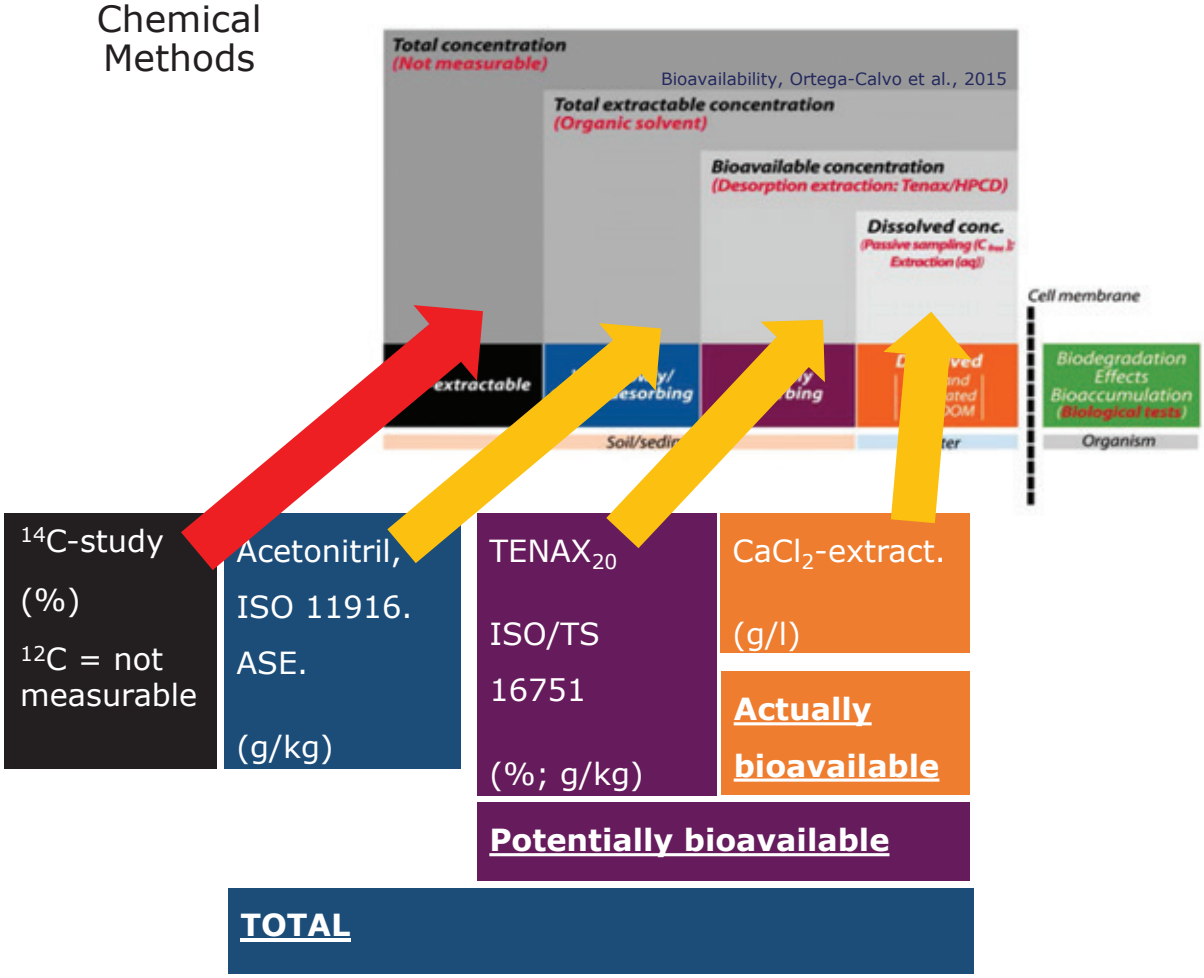
Chemical Methods



Ecotoxicological Methods

Advances in the Development of Procedures to Establish the Toxicity of Non-Extractable Residues (NER) in Soil. LRI-ECO25

Joop Harmsen, Dieter Hennecke, Kerstin Hund-Rinke, Joost Lahr, John Deneer

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Joop Harmsen[1], Dieter Hennecke[2], Kerstin Hund-Rinke[2], Joost Lahr[1], John Deneer[1]

1 Wageningen Environmental Research

2 Fraunhofer Institute for Molecular Biology and Applied Ecology IME

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Abstract
There is already a long discussion around the bioavailability and ecotoxicological relevance of Non Extractable Residues (NER) in soil. Is NER formation a detoxification process or should it be considered a hidden hazard? NER can only be established using labelled chemicals (e.g. ^{14}C) and cannot be measured with conventional chemical analytics.
Regulations ask for understandable and measurable parameters. Considered in the developed tool are three measurable parameters: 1) Chemical present in the water phase, 2) A potentially available fraction in equilibrium with the water phase. 3) The total extractable amount. NER is considered, but mentioned as non-measurable and non-bioavailable.
The fates of three NER-forming chemicals were followed in a period of 6 months after addition also using ^{14}C chemicals. For the chemical Tri-NitroToluene (TNT), NER-formation was reproducible and NER formation during aging removed toxicity. By removing the bioavailable fractions directly after spiking and after aging it was also possible to remove toxicity. The experiments showed that toxicity was caused by the bioavailable chemical and not by NER. With Cypermethrin and Carbendazim, results were less clear, because there was a large uncertainty in NER-formation. The degree of biodegradation was not reproducible for Cypermethrin and unexpected losses occurred with Carbendazim and it is not possible to draw conclusions from only a non-labelled experiments.

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Keywords: Non extractable residue, NER, bioavailability, soil ecotoxicology, risk assessment

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Contents

List of abbreviations

°C	degree Celsius
µg	microgram
^{14}C	carbon 14 (radioactive)
ACN	Acetonitrile
AR	Applied Radioactivity
ASE	Accelerated Solvent Extraction
B(a)P	Benzo(a)pyrene
Bq	Becquerel
$CaCl_2$	Calciumchloride
cm	centimeter
CO_2	Carbon Dioxide
d	days
dm	dry mass
DOC	Dissolved Organic Carbon
DT50	Dissappearance time (50=half life)
EC	Effect Concentration
EU	European Union
FNU	formazin nephelometric units
g	gram
GLP	Good Laboratory Practise
h	hour
kBq	kiloBecquerel
kg	kilogram
K_{OW}	Sorption Coefficient
l	litre
LSC	Liquid scintillation counting
M	Molar
MBq	Megabecquerel
MeOH	Methanol
mg	milligram
min	minute
ml	millilitre
NER	Non Extractable Residues
ng	nanogram
NOEC	No Observed Effect Concentration
OC	Organic Carbon
PAH	Polycyclic Aromatic Hydrocarbons
PCB	PolyChlorinated Biphenyls
rpm	Rotations per minute
SFE	supercritical fluid extraction
t	time
TLC	Thin Layer Chromatography
TNT	2,4,6-TriNitroToluene
TR	Technical Report
WHCmax	maximum water holding capacity

Summary

There is already a long discussion concerning the bioavailability and ecotoxicological relevance of non-extractable residues (NER) in soil. Is NER formation a detoxification process or should it be considered a hidden hazard? NER can only be established using labelled chemicals (e.g. 14C) and cannot be measured with conventional chemical analytics. However, even using labelled compounds uncertainty exists about the identity of measured radioactivity. Do we measure: 1) Association of the parent chemical or breakdown product with mineral and/or organic matter, and/or 2) Mineralisation and incorporation of carbon into microbial biomass and carbonates?

Regulations ask for understandable and measurable parameters. The approach of Ortega-Calvo et al. (2015) has been followed, because this approach defines clear and measurable fractions. The only fraction not measurable is NER, but this can be considered as a residual fraction if all others are measured. Considered fractions are:

- Chemical present in the water phase, actually available (passive sampling or CaCl2-extraction);
- A potentially available fraction in equilibrium with the water phase (Tenax, ISO TS-16751);
- The total extractable amount, measured with a (standard) method;
- NER is considered, but mentioned as non-measurable and also non-bioavailable.

We applied a recently standardized method using Tenax (in accordance to ISO TS-16751) to remove the bioavailable fraction. Tenax is an extra solid phase that can be removed easily leaving the soil without a bioavailable fraction. Solvents can also be used to remove the bioavailable fraction, but these were not applied because residual solvent in the soil may cause toxicity, which would make it impossible to establish the role of NER.

We studied three NER-forming chemicals and followed their fate for a period of 6 months after addition. An important part of the study were experiments using ^{14}C chemicals. During these experiments, formation of non-extractable ^{14}C was observed for all chemicals.

Toxicity of NER-forming compounds is mostly low. Acute toxicity tests using Earthworm avoidance, Vibrio fischeri and Daphnia magna were found to be suitable for the selected chemicals, because they can be performed within 2 days, a period that NER-formation is still limited. Longer lasting chronic toxicity test were not applied. At the end of a chronic test, most of the spiked chemical can be present as NER, which makes interpretation of the results uncertain. A long-lasting test such as the earthworm reproduction test (incubation period 8 weeks) cannot answer the question whether toxicity is caused by the initial spiked concentration or by the bioavailable concentration at the end of the experiment.

For the chemical trinitrotoluene (TNT), NER-formation was reproducible and NER formation during aging removed toxicity. By removing the bioavailable fractions directly after spiking and after aging it was also possible to remove toxicity. The experiments with and without labelled TNT clearly showed that toxicity was caused by the bioavailable chemical and not by NER. With the other two selected chemicals, cypermethrin and carbendazim, results were less clear, because there was a large uncertainty in NER-formation. The degree of biodegradation was not reproducible for cypermethrin and unexpected losses occurred with carbendazim. This resulted in a very large uncertainty about NER-formation using non-labelled compounds. For these compounds, it is not possible to draw conclusions from non-labelled experiments only.

In conclusion, we developed a tool that can be used if the fate of the chemical including NER formation is well known. Additional experiments using labelled compound remain necessary if uncertainties about the fate of the chemical exist.

Extensive summary

The Cefic-LRI ECO-25 project aimed to develop procedures to establish the toxicity of non-extractable residues (NER) in soil. These procedures are necessary to support the risk assessment of NER-forming chemicals within a prospective regulatory context. There is already a long discussion concerning the bioavailability and ecotoxicological relevance of NER in soil. Is NER formation a detoxification process or should it be considered as a hidden hazard? The formation and presence of NER may be attributable to: 1) Association of the parent chemical or breakdown product with mineral and/or organic matter, and/or 2) Mineralisation and incorporation of carbon into microbial biomass and carbonates.

NER can only be established using labelled compounds (e.g. ^{14}C). Because it is non-extractable, it cannot be measured with conventional chemical analytics. Regulations ask for understandable and measurable parameters. The approach of Ortega-Calvo et al., (2015) is followed in this research project. Considered are both sides of the cell membrane of an organism, soil side and the internal side of an organism:

Soil side of the cell membrane

- The water phase in direct contact with the organism, in which concentrations/activity can be measured using passive sampling or extractions with 0.01 $CaCl_2$ (actual availability);
- A potentially available fraction in equilibrium with the water phase, measured using ISO/TS 16751: 2018, Soil quality — Environmental availability of non-polar organic compounds — Determination of the potentially bioavailable fraction and the non-bioavailable fraction using a strong adsorbent or complexing agent;
- The total extractable amount, to be measured with a (standard) method designed to measure the total substance amount;
- NER is considered, but mentioned as non-measurable and non-bioavailable. All other fractions are measurable and bioavailable and non-bioavailable. If the fate of a chemical is known, NER in addition experiments can be quantified as 'Amount added minus amount extracted and minus all losses (e.g. biodegradation, volatility)'.

We applied a recently standardized method using Tenax (ISO/TS 16751, up-scaled version for the project) to remove the bioavailable fraction. Tenax is an extra solid phase that can be removed easily after application, leaving the soil without a bioavailable fraction. Solvents can also be used to remove the bioavailable fraction, but these were not applied because the solvent would kill any biological activity in soil and even residual solvent in the soil may cause toxicity, which would make it impossible to establish the role of NER with regard to toxicity. Nevertheless, it turned out that the combination of Tenax-extraction and subsequent treatment of the soil had effect on microbial ammonium oxidation activity. Thus, this method is not further used to establish the toxicity of the spiked and aged soil. However, it clearly indicates that it is always necessary to check the effect of any soil treatment on the bioassay used.

Organism side of the cell membrane (organism)

- Measurement of the effects of the bioavailable chemical passing the cell membrane, using an organism.

The experimental part of the project contained the following steps:
Selection of chemicals and ecotoxicological tests, which was challenging, since the toxicity of known NER-forming chemicals is often limited. Within this project, it was necessary to select NER-forming chemicals that cause effects in well applicable tests or bioassays. Because NER formation occurs in the first months after addition it was not possible to apply chronic toxicity tests lasting several weeks, to explain difference in toxicity between freshly spiked and aged soils. At the end of a chronic test, most of the spiked chemical can be present as NER, which makes interpretation of the

results uncertain. Is toxicity caused by the initial spiked concentration or by the bioavailable concentration at the end of the experiment?

Bioassays had to be used which can be performed within a few days. The applicability of the following tests was investigated. The underlined acute toxicity tests were found to be applicable for the three selected chemicals and applied in further investigations. The chemicals TNT, cypermethrin and carbendazim were selected.

- Earthworm Avoidance
- Potential ammonium oxidation activity
- Luminescence bacteria test
- Daphnia test
- UMU test

The used test approach is not suitable to show reduction of chronic toxicity, because spiked chemical will change into NER during the chronic toxicity test, which usually have a longer test duration. Aged soil material can be used for a chronic toxicity test in future research. However, as the reference (toxicity on day 0 = date of spiking) will be missing, the information concerning the reduction of toxicity is limited. Therefore, a combined approach can be useful. Tests with short test duration (usually acute tests) to show the reduction of toxicity, combined with chronic tests to rebut the long-term exposure concern.

Transformation kinetics and mass balance experiments were applied using radiolabelled chemicals, which were monitored over a period of 6 months. The formation of NER could be determined and was high for TNT (60-75%), low for cypermethrin (15-30%) and intermediate for carbendazim (25-50%). The fate of TNT was reproducible but the other compounds showed non-reproducible behaviour; different degradation rates were observed for cypermethrin, and high losses were observed for carbendazim in a second experiment, due to formation of a volatile carbendazim transformation product.

Sequential extraction and ecotoxicological testing. To limit uncertainties, radiolabelled experiments were also included. 0.01M $CaCl_2$ solutions were used to extract the actual available concentration, followed by Tenax extraction to extract the potential bioavailable or rapidly desorbing portion, and the slowly desorbing portion (respectively, extraction for 20 hours and 1 week). An exhaustive extraction with an organic solvent and accelerated solvent extraction (ASE) was used to extract the extractable portion. The remaining fraction was assumed to be the NER.

Freshly spiked and aged soils, before and after extraction of the bioavailable fraction (0.01M $CaCl_2$ + Tenax), were used in bioassays (earthworm avoidance, Microtox, daphnia). Results of TNT were well defined and showed that toxicity was caused by the bioavailable fraction and a high NER did not cause toxicity. Also in a follow-up test without radiolabelled TNT and designed to be generally applicable, the same conclusion could be drawn. In addition, the distribution of the chemical over the fractions and the residual amount (still present after removing of the total extractable amount) was measured using ^{14}C-labelled compounds. With ^{14}C-experiments radioactivity was measured and not the substance identity.

The low NER formation combined with high toxicity of cypermethrin made it unfeasible to explain the role of NER for cypermethrin. Toxicity did not change during aging. The observed differences in degradation rate will cause high uncertainties if no radiolabelled cypermethrin is used. For cypermethrin it will be necessary to use labelled chemical in further studies.

In the radiolabelled experiment, it was possible to show that NER from carbendazim did not cause toxicity. However, large differences in losses were observed in different experiments. In the second experiment, most of the carbendazim was lost as a volatile intermediate. This high uncertainty in the mass balance will make it impossible to estimate the amount of NER in a non-labelled study.

The results of this project show that, if the fate of a chemical is known, it is possible to quantify NER as "Amount added minus amount extracted minus quantified other losses". Tenax can be used to remove and to estimate the bioavailable fraction. For more soluble chemicals, the 0.01M $CaCl_2$ extractable concentration is also a good measure of bioavailability. Results are in line with the approach of Ortega-Calvo et al., 2015. In such cases, chemistry including NER can be used to explain the results of bioassays in freshly spiked and aged soil, both before and after the removal of the bioavailable fraction. The final design of the test presented without radiolabelled chemical explains the role of different fractions, including NER, on toxicity.

However, if uncertainties exist concerning the fate and a proper mass-balance cannot be made, it is not possible to quantify NER in a non-labelled study. For such chemicals, a toxicity study on aged soils has to be supported by a study using radiolabelled material. If NER formation is limited and the chemical is very toxic, differences before and after aging will be small and the role of NER will be difficult to recognize.

Results of this project show that it is not yet possible to propose a generic way to assess NER-forming chemicals. As mentioned, the methodology is suitable for chemicals with a well-known behaviour. For other chemicals, it is a good starting point, but additional gathered information will be necessary to prove the presence of NER. Further research should be focussed on how this extra information has to be gathered. Additional experiments using radiolabelled chemicals seem to be the best way.

Results of this project need to be communicated with stakeholders, to show that if the presence of NER is proven, the developed methodology is able to show whether toxicity is present or not. With the investigated chemicals, NER did not result in acute toxicity. Important for the communication is that the approach suggested is understandable and based on measurable parameters. This will make it easier to implement the developed method into regulations. Appropriate ways of communication should be used to share the results of this project with regulators.

1 Introduction

1.1 Non Extractable Residues

The term non-extractable residues (NER) originates from pesticide regulation where the use of ^{14}C-radiolabelled substances is mandatory to determine transformation pathways. The original definition was: *'chemical species originating from pesticides, used according to good agricultural practice, that are non-extractable by methods which do not significantly change the chemical nature of these residues'*. In 1996 the definition was modified to: *'substances in soils, plants or animals which persist in the matrix after extraction in the form of the parent substance or its metabolites that are indistinguishable from naturally occurring substances. The extraction must not substantially change the substances themselves nor the nature of the matrix'*. (Statement on the FERA guidance proposal: "Guidance on how aged sorption studies for pesticides should be conducted, analysed and used in regulatory assessments" (FERA, 2012; EFSA, 2015)).

In practise, extraction of chemicals occurs using solvents (see section 1.2.1). Several methods are available and also standardized. In contradiction to the extraction of heavy metals using *aqua regia*, the solvents do not change the matrix or the chemical. The extractable amount is considered to be the total concentration within a perspective regulatory context (assessment of contaminated sites). Having the knowledge that part of spiked radioactivity is not extracted makes the assessment in prospective regulation (risk assessment of chemicals) more difficult.
There is already a long discussion concerning the bioavailability and ecotoxicological relevance of NER in soil. Is NER formation a detoxification process or should it be considered to be a hidden hazard? The formation and presence of NER may be attributable to:

1. Association of the parent chemical or breakdown product with mineral and/or organic matter of the soil matrix, and/or
2. Mineralisation and incorporation of carbon into microbial biomass and carbonates.

NER can only be established using labelled compounds (e.g. ^{14}C). Because it is non-extractable, it cannot be measured with conventional chemical analytics and consequently they appear invisible to environmental monitoring.
Starting point of the current research were the two ECETOC-reports:

- TR 117: Understanding the Relationship between Extraction Technique and Bioavailability; (ECETOC, 2012-a).
- TR 118: Development of interim guidance for the inclusion of non-extractable residues (NER) in the risk assessment of chemicals. (ECETOC, 2012-b).

The original objectives of the project were:

- Develop a "soup test" to assess the toxicity of the different fractions of the test chemicals in the soil for different test organisms (plant, soft bodied soil organism, microorganisms) – ECETOC TR118
- Validate the extraction regime as proposed in ECETOC TR 117.
- Validate the "definition" of bioavailability (as in ECETOC TR117) in toxicity tests in relation to exposure scenarios in tests (time scales)

As described in the following section, it was necessary to adjust the extraction regime of ECETOC TR 117. Newly developed ideas made it possible to simplify the regime in such a way that it became easier to incorporate into regulation. As summarized by Kästner et al. (2017) "Criteria for the assessment of chemical properties and toxicological and environmental behaviour of industrial chemicals in general, and particularly for biocidal products, plant protection products, and veterinary medicines are described in specific European legislations, i.e. regulations (EC_1907_2006), (EC_528_2012), (EC_1107_2009), and (EMA_2014_PBT_vPvB), respectively. One of the critical issues in the environmental risk assessment refers to the formation of non-extractable residues (NER) and the assessment of persistency, bioaccumulation potential, and toxicity (PBT) and very persistent and

very bioaccumulative (vPvB) properties. For PBT and vPvB properties there are guidance documents available (ECHA 2017a, 2017b) but for the assessment of NER there is no unified guidance based on a scientific background available."

1.2 Organic chemicals in soils

1.2.1 General

If an anthropogenic organic chemical is added to soil, it can be present as:

- **Pure material**, which will occur, at least temporarily, depending upon stability, if high amounts are added to soil and the solubility is low. Bulk chemicals like mineral oil products can be present as a separate phase or in the form of small droplets (Brils et al., 2002). Because the amount applied to soil of most chemicals is low, the presence of pure material does not have to be considered for most chemicals.
- **Adsorbed to soil,** organic chemicals adsorb to the soil matrix**.** How strongly they are adsorbed, depends on different factors like chemical structure of the substance, polarity and on soil properties. For ionic substances, other processes are relevant (Franco and Trapp, 2008)
- **Solved in the pore water,** depending on its solubility. Because solubility of most organic chemicals is limited, the amount present in the pore water will be low. In a soil/water slurry, the amount present in the water phase can be considerable if the solubility is relatively high.

To analyze a chemical in soil it has to be isolated from the matrix. A very polar compound can be extracted from the soil with a polar solvent, e.g. water. Instead of pure water, 0.01 M $CaCl_2$ (ISO 14255) or 0.001M $CaCl_2$ (ISO 21268-1) are often used in standard methods. The advantage of the presence of $CaCl_2$ is that the formation of colloids is suppressed which supports the solid / liquid separation step in sample work up for further analysis. Less polar organic chemicals are usually extracted with appropriate organic solvents; however, this has to be developed for each individual chemical. Validated standard procedures are available for most environmentally relevant chemicals.

