng *et al*, 2012). This phenomenon can also occur in the internal surfaces of porous minerals.

After initial introduction of a contaminant into the matrix, a rapid and reversible equilibrium is established involving adsorption between the chemical and the matrix. A subsequent step following this adsorption can involve a reaction like covalent binding, resulting in much stronger binding of the chemical to the matrix. Another mechanism is the sequestration of the residues into the matrix.

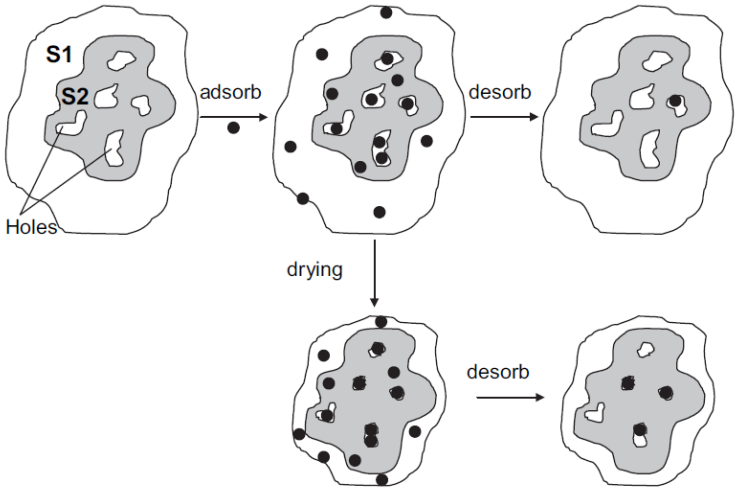
Nanometer-scale pores are ubiquitous in porous geologic media and may account for > 90% of the total mineral surface area. They can be present in the surface micro-structures of minerals resulting from weathering, precipitation, turbostatic stacking of nano-sized particles, and in the forms of nanometer-scale structural pores, cavities, and channels in both crystalline and amorphous minerals (Cheng *et al*, 2012).

Soil has a complex structure which is composed of particles and pores of various sizes. Nanometer-scale pores have dimensions comparable to the sizes of organic molecules. According to the International Union of

Pure and Applied Chemistry (IUPAC), pores can be classified as: micropore (< 2 nm), mesopore (2-50 nm) and macropore (> 50 nm).

The majority of micropores, e.g. of soil are located in the clay fraction and/or in the organic matter fraction of the soil. Dissolved substances are transferred into the nanometric cavities with their reactive inner surfaces with polar and hydrophobic regions. These transport processes are controlled by irreversible thermodynamic processes (non-equilibrium diffusion processes), and therefore could lead to irreversibly encapsulated or entrapped residues.

Figure 6: A conceptual diagram for the effect of drying on interactions between pesticide molecules and SOM (adapted by Lennartz and Louchart (2007) from Lu and Pignatello (2002))



A conceptual diagram of the proposed interactions between SOM and pesticide molecules upon water removal is shown in Figure 6. Adsorption to the inner more rigid phase of the SOM is considered as a hole filling process (Xing and Pignatello, 1997; Lu and Pignatello, 2002). In the frame of the concept of serial sorption sites, the S1 site surface (instantaneous sorption reaction) decreases along with the decrease of the inner pore space which leads to a hindered diffusion and trapped molecules.

This process is often described as 'ageing' (Förster *et al*, 2009). However, the term ageing may mean that the chemical is modified rather than intact. Because some of the compounds are still intact and become inaccessible, Alexander (1995) introduced the term 'sequestration' as distinct from a compound complexed with soil components. The term sequestration refers to a loss in availability of a compound, and the term 'ageing' refers to the time required. With increasing time the entrapped, sequestered residues are less bioavailable and therefore less harmful (Gevao *et al*, 2003). Nevertheless those encapsulated or entrapped residues could be released again if the compound was not transformed during the ageing or sequestration process e.g. if those mesopores were broken up (Ye *et al*, 2003).

During the 'ageing' process sequestration of chemicals often can be described by 'hockey stick-shaped' kinetics (Alexander, 1995). In general, the compound disappears rapidly in the initial stage and the disappearance slows down with increasing residence, and finally its disappearance almost stops. However,

the extent of the remaining compound as well as the rate of disappearance of the compound is expected to be different with dissimilar soils.

Sorption into mineral micropores has been studied for halogenated hydrocarbons (Ball and Roberts, 1991; Cornelissen *et al*, 1998a; Steinberg *et al*, 1987), while there are also limited reports for aromatic hydrocarbons (Weissenfels *et al,* 1992) and pesticides (Ahmad *et al*, 2004).

Cornelissen *et al* (1998a) studied the desorption kinetics of three PCBs (polychlorinated biphenyls) and three chlorobenzenes at varying temperature (5, 20 and 60°C) using a range of sorbent materials. They used the microporous materials zeolite, clay mineral montmorillonite and the porous resin XAD-8 as sorbents to investigate the effects of micropore diffusion on desorption kinetics. Organic polymer macromolecular matrix materials (rubbery polyacetal and glassy polystyrene) were used as sorbents to investigate the effects of entrapment in voids on desorption kinetics. They also studied desorption of the chlorinated compounds from a natural sediment and a natural sediment whose organic matter (OM) had been completely removed. It was found that sorbent-water distribution ratios of the microporous sorbents (zeolite, montmorrilonite and XAD-8) and the sediment without OM were 10-100 times lower than the ones of the original sediment. Cornelissen *et al* suggests that although the presence of both mineral micropores and/or OM can result in the slow desorption behaviour of the chlorinated compounds from soils and sediments, OM is more important for slow desorption than mineral micropores in sediments with more than about 0.1-0.5% OM.

Steinberg *et al* (1987) compared the availability of the soil fumigant 1,2-dibromoethane (EDB) aged in two agricultural top soils for 0.9 and 3 years with that of freshly added EDB (spiked). They found that while ~90% of the freshly added EDB was degraded in 4 and 22 days in each soil, respectively, no degradation of EDB was observed in either of the aged soils. The release of EDB into aqueous solution was studied using a gas purging technique to recover desorbed EDB from the aqueous solution. Freshly added EDB was easily removed from the soil, while EDB in soil aged for 0.9 years was removed at a much slower rate. The authors suggested entrapment in soil micropores was a possible mechanism for the sequestration of EDB.

Weissenfels *et al* (1992) investigated the biodegradation of polycyclic aromatic hydrocarbons (PAHs) in soils obtained from two different industrial sites under simulated land-treatment conditions. PAHs were readily degraded in soil A but not in soil B. The addition of PAH-degrading bacteria to soil B did not enhance the biodegradation. However, when extracted PAHs from soil B were added to a clean soil or back to soil B, PAH-degrading inoculum degraded ~72% of the total PAHs. These results indicated that aged PAHs in soil B might penetrate to remote sites (e.g. nanopores) which restrict availability to bacteria for biodegradation. These facts also indicated that freshly added chemicals do not accurately mimic the behaviour of chemicals in soils found in a contaminated field that have been exposed to the chemicals for a long period of time.

Ahmad *et al* (2004) investigated the bioavailability and biodegradation of the pesticide carbaryl (1-naphthyl methylcarbamate) in a soil with a long history of exposure and contamination. A series of experiments revealed that 49% of the total carbaryl was not water extractable and also considered non-biodegradable, as demonstrated by the addition of mixed bacterial inoculum to the soil which showed the bacteria were only capable of degrading the available (i.e. water extractable) portion of the pesticide. When the soil was pulverised to enhance release of the residue an additional 19% of carbaryl became bioavailable. However, a significant portion of the pesticide (~33%) remained unavailable. Ahmad *et al* suggested that the combination of high concentration (88 mg kg^{-1}) and long contact time or 'ageing' (> 12 years) between the

pesticide and the soil allowed for sequestration and entrapment in the soil nanopores, rendering carbaryl inaccessible to bacterial microorganisms and significantly reducing its bioavailability.

The structural characteristics and type of soil or sediment play an important role in the extent of sequestration / entrapment of organic contaminants. In terms of the physico-chemical properties of the organic contaminant itself, molecular size / volume, polarity, hydrophobicity and diffusivity potential all play an important role in determining the sorption into mineral micropores.

The enhancement of adsorption potentials in soil matrix pores depends upon the size ratio of the pore dimension to the molecular diameter, and appreciable enhancement can occur in pores of six molecular diameters or even larger. The sorption capacity of micropores is extremely high because molecule sorption in micropores follows a mechanism of pore filling of the adsorption cavities rather than surface coverage (Cheng *et al*, 2012 and references therein).

Because of size restrictions, micropores (< 2 nm) can be accessed by organic contaminants, but not the much larger molecules e.g. proteins (average diameter: 2-6 nm). Mesopores (2-50 nm) with smaller pore sizes (2-10 nm), however, are expected to be important for the sorption of larger molecules (e.g. proteins).

Brusseau *et al* (1991) found that descriptors of molecular size of organic contaminants appear to be correlated with sequestration. Later, Piatt and Brusseau (1998) studied rate limited sorption kinetics of a series of PAHs, alkyl benzenes, chlorinated benzenes and chlorinated alkenes in two soils. The OM in one soil was dominated by fulvic acid and the other by humic acid. It was found that the compounds sorbed strongest to the soil with high humic acid content. The authors then established the degree of correlation between the determined equilibrium sorption coefficients with the first-order molecular connectivity index (MCI or $^{1}X^{v}$; a molecular solute descriptor) and log K_{ow} of the compounds. The degree of correlation between MCI and the equilibrium sorption coefficients proved to be better than using the log K_{ow} parameter. It was therefore suggested that the shape/structure of the compounds may have an influence on the overall magnitude of equilibrium sorption and have a significant effect on the sorption kinetics.

Kottler and Alexander (2001) examined the behaviour of a series of 21 PAHs in soil to determine whether sequestration could be correlated with their properties. A series of extraction experiments using *n*-butanol revealed that 22-58% of the PAHs were not recoverable after their addition to soil which increased to 47-77% after 28 days of soil ageing. The correlations were based on the amounts of aged compound extracted with *n*-butanol against log K_{ow}, molecular length and molecular-connectivity index (MCI) properties of each compound. It was found that not one property, including log K_{ow}, resulted in an r^2 value greater than 0.26. Because no single compound property descriptor appeared to be highly correlated with sequestration, regressions were developed using multiple descriptors. However, even when multiple descriptors were used, a maximum r^2 value of 0.54 was derived. The results suggested that the properties tested were poorly correlated with sequestration of the PAHs in soil and that predictions using these descriptors are not possible.

White *et al* (1999) investigated factors affecting sequestration and bioavailability of phenanthrene in several soils. However, no specific effect was observed with soil aggregates, clay content, and organic matter content, possibly due to the complexity and heterogenicity of the soils.

It has been suggested that the OM content of soil is a major determinant of sequestration (Nam and Alexander, 1998; Nam *et al*, 1998). Later, Chung and Alexander (1998) studied the effect of dissimilar soils on the sequestration of atrazine and phenanthrene. They found that the two compounds became sequestered in each of the soils but the rate and extent of sequestration varied markedly among the soils. In addition, the extent of sequestration of the two compounds in the 16 soils tested was not found to be highly correlated and the decline in bioavailability of the compounds in the soils was not highly correlated with the decrease in extractability.

Mayer (1994) proposed that organic matter in marine sediments was protected by its location inside pores that are too small to allow for the entrance or functioning of hydrolytic enzymes. Organic compounds introduced into soil slowly diffuse into nanopores while they repeatedly sorb to and desorb from soil components such as organic matter. The more time is allowed to pass the more the compounds move into remote sites between particles or within particles. In the SOM, the rate of diffusion is orders of magnitude slower than in water (Brusseau *et al*, 1991). Thus, once sequestered (or aged) in the remote sites, the organic compounds would move very slowly through the three-dimensional matrix to the outer surfaces where they could become bioavailable.

Chung and Alexander (1999a) reported most of the larger nanopores (10^4 - 10^2 nm) exist in the organic fraction and that the smaller pores (< 10^2 nm) are mainly present in the clay fraction of soil. They also suggest that qualitative differences in organic matter and clay types should be considered as a factor impacting on sequestration. The concentration of a compound often affects its behaviour in soil. In agreement with the expectation, Chung and Alexander (1999b) reported that, although the sequestration of phenanthrene and pyrene occurred at both low and high concentrations, the amount sequestered increased but the percentage decreased with increasing concentrations.

Hatzinger and Alexander (1997) designed an experiment where synthetic polymers, waxes, and alkanes were selected as models for organic solids and silica particles within an artificial nanoporous network. The results showed that the degree of protection from biodegradation was dependent on characteristics of the sorbents. The rate and extents of phenanthrene partitioning varied markedly between the solids. The rates of partitioning and degradation of phenanthrene initially present in the solid alkanes were positively correlated. The rates and extents of phenanthrene degradation decreased as the % of the compound sequestered in the porous silica particle nanopores increased. However, significant reductions in rate and/or extent of phenanthrene biodegradation were less pronounced than with the nonporous solid sorbent materials. The biodegradation rate of 4-nitrophenol was also found to decrease with increasing concentrations of the compound present in nanoporous cavities.

In another study, Nam and Alexander (1998) employed model systems to test what factors are responsible for sequestration and ageing. The results reported that the rates of biodegradation in the presence of beads having no hydrophobic surfaces were essentially the same as particles in solution. Very little biodegradation occurred when phenanthrene was sorbed to hydrophobic beads made of polystyrene that contained 5 or 300 - 400 nm pores, and the sorption of the compound to the bead was rapid and the desorption very slow. This suggested that the bioavailability of a hydrophobic compound can be markedly reduced by soil types bearing nanopores having hydrophobic surfaces.

Under natural environmental conditions, the time scale of the release of recalcitrant, sequestered or aged residues will be in the same range as the turnover of the humic material they are sequestered in. The turnover of fulvic acid, humic acid and humin in soil is less than 1% (Barraclough *et al*, 2005), therefore the release of a sequestered chemical will be years and as a consequence of the low percentage of turnover the amounts released will be small to negligible.

2.1.5 Mixed mode sorption

Lerch *et al* (1997) demonstrated using atrazine and its degradation products that non-extractable residues can be formed by mixed-mode sorption involving a combination of different binding mechanisms. It was found that hydroxylated atrazine degradation products (HADP) could bind to SOM via both cationic exchange (ionic binding) and hydrophobic interactions (Figure 5). Tightly soil bound residues of triazines have been attributed to initial hydrophobic interactions with SOM followed by ageing of the residues to form irreversibly bound residues. This mixed-mode sorption which involves a combination of different binding sites can, in part, explain the 'ageing' effect, which is characterised by a decrease in extractability of residues with time. It can also explain why some substances could appear to bind more strongly than might have been anticipated due to the addition of a number of interactions (i.e. binding forces) with SOM.

Generally, a higher amount of extractable residues is achieved when multi-step or multi-solvent methods are used. A clear example of this is shown in a study carried out by Cheng (1990) with atrazine. The use of different solvents produced similar extraction amounts between experiments, however, the highest quantity of residue was extracted when all solvents were combined in a multi-step extraction; this was found in four separate experiments under the same conditions. The use of a single extraction method may only remove residues sorbed or bound by a single mechanism, results may therefore indicate that more residue is sorbed or bound than actually is. Those bound by mixed-modes of action could still be left and wrongly assigned as the NER fraction; one mechanism of binding may be mitigated by the extraction method, however the other may still be intact, resulting in inefficient extraction. It is therefore important to select appropriate extraction methods, as discussed within the extraction framework model (presented in Section 4).

Initial hydrophobic interactions can provide a greater chance for further binding to occur via either ageing and sequestration or the formation of more permanent interactions such as covalent bonds. The longer a compound is in proximity with SOM the more likely suitable conditions will occur allowing other interactions or bonds to occur. The soil organic carbon-water partition coefficient (K_{oc}) is currently used to determine the mobility of compounds within soil. Compounds with high K_{oc} values are more likely to be retained in soil and a higher proportion spend more time on soil surfaces than dissolved in pore water. The K_{oc} value may therefore indicate which compounds are likely to spend a longer period in contact with SOM and therefore have the potential to form higher amounts of NER.

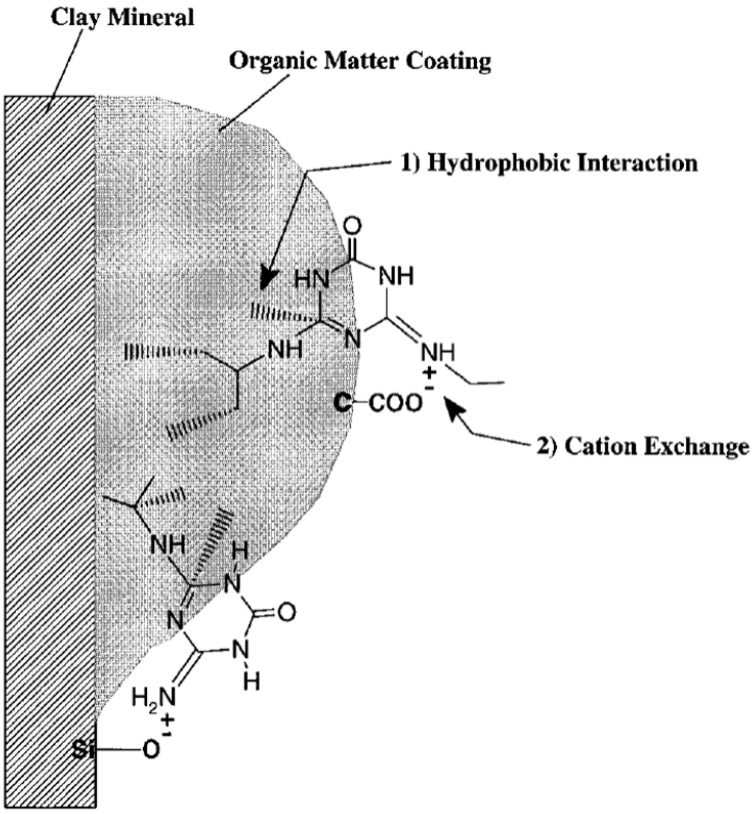
Lerch *et al* (1997) demonstrated, using HPLC (High Performance Liquid Chromatography) with a stationary phase that included silanol groups, how hydroxyl groups on SOM can act as sites of cationic exchange or hydrogen bonding. These groups donated a proton to hydroxylated atrazine products creating a cationic exchange site (the HADP acting as a base or proton acceptor) (see Figure 7). When buffer (pH 7.5) was added the silanol group hydrogen did not dissociate from the hydroxyl group, however, hydrogen bonding occurred

as an alternative to cationic exchange at these sites. Soil water pH and the dissociation constant (K_d) of compounds entering the soil (as well as dissociation constants of SOM groups) have an effect on the reactivity of structures such as hydroxyl groups. These values may affect the NER formation.

In summary, compounds or their metabolites which have 'free' (accessible) reactive chemical groups (-NH_2 and -OH) appear more likely to form larger proportions of non-extractable residues.

Biodegradation of compounds via microorganisms which create metabolites with hydroxyl or amine groups may lead to higher proportions of NER formation. Predicting the likelihood a compound will form non-extractable residues (and in what quantity) based on structural alerts may be a useful tool in the risk assessment of compounds, however, the subject is a very complex one. Whilst the current literature allows discussion on some general trends and mechanisms relating molecular structures to NER formation, it is not extensive enough to allow the prediction of residue formation characteristics such as the type of bonding, etc.

Figure 7: The mixed-mode model for sorption of hydroxylated atrazine degradation products (HADP) to soil (from Lerch et al, 1997)



2.1.6 Influence of activated carbon

Soil organic matter consists of amorphous organic matter (AOM) as well as carbonaceous materials such as black carbon, coal and kerogen. Black carbon is reported to make up to on average 1-20% of soil total organic carbon (TOC) (Nam *et al*, 2008). Generally, black carbon structures are three-dimensional with high

carbon content and low numbers of functional groups (when compared with AOM) (Wang *et al*, 2012). The effects of black carbon (activated charcoal/carbon) on organic contaminant and soil/ soil biota relationships has recently become of great interest due to studies showing its 'super sorbent' like abilities. It is reported to have sorption capacity (for some HOCs) some 10-100 times greater than other types of organic carbon (Cornelissen and Gustafsson, 2004; Jonker *et al*, 2004). Activated Carbon (AC), a form of black carbon, is currently a subject of intense research due to its potential use in soil and sediment remediation. Whilst most studies have focussed on bioremediation uses, there is also interest in natural black carbon and its effects on contaminant bioavailability. The addition of AC (in powdered or granular form, PAC/GAC) to contaminated soil causes hydrophobic organic chemicals to desorb from the soil/ sediment matrix and repartition to the AC particles. Lysimeter (or passive sampler) field trials have mainly focussed on contaminated sediments where amendment of the sediment with low percentages of AC has been shown to sequester PAHs and PCBs and reduce dissolved concentrations and therefore bioavailability (Ghosh *et al*, 2011). Zimmerman *et al* (2004) have also shown the effectiveness of AC amendment in sediments, with just a 3.4% addition reducing dissolved PCB and PAH concentrations by 92% and 84%, respectively. AC amendments to soil have also been studied albeit to a lesser extent. Oleszczuk *et al* (2012) have shown that amendments with AC or biochar could reduce dissolved PAH concentrations by up to 95% and 57%, respectively depending on the treatment dose. Recent field studies show that PAH concentrations in drainage water were reduced by up to 93% with AC amendment when measured with polyoxymethylate (POM) passive samplers. This percentage reduction was also noted in soil pore water measurements (Hale *et al*, 2012). Such studies demonstrate the possible uses for AC/black carbon in soil remediation and for controlling residue bioavailability.