Literature gives several solvents that can be used to extract chemicals from soil and results are assumed to present the "total content". Standard methods like ISO are the product of international discussion and combine broad experience. Administrations prefer to prescribe standard methods to measure total concentrations in regulations and results are used without doubt. Extraction solvents used in standard methods are often based on a mixture of acetone/petroleum ether to extract hydrophobic chemicals (e.g. ISO13859 for PAH and ISO18287 for PCB) and acetonitrile (e.g. ISO 11916-1 for explosives including TNT) for more polar chemicals. For an efficient extraction, the solvent should combine the functions of reaching the chemical in small pores as well as dissolving the chemical (Harmsen and Frintrop, 2003). For the first extraction step, a water miscible solvent like acetone or acetonitrile is needed to reach the chemical, because the soil particle surface is covered with water. To extract hydrophobic chemicals this should be followed by a second step using a non-polar solvent, e.g. petroleum ether. It should be realized, that, if NER-formation is not recognized, the recovery might be low and the solvent used will be classified inappropriate.

1.2.2 Aging of a chemical in soil matrix

Visualization may aid in understanding the sorption process. In Figure 1, soil is presented as a block. At the surface of the soil (right surface of the block), adsorbed chemicals are in equilibrium with the water phase. In the middle of a soil particle, but also on specific adsorption sites, chemicals are bound very strongly. These sites are represented by the left part of the block. Directly after spiking, the chemical will be adsorbed to the surface and it will be in equilibrium with the water phase. Part of the chemical in the water phase will be freely dissolved and another part might bind to the dissolved organic carbon (DOC). The higher the K_{ow} of the chemical, the larger is the amount of the chemical that might build complexes with the DOC. It should be realized that there is not always a linear relation between the concentration in the soil and the free dissolved concentration in the water phase. For example, the solubility of the chemical will limit its final concentration in water.

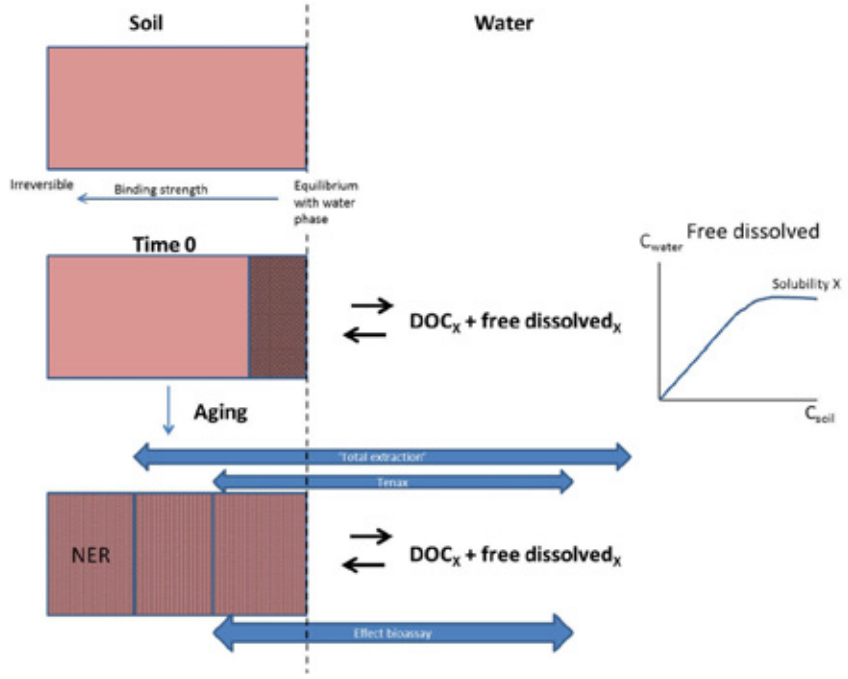


Figure 1 *Extractable and non-extractable fraction of a chemical in soil and water. Upper: Binding places in soil. Middle: Chemical directly after adding to the soil. Lower: Chemical after aging*

Aging of a chemical in soil changes its distribution. The right side of the box represents the part that is still in equilibrium with the water phase, but the other boxes, which are not in equilibrium with the water phase, fill up with the chemical. The chemical in those boxes are more strongly adsorbed or absorbed. The left most box represents chemical which, is adsorbed in a way making it non-extractable anymore (NER). The middle part is extractable using harsh extraction methods, which are developed to measure the total concentration for use in regulation and monitoring. These extraction methods will determine the sum of chemical present in the water phase and the extractable portion in the soil.

Using Tenax or Cyclodextrine (e.g. ISO/TS 16751, 2018), only the sorbed amount in equilibrium with the water phase can be extracted. The Tenax / Cyclodextrine adsorbs the chemical from the water phase and acts as an infinite sink. This process disturbs the sorption equilibrium between chemicals on the soil surface and in the water phase. This results in enhanced desorption of the chemical from soil to restore the equilibrium. In the ISO standard method, the desorption time is set to 20 hours (hereafter named Tenax$_{20}$). In the present study, it was decided to work with Tenax rather than with Cyclodextrine. The reason for this decision is that Cyclodextrine is a water-soluble compound and thus, after the soil extraction, the soil might contain significant amounts of Cyclodextrine. This may interfere with the subsequent ecotox-testing of the soil. Tenax forms a real third phase, which can be recovered completely from the mixture and is therefore considered an extraction technique, which imputes only a limited impact on soil structure during extraction. In ISO/TS 16751 (2018), this distinction is of no concern, since the focus is on the desorbable (= available) fraction. However, in this study, biological testing was to be conducted with the soil residue, and therefore Tenax was better suited as an extractant.

For a chemical with a high K_{ow}, the recovery from the water phase by Tenax will be close to 100%. Chemicals with a lower K_{ow} might leave a low residual concentration in the water phase. In Figure 1, this is represented by an arrow that does not fully overlap with the water phase. In the present study, we selected test substances that cover a wide range of K_{ow} in order to see both effects. The effect observed in bioassays is explained by the bioavailable fraction, which is considered equal to the fraction extracted with Tenax and equal to the maximum amount in the water phase. Very often, the amount complexed by DOC is not considered toxic; therefore, the arrow representing toxicity does not fully overlap the water phase.

1.2.3 The toxic fraction

The bioavailable fraction is considered to be the fraction that is responsible for toxic effects. In theory, it should be possible to remove this fraction from the soil, using Tenax (see 1.2.2). It is assumed that the amount left in the soil does not cause toxic effects (Figure 2). The total concentration is reduced but NER should not change.

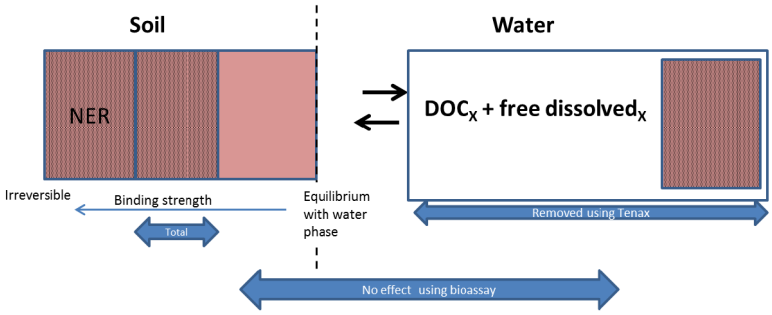


Figure 2 *Toxicity after removal of rapid desorbing fraction (Tenax$_{20}$)*

1.2.4 Rapid and slow desorbing fractions: Tenax at 20h and 1 week

The Tenax/Cyclodextrine extraction, as performed according to ISO/TS 16751-1, enables quantification of the amount that desorbs during 20 hours at 20°C. This amount is considered the rapidly desorbing fraction in the mathematical model. In this model, we define the desorption by a sum of three, first order decreases, as also used by Cornelissen et al., 1997, Harmsen, 2004, ISO17402 and Rhodes et al., 2010.

$$\frac{c_t}{c_0} = F_{rapid} \cdot e^{-k_{rapid} \cdot t} + F_{slow} \cdot e^{-k_{slow} \cdot t} + F_{very\,slow} \cdot e^{-k_{very\,slow} \cdot t}$$

Where:

c_t	=	soil sorbed amount (mg/kg dm) at time *t* (y)
c_0	=	soil sorbed amount (mg/kg dm) at time 0
F_{rapid}	=	fraction of contaminant in rapidly desorbing compartment
F_{slow}	=	fraction of contaminant in slowly desorbing compartment
$F_{very\ slow}$	=	fraction of contaminant in very slowly desorbing compartment
K_{rapid}	=	rate constant rapid desorption (y^{-1})
k_{slow}	=	rate constant slow desorption (y^{-1})
$k_{very\ slow}$	=	rate constant very slow desorption (y^{-1}).

The Tenax method is a slurry method, which means that mass transfer is very rapid. Desorption times and desorption of the rapidly desorbing fraction correlated to the desorption coefficient are given in Table 1 for the slurry system and the unsaturated system. The water content in a soil is much lower and consequently the mass transfer is much slower (Harmsen et al., 2007). Instead of 20 hours, weeks to months are necessary. A period of weeks to months is also a period used for many bioassays, which means that the rapidly desorbing fraction may desorb during the testing period. The model also includes a slowly desorbing fraction, which desorbs in a period of months to years. On a human scale, this is a relevant time frame. For this reason, this amount should be considered. Table 1 also contains the very slowly desorbing fraction and the irreversibly sorbed (NER) fraction. The desorption rates of these fractions are very low and they are not expected to be responsible for toxicological effects, even on a long-term chronic basis.

Table 1 *Operational characteristics of different desorbing fractions*

Fraction	Slurry system (ISO/TS 16751)		Unsaturated system (soil)	
	Desorption time	Desorption coefficient (year^{-1})	Desorption time	Desorption coefficient (year^{-1})
Dissolved	0		0	
Rapidly desorbing	20 hours	2600	Weeks to months	>3
Slowly desorbing	Week	7	Months to years	0.33
Very slowly desorbing	Months to year	0.1-0.3	Decade	0.03
Irreversible sorbed	Years to decades	<0.01	Centuries	<0.001

The slowly desorbing fraction can be measured by using Tenax/Cyclodextrine during an extraction period of a week at 20°C ($Tenax_{1\ week}$) or 20 hours at 60°C (Tenax 60°C). The slowly desorbing fraction is illustrated in Figure 3. For this study, $Tenax_{1\ week}$ was selected rather than Tenax 60°C, because if a soil is heated to 60°C it is not possible to do any meaningful ecotox-tests with that material any more. The extraction method, therefore, used $Tenax_{20}$ followed by $Tenax_{1\ week}$, both at 20°C.

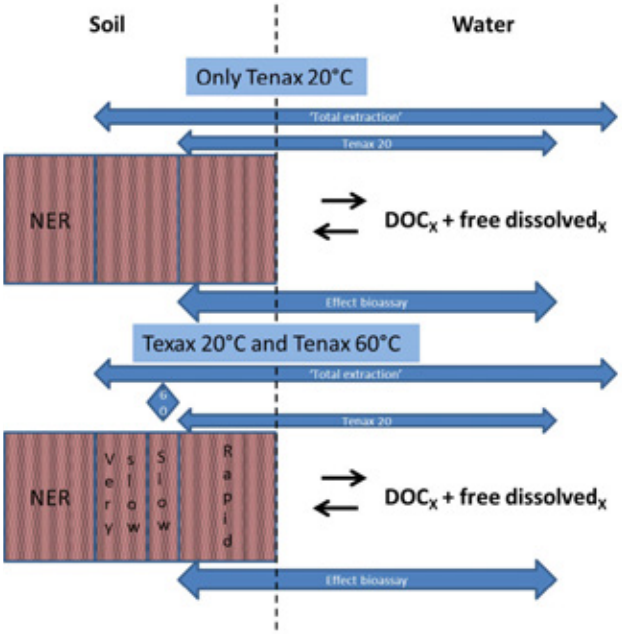


Figure 3 *Fractions to be considered using $Tenax_{20}$ and Tenax 60°C*

In Ortega-Calvo et al. (2015), the ideas outlined above were used, leading to Figure 4. In this approach, chemicals are either bioavailable or non-bioavailable. Bioavailability is represented by the concentration in the water phase and/or the amount in equilibrium with the water phase as can be measured using the standardized Tenax at 20°C. The total concentration is measured with extraction methods using organic solvents, which are often standardized and accepted by regulation. Figure 4 is a well-accepted representation of the presence of a chemical in soil and will be used as a basis in this project.

The terminology used for extraction of the total concentration is confusing. The ECETOC TR 17 report uses **exhaustive** extraction. The terms **harsh** extraction and **destructive** extraction methods are also in use. To measure a chemical, we distinguish only

- Extractions that intend to extract the total concentration. This includes exhaustive and harsh extraction methods;
- Extractions that have the intention to extract a biologically available fraction of the chemical. This includes extraction methods using water and salt solution for the water-soluble part and additional methods for the rapidly desorbing fraction

Destructive extractions cannot be used for most of organic chemicals, because the destruction will also destroy the chemical. Destructive methods can only be used in radiolabelled experiments. Destruction and measurement will show the presence of the ^{14}C-label, but is no proof of presence of the original chemical.

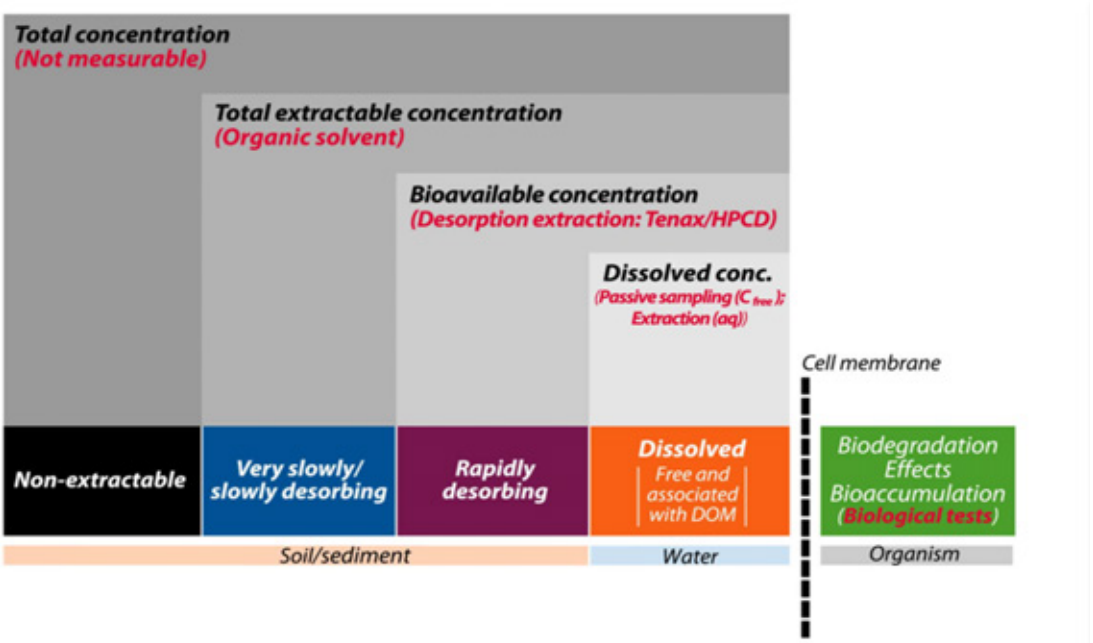


Figure 4 *Bioavailability and NER according to Ortega-Calvo et al., 2015*

1.2.5 Extraction of the bioavailable amount

The determination of the bioavailable amount is a key issue in this project. Results from literature suggest that some solvents (e.g. acetonitrile) can be used as weak extractant to measure the bioavailable fraction. The same solvent, however, is also suggested to extract the total concentration. Formation of NER and not recognizing this phenomenon may be the explanation for this discrepancy. Several weak extractants are suggested to extract the bioavailable fraction. Starting with the group of Alexander at Cornell University using solvents and use of supercritical fluid extraction (SFE) by Hawthorne and Grabanski, 2000. In order to extract only the bioavailable part, they and others (see also ECETOC-Report 117) created milder conditions by:

- Decreasing the extraction time
- Diluting the solvent with water
- Decreasing temperature
- Decreasing pressure (SFE)

Calibration of the method is always necessary and small changes can have a large effect on the result. Creation of milder conditions for extraction is the common factor. Besides the milder conditions, the methods do not have a chemical, physical or biological explanation; they are empiric. Using a mild solvent, it will always be possible to find conditions for a specific chemical that will give results representative of the bioavailable fraction. However, it was not the aim of the project to develop a new empirical extraction method. The mild extractions were important for the development of ideas on bioavailability, but lacking a proper mechanistically base, they are not the methods for the future (Ortega-Calvo et al., 2015).

In the approach of Ortega-Calvo et al., 2015, adopted in this project, the biological fraction is defined as the amount desorbed in a certain exposure time. In publications of Semple et.al. (2007) and in the ECETOC-Report 117 the same definition is used. The water phase in soil becomes the explaining physical factor. Methods to be used should therefore be based on the water phase. The ISO-guideline on bioavailability (ISO 17402: 2008) also recommends the use of chemical-physical based methods.

We followed this recommendation and did not apply the mild extraction in this investigation. A second argument was that after application of a mild solvent, residuals of the solvent will be present in the extracted soil. The residual solvent will have an impact on the result of ecotoxicity tests applied to the soil after removal of the bioavailable fraction.

Two types of measurement are used to measure the rapidly desorbing fraction. The first makes use of an infinite sink that is applied for one day. The infinite sink used is Cyclodextrine or TENAX. In our project, we applied TENAX and results can be expressed as mg/kg or percentage of the total. NER can be expressed in the same way. The second type of measurement, which was applied here, establishes the concentration in the water (mg/l) directly, after a separation and an extraction step. Another way, not applied in this investigation, is the application of passive sampling (Reichenberg et al., 2006) which measures the freely dissolved concentration (mg/l).

1.3 Objectives

As mentioned, the original objective of this research as described in the research proposal was to validate the extraction scheme proposed in ECETOC TR117 and to **assess the (potential) toxicity of Non-Extractable Residues of chemicals in soils**, as defined in ECETOC Technical Report 118. For this, the following topics were addressed:

- Develop a "soup test" to assess the toxicity of the different fractions of the test chemicals in the soil for different test organisms (plant, soft bodied soil organism, microorganisms) – ECETOC TR118
- Validate the extraction regime as proposed in ECETOC TR 117.
- Validate the "definition" of bioavailability (as in ECETOC TR117) in toxicity test in relation to exposure scenarios in tests (time scales)

The basic approach was to relate the extraction regime to the most important mechanistic route of uptake of chemicals by soil organisms, which is the pore water. Therefore, the gist of our approach is to link extractability via the water phase to exposure through the water phase. Based on this, it was expected to provide a clear, mechanistically driven definition of NER and residual toxicity caused by NER.

During the study, it turned out to be necessary to modify the objectives. The 10th SETAC Europe Special Science Symposium, BIOAVAILABILITY OF ORGANIC CHEMICALS (14-15 October 2014 in Brussels), played a very important role. The project team and monitoring team of this project were strongly involved in the organization, presentation, leading and participation in discussions and formation of the resulting paper (Ortega-Calvo et al., 2015). An important result was that the explanation of bioavailability and of NER was improved, and the relatively complex Figure 5 could be replaced by a simpler Figure 4.

The extraction regime that originally needed to be validated is provided in Figure 5. In this figure, the non-extractable residue (NER) of a chemical in the soil is defined as the fraction that is irreversibly sorbed to the soil matrix, i.e. the fraction of a chemical that can only be extracted by the use of organic solvents with elevated temperature or pressure (e.g. by ASE, Soxhlet, SFE, microwave). This part of the figure (lower left-hand side) is in contradiction with the definition. Soxhlet and ASE are not usually considered matrix-destroying methods; this amount should therefore never be attributed to NER because it is extractable. NER is only what you get from combustion after the last, non- matrix-destroying extraction. Combustion also destroys the organic chemicals. Presence of the chemical can only be established using labelled chemicals. We approached NER in this way and included all extractable chemical in the box 'Slowly/Very Slowly Desorbed'. In contradiction to organic chemicals, heavy metals 'survive' a matrix destructive method and can be measured afterwards. NER is therefore not an issue for heavy metals.

As mentioned already in the adopted approach of Ortega-Calvo, the total concentration is measured using extraction methods that are preferably standardized to have them accepted for use within regulation. During development and validation of standard methods their extraction efficiency has been investigated. In this project, no distinction is made between harsh and destructive extraction

methods. Only methods developed to measure the total concentration are considered and, as described by Harmsen and Frintrop (2003), these methods use the principle of first dissolving the chemical and then reaching the chemical in order to maximise the extraction efficiency. Although solvents will have an effect on the soil structure, which makes e.g. the residual soil unsuitable for further ecotoxicological testing, these methods are considered as "non-destructive" in regulatory context. A small amount of solvent soluble organic matter may give coloured extracts.

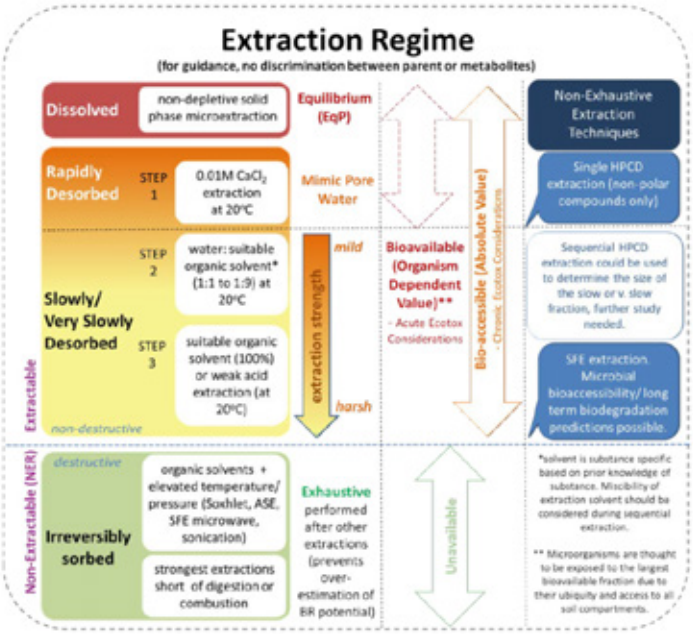


Figure 5 *Extraction methodology framework (taken from ECOTOC TR 117)*

ECHA, 2017-a warns that formation of NER should not be confused with degradation. Within NER, ideally a distinction between remobilizable and irreversibly bound fractions should be possible. While the irreversibly bound part (e.g. biogenically bound) can be considered as a potential removal pathway, the remobilizable fraction (heavily sorbed, physical inclusion) may pose a potential risk for the environment. The environmental significance of NERs is related precisely to the extent to which they become "indistinguishable" from existing soil, sediment or organic matter. However, the term "indistinguishable" cannot currently be defined because the relationship between extraction by the different individual extraction methods and the type of chemical binding to soil/sediment is not simple to understand or to describe. Indirect measures may be used, to show the role of biodegradation in NER-formation. ECHA 2017-b recommends the use of severe extractions. Both ECHA publications describe a grey area between extractable/available and non-extractable/non-available. Grey areas are not easy to implement within regulation. This study should give a proper clarification and reduce the uncertainties related to NER.

2 Working program

In order to address the objectives mentioned in chapter 1.3, the following experimental program was developed. The program was applied to three soils and three different test chemicals. The working program consisted of the following activities:

1. Selection of reference chemicals to be used for the planned experiments, see 2.1
2. Determination of NER formation kinetics in a simplified approach with the ^{14}C-radiolabelled chemicals, see 2.2
3. Development of a sequential extraction procedure and application of ecotoxicological tests to the different fractions, see 2.3
4. Spiking of larger amounts of soils with non-radiolabelled chemicals at the same application rate as employed in the radio-labelled studies, for ecotoxicological testing (earthworm avoidance test), which required large amounts of soil, see 2.4
5. The developed method, based on the results of step 1-4, was then tested with non-radiolabelled chemicals

2.1 Selection of chemicals

As a result of an extensive literature survey, it turned out that selection of the test chemicals was much more complex than expected. The toxicities of known NER forming chemicals are often limited. Most NER formation occurred in a period of weeks to a month. To measure toxicity, it was therefore necessary to select bioassays that could be performed within a few days. Otherwise, ongoing NER formation would make a long- term bioassays impossible to interpret. In order to explain differences between freshly spiked and aged soils it was necessary to do some practical studies addressing toxicity. For details, see chapter 3.

2.2 Transformation kinetics and mass balance experiment

The focus of the present study was the assessment of the NER-fraction. As already pointed out in chapter 1.1 there is no practical way to quantify / characterise the non-extractable fraction of an organic chemical in soil without using ^{14}C-radiolabelled chemicals. Therefore, the strategy was to start spiking experiments with ^{14}C-radiolabelled test chemicals in order to understand the fate of the chemical better and to use the results as a basis for parallel experiments with non-radiolabelled chemicals. The spiked soil was incubated at standard conditions (aerobic, 20°C in the dark) and the distribution of the chemical was followed over a period of 6 months by monthly sampling. As relevant fractions the extractable radioactivity, the mineralisation ($^{14}CO_2$-trapping), and the non-extractable radioactivity (combustion analysis) were quantified. In addition, the extractable radioactivity was analysed to distinguish between parent chemical substance and transformation product. The following Figure 6 shows the schematic of the activities A:

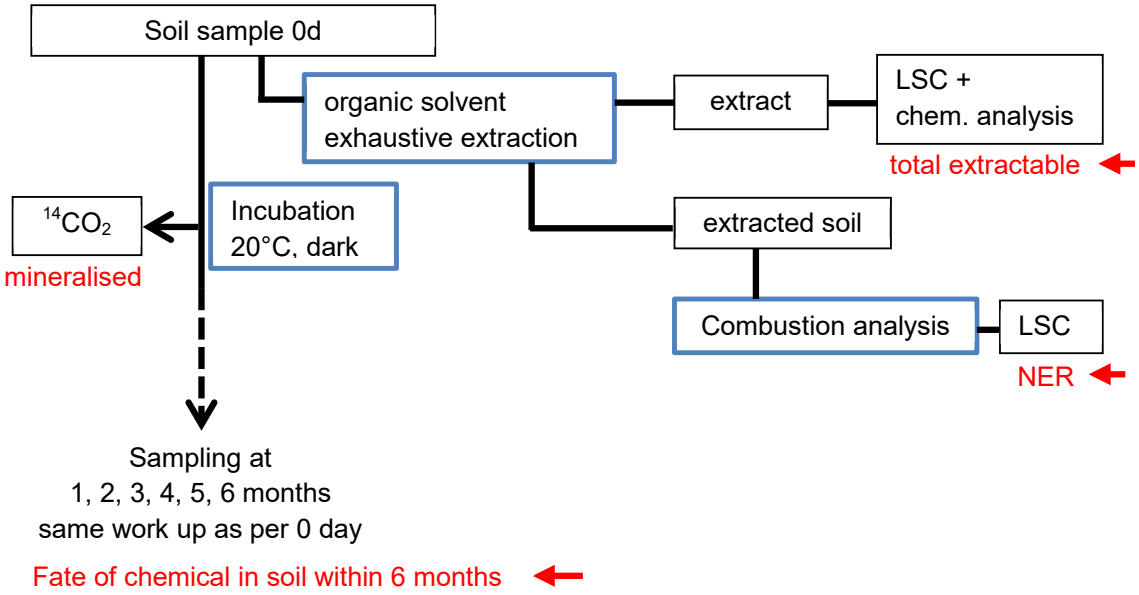


Figure 6 *Scheme for the measurement of NER-formation kinetics*

2.3 Sequential extraction and ecotoxicological testing

This part represents the main test battery of the project. Soil was spiked and incubated at the same rate and conditions as in 2.2, but no transformation kinetic was established. Instead, a sequential extraction procedure was applied at the end of the incubation to assign the initially applied chemical into the fractions as defined in Figure 4. At the same time, ecotoxicological tests were performed with the extraction residues, in order to determine whether the fraction remaining in the soil did still cause adverse effects to aquatic test organisms. The extracts, representing different fractions as defined in Figure 4 were also tested for ecotoxicological effects. In order to determine changes over the incubation period of 6 months the same procedure was conducted with the freshly spiked soil. In detail:

1. 0.01 M $CaCl_2$ - extraction:
   the soil is extracted for 24 hours with 0.01 M $CaCl_2$, at 2:1 liquid / solid ratio.
   Extracted: the actual bioavailable portion ("Dissolved" according to Figure 4).
   The soil residue is split in two portions, one portion for ammonium oxidation test, the other portion is used in the next extraction step 2.
2. $Tenax_{20}$ – extraction:
   the extraction residue from 1. is mixed with Tenax and water, ratio soil/Tenax/water 1/1/10 by weight and put in an overhead shaker at 20°C for 20 hours at about 10 rpm.
   Extracted: the potential bioavailable portion ("Bioavailable concentration rapidly desorbing" in Figure 4).
   The soil residue remaining after separation of the Tenax is used in the next extraction step 3.
3. $Tenax_{1\ week}$ – extraction:
   the extraction residue from 2. is mixed with fresh Tenax and water, ratio soil/Tenax/water 1/1/10 by weight and put in an overhead shaker at 20°C for 1 week at about 10 rpm.
   Extracted: the potential bioavailable portion 2 ("Bioavailable concentration slowly desorbing" in Figure 4)
   The soil residue remaining after separation of the Tenax is split into two portions, one portion for ammonium oxidation test **representing soil containing only NER + difficult to extract / non-bioavailable fraction**, the other portion is used in the final exhausting extraction step.
4. Exhaustive extraction:
   the extraction residue from 3 is extracted with organic solvent in a shaking procedure and then by ASE. The extracted fraction represents the **Total extractable portion reduced by the bioavailable portion** in Figure 4.

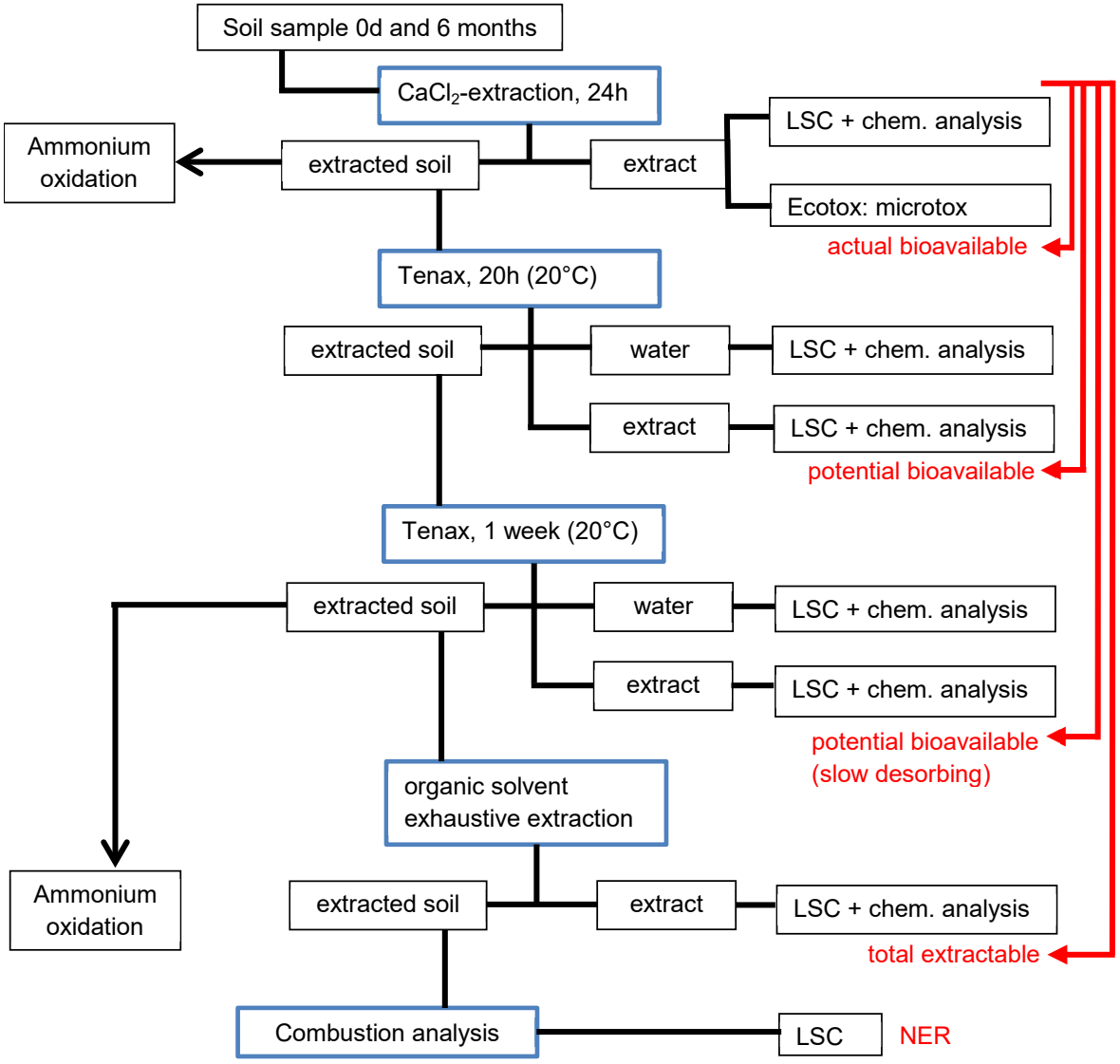


Figure 7 *Scheme for sequential extraction and ecotoxicological testing*

According to the initial planning, this package should have been performed with non-labelled test chemicals only. The amount of NER should be estimated from the results of 2.2 to have an idea how much NER is present for the final ammonium oxidation testing. However, we finally decided to perform this package with ^{14}C-radiolabelled substance as well, to have a direct measure of the NER, since the entire study focuses on that topic and this makes the results significantly more reliable. It might not be possible to conduct such work with ^{14}C-radiolabelled substances in everyday practice, but in this study, it turned out to be crucial for understanding of the results obtained. The work package was performed with three test chemicals and three soils each.

2.4 Ecotoxicological testing with soils spiked with non-labelled test chemicals

In addition to the ecotox testing in 2.3 another approach was taken in parallel. A much bigger portion of soil (5 to 7 kg per soil) was spiked with the non-labelled test chemical and incubated in the same conditions as in 2.3. At time 0 and after 6 month of incubation the soils were used to perform the earthworm avoidance test and the ammonium oxidation test. Because of the amount of soil necessary for this test, it was not possible to perform the sequential extraction as described in 2.3 prior to the ecotox testing. Thus, the test was performed with the spiked soil without any further soil treatment.

Consequently, the test result only compares the effects on earthworms and ammonium oxidation at test start and after 6 months.

2.5 Control soils

Effects determined in ecotoxicological testing represent an integrating result of the test. Without an uncontaminated control soil, observed effects cannot be reliably assigned to a soil contamination. This was even more significant in the current project as the key question was whether NER still contributes to ecotoxicity or not. Although the sequential extraction as described in 2.3 was selected in order not to change the soil matrix, effects to the test organisms could not be excluded. Thus, to have robust data, we decided to perform the extractions as described in 2.3 in an identical manner with non-spiked soil as well, without performing the chemical analysis. The material produced served as control within the ecotox tests.

2.6 Applicable Method for NER assessment

One of the objectives of the project was the development of a procedure applied to test the toxicity of the different fractions of a chemical. To be applicable in different laboratories, this procedure should be simpler than the one presented in Figure 7. Based on the results obtained and the necessity to present results in line with Figure 4, we designed an approach using an unlabelled chemical. The procedure is presented in Figure 8.

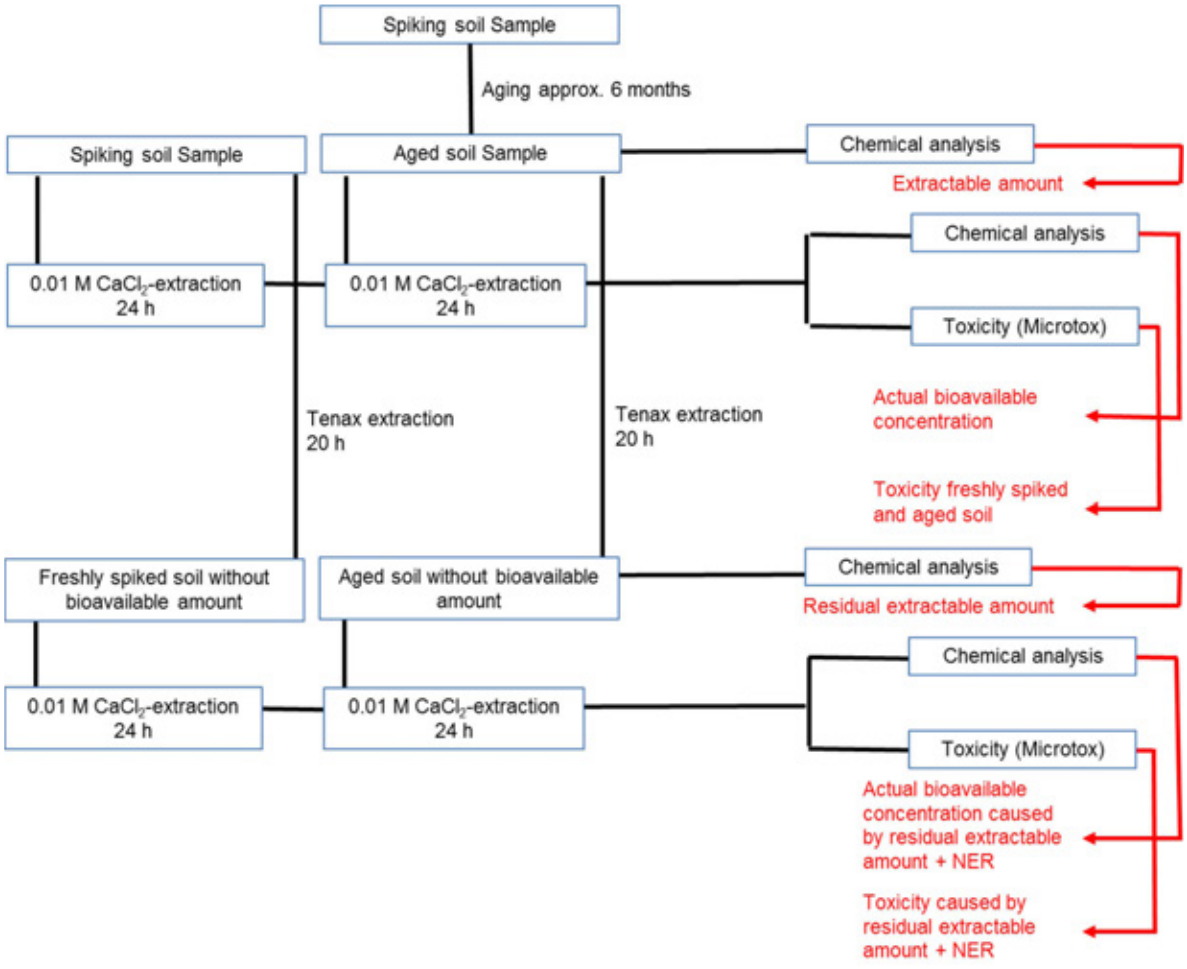


Figure 8 *Scheme for sequential extraction and ecotoxicological testing with non-labelled chemical*

To reduce any temporal bias in the bioassays, we did all tests simultaneously. After the aging period, a new batch of stored soil was spiked to represent the fresh spike (T=0). Both, the aged soil and fresh spiked soil were extracted using 0.01 M $CaCl_2$ and $Tenax_{20}$. The concentration in the 0.01M $CaCl_2$ and amount removed by Tenax were measured. The concentration in 0.01 M $CaCl_2$ represents the actual bioavailability and the Tenax–results the potential bioavailability. Especially for chemicals having a high solubility like TNT, the use of 0.01 M $CaCl_2$ in different parts of the scheme supplies relevant data. Toxicity tests (see section 3.3) were performed with the freshly spiked and aged soils before and after application of the extractions.

3 Materials and methods

3.1 Selection of test soils

Fraunhofer IME holds the German reference soil set (www.refesol.de), which is a set of 12 soils representing the variety of soils found throughout Germany. The sites were selected together with the German Federal Agency for Environment and soils from these sites are widely used for testing of chemicals for registration. It's currently the only set of soils officially accepted as reference soils by respective authorities. Fraunhofer IME has access to the sites to sample fresh soils any time of the year. Soils 01-A, 02-A and 03-G were used in this project. These soils cover a wide range of OC-content (1% to 4%), texture (sandy, silty and loamy) and water capacity (264 to 768 mL per kg). Characterisation of the soils is done frequently under GLP conditions (GLP IME-010/7-85) and characteristics are given in Table 2. The analytical methods used are: soil texture: DIN ISO 11277, USDA soil texture classification; total carbon, organic carbon, inorganic carbon, organic matter: DIN EN 15936; total N: Kjeldahl extraction according VDLUFA; pH: DIN EN 15933; CEC_{eff}: DIN ISO 11260; WHCmax: SOP V3-370.

Table 2 *Characteristics of the used soils*

RefeSol number	according to DIN			according to USDA							
	Sand	Silt	Clay	Sand	Silt	clay	organic C	total N	pH ($CaCl_2$)	CEC_{eff}	WHC
	%	%	%	%	%	%	%	g/ kg		mmol/kg	g/kg
01-A	76.7	17.2	6.1	76.6	17.7	5.7	0.80	0.71	4.72	7.60	291
02-A	2.3	82.0	15.7	4.1	80.1	15.8	0.92	1.18	6.19	59.10	457
03-G	20.2	57.8	22.0	22.3	55.2	22.6	2.46	3.26	5.71	66.90	714

Sol identification	Soil type	Attributes
01-A	Dystric Cambisol	loamy sand, medium acid, very light humic
02-A	Stagnic Luvisol	silt loam, sub-acid, light humic
03-G	Eutric Cambisol	silt loam, medium acid, medium humic

3.2 Selection of test chemicals

3.2.1 Introduction

For the study, it was agreed to select three test chemicals with different properties. As several points had to be considered this step turned out to be far more complex than expected. Nevertheless, the choice of proper test chemicals is one of the most important steps for a study like this. The substances should:

- form significant amounts of NER in soils within a period of 6 months;
- have a high toxicity to terrestrial test organisms;
- be available as ^{14}C-labelled substance at reasonable cost;
- be non-ionisable so as not to complicate the experimental part of the study.

In this chapter, the selection process is described. Literature data gave ideas but as a matter of fact, pre-experiments were necessary to learn more about substance behavior.

3.2.2 Selection based on existing data

The first selection (Annex 1) made of plant protection products and personal care products showing NER is largely based on findings reported by Barriuso et al. (2008). This paper represents one of the most comprehensive summaries available on NER formation in soil, and is based on analysis of literature and EU pesticide registration dossiers. They found that many studies on trends of NER formation were of limited use due to the high variability in the quality of data. However, one of their conclusions was that pesticides and metabolites containing aniline or phenol groups often tend to form larger proportions of NER than compounds without these groups (Barriuso et al., 2008).

From an experimental point of view, compounds of relatively high hydrophobicity are to be preferred in view of the Tenax extractions intended to remove compounds from soil matrix. Moreover, compounds with high toxicity to soil organisms are preferred over compounds with low toxicity, because of the experimental aim to demonstrate effects, or a decrease thereof. Where data on soil toxicity for compounds was not readily available, the toxicity to Daphnids was used as an alternative indicator. An additional requirement was sufficient stability of the compound in the soil matrix. Compounds, which e.g. rapidly undergo hydrolysis, were not suitable in this study. The same considerations were made for the potential selection of pharmaceuticals from an elaborate list of compounds provided by Sanderson and Thomsen (2009). The combination of NER-formation and terrestrial toxicity does not occur frequently. A refined selection was compiled from the first one and includes chemicals that had shown terrestrial toxicity only (Table 3). In addition, the availability of ^{14}C-radiolabelled chemical was considered.

From this list, finally TNT, cypermethrin and carbendazim were selected to be used for pre-tests. Benzo(a)pyrene (B(a)P) was added for consideration because it is also a well investigated compound at IME and Wageningen Environmental Research, but also at Lancaster University (group of Kirk Semple). From a chemical point of view, it is an interesting compound for the study with high Kow. It is however not possible to demonstrate toxicity of B(a)P and thus it was not selected. The results of the toxicity pre-experiments are presented in chapter 3.3.7.