A study by Vasilyeva *et al* (2010) showed that soil amended with AC had reduced PCB concentrations resulting in 71% reduced bioaccumulation. Interestingly they also found that the AC amendment did not reduce the amount of mineralisation of the PCBs by microorganisms (compared with non-amended soils) and so they speculated that the activated carbon may have provided a surface for the reductive degradation of these contaminants. This finding contrasts with other studies which show that AC/black carbon amendment to soils contaminated with ^{14}C-phenanthrene and isoproturon, were subject to decreased contaminant mineralisation (Rhodes *et al*, 2010a; Towell *et al*, 2011; Sopeña *et al*, 2012). Rhodes *et al* (2010a) speculated that the reduction in bioaccessibility (due to residue binding to AC) caused the retardation of catabolic development and thus an overall decrease in mineralisation had incurred. The mineralisation of contaminant residues has been shown to be strongly influenced by previous exposure of a microbial community to contaminants (Macleod and Semple, 2006; Couling, 2010). AC may reduce the bioavailability of contaminants so much as to prevent the development of the microbial biomass's ability to degrade such contaminants. The mineralisation of contaminants in soils amended with AC is likely to be dependent on the amount applied and whether sufficient contaminant is bioavailable for microorganism communities to adapt to.

2.2 Mechanisms which may cause the release of residues

As discussed earlier in this report, irreversibly sorbed residues are residues of a defined chemical entity which are not available via the dissolved phase on a timescale relevant for risk assessment and generally cannot be extracted from soil via the use of non-destructive methods. As summarised in Gevao *et al* (2003), these residues become increasingly unavailable to the biota, less toxic and less likely to desorb from humic

substances in a process termed aging. The reduction in bioavailability and accessibility is beneficial to the environment. However there might be processes which cause a release of irreversibly sorbed residues over time (Khan and Ivarson, 1981, 1982; Yee *et al*, 1985). The current literature considers a number of release mechanisms both abiotic and biotic, and the current scientific knowledge of processes which can cause the release of irreversibly sorbed residues will be discussed. These will be considered in the light of the likely use or exposure pattern of the compounds.

2.2.1 Microorganism release mechanisms

Various studies have investigated the effect soil microorganisms have on the release of slowly desorbable or irreversibly sorbed residues. The majority of studies have focussed on the effect of bacteria on the enhanced release of these residues; however fungi such as *Penicillium frequetans* which degrade soil organic matter (SOM) were also investigated (Mathur and Paul, 1967). Such studies give an insight into the mechanisms and potential rate of release of residues but the use of cultures and laboratory soils mean that the wider significance of the findings has to be carefully contextualised. For instance, sieving soils prior to use in laboratory studies may impair certain microbial communities, the influence of introduced species may therefore not reflect what would happen under realistic use conditions in the field.

The methods most frequently used involved tagging organic molecules with ^{14}C to allow quantitative study of their release over a set incubation period. Multiple studies have focussed on the release of chlorophenol residues from humic material over time (Dec and Bollag, 1988; Dec *et al*, 1990; Bollag, 1991). Dec and Bollag (1988) bound four different chlorophenols to a synthetic humic acid polymer, these were then incubated with microbial cultures naturally found in soil for a period of 13 weeks. The amount of residue released was between 0.4 - 12.4%, varying with the chemical structure and inoculate. In addition to this, between 1.2% and 10% was mineralised to CO_2. Further studies by Dec *et al* (1990) investigated ^{14}C-labelled-2,4-dichlorophenol that was sorbed to both natural and synthetic organic matter. The amount of ^{14}C labelled residue released was very low with a maximum of 2.2% released over a 12-week incubation period (incubated with forest soil microorganisms). In addition, the amount of CO_2 evolution reached 0.5 - 9.4% of the initial NER. Both papers suggest that the release of sorbed chlorophenols is not significant enough to be a health hazard and occurs slowly over a long period of time.

Khan and Ivarson (1982) compared the amount of slowly and irreversibly sorbed ^{14}C (prometryn) residues released by four types of microorganism found in different types of soil. Little difference was found between the physiological groups of organism. After 28 days, 23.5 - 27.1 % of sorbed ^{14}C was released into the soil substrate with between 1.4 - 3.0 % of the total NER being mineralised to $^{14}CO_2$. The similarity in the release between the various species of microorganism indicates a common mechanism for releasing slowly or irreversibly sorbed residues. In this case Khan and Ivarson showed evidence that the sorbed residues were metabolised via hydrolysis and partial N-dealkylation and that various classes of microbes found in soil have the ability to release sorbed residues. Other work by this research group (Khan and Ivarson, 1981) on the herbicide prometryn, similarly resulted in 27% of slowly and irreversibly sorbed residues being released by microbes over a 22 day incubation period. Results indicated that microbes had attacked the bonding between prometryn and SOM leaving the labelled triazine ring intact within the substrate. The release of the residues was succeeded by mineralisation to CO_2 via hydrolysis and dealkylation. Whilst newly released

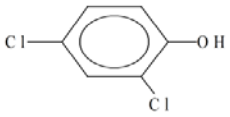
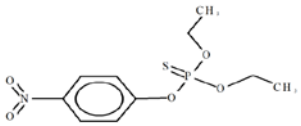
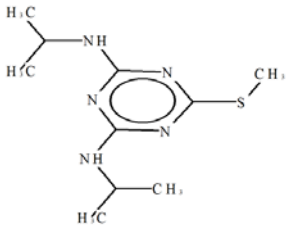
residue is degraded by microbes as any other, it is also accessible to other biota within the proximate environment and can therefore be considered as bioavailable and bioaccessible as previously defined by this report. Interestingly Khan and Ivarson (1981) found that a small percentage of the irreversibly sorbed and/or slowly desorbed residues quantified at the end of the study were derived from biodegradation of prometryn.

Bartha *et al* (1983) showed that via the action of microbes, previously sorbed residue of 3,4-dichloroaniline (DCA) was released and taken up by crops. Racke and Lichenstein (1985) carried out experiments on ^{14}C parathion treated soils and found that again, microorganisms in soil were key to the release and metabolism of slowly desorbed or irreversibly sorbed residues. They showed that sterilised soils did not release any $^{14}CO_2$, whereas soils inoculated with microorganisms mineralised 22.74% of the initial sorbed residues to CO_2 in four weeks. Further to this, soils treated with compounds shown to increase or decrease microbial activity produced 10 - 22% more or 9 - 35% less $^{14}CO_2$ evolution than control soils. After four weeks incubation 5.7 - 6.4% of initially sorbed residues were extractable. Follow up investigation into the effects of manure on the release of initially sorbed residues and subsequent uptake into oat plants was made (Racke and Lichenstein, 1985). The addition of manure increased the amount of extractable residues by 7% in addition to an increase to 27.8% (from 16.6%) of initially sorbed residues mineralised and evolved as CO_2. This is most probably attributable to the increase in microorganism number after manure application.

Research on various organic compounds suggest that a variety of microorganisms have the ability to mineralise initially sorbed residues either *in situ* and/or still bound to the matrix; the exact mechanisms are however not clear and are probably specific to the combination of microorganism, the matrix, e.g. soil organic matter and the kind of sorption or bonding of the sorbed residues to the matrix (Racke and Lichenstein, 1985; Bartha *et al*, 1983; Khan and Ivarson, 1981, 1982).

Eschenbach *et al* (1998) investigated various factors to be taken into account when assessing the risk of slowly desorbed or irreversibly sorbed residues, in this instance using polycyclic aromatic hydrocarbons (PAH). They found that residues that were released were predominantly mineralised to CO_2 by microorganisms found in soil and that the addition of organic supplements such as PAH specific metabolising bacteria did not enhance the mineralisation rate. The residues which were not released (e.g. still bound to humic substances) were mineralised at a rate similar to the natural metabolic rate of soil organic matter (SOM). These findings as well as the differing residue release / mineralisation ratios between studies suggest that the release of residues and/or the mineralisation of initially sorbed residues are by-products of normal soil microorganism activity rather than microorganisms specifically targeting the sorbed residues. The lack of an increase in the release / mineralisation of sorbed residues with the addition of specific PAH targeting microorganisms may suggest that within a soil structure there are a limited number of 'sites' that soil microorganisms may attack at any moment in time. Whether those residues are released depends on how and where (in relation to these sites) they are sorbed to the matrix (in this case SOM). There appears to be a distinction between residues which are released into an extractable form and those that are metabolised *in situ* and thereafter sorbed to the SOM which may reflect the differing metabolisms of microbial species. A summary of the effects of microorganisms on various chemical compounds is shown in Table 2. Whilst the methods used do not provide comparable data, it is still clear that some sorbed residues are more susceptible to release than others and the large variety highlights the complexity of the issue.

Table 2: A comparison of release and mineralisation of NER

Chemical class	Chemical structure	Time period	IBR Released (%)	IBR mineralised (%)	Reference
Chlorophenol		13 weeks	0.4-12.4	1.2-10	Dec and Bollag (1988)
2,4-Dichlorophenol		12 weeks	2.2	0.5-9.4	Dec *et al* (1990)
Parathion		4 weeks	5.7-6.4	22.74	Racke and Lichtenstein (1985)
Prometryn		4 weeks	23.5-27.1	1.4-3.0	Khan and Ivarson (1982)
		3 weeks	27	-	Khan and Ivarson (1981)

2.2.2 Physical release mechanisms

Whilst research indicates microorganisms have an effect on the release of NER, there are other mechanisms of release to consider. Eschenbach *et al* (1998) investigated the effects of mechanical disruption on the release of slowly desorbed and/or irreversibly sorbed residues using ultrasonic waves. Whilst this appeared to increase the bioavailability of residues which were in a reversibly bound state (i.e. slowly desorbed), irreversibly sorbed residues were not released in greater quantities. Scientific literature on the mechanical release of irreversibly sorbed residues (such as tilling, freeze-thaw or wet-dry cycling), is scarce, however there are studies which investigate the effects these processes have on general soil structure, especially on SOM.

The incorporation of NER into SOM (covalently bound or physically sequestered) has been discussed previously. Whilst studies on the effects of tilling on NER release are few and far between, studies into the effect of tilling on SOM turnover have been conducted; the possible effects of tillage on NER release through this relationship can therefore be inferred. Balesdent *et al* (2000) investigated the relationship between SOM, tillage and wet-dry cycling. The authors reported that conventional tilling doubled the mineralisation rate of SOM over a period of 17 years and credited the breakup of physically protected SOM 'pools' with this increase. The breakup of the protected SOM by tilling could therefore release physically sequestered NER (via physical release). Evidence suggests that SOM breakdown is faster within a soil under field conditions than under artificial conditions, i.e. in laboratory (Balesdent *et al*, 2000). Additionally, an important factor in the breakdown of SOM is its position within the soil matrix. Therefore any physical processes such as tilling or wet-dry cycles have the potential to expose previously protected SOM (and by association NER which were entrapped) to biodegradation and mineralisation.

It is already known that, over time, organic compounds can become increasingly unavailable and sequestered in soil in a process termed ageing (Gevao *et al*, 2003). A possible mechanism for this is highlighted in the work of Lennartz and Louchart (2007). Whilst this study did not address the issue of irreversibly sorbed residues, *per se*, it does highlight possible mechanisms for the sequestration of residues during repeated wetting and drying cycles. "*Water content variations and corresponding hydraulic pressure effects modify the structure of the SOM in a shrinking-like process and diffusion is hindered with molecules getting trapped"* (Lennartz and Louchart, 2007). It would appear as though the process of drying 'locks in' residues which may be within the soil pores at the time of drying (increasing the time for strong bond formation and irreversibly sorbed residues formation) or 'lock out' those which would be considered desorbable, releasing them into the soil matrix. The study goes on to show that each drying cycle adds to this shrinking effect allowing further sequestration of residues. During wet periods, slow re-wetting of the SOM can lead to recovery and swelling, reducing the pore shrinking effect and potentially leaving irreversibly sorbed residues more vulnerable to release. The process of soil ageing may be a consequence of the repeated exposure of soil to drying conditions over time.

The hypothesised sequestration of pesticide residues via 'ageing' through natural processes such as wet-dry cycling, promotes thoughts as to whether similar natural processes can also cause the release of irreversibly sorbed residues. Jablonowski *et al* (2012 a,b) found that previously aged soils treated with repeated dry-wet cycles released higher amounts of pesticide residue than those which were maintained consistently moist after water extractions (3.45% compared with 1.45% of initial parent compound ETD (Ethidimuron) and 8.2% versus 1.9% with atrazine). Therefore, constant drying and re-wetting appears to cause more structural changes within the soil, releasing more of the sequestered NER proportion. It is important to consider that pesticide residue was still released even in the constantly moistened sample; this release may have been the baseline microorganism release rate, or maybe a slower physical release of NER through slow restructuring of the soil whilst moist. It is unclear whether the increase in release of NER with dry-wet cycling is due to physical release processes of physically sequestered NER or through the exposure of irreversibly sorbed residues to microorganism attack.

Fierer and Schimel (2002) investigated the effect of wet-dry cycles on the mineralisation of SOM in two varieties of soil (grassland soil and soil under tree cover). It has been observed that wet-dry cycles can either increase or decrease the mineralisation of SOM depending upon the soil type. In either case, after repeated rapid re-wetting, the amount of CO_2 released in a 'pulse' (after multiple dry-wet cycles) decreases. It is not clear why this is the case but Fierer and Schimel suggest two explanations; microbes which can withstand the osmotic shock may over time be selected for, reducing microbial cell death (reducing substrate for surviving microbes). The other theory is that the dry-wet cycle may release previously sequestered SOM (including physical and chemical NER); repeated cycles over time may mean less is available during subsequent cycles, reducing CO_2 mineralisation. Either hypothesis is important to consider within the context of NER release. Constant drying and re-wetting can cause microbial community changes resulting in an overall decrease in respiration rates and mineralisation of SOM (including NER). Whilst drying and re-wetting may release more SOM (and therefore NER), this should coincide with a microbial mineralisation 'pulse' brought about by the influx of resources. The ratio of NER release to the rate of its mineralisation would therefore be an important relationship to consider in risk assessment. This ratio will depend on the soil type and the capabilities of the microbial communities within it to respond to repeated dry-wet cycles (Fierer and Schimel, 2002).

As well as wet-dry cycles, soils can also be exposed to freeze-thaw processes. Zhao *et al* (2009) found when investigating two varieties of Chinese soil, freeze-thaw cycles had a variety of effects on the release of PAH. In one type of soil, less PAH was released for extraction (2.9 - 14.4% more NER) whereas another type of soil had an increased amount of extractable PAH (0.03 - 10.1% less NER). Other studies have shown similarly disparate results demonstrating that freeze-thaw cycles can inhibit hydrocarbon biodegradation temporarily, but ultimately lead to an overall increase compared with soils kept above freezing (Eriksson *et al*, 2001; Feng *et al*, 2007). This is probably attributable to the structural alteration of the soil (upon freezing) releasing previously protected SOM (and NER); it is of note that Eriksson *et al* (2001) studied low carbon number alkanes, more volatile than PAHs and potentially more easily released from soil pores. It was found that the freeze-thaw cycle treated soils had altered microbial communities; at least one species of bacteria with advanced tolerance of freezing conditions became more prevalent within the community. Both studies found no change in the overall bacterial biomass; however, Feng *et al* (2007) discovered a reduction in the fungal biomass. This demonstrates the potential of microorganism communities to adapt to a variety of conditions and maintain similar levels of biodegradation. Desorption of residues due to wet-dry or freeze-thaw cycles may mean that release of residues in laboratory experiments is underestimated. However, there is no real evidence from field studies that such processes lead to huge flashes of desorbed material being released.

2.2.3 Other release mechanisms

Other processes which can affect the release of slowly desorbed or irreversibly sorbed residues have also been investigated (Yee *et al*, 1985). Large pH changes were shown to release up to 9.3% more ^{14}C than small pH changes. Both the size and shape of organic soil materials have been shown to be affected by changes in pH (Chen and Schnitzer, 1976). Yee *et al* (1985) hypothesised that residues previously sequestered in soil voids may be released by structural changes in the soil caused by large pH changes. It was suggested that this was due to both ionic interactions (from buffer solutions) which act to disrupt humic acid structure, and a larger scale soil structural shift brought about by the overall soil pH. Within the same study, experiments involving the addition of both ionic and non-ionic fertiliser suggest ionic interactions are important. Ionic fertiliser caused a much greater release (16.7%) of sorbed residues than non-ionic (0.4%) fertiliser compared with controls.

Plants have been shown to cause the release of slowly desorbed or irreversibly sorbed residues. Studies have been carried out using soy bean, oat and wheat seedlings. All have been shown to illicit the release of sorbed residues, with an increased release range of 0.14 to 5.1% above controls (Fuhremann and Lichtenstein, 1978; Helling and Krivonak, 1978; Roberts and Standen, 1981). Whilst these studies have shown a release of slowly desorbed or irreversibly sorbed residues associated with plants, the mechanism of release was not clear. The studies of Yee *et al* (1985) and Weinberger and Yee (1984) show that plants can effect (albeit small) pH changes in soil proximate to their root and that changes in pH can increase the release of sorbed residues as discussed previously. Another hypothesis is that plant roots induce increased microbial activity which has been previously correlated with the release of sorbed residues. The increase in release may be a function of both the increased microbial activity and the pH changes.

2.2.4 Further studies

A number of studies have shed light on possible mechanisms of the release of slowly desorbed or irreversibly sorbed residues. Whilst investigations have been undertaken into the effect of microorganisms, plants, pH changes and the addition of fertiliser on the release of sorbed residues, other common farm practices which would cause mechanical disruption like tilling have not been specifically studied. There are studies which address the effect these physical processes have on SOM, however, using our understanding of the interactions of sorbed residues with SOM the probable effects on the release of sorbed residues can be inferred. The current literature suggests the release of slowly desorbed or irreversibly sorbed residues is minimal and that what is released is quickly mineralised or degraded into harmless products via microorganisms, therefore posing no risk to the environment. However, it is unclear what effect large scale practices can have on the release of sorbed residues. It has been shown that tilling instead of ploughing can in fact reduce the leaching of atrazine under heavy rainfall by up to 75% (Warnemuende *et al*, 2007).

Currently, studies on the different mechanisms of release of slowly desorbed or irreversibly sorbed residues are generally not comparable. Data on the release of residues by microorganisms are varied, with studies carried out over differing time periods and conditions.

2.3 Chemical structures and their relationship with binding mechanisms (i.e. 'structural alerts')

Due to their extensive registration requirements, plant protection products are the best evaluated chemicals. Due to the huge variety of compounds in use, a wide range of functional moieties are present in their molecular structures. In a review, Barriuso *et al* (2008) tried to determine the relationship between different plant protection product families (with common structural moieties) and the formation of NER.

The data analysed was obtained from a wide variety of studies which used a range of extraction techniques. There is therefore large variability in what each study defines as extractable residue and as the authors noted, the percentage not extracted cannot necessarily be differentiated into slowly desorbed, irreversibly sorbed or assimilated residues (as defined in this report). Instead trends and patterns for NER within these data sets were reviewed.

After review of the literature and EU plant protection product registration dossiers it was found that NER formation was greatest for compounds in the carbamate and dithiocarbamate families. These molecules have electron-rich functional groups, such as a double bonded oxygen or sulphur. These reactive groups may be more likely to interact with similar sites in SOM. These reactive groups are generally polarised in some form and are therefore more likely to interact with SOM via electrostatic forces (ionic bonding) or hydrogen bonding. It was found that dinitroanilines formed the lowest proportion of NER when compared with other families. The abundance of electronegative (electron withdrawing) functional groups within the dinitroanilines may reduce any polarising effect. In agreement with Berry and Boyd (1984) it was found that the presence of reactive groups such as aniline or phenol generally led to a higher percentage of NER formation. Similar compounds containing N-heteroatomic rings as opposed to phenyl structures formed less NER in comparison. The N-heteroatomic ring is more electronegative than the phenyl ring and draws

electron density away from the external functional groups, which can reduce polarisation and therefore the reactivity of the functional groups. Barriuso *et al* (2008) stated that when calculating NER formation it is important to consider the position of radioactive (usually ^{14}C) labelling. In molecules with both phenol and pyrimidine rings, labelling on the latter caused an apparent under recording of NER formation as the pyrimidine is less conducive to NER formation than the phenol (due to their respective reactivities). No attempt was made by the authors to assess the extent to which NER formation was influenced by the rate and extent of mineralisation and potential for incorporation into the biomass. The formation of incorporated residues is considered in more detail in Section 3.7.

In other studies PAHs are described as relatively inert in terms of forming large proportions of NER, however, microbial biodegradation can result in more reactive hydroxylated polyaromatic compound metabolites. With respect to the formation of NER, hydroxylated metabolites of PAHs are generally more reactive than the parent molecule and have been shown to be involved in covalent bonding with soil organic matter (SOM) (Richnow *et al*, 1997). When considering compounds such as PAHs, it could be suggested that NER formation is directly related to microorganism biodegradation rates. As well as generally increasing the amount of reactive metabolites, there is also some evidence to suggest microorganisms play a role in some mechanisms of covalent bond formation between residues and SOM.

Berry and Boyd (1984, 1985) suggest other enzyme mediated reactions are important. Phenols and anilines may undergo oxidative coupling via peroxidise enzymes. This reaction is shown to be strongly influenced by proximity to nucleophilic and electrophilic substituents. Electron donating nucleophilic groups can enhance the reaction whilst electron withdrawing groups may hinder it. In addition, reactive groups on aromatic rings such as hydroxyl can be deactivated to an extent by electron withdrawing groups attached in the *ortho* position.