Table 3 *Candidate chemicals for testing with reported terrestrial toxicity*

Compound name	^{14}C available*	Assessment of suitability of compounds by ECO-25 Monitoring Team	
		Positive aspects	Negative aspects
Chlorpropham	++	NER fraction = 54-78%	Only moderately toxic to earthworms = 132 mg/kg
Fluoxetine	++	High K_{ow} value Persistent, adsorbs readily to soil	No data on NER binding No earthworm toxicity data Not suited to Tenax/HPCD extraction at neutral pH
Sulfadiazine	++	NER fraction = 20-104%	Low K_{ow} so less suitable to Tenax/HPCD No earthworm toxicity data
TNT	++	NER fraction = 98%	Only moderately toxic to earthworms = 68 mg/kg. Also 7 day earthworm data (LC_{50} = 21-97 mg/kg)
Pyraclostrobin	++	NER fraction = 54-56% Chronic toxicity to earthworms = 0.44 mg/kg	Conflict of interest at IME
3,4-Dichloroaniline	+	Forms covalent bonds with organic fraction is soils and sediments	Log K_{ow} = 2.7 Only moderately toxic to earthworms (LC_{50} = 132 mg/kg, NOEC = 100 mg/kg)
Triclosan	++	Toxic to cucumbers/plants NOEC ≤ 1 mg/kg Toxic to predatory mites (14 d EC_{50} = 52 mg/kg, NOEC = 1.96 mg/kg) NER data (>50% after 42 d) plus extractability data (Butler, 2012)	Low toxicity to earthworms – NOEC = >1026 mg/kg
Sulfamethoxazole	++	High NER formation (73-99%) Toxicity to plants (EC_{50} = 12-20 mg/kg)	No earthworm data
Carbendazim	+	Used as toxic reference in many OECD terrestrial toxicity testing guidelines Related benzimidazole structure (thiophanate-methyl) has 40-73% NER at day 120 33% of radioactivity remained in upper soil layers after 4 years. NER = 65% after 4 months (Lewandowska and Walorcyzk, 2010). LC_{50} to *E. fetida* = 5.7-9.3 mg/Kg soil, NOEC values 0.6 - 2.0 mg/kg. EC_{50} and NOEC data for reproduction of *E. Andrei* are< 1 mg/kg and 0.58 mg/kg (Chelinho et al., 2014)	Log K_{ow} = 1.49
Ivermectin	-	High experimental K_{oc}. Elevated level of sorption (Löffler et al., 2005)	Not available as ^{14}C Degrades rapidly under light Earthworms accumulated under a pellet of sheep dung spiked with Ivermectine.
Carbofuran	+	Earthworm toxicity ranges from 0.6 - >64 mg/kg (Van Gestel, 1992). In field studies 50% reduction in earthworm populations observed at 1.4-16 mg/kg	No NER binding data available
Carbaryl	+	Earthworm toxicity ranges from <4-263 mg/kg (Van Gestel, 1992)	No NER binding data available
Cypermethrin	+	Very toxic to earthworms LC_{50} = 11 µg/cm (Wang et al., 2012) NER formation = >20 - <60% (Barriuso et al., 2008)	-Different isomers, difficult to analyze. High adsorbtion. However, this can also be explained as advantage, formation of NER. Low application rate

++ available at IME for pretests; + Likely available at suppliers: - not available

3.3 Ecotoxicological testing

3.3.1 General remarks

Because NER-formation starts in days to weeks, we selected tests with short incubation periods. The results of these tests provide information on the bioavailability of the contaminants at the date of sampling. In case of tests with long incubation periods you do not know if the result represents T = 0 with no NER or for instance T= 8 weeks with NER. Therefore, instead of the more common earthworm reproduction test with 8 weeks testing period we applied the earthworm avoidance test (2 days testing period). We further used the daphnia test, luminescence bacteria test, ammonium oxidation test and umu-test. These tests have a testing period of two days (daphnia) or less. The terrestrial ecotoxicological tests were performed within one week after sampling. The maximum storage of the soil eluate for the aquatic ecotoxicological tests was one week at 4 °C following the test guideline ISO 18512 (2007).

Ecotoxicity tests make use of living material and to test the applicability of the used organisms, controls are used. In the method to be developed, the situation before and after NER-formation has to be investigated. The differences in toxicity are more important than absolute values. In the final test used, it was decided to spike the soil, wait for NER-formation during aging and prepare a fresh spiked soil on the moment that the aged soil was to be tested. Doing this, freshly spiked and aged soils are tested at the same moment, which will increase the reliability of any observed differences.

3.3.2 Avoidance test

The test was performed according to the guideline ISO 17512-1:2008 "Soil quality -- Avoidance test for determining the quality of soils and effects of chemicals on behaviour -- Part 1: Test with earthworms (*Eisenia fetida* and *Eisenia andrei*)" using the two-chamber system. We filled polypropylene containers (Bellaplast GmbH, Alf, Germany) to a depth of approximately 5 cm with 640 g dry mass of soil (55% WHC_{max}). Adult worms were derived from a synchronized culture (age about 3 – 4 months). At the beginning of the test, the vessels were divided into two equal sections by a vertically introduced plexiglass divider. One half of the vessel was filled with test soil (Section A) and the other half with control soil (Section B). Then the separator was removed, and ten adult worms of the species *Eisenia andrei* (weight: 300 - 600 mg) were placed on the separating line of each test vessel. To prevent the worms from escaping from the vessels, these were covered with gauze permeable to light and air. The vessels were incubated at 20 ± 2°C, with a day/night rhythm of 16/8 hours for 48 h. At the end of the test period, the control and test soils were separated by inserting the plexiglas divider. The number of worms was determined for both sections of the vessels. Worms divided due to the introduction of the plexiglas divider were counted as 0.5 independent of the length of the remaining body. The tests were run using five replicates. The results are presented as percent worms in the two soils (control and test soil). The results at test start and test end were compared to get information on the bioavailability of the contaminants and the changes during the incubation period of the soil of several months.

The test is valid if the number of dead worms does not exceed 10%. The homogeneity of the distribution of the worms in the test vessels if the same soil was filled in both chambers should be within the range of 60% - 40% (Table 4). Additionally, a boric acid as reference substance was tested to prove the sensitivity of the test. According to the guideline an obvious effect should be at a concentration of 750 mg/kg (Table 5). All validity criteria were fulfilled.

Table 4 *Distribution of the worms if the same soil is filled in both test chambers*

Soil	Earthworm avoidance test: distribution of worms – test 1		Earthworm avoidance test: distribution of worms – test 2	
Vessel (Replicates)	Chamber 1	Chamber 2	Chamber 1	Chamber 2
1	4	6	6	4
2	5.5	4.5	5.5	4.5
3	4	6	5.5	4.5
4	6	4	4	6
5	5	5	6	4
Mean value [%]	49	51	54	46

Table 5 *Effect of the reference substance boric acid on earthworm avoidance*

Soil	Earthworm avoidance test: distribution of worms – test 1		Earthworm avoidance test: distribution of worms – test 2	
Vessel (Replicates)	Control soil	Boric acid (750 mg/kg)	Control soil	Boric acid (750 mg/kg)
1	8	2	7	3
2	8	2	7	3
3	7,5	2,5	8	2
4	8	2	9	1
5	7	3	7	3
Mean value [%]	78	22	76	24

3.3.3 Potential ammonium oxidation activity

In contrast to the nitrification test (OECD TG 216) in which a complex organic nitrogen source is added and the formation of ammonium, nitrite and nitrate investigated over a period of at least 28 days, in the test on the potential ammonium oxidation activity, ammonium is added and the formation of nitrite is determined after a six-hour incubation period. The step from nitrite to nitrate is inhibited in this test. The advantage of the test is its high sensitivity and the fact that information on current microbial activity is obtained due to the short incubation period.

The potential ammonium oxidation activity (ISO 15685) was measured in a slurry of 25 g dry soil matter in 100 mL mineral test medium. The slurries were incubated on an orbital shaker at 25°C ± 2°C, and 10-mL samples were removed after 2 and 6 h. The samples were mixed with 10 mL 4 mol/L KCl, and after centrifugation, the nitrite levels in the filtrate were measured photometrically. Validity criteria or results of reference substances are not described in the test guideline.

The results at test start and test end were compared to get information on the bioavailability of the contaminants and the changes during the incubation period of the soil of several months. It was expected that the microorganisms, which perform the transformation from ammonium to nitrite, would recover if the bioavailability of the contaminants is reduced due to the formation of NER. During this project, it became obvious that the assumption was not correct. A recovery was not observed.

3.3.4 Luminescence bacteria test

The test was performed according to ISO 11348:2007 "Water quality -- Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test)". The luminescence of a culture of *Vibrio fischeri* is determined at test start and after an incubation period of 30 min. Several dilutions of the aqueous sample were investigated (2% NaCl - solution) and the inhibition compared to the control soil calculated. A test is valid if the replicates do not differ by more than 3% from the mean value and if the f_{kt} value for the controls is in the range of 0.6 – 1.8. The results at test start and test end were compared to get information on the bioavailability of the contaminants and the changes during the incubation period of the soil of several months.

3.3.5 Daphnia test

The test was performed according to OECD test guideline 202 (2004). Young daphnids (*Daphnia magna*), aged less than 24 hours at the start of the test, were exposed under static conditions to the soil eluate ($CaCl_2$ extraction) at a range of concentrations for a period of 48 hours. For the various concentrations (undiluted; 50%, 25; 12.5; 6.25%) the soil eluate was diluted with purified tap water. Immobilization was recorded at 24 hours and 48 hours and compared with control values. A test is valid if the immobilization rate in the control does not exceed 10% and the dissolved oxygen concentration at the end of the test is ≥ 3 mg/L. Twice per year the test is performed with a reference substance ($K_2Cr_2O_7$) to assure the reliability of the test conditions. The results at test start and test end were compared to get information on the bioavailability of the contaminants and the changes during the incubation period of the soil of several months.

3.3.6 Umu test

The test was performed according to ISO 13829:2000 "Water quality - Determination of the genotoxicity of water and waste water using the umu-test". A genetically engineered bacterium *Salmonella typhimurium* TA1535/pSK1002 serves as test organism. The test is based on the capability of genotoxic agents to induce the umuC-gene in the Salmonella strain in response to genotoxic lesions in the DNA. The test organisms are exposed to the soil extract with and without metabolic activation system using microplates. After 4 h of incubation, the genotoxic-dependent induction of the umuC-gene was compared to the spontaneous activation of the untreated, control culture. The test is considered valid, if the positive controls reach an induction ration of at least 2 under the given test conditions. Minimum growth of the negative controls is 140 FNU (formazin nephelometric units). The results at test start and test end were compared to get information on the bioavailability of the contaminants and the changes during the incubation period of the soil of several months.

3.3.7 Ecotoxicological pre-experiments

In pre-experiments, the sensitivity of the test systems to the selected test substances were studied. One concentration and an uncontaminated control soil were tested. The results are presented in Table 6. There were no effects in the control soil.

Table 6 *Results on the sensitivity of the selected test systems on the initial selection of test substances*

Test system	TNT Spiking of soil: stock solution in acetone	B(a)P Spiking of soil: dry spiking	Cypermethrin Spiking of soil: stock solution in acetone	Carbendazim Spiking of soil: dry spiking
Test with soil				
Avoidance test	100 mg/kg: significant avoidance behavior 3% of organisms in contaminated soil	100 mg/kg: no obvious avoidance behavior; 62% of organisms in contaminated soil	100 mg/kg: slight avoidance behavior; 31% of organisms in contaminated soil	20 mg/kg: obvious avoidance behavior; 24% of organisms in contaminated soil
Ammonium oxidation	100 mg/kg: 20% effect	Not determined	No effect	No effect
Luminescent bacteria	nominal in soil: EC_{50}: 40 mg/kg EC_{20}: <6.25 mg/kg	No effect (highest test concentration: 80% eluate)	No effect (highest test concentration: 80% eluate)	No effect (highest test concentration: 80% eluate)
Daphnia test	No effect	About 30% effect in highest test concentration (80% eluate)	50% effect between 25 – 50% soil eluate	50% effect at about 80% soil eluate
Umu test	Not determined	No effect (highest test concentration: 80% eluate)	Not determined	Not determined

From the ecotoxicological point of view B(a)P proved to be inappropriate for the project (Table 7) due to its low ecotoxicity. Even with the test on mutagenicity, effects of this test substance could not be detected. For TNT, cypermethrin and carbendazim different ecotoxicological tests were considered as suitable:

- TNT:
  - Soil
    - Avoidance test
    - Potential ammonium oxidation activity
  - Soil eluate
    - Luminescent bacteria test
- Cypermethrin and carbendazim
  - Soil
    - Avoidance test
  - Soil eluate
    - Daphnia test

Table 7 *Schematic overview on the suitability of the test systems regarding the initial selected test substances*

Test system	TNT Spiking of soil: stock solution in acetone	B(a)P Spiking of soil: dry spiking	Cypermethrin Spiking of soil: stock solution in acetone	Carbendazim Spiking of soil: dry spiking
Test with soil				
Avoidance test	+++	-	+	++
Ammonium oxidation	+	Not determined	-	-
Test with soil eluate ($CaCl_2$ extract)				
Luminescent bacteria	+++	-	-	-
Daphnia test	-	+	+++	++
Umu test	Not determined	-	Not determined	Not determined

Additionally, we performed ecotoxicological tests with the soil after Tenax extraction. This will be possible for the test on potential ammonium oxidation activity due to the low amount of soil, which could be provided for the test. Although we extracted in our experiments higher amounts of soil, in daily practise Tenax can be used for up to 200 g of soil due to the size of the extraction vessels required. In the earthworm avoidance test, about 2 kg of soil are required. It would have been impossible to perform a Tenax extraction with such an amount of soil; in addition, this would have cost about € 50.000 for the Tenax material alone. It should be realized that other extraction methods are also not applicable for 2 kg of soil. High amounts of solvents (> 10 l) will be needed, which will not be practicable under laboratory test conditions. As the potential ammonium oxidation activity proved to be unsuitable in the scope of the project, the approach of testing soil after Tenax extraction had to be skipped (see 4.5).

3.4 Chemical methods

3.4.1 Solvent extraction, literature review

For 2,4,6 Trinitrotoluene (TNT) the solvent acetonitrile is presented as a good solvent for extraction with recoveries >95% (Robertson and Jjemba, 2005), Williford and Bricka (1999) showed that most of the extractable TNT was extracted in the first extraction step if subsequent extractions using acetonitrile were used. Acetonitrile is on the other hand also presented as a weak solvent with recoveries below 60% (Gong et al., 1999). The difference might be explained by the different ways of spiking reported in the two publications. Robertson and Jjemba (2005) used a water slurry while Gong et al. used a high amount of acetonitrile (equal to the amount of soil).

Acetone and methanol also showed high recoveries which decreased during aging, which was attributed to NER formation (Cataldo et al., 1989; Campbell et al., 2003). According to their results (see Figure 9), a stable NER-fraction of about 50% was formed after 50 days. This is in line with results of IME, where a DT_{50} of 32 days was determined in previous studies and no mineralisation but mostly turnover into NER (Joos et al., 2008).

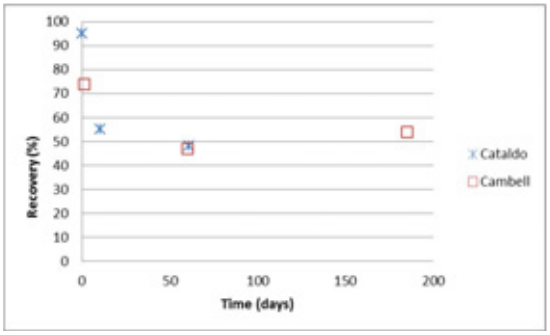


Figure 9 *Change of recovery in time using literature data*

Water was used to determine the available fraction. About 20% could be extracted with a buffer solution (Bordelon et al., 1989). They used extraction with acetone/hexane to extract 100%.
In regulation, standard methods are preferred and results of these methods are considered reliable for use in assessments. Knowing the regulatory interest of NER, we therefore used standard methods if available. According to ISO 11916-1, TNT can be extracted from soil with acetonitrile or methanol. This extraction was validated by an international Ring test, which was organised and evaluated by Fraunhofer IME (see ISO 11916-1).

Babić et al. (1998) used several solvents for the extraction of α-cypermethrin. Best recovery was with acetone, a solvent that mixes well with water. Lower recoveries were found with acetonitrile and for solvents that do not mix with water (Table 8). Hexane, a lipophilic solvent and less mixable with water has the lowest recovery. These solvents are not able to enter small soil pores, which may be filled with water and consequently cannot extract chemicals like cypermethrin (Harmsen and Frintrop 2003). In contrast, the combination acetone/hexane gave a high recovery (Braun and Stanek, 1982). Other mixtures, like toluene and methanol also gave high recoveries.

Table 8 *Extraction recoveries for α-cypermethrin from soil using different solvents*

Solvent	cypermethrin recovery in %
Diethylether	75.9 ± 3.2
Chloroform	83.2 ± 3.5
Hexane	66.7 ± 2.7
Benzene	77.0 ± 3.4
Acetonitrile	69.4 ± 2.9
Dichloromethane	76.8 ± 3.3
Acetone	97.2 ± 4.4

Austin and Briggs (1976) showed that addition of ammonium chloride increased the recovery of carbendazim from soil. Most effective solvent was the combination of acetone and ammonium chloride, while an aqueous solution of ammonium chloride extracted 15% only. Matser and Leistra (2000) used water and reported the decrease of the measured concentration to about zero in 60 days. Virag and Kiss (2009) used buffer and salt solutions to estimate bioavailability.
Combining these three observations, water or a buffer or salt solution should be appropriate as a weak solvent for carbendazim. In more recent publications, ethyl acetate was used to obtain high recoveries. Virag and Kiss (2009) had a high recovery using methanol, but a low recovery using chloroform.

In Annex 3 more data are presented about potential solvents for extraction of bioavailable residues of TNT, benzo(a)pyrene, carbendazim and cypermethrin from soils/plants.

3.4.2 Pre-experiments

3.4.2.1 Extraction from the water phase using petroleum ether

A general procedure to isolate a non-polar chemical from the water phase is liquid / liquid extraction with a non-polar solvent (e.g. petroleum ether). This has been tested by extracting 100 ml spiked water with 50 ml of petroleum ether. Recoveries are presented in Table 9. During these experiments benzo(a)pyrene was still being considered.

Table 9 *Recoveries of chemical from water by extraction with petroleum ether*

Chemical	Type	Spike (mg/l)	Recovery (%)
TNT	Explosive	4.44	64.7
Benzo(a)pyrene	PAH	2.00	98.6
Cypermethrin	Insecticide	1.90	103.7
Carbendazim	Fungicide	2.00	100.0

From Table 9 can be concluded that all substances except TNT can be fully recovered from spiked water. The low recovery for TNT can be explained by the higher polarity and thus better water solubility of TNT. It is better to analyse TNT by direct measurement of the water phase.

3.4.2.2 Extraction from the water phase using Tenax

In order to try the Tenax extraction, an experiment was conducted where Tenax was added to the spiked water and the concentration in the water phase was measured after different times. By this procedure, sorption kinetics to the Tenax was determined. Results are presented in Table 10.

Table 10 *Removal kinetics of chemicals from the water phase by Tenax*

Time (hours)	TNT (ng/ml)	Benzo(a)pyrene (ng/ml)	Cypermethrin (ng/ml)	Carbendazim (ng/ml)
0	9155	4160	4334	4121
1	532	58	12	1961
2	275	77	29	1468
4	124	47	19	1411
6	82	40	30	1286
24	78	50	50	1294
Extracted after 1 hour	94%	98.7%	99.7%	52.4%

Table 10 shows that the suitability of Tenax for determining extraction of cypermethrin and benzo(a)pyrene was confirmed with >98% recovered after 1 hour. Tenax also adsorbs TNT, but kinetics are slightly slower. Between 2 and 4 hours are necessary to adsorb >98%. Tenax less easily extracted carbendazim and part of it remained in the water phase.

The Tenax was isolated and the amount of chemical, which could be recovered by repeated back extraction with petroleum ether in a column, was determined. Table 11 shows the results from the different extracts. Extracts 1 and 2 recovered the main part of TNT, benzo(a)pyrene and cypermethrin. Carbendazim was less extractable from Tenax.

Table 11 *Extraction of chemicals from Tenax. Results from different fractions*

Petroleum ether Extract	TNT (ng)	Benzo(a)pyrene (ng)	Cypermethrin (ng)	Carbendazim (ng)
1	57.8	89.3	58.9	20.7
2	9.3	7.3	4.3	7.8
3	1.3	1.2	0.2	3.8
4	0.5	0.3	0.05	3.3
5	0.04	0.1	0.02	3.2
Total	68.9	98.3	63.5	38.7

3.4.2.3 Extraction from soil / water mixture, introductory experiments

The test system consisted of 2 g soil and 100 ml water and was shaken for 24 hours. Soil and water were extracted separately with acetone/petroleum ether (2g soil with 10 ml acetone and 20 ml petroleum ether) and petroleum ether and 75 ml water with 20 ml petroleumether), respectively. Results are given in Table 12.

Table 12 *Extraction of chemicals from a soil / water system*

Test Chemical	Measured in water (ng)	Measured in soil (ng)	Recovery (%)
TNT	6332	2177	37.5
Benzo(a)pyrene	685	13917	71.2
Cypermethrin	2282	10061	63.3
Carbendazim	5804	8982	73.1

Most of the spiked TNT was present in the water phase, even knowing that the extraction of the water phase was not optimal. Also, a large fraction of carbendazim was extracted from the water phase. Cypermethrin and benzo(a)pyrene, both having a large K_{ow} (log K_{ow} respectively 6.60 (Toxnet, 2017) and 5.13 (Otte et al., 2001)), were mainly present in the soil phase.

Recoveries were not optimal in general. This can be explained by the system and the solvent used. In this experiment, we have used a slurry of 2 g of soil and 100 ml of water. The non-polar solvent petroleum ether was not optimal for this system. Enhanced biodegradation as well as transport processes might also lead to lower recoveries. However, from this experiment it cannot be concluded if the low recovery was caused by NER-formation, biodegradation or other processes.

3.4.2.4 Extraction from soil / water mixture, advanced experiments

The experiments from section 3.4.2.3 were repeated in four-fold to obtain an impression of repeatability and different conditions were used to observe if kinetics of the adsorption plays a role. For this purpose, field-moist spiked soil was stored under atmospheric conditions and in parallel in a slurry. In the slurry system, 4 g of soil were mixed with 100 ml of water. If adsorption kinetics are relevant, differences would be observed in the two setups. Autoclaved soil was used in order to suppress biodegradation. The amount of test chemical in soil and the water phase was measured after various treatments.

Table 13 shows a very good recovery from soil directly after spiking. The decrease after 3 days may indicate some abiotic degradation / NER-formation. The water solubility of TNT is visible in the slurry systems (row 3 and 4), because most of the TNT could be detected in the water phase. The total recovery was below 100%, which may indicate NER formation in the slurry system. Application of Tenax removed most of the available TNT from the water phase and the available part in the soil.