Bollag *et al* (1992) suggest that the formation of covalently bound residues is a similar process to that of humification. Plant protection products which are most likely to bind to SOM have chemical functionalities similar to humus components such as fulvic or humic acids. It is believed that oxidative coupling reactions can incorporate organic pesticides and their metabolites (phenols, anilines) just as in humification (via covalent bonding). Oxidative coupling can happen in a variety of ways, however, it is generally when compounds are linked together after they have been oxidised, either by enzymes or chemical agents. The oxidation of phenols and anilines leads to the formation of free radicals; these have the ability to react with other molecules creating free radical intermediates. These reactions can result in C-C and C-O bonds (phenols) or C-N and N-N bonds (aromatic amines). Generally, OH groups are oxidised and turned into free radicals by the removal of the H^+ and its electron, this leaves an oxygen free radical which is very reactive towards similar groups on other plant protection product molecules (forming oligomers and polymers) as well as similar groups within the SOM. These reactions can be initiated by microbial and plant enzymes as well as metal ions with oxidising potential. Coupling can also occur randomly, only requiring a neutral to alkaline pH and free oxygen. Once a free radical is created it is more likely to attack electron rich regions such as double bonds (such as in carbonyl groups) via mechanisms depending on its structure (these may include nucleophilic addition, nucleophilic conjugation addition or nucleophilic acyl substitution). The usual result is the creation of a nucleophilic oxygen atom which can attack double bonds via nucleophilic mechanisms, attaching whatever compound it is part of, for example; a radicalised phenol group may attack an oxygen double bond within SOM, and form a covalently bound residue (Bollag *et al,* 1992).

Besides covalent bonding, molecules can also adsorb to the matrix, e.g. SOM, via a variety of mechanisms depending on their structure.

Adsorption strength can vary from rapidly desorbable to total irreversibility; this depends on the properties of the matrix, e.g. SOM, and the molecule itself. Properties like shape, size, polarity, configuration, solubility, presence of functional groups, all have an influence (Senesi, 1992). The adsorption properties of SOM and plant protection products can affect both the biodegradability and percentage of NER formation as discussed earlier.

2.4 Conclusion

Many different mechanisms are responsible for the formation of irreversibly sorbed residues. The nature and the amount of interactions between a chemical and the matrix will determine the binding strength of the residues. These interactions depend on the compound itself, as well as matrix constituents and the physical / chemical properties of the matrix, and the period of ageing.

The three main types of bindings involved in the process of NERs are:

- sorption between the chemical compound and/or its degradation products and the matrix, i.e. soil organic matter:

  This process should be reversible. In other words the chemical should be extractable. In general the binding energies will be low (< 50 KJ/mol). Due to its chemical nature (polar, non-polar) the compound should be extractable with the corresponding solvent. There are only a few examples where binding energies were too high and therefore the extraction was not possible or insufficient.

- the covalent binding of chemical compound and/or its degradation products to the matrix:

  This process should be irreversible. High energies will be necessary to break the covalent bonds (> 100 KJ/mol). Therefore the covalent bond chemical is not extractable. Harsh extraction methods like strong acids or bases will be needed to break down these bonds. Those harsh extraction methods will most probably also destroy the matrix and the risk of producing processing artefacts with no environmental relevance is unavoidable.

- the physical sequestration of chemical compound and/or its degradation products into the matrix:

  This is also considered to be an irreversible process. The altered or sequestered compound could only be released from the matrix into the environment by destroying the matrix itself. Since less than 1% of the soil organic matter is converted in one year, release of an entrapped compound from soil into the environment will most probably be ‘very small to negligible’. To obtain information about the potentially entrapped unaltered compound different techniques, i.e. silylation, were applied. The harsh methods which are needed to destroy the matrix mean that such techniques, however, do carry the inherent risk of producing experimental artefacts.

3. EXTRACTION METHODS AND THEIR RELATIONSHIP WITH BIOAVAILABILITY

3.1 Introduction

In order to design a suitable extraction method to identify when a chemical will pose a risk to the environment, a number of different methodologies were investigated. Currently extraction methods are performed with the main aim of removing the maximum amount of test material from the matrix in question. However, during a risk assessment the values are used to refine the predicted concentration of the chemical in environmental matrices, and to relate this to the exposure of species in the environment. Extraction regimes are variable and little consideration is generally given to the biological relevance of the extraction methods employed when performing environmental studies. This investigation was performed to enable reasoned and rational choice of extraction method during environmental studies, in order to provide a realistic representation of the exposure scenario. This review of methods suggests a pathway for scientists to utilise, in order to assist in relating the extraction method to 'real world' situations. However, a combination of extraction methods should be used to inform the specific environmental investigation and endpoint being investigated.

3.2 General considerations for soil extraction methods

The distinction between 'bound' residues, and extractable residues is dependent on the extraction regime employed (Gevao *et al*, 2000; Northcott and Jones, 2000; Frische *et al,* 2003). An extraction regime should be selected after consideration of the physico-chemical properties of the test substance being investigated. Information including, (but not limited to): solubility in different solvents; pK_a; chemical structure; partitioning data (K_{ow}/K_d) can be utilised to aid the development of a robust extraction strategy. Similarly, the characteristics of the matrix being extracted require consideration, particularly in respect to: organic carbon (OC) content, cation exchange capacity (CEC), clay type and proportion, pH and moisture content. Through identification of significant drivers present in a test system and inherent to the test substance, the partitioning and subsequent availability (Frische *et al,* 2003) the test design can be tailored to the test conditions. Prior to designing an extraction regime, collation of all available chemical data from past studies is suggested, and investigation into methods utilised for similar chemical classes and structures is advised to aid selection of suitable extraction solvents. In many cases, development of a suitable extraction method can be performed prior to commencing an expensive regulatory study in order to optimise the extraction regime.

One of the major challenges of designing suitable extraction regimes for test substances is the potential for the extraction process to break down the parent test substance (Fomsgaard, 2004). The necessary method development should be performed to understand this potential.

The extraction methodology should provide an empirical basis to understanding the behaviour of residues in soils as related to bioavailability and bioaccessibility. In that respect, a number of authors have compared the quantities of compounds removed by solvent extraction with those mineralised by specific degrading

microbes. Microbes provide a good estimate of the total bioaccessible (total reversibly sorbed residue) residue. The advantage of this approach is that the two approaches can be directly compared as the microbes can access a similar volume of the soil as the solvent, which is not the case when larger organisms such as earthworms are utilised.

A further area of uncertainty is where the boundary between the slowly desorbed and irreversibly sorbed fractions lies and hence the boundary between bioaccessible and non-bioaccessible residues. This boundary can be determined by sophisticated batch equilibrium experiments utilising isotope exchange techniques to differentiate between reversible and irreversible fractions. However, these approaches are time consuming and complex and it is therefore likely that solvent extraction based approaches would still be required.

Soil moisture levels can also influence sorption of chemicals to soil (and therefore the amount that can be extracted) (García-Valcárcel and Tadeo, 1999; Schroll *et al*, 2006). García-Valcárcel and Tadeo (1999) demonstrated that repeated wetting and re-drying increased the sorption of hexazinone and simazine herbicide residues to soil and that this process also reduced residue degradation. Other studies have shown increased sorption of imazaquin to drier soils (Goetz *et al*, 1986).

The effects of soil moisture on extraction efficiencies are dependent on the type of extraction technique employed. With microwave assisted extraction (MAE), extraction of PAHs was observed to increase with increasing soil moisture. This is due to the ability of the localised superheating to form gas bubbles from existing water residues in soil and cause expansion of pores, allowing solvent penetration into the matrix. On the other hand, supercritical fluid extraction (SFE) and Soxhlet extraction studies have shown that the presence of soil moisture decreased or did not affect the efficiency of PAH removal from soil (Lau *et al*, 2010). These authors propose that soil drying is carried out to eliminate the influence of moisture on the extraction efficiencies of PAHs. Chiba and Morley (1968), however, show lower residues are extractable from drier soils when using an acetone/n-hexane solvent system. This relationship, however, was not noticed when dimethylformamide was used as an extractant. It would therefore appear that the relationship between soil moisture and residue extractability is relatively complex.

3.3 Freely dissolved fraction

Within the bioavailability definition, the dissolved residue pool is that which exists in equilibrium with the rapidly desorbable fraction. Freely dissolved residues in soil or sediment pore water are available for partitioning and considered the most bioaccessible residue pool. Reichenberg and Mayer (2006) have equated freely dissolved concentrations with chemical activity (fugacity) and discuss the relation in terms of overall bioaccessibility. Subsequent discussions relating freely dissolved residues to organism uptake are based on Equilibrium Partitioning (EqP) theory which was initially proposed by Shea (1988) and further developed by DiToro *et al* (1991). EqP was developed to explain the variation in sorption behaviour of organic chemicals in environmental compartments. The theory proposes that organic chemicals that are sorbed to soil or sediment are in equilibrium with the aqueous phase or pore water and is the same aqueous phase to which benthic and terrestrial organisms are exposed.

Extraction techniques which replicate partitioning between the dissolved and rapidly desorbable residue pools are best suited for measuring freely dissolved concentrations and are commonly referred to as biomimetic extractions. Solid phase microextraction (SPME) is the most often cited extraction technique for isolating dissolved concentrations in pore-water at equilibrium with surrounding soils and sediments. In their evaluation of biomimetic extraction techniques Sijm *et al* (2000), consider SPME to be one of the principle means of estimating bioavailability to soil and benthic organisms. Patented by Pawlisyn in 1990 and commercialised by Supelco some years later, SPME is a partition-based extraction technique well suited to differentiate between truly dissolved concentrations and those present in bound, less bioaccessible residue pools. SPME can be applied in a variety of formats including fibres, thin films and stirrer bars. SPME devices are commercially available or can be prepared for specific applications. Typical applications utilise small lengths (1 - 3 cm) of narrow diameter (100 - 200 µm) glass fibres coated with a non-polar polymeric phase ranging from 7 - 100 µm in thickness. Common stationary phases include polydimethylsiloxane (PDMS), polyacrylate and polyoxymethylenes (POM). After the SPME device is equilibrated with a sample, it is thermally desorbed in the injection port of a gas chromatograph or desorbed with a small volume of organic solvent for analysis.

A number of publications describe the application of SPME to measure freely dissolved concentrations for correlation with bioaccessibility. Hunter *et al* (2006) and Bondarenko *et al* (2006) used disposable polymeric coated fibres to measure freely dissolved concentrations of pyrethroids in water and sediments to assess bioavailability and acute sediment toxicity. A number of researchers refer to 'matrix-SPME' to emphasise that the fibre is deployed directly in the sample so the entire soil or sediment matrix is available as a reservoir for equilibrium extraction. Mayer *et al* (2000) and You *et al* (2007) both applied this technique to measure freely dissolved concentrations in pore water to measure the chemical activity of a series of hydrophobic and persistent, bioaccumulative compounds in both spiked and field contaminated sediments. The latter also used Tenax extractions to quantify the rapidly desorbed fraction. Yang *et al* (2008) used a porewater sampler to measure freely dissolved concentrations of twelve representative hydrophobic organic compounds including PAHs, PCBs and chlorinated insecticides spiked into sediment. The sampler consisted of commercially available SPME fibres of increasing film thicknesses secured in a protective mesh housing to permit aqueous exchange while eliminating direct contact with the sediment. They also compared SPME recoveries with those of liquid-liquid extraction (LLE) of pore water. In general, SPME yielded lower recoveries relative to LLE. This was attributed to SPME measuring truly dissolved concentrations while LLE also extracted compounds associated with dissolved organic matter.

Though the majority of published applications deal with sediment pore water, additional work has been described using SPME to measure dissolved concentrations in soil pore water. Fang *et al* (2010) compared matrix SPME with hexane-Soxhlet, butanol agitation and water agitation extractions for measuring the estimated the bioavailability of DDT (dichlorodiphenyltrichloroethane) in soil to earthworms and vegetables. Van der Wal *et al* (2004) used 30 µm PDMS SPME fibres to measure freely dissolved concentrations in pore water from field soils contaminated with HCB (hexachlorobenzene), dieldrin and a series of PCBs. They also correlated dissolved concentrations to bioavailability in earthworms. Styrishave *et al* (2008) used SPME to predict the availability and toxicity of pyrene to springtails in soils with varying amounts of organic matter and soil ageing durations.

When assessing freely dissolved concentrations as a measure of bioavailability, it is important that SPME be applied in a non-depletive or negligible depletion mode (nd-SPME). Negligible depletion generally refers to a decrease of less than five percent of the concentration of a specific compound in pore water. The ratio of SPME stationary phase relative to the mass of soil/sediment and associate pore water must be sufficiently small so as not deplete the aqueous phase which would result in active replenishment from the contaminated matrix. Hermens *et al* (2001) introduced the application of nd-SPME for estimating bioavailability and bioaccumulation of individual chemicals and mixtures. Heringa and Hermens (2003) provide a review of theoretical and experimental details associated with negligible depletion SPME for measuring bioavailable quantities of chemicals. Yang *et al* (2008) refers to negligible depletion SPME in experimentally verifying a model predicting freely dissolved pore water concentrations of sediments spiked with radiolabelled phenanthrene, DDE and two PCB congeners.

3.4 Rapidly desorbed fraction

Extraction methods employed to identify the 'rapidly desorbed' fraction of the test system are selected to consider the material that partitions from the solid matrix into the soil pore water, or aqueous fraction of the environment, since soil organisms live mainly in the soil pore water, or utilise this matrix as a substrate. The rapidly desorbed fraction is most suitable for assessment of bioavailable test material to soil organisms by utilising 'mild' solvent extraction (Katayama *et al,* 2010), and less exhaustive extraction methods. These extractions are designed to be simple and non-destructive, appropriate agitation methods include shaking or rolling at nominal room temperature.

The initial extraction solvent should utilise extraction solutions that mimic the composition of pore water, comprising of weak salt solutions. Reported salts and solution concentrations vary (Table 3).

Table 3: Soil pore water extraction solution

Salt solution	Concentration (M)	Reference
Calcium chloride ($CaCl_2$)	0.01	Smith *et al* (2010) Krishnamurti (2008) Peijnenburg *et al* (2007) Barriuso *et al* (2004) Houba *et al* (2000)
	0.05	Krishnamurti (2008) Cheng (1990)
	0.1	Peijnenburg *et al* (2007)
Calcium nitrate ($Ca(NO_3)_2$)	0.1	Peijnenburg *et al* (2007) Lanno *et al* (2004)
Ammonium acetate (NH_4Ac)	1.0	Peijnenburg *et al* (2007)
Mg-salts	n/a[1]	Peijnenburg *et al* (2007)
Barium chloride ($BaCl_2$)	n/a[1]	Peijnenburg *et al* (2007)
Sodium nitrate ($NaNO_3$)	0.01	Yin *et al* (2002)
	0.1	Peijnenburg *et al* (2007)
Ammonium nitrate (NH_4NO_3)	0.1	Peijnenburg *et al* (2007)

[1] n/a: not available

A 0.01M $CaCl_2$ solution has been documented as the preferential extraction solution because the concentration of Ca^{2+} ions is similar to that measured in soil pore water solutions (Peijnenburg *et al*, 2007) and a similar ionic strength (Houba *et al,* 2000).

The application of solvent extraction as a method to estimate the bioavailability of atrazine was investigated by Barriuso *et al* (2004). Atrazine was aged in two soils for up to 8 weeks. Samples were either not extracted, extracted with 0.01M $CaCl_2$, or extracted with 0.01M $CaCl_2$/aqueous methanol, then inoculated with *Pseudomonas* sp. strain ADP. In both soils, samples that were not pre-extracted, more atrazine was mineralised than was extracted by 0.01M $CaCl_2$ but less than was extracted by 0.01M $CaCl_2$ followed by aqueous methanol (80:20 v/v).

The same group subsequently used a similar approach with simazine (Regitano *et al*, 2006). Six contrasting soils were incubated with simazine. At various timepoints they were either twice extracted with 0.01M $CaCl_2$ followed by aqueous methanol (80:20) or incubated with atrazine degrading *Pseudomonas* (ADP), which can also mineralise simazine. The soils incubated with ADP were subsequently extracted with the same solvent system used for the non-inoculated samples. As demonstrated previously with atrazine, the entire $CaCl_2$ extractable residue was mineralised as was most, but not all, of the solvent extractable residue. With time, the aqueous methanol available residue increased in the ADP inoculated soils in contrast to the non-ADP treated samples in which the methanol extractable residue continued to decline. This suggests that the residue available to ADP was declining with time and that aqueous methanol was removing more of the residue with time than was bioaccessible to ADP.

3.4.1 Extraction methods in relation to bioavailability

Several authors have tried to relate an extraction method which could reflect the amount of residues bioavailable for earthworms. Ehlers and Loibner (2006) and Lanno *et al* (2004) have reviewed these methods.

Chemical extractants

Kelsey *et al* (1997) has conducted extraction with chemical mixtures and has found that methanol-water and n-butanol extraction of phenanthrene and atrazine were the methods providing the best correlation with uptake by earthworms. The same was observed for PAH in the study of Tang and Alexander (1999) with the use of n-butanol, propanol or ethyl acetate. Other studies report butanol and cyclodextrin extraction to be better predictors of the bioavailable fraction of PAH for earthworms (Gomez-Eyles *et al*, 2010, 2011).

Solid-Phase Extraction (SPE) and Solid-Phase Micro-Extraction (SPME)

SPME fibres (van der Wal *et al*, 2004; Jonker *et al*, 2007), triolein embedded cellulose acetate membrane (TECAM) (Tao *et al*, 2008, 2009), Tenax devices (Morrison *et al*, 2000; Gomez-Eyles *et al*, 2010; Liang *et al*, 2010) and C18 membrane disks (Tang *et al*, 1999; Liste and Alexander, 2002) have been placed in soil to assess the bioavailability of chemicals.

For most of the experiments, the amount of residues adsorbed on the devices correlated well with the uptake in earthworms. In the studies of Tao *et al* (2008, 2009) the relationship between concentrations of PAHs in TECAM and uptake in earthworm was close to 1:1.

Supercritical Fluid Extraction (SFE)

The SFE technique is used for the extraction of chemicals from environmental matrices, such as soil, without altering the soil organic matrix. The amount of PCBs extracted by SFE with CO_2 as extraction gas (50°C, 350 bar, 60 min extraction) was very close to the calculated bioavailable fraction (Hallgren *et al*, 2006). Sun and Li (2005) also observed a good linear relationship between extractability of pyrene by SFE with CO_2 as extraction gas (40°C, 138 bar, 20 min extraction) and uptake by earthworms in aged soils.

According to Sijm *et al* (2000), the most relevant method for estimating the bioavailability of a chemical is SPME and C18 membranes. This biomimetic approach mimics the partitioning of contaminants between the pore water and the organism. In these methods only the freely dissolved contaminants will partition between the aqueous phase and the fibre or membrane device. However it can lead to some mis-estimation (Johnson *et al*, 2002; Kelsey and Alexander, 1997; Bergknut *et al*, 2007) because:

- only the freely dissolved contaminant concentrations will represent bioavailability, which is not the case in the earthworm uptake process;
- these methods will underestimate the concentration in earthworms which have other routes of uptake than pore water;
- these methods do not take into account the organism or its feeding behaviour.

An important observation was made in the study of Gomez-Eyles *et al* (2010, 2011) where the profile of the PAHs extracted by butanol, cyclodextrin or Tenax was different from the one found in earthworms, even though the correlation was good between the amount of residues extracted by butanol and the amount in the organism. The soil was spiked with a mixture of naphthalene and acenaphthene (2-ring PAHs), fluorene and phenanthrene (3-ring PAHs) and fluoranthene and pyrene (4-ring PAHs). Results confirmed that earthworms accumulated a higher proportion of the heavier 4-ring PAHs than those found in the chemical extractions.

It must be kept in mind that since bioavailability is dependent on the organism and the species, and the uptake mechanism is complex, it is very difficult to exactly mimic the bioavailability of residues to earthworms. As model predictors, these extraction techniques can only give an idea of the real amount contained in the organisms.

Chemical techniques + EqP theory

Most of the papers referenced above are meant to provide a measure of bioavailability, not bioaccumulation. The final concentration found in the earthworms will depend also on the metabolic fate of the contaminant within the organism and the partitioning properties of the contaminant. This explains why this alternative method is based on predicting accumulation from concentration (measured by chemical methods) and accounting for contaminant partitioning properties.

Van der Wal *et al* (2004) has found a linear relation between uptake in earthworms and concentrations of chemical in SPME fibres. Estimated concentrations from SPME fibre measurements calculated via the EqP theory were close to the measured uptake in earthworms. Jonker *et al* (2007) has observed that the SPME predicted concentration was within a factor of 10 of measured bioaccumulation.

As well as being used to measure the bioavailability of organic residues (freely dissolved) based on equilibrium partitioning using non-depletive SPME techniques (see Section 3.3) biomimetic extraction methodology can also be applied to measure the rapidly desorbed fraction which quickly replenishes the aqueous phase as it is depleted. Examples of these include Tenax, cyclodextrin and depletive SPME techniques.

Hartnik *et al* (2008) demonstrated the use of non-exhaustive cyclodextrin extraction to estimate the bioavailability of two insecticides, alpha-cypermethrin and chlorfenvinphos in soil to earthworms. They specifically found that the use of hydroxyl-β-cyclodextrin mimics the uptake of hydrophobic organic compounds in earthworm when there is 3.5 times the quantity of cyclodextrin used compared to the amount of soil and equilibrium appears to be reached within 48 hours. ten Hulscher *et al* (2003) found that Tenax extraction of soils contaminated with PAHs, PCBs and organochlorine pesticides mimicked bioavailability to worms when the Tenax extracted amounts were normalised for soil organic carbon content.