Table 13 *TNT measured in the spiked autoclaved soil (in percentage of initial amount. Number of replicates n= 4)*

Treatment	soil		water		Total	
	average	stdev	average	stdev	average	stdev
extraction soil, 1 hour after spiking	97.9	10.5				
extraction soil, 72 hours after spiking	82.6	12.7				
24 hours after spiking, adding water and shaking for 20 hours. Extraction of soil and water phase	15.3	1.2	55.2	17.1	70.5	18.1
72 hours after spiking, adding water and shaking for 20 hours. Extraction of soil and water phase	16.4	1.0	56.0	3.0	72.4	3.5
24 hours after spiking, adding water and Tenax and shaking for 20 hours Extraction of soil, water and Tenax	4.8	1.2	1.2	0.3	6.1	1.2
72 hours after spiking, adding water and Tenax and shaking for 20 hours Extraction of soil, water and Tenax	6.0	1.9	1.4	0.3	7.4	2.2

The recovery of cypermethrin after spiking was below 100% but still acceptable (Table 14). In the slurry, the recovery dropped drastically. Biodegradation was unlikely because of the use of autoclaved soil. With Tenax, the extractable amount did not change significantly. Because of the poor water solubility, the amount recovered from the water phase was low. The recovery from the wet soil system was always difficult with a non-polar solvent, which might be the reason for the low recovery. The test with the radiolabelled cypermethrin showed a better recovery, but there a different solvent was used (see 3.5.4).

Table 14 *Cypermethrin measured in the spiked autoclaved soil (in percentage of initial amount. Number of replicates n= 4)*

Treatment	soil		water		Total	
	average	stdev	average	stdev	average	stdev
extraction soil, 1 hour after spiking	88.9	4.1				
extraction soil, 72 hours after spiking	86.3	7.3				
24 hours after spiking, adding water and shaking for 20 hours. Extraction of soil and water phase	15.5	1.1	1.7	0.2	13.3	7.9
72 hours after spiking, adding water and shaking for 20 hours. Extraction of soil and water phase	17.9	3.4	2.3	0.2	20.2	3.4
24 hours after spiking, adding water and Tenax and shaking for 20 hours Extraction of soil, water and Tenax	12.9	2.0	2.3	0.2	15.2	2.2
72 hours after spiking, adding water and Tenax and shaking for 20 hours Extraction of soil, water and Tenax	14.1	4.2	0.7	0.0	14.6	3.9

The recovery of carbendazim was low from the very beginning (Table 15). The substance was difficult to extract as it is neither very soluble in water (very polar solvent) nor in non-polar organic solvents. The recovery of carbendazim decreased quickly. With Tenax, about 10% should be trapped (indirect assessment from the comparison of the recoveries with and without Tenax). The low recoveries could not be explained. The experiments with the ^{14}C-radiolabelled carbendazim gave better information on the fate of the chemical (see 4.3).

Table 15 *Carbendazim measured in the spiked autoclaved soil (in percentage of initial amount. Number of replicates n= 4)*

Treatment	soil		water		Total	
	average	stdev	average	stdev	average	stdev
extraction soil, 1 hour after spiking	61.0	5.0				
extraction soil, 72 hours after spiking	29.0	6.2				
24 hours after spiking, adding water and shaking for 20 hours. Extraction of soil and water phase	11.6	2.3	0.4	0.0	10.1	4.1
72 hours after spiking, adding water and shaking for 20 hours. Extraction of soil and water phase	11.7	1.8	0.3	0.1	11.9	1.9
24 hours after spiking, adding water and Tenax and shaking for 20 hours Extraction of soil, water and Tenax	3.2	0.9	0.3	0.1	3.5	0.9
72 hours after spiking, adding water and Tenax and shaking for 20 hours Extraction of soil, water and Tenax	2.0	0.5	0.2	0.1	2.3	0.5

Benzo(a)pyrene (B(a)P) was tested in the same system. This chemical was investigated in several studies by Wageningen Environmental Research and Fraunhofer IME and the fate is well understood. B(a)P is a substance that strongly adsorbs to organic matter. After spiking, B(a)P was fully recovered from the soil (Table 16). The recovery was still high after the slurry treatment. Tenax removed about 55% of the B(a)P (difference in recovery to the non-Tenax experiment). This part is considered bioavailable. Approx. 45% is still present in the soil after Tenax treatment and is not bioavailable. A low amount is measured in the water phase. This is likely to be associated with organic matter and colloids, which are not removed by centrifugation. The results are in line with the expectations, showing that the method applied was working.

Table 16 *B(a)P measured in the spiked autoclaved soil (in percentage of initial amount. Number of replicates n= 4)*

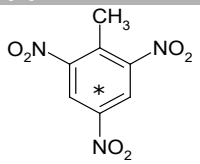
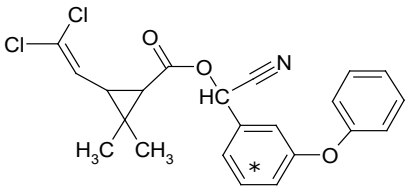
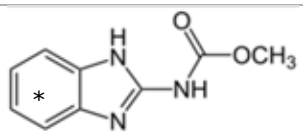
Treatment	soil		water		Total	
	average	stdev	average	stdev	average	stdev
extraction soil, 1 hour after spiking	93.0	6.9				
extraction soil, 72 hours after spiking	100.3	2.2				
24 hours after spiking, adding water and shaking for 20 hours. Extraction of soil and water phase	109.5	4.2	2.2	0.4	111.8	4.0
72 hours after spiking, adding water and shaking for 20 hours. Extraction of soil and water phase	105.6	1.0	2.0	0.5	107.6	1.0
24 hours after spiking, adding water and Tenax and shaking for 20 hours. Extraction of soil, water and Tenax	45.0	6.4	2.0	0.5	46.9	5.9
72 hours after spiking, adding water and Tenax and shaking for 20 hours. Extraction of soil, water and Tenax	42.8	11.0	0.7	0.1	43.6	11.1

3.5 ^{14}C-radioactive experiments

3.5.1 Test chemicals

For the ^{14}C-radiolabelled experiments, the following test substances were used (Table 17).

Table 17 *List of ^{14}C-radiolabelled chemicals used for the study*

Substance name	label position (*) denotes ^{14}C labelling position	supplier	specific radioactivity
2,4,6-TNT IUPAC: 2-Methyl-1,3,5-trinitrobenzen CAS-No 118-96-7		University Paderborn	4.39 MBq/mg Purity 94.5%
Cypermethrin IUPAC: (RS)-α-cyano-3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate CAS-No 52315-07-8		Quotient Bioresearch	1.97 MBq/mg Purity 99.8%
Carbendazim IUPAC: methyl benzimidazol-2-ylcarbamate CAS-No 10605-21-7		Quotient Bioresearch	21.7 MBq/mg Purity 99.9%

3.5.2 Spiking of soils

For soil spiking, a solution of ^{14}C-radiolabelled and non-labelled test chemical at a known ratio was prepared in acetone. Very high concentrations of 120 mg chemical per kg soil each were selected to ensure that the NER at test end were present at a concentration where ecotoxicological effects could eventually be measured in case they still contributed to ecotoxicity. However, this could not be done with the pure ^{14}C-labelled test chemicals due to costs and availability. In the mixture of ^{14}C-radiolabelled and non-labelled test chemical, the applied radioactivity ensured a sufficient sensitivity for the intended work (mass balance, NER detection, radio-TLC for parent analysis).

For spiking, moist soil was spread on a stainless-steel surface. TNT and cypermethrin were dissolved in acetone and applied as homogeneously as possible in little droplets with a syringe to the soil surface. The solvent volume was below 1% of soil weight. Carbendazim does not dissolve completely in any solvent in the desired amount and thus a suspension was prepared in methanol and applied to the soil in the same way as described above. For details of the application, see Table 18.

After application, the solvent was allowed to evaporate for 20 min and then the soil was mixed intensively. Soil used for the sequential extraction (see 2.3) was stored in a container at 20°C in the dark. The container was covered with aluminium foil to prevent the soil drying out. The moisture was frequently controlled by weighing and re-adjusted with distilled water if necessary. For the transformation kinetics and mass balance experiments (refer to 2.2), samples were prepared individually for later sacrificial sampling. Each single sample was applied from the same solution as described above with an appropriate pipette directly in the test vessel. By this procedure and with sacrificial sampling it is assured that each sample received the same amount of radioactivity without variation due to unsatisfactory homogenisation. This was important for a valid and reproducible mass balance.

Table 18 *Details of application volumes and concentration in the different experiments. All soils are treated equal*

substance	Mass balance experiment (see 2.2)				Sequential extraction (see 2.3)				Ecotoxicology (see 2.4)		
	sample [g dm]	Vol [µl] solvent	Conc [µg/g]	Activity [kBq/g]	sample [g dm]	Vol [µl] solvent	Conc [µg/g]	Activity [kBq/g]	sample [g dm]	Vol [ml] solvent	Conc [µg/g]
TNT	20 g per sample	200, A	128	19.6	800	6196, A	120	20.475	7000	28.3, A	120
Cypermethrin		205, A	120	10.1	800	5602, A	120	16.647	5000	28.2, A	120
Carbendazim		207, M	120	10.0	800	10000, M	119.6	19.064	5000	30.0, M	120

A = Acetone, M = Methanol

3.5.3 Sequential extraction

3.5.3.1 $CaCl_2$-eluate

A 200 g aliquot of soil dry matter was added to each of 4 centrifuge tubes and 100 ml of 0.01M $CaCl_2$-solution were added to get a soil /solution ratio of 2:1. The tubes were shaken overhead at 5-7 rpm for 24 hours. After 24h of shaking, the supernatant was separated by centrifugation (15 min at 2100 rpm / 1218 x g) (Figure 10). The volume of the supernatant was determined and the radioactivity concentration measured by LSC (liquid scintillation counting).

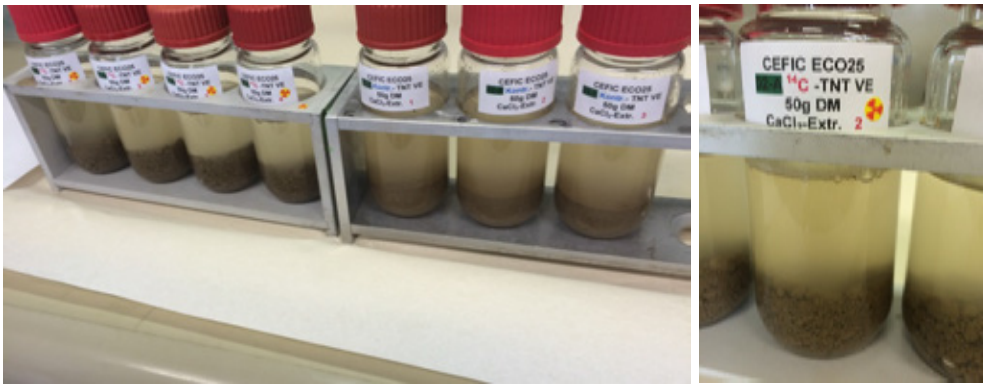


Figure 10 *Soil samples for $CaCl_2$-extraction*

3.5.3.2 $Tenax_{20}$ - extraction

The soil residue from 3.5.3.1 was transferred into two 1 litre Schott bottles (100 g soil per bottle). To each bottle, 100 g of Tenax and 1000 ml of distilled water were added. The bottles were shaken on an overhead shaker at low rpm for 20 hours (Figure 11). After shaking, the content of the bottles was transferred to centrifugation tubes and centrifuged at 2500 rpm / 1726 x g for 20 min at room temperature. This procedure separates the Tenax from the mixture and can be isolated (Figure 12). The Tenax was washed with water until the water looked clear. All water phases from the extraction step (supernatant from mixture plus washing water) were combined and analysed for radioactivity by LSC.

The Tenax was transferred to a glass column specially designed for this purpose (Figure 13). After draining the residual water, the Tenax was extracted four times with 250 mL acetone (for carbendazim: methanol) followed by four extractions with 250 mL cyclohexane. The last extract was controlled by LSC and in case there was still some radioactivity present, the extraction was repeated until no further radioactivity (below 0.1 Bq per ml) could be recovered from the Tenax. By this procedure, in some cases up to 3000 mL of cyclohexane extract were produced. The combined extracts were later analysed for radioactivity and parent test substance.

Figure 11 *Bottles prepared for Tenax$_{20}$-extraction*

Figure 12 *Separation of Tenax for further processing*

Figure 13 *Transfer of Tenax into the glass column and extraction of Tenax*

3.5.3.3 $Tenax_{1\ week}$ - extraction

The soil residue from 3.5.3.2 was treated again with Tenax as described there. The shaking duration was extended to 7 days instead of 20 hours. All following procedures were similar to those described in 3.5.3.2. After cleaning, the Tenax could be recycled for the next extraction.

3.5.4 Exhaustive solvent extraction

Samples from the mass balance experiment were transferred into a centrifuge tube and 40 ml acetonitrile (ACN) (for carbendazim: methanol (MeOH)) were added. The vessels were shaken overhead for 20 hours in the dark. After centrifugation at 2000 rpm / 1105 x g for 20 min at room temperature the supernatant was separated. The volume was determined and an aliquot used for LSC-analysis (liquid scintillation counting) in order to quantify the radioactivity in the extract.
The extraction residue was further extracted by means of ASE (Accelerated Solvent Extraction), ASE 350, Thermo) in order to measure the entire extractable chemical. For this purpose, the soil was mixed with diatomaceous earth in a ratio 4:1 and filled into the ASE extraction tubes. Extraction was performed at 100°C with 2000 psi for 15 min with acetonitrile (for carbendazim: methanol) (Figure 14). The volume of the ASE-extract was determined and the extracted radioactivity was measured by LSC.
The same procedures as described above for the mass balance samples, were applied to an aliquot of the soil residue from 3.5.3.3 in order to recover the remaining chemical from the soil.

Figure 14 *Extraction with ASE, program parameter setting*

3.5.5 NER determination

NER represent the fraction remaining in the soil matrix after an exhaustive, harsh, but not matrix destructing extraction (see 1.2.4 for definitions). For the determination of NER, the soil residue after ASE-extraction was air dried at room temperature and then combusted in a Zinsser OX700 Oxidizer. In this device, the sample is combusted in an oxygen atmosphere at 650°C. Each organic C-atom will be transformed to CO_2 or $^{14}CO_2$ by this procedure. The combustion gases are trapped in an appropriate LSC-cocktail and quantified by subsequent LSC (Figure 15). Every 10^{th} sample is followed by a standard and every fifth sample is a blank to ensure quality by testing recovery and carryover. Combustion is the only common technique to quantify NER from a matrix. With non-labelled organic chemicals, this determination is not possible.

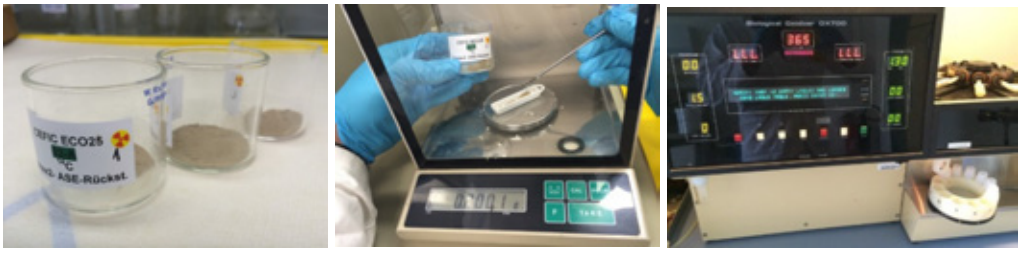


Figure 15 *NER determination by combustion*

3.5.6 Radio-TLC chemical analysis

In order to differentiate, in the extractable radioactivity, between the parent test chemicals and potential transformation products, all extracts were subject to radio-TLC (thin layer chromatography). Known amounts of the extracted radioactivity were applied to TLC-plates and after evaporation of the solvent, the plates were placed in a closed chamber into which 100 ml of a specific solvent mixture was added. The solvent runs up the plate driven by capillary forces and due to interactions of the sample with the plate material (e.g. RP18 or KG60) and the solvent substances separate on the plate. For detection, the plate was exposed to a special film, which is sensitive to radioactivity and can be read later (plate reader: Typhoon FLA 700). A software converts the TLC-plate into a chromatogram and the distribution of the radioactivity is evaluated.

The advantage of the method is a very sensitive detection due to the free selectable exposure time, which can be up to 4 weeks. In addition, the chromatogram shows the entire applied radioactivity, which is very important if the composition of the sample is unknown. Even if the system is optimised for the parent chemical it might happen that transformation products have different polarity or for some other reason they do not move on the plate. In HPLC, this fraction will stay invisible. On TLC, this fraction is detected as a start peak, which allows determining relative amounts in percentage of the total radioactivity. TLC is however, not applicable for volatile substances as these are driven off with the solvent evaporation. Table 19 shows the TLC-system used for the test chemicals:

Table 19 *Radio-TLC-methods used in the project*

Test chemical	solvent mixture v/v in ml	TLC-plate
TNT	ACN/water 70/30	RP18
Cypermethrin	n-hexane/acetone 80/20	KG60
Carbendazim	n.hexane/EA/TCM/THF/AcOH 30/30/30/6/2	KG60

With:	KG60	=	Merck TLC Silica gel 60, F254, 20 x 20 cm
	RP18	=	Merck TLC Silica gel 60 RP18, F254, 20 x 20 cm
	ACN	=	Acetonitrile
	EA	=	Ethylacetate
	TCM	=	Trichloromethane
	THF	=	Tetrahydrofurane
	AcOH	=	Acetic acid

Figure 16 shows an example TLC-plate (carbendazim, mass balance 01-A) and the chromatogram from the 2 months sampling. It shows that each sample has about the same intensity. If significant amounts of radiolabel evaporate, the signal will be much pale – which we never observed.

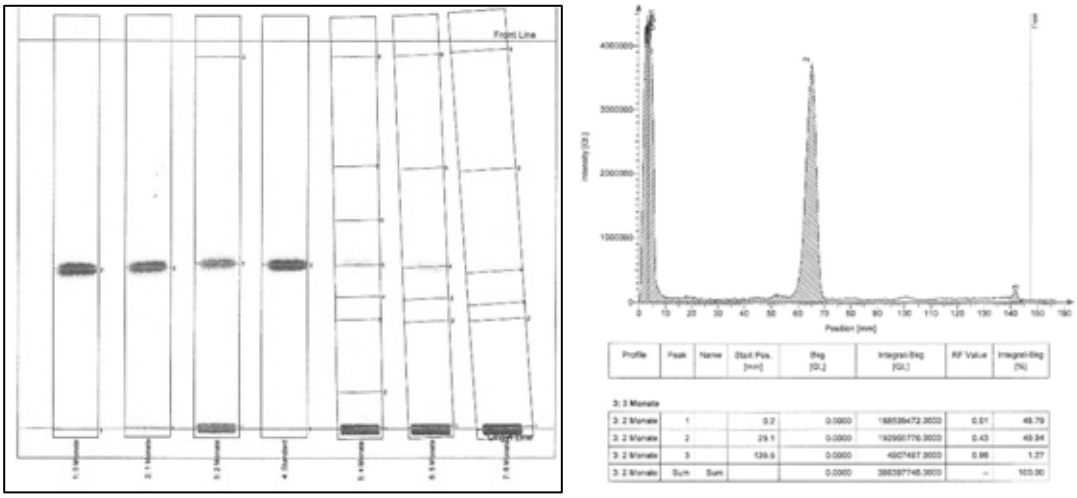


Figure 16 *Radio-TLC analysis, example carbendazim, mass balance 01-A, 2 months profile (right side)*

4 Results

4.1 2,4,6-Trinitrotoluene (TNT)

4.1.1 Mass balance control

Table 20 gives an overview on the mass balance and the distribution of the radioactivity after the application of ^{14}C-labelled TNT to the test soils. The extraction used was the extraction described in ISO11916, whose result is considered to represent the total TNT concentration. ASE is generally considered the harshest non-destructive extraction method, though from our point of view that cannot be generalized but is matrix and chemical specific. However, we did the ASE extraction for all samples to comply with external expectations.

The radioactivity recovered is presented in Table 20, which shows that the extractability decreased with time and the radioactivity as NER in the soil increased. The results of the ASE showed that most of the extractable radioactivity was recovered with the shaking extraction. At the t=0 sampling residual solvent in the soil containing still radioactivity may contribute significantly to the ASE fraction.

The major fraction observed were NER, which reached a plateau after about 3 months and ranged around a value of 70% from then. The mineralisation, determined as $^{14}CO_2$ was negligible and had reached a steady level by 4 months. The mass balance ranged mostly above 90%, which is an OECD validity criterion in GLP-testing with ^{14}C-radiolabelled substances.

Table 20 *Recovered radioactivity and mass balance of ^{14}C-TNT, initially applied radioactivity of 392 kBq per 20 g soil dry matter (= 100%) corresponding to 120 mg TNT per kg soil dm*

Time [months]	Soil	Extractable ACN [%]	Extractable ASE [%]	$^{14}CO_2$ [%]	NER [%]	recovery [%]
0	01-A	86.5	5.0		14.6	106.0
	02-A	91.3	4.2		23.0	118.4
	03-G	85.5	5.9		20.6	112.0
1	01-A	52.7	6.7	0.7	41.9	102.0
	02-A	32.4	9.3	2.7	56.0	100.3
	03-G	26.1	10.1	3.0	63.6	102.8
2	01-A	35.6	9.7	1.1	49.8	96.2
	02-A	23.2	7.1	3.8	68.8	102.9
	03-G	18.5	6.6	4.9	70.3	100.3
3	01-A	31.9	7.4	1.3	57.7	98.3
	02-A	18.9	6.0	4.2	68.1	97.2
	03-G	14.0	5.4	6.3	69.6	95.3
4	01-A	25.9	6.9	2.0	52.6	87.4
	02-A	16.3	6.0	4.7	72.6	99.6
	03-G	11.2	5.0	7.0	72.0	95.2
5	01-A	27.0	5.2	2.1	57.5	91.8
	02-A	14.6	4.3	5.3	73.2	97.4
	03-G	10.1	4.9	7.0	71.3	93.3
6	01-A	22.0	5.2	2.1	64.1	93.4
	02-A	13.5	4.5	5.4	74.6	98.0
	03-G	9.5	3.8	7.3	77.2	97.8

All observations described in Table 20 are in perfect accordance with our experience with TNT from previous projects. The main process for the fate of TNT in soil is the reduction of nitro-groups to amino groups and subsequent irreversible formation of covalent bonds to the soil matrix. This has been shown in a number of previous studies (Joos et al., 2008) and can also be observed in the present study. The ^{14}C-experiments prove that the amount of TNT, which cannot be extracted, is stored in the soil as NER.

From the experimental results we conclude that the amount of non-labelled TNT, extracted using the standard method ISO 11916-1 (2013) is high and ASE add a relatively low extra amount. The residual amount after extraction with the standard method is a good indication of NER.

The results from Table 20 are summarized in Figure 17, showing that after 3 to 4 months a stable situation is obtained where most of the TNT is present as NER. This time is necessary for the formation of NER after spiking. This is in agreement with results of Cataldo et al. (1989) and Campbell et al. (2003). Differences between the three soils (see Table 2) demonstrate that soil properties (e.g. organic matter and porosity) also have an influence on the formation of NER.