Fang *et al* (2010) compared matrix SPME with hexane-Soxhlet, butanol agitation and water agitation extractions for estimating the bioavailability of DDT in soil. They concluded that matrix-SPME is a better approach compared to the chemical based extraction techniques in predicting bioavailability of DDT to earthworms and vegetables from both freshly spiked and aged soils. They also observed significantly greater

assimilation of DDT in earthworms compared to vegetables. They cite previous work by Krauss *et al* (2000) who attributed the higher body-lipid content of earthworms and their multiple uptake routes including passive diffusion across the dermis and diffusion across the gastrointestinal tract after ingestion of contaminated soil. Also regarding uptake routes in earthworms, Qi and Chen (2010) examined the uptake of naphthalene from soil and its implications for biological uptake routes. They proposed that for rapidly desorbable contaminants, the primary uptake route is via pore water (i.e. dissolved), but for desorption-resistant contaminants enhanced uptake from ingested soil particles is also important.

3.4.2 Non-exhaustive extraction techniques

A number of non-exhaustive extraction techniques (NEETs) have been used to investigate the relationship between contaminant residues, bioaccessibility and desorption kinetics (Stroud *et al*, 2008b).

Initial studies by Reid *et al* (1998, 1999, 2000a) sought to provide a method to predict the bioavailability of PAHs in soil using aqueous hydroxypropyl-β-cyclodextrin (HPCD). They demonstrated that PAH extraction via this method was closely related to microorganism mineralisation. Cyclodextrins are macrocyclic oligosaccharide compounds which are highly soluble in water (due to a number of external hydroxyl groups) but have an apolar cavity at their centre. Organic moieties with low aqueous solubility can form inclusion complexes with cyclodextrins at a 1:1 ratio (providing they are a suitable size and shape), allowing them to dissolve in quantities exceeding their usual aqueous solubility limit (allowing extraction). A study done by Cuypers *et al* (2002) agreed with the earlier work of Reid *et al* (1998, 1999, 2000), showing the HPCD method allows the determination of the size of the rapidly desorbing PAH fraction by extracting only what is available to microorganisms. This was confirmed when HPCD was included in biodegradation studies and did not enhance PAH biodegradation when compared with controls. Stroud *et al* (2008a) demonstrated the applicability of the HPCD technique by using hydroxypropyl-α-cyclodextrin (HP-α-CD) to predict the microbial bioaccessibility of aliphatic hydrocarbon contaminant hexadecane with extractions correlating very well with mineralisation data. As identified by Stroud *et al* (2009), hydroxypropyl-β-cyclodextrin extraction has been tested for a range of hydrocarbons including naphthalene, phenanthrene, pyrene, linear alkyl benzenes and hexadecane. They went on to test HP-α-CD (hexadecane extraction) and HP-β-CD (phenanthrene extraction) predictive capabilities under single co-contaminant and mixed contaminant conditions. Both HP-α-CD and HP-β-CD extractions predicted the microbially accessible fraction reliably under single co-contaminant conditions. However, only extraction of phenanthrene using HP-β-CD was able to predict microbial biodegradation under mixed contaminant conditions.

Cornelissen *et al* (1997) has applied solid-phase extraction of samples using Tenax, a solid porous polymer. This method was shown to extract rapidly desorbing PAHs from sediment samples (Cornelissen *et al*, 1998b). Cuypers *et al* (2002) achieved similar results as well as noting that similar performance occurred between both HPCD and SPE methods. It was concluded that SPE and HPCD act as ‘sinks’ for contaminant PAHs in the aqueous phase reducing their concentration. This maximises the concentration gradient between the solid and aqueous phases, enhancing desorption of the rapidly desorbable residue into the aqueous phase.

In the same study, Cuypers *et al* (2002) investigated the use of a non-ionic surfactant Triton X-100 to assess the contaminant fraction available to microorganisms. This surfactant forms micelles above its critical

micelle concentration (CMC) into which PAH molecules can partition. As with HPCD this action increases the solubility of hydrophobic contaminants such as PAHs, allowing extraction. However, it was discovered that Triton X-100 was not suitable for quantifying the rapidly desorbing contaminant fraction as it also enhanced the availability of the slowly desorbed PAH fraction to microorganisms. They postulated that the increased availability of the slowly desorbed fraction, was due to interaction of the surfactant with the solid matrix.

The NEETs described above (HPCD and SPE) were developed in response to increasing knowledge about the risks associated with organic environmental contaminants. Previous efforts have focussed on methods that were designed to exhaustively extract contaminant (e.g. Soxhlet extraction). However recent studies have placed emphasis on assessing the bioaccessible fraction. The HPCD and SPE methods attempt to extract only those residues that microorganisms can access; it has been demonstrated that these two methods correlate well with the microbial bioavailable contaminant fraction with the SPE method being assessed as more time consuming when both methods were compared. Currently, the SPE and HPCD techniques are being considered for inclusion within a standard method. The scope of this new standard method would be to provide an estimation of the potential environmental availability of organic contaminants to the aqueous phase. If accepted the method would be applicable for non-polar organic contaminants with a log $K_{ow} > 3$ which may exist in soil or sediments (Reid *et al*, 2000a; Cuypers *et al*, 2002; Bernhardt *et al*, 2013).

The supercritical fluid extraction (SFE) method is another NEET that has been used to gain insight into the desorption kinetics of contaminants in soil (Hawthorne and Grabanski, 2000). SFE involves passing supercritical CO_2 through an extraction chamber containing the soil sample. It has been shown that using CO_2 for extraction does not affect the soil organic matter composition (Bjorklund *et al*, 1999). The supercritical fluid solubilises the contaminants within the sample before transporting them to a collection solvent. The temperature and pressure of the supercritical fluid can be varied to extract residues from fast to very slow desorption sites, however the conditions to extract must be refined/optimised for different classes of compound. Hawthorne and Grabanski (2000) identified the conditions required to extract PAHs from fast, moderate, slow and very slow desorption sites in soil and were able to predict the extent of biodegradation of PAHs in soils undergoing bioremediation. It was found that the amount of PAH residue extracted using the mildest SFE conditions (120 bar, 50˚C) was comparable to the amount removed by a year of biodegradation in the field. In addition, they learned that bioremediation after one year removed PAHs found only in the 'fast' fraction of the soil and that this did not encourage the movement of residue from 'slower' to 'faster' sites. They concluded that mild SFE conditions could be used to predict the bioavailability of PAHs in contaminated soils (Hawthorne and Grabanski, 2000). In agreement with these conclusions Hawthorne *et al* (2005) used mild SFE extraction conditions to extract the rapidly desorbable PAH fraction from a soil previously causing 100% mortality to terrestrial oligochaetes. After extraction, no toxicity to oligochaetes was observed, indicating that the biologically relevant PAH fraction had been completely extracted under these conditions. The characterisation of residues into 'fast' to 'very slow' sites is defined by the extraction parameters, with increasingly harsh conditions being used to extract residues from slowly desorbing sites. Stroud *et al* (2008b) investigated the use of SFE to measure desorption and bioaccessibility of phenanthrene in soils. They carried out SFE extractions of samples at 5-10 minute time intervals up to 30 minutes for each desorbable fraction producing an extraction rate curve for each. Overall, they found that the SFE technique shows residue behaviour and kinetics that agree with the current literature with residues migrating from fast to slow sites as time goes by (Pignatello and Xing, 1995).

The SFE method describes four compartments of desorption based on the extraction conditions used (temperature and pressure). Compared to the HPCD method the SFE technique has some drawbacks in that it requires specialised expertise and equipment, in comparison, the HPCD technique is very straightforward and is highly suitable for soils (Rhodes *et al*, 2010b). Rhodes *et al* used HPCD extraction to measure desorption kinetics of phenanthrene by taking consecutive extractions over time. They found that fitting desorption data to a tri-phase model (rapidly, slowly and very slowly desorbing) gave a statistically significant improvement over the two-phase model (rapidly and slowly desorbing) which has been used previously. They found that a single HPCD extraction after 24 hours was equivalent to the rapidly desorbed fraction (when calculated using the three-compartment model) with a ratio of almost 1:1. These findings are consistent with other studies which show that a single HPCD extraction removes only the rapidly desorbed / biodegradable fraction (Doick *et al*, 2005, 2006; Stokes *et al*, 2005; Allan *et al*, 2006). In addition to this work, Rhodes *et al* (2010b) also compared the relationship between the rapidly desorbed fraction (calculated through two-compartment and three-compartment models), with the total mineralisable fraction after 10 days. They found that both models correlated well with the total mineralisable fraction (r^2 = 0.84, 0.89 respectively); however, it was found that the two-compartment model slightly overestimated the mineralisable fraction and rapidly desorbed fractions estimated by the two-compartment first-order model may have been overestimated in the past. They conclude that HPCD extraction (24 h) and three-compartment modelling could be used for commercial PAH bioremediation applications by predicting / estimating the amount of contaminant biodegradation. They also highlight the simplicity / suitability of this technique in comparison to others currently available (SFE, SPE).

After careful assessment, the current literature suggests that a single HPCD soil extraction allows quick assessment of the rapidly desorbed fraction which has been documented to represent the microbially bioavailable fraction. Additionally, current research highlights SFE as a method which is able to characterise all but the irreversibly bound fraction and with further development may be used to assess the total bioaccessible fraction.

3.5 Slowly desorbed fraction

Methods used to extract the slowly (reversibly) desorbed fraction utilise harsher techniques than those described in the previous section for rapidly desorbed residues. However, they stop short of exhaustive extraction techniques. Slowly desorbed fraction methods are not intended to be substantially destructive and encompass extractions using mostly polar organic solvents. The methods can also include modification of pH utilising organic solvents and weak acids in addition to more vigorous agitation. More extreme extraction methods typically include a combination of organic solvents, elevated temperature and/or pressure and extended extraction time and represent destructive methods intended to recover strongly sorbed residues that are considered unrepresentative of the bioavailable fraction.

Hatzinger and Alexander (1995) compared the amounts of phenanthrene and 4-nitrophenol that were mineralised to the quantities extracted from sterile soil. Phenanthrene was extracted using a short (two minute) butanol shake followed by Soxhlet extraction using dichloromethane. Mineralisation was measured by incubation with *Pseudomonas* strain R over 33 days. Extraction of 4-nitrophenol was conducted using butanol extraction only and mineralisation was achieved by incubation with bacterium WS-5 for

73 hours. With time, both compounds became more resistant to biodegradation and extraction. For all combinations of soil type, chemical concentration and ageing period; butanol extraction removed significantly more of each chemical than was mineralised.

The Alexander group (Kelsey *et al*, 1997) used a similar approach with phenanthrene and atrazine to compare mineralisation by bacteria, uptake by earthworms and extractabilty using a range of solvents, with and without agitation. After each ageing period, the relevant bacterium or earthworms were added to the soil. Subsequent mineralisation took place over 15.4 days for atrazine and 18 days for phenanthrene while uptake into earthworms took place over 8 days. As in the previous study, both compounds became more resistant to biodegradation and extraction with time. In the case of atrazine, 66.8% was mineralised after 0 days ageing and 5.7% taken up into earthworms. For phenanthrene, mineralisation was lower after 0 days ageing with 23% removed while earthworms took up 8.7%. The relatively low amounts taken up by earthworms illustrate the challenge of comparing methods with high 'accessibility' to those with low 'accessibility'. All of the extraction methods used for atrazine without agitation removed less of the compound than was mineralised. Conversely, extraction using agitation (two hours shaking) with 25 mL methanol/water (1:1) or methanol extracted more atrazine than was mineralised. For phenanthrene, extraction using butanol with and without agitation and extraction with acetonitrile/water (1:1) without agitation removed more compound than was mineralised. Use of more polar solvents such as ethanol and methanol mixtures with water, all removed less phenanthrene than was mineralised.

A novel approach using aqueous based extraction of phenanthrene was used by Latawiec *et al* (2008). The quantities extracted by hot and superheated water (at 40°C, 80°C, 120°C, 160°C, 180°C and 200°C) from two contrasting soils were compared to the amount mineralised by *Pseudomonas putida*. Extractions at 40°C and 80°C underestimated mineralisation, extraction at 160°C removed similar amounts as were mineralised in four of six aged samples and extraction at 180 and 200°C overestimated mineralisation in Moulton soil.

The extraction of slowly desorbed material can include acid extractions using weak acids (e.g. dilute acetic, citric or hydrochloric acid) (Peijnenburg *et al*, 2007). Weak alkaline extractions should be avoided as these can fractionate and thus destroy organic matter constituents of the solid environmental matrix.

Reid *et al* (2000b) demonstrated using dichloromethane Soxhlet extractions and extraction by shaking with butanol overestimated bioavailability of phenanthrene by greater than 60% (Katayama *et al*, 2010). More destructive methods also pose a greater risk of altering the chemical form of the test substance (Fomsgaard, 2004).

3.6 Irreversibly sorbed fraction

Irreversibly sorbed or non-extractable residues are those that are most recalcitrant to extraction and are not bioaccessible. Along with residues incorporated into the soil, the sorption of these residues may, in part, be reversible but the partitioning is much favoured towards binding to the matrix. Though these 'total' residue concentrations have historically been the most frequently quantified with regard to risk assessments, they typically pose minimal bioavailability to indigenous organisms.

Extraction of irreversibly sorbed residues almost always utilise organic solvents, in contrast to the mild solutions described for slowly (reversibly) desorbed residues discussed in the previous section. The use of solvents is typically accompanied by harsh extraction conditions including extraction duration, elevated temperature and/or pressure, application of high energy or the use of sub- and supercritical fluids. The current roster of harsh extraction techniques includes but is not limited to: reflux (i.e. Soxhlet) (Hawthorne *et al*, 2000; Ramos *et al*, 2002; Martinez *et al*, 2004), microwave assisted extractions (MAE), sonication (Martinez *et al*, 2004), pressurised liquid extractions such as accelerated solvent extractions (ASE) and sub/supercritical fluid extractions (SFE) (Hawthorne *et al*, 2000; Ramos *et al*, 2002; Rodil and Popp, 2006). The objectives of these techniques are to recover as much of the sorbed residues as possible without regard to the significance of how these concentrations relate to bioavailability or bioaccessibility. Semple *et al* (2007) relate such in their comprehensive review of the relationship of contemporary extraction techniques and bioavailability as related to soil microbes. They specifically cite the weakness of exhaustive organic solvent extractions in that they overestimate bioaccessibility. However, they also indicate bioaccessibility should be normalised to the total extractable fraction based on an exhaustive extraction technique.

A final methodology that can be used to assess the irreversibly sorbed material is accelerated solvent extraction (ASE). Vazquez-Roiga *et al* (2010) determined that extraction using aqueous ASE methods to extract pharmaceuticals from soils and sediments was comparable to using a range of co-solvent, acid and complexing agent (e.g. EDTA (ethylenediaminetetraacetic acid). Evidence has shown that using exhaustive methods can overestimate the bioavailability of test substances (Katayama *et al*, 2010). The method developed by Vazquez-Roiga *et al* (2010) used extraction with water at 90°C; however there are many ASE methods that use organic solvent: water mixtures to extract chemicals from soil and sediment (US EPA, 2007a; Ramos *et al*, 2002; Jelíc *et al*, 2009). For solid samples that are not heavily contaminated, ASE has shown similar extraction efficiencies despite the variety of extraction solvent selected (Ramos *et al*, 2002).

A review of contemporary extraction methods utilised to recover non-extractable residues indicate that many researchers have also applied milder versions of otherwise harsh extraction procedures in an attempt to relate extracted concentrations to bioavailability. This typically includes substituting polar or aqueous extraction solvents and reduced temperature and pressure conditions. Kramer and Ryan (2000) examined the use of Soxhlet and MAE for determining the bioaccessibility of pesticides in soils. They observed that while Soxhlet and MAE yielded similar quantitative results, neither technique accurately measured the bioaccessible fraction. They did report that MAE modified using water as the solvent was a much better predictor of bioavailability.

Hawthorne *et al* (2007) examined the relationship between several extraction techniques and the bioavailability and toxicity of PAHs in some 97 sediment samples taken from six former manufactured gas sites and two aluminium smelter sites. They compared Soxhlet extractions using methylene chloride and acetone, mild SFE to measure the rapidly released fraction and SPME to measure dissolved pore water PAHs. Prediction efficiencies were calculated for each technique based on how accurately the toxicity of each sample was predicted by the respective extraction procedures based on observed toxicity to *Hyalella azteca.* Soxhlet extraction resulted in an overall prediction efficiency of less than 50% but increased to 80% if the alkylated PAH analogs (PAH_{34}) were included in the quantification beyond the US EPA (United States Environmental Protection Agency) regulated sixteen parent PAHs (PAH_{16}). The prediction

efficiency of mild SFE was 71% which the authors considered disappointing. Measurement of dissolved PAH_{34} concentrations by SPME yielded a prediction efficiency of 90% and was considered the best predictor of PAH bioavailability and toxicity in sediments.

Hu and Zhou (2011) compared MAE, simultaneous distillation-solvent extraction (SDSE - i.e. steam distillation), Soxhlet and ultrasonic probe (UP) for extraction recoveries and relation to bioavailability of several polycyclic musks (tonalide and galaoxide) from sediments. All of the techniques yielded quantitative recoveries (greater than 80%) with the order of recovery being Soxhlet > SDSE > MAE > UP. However, only UP extractions strongly correlated ($r^2 > 0.92$) with bioavailability to wheat root and associated changes in plant chlorophyll, malondialdehyde and peroxidases. It is unclear whether UP extractions actually mimic bioavailability or if this was simply an artifact of lower recoveries compared to the other extraction techniques.

One of the confounding factors in using harsh extraction techniques to estimate bioavailability is that it is very much organism dependent. For example, several publications (Bosma and Harms, 1996; Meharg, 1996) suggest the rapidly desorbed and to a lesser extent, the slowly desorbed fractions, (typically extracted using aqueous or other mild extraction techniques) best represent that available for microbial degradation. However, Gevao *et al* (2001) demonstrated that even after methanol and dichloromethane Soxhlet extractions of aged soils, significant activity of ^{14}C-labelled isoproturon, dicamba, and atrazine remained in the soil and actually increased as earthworms were introduced to contaminated soils.

3.6.1 Irreversibly sorbed fraction – characterisation of the solid matrix

Many extraction methods routinely employed during laboratory studies employ techniques which release irreversibly sorbed material by acting to disassemble the fractions comprising the solid matrix. These methods include: soil fractionation, acid digestion and the use of surfactants. These extraction methods will extract material which would not be available in the natural environment, and would not be applicable to characterise material that would be biologically available.

Soil organic matter fractionation is performed routinely in many laboratory studies. The soil organic matter is fractionated into humin, humic acids and fulvic acids. The fulvic and humic acids are extracted from the solid matrix using a strong alkali extraction, to leave the humin fraction. Then the humic acids are precipitated from the aqueous phase using acid, leaving the fulvic acids in solution (Carter and Gregorich, 2007). This is a highly destructive extraction method as it is designed to destroy the soil matrix.

Extractions involving acid digests have been used to characterise the constituents of solid matrices (US EPA, 1996). This aggressive extraction methodology is not appropriate for investigating availability to natural environmental systems.

Surfactants have been used routinely to enhance biodegradation by increasing bioavailability (i.e. ECHA, 2008). There has been little reported in terms of extraction efficiency of surfactants. Beigel *et al* (1999) noted that using an anionic surfactant provided no observed enhancement of soil desorption for triticonazole. Introduction of surfactants in an extraction procedure would be destructive as any charge of the surfactant would disrupt the interactions within clay colloids. Surfactants may have utility in

characterising the irreversibly sorbed residues as surfactants of different ionic charge will complex with associated ionic entities present within the chemical of interest. However, this would be a supplementary extraction as information relating to biological endpoints would not be appropriate due to the destruction of the solid matrix extracted.

These extreme extraction methodologies can be used to further characterise the irreversibly sorbed material in terms of the fraction of the solid matrix associated with the material of interest and may assist in characterising the binding exhibited during this interaction.

3.7 Assimilated residues

'Assimilated' residues are those where individual atoms or fragments of the applied xenobiotic are incorporated by microbes into bio-molecules such as proteins (amino acids), carbohydrates (sugars) and lipids. This can result either from the utilisation of the parent compound or a metabolite in the normal biochemical pathways of soil microorganisms or from the uptake of evolved CO_2 from mineralisation via such pathways (Miltner *et al*, 2005). The purpose of this Section is to review the methods that have been used for the determination of assimilated residues and to assess the extent and significance of assimilated residue formation.

Most definitions of bound and non-extracted residues specifically exclude such components on the basis that they are not relevant to risk assessment. However in studies conducted using radiolabelled compounds, assimilated residues can be inadvertently included in the quantification of extracted and bound and/or non-extracted residues. Typical extraction methodologies may solubilise some natural products containing assimilated residues from environmental matrices particularly when harsh conditions are used. Furthermore, because non-extracted residues are quantified by destructive sample combustion the contribution of assimilated residues often cannot be differentiated from remaining parent and degradate residues. This inconsistency in the treatment of assimilated residues is why it is important that they are explicitly delineated (as shown earlier in the framework model) from other residue pools in environmental matrices. Understanding the scale of assimilated residue formation is therefore important to ensure that the contribution they may make to both uncharacterised extracted residues and to unextracted residues is properly accounted for when considering the significance of such residues.

Interest in assimilated residues has revived somewhat in recent years but it has been known for many years that parts of xenobiotic molecules can be reconstituted into bio-molecules. Helweg (1975) used boiling 6N HCl to extract the amino acids from the soil. Neutralised extracts were passed through an H^+ saturated cation exchange column and the amino acids eluted using ammonia. The eluate was reduced to dryness then redissolved in water. Up to 4.2% of the ^{14}C from radiolabelled maleic hydrazide was incorporated into amino acids after 9 days incubation in a sandy loam soil. This represented 7.4% of the radioactivity remaining in soil at that timepoint.