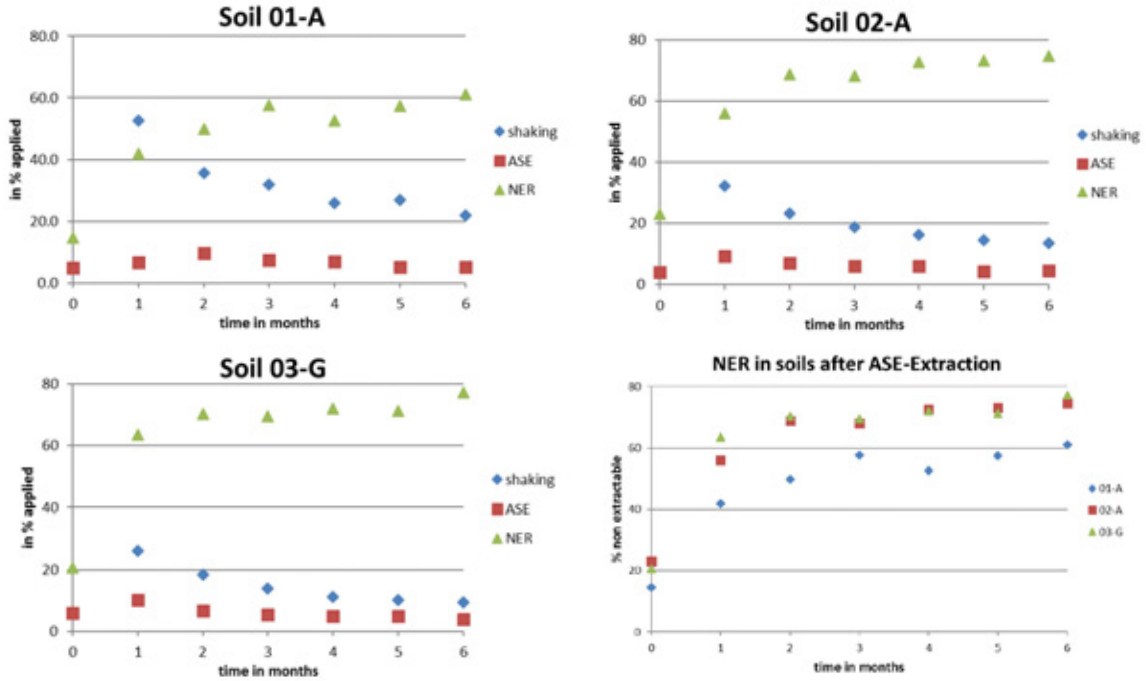


Figure 17 *Extractability of radioactivity in soil spiked with radiolabelled TNT*

4.1.2 Sequential extraction TNT

Table 21 *Presence of radioactivity in the different extracts of the sequential extraction (applied radioactivity 4095 kBq ^{14}C-TNT to 800 g soil dry matter corresponding to 120 mg TNT per kg soil dm)*

Soil	0.01 M $CaCl_2$	$Tenax_{20}$		$Tenax_{1\ week}$		Acetonitrile shaking	ASE	NER	Total recovery
		Water*	Tenax	water	Tenax				
Test start									
01-A	47.6%	4.6%	28.0%	1.5%	5.2%	2.3%	0.2%	2.8%	92.2%
02-A	40.3%	4.8%	36.9%	2.5%	3.8%	2.8%	0.6%	9.3%	101.0%
03-G	23.5%	4.9%	44.7%	3.5%	5.6%	3.6%	0.8%	13.4%	100.0%
T= 6 months									
01-A	6.1%	4.3%	9.3%	6.6%	5.4%	3.7%	n.d.	50.4%	85.8%
02-A	3.4%	3.5%	7.1%	3.7%	2.4%	6.2%		54.1%	80.4%
03-G	1.3%	2.4%	5.7%	2.7%	2.9%	5.8%		63.0%	83.8%

n.d. not determined; * see 3.5.3.2

TNT has a relatively high water solubility (approximately 100 mg/l (Joos et al., 2008)). For this reason it is important to know the concentration present in the water phase when trying to explain toxicity. The 0.01M $CaCl_2$ extraction and $Tenax_{20}$ extract show that directly after spiking most of the TNT is bioavailable (see ISO/TS 16751 (2017)) (Table 21). As described in chapter 1, 0.01M $CaCl_2$ extraction and $Tenax_{20}$ extraction are used to estimate bioavailability (see ISO 17402: 2008, Ortega-Calvo et al., 2015). The slowly available fraction ($Tenax_{1\ week}$) is small compared to that part. After the Tenax extractions, only a small fraction can still be extracted with acetonitrile. The mass balance is close to 100%.

After 6 months the amount in 0.01 M $CaCl_2$ and $Tenax_{20}$ had decreased dramatically whereas the slow desorbable fraction stayed almost constant – though at a low level. This makes sense if it represents the portion, which is only slowly available for degradation processes. The increase in the ACN extract is only moderate and without chemical analysis, it cannot be judged if it is due to TNT or a degradation product.

The NER levels did not reach the levels that were obtained in the mass balance testing, but is still above 50% for all soils. The mass balance dropped to around 80%, but this was not the focus of the experiment. Since mineralisation was not monitored in this experiment, one possible loss is volatilisation of $^{14}CO_2$. This was not found to be a very significant process in the mass balance approach but still contributed with up to 7.3%, which would lift the recovery over the 90% trigger for two soils.

4.2 Cypermethrin

4.2.1 Mass balance control

Most of the ^{14}C-labelled cypermethrin was extractable until the end of the 6 months incubation. Only in one sample, the extractable radioactivity dropped below 50%. ASE, to complete the extraction procedure, still extracted up to 20% of the applied radioactivity. Both extracts should be assessed as a sum for further consideration.

Mineralisation was, similar to the TNT experiment, not a significant fate pathway. Maximum mineralisation over 6 month was measured at soil 03-G with 4%. NER varied between 16% and 37%, which was a bit below expected values. Table 22 shows that longer incubation time might have led to a higher amount of NER. The almost perfect mass balance proves that no significant losses occurred during the experiment and all relevant pathways were recorded.

Table 22 *Recovered radioactivity and mass balance of ^{14}C-cypermethrin, initially applied radioactivity of 201 kBq per 20 g soil dry matter (= 100%) and 120 mg cypermethrin per kg soil dm*

Time [months]	Soil	Extractable ACN [%]	Extractable ASE [%]	$^{14}CO_2$ [%]	NER [%]	recovery [%]
0	01-A	87.8	10.4		1.6	99.8
	02-A	78.6	19.3		2.2	100.1
	03-G	74.6	21.6		4.1	100.3
1	01-A	84.2	10.1	0.6	6.0	100.9
	02-A	76.4	9.0	0.7	12.6	98.7
	03-G	69.5	21.3	0.7	14.6	106.1
2	01-A	83.3	10.0	0.4	8.1	101.8
	02-A	71.8	13.7	0.7	19.3	105.5
	03-G	62.2	18.5	0.2	23.4	104.3
3	01-A	78.8	10.4	0.4	11.6	101.2
	02-A	64.9	8.2	0.1	22.5	95.7
	03-G	52.9	16.8	1.7	29.8	101.2
4	01-A	80.8	9.9	0.3	12.1	103.1
	02-A	61.6	12.9	0.7	22.3	97.5
	03-G	58.9	17.1	0.2	26.3	102.5
5	01-A	76.5	9.5	1.8	15.7	103.5
	02-A	62.1	13.1	2.4	23.2	100.8
	03-G	53.8	16.7	3.2	31.5	105.2
6	01-A	78.2	9.1	1.9	16.5	105.7
	02-A	59.5	12.8	2.9	22.0	97.2
	03-G	47.5	15.1	4.0	37.6	104.2

The results from Table 22 are summarized in Figure 18. It shows that after about 3 months a more stable situation is obtained. However, the NER formation was smaller than expected. The soil with the highest organic carbon content also had the highest NER.

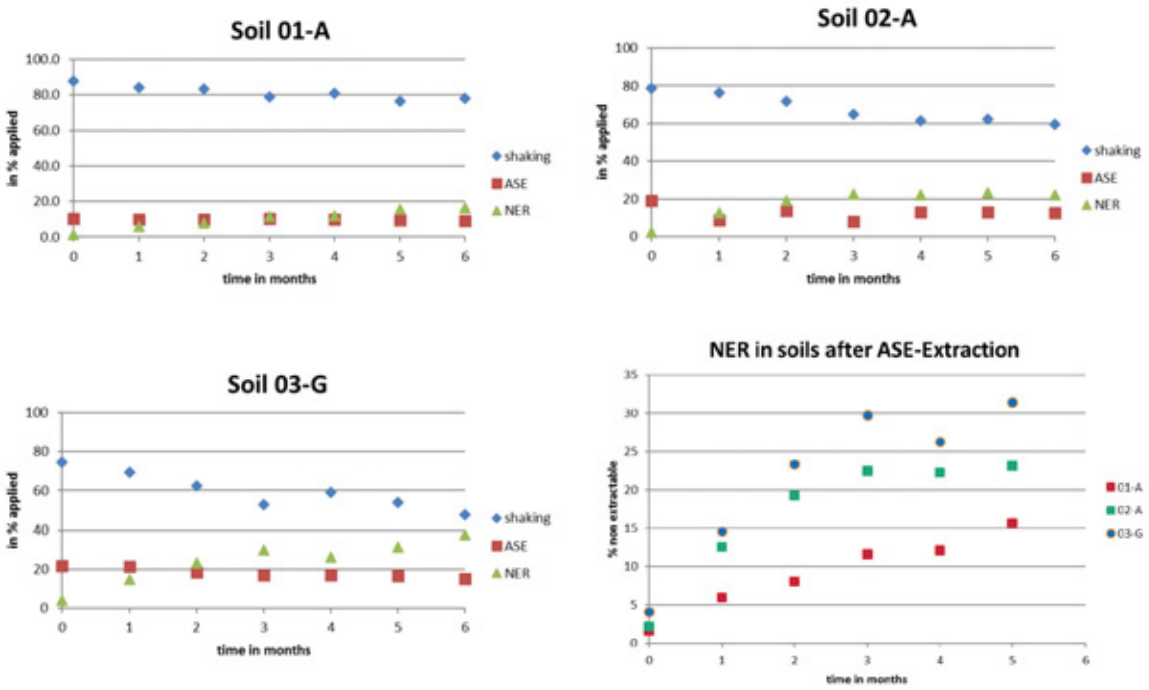


Figure 18 *Extractability of radioactivity in soil spiked with radiolabelled cypermethrin*

4.2.2 Sequential extraction cypermethrin

Table 23 *Presence of radioactivity in the different extracts of the sequential extraction (applied radioactivity 3329 kBq ^{14}C-cypermethrin to 800 g soil dm corresponding to 120 mg cypermethrin / kg soil)*

Soil	0.01 m $CaCl_2$	$Tenax_{20}$		$Tenax_{1\ week}$		Acetonitrile shaking	ASE	NER	Total recovery
		7	Tenax	water	Tenax				
Test start									
01-A	0.1%	0.3%	41.5%	0.4%	22.5%	17.6%	1.0%	0.3%	83.7
02-A	0.2%	0.3%	80.6%	0.1%	5.9%	4.1%	0.8%	0.3%	92.3
03-G	0.2%	0.5%	42.0%	0.3%	26.4%	21.3%	2.9%	0.5%	94.1
T= 6 months									
01-A	0.8%	0.0%	15.6%	0.7%	26.5%	30.7%	1.2%	1.4%	76.9%
02-A	0.6%	0.0%	16.5%	0.8%	34.0%	34.7%	2.7%	2.3%	91.6%
03-G	2.0%	0.4%	13.7%	0.5%	17.5%	31.8%	5.4%	6.4%	77.7%

The solubility of cypermethrin is very low (9 µg/L, IUPAC database), which makes the water phase less important for the mass balance. Although the concentration in the water phase was low, it was still considered one of the explaining parameters for toxicity. Surprisingly the concentration in the $Tenax_{20}$ extract was very high at test start, indicating that most of the cypermethrin applied would be available to organisms (Table 23). Even in the $Tenax_{1\ week}$, still considerable concentrations were measured. This demonstrates the good performance of the Tenax extraction which shows orders of magnitude more cypermethrin available compared to the 0.01M $CaCl_2$ extraction. Almost no NER were generated immediately after spiking.

After 6 months, the amount of radioactivity extracted in $Tenax_{20}$ had decreased dramatically as already shown for TNT. At the same time, the slowly available ($Tenax_{1\ week}$) and the non-available (Acetonitrile) amount had increased, and significant differences were observed between the soils. In soil 01-A these fractions almost doubled in sum, in soil 02-A the increase is by a factor of seven and in soil 03-G the amount stays more or less constant with a shift to the non-available part.

The most dramatic difference to the mass balance experiments were the significantly lower amounts of NER detected. NER in this experiment were lower by a factor of 5 to 10 after 6 months compared to the mass balance experiment. Further, the measured data do not match with data for cypermethrin found in the literature survey. Experimental errors of this significance can be excluded as the NER determination is one of the most robust measurements in the entire project and the mass balance – at least for soil 02-A – was above 90%. Therefore, the difference between NER results shown in Table 22 and Table 23 cannot be explained. The focus of the experimental work was not on the mass balance and the shift of the test chemical from the available fraction to the less available fraction was shown pretty good by the experiment. Nevertheless, the very limited NER formation was subject of concern for the project targets. Thus, an additional experiment was conducted with cypermethrin at much lower starting concentration, see 4.2.3.

4.2.3 Mass balance control, additional experiments

Even in the mass balance experiments, the observed NER formation was below the expectations found in the literature survey (see Table 3) Fate data for cypermethrin in soils from literature were always determined in approaches with two order of magnitudes lower cypermethrin starting concentrations in the soils. So there was concern whether the high starting concentration might have influenced the fate, e.g. by toxic effects or saturation of sorption sites in the soil. It was decided to perform additional experiments with a low starting concentration of 5 mg/kg instead of 120 mg/kg. Initially, it was intended to start with1 mg/kg, but with the specific radioactivity adjusted for the previous experiment the radio-analytical sensitivity would have been too low.

Table 24 *Recovered radioactivity and mass balance of ^{14}C-cypermethrin, initially applied radioactivity of 15.6 kBq per 20 g soil dry matter (= 100%) and 5 mg cypermethrin per kg soil dm*

Time [months]	Soil	Extractable ACN [%]	Extractable ASE [%]	$^{14}CO_2$ [%]	NER [%]	recovery [%]
0	01-A	91.3	12.5		0.0	103.8
	02-A	85.4	18.9		0.0	104.3
	03-G	80.8	25.9		11.0	117.7
2	01-A	67.2	27.3	9.9	11.7	116.1
	02-A	23.4	5.8	16.9	28.2	74.3
	03-G	38.0	9.7	20.1	26.4	94.2
4	01-A	49.8	20.4	19.0	14.9	104.1
	02-A	9.5	2.9	44.8	36.3	93.5
	03-G	19.2	6.2	56.4	30.5	112.3
6	01-A	43.8	6.7	24.8	16.0	91.3
	02-A	7.0	2.0	49.7	34.0	92.7
	03-G	12.9	4.4	65.2	31.7	114.2

Interestingly, results differing from previously obtained results were obtained (Table 24). In contrast to the mass balance experiments using 120 mg/kg significant mineralisation was observed when using 5 mg/kg. For soils 2 and 3, this became by far the most relevant pathway. The NER stayed more or less the same after 6 months and the order among the soils could be confirmed (soil 1 just half the NER of the others). The mass balance showed a bit more variation and one outlier (02-A after 2 months) was observed, but generally ranged around 100%.

Thus, in three experiments with cypermethrin three very different results were obtained. The first experiment showed moderate amounts of NER, no mineralisation and a perfect mass balance. In the second experiment almost no NER were found, mineralisation was not determined as it was not of interest and consequently the mass balance was not at 100% but still acceptable. In the third experiment, a very high mineralisation rate was found accompanied by a moderate NER formation and again acceptable mass balances. An easy explanation for these results cannot be presented. However, this was not focus of the experiments though it is unfortunate that in particular in the second experiment, where the focus was on NER formation, almost no NER were formed.

4.3 Carbendazim

4.3.1 Mass balance control

Table 25 *Recovered radioactivity and mass balance of ^{14}C-carbendazim, initially applied radioactivity of 200 kBq per 20 g soil dry matter (= 100%) and 120 mg carbendazim per kg soil dm*

Time [months]	Soil	Extractable MeOH [%]	Extractable ASE [%]	$^{14}CO_2$ [%]	NER [%]	recovery [%]
0	01-A	100.7	1.1		5.0	106.8
	02-A	102.1	2.3		8.8	113.2
	03-G	101.1	4.1		13.4	118.6
1	01-A	76.7	10.8	0.3	10.1	97.9
	02-A	77.1	5.4	0.2	15.1	97.8
	03-G	62.4	6.9	1.6	26.5	97.4
2	01-A	64.3	8.3	0.4	22.1	94.2
	02-A	35.5	3.1	7.4	36.4	82.4
	03-G	36.3	6.3	6.3	39.0	87.9
3	01-A	7.8	1.8	7.2	30.9	47.7
	02-A	2.5	1.5	20.9	36.1	61.0
	03-G	25.6	5.8	7.4	42.7	81.5
4	01-A	1.9	1.5	8.5	26.5	38.4
	02-A	1.0	0.9	22.8	31.2	55.9
	03-G	11.3	4.0	7.7	39.4	62.4
5	01-A	1.6	1.4	8.7	25.0	36.7
	02-A	0.8	0.7	23.3	29.8	54.6
	03-G	17.8	4.9	7.7	50.2	80.6
6	01-A	1.3	1.1	9.0	26.5	37.9
	02-A	0.8	0.6	23.4	31.5	56.3
	03-G	8.2	3.2	7.8	56.1	75.3

Table 25 shows the results of the mass balance experiments with carbendazim. In contrast to the two other substances, the mass balance dropped significantly after 3 months of incubation. The massive losses in all soils can be explained only by volatilization of a degradation product other than $^{14}CO_2$. As the test system was not set up to trap volatiles other than $^{14}CO_2$, this could not be monitored. However, comparing to data from our Literature survey (see Table 3). This is a very surprising result as no volatile metabolites are described anywhere.

The process of NER formation was finished after 3 months for soils 01-A and 02-A and the level of the extractable portion had also stabilised after this time. In soil 3, processes were apparently a bit slower. Also NER formation continued until the end of the experiment though finally after 6 months the extractable portion also dropped below 10%.

Chemical analysis showed later that the drop of the recovery and the end of the NER formation process goes along with almost complete disappearance of the parent carbendazim in the extracts (see Table 36, Table 37 and Table 38). The differences observed in the soils were observed in the chemical analysis of the soil extracts as well.

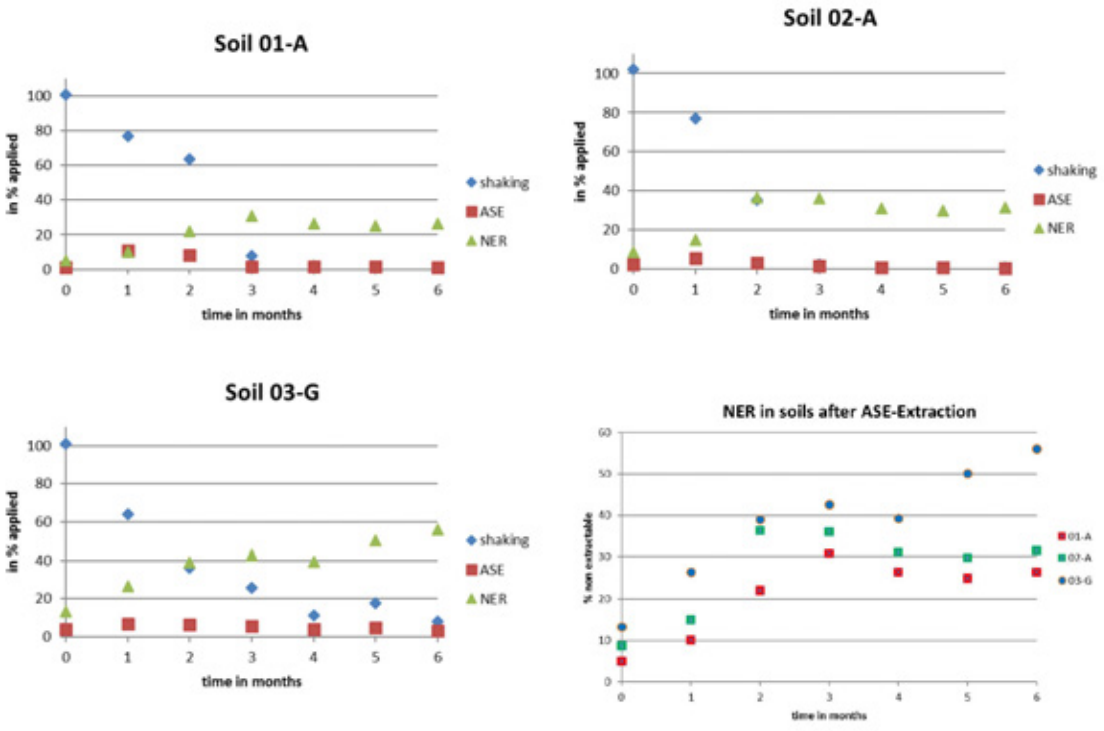


Figure 19 *Extractability of radioactivity in soil spiked with radiolabelled carbendazim*

4.3.2 Sequential extraction carbendazim

Carbendazim has a moderate water solubility (8.0 mg/L IUPAC database) and thus at 0d the major part was found to be extracted by 0.01M $CaCl_2$ and $Tenax_{20}$ extractions. The mass balance after spiking was still good, which demonstrates that the analytical procedures applied, worked well with parent carbendazim (Table 26).