As part of an investigation into reasons for accelerated pesticide biodegradation Robertson and Alexander (1994) quantified the extent of incorporation of 2,4-D, carbofuran, propham, glyphosate and simazine, into both microbial biomass and amino acids. To demonstrate incorporation into amino acids soil samples were

initially refluxed with 0.5M HCl for 16 hours, then for 16 hours with 6M HCl. The filtrates and washings were evaporated to near dryness then dissolved in water. To quantify incorporation into biomass a modification of the chloroform-fumigation extraction method of Jenkinson and Powlson (1976) was used. The quantity of ^{14}C from 2,4-D (11.5%), propham (10.3%) and glyphosate (10.8%) incorporated into the microbial biomass was similar to that incorporated from glucose (12.4%). For carbofuran and simazine less than 1% of the ^{14}C was incorporated into the biomass. This was attributed to these compounds being cometabolised, rather than being used as energy sources, although appreciable mineralisation of both compounds was observed. Incorporation into (bound) amino acids accounted for just under 5% of the ^{14}C in the cases of 2,4-D and glyphosate but less than 1% of for the other compounds.

Utilisation of ^{13}C labelled phenanthrene enabled Richnow *et al* (2000) to track its incorporation into amino acids as part of an investigation into the transformation of the compound in a soil bioreactor. Soil was extracted using 6M HCl for 22 hours at 110°C. Following derivatisation the amino acids were quantified by HPLC and identified using GC-MS. The isotopic composition was established by use of isotope monitoring gas chromatography. Phenanthrene derived ^{13}C incorporated into amino acids accounted for 11% of the unextracted soil residue after 50 days. The authors also noted that phenanthrene derived carbon may have also have been incorporated into fatty acids, carbohydrates, glycerols and other cell components.

The microbial utilisation of nitrogen derived from atrazine was demonstrated by Bichat *et al* (1999) using ^{15}N labelled atrazine. After extraction, the hydrolysable soil amino acid fraction was demonstrated to contain atrazine derived ^{15}N.

A modified version of the fumigation extraction approach was used by Ghani and Wardle (2001) to measure the incorporation of ^{14}C into the soil microbial biomass. This was part of an investigation into the fate of the herbicide metsulfuron in soil microcosm systems receiving different treatments (with and without; weeds, nitrogen or phosphorus). A maximum of 18.1% of the ^{14}C was incorporated after 131 days. Incorporation was generally higher in those systems that received amendments.

The distribution of ^{14}C incorporated into bacteria and fungi in cultures following treatment with 2,4-D and glyphosate was examined by Charnay *et al* (2004). Biomass fractionation into lipids, polysaccharides and proteins was accomplished using a selective extraction scheme. In the case of 2,4-D, 12% and 4% of the ^{14}C was incorporated into the biomass of the fungi and bacterium respectively. Incorporation of ^{14}C from glyphosate into fungal biomass accounted for 16.5%. Incorporation of ^{14}C into all of the biomolecules was observed for both compounds. The fungi incorporated these compounds mainly in the polysaccharide fraction extracted from the cytoplasm. In contrast the bacterium incorporated more ^{14}C derived from 2,4-D into the lipid and polysaccharide from cell walls.

More recently, techniques using isotopically labelled biomarkers such as fatty acids (FA), fatty acid methyl esters (FAME) and phospholipid fatty acids (PLFA) have been used to elucidate the formation of assimilated residues. An advantage of these approaches is that, as well as providing data on the extent of incorporation into the specific microbial biomarker, they can be used to estimate incorporation into the total biomass. Use of biomarker analysis also provides some information on the type of microorganisms involved in the degradation processes. Furthermore, used in combination these biomarkers can provide some differentiation of incorporation into the living and dead biomass.

These approaches along with extraction of amino acids were used by Nowak *et al* (2011) and Nowak (2011) to investigate the formation of incorporated residues formed during 2,4-D and ibuprofen degradation in soil. Differential extraction methods were used to extract total FA containing FA from living (PLFA) and dead biomass. A similar approach was used to extract amino acids (AA) from the living biomass (bioAA) and the living plus dead biomass (tAA). At the end of the incubation experiments ^{13}C incorporated into the total amino acids accounted for 22% and 27% of the initial ^{13}C added for 2,4-D and ibuprofen, respectively. These values corresponded to 61% of the total 2,4-D non-extracted residue and equate to 90% of the ibuprofen non-extracted residue. Using data from the incorporation of ^{13}C from 2,4-D into total amino acids in a culture of *C. necator* JMP 134, the author suggested that approximately half of the total amino acids were extracted and that a factor of 2 be applied to estimate total incorporation into amino acids in the soil experiments. Hence, total assimilated residues were estimated as 44% applied ^{13}C-2,4-D equivalents and 54% applied ^{13}C-ibuprofen equivalents. In comparing these values to previous studies the author noted that differences in the extraction methods would result in variations in quantification of non-extracted residues and that some of the unidentified radioactivity that was extracted in previous studies could have been incorporated into bio-molecules.

Conclusion

It has been known for many years that carbon and nitrogen from xenobiotics can be incorporated into microbial bio-molecules. As more sophisticated approaches have been developed to investigate assimilated residues, evidence has increased that they can form a substantial part of the unextracted residues and potentially account for some of the unidentified extracted material depending on the extraction methodology used. Assimilated residues are of no toxicological or ecotoxicological concern. It is therefore essential that the potential for the extensive formation of such residues is taken into account when considering the significance of ‘non-extracted’ and ‘bound’ residues in risk assessment. On this basis, it is proposed in this report that assimilated residues are explicitly considered separately from residues that comprise the parent compound or it’s degradates. It also has to be recognised that the continued use of the operationally defined terms ‘non-extracted’ and ‘bound’ residues, irrespective of how they are defined, do not reflect the nature of the residues *in situ* and are therefore unhelpful when assessing the relevance of residues for risk assessment.

4. INTELLIGENT EXTRACTION STRATEGY FRAMEWORK

A review of literature conducted in Chapter 2 suggested many of the techniques currently employed for the extraction of a range of chemical classes are performed to maximise the recovery of the compound (and its metabolites) from the soil or sediment matrix. Techniques such as Soxhlet extraction, accelerated solvent extraction, supercritical fluid extraction, microwave extraction and sonication, which employ organic solvents at elevated temperatures and pressures will remove irreversibly sorbed residues, which would normally only be released under typical environmental conditions in extremely small percentages. Other methods include use of concentrated acids or alkalis in conjunction with digestion and combustion techniques. Such harsh extraction approaches result in destruction of the organic matrix and an overestimation of the material potentially available to environmental exposure scenarios.

The proposed extraction strategy framework has been based on extraction and quantitation of the dissolved and rapidly desorbed fraction (for the bioavailable residues) and, in addition, the slowly desorbed fraction (for the bioaccessible fraction) is considered more appropriate for assessing the chronic risk to the environment of such residues. Further details of selection of the various extraction parameters are detailed in the following sections. Use of such solvents under these conditions, which do not result in destruction of the organic soil or sediment matrix, are aimed at extracting and quantifying environmentally relevant residues.

The framework is designed to provide a logical and reasoned sequence of extraction to enable the relationship between extraction and bioavailability or bioaccessibility to be considered. Many of the extraction steps are conservative, providing enhanced extraction from the matrix than would be available to organisms in the environment. The study endpoint and purpose should be considered when designing the extraction regime for an environmental study. The test operator following the scheme has the option to continue to use more destructive methods, if the study design requires further investigation of the solid matrix. However, the applicability of this methodology to environmental situations should be considered and evaluated separately from extraction to enable efficient recovery of material from a matrix.

The steps and solvent choices within the extraction framework should be selected to complement the chemical of interest. It is important to utilise all available physico-chemical property information available and, in particular, any knowledge gained during the conception and design of a specific extraction method from previously performed or published studies. Useful information to assist selection of the solvent of choice includes the solubility of the material in different solvents, partition coefficient (log K_{ow}) and pKa. The log K_{ow} value is a measurement that acts to as a guide to the hydrophobicity of the molecule and hence the choice of extraction solvent. Molecules with a pKa value will contain ionisable groups depending on the pH of the media. If the pKa value is known, a test operator should aim to use an optimised pH value two units from the pKa to enable a single ionised species of the molecule to be present. Additionally, NEETs may be considered, providing the compound meets the criteria for use.

The boundary between bioaccessible and inaccessible fractions for a particular chemical is of primary concern in many environmental studies. The extraction framework provides a conservative evaluation of bioaccessible residues providing the framework is applied using considered and rational methodology.

Through utilising this intelligent extraction regime within a well-designed study, robust laboratory data to assess the bioavailable material in environmental matrices will be measured.

4.1 Solid: extraction solution ratio

Prior to performing extractions, consideration should be given to the ratio of solid matrix to extraction solution. Most studies reviewed utilise a ratio of solid to extractant, using the dry weight equivalent of the soil sample. This is preferred to using a wet solid phase weight, as typified by Jakher *et al* (2006) who compared the extraction efficiency from two soils using soil to solution ratios of 1:1 and 1:3, and determined the optimal extraction ratio was 1:3. However, they based their extraction ratios on wet weights of solid matrices. When corrected for moisture contents, the ratios approximated 0.5:1 and 0.6:1, 1.5:1 and 1.9:1 for each soil. Therefore, the reported ratios were inconsistent between the soil types and were not comparable. A more consistent approach selects the solid to extractant ratio based on dry weight equivalents of a moist sample. Thus extracting a moist sample, and not subjecting the matrix to be extracted to drying.

Documented solid to extractant ratios vary widely (Table 4). Most identified were extracting chemicals from soil. However, it is recognised that consistency in extraction methods with respect to contact time and soil to solution ratio is required (Hoskins and Ross, 2011). There are some suggestions to improve through incorporation of an element of 'realism' to use a relatively high soil to solution ratio to be more applicable to conditions in the natural environment (Yin *et al*, 2002). A review of current methods indicates that an extraction ratio of 1:5 is most frequently utilised and is more environmentally applicable than using other ratios reported. It is recommended that during experimental work a solid to extractant ratio of 1:5 is preferred.

Table 4: Review of solid to extractant (w/v) ratios

Solid phase: extraction solution ratio	Test substance	Reference
1:10	Atrazine	Cheng (1990)
1:2.5	Heavy metals	Esnaola and Millán (1998)
1:2, 1:10 and 1:3	Herbicides	Diez *et al* (2006)
1:5 and 1:100	Phosphorus	McDowell and Sharpley (2001)
1:2, 1:1.25, 1:5, 1:10, 1:25 and 1:50	Heavy metals	Yin *et al* (2002)
1:5	PAH	Gomez-Eyles *et al* (2011)
1:5	Atrazine	Barriuso *et al* (2004)
1:5	Pharmaceuticals and personal care products	US EPA (2007b)

4.2 Contact time

Another important consideration when designing an extraction experiment, is the contact time between the extraction solvent and the solid phase. This has not been standardised between methods or laboratories and reviewed studies generally do not investigate the preferential contact time for their methods. Jakher *et al* (2006) investigated the effect of contact time on extractability of PCBs and found that greater than

26 minutes was optimal for consistency. Many methods utilise 30 minutes contact time (McDowell and Sharpley, 2001; US EPA, 2007b), whilst others extract for longer, Gomez-Eyles *et al* (2011) used two hours and Barriuso *et al* (2004) used four hours and 16 hours for different extractions. It is recommended that the minimum contact time between the solid phase being extracted and the extraction solvent should be 30 minutes.

4.3 Method of agitation

The method of agitation used with this extraction framework is selected to minimise the input of energy to extract the test substance, and to ensure that the mixing occurs between the phases. Suitable agitation methods include rolling or shaking at a sufficient speed to observe the phases combining. Methods that involve energy input or destruction of the solid matrix are to be avoided (e.g. microwave extraction).

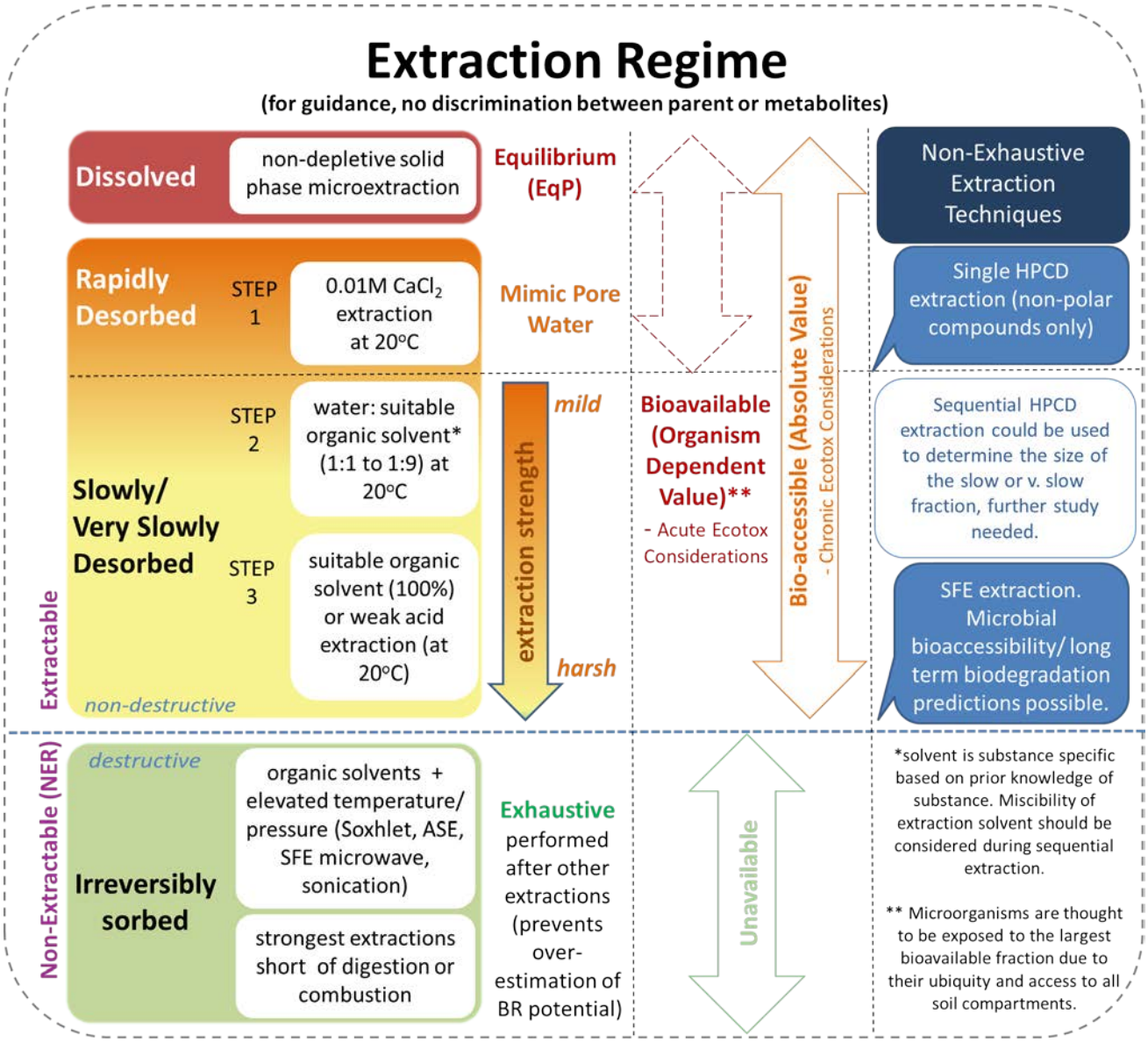
4.4 Temperature

The temperature of the extractions is suggested as nominal room temperature i.e. the temperature at which the exposure is conducted. The use of elevated temperatures to enhance extractability should be avoided.

4.5 Extraction framework

The following extraction methodology framework (Figure 8) was designed to provide an empirical basis to understanding the behaviour of residues in soils as related to bioavailability and bioaccessibility. The extraction solutions selected were considered alongside the observations summarised during this report, and are based on the definitions of each of the compartments as identified in Chapter 2.

Figure 8: Extraction methodology framework



If extraction from the aqueous phase is required, because direct analysis may not be performed, then solid phase microextraction (SPME) may be used for certain chemical classes and has been shown particularly successful with hydrophobic organic compounds. If this method is utilised, then it must be used in non-depletive mode (Section 2.2). The first extraction of the soil or sediment should be performed in an aqueous solution, designed to mimic the composition of soil or sediment pore water to release the rapidly desorbable fraction (as discussed in Section 2.3).

The second extraction utilised as a surrogate to mimic the uptake by soil organisms (Katayama *et al*, 2010) utilises a weak water miscible organic co-solvent for extraction, the addition of a proportion of solvent is designed to increase solubility and improve partitioning of the test substance from the solid matrix into the extractant (Kookana *et al*, 1990). Successful reported co-solvent to water ratios suitable for use in this context range from 1:1 to 9:1 when investigating the bioavailability of atrazine to bacteria and earthworm, respectively (Kelsey *et al,* 1997) and 8:2 when investigating the bioavailability of atrazine to bacteria in soil (Barriuso *et al,* 2004). Therefore, suitable ranges of co-solvent: water can be selected between 1:1 and 9:1.

To extract in pure organic solvent would be considered a 'harsh' extraction, and therefore applicable as the last non-destructive stage of the extraction regime.

The techniques that are listed as destructive involve the use of elevated temperature (e.g. reflux), elevated pressure (e.g. ASE) or extreme agitation or disruption of the solid matrix (e.g. microwave extraction). These methods may be required to further characterise the un-extractable material, as a requisite for regulations of specific industrial sectors (e.g. pesticide regulation). However, for the purposes of determining the bioavailability of the test substance, these extraction methods would significantly overestimate the bioavailable fraction. A correlation of the main types of binding (with respective bond energies) and the extraction techniques and strengths required to extract residues bound in this manner is provided in Figure 9.

Figure 9: Relationship between the strength of interaction between chemical moieties and the soil / sediment matrix and the extraction strength of solvent systems to desorb them

4.6 Limitations and further work

With the extraction framework established, validation is recommended using a series of model compounds. Ideally, the selected compounds will capture a range of predicted binding strengths and properties and be representative of various chemical segments (e.g. agrochemical, pharmaceuticals, petroleum hydrocarbons, other industrial chemicals). Depending on the compounds selected, the evaluation may be performed with either ^{14}C-labelled or unlabelled (cold) products dosed to soils or sediments according to current OECD test guidelines. The objective will be to challenge the framework by progressing through the extraction regime in terms of, extraction technique, solvent strength and temperature and pressure conditions aimed at obtaining the respective dissolved, rapidly desorbed, slowly desorbed and irreversibly desorbed fractions.

In the instances where radiolabelled products are utilised, radio-TLC or radio-HPLC can be employed to confirm parent and metabolite profiles. Soil and sediment samples from these studies can be combusted and analysed In order to provide mass balances. For evaluations where ^{14}C-labelled are not practically available and unlabelled compounds are utilised, analysis may be limited to tracking of only the parent compound. Studies could also investigate the effect of increasing equilibration period (ranging from freshly spiked soils / sediments to those equilibrated for periods of up to several months) on both extractability, as an indicator of bioavailability, and ecotoxicity to relevant organisms. Organisms for consideration should include relevant soil / sediment dwelling invertebrates, plants and microorganisms. Additionally, more extensive investigation of the fractionation of irreversible bound moieties in humin, humic and fulvic acids may also be carried out.

Additionally, a number of research proposals (Table 5) were generated as a result of the previous ECETOC workshop on the "Significance of bound residues in environmental risk assessment" (ECETOC, 2010). Some or parts of these proposals have been addressed by the work of this Task Force, whilst others have not (shown in italics).

Table 5: Progress to date against the initial research proposals identified at bound residues workshop (ECETOC, 2010)

Research proposal	Summary of what this Task Force (TF) has covered in the report in relation to the various RfPs
RfP 1 - Guidance document for extraction schemes	A common extraction scheme for compounds based on their bioavailability and bioaccessibility has been proposed by the TF. This also identifies some functional groups which will lead to irreversible adsorption.
RfP 2 - Characterisation of bonding	A review of the bonds and their binding energies that can be formed between the test compounds and the matrices has been carried out. This includes binding mechanisms due to physical entrapment or chemical binding. We have also linked the Framework model to a solvent extraction scheme to identify whether binding would be reversible or irreversible. It has also looked at the changes in the matrix (e.g. effect of changes of fertiliser, ploughing, wet-dry cycle, freeze-thaw cycle).
RfP 3 - Comparison of bioassay bioavailability versus chemical extraction methods	The Task Force have reviewed the current literature to assess if chemical methods from extraction schemes compare to uptake in organisms. The comparison between various extraction solvents, membrane devices or SPME fibres and uptake into earthworms has been reviewed. Also the comparison of solvent extraction to mineralisation by bacteria or uptake by earthworms has been shown.
RfP 4 - *Develop simple and rapid assays to evaluate irreversible binding of chemicals to soil and sediment*	The Task Force have identified certain binding mechanisms that will give rise to 'irreversible sorption'. *However, experimental work on the assessment or development of high throughput assays to evaluate different types of irreversible binding to clay minerals, iron/aluminium oxihydroxides, natural organic matter in soil, sediment and sewage sludge has not been covered in this report. Neither has the influence of boundary conditions (pH, ionic strength, types of ions) been investigated, nor the validation of such assays using chemicals of known binding behaviour. This experimental work is better suited to a research institute or University.*
RfP 5 - Develop understanding of major mechanisms of no-covalent and covalent binding to soil and sediment matrices	The current literature review has developed an understanding of the major mechanisms of non-covalent and covalent binding to soil and sediment. Structural rules for differentiating between high and low extent of non-extractable residue formation have also been developed.
RfP 6 - How will environmental change affect non-extractable residues of chemicals in soil or sediment?	This is aimed at better understanding of the impact of environmental changes (such as changes in temperature, humidity, redox conditions) in response to flooding on the availability / release of irreversibly sorbed residues. Our review has addressed changes in the matrix (e.g. effect of changes of fertiliser, ploughing, wet-dry cycle, freeze-thaw cycle) and tried to quantitate the magnitude of these.
RfP 7 - *Will a better understanding of soil organic matter pool dynamics be the key to understanding the non-extractable pool dynamics?*	The aim is to better understand the impact of changes in soil management (e.g. agriculture) or changes in weather conditions on the soil organic matter pool and how this impacts the non-extractable pool of chemicals. *This has not been considered in the current report.*
RfP 8 - *How do changes in land use affect the non-extractable pool of chemicals in soils?*	The aim is to better understand how changes in land use (such as minimal tillage, fertilisation, increased chemical application, crops or afforestation/deforestation) affect the non-extractable pool of chemicals in soil. *This has not been considered in the current report.*

5. DISCUSSIONS AND CONCLUSIONS

A workshop was commissioned by ECETOC in 2009 to investigate the "Significance of bound residues in environmental risk assessment (ERA)". One outcome from the workshop was the formation of this Task Force and preparation of this report. The goal of this report was to address knowledge gaps in the relationship between bioavailability and extraction technique with regards to bound or non-extractable residues with the ultimate goal being the development of a standard framework for intelligent extraction strategies. After searching the available literature on bound residues it was decided that, due to its complicated and often conflicting nature, it was vital to define the key terms to be used in any new framework being produced. A number of residue 'categories' were defined (dissolved, rapidly desorbed, slowly desorbed, irreversibly sorbed and assimilated) as well as the terms bioavailable and bioaccessible which were aligned with each type of residue within the framework model.

The definition and identification of the concept of 'total residue' within a solid matrix was also discussed. After much discussion it was decided that Reichenburg and Mayer (2006) definition of 'Total Residue' as 'freely dissolved + reversibly bound + irreversibly bound' was the most agreeable with the current science. In order to develop a standard framework and an extraction regime it was decided to differentiate residues termed reversibly bound into those 'rapidly desorbed' and 'slowly desorbed'. This differentiation was based on the extraction method necessary to extract each type of residue and led to the development of the extraction regime.

The next key step for the ERA of NER was to consider the amount of residue available for uptake by organisms and the potential availability of residue in the future. After considering the literature the Task Force decided to utilise the terms 'bioavailable' and 'bioaccessible', and define them based on Semple *et al* (2004).

Bioavailable: Is freely available to cross an organism's cellular membrane from the medium the organism inhabits at a given time e.g. available now (no constraints).

Bioaccessible: 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 separated from the organism or only bioavailable after a period of time, i.e. available, but not within reach from a given place and/or time (constrained).

In order to justify the relevance of these terms in relation to how the soil biota could be affected, research into the uptake of residues in microorganisms and earthworms was undertaken. It was found that worms were capable of taking up both freely available residues (via passive diffusion) as well as NER (via ingestion of soil). It was concluded that modelling the uptake of residues by earthworms (as an example of soil biota) was very complex and unpredictable due to its reliance on a large number of factors including the behaviour of the organism, the type of organism, the nature of the soil and chemical. Chemical uptake and bioaccumulation were found to be well correlated with a variety of solvent extraction techniques showing that the extraction regime produced is appropriate to a typical soil organism allowing extraction techniques to estimate the amount of bioavailable or bioaccessible residue.

To better understand the relationship between extraction technique and bioavailability, research was carried out on extraction methodologies and how representative each is of the bioavailable and bioaccessible chemical fractions. The majority of studies within the literature used microorganism mineralisation to determine how much chemical was bioaccessible. This baseline level was then compared with the amount of chemical obtained using a specific technique, to determine if the amount recovered was representative of the bioavailable/bioaccessible fraction i.e. which technique produced the value closest to the 'actual' fraction available to the soil biota (as represented by the microorganism mineralisation). Using previous research and the created framework model as a guide, an extraction regime was created which aligned the sorbed state of the chemical residues with suitable techniques for extracting them, for example the rapidly desorbed residue fraction is most closely represented by extraction techniques involving 'mild' solvents such as weak salt solutions designed to mimic soil pore water or a single HPCD extraction. A clear definition was made between those techniques and sorbed states which would cause destruction of the soil matrix.

An important consideration in predicting the behaviour of chemicals entering the soil is to understand their interaction with the soil matrix. A complex set of factors determines how much of the initial application or release forms NER, how much residue is freely dissolved and how much is in equilibrium between being sorbed and desorbed. To be able to better predict the chemical dynamics once a chemical enters the soil, it is necessary to understand the processes which govern these interactions. After extensive research it was determined that generally, chemicals which were most strongly associated with the soil (and least bioaccessible) were either covalently bound to the soil, or physically sequestered and trapped in soil pores. Other interactions which were shown to lead to NER or slowly desorbed residues included ionic and ligand exchange. Chemicals were also shown to interact with the soil matrix via van der Waals' forces, hydrophobic partitioning, charge transfer complexes and hydrogen bonds, these interactions are generally thought of as weaker and most likely to lead to desorbable residues. The various interactions studied (and their bond strength ranges) were aligned with the extraction regime and framework model. Research into this area has led to a better understanding of how chemical properties can influence residue characteristics e.g. van der Waals' forces and hydrophobic interactions are more important in large neutral organic molecules, as well as molecules with reactive groups such as hydroxyl being more prone to covalent bond formation.

One of the major issues of particular concern with regards to environmental risk assessment is the future re-release of NER. A comprehensive review of the literature identified several NER release mechanisms, both physical and chemical, which could cause releases in varying amounts. It was found that physical processes such as freeze-thaw and wet-dry cycling can cause the release of sequestered residues via the breakup of the soil matrix and SOM. Additionally, chemical and biological processes such as pH changes and microorganism metabolism have been found to cause the release of NER. The current literature suggests that the amounts of NER released do not pose an environmental risk, however, it was identified that further research is necessary in this area, especially with regards to release caused by physical processes, on which very few studies exist. Black carbon was identified as a 'super-sorbent' material which could potentially be used (after further study) to reduce the bioavailable fraction of residues in contaminated soils.

In conclusion, the issue of non-extractable residue formation and release is a very complex one. The interaction of chemicals released to the environment with the soil is reliant on a number of factors, not least of all the nature of the soil. Soil organic matter is a key component of soil, this complex soil constituent and the potential interactions it may have with chemicals is not very well understood and needs further research. However, this Task Force from literature review has developed a framework model and extraction regime (see Figure 8) designed to identify bioavailable and bioaccessible residues. It has also identified avenues of work where additional research is needed as environmental risk assessment becomes an increasingly important issue.

ABBREVIATIONS

AA	Amino Acids
AC	Activated Carbon
ADP	Adenosine diphosphate
AOM	Amorphous Activated Matter
ASE	Accelerated Solvent Extraction
BR	Bound Residue
CEC	Cation Exchange Capacity
CMC	Critical Micelle Concentration
DCA	Dichloroaniline
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
EDB	1,2-dibromoethane
EDTA	Ethylene Diamine Tetra Acetic Acid
EqP	Equilibrium Partitioning
ER	Extractable Residue
ERA	Environmental Risk Assessment
ETD	Ethidimuron
FA	Fatty Acids
FAME	Fatty Acid Methyl Esters
HADP	Hydroxylated Atrazine Degradation Products
HCB	Hexachlorobenzene
HPLC	High Performance Liquid Chromatography
HPCD	Hydroxypropyl Cyclodextrin
IUPAC	International Union of Pure and Applied Chemistry
LLE	Liquid-Liquid Extraction
MAE	Microwave-Assisted Extraction

MCI	Molecular Connectivity Index
nd-SPME	Negligible Depletion Solid Phase Micro Extraction
NER	Non-Extractable Residue
OC	Organic Carbon
OM	Organic Matter
PAH	Polycyclic Aromatic Hydrocarbon
PCB	Polychlorinated Biphenyls
PDMS	Polydimethylsiloxane
PLFA	Phospholipid Fatty Acids
POM	Polyoxymethylenes
SDSE	Simultaneous Distillation-Solvent Extraction
SFE	Sub/Supercritical Fluid Extraction
SOM	Soil Organic Matter
SPE	Solid Phase Extraction
SPME	Solid Phase Micro Extraction
TECAM	Triolein Embedded Cellulose Acetate Membrane
TF	Task Force
TOC	Total Organic Carbon
US EPA	United States Environmental Protection Agency
UP	Ultrasound Probe

BIBLIOGRAPHY

Aharonson N, Kafkafi U. 1975. Adsorption of benzimidazole fungicides on montmorillonite and kaolinite clay surfaces. *J Agric Food Chem* 23(3):434-437.

Ahmad R, Kookana RS, Megharaj M, Alston AM. 2004. Aging reduces the bioavailability of even a weakly sorbed pesticide (carbaryl) in soil. *Environ Toxicol Chem* 23(9):2084-2089.

Alexander M. 1995. How toxic are toxic chemicals in soil? *Environ Sci Technol* 29(11):2713-2717.

Allan IJ, Semple KT, Hare R, Reid BJ. 2006. Prediction of mono- and polycyclic aromatic hydrocarbon degradation in spiked soils using cyclodextrin extraction. *Environ Pollut* 144(2):562-571.

Andreux F, Menoret M, Balesdent J, Groussard M, Robert M, Lesaffre M, Jacquin M, Duchaufour M, Feller M, Henin M. 1997. La matière organique des sols. Interactions avec les polluants [Soil organic matter. Interactions with pollutants]. *C R Acad Agric Fr* 83(6):111-123.

Bailey GW, White JL, Rothberg T. 1968. Adsorption of organic herbicides by montmorillonite: Role of pH and chemical character of adsorbate. *Soil Sci Soc Am J* 32:222-234.

Balesdent J, Chenu C, Balabane M. 2000. Relationship of soil organic matter dynamics to physical protection and tillage. *Soil Till Res* 53(3):215-230.

Ball WP, Roberts PV. 1991. Long-term sorption of halogenated organic chemicals by aquifer material. 2. Intraparticle diffusion. *Environ Sci Technol* 25(7):1237-1249.

Barraclough D, Kearney T, Croxford A. 2005. Bound residues: Environmental solution or future problem? *Environ Pollut* 113(1):85-90.

Barriuso E, Koskinen WC, Sadowsky MJ. 2004. Solvent extraction characterization of bioavailability of atrazine residues in soils. *J Agric Food Chem* 52(21):6552-6556.

Barriuso E, Benoit P, Dubus IG. 2008. Formation of pesticide nonextractable (bound) residues in soil: Magnitude, controlling factors and reversibility. *Environ Sci Technol* 42(6):1845-1854.

Bartha R, You IS, Saxena A. 1983. Microbial mineralization of humic acid-3,4-dichloroaniline complexes. In Miyamoto J, Kearney PC, eds, *IUPAC Pesticide Chemistry*. Pergamon Press, New York, USA, pp 345-350.

Beigel C, Charnay M-P, Barriuso E. 1999. Degradation of formulated and unformulated triticonazole fungicide in soil: Effect of application rate. *Soil Biol Biochem* 31(4):525-534.

Belfroid AC, Meiling J, Sijm DTHM, Hermens JLM, Seinen W, van Gestel KCAM. 1994. Uptake of hydrophobic halogenated aromatic hydrocarbons from food by earthworms (*Eisenia andrei*). *Arch Environ Contam Toxicol* 27(2):260-265.

Belfroid AC, Seinen W, van Gestel KCAM, Hermens JLM, van Leeuwen KJ. 1995. Modelling the accumulation of hydrophobic organic chemicals in earthworms. *Environ Sci Pollut Res* 2(1):5-15.

Belfroid AC, Sijm DTHM, van Gestel KCAM. 1996. Bioavailability and toxicokinetics of hydrophobic aromatic compounds in benthic and terrestrial invertebrates. *Environ Rev* 4(4):276-299.

Bergknut M, Sehlin E, Lundstedt S, Andersson PL, Haglund P, Tysklind M. 2007. Comparison of techniques for estimating PAH bioavailability: Uptake in *Eisenia fetida*, passive samplers and leaching using various solvents and additives. *Environ Pollut* 145(1):154-160.

Bernhardt C, Derz K, Kördel W, Terytze K. 2013. Applicability of non-exhaustive extraction procedures with Tenax and HPCD. *J Hazard Mater* (In Press).

Berns A, Vinken R, Bertmer M, Breitschwerdt A, Schäffer A. 2005. Use of ^{15}N-depleted artificial compost in bound residue studies. *Chemosphere* 59(5):649-658.

Berry DF, Boyd SA. 1984. Oxidative coupling of phenols and anilines by peroxidase: Structure-activity relationships. *Soil Sci Soc Am J* 48(3):565-569.

Berry DF, Boyd SA. 1985. Decontamination of soil through enhanced formation of bound residues. *Environ Sci Technol* 19(11):1132-1133.

Beyer WN. 1996. Accumulation of chlorinated benzenes in earthworms. *Bull Environ Contam Toxicol* 57(5):729-736.

Bichat F, Sims GK, Mulvaney RL. 1999. Microbial utilization of heterocyclic nitrogen from atrazine. *Soil Sci Soc Am J* 63(1):100-110.

Bitton G. 2011. Wastewater microbiology. Wiley-Blackwell, Online ISBN 9780470901243.

Björklund E, Bøwadt S, Mathiasson L, Hawthorne SB. 1999. Determining PCB sorption / desorption behavior on sediments using selective supercritical fluid extraction. 1. Desorption from historically contaminated samples. *Environ Sci Technol* 33(13):2193-2203.

Boesten JJTI. 1993. Bioavailability of organic chemicals in soil related to their concentration in the liquid phase: A review. *Sci Total Environ* 134(S1):397-407.

Bollag JM. 1991. Enzymatic binding of pesticide degradation products to soil organic matter and their possible release. In ACS Symposium Series, *Pesticide Transformation Product*, Vol 459. American Chemical Society, pp 122-132.

Bollag JM, Myers CJ, Minard RD. 1992. Biological and chemical interactions of pesticides with soil organic matter. *Sci Total Environ* 123-124:205-217.

Bondarenko S, Hunter W, Gan J. 2006. Solid-phase microextraction (SPME) measurement of pyrethroids in sediment porewater. Abstracts of Papers, 232nd ACS National Meeting, San Francisco, CA, USA, Sept. 10-14.

Bosma TNP, Harms H. 1996. Bioavailability of organic pollutants. *EAWAG News* 40E:28-31.

Brusseau ML, Jessup RE, Rao PSC. 1991. Nonequilibrium sorption of organic chemicals: Elucidation of rate-limiting processes. *Environ Sci Technol* 25(1):134-142.

Calderbank A. 1989. The occurrence and significance of bound pesticide residues in soil. *Rev Environ Contam Toxicol* 108:71-103.

Calvet R. 1989. Adsorption of organic chemicals in soils. *Environ Health Perspect* 83:145-177.

Carter MR, Gregorich EG, eds. 2007. Soil Sampling and Methods of Analysis, 2nd Ed. Taylor & Francis.

Charnay M-P, Mougin C, Farrugia A, Barriuso E. 2004. Incorporation of pesticides by soil micro-organisms as a way of bound residues formation. *Environ Chem Lett* 2(1):27-30.

Chen Y, Schnitzer M. 1976. Scanning electron microscopy of a humic acid and of a fulvic acid and its metal and clay complexes. *Soil Sci Soc Am J* 40(5):682-686.

Cheng HH. 1990. Organic residues in soils: Mechanisms of retention and extractability. *Int J Environ Anal Chem* 39(2):165-171.

Cheng H, Hu E, Hu Y. 2012. Impact of mineral micropores on transport and fate of organic contaminants: A review. *J Contam Hydrol* 129-130:80-90.

Chiba M, Morley HV. 1968. Factors influencing extraction of Aldrin and Dieldrin residues from different soil types. *J Agric Food Chem* 16(6):916-922.

Chung N, Alexander M. 1998. Differences in sequestration and bioavailability of organic compounds aged in dissimilar soils. *Environ Sci Technol* 32(7):855-860.

Chung N, Alexander M. 1999a. Effect of concentration on sequestration and bioavailability of two polycyclic aromatic hydrocarbons. *Environ Sci Technol* 33(20):3605-3608.

Chung N, Alexander M. 1999b. Relationship between nanoporosity and other properties of soil. *Soil Sci* 164(10):726-730.

Collins C, Fryer M, Grosso A. 2006. Plant uptake of non-ionic organic chemicals. *Environ Sci Technol* 40(1):45-52.

Connell DW, Markwell RD. 1990. Bioaccumulation in the soil to earthworm system. *Chemosphere* 20(1-2):91-100.

Cornelissen G, van Noort PCM, Govers HAJ. 1997. Desorption kinetics of chlorobenzenes, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls: Sediment extraction with Tenax® and effects of contact time and solute hydrophobicity. *Environ Toxicol Chem* 16(7):1351-1357.

Cornelissen G, van Noort PCM, Govers HAJ. 1998a. Mechanism of slow desorption of organic compounds from sediments: A study using model sorbents. *Environ Sci Technol* 32(20):3124-3131.

Cornelissen G, Rigterink H, Ferdinandy MMA, van Noort PCM. 1998b. Rapidly desorbing fractions of PAHs in contaminated sediments as a predictor of the extent of bioremediation. *Environ Sci Technol* 32(7):966-970.

Cornelissen G, Gustafsson Ö. 2004. Sorption of phenanthrene to environmental black carbon in sediment with and without organic matter and native sorbates. *Environ Sci Technol* 38(1):148-155.

Couling NR, Towell MG, Semple KT. 2010. Biodegradation of PAHs in soil: Influence of chemical structure, concentration and multiple amendment. *Environ Pollut* 158(11):3411-3420.

Cox L, Koskinen WC, Celis R, Hermosin MC, Cornejo J, Yen PY. 1998. Sorption of imidacloprid on soil clay mineral and organic components. *Soil Sci Soc Am J* 62(4):911-915.

Cuypers C, Pancras T, Grotenhuis T, Rulkens W. 2002. The estimation of PAH bioavailability in contaminated sediments using hydroxypropyl-beta-cyclodextrin and triton X-100 extraction techniques. *Chemosphere* 46(8):1235-1245.

Dec J, Bollag J-M. 1988. Microbial release and degradation of catechol and chlorophenols bound to synthetic humic acid. *Soil Sci Soc Am J* 52(5):1366-1371.

Dec J, Haider K, Rangaswamy V, Schäffer A, Fernandes E, Bollag J-M. 1997. Formation of soil-bound residues of cyprodinil and their plant uptake. *J Agric Food Chem* 45(2):514-520.

Dec J, Shuttleworth KL, Bollag J-M. 1990. Microbial release of 2,4-dichlorophenol bound to humic acid or incorporated during humification. *J Environ Qual* 19(3):546-551.

Dickinson E. 1997. Enzymic crosslinking as a tool for food colloid rheology control and interfacial stabilization. *Trends Food Sci Technol* 8(10):334-339.

Díez C, Traag WA, Zommer P, Marinero P, Atienza J. 2006. Comparison of an acetonitrile extraction/partitioning and “dispersive solid-phase extraction” method with classical multi-residue methods for the extraction of herbicide residues in barley samples. *J Chromatogr A* 1131(1-2):11-23.

Ding Q, Wu HL, Xu Y, Guo LJ, Liu K, Gao HM, Yang H. 2011. Impact of low molecular weight organic acids and dissolved organic matter on sorption and mobility of isoproturon in two soils. *J Hazard Mater* 190(1-3):823-832.

Di Toro DM, Zarba CS, Hansen DJ, Berry WJ, Swartz RC, Cowan CE, Pavlou SP, Allen HE, Thomas NA, Paquin PR. 1991. Technical basis for establishing sediment quality criteria for nonionic organic chemicals using equilibrium partitioning. *Environ Toxicol Chem* 10(12):1541-1583.

Doick KJ, Dew NM, Semple KT. 2005. Linking catabolism to cyclodextrin extractability: Determination of the microbial availability of PAHs in soil. *Environ Sci Technol* 39(22):8858-8864.

Doick KJ, Clasper PJ, Urmann K, Semple KT. 2006. Further validation of the HPCD-technique for the evaluation of PAH microbial availability in soil. *Environ Pollut* 144(1):345-354.

Ebert D. 1992. Differences in uptake of bound residues of bentazon, a herbicide, by two different earthworm species, *Eisenia foetida* (Sav.) and *Lumbricus rubellus* (Hoffm.). *Soil Biol Biochem* 24(12):1715-1718.

ECETOC. 2010. Significance of Bound Residues in Environmental Risk Assessment. 14-15 October 2009, Brussels. Workshop Report No. 17. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.

ECHA. 2008. Guidance on information requirements and chemical safety assessment, version 1.1. Chapter R.7b: Endpoint specific guidance. European Chemicals Agency, Helsinki, Finland.

Ehlers GA, Loibner AP. 2006. Linking organic pollutant (bio)availability with geosorbent properties and biomimetic methodology: A review of geosorbent characterisation and (bio)availability prediction. *Environ Pollut* 141(3):494-512.

Eriksson M, Ka J-O, Mohn WW. 2001. Effects of low temperature and freeze-thaw cycles on hydrocarbon biodegradation in Arctic Tundra soil. *Appl Environ Microbiol* 67(11):5107-5112.