Table 26 *Presence of radioactivity in the different extracts of the sequential extraction (applied radioactivity 3813 kBq 14C-carbendazim to 800 g soil dm corresponding to 120 mg carbendazim / kg soil)*

Soil	0.01 M	$Tenax_{20}$		$Tenax_{1\ week}$		Methanol	ASE	NER	Total
	$CaCl_2$	water	Tenax	water	Tenax	shaking			recovery
Test start									
01-A	19.2%	5.5%	45.3%	0.3%	8.7%	0.9%	0.3%	0.6%	80.8%
02-A	17.3%	4.7%	71.7%	0.5%	2.3%	0.5%	0.2%	0.7%	97.9%
03-G	10.8%	12.2%	69.4%	0.2%	6.3%	1.3%	0.8%	1.3%	102.3%
T= 6 months									
01-A	0.4%	0.7%	0.2%	0.8%	0.2%	0.4%	0.5%	4.2%	7.4%
02-A	0.2%	0.3%	0.1%	0.5%	0.1%	0.5%	0.7%	12.2%	14.6%
03-G	0.9%	0.9%	0.1%	1.0%	0.5%	0.5%	1.9%	30.3%	36.1%

After 6 months, the picture changed completely. There was almost no extractable radioactivity at all present in the soils, neither with 0.01M $CaCl_2$ and Tenax, nor with organic solvent. Some radioactivity was detected as NER but only soil 3 still contained NER above 15%. Generally, the picture was similar to the mass balance experiment, but somewhat more extreme. The recovery of radioactivity decreased even more and less NER were determined, though the order of soils is the same in both experiments. The results indicate a poor mass balance, which may suggest the occurrence of a volatile transformation product. The size difference of the systems (mass balance experiment used 20 g soil, while the sequential extraction experiment used 800 g soil) may have caused differences in

microbiology, which may explain the higher level of degradation compared to the mass balance experiment.

The data demonstrate once again that one has to be very careful to conclude on NER formation while working with non-labelled test substances. In this particular case, it would have been concluded that almost 98% should be present as NER in each soil after 6 months incubation time. Volatilization has not previously been reported for this compound and volatilization of transformation products has not been reported either in the reviewed literature. CO_2, which could be collected theoretically also in unlabelled experiments wouldn´t have provided any further indication. Only the radioactive mass balance can show that most of the radioactivity left the system for some reason. That is an important result for further research.

4.4 Chemical analysis of extracts

4.4.1 2,4,6-TNT

For the TLC analysis, the organic extracts were pooled with the ASE-extracts in order to keep the effort as low as possible. This applies to the mass balance experiments as well as to the sequential extraction experiments. The $Tenax_{20}$ extracts and the $Tenax_{1\ week}$ extracts were analysed separately to detect potential differences in the nature of the fast and slow desorbable radioactivity.

As shown already in Table 20 and Figure 17, the extractable radioactivity decreased rapidly in the mass balance experiment. Table 27 shows in addition the amount of parent TNT present in the extracts. Chemical analysis showed that, in the first month, the extractable radioactivity was predominantly due to parent TNT. This changed in the 6 months sampling, where only about 50% of the extractable radioactivity was due to parent TNT in all soils. This indicates the ongoing transformation processes which finally result in the formation of NER.

Table 27 *Results of extract analysis from the mass balance experiment for TNT*

Sample	soil 01-A total extractable radioactivity in % AR	% parent in extract	soil 02-A total extractable radioactivity in % AR	% parent in extract	soil 03-G total extractable radioactivity in % AR	% parent in extract
0d	86.5	95.1	91.2	98.4	85.5	95.4
1M	52.7	84.0	32.4	74.0	26.1	69.8
2M	35.6	78.0	23.2	68.1	18.5	64.0
3M	31.9	72.3	18.9	61.1	14.0	58.9
4M	25.9	69.4	16.3	58.5	11.2	56.5
5M	27.0	65.6	14.6	55.3	10.1	51.6
6M	22.0	62.6	13.5	53.5	9.5	47.6

%AR = percent of the initially applied radioactivity

In the sequential extraction experiment, on the one hand two different Tenax extracts were analysed and on the other hand two different sampling points can be compared. Results are summarised in the Tables 28, 29 and 30. At test start, a significant difference can be observed between $Tenax_{20}$ and $Tenax_{1\ week}$. In the $Tenax_{20}$ close to 100% of the radioactivity is due to unchanged TNT whereas in the $Tenax_{1\ week}$ only about 2/3 of the extracted radioactivity is due to TNT. This finding already indicates the good degradability of TNT. At test end this effect cannot be observed any more as most of the parent TNT is transformed already and only around 50% of the very little remaining extractable radioactivity are due to TNT.

Table 28 *Amount of parent TNT in Tenax extracts at test start t = 0*

soil	extract	[%AR] extractable	[%Area] Parent	[%AR] Parent
01-A	$Tenax_{20}$	28.0	90.1	25.2
02-A	$Tenax_{20}$	36.9	96.0	35.4
03-G	$Tenax_{20}$	44.7	100.0	44.7
01-A	$Tenax_{1\ week}$	5.2	64.2	3.3
02-A	$Tenax_{1\ week}$	3.8	71.3	2.7
03-G	$Tenax_{1\ week}$	5.6	66.4	3.7

%AR = percent of the initially applied radioactivity

Table 29 *Amount of parent TNT in Tenax extracts at test end t = 6 months*

soil	extract	[%AR] extractable	[%Area] Parent	[%AR] Parent
01-A	$Tenax_{20}$	9.3	76.0	7.1
02-A	$Tenax_{20}$	7.1	40.6	2.9
03-G	$Tenax_{20}$	5.7	56.5	3.2
01-A	$Tenax_{1\ week}$	5.4	55.1	3.0
02-A	$Tenax_{1\ week}$	2.4	35.8	0.9
03-G	$Tenax_{1\ week}$	2.9	39.3	1.1

%AR = percent of the initially applied radioactivity

Table 30 *Amount of parent TNT in organic extracts at test start and test end*

soil	Organic extract	[%AR] extractable	[%Area] Parent	[%AR] Parent
01-A	Test start	7.5	34.7	2.6
02-A	Test start	6.6	29.6	2.0
03-G	Test start	9.2	32.1	3.0
01-A	Test end	3.7	40.8	1.5
02-A	Test end	6.2	45.7	2.8
03-G	Test end	5.8	23.8	1.4

%AR = percent of the initially applied radioactivity

In the final organic extract, which represents the very slow desorbing fraction, only about 30% to 40% are due to unchanged parent. As a summary, it can be concluded that for degradable ^{14}C-labelled chemicals, the radio-analysis is just the first step. For further assessment, specific chemical analysis is necessary to make sure whether the recovered radioactivity is still due to the parent test chemical or represent predominantly degradation products, which might have completely different properties compared to the parent test chemical.

4.4.2 Cypermethrin

Table 31 *Results of extract analysis from the mass balance experiment for cypermethrin (120 mg/kg)*

Sample	soil 01-A total extractable radioactivity in % AR	% parent in extract	soil 02-A total extractable radioactivity in % AR	% parent in extract	soil 03-G total extractable radioactivity in % AR	% parent in extract
0d	87.8	100.0	78.6	100.0	74.6	100.0
1M	84.2	100.0	76.4	100.0	69.5	100.0
2M	83.3	100.0	71.8	100.0	62.2	100.0
3M	78.8	100.0	64.9	100.0	52.9	100.0
4M	80.8	96.1	61.9	100.0	58.9	98.8
5M	76.5	96.0	62.1	100.0	53.8	100.0
6M	78.2	94.4	59.5	100.0	47.5	100.0

%AR = percent of the initially applied radioactivity

Table 31 shows that the extractable radioactivity in the cypermethrin mass balance experiment, decreases slowly over time. The chemical analysis showed that the extractable part was more or less 100% unchanged parent cypermethrin in all soils until the end of incubation (Table 32, 33 and 34). In contrast to TNT, cypermethrin degraded only slowly in the mass balance experiment. Thus, no rapid decrease of the extractable portion occurred and as a result, the very good mass balance was observed. The extraction procedure, which was developed for the parent test chemicals only, consequently worked well until the test end at 6 months incubation time.

Table 32 *Amount of parent cypermethrin in Tenax extracts at test start t = 0*

soil	extract	[%AR] extractable	[%Area] Parent	[%AR] Parent
01-A	$Tenax_{20}$	41.5	96.5	40.0
02-A	$Tenax_{20}$	80.6	98.3	79.2
03-G	$Tenax_{20}$	42	98.2	41.3
01-A	$Tenax_{1\ week}$	22.5	95.8	21.5
02-A	$Tenax_{1\ week}$	5.9	94.4	5.6
03-G	$Tenax_{1\ week}$	26.4	96.2	25.4

%AR = percent of the initially applied radioactivity

Table 33 *Amount of parent cypermethrin in Tenax extracts at test end t = 6 months*

soil	extract	[%AR] extractable	[%Area] Parent	[%AR] Parent
01-A	$Tenax_{20}$	15.6	92.2	14.4
02-A	$Tenax_{20}$	16.5	97.1	16.0
03-G	$Tenax_{20}$	13.7	93.3	12.8
01-A	$Tenax_{1\ week}$	26.5	98.4	26.1
02-A	$Tenax_{1\ week}$	34	94.8	32.2
03-G	$Tenax_{1\ week}$	17.5	93.6	16.4

%AR = percent of the initially applied radioactivity

Table 34 *Amount of parent cypermethrin in organic extracts at test start and test end*

soil	Organic extract*	[%AR] extractable	[%Area] Parent	[%AR] Parent
01-A	Test start	18.6	98.3	18.3
02-A	Test start	4.9	99.1	4.9
03-G	Test start	24.2	100.0	24.2
01-A	Test end	31.9	99.0	31.6
02-A	Test end	37.4	100.0	37.4
03-G	Test end	37.2	100.0	37.2

%AR = percent of the initially applied radioactivity

* sum of columns "Acetonitril shaking" and "ASE" from Table 23

Chemical analysis of the extracts from the sequential extraction experiment, gave the same results as from the mass balance experiments. Almost 100% of the extractable radioactivity in any extract was due to unchanged cypermethrin. A very low decrease can be observed in the 6 months Tenax extracts with up to 8% degradation products in the extracts. This indicates that the degradation products were more water soluble than parent cypermethrin and consequently are stripped out already in the more aqueous Tenax extraction procedure.

4.4.3 Carbendazim

In section 4.3.1 results from the mass balance experiment, indicated significant differences between the test soils. Chemical analysis of the extracts as shown in Table 35 confirms this result.

Table 35 *Results of extract analysis from the mass balance experiment for carbendazim*

Sample	soil 01-A total extractable radioactivity in % AR	% parent in extract	soil 02-A total extractable radioactivity in % AR	% parent in extract	soil 03-G total extractable radioactivity in % AR	% parent in extract
0d	100.7	89.9	102.1	99.4	101.1	99.4
2M	63.4	85.8	35.5	97.7	36.3	91.7
3M	7.8	49.9	2.5	69.8	25.6	87.7
4M	1.9	21.2	1	59.2	11.9	82.4
5M	1.6	19.4	0.8	20.8	17.8	82.9
6M	1.3	16.1	0.8	20.2	8.2	77.7

%AR = percent of the initially applied radioactivity, 1M samples lost

Results are very much in line with the result shown in Figure 19. In the first two months, the extractable radioactivity is mostly due to unchanged parent carbendazim in all soils. At later points in time the picture changes:

- In soil 01-A at 3 months, just 50% of the extractable portion is unchanged parent compound and the extractable portion itself drops to 7.8%. From 4 months, less than 20% of the extractable portion is still parent compound or, expressed as %AR, far less than 1% parent can be recovered.
- In soil 02-A the extractable portion drops even faster. Even if at 4 months still 60% of the extractable portion is unchanged parent carbendazim, this means only about 1% AR. This is similar to soil 01-A. In the following samplings, the amount of unchanged parent decreased to 20% which was also similar to soil 01-A.
- In soil 03-G, processes are slower. The decrease of the extractable portion slows down after 3 months and by the test end at 6 months, up to 80% of the extracted radioactivity is due to unchanged parent carbendazim.

The different transformation speeds also reflect the drop of the mass balance as shown in Figure 19. The more parent is found in the extract, the better is the mass balance. This strongly indicates that the degradation of carbendazim leads to a product, which escapes from the system.

Chemical analysis of the extracts of the sequential extraction experiment with carbendazim are interesting, even if the extractable amounts were only significant at test start (Table 37, 37 and 38). At test start, both Tenax fractions represented more than 90% unchanged parent carbendazim. At test end, even though almost no radioactivity was extractable any more, $Tenax_{20}$ still contained mostly unchanged parent but the $Tenax_{1\ week}$, predominantly contained degradation products. Similar results were obtained for the exhaustive organic solvent extraction. Though extractable amounts were very low, differences in the composition at test start and test end could be determined. At test end, the amount of non-parent radioactivity increased significantly compared to test start. All those results indicate considerable degradation processes which finally was proven by the mass balance.

Table 36 *Amount of parent carbendazim in Tenax extracts at test start t = 0*

soil	extract	[%AR] extractable	[%Area] Parent	[%AR] Parent
01-A	$Tenax_{20}$	45.3	96.2	43.6
02-A	$Tenax_{20}$	71.7	97.3	69.7
03-G	$Tenax_{20}$	69.4	96.9	67.3
01-A	$Tenax_{1\ week}$	8.7	95.6	8.3
02-A	$Tenax_{1\ week}$	2.3	89.1	2.0
03-G	$Tenax_{1\ week}$	6.3	94.6	6.0

%AR = percent of the initially applied radioactivity

Table 37 *Amount of parent carbendazim in Tenax extracts at test end t = 6 months*

soil	extract	[%AR] extractable	[%Area] Parent	[%AR] Parent
01-A	$Tenax_{20}$	0.2	65.9	0.1
02-A	$Tenax_{20}$	0.1	98.3	0.1
03-G	$Tenax_{20}$	0.1	98.5	0.1
01-A	$Tenax_{1\ week}$	0.2	33.0	0.1
02-A	$Tenax_{1\ week}$	0.1	25.9	0.0
03-G	$Tenax_{1\ week}$	0.5	16.8	0.1

%AR = percent of the initially applied radioactivity

Table 38 *Amount of parent carbendazim in organic extracts at test start and test end*

soil	Organic extract	[%AR] extractable	[%Area] Parent	[%AR] Parent
01-A	Test start	1.2	94.1	1.1
02-A	Test start	0.7	94.0	0.7
03-G	Test start	2.1	93.1	2.0
01-A	Test end	0.9	59.3	0.5
02-A	Test end	1.2	44.2	0.5
03-G	Test end	2.4	68.5	1.6

%AR = percent of the initially applied radioactivity

4.5 Ecotoxicity

4.5.1 General remarks

Two tests using the test soil (potential ammonium oxidation of the soil microflora; avoidance of earthworms) and two tests using aqueous extracts (assay with luminescence of the bacterium *Vibrio fischeri* for TNT and the acute daphnia tests for cypermethrin and carbendazim) had been selected. The tests were performed at test start immediately after spiking of the soil and at test end. The aquatic tests were performed in the assays with radioactive labelled compound. Due to the large amount of soil required for the test with earthworms, the test was performed with soil of a set-up using non-radiolabelled compound (see chapter 2.4). The test with soil microflora was performed with radiolabelled and non-radiolabelled test compound. The principle of the test with soil microflora differs from the principles of the assays with earthworm, luminescent bacteria and daphnia. In the latter three tests, the test organisms are added to the soil or aqueous extract at the individual times of measurement. In the soil microflora test, the activity of the natural community is determined and no fresh microorganisms are added. With this test, additional information on the recovery of the natural soil microflora is obtained. During the experiments, we observed peculiarities, which resulted in modifications of the original planned procedures. These specific observations are described in the following chapter. Chapter 4.5.3 focusses on the results, which are suitable to explain the bioavailability and NER formation of TNT, cypermethrin and carbendazim.

4.5.2 Suitability of selected test procedures regarding bioavailability

4.5.2.1 Microbial ammonium oxidation activity

We started with the testing of TNT and observed a significant inhibition of the microorganisms which transform ammonium to nitrite (= potential ammonium oxidation). At test end in the treated samples there was no activity at all or only a very low activity. The microbial activities in the control samples and treated samples at test start and test end were comparable. We assume that the added TNT concentration resulted in a complete or nearly complete inhibition of the microbial group, which was responsible for the transformation. As the microorganisms responsible for the transformation from ammonium to nitrite are a relatively homogenous group, with only a few different species able to perform the transformation, the capability of recovery is small. If there were no resistant microorganisms left or only a very low number of microbial cells, no recovery would have been

possible. To prove this hypothesis, an additional experiment was performed in the scope of the set-up with soil 03-G by mixing the incubated soil with fresh soil (Table 39). The fresh soil had a high microbial activity compared to the incubated control soil. The addition of this soil to the incubated control soil and spiked soil increased the activity of the incubated soils significantly. In the mixtures with 50% fresh soil, the measured and calculated activities were comparable. In the mixtures with 25% fresh soil, the measured activities even exceeded the calculated values. If there were significant amounts of bioavailable contaminants, the measured values should have been lower, as the microorganisms in the fresh soil should have been inhibited. These findings support the hypothesis that limited microbial recovery due to limited microbial resources is the reason for the low microbial activity in the spiked and incubated soil. Bioavailable TNT as the reason for the low microbial activity is therefore excluded. The results of the microbial activity in the experiments with TNT were therefore not used for the interpretation of the results on bioavailability and formation of NER.

For cypermethrin and carbendazim, the assay on potential ammonium oxidation failed entirely. The microbial activity was variable, irrespective of approach and the results were not repeatable. Each time the test was repeated different results regarding the inhibition of the test substance, were obtained. In one experiment, only the cold substance affected the microorganisms, in the repeat experiment it was only the radioactive substance that exhibited inhibition. Due to the limited amount of soil, we had to stop the tests on microbial activity and focus on the terrestrial test with earthworms. The observed difficulties with the test on potential ammonium oxidation activity are mentioned in the following chapter.

Table 39 *Control experiment with soil mixtures*

	NO_2-N [ng/g dm/h] [1]	Expected activity [2] [ng/g dm/h]
Control soil of experiment (test end)	196 ± 10	---
100% soil treated with TNT (test end)	47 ± 12	---
Fresh soil	822 ± 73	---
50% control soil + 50% fresh soil	538 ± 31	509
50% treated soil + 50% fresh soil	433 ± 39	435
75% control soil + 25% fresh soil	409 ± 39	353
75% treated soil + 25% fresh soil	332 ± 44	241

[1] Mean value ± standard deviation; [2] activity calculated based on the measured values of the control soil of the experiment, of the 100% soil treated with TNT and of the fresh soil.

4.5.2.2 Suitability on testing soil after Tenax extraction

To prove that NER in soil have no inhibitory effects, the soil after Tenax extraction should be tested. The structure of the soil after Tenax extraction differed significantly from the structure before the extraction. It was hard and clumpy and was difficult to handle. The wet soil had to be dried before the ecotoxicological testing. Drying was performed careful to avoid the damage of the soil microflora. The soil was turned periodically to guarantee homogenous drying. Nevertheless, the resulting soil was difficult to handle. In the test on potential ammonium oxidation (Table 40) 100% control soil and 100% TNT-contaminated soils were tested. The results with the soil after Tenax-extraction were not satisfying. The relationship between the three control soils and the corresponding TNT-treated soils after Tenax-extraction differed as well as the determined microbial activity at test end and at test start after Tenax-extraction. At test start in all three soils (01-A, 02-A, 03G) the microbial activity after Tenax extraction was much higher than in the pure soil and the inhibition by TNT was lower compared to the soil before the extraction. At test end the results of the three soils differed. In soil 01-A, almost no activity could be measured after Tenax extraction in the control soil as well as in the spiked soil. Therefore, no information on bioavailability of TNT was possible. In soil 02-A the microbial activity in the treated soil before and after Tenax extraction was similar and there was still a strong inhibition of the activity in the treated soil compared to the control soil. For soil 03-A the inhibition after Tenax extraction was 25% higher compared to the activity in the soil before the extraction. There were no logical explanations for these observations. Due to the inconsistencies regarding microbial activity and due to the modification of the soil structure, which is assumed, to modify the microbial activity (e.g.

lower soil pores for oxygen and therefore reduced aerobic microbial activity), no further experiments such as addition of fresh soil were performed.

Therefore, the approach failed to directly demonstrate that NER formation resulted in no ecotoxicity and that NER are no longer bioavailable.

Table 40 *Activity of ammonium oxidizing bacteria*

	Control	TNT treated soil	Inhibition to control [%]	Control	TNT treated soil	Inhibition to control [%]
	Pure soil			After Tenax extraction		
01-A – test start	26.0 ± 7.9 ng/gTM/h	18.6 ± 7.8 ng/gTM/h	29	100.8 ± 4.6 ng/gTM/h	97.0 ± 11.3 ng/gTM/h	4
01-A – test end	23.0 ± 1.9 ng/gTM/h	2.7 ± 1.0	88	3.91 ± 0.99 ng/g TM/h (nearly no activity)	0.51 ± 1.02 ng/g TM/h	87
02-A – test start	58.7 ± 9.6 ng/gTM/h	3.4 ± 2.4 ng/gTM/h	94	134.5 ± 13.2 ng/gTM/h	73.7 ± 4.1 ng/gTM/h	45
02-A – test end	165.8 ± 4.3 ng/gTM/h	49.7 ± 5.9 ng/gTM/h	70	103.6 ± 2.2 ng/gTM/h	31.9 ± 11.6 ng/gTM/h	69
03-G – test start	76.4 ± 17.6 ng/gTM/h	9.3 ± 10.6 ng/gTM/h	-14	154.5 ± 13.5 ng/gTM/h	176.3 ± 10.2 ng/gTM/h	-14
03-G – test end	365.1 ± 9.6 ng/gTM/h	180.3 ± 27.2 ng/gTM/h	51	196.4 ± 10.3 ng/gTM/h	47.4 ± 12.5 ng/gTM/h	76

Conclusions regarding bioavailability based on aquatic tests and earthworm test using Tenax extracted soil

As mentioned in the previous paragraph, testing of Tenax extracted soil was not possible. Nevertheless, we think that conclusions regarding NER and bioavailability can be drawn based on the results of the aquatic tests and the earthworm tests. This is shown for TNT. Toxicity at test start in the aquatic test (Luminescent bacteria) and in the earthworm test (results see Annex 2) shows that TNT was bioavailable and toxic for the test organisms. At test start no NER were present. At the test end NER in the soil were formed (Table 21) and no effect in the earthworm test and no toxicity in 0.01M $CaCl_2$ eluate (Luminescent bacteria assay) was observed. This demonstrated that the amount of dissolved toxic contaminants (TNT) was too low to cause ecotoxicity and that the NER formed from TNT were not bioavailable (Figure 20).

Suitability of testing the test substance removed by $Tenax_{20}$

We tested the amount of TNT removed by $Tenax_{20}$ after solubilisation in organic solvent and transfer to water with the Luminescent bacteria assay. We observed a comparable and high inhibition in the control as well as in the spiked sample and assume that the inhibition results from small amounts of solvent still present in the aqueous sample. This confirms that use of solvents has to be avoided in tests were chemical measures are combined with ecotoxicity tests. Therefore, this approach of testing was deleted from the test programme.