Eschenbach A, Wienberg R, Mahro B. 1998. Risk assessment of PAH in soil. Behavior and fate of nonextractable ^{14}C-PAH-residues under environmental stress conditions. Contaminated Soil ’98, Proceedings of the International FZK/TNO Conference on Contaminated Soil 2:821-822.

Esnaola MV, Millán E. 1998. Effect of acid precipitation on the mobilisation and leaching of heavy metals from contaminated soils. *Environ Technol* 19(6):567-578.

Fang H, Chu X, Wang X, Pang G, Yu Y. 2010. Using matrix solid-phase microextraction (matrix-SPME) to estimate bioavailability of DDTs in soil to both earthworm and vegetables. *Arch Environ Contam Toxicol* 58(1):62-70.

Feng X, Nielsen LL, Simpson MJ. 2007. Responses of soil organic matter and microorganisms to freeze-thaw cycles. *Soil Biol Biochem* 39(8):2027-2037.

Fierer N, Schimel JP. 2002. Effects of drying-rewetting frequency on soil carbon and nitrogen transformations. *Soil Biol Biochem* 34(6):777-787.

Fomsgaard IS. 2004. The influence of sorption on the degradation of pesticides and other chemicals in soil. Environmental Project No. 902. Danish Environmental Protection Agency.

Förster M, Laabs V, Lamshöft M, Groeneweg J, Zühlke S, Spiteller M, Krauss M, Kaupenjohann M, Amelung W. 2009. Sequestration of manure-applied sulfadiazine residues in soils. *Environ Sci Technol* 43(6):1824-1830.

Frische T, Mebes KH, Filser J. 2003. Assessing the bioavailability of contaminants in soils: A review on recent concepts. Environmental research of the Federal Ministry of the environment, nature conservation and nuclear safety. Research report 201 64 214, UBA-FB 000405. On behalf of the Federal Environmental Agency, UBA, Berlin, Germany.

Führ F. 1987. Non-extractable pesticide residues in soil. In Greenhalgh R, Roberts TR, eds, Pesticide Science and Biotechnology, Proceedings 6th Int Congr Pestic Chemistry, IUPAC, Blackwell Scientific Publications, London, pp 381-389.

Führ F, Ophoff H, Burauel P, Wanner U, Haider K. 1998. Modification of the definition of bound residues. In Führ F, Ophoff H, eds, *Pesticide Bound Residues in soil*. Wiley-VCH, Weinheim, Germany pp 175-176.

Fuhremann TW, Lichtenstein EP. 1978. Release of soil-bound methyl [^{14}C]parathion residues and their uptake by earthworms and oats plants. *J Agric Food Chem* 26(3):605-610.

García-Valcárcel AI, Tadeo JL. 1999. Influence of soil moisture on sorption and degradation of hexazinone and simazine in soil. *J Agric Food Chem* 47(9):3895-3900.

Gevao B, Semple KT, Jones KC. 2000. Bound residue residues in soils: A review. *Environ Pollut* 108(1):3-14.

Gevao B, Mordaunt C, Semple KT, Piearce TG, Jones KC. 2001. Bioavailability of nonextractable (bound) pesticide residues to earthworms. *Environ Sci Technol* 35(3):501-507.

Gevao B, Jones KC, Semple KT, Craven A, Burauel P. 2003. Peer reviewed: Nonextractable pesticide residues in soil. *Environ Sci Technol* 37(7):138A-144A.

Ghani A, Wardle DA. 2001. Fate of ^{14}C from glucose and the herbicide metsulfuron-methyl in a plant-soil microcosm system. *Soil Biol Biochem* 33(6):777-785.

Ghosh U, Luthy RG, Cornelissen G, Werner D, Menzie CA. 2011. In-situ sorbent amendments: A new direction in contaminated sediment management. *Environ Sci Technol* 45(4):1163-1168.

Goetz AJ, Wehtje G, Walker RH, Hajek B. 1986. Soil solution and mobility characterization of imazaquin. *Weed Sci* 34(5):788-793.

Gomez-Eyles JL, Collins CD, Hodson ME. 2010. Relative proportions of polycyclic hydrocarbons differ between accumulation bioassays and chemical methods to predict bioavailability. *Environ Pollut* 158(1):278-284.

Gomez-Eyles JL, Collins CD, Hodson ME. 2011. Using deuterated PAH amendments to validate chemical extraction methods to predict PAH bioavailability in soils. *Environ Pollut* 159(4):918-923.

Hale SE, Elmquist M, Brändli R, Hartnik T, Jakob L, Henriksen T, Werner D, Cornelissen G. 2012. Activated carbon amendment to sequester PAHs in contaminated soil: A lysimeter field trial. *Chemosphere* 87(2):177-184.

Hallgren P, Westbom R, Nilsson T, Sporring S, Björklund E. 2006. Measuring bioavailability of polychlorinated biphenyls in soil to earthworms using selective supercritical fluid extraction. *Chemosphere* 63(9):1532-1538.

Hamaker JW, Thompson JM. 1972. Adsorption in organic chemicals. In Goring CAI, Hamaker JW, eds, *Organic chemicals in the soil environment*, Vol 1. Marcel Dekker Inc, New York, NY, USA, pp 49-143.

Harms H, Bosma TNP. 1997. Mass transfer limitation of microbial growth and pollutant degradation. *J Ind Microbiol Biotechnol* 18(2-3):97-105.

Harms H, Wick LY. 2004. Mobilisation of organic compounds and iron by microorganisms. In van Leeuwen HP, Koster W, eds, *Physicochemical kinetics and transport at biointerfaces*, Vol 9. John Wiley & Sons, Chichester, UK.

Hartnik T, Jensen J, Hermens JLM. 2008. Nonexhaustive β-cyclodextrin extraction as a chemical tool to estimate bioavailability of hydrophobic pesticides for earthworms. *Environ Sci Technol* 42(22):8419-8425.

Hatzinger PB, Alexander M. 1995. Effect of aging chemicals in soil on their biodegradability and extractability. *Environ Sci Technol* 29(2):537-545.

Hatzinger PB, Alexander M. 1997. Biodegradation of organic compounds sequestered in organic solids or in nanopores within silica particles. *Environ Toxicol Chem* 16(11):2215-2221.

Hawthorne SB, Grabanski CB, Martin E, Miller DJ. 2000. Comparisons of Soxhlet extraction, pressurized liquid extraction, supercritical fluid extraction and subcritical water extraction for environmental solids: Recovery, selectivity and effects on sample matrix. *J Chromatogr A* 892(1-2):421-433.

Hawthorne SB, Grabanski CB. 2000. Correlating selective supercritical fluid extraction with bioremediation behavior of PAHs in a field treatment plot. *Environ Sci Technol* 34(19):4103-4110.

Hawthorne SB, Lanno R, Kreitinger JP. 2005. Reduction in acute toxicity of soils to terrestrial oligochaetes following the removal of bioavailable polycyclic aromatic hydrocarbons with mild supercritical carbon dioxide extraction. *Environ Toxicol Chem* 24(8):1893-1895.

Hawthorne SB, Azzolina NA, Neuhauser EF, Kreitinger JP. 2007. Predicting bioavailability of sediment polycyclic aromatic hydrocarbons to Hyalella azteca using equilibrium partitioning, supercritical fluid extraction, and pore water concentrations. *Environ Sci Technol* 41(17):6297-6304.

Hayes MHB, Swift RS, Wardle RE, Brown JK. 1975. Humic materials from an organic soil: A comparison of extractants and of properties of extracts. *Geoderma* 13(3):231-245.

Hayes MHB, Himes FL. 1986. Natural properties of humus mineral complexes. In Huang PM, Schnitzer M, eds, *Interactions of soil minerals with natural organics and microbes*. Soil Science Society of America, Madison, Wisconsin, USA, pp 103-158.

Helling CS, Krivonak AE. 1978. Biological characteristics of bound dinitroaniline herbicides in soils. *J Agric Food Chem* 26(5):1164-1172.

Hellstrom A. 2004. Uptake of organic pollutants in plants. Department of Environmental Assessment, Swedish University of Agricultural Sciences, Upsalla, Sweden.

Helweg A. 1975. Degradation of ^{14}C-labelled maleic hydrazide in soil as influenced by sterilization, concentration and pretreatment. *Weed Res* 15(1):53-58.

Heringa MB, Hermens JLM. 2003. Measurement of free concentrations using negligible depletion-solid phase microextraction (nd-SPME). *Trends Analyt Chem* 22(9):575-587.

Hermens JLM, Freidig AP, Ramos EU, Vaes WHJ, Van Loon WMGM, Verbruggen EMJ, Verhaar HJM. 2001. Application of negligible depletion solid-phase extraction (nd-SPE) for estimating bioavailability and bioaccumulation of individual chemicals and mixtures. In ACS Symposium Series 773, *Persistent, bioaccumulative, and toxic chemicals* II, Chapter 6, pp 64–74. ACS Publications, Washington, DC, USA.

Hoskins BR, Ross D. 2011. Soil sample preparation and extraction. In Recommended soil testing procedures for the Northeastern United States, 3rd edition. Northeastern Regional Publication No. 493.

Houba VJG, Temminghoff EJM, Gaikhorst GA, van Vark W. 2000. Soil analysis procedures using 0.01 *M* calcium chloride as extraction reagent. *Commun Soil Sci Plant Anal* 31(9-10):1299-1396.

Hu X, Zhou Q. 2011. Comparisons of microwave-assisted extraction, simultaneous distillation-solvent extraction, Soxhlet extraction and ultrasound probe for polycyclic musks in sediments: Recovery, repeatability, matrix effects and bioavailability. *Chromatographia* 74(5-6):489-495.

Hunter W, Yang W, Gan J, Bondarenko S, Spurlock F. 2006. Bioavailability of pyrethroids in surface water and sediment. Abstracts of Papers, 232nd ACS National Meeting, San Francisco, CA, USA, Sept 10-14.

Israelachvili JN. 1991. Intermolecular and surface forces. Academic Press, New York, NY, USA.

Jablonowski ND, Yu K, Koeppchen S, Burauel P. 2012a. Release of aged ^{14}C-atrazine residues from soil facilitated by dry-wet cycles. *Geophys Res Abstr* 14:EGU2012-9514-1.

Jablonowski ND, Mucha M, Thiele B, Hofmann D, Burauel P. 2012b. Influence of alternating soil drying and wetting on the desorption and distribution of aged ^{14}C-labeled pesticide residues in soil organic fractions. *Geophys Res Abstr* 14:EGU2012-9673.

Jager T. 2003. Worming your way into bioavailability. Modelling the uptake of organic chemicals in earthworms. PhD Thesis.

Jager T, Fleuren RHLJ, Hogendoorn EA, de Korte G. 2003. Elucidating the routes of exposure for organic chemicals in the earthworm, *Eisenia andrei* (Oligochaeta). *Environ Sci Technol* 37(15):3399-3404.

Jager T. 2004. Modelling ingestion as an exposure route for organic chemicals in earthworms (Oligochaeta). *Ecotoxicol Environ Saf* 57(1):30-38.

Jakher A, Achari G, Langford CH. 2006. An investigation into solvent extraction of PCBs from weathered soils. *Environ Technol* 28(1):49-57.

Jelić A, Petrović M, Barceló D. 2009. Multi-residue method for trace level determination of pharmaceuticals in solid samples using pressurized liquid extraction followed by liquid chromatography/quadrupole-linear ion trap mass spectrometry. *Talanta* 80(1):363-371.

Jenkinson DS, Powlson DS. 1976. The effects of biocidal treatments on metabolism in soil – V: A method for measuring soil biomass. *Soil Biol Biochem* 8(3):209-213.

Johnsen AR, Wick LY, Harms H. 2005. Principles of microbial PAH-degradation in soil. *Environ Pollut* 133(1):71-84.

Johnson DL, Jones KC, Langdon CJ, Piearce TG, Semple KT. 2002. Temporal changes in earthworm availability and extractability of polycyclic aromatic hydrocarbons in soil. *Soil Biol Biochem* 34(9):1363-1370.

Jonker MTO, Hoenderboom AM, Koelmans AA. 2004. Effects of sedimentary sootlike materials on bioaccumulation and sorption of polychlorinated biphenyls. *Environ Toxicol Chem* 23(11):2563-2570.

Jonker MTO, van der Heijden SA, Kreitinger JP, Hawthorne SB. 2007. Predicting PAH bioaccumulation and toxicity in earthworms exposed to manufactured gas plant soils with solid-phase microextraction. *Environ Sci Technol* 41(21):7472-7478.

Käcker T, Haupt ETK, Garms C, Francke W, Steinhart H. 2002. Structural characterisation of humic acid-bound PAH residues in soil by 13C-CPMAS-NMR-spectroscopy: Evidence of covalent bonds. *Chemosphere* 48(1):117-131.

Kahle M, Stamm C. 2007. Time and pH-dependent sorption of the veterinary antimicrobial sulfathiazole to clay minerals and ferrihydrite. *Chemosphere* 68(7):1224-1231.

Katayama A, Bhula R, Burns GR, Carazo E, Felsot A, Hamilton D, Harris C, Kim Y-H, Kleter G, Koedel W, Linders J, Peijnenburg JGMW, Sabljic A, Stephenson RG, Racke DK, Rubin B, Tanaka K, Unsworth J, Wauchope RD. 2010. Bioavailability of xenobiotics in the soil environment. *Rev Environ Contam Toxicol* 203:1-86.

Kaufman DD. 1976. Bound and conjugated pesticide residues. In Kaufman DD, Still GG, Paulson GD, Bandal SK, eds, *Bound and Conjugated Pesticide Residues*. ACS Symposium Series 29:1-10.

Kearney PC. 1982. IUPAC Pesticide Commission Report. *J Assoc Off Anal Chem* 65:1030-1032.

Kelsey JW, Alexander M. 1997. Declining bioavailability and inappropriate estimation of risk of persistent compounds. *Environ Toxicol Chem* 16(3):582-585.

Kelsey JW, Kottler BD, Alexander M. 1997. Selective chemical extractants to predict bioavailability of soil-aged organic chemicals. *Environ Sci Technol* 31(1):214-217.

Khan SU. 1980. Plant uptake of unextracted (bound) residues from an organic soil treated with prometryn. *J Agric Food Chem* 28(6):1096-1098.

Khan SU. 1982. Bound pesticide residues in soil and plants. *Residue Rev* 84:1-25.

Khan SU, Ivarson KC. 1981. Microbiological release of unextracted (bound) residues from an organic soil treated with prometryn. *J Agric Food Chem* 29:1301-1303.

Khan SU, Ivarson KC. 1982. Release of soil bound (nonextractable) residues by various physiological groups of microorganisms. *J Environ Sci Health B* 17(6):737-749.

Klaus U, Mohamed S, Volk M, Spiteller M. 1998. Interaction of aquatic humic substances with anilazine and its derivatives: The nature of the bound residues. *Chemosphere* 37(2):341-361.

Klein W, Scheunert I. 1982. Bound pesticide residues in soil, plants and food with particular emphasis on the application of nuclear techniques. International symposium on agrochemicals: Fate in food and the environment using isotope techniques. Rome (Italy). 7-11 Jun 1982. Report IAEA-SM-263-38. Proceedings series.

König B. 2011. Vorlesung Supramolekulare Chemie. Regensburg University, Institut für Organische Chemie, Germany. www-oc.chemie.uni-regensburg.de

Kookana RS, Gerritse RG, Aylmore LAG. 1990. Effect of organic cosolvent on adsorption and desorption of linuron and simazine in soil. *Aust J Soil Res* 28(5):717-725.

Kottler BD, Alexander M. 2001. Relationship of properties of polycyclic aromatic hydrocarbons to sequestration in soil. *Environ Pollut* 113(3):293-298.

Kramer BK, Ryan PB. 2000. Soxhlet and microwave extraction in determining the bioaccessibility of pesticides from soil and model solids. Proceedings of the Conference on Hazardous Waste Research: Environmental Challenges and Solutions to Resource Development, Production, and Use, Denver, CO, United States, May 23-25, 2000, 196-210.

Krauss M, Wilcke W, Zech W. 2000. Availability of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) to earthworms in urban soils. *Environ Sci Technol* 34(20):4335-4340.

Krishnamurti GSR. 2008. Chapter 20: Chemical methods for assessing contaminant bioavailability in soils. *Dev Soil Sci* 32:495-520.

Lanno R, Wells J, Conder J, Bradham K, Basta N. 2004. The bioavailability of chemicals in soil for earthworms. *Ecotoxicol Environ Saf* 57(1):39-47.

Latawiec AE, Swindell AL, Reid BJ. 2008. Environmentally friendly assessment of organic compound bioaccessibility using sub-critical water. *Environ Pollut* 156(2):467-473.

Lau EV, Gan S, Ng HK. 2010. Extraction techniques for polycyclic aromatic hydrocarbons in soils. Int J Anal Chem, Vol 2010, Article ID 398381, 9 pp, doi: 10.155/2010/398381.

Lennartz B, Louchart X. 2007. Effect of drying on the desorption of diuron and terbuthylazine from natural soils. *Environ Pollut* 146(1):180-187.

Leone P, Nègre M, Gennari M, Boero V, Celis R, Cornejo J. 2002. Adsorption of imidazolinone herbicides on smectite-humic acid and smectite-ferrihydrite associations. *J Agric Food Chem* 50(2):291-298.

Leppänen MT, Kukkonen JVK. 1998. Relative importance of ingested sediment and pore water as bioaccumulation routes for pyrene to oligochaete (*Lumbriculus variegatus*, Müller). *Environ Sci Technol* 32(10):1503-1508.

Lerch RN, Thurman EM, Kruger EL. 1997. Mixed-mode sorption of hydroxylated atrazine degradation products to soil: A mechanism for bound residue. *Environ Sci Technol* 31(5):1539-1546.

Liang X, Zhu S, Chen P, Zhu L. 2010. Bioaccumulation and bioavailability of polybrominated diphenyl ethers (PBDEs) in soil. *Environ Pollut* 158(7):2387-2392.

Liste H-H, Alexander M. 2002. Butanol extraction to predict bioavailability of PAHs in soil. *Chemosphere* 46(7):1011-1017.

Lord KA, Briggs GG, Neale MC, Manlove R. 1980. Uptake of pesticides from water and soil by earthworms. *Pestic Sci* 11(4):401-408.

Lu Y, Pignatello JJ. 2002. Demonstration of the “conditioning effect” in soil organic matter in support of a pore deformation mechanism for sorption hysteresis. *Environ Sci Technol* 36(21):4553-4561.

Macleod CJA, Semple KT. 2006. The influence of single and multiple applications of pyrene on the evolution of pyrene catabolism in soil. *Environ Pollut* 139(3):455-460.

Martinez E, Gros M, Lacorte S, Barceló D. 2004. Simplified procedures for the analysis of polycyclic aromatic hydrocarbons in water, sediments and mussels. *J Chromatogr A* 1047(2):181-188.

Markovitch O, Agmon N. 2007. Structure and energetics of the hydronium hydration shells. *J Phys Chem A* 111(12):2253-2256.

Mathur SP, Paul EA. 1967. Microbial utilization of soil humic acids. *Can J Microbiol* 13(5):573-580.

Mayer LM. 1994. Relationships between mineral surfaces and organic carbon concentrations in soils and sediments. *Chem Geol* 114(3-4):347-363.

Mayer P, Vaes WHJ, Wijnker F, Legierse KCHM, Kraaij RH, Tolls J, Hermens JLM. 2000. Sensing dissolved sediment porewater concentrations of persistent and bioaccumulative pollutants using disposable solid-phase microextraction fibers. *Environ Sci Technol* 34(24):5177-5183.

McDowell RW, Sharpley AN. 2001. Soil phosphorus fractions in solution: Influence of fertiliser and manure, filtration and method of determination. *Chemosphere* 45(6-7):737-748.

Meharg AA. 1996. Bioavailability of atrazine to soil microbes in the presence of the earthworm *Lumbricus terrestris* (L.). *Soil Biol Biochem* 28(4-5):555-559.

Miltner A, Kopinke F-D, Kindler R, Selesi D, Hartmann A, Kästner M. 2005. Non-phototrophic CO_2 fixation by soil microorganisms. *Plant Soil* 269(1-2):193-203.

Morrison DE, Robertson BK, Alexander M. 2000. Bioavaibility to earthworms of aged DDT, DDE, DDD, and Dieldrin in soil. *Environ Sci Technol* 34(4):709-713.

Mostafa IY, Zayed SMAD, Adam YM, Attaby HSH. 1982. Investigations on trifluralin binding to soil and possible uptake of bound residues by plants. *J Environ Sci Health B* 17(3):265-275.

Nam K, Alexander M. 1998. Role of nanoporosity and hydrophobicity in sequestration and bioavailability: Tests with model solids. *Environ Sci Technol* 32(1):71-74.

Nam K, Chung N, Alexander M. 1998. Relationship between organic matter content of soil and the sequestration of phenanthrene. *Environ Sci Technol* 32(23):3785-3788.

Nam JJ, Gustafsson O, Kurt-Karakus P, Breivik K, Steinnes E, Jones KC. 2008. Relationships between organic matter, black carbon and persistent organic pollutants in European background soils: Implications for sources and environmental fate. *Environ Pollut* 156(3):809-817.

Northcott GL, Jones KC. 2000. Experimental approaches and analytical techniques for determining organic compound bound residues in soil and sediment. *Environ Pollut* 108(1):19-43.

Nowak KM, Miltner A, Gehre M, Schäffer A, Kästner M. 2011. Formation and fate of bound residues from microbial biomass during 2,4-D degradation in soil. *Environ Sci Technol* 45(3):999-1006.

Nowak KM. 2011. "Bound" residues from biomass and CO_2 in soils – formation, fate and stability during biotic incubation. Thesis. Helmholtz-Zentrum fur Umweltsforschung-UFZ, Bibliothek, ISSN 1860-0387.

Oleszczuk P, Hale SE, Lehmann J, Cornelissen G. 2012. Activated carbon and biochar amendments decrease pore-water concentrations of polycyclic aromatic hydrocarbons (PAHs) in sewage sludge. *Bioresour Technol* 111:84-91.

Oliver BG. 1984. Uptake of chlorinated organics from anthropogenically contaminated sediments by oligochaete worms. *Can J Fish Aquat Sci* 41(6):878-883.

Parales RE, Ju KS, Rollefson JB, Ditty JL. 2008. Bioavailability, chemotaxis and transport of organic pollutants. In Diaz E, ed, *Microbial biodegradation: Genomics and molecular biology*. Caister Academic Press, Norfolk, UK, pp 145-187.

Pandey G, Jain RK. 2002. Bacterial chemotaxis toward environmental pollutants: Role in bioremediation. *Appl Environ Microbiol* 68(12):5789-5795.

Peijnenburg WJ, Zablotskaja M, Vijver MG. 2007. Monitoring metals in terrestrial environments within a bioavailability framework and a focus on soil extraction. *Ecotoxicol Environ Saf* 67(2):163-179.

Piatt JJ, Brusseau ML. 1998. Rate-limited sorption of hydrophobic organic compounds by soils with well-characterized organic matter. *Environ Sci Technol* 32(11):1604-1608.

Pignatello JJ. 1989. Sorption dynamics of organic chemicals in soils and sediments. In *Reactions and movements of organic chemicals in soils*, SSSA special publication 22:45-80.

Pignatello JJ, Xing B. 1995. Mechanisms of slow sorption of organic chemicals to natural particles. *Environ Sci Technol* 30(1):1-11.

Postma J, van Veen JA. 1990. Habitable pore space and survival of *Rhizobium leguminosarum* biovar *trifolii* introduced into soil. *Microb Ecol* 19(2):149-161.

Qi Y, Chen W. 2010. Comparison of earthworm bioaccumulation between readily desorbable and desorption-resistant naphthalene: Implications for biouptake route. *Environ Sci Technol* 44(1):323-328.

Racke KD, Lichtenstein EP. 1985. Effects of soil microorganisms on the release of bound ^{14}C residues from soils previously treated with [^{14}C]parathion. *J Agric Food Chem* 33(5):938-943.

Ramos L, Kristenson EM, Brinkman UA. 2002. Current use of pressurised liquid extraction and subcritical water extraction in environmental analysis. *J Chromatogr A* 975(1):3-29.

Regitano JB, Koskinen WC, Sadowsky MJ. 2006. Influence of soil aging on sorption and bioavailability of simazine. *J Agric Food Chem* 54(4):1373-1379.

Reichenberg F, Mayer P. 2006. Two complementary sides of bioavailability: Accessibility and chemical activity of organic contaminants in sediments and soils. *Environ Toxicol Chem* 25(5):1239-1245.

Reid BJ, Semple KT, Jones KC. 1998. Prediction of bioavailability of persistent organic pollutants by a novel extraction technique. ConSoil 98, Vol 2. Lancaster EPrints, pp 889-990.
http://eprints.lancs.ac.uk/id/eprint/18598

Reid BJ, Jones KC, Semple KT. 1999. Can bioavailability of PAHs be assessed by a chemical means. In Leeson A, Alleman BC, eds, Bioremediation technologies for polycyclic aromatic hydrocarbon compounds, Proceedings of the 5th *In Situ* and On-Site Bioremediation Symposium, Vol 8. Battelle Press, Columbus, OH, USA, pp 253-258.

Reid BJ, Stokes JD, Jones KC, Semple KT. 2000a. Nonexhaustive cyclodextrin-based extraction technique for the evaluation of PAH bioavailability. *Environ Sci Technol* 34(15):3174-3179.

Reid, BJ, Jones KC, Semple KT. 2000b. Bioavailability of persistent organic pollutants in soils and sediments – A perspective on mechanisms, consequences and assessment. *Environ Pollut* 108(1):103-112.

Rhodes AH, McAllister LE, Chen R, Semple KT. 2010a. Impact of activated charcoal on the mineralisation of ^{14}C-phenanthrene in soils. *Chemosphere* 79(4):463-469.

Rhodes AH, McAllister LE, Semple KT. 2010b. Linking desorption kinetics to phenanthrene biodegradation in soil. *Environ Pollut* 158(5):1348-1353.

Richnow HH, Seifert R, Hefter J, Link M, Francke W, Schaefer G, Michaelis W. 1997. Organic pollutants associated with macromolecular soil organic matter: Mode of binding. *Org Geochem* 26(11-12):745-758.

Richnow HH, Annweiler E, Koning M, Lüth J-C, Stegmann R, Garms C, Francke W, Michaelis W. 2000. Tracing the transformation of labelled [1-^{13}C]phenanthrene in a soil bioreactor. *Environ Pollut* 108(1):91-101.

Roberts TR, Standen ME. 1981. Further studies of the degradation of the pyrethroid insecticide cypermethrin in soils. *Pestic Sci* 12(3):285-296.

Robertson BK, Alexander M. 1994. Growth-linked and cometabolic biodegradation: Possible reason for occurrence or absence of accelerated pesticide biodegradation. *Pestic Sci* 41(4):311-318.

Rodil R, Popp P. 2006. Development of pressurized subcritical water extraction combined with stir bar sorptive extraction for the analysis of organochlorine pesticides and chlorobenzenes in soils. *J Chromatogr A* 1124(1-2):82-90.

Ronday R, Van Kammen-Polman AMM, Dekker A, Houx NWH, Leistra M. 1997. Persistence and toxicological effects of pesticides in topsoil: Use of the equilibrium partitioning theory. *Environ Toxicol Chem* 16(4):601-607.

Schroll R, Becher HH, Dörfler U, Gayler S, Grundmann S, Hartmann HP, Ruoss J. 2006. Quantifying the effect of soil moisture on the aerobic microbial mineralization of selected pesticides in different soils. *Environ Sci Technol* 40(10):3305-3312.

Semple KT, Doick KJ, Jones KC, Burauel P, Craven A, Harms H. 2004. Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated. *Environ Sci Technol* 38(12):228A-231A.

Semple KT, Doick KJ, Wick LY, Harms H. 2007. Microbial interactions with organic contaminants in soil: Definitions, processes and measurement. *Environ Pollut* 150(1):166-176.

Senesi N. 1992. Binding mechanisms of pesticides to soil humic substances. *Sci Total Environ* 123-124:63-76.

Shea D. 1988. Developing national sediment quality criteria. *Environ Sci Technol* 22(11):1256-1261.

Sijm D, Kraaij R, Belfroid A. 2000. Bioavailability in soil or sediment: Exposure of different organisms and approaches to study it. *Environ Pollut* 108(1):113-119.

Smith BA, Greenberg B, Stephenson GL. 2010. Comparison of biological and chemical measures of metal bioavailability in field soils: Test of a novel simulated earthworm gut extraction. *Chemosphere* 81(6):755-766.

Sopeña F, Semple KT, Sohi S, Bending G. 2012. Assessing the chemical and biological accessibility of the herbicide isoproturon in soil amended with biochar. *Chemosphere* 88(1):77-83.

Stegmann R, Brunner G, Calmano W, Matz G, eds. 2001. Treatment of contaminated soil. Fundamentals, analysis, applications. XVI, Springer pp 658. http://www.springer.com/978-3-540-41736-1

Steinberg SM, Pignatello JJ, Sawhney BL. 1987. Persistence of 1,2-dibromoethane in soils: Entrapment in intraparticle micropores. *Environ Sci Technol* 21(12):1201-1208.

Stokes JD, Wilkinson A, Reid BJ, Jones KC, Semple KT. 2005. Prediction of polycyclic aromatic hydrocarbon biodegradation in contaminated soils using an aqueous hydroxypropyl-β-cyclodextrin extraction technique. *Environ Toxicol Chem* 24(6):1325-1330.

Stroud JL, Paton GI, Semple KT. 2008a. Linking chemical extraction to microbial degradation of ^{14}C-hexadecane in soil. *Environ Pollut* 156(2):474-481.

Stroud JL, Rhodes AH, Semple KT, Simek Z, Hofman J. 2008b. Using supercritical fluid extraction to measure the desorption and bioaccessibility of phenanthrene in soils. *Environ Pollut* 156(3):664-670.

Stroud JL, Paton GI, Semple KT. 2009. Predicting the biodegradation of target hydrocarbons in the presence of mixed contaminants in soil. *Chemosphere* 74(4):563-567.

Strynar M, Dec J, Benesi A, Jones AD, Fry RA, Bollag J-M. 2004. Using ^{19}F NMR spectroscopy to determine trifluralin binding to soil. *Environ Sci Technol* 38(24):6645-6655.

Styrishave B, Mortensen M, Krogh PH, Andersen O, Jensen J. 2008. Solid-phase microextraction (SPME) as a tool to predict the bioavailability and toxicity of pyrene to the Springtail, *Folsomia candida*, under various soil conditions. *Environ Sci Technol* 42(4):1332-1336.

Sun H-W, Li J-G. 2005. Availability of pyrene in unaged and aged soils to earthworm uptake, butanol extraction and SFE. *Water Air Soil Pollut* 166(1-4):353-365.

Suss A, Grampp B. 1973. The uptake of adsorbed monolinuron in the soil by mustard plants. *Weed Res* 13(3):254-266.

Szecsody JE, Bales RC. 1991. Temperature effects on chlorinated-benzene sorption to hydrophobic surfaces. *Chemosphere* 22(12):1141-1151.

Tang J, Robertson BK, Alexander M. 1999. Chemical-extraction methods to estimate bioavailability of DDT, DDE and DDD in soil. *Environ Sci Technol* 33(23):4346-4351.

Tang J, Alexander M. 1999. Mild extractability and bioavailability of polycyclic aromatic hydrocarbons in soil. *Environ Toxicol Chem* 18(12):2711-2714.

Tang J, Liste HH, Alexander M. 2002. Chemical assays of availability to earthworms of polycyclic aromatic hydrocarbons in soil. *Chemosphere* 48(1):35-42.

Tao Y, Zhang S, Wang Z, Ke R, Shan XQ, Christie P. 2008. Biomimetic accumulation of PAHs from soils by triolein-embedded cellulose acetate membranes (TECAMs) to estimate their bioavailability. *Water Res* 42(3):754-762.

Tao Y, Zhang S, Wang Z, Christie P. 2009. Predicting bioavailability of PAHs in field-contaminated soils by passive sampling with triolein embedded cellulose acetate membranes. *Environ Pollut* 157(2):545-551.

ten Hulscher TEM, Postma J, den Besten PJ, Stroomberg GJ, Belfroid A, Wegener JW, Faber JH, van der Pol JJ, Hendricks AJ, van Noort PC. 2003. Tenax extraction mimics benthic and terrestrial bioavailability of organic compounds. *Environ Toxicol Chem* 22(10):2258-2265.

Tolls J. 2001. Sorption of veterinary pharmaceuticals in soils: A review. *Environ Sci Technol* 35(17):3397-3406.

Towell MG, Browne LA, Paton GI, Semple KT. 2011. Impact of carbon nanomaterials on the behaviour of ^{14}C-phenanthrene and ^{14}C-benzo-[a]pyrene in soil. *Environ Pollut* 159(3):706-715.

US EPA. 1975. Guidelines for registering pesticides in the United States. Federal Register, vol. 40, No. 123, 25 June 1975.

US EPA. 1996. Method 3050B: Acid digestion of sediments, sludges, and soil. Revision 2. http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3050b.pdf

US EPA. 2007a. Method 3545A: Pressurized fluid extraction (PFE). Revision 1. http://www.epa.gov/epawaste/hazard/testmethods/sw846/pdfs/3545a.pdf

US EPA. 2007b. Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS.
http://nepis.epa.gov/EPA/html/DLwait.htm?url=/Adobe/pdf/P10070fj.pdf
http://water.epa.gov/scitech/methods/cwa/bioindicators/upload/2008_01_03_methods_method_1694.pdf

van der Wal L, Jager T, Fleuren RHLJ, Barendregt A, Sinnige TL, van Gestel CAM, Hermens JLM. 2004. Solid-phase microextraction to predict bioavailability and accumulation of organic micropollutants in terrestrial organisms after exposure to a field-contaminated soil. *Environ Sci Technol* 38(18):4842-4848.

Van Eldik R, Hubbard CD. 2006. The interpretation and mechanistic significance of activation volumes for organometallic reactions. In Richard JP, ed, *Advances in Physical Organic Chemistry*, Vol 41:1-78. Elsevier, Amsterdam, the Netherlands. http://www.sciencedirect.com/science/article/pii/S0065316006410017

van Gestel CAM, Ma W-C. 1988. Toxicity and bioaccumulation of chlorophenols in earthworms, in relation to bioavailability in soil. *Ecotoxicol Environ Saf* 15(3):289-297.

van Gestel CAM, Ma W-C. 1990. An approach to quantitative structure-activity relationships (QSARs) in earthworm toxicity studies. *Chemosphere* 21(8):1023-1033.

Van Leeuwen CJ, Vermeire TG. 1995. Risk assessment of chemicals: An introduction, 2nd ed. Springer, Doordrecht, The Netherlands.

Vasilyeva GK, Strijakova ER, Nikolaeva SN, Lebedev AT, Shea PJ. 2010. Dynamics of PCB removal and detoxification in historically contaminated soils amended with activated carbon. *Environ Pollut* 158(3):770-777.

Vazquez-Roig P, Segarra R, Blasco C, Andreu V, Picó Y. 2010. Determination of pharmaceuticals in soils and sediments by pressurized liquid extraction and liquid chromatography tandem mass spectrometry. *J Chromatogr A* 1217(16):2471-2483.

von Oepen B, Kördel W, Klein W. 1991. Sorption of nonpolar and polar compounds to soils: Processes, measurements and experience with the applicability of the modified OECD-Guideline 106. *Chemosphere* 22(3-4):285-304.

Wang P, Wang H, Wu L, Di H, He Y, Xu J. 2012. Influence of black carbon addition on phenanthrene dissipation and microbial community structure in soil. *Environ Pollut* 161:121-127.

Warnemuende EA, Patterson JP, Smith DR, Huang C-H. 2007. Effects of tilling no-till soil on losses of atrazine and glyphosate to runoff water under variable intensity simulated rainfall. *Soil Till Res* 95(1):19-26.

Weinberger P, Yee D. 1984. The influence of nitrogen sources on root-mediated changes in substrate pH. *Can J Bot* 62(1):161-162.

Weissenfels WD, Klewer H-J, Langhoff J. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: Influence on biodegradability and biotoxicity. *Appl Microbiol Biotechnol* 36(5):689-696.

White JC, Alexander M, Pignatello JJ. 1999. Enhancing the bioavailability of organic compounds sequestered in soil and aquifer solids. *Environ Toxicol Chem* 18(2):182-187.

White JC. 2001. Plant-facilitated mobilization and translocation of weathered 2,2-bis(*p*-chlorophenyl)-1,1-dichlororethylene (*p,p'*-DDE) from an agricultural soil. *Environ Toxicol Chem* 20(9):2047-2052.

White JC. 2002. Differential bioavailability of field-weathered *p,p'*-DDE to plants of the *Cucurbita* and *Cucumis* genera. *Chemosphere* 49(2):143-152.

Witte EG, Philipp H, Vereecken H. 1998. Binding of 13C-labelled 2-aminobenzothiazoles to humic acid as derived from 13C NMR spectroscopy. *Org Geochem* 29(5):1829-1835.

Wood M. 1995. Environmental Soil Biology, 2nd ed. Blackie Academic and Professional, Glasgow, UK.

Xing B, Pignatello JJ. 1997. Dual-mode sorption of low-polarity compounds in glassy poly(vinylchloride) and soil organic matter. *Environ Sci Technol* 31(3):792-799.

Yang ZY, Maruya KA, Greenstein D, Tsukada D, Zeng EY. 2008. Experimental verification of a model describing solid phase microextraction (SPME) of freely dissolved organic pollutants in sediment porewater. *Chemosphere* 72(10):1435-1440.

Ye Q, Sun J, Wu J. 2003. Causes of phytotoxicity of metsulfuron-methyl bound residues in soil. *Environ Pollut* 126(3):417-423.

Yee D, Weinberger P, Khan SU. 1985. Release of soil-bound prometryne residues under different soil pH and nitrogen fertilizer regimes. *Weed Sci* 33(6):882-887.

Yin Y, Impellitteri CA, You S-J, Allen HE. 2002. The importance of organic matter distribution and extract soil:solution ratio on the desorption of heavy metals from soils. *Sci Total Environ* 287(1-2):107-119.

Yoshitomi KJ, Shann JR. 2001. Corn (*Zea mays* L.) root exudates and their impact on ^{14}C-pyrene mineralization. *Soil Biol Biochem* 33(12-13):1769-1776.

You J, Pehkonen S, Landrum PF, Lydy MJ. 2007. Desorption of hydrophobic compounds from laboratory-spiked sediments measured by tenax absorbent and matrix solid-phase microextraction. *Environ Sci Technol* 41(16):5672-5678.

Zarfl C, Klasmeier J, Matthies M. 2009. Non-extractable residues are not necessarily bound residues. Poster presented at SETAC Europe 19th Annual Meeting, Göteborg, Sweden.

Zhao Q, Li P, Stagnitti F, Ye J, Dong D, Zhang Y, Li P. 2009. Effects of aging and freeze-thawing on extractability of pyrene in soil. *Chemosphere* 76(4):447-452.

Zhu Y, Zhang S, Huang H, Wen B. 2009. Effects of maize root exudates and organic acids on the desorption of phenanthrene from soils. *J Environ Sci (China)* 21(7):920-926.

Zimmerman JR, Ghosh U, Millward RN, Bridges TS, Luthy RG. 2004. Addition of carbon sorbents to reduce PCB and PAH bioavailability in marine sediments: Physicochemical tests. *Environ Sci Technol* 38(20):5458-5464.

MEMBERS OF THE TASK FORCE

C.V. Eadsforth (Chairman)	Shell UK - Chester
D. Letinski	ExxonMobil USA - Annandale
H. Tungate	AstraZeneca UK - Devon
R. Oliver	Syngenta UK - Bracknell
K. Semple	Lancaster University UK - Lancaster
M. Telscher	Bayer CropScience D - Monheim
M. Galay Burgos	ECETOC B - Brussels

Acknowledgement

This Task Force is indebted to Tom Austin (Shell, UK) for his substantial contributions to the report.

MEMBERS OF THE SCIENTIFIC COMMITTEE

(Peer Review Committee)

F. Lewis (Chairman)* Global Platform Lead	Syngenta UK - Bracknell
B. van Ravenzwaay (Vice Chairman) Senior Vice President, Experimental Toxicology and Ecology	BASF D - Ludwigshafen
R. Bars Team Leader, Toxicology Research	Bayer CropScience F - Sophia Antipolis
D. Farrar Occupational Health Business Manager	Ineos Chlor UK - Runcorn
A. Flückiger Chief Occupational Health Officer	F. Hoffmann - La Roche CH - Basel
H. Greim Institute of Toxicology and Environmental Hygiene	Technical University München D - München
G. Malinverno Global Government & Regulatory Affairs Manager	Solvay I - Milano
L. Maltby Professor of Environmental Biology	University of Sheffield UK - Sheffield
S. Marshall Environmental Science Leader	Unilever SEAC UK - Bedford
M.L. Meisters Manager Health and Environmental Sciences EMEA	DuPont de Nemours B - Mechelen
C. Money Distinguished Scientific Associate	ExxonMobil UK - Hythe, Hants
M. Pemberton Director	Systox UK - Wilmslow

* Responsible for primary peer review.

MEMBERS OF THE SCIENTIFIC COMMITTEE (cont'd)

C. Rodriguez Principal Toxicologist, Corporate Central Product Safety	Procter and Gamble B - Strombeek-Bever
L. Rushton Principal Research Fellow	Imperial College London UK - London
D. Salvito* Vice President, Environmental Sciences	RIFM USA - Woodcliff Lake, NJ
J. Snape Principal Scientist	AstraZeneca UK - Brixham
G. Swaen Senior Epidemiologist	Dow Chemical NL - Terneuzen
J. Tolls Director, Environmental Safety Assessment	Henkel D - Düsseldorf
S. van der Vies Professor of Biochemistry	VU University Medical Center NL - Amsterdam
C.J. van Leeuwen Principal Scientist	KWR Watercycle Research Institute NL - Nieuwegein
H.-J. Wiegand Global Coordination for Product Stewardship	Evonik Industries D – Essen

* Responsible for primary peer review.

ECETOC PUBLISHED REPORTS

The full catalogue of ECETOC publications can be found on the ECETOC website:
http://www.ecetoc.org/publications

Responsible Editor:
Dr Alan Poole
ECETOC AISBL
Av. E. Van Nieuwenhuyse 2 (box. 8)
B-1160 Brussels, Belgium
VAT: BE 0418344469
www.ecetoc.org
D-2013-3001-225

Established in 1978, ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) is Europe's leading industry association for developing and promoting top quality science in human and environmental risk assessment of chemicals. Members include the main companies with interests in the manufacture and use of chemicals, biomaterials and pharmaceuticals, and organisations active in these fields. ECETOC is the scientific forum where member company experts meet and co-operate with government and academic scientists, to evaluate and assess the available data, identify gaps in knowledge and recommend research, and publish critical reviews on the ecotoxicology and toxicology of chemicals, biomaterials and pharmaceuticals.