4.5.3 Ecotoxicity of TNT, Cypermethrin and Carbendazim regarding bioavailability

Table 41 to Table 43 give an overview of the results (presented as percent inhibition). The original data are given in Annex 2. In the tables in Annex 2, results of further determinations are presented (e.g. test with aqueous extract of Tenax extracted soil; soil microflora in soil after Tenax extraction). As these results do not provide additional information within the scope of the project these results are not presented in the summary table.

Table 41 *Effect of TNT on earthworms and luminescence bacteria (results presented as percent inhibition compared to the control)*

Soil	Earthworm avoidance test: inhibition [%] by highest test concentration (100% soil)		Luminescent bacteria: Luminescence inhibition [%] at 33% soil eluate (0.01M $CaCl_2$)	
	Test start	Test end	Test start	Test end
01-A	100	0	81	0
02-A	100	0	71	0
03-G	100	16	57	0

Table 42 *Effect of Cypermethrin on earthworms and daphnids*

Soil	Earthworm avoidance test: inhibition [%] by highest test concentration (100% soil)		Daphnids: immobilization [%] in 25% soil eluate (0.01M $CaCl_2$)	
	Test start	Test end	Test start	Test end
01-A	94	72	60	90
02-A	84	60	15	50
03-G	92	96	80	90

Table 43 *Effect of Carbendazim on earthworms and daphnids*

Soil	Earthworm avoidance test: inhibition [%] by highest test concentration (100% soil)		Daphnids: immobilization [%] in 25% soil eluate (0.01M $CaCl_2$)	
	Test start	Test end	Test start	Test end
01-A	20	8	100	0
02-A	4	0	100	5
03-G	28	4	100	5

The results regarding bioavailability of the three test substances and the various suitable test organisms can be summarized as follows:

- For TNT (Table 41) the tests with luminescent bacteria and earthworms proved to be suitable. Based on these tests, there is a clear reduction of toxicity during the incubation.
- For cypermethrin (Table 42), a high toxicity was still determined at test end. This can be explained by the high ecotoxicity and low water solubility (9 µg/L, IUPAC database) of this substance. An EC_{50} of 0.3 µg/L is reported (Yordanova et al., 2009; BASF, 2008) in the 48 h daphnia immobilization test which corresponds to 10^{-4}% of the nominal application concentration. Due to the low water solubility, even low remaining concentrations of this substance exceed the water solubility and a reduction of the available amount is not determinable.
- Only small or low inhibition of the earthworms was observed for carbendazim (Table 43) at test start and test end. Although carbendazim is the recommended reference substance in the earthworm reproduction test and therefore, the earthworms were considered suitable test organisms. Additionally, avoidance behaviour is reported for this chemical. At a concentration of 10 mg/kg, about 75% to 100% avoidance was observed (Chelinho et al., 2014). In our experiments, the avoidance behaviour was lower. Nevertheless, a difference between test start and test end was detected. The missing toxicity at test end can be explained by a degradation/NER formation of the toxic metabolite (Figure 22).
  There was a high toxicity for daphnids at test start and a much lower at test end.
  The concentration of bioavailable contaminants detected by daphnids decreased during the incubation period. This can be due to degradation, mineralization or lower bioavailability (formation of NER) (Figure 22).

5 Combination of chemical (radiolabelled) analyses and ecotoxicological results

5.1 Introduction

According to Figure 4 (approach of Ortega-Calvo et al., (2015)) both sides of the cell membrane of a soil living organism have to be considered.

To explain toxicity from the soil matrix, the bioavailability measured using the water phase (0.01M $CaCl_2$ extract) and/or the bioavailable fraction ($Tenax_{20}$) are used. As stated in ISO 17402 and Ortega Calvo et al., the 0.01M $CaCl_2$ extract represents the actual availability and $Tenax_{20}$ the potential availability. In the experiments presented in this chapter, we applied a sequential extraction (see chapter 2.3) and the actual available amount of the test chemical determined by 0.01M $CaCl_2$ extraction is given in percentage of the initially applied test substance. In the residual soil, we measured the potential bioavailable amount by $Tenax_{20}$ (in percentage of initially applied test substance). The residue is the amount left after $Tenax_{20}$ and extracted using $Tenax_{1\ week}$ and the organic solvent. This is not entirely in line with the definition given in chapter 2.3, but the $Tenax_{1\ week}$ attempts to represent a slow desorbing fraction (Cornelissen et al., 1997; Harmsen 2004) which should not be relevant for the acute ecotox-tests where the test duration is far below 1 week. NER represent the non-extractable amount, which is left after all extractions and can be determined with ^{14}C-label and combustion analysis only.

5.2 TNT

The results of the radiolabel analyses and the respective ecotox tests for TNT are given in Figure 20.

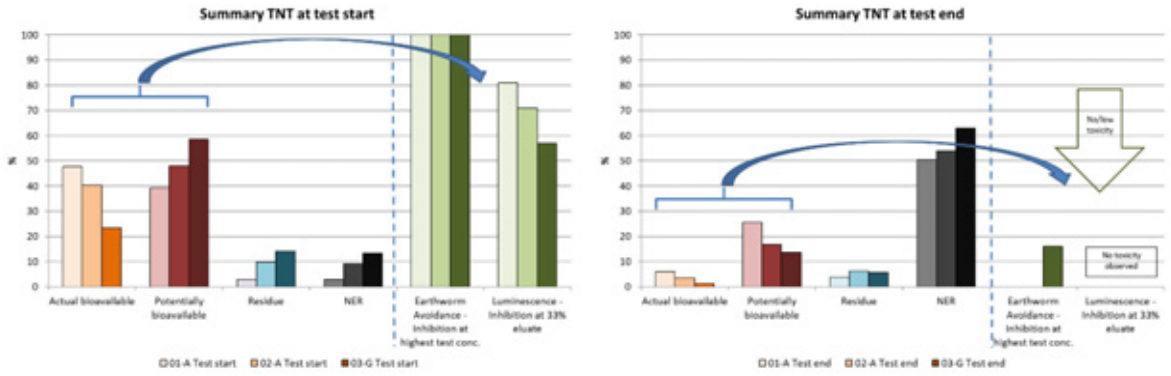


Figure 20 *Results of test with TNT in the three test soils. NER, Actual available, residue and potential available expressed as % of added TNT, toxicity as percent inhibition (earthworm avoidance: 100% test soil; luminescence inhibition: at 33% soil eluate)*

The results show that toxicity can be explained by the bioavailable part of the TNT. Both, a measurable actual bioavailable concentration and a potential bioavailable fraction can explain the toxicity at test start. For the aquatic test performed with the $CaCl_2$-eluate, the actual bioavailable fraction, is responsible for the ecotoxicity. Earthworms are exposed in soil and therefore both fractions (actual and potentially bioavailable) can affect the organisms. At test end, the actual bioavailable and potentially bioavailable fractions turned mostly into NER, as proven by radio-analysis. The toxicity at the same time had dropped dramatically, indicating that even high NER in the absence of bioavailable

TNT does not cause toxicity. A low toxicity using the earthworm avoidance test was only determined for soil 03-G which is much lower compared to test start. NER formation from TNT degradation in soils consequently can be assessed as a detoxification process. This has been proven in a previous project (Hund-Rinke et al., 2003).

5.3 Cypermethrin

Figure 21 shows the data determined for cypermethrin (120 mg/kg). The actual bioavailable concentration of cypermethrin is limited by its very low water solubility (9 µg/L, IUPAC database). Nevertheless, there is a considerable effect on the test organisms, due to the high ecotoxicity of the substance. An interesting result is the very high amount of potentially bioavailable substance despite the low water solubility. At the test end the potentially bioavailable fraction turns into the "Residue" fraction, most likely by physical effects, since no degradation products were detected. Chemical analysis (see 4.4.2) showed that all radioactive fractions recovered were almost 100% identified as parent cypermethrin. That might also be the reason that only very low amounts of NER were formed. NER are normally assumed to form by covalent bonding between a substance and the matrix. This requires a change of the chemical structure or – in other words – degradation. If no significant degradation is observed, formation of NER is unlikely and results obtained are reasonable. As already described in chapter 4.5.3 cypermethrin is very toxic (EC_{50} for immobilization of *D. magna*: 0.3 µg/L). Therefore even low remaining concentrations of cypermethrin cause toxic effects. Due to the low water solubility of the test substance, water saturation is achieved and a reduction of the available amount is not determinable. Due to the high sensitivity of the test organisms, the remaining potentially available substance fraction was sufficient to cause the observed effects. Thus, no evaluation with regard to NER toxicity was possible from this approach. The observed difference in behaviour of soil 02-A from the other soils cannot be explained from the current experiments.

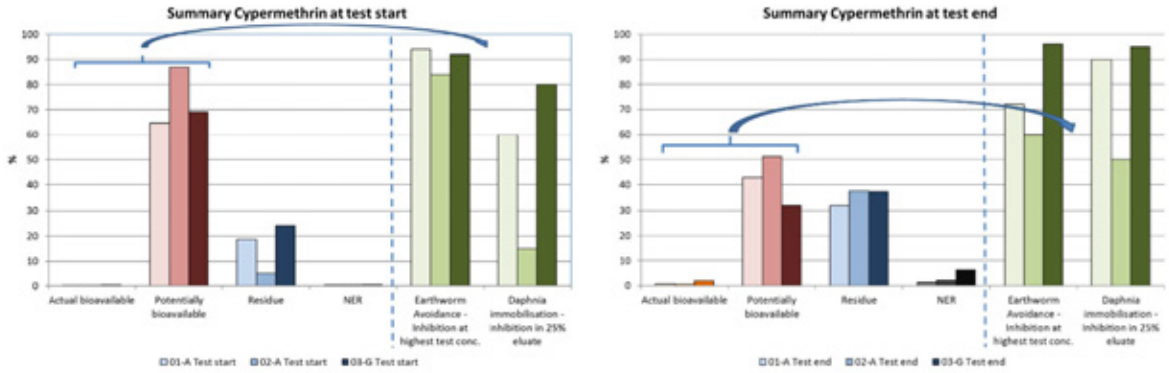


Figure 21 *Results of test with Cypermethrin in the three test soils. NER, Actual available, residue and potential available expressed as % of added TNT, toxicity as percent inhibition (earthworm avoidance: 100% test soil; Daphnia magna immobilization: at 25% soil eluate)*

5.4 Carbendazim

The results of carbendazim are again very different from findings for cypermethrin. At test start, a significant amount of actually bioavailable substance and a larger amount (up to 81%) of potentially bioavailable substance was determined. Carbendazim is very toxic for daphnids (EC_{50} values on the immobilization of daphnids: 10 – 40 µg/L; Sigma-Aldrich, 2013). The daphnia test was performed with the $CaCl_2$-eluate, which represents the actual bioavailable concentration. 10 – 20% of the total concentration correspond to 12 – 24 mg carbendazim/l. Accordingly, the toxicity measured was high, at least in the daphnia test. The lower toxicity compared to the EC_{50} value reported by Sigma-Aldrich (2013) can be due to the difference in the test medium (mineral medium for the tests performed according to guidelines; soil eluate with higher amounts of soil components which reduce the toxicity

of extracted chemicals. At test end, the applied radioactivity disappeared almost completely except soil 03-G, where up to 30% NER were still found. This was very surprising and different from the results of the mass balance experiment, where these significant losses of radioactivity were also observed, but to a lesser extent. However, chemical analyses of the recovered radioactivity showed that the disappearance of the radioactivity over time was consistent with a high degradation rate of the parent carbendazim (see Table 35). Soil 03-G always showed the lowest degradation rate, the best mass balance and approx. 30% NER formation. We could not explain this from soil properties (Table 2). As already seen for TNT the toxicity disappeared from all test soils, independent of whether they had 30% NER or only 4% NER. This indicated that NER formed from carbendazim was not responsible for any toxic effects. Thus, NER formation of carbendazim in soils can be assessed as a detoxification process.

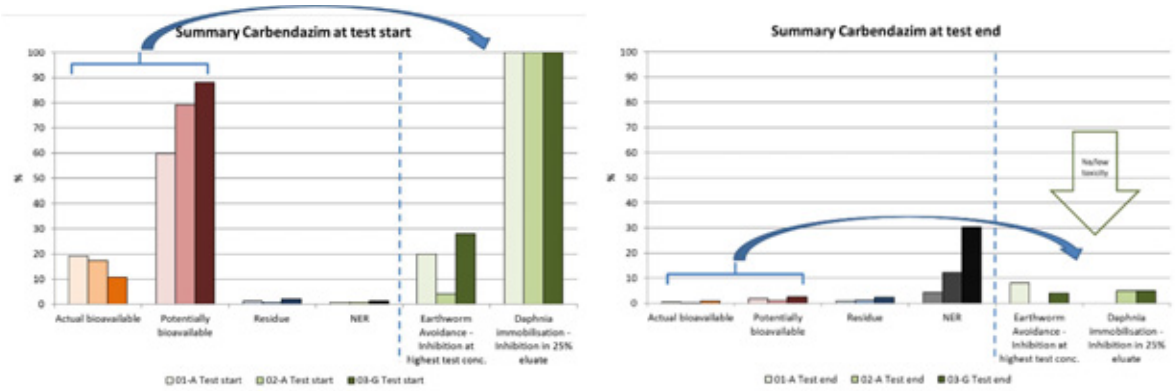


Figure 22 *Results of test with Carbendazim in the three test soils. NER, Actual available, residue and potential available expressed as % of added TNT, toxicity as percent inhibition (earthworm avoidance: 100% test soil; Daphnia magna immobilization: at 25% soil eluate)*

5.5 Test system to be used in assessment of NER

5.5.1 Introduction

The results described in chapter 3- 5.4 were used to design a test system applicable to most laboratories. It this system, non-labelled chemicals have to be used. This requires certainty on the fate of the chemical. The radiolabelled experiments on TNT had shown that the major pathway of TNT in soil is the formation of NER. Mineralization is below 10%. This made it possible to design a test system and to explain with measured data the importance of different fractions of chemical, including NER, for toxicity. For cypermethrin there is still an uncertainty regarding to the degradation rate, which results in high uncertainty on the amount of NER. For cypermethrin, the test has been limited to chemical part to underline the impossibility to quantify NER. Because the losses of radioactivity with carbendazim can be very high. It was decided not to use the test system with non-labelled carbendazim.

5.5.2 Test system applied for TNT

Following the approach of Ortega-Calvo et al., (2015), the test system to be used in assessments should be based on measurable values and easy to understand. This version of the test can be simpler than the system applied during method development as described in the first part of this chapter. Again, both sides of the cell membrane are considered (see Figure 4).

Left side of the cell membrane (soil/water)

- The water phase, in which concentrations[1] can be measured using extractions with 0.01M $CaCl_2$ (actual bioavailability);
- A potentially bioavailable fraction in equilibrium with the water phase, measured using (ISO/ TS 16751). We used Tenax ($Tenax_{20}$) to adsorb the fraction of bioavailable chemical, which is able to diffuse to the water phase. This fraction can be estimated by extraction of the Tenax or as done in this experiment as the difference of TNT present in soil before and after TENAX extraction. Reason was that more water had to be used to obtain a good separation of the Tenax. Resolubilisation of TNT in the water phase might lead to an underestimation of the potential bioavailable fraction;
- The total extractable amount was extracted with acetonitrile (ISO 11916). Results of this standard extraction are assumed to represent the total extractable concentration. This is confirmed in our experiments. Also application of ASE gave less than 1% extra recovery (Table 21);

NER was considered, but were non-measurable in these tests using non-labelled material, and also considered non-bioavailable. All other fractions were measurable. As a conclusion of the radiolabelled experiments, NER was quantified as 100% minus the extractable TNT in the soil. In this assumption, the mineralization of TNT of approx. 5% (Table 20) is ignored. It was shown (Table 27) that after aging not all extracted radioactivity could be contributed to the parent TNT, which could lead to an overestimation of NER. After 5 months the extractable amount was low and the mistake in NER, quantified as amount added minus amount measured could also be ignored. In the three soils most of the added TNT could be quantified as NER and ignoring biodegradation and extracted but not measured non-parent compounds will not change this conclusion.

Right side of the cell membrane (organism)

- Measurement of the bioavailability using organism at the right side of the membrane. We used *Vibrio Fischeri* (see 3.3.4)

Results are given in Figure 23**,** Figure 24 and Figure 25. Two situations are considered. A freshly spiked soil (T=0) in which the 'Total' extractable soil concentration, actually bioavailable and potentially bioavailable fractions were measured. The soil was also used for a toxicity test. The same ecotoxicity test measurement was performed before and after the removal of the potentially bioavailable fraction with TENAX. The same procedure was applied in aged soils (T= 5 months for soil 1 and T= 7 months for soil 2 and 3).

- NER was estimated as TNT added, minus the Total Extractable concentration (%);
- Residue was estimated as Total extracted TNT concentration minus potentially bioavailable concentrations (%);
- Potential bioavailable concentration was the amount removed with the TENAX extraction (difference of extractable TNT present in soil before and after TENAX extraction) (%);
- Actual bioavailability was the concentration in the 0.01M $CaCl_2$ extract of the soil. This value is presented as concentration[2] (mg/l);
- Actual and potential bioavailable were measured in parallel, not sequential;
- Toxicity is presented such that a high toxicity gives also a high value (i.e.$100/EC_{20}$). The method used delivered an EC_{20}-value, which is presented as $100/EC_{20}$.

[1] Measurement of activity using passive sampling is another possibility. Passive samplers are not used in this project.

[2] Actual availability is expressed as mg/l and not as % according Ortega-Calvo et al., (2015). The amount extracted with $CaCl_2$ depends on the volume used, the concentration not.

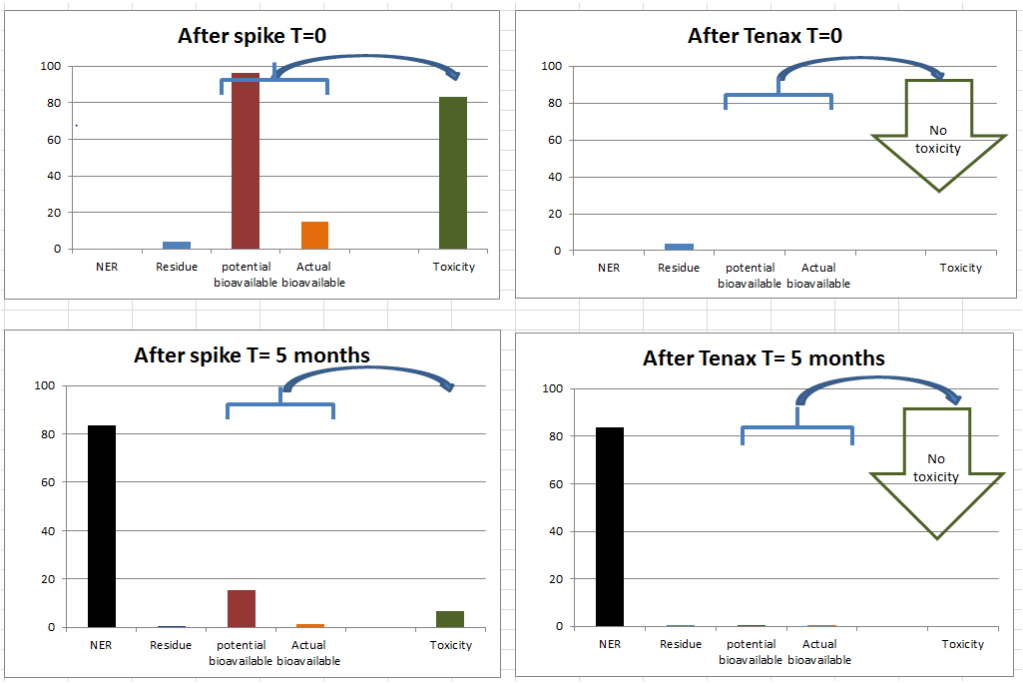


Figure 23 *Results of test with TNT in soil 1. NER, Residue and potential available expressed as percentage of added TNT, Actual available as mg/l TNT and toxicity as 100/EC20. Potential available is calculated as difference of TNT present in soil before and after TENAX-extraction. The same Y-axis can be used for all parameters*

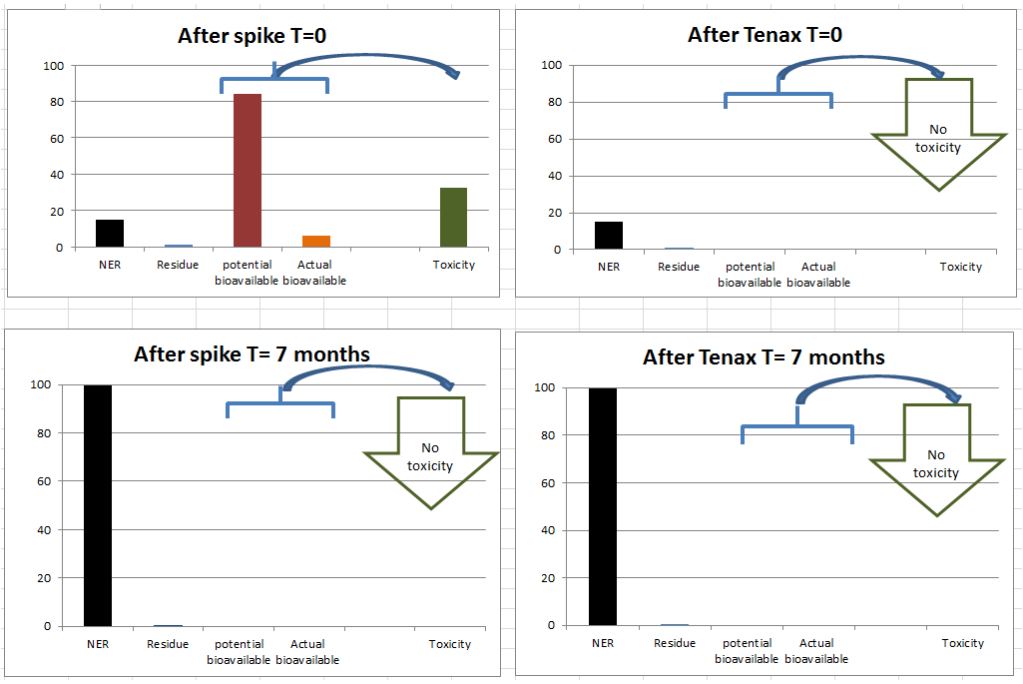


Figure 24 *Results of test with TNT in soil 2. NER, Residue and potential available expressed as percentage of added TNT, Actual available as mg/l TNT and toxicity as 100/EC_{20}. Potential available is calculated as difference of TNT present in soil before and after TENAX-extraction. The same Y-axis can be used for all parameters*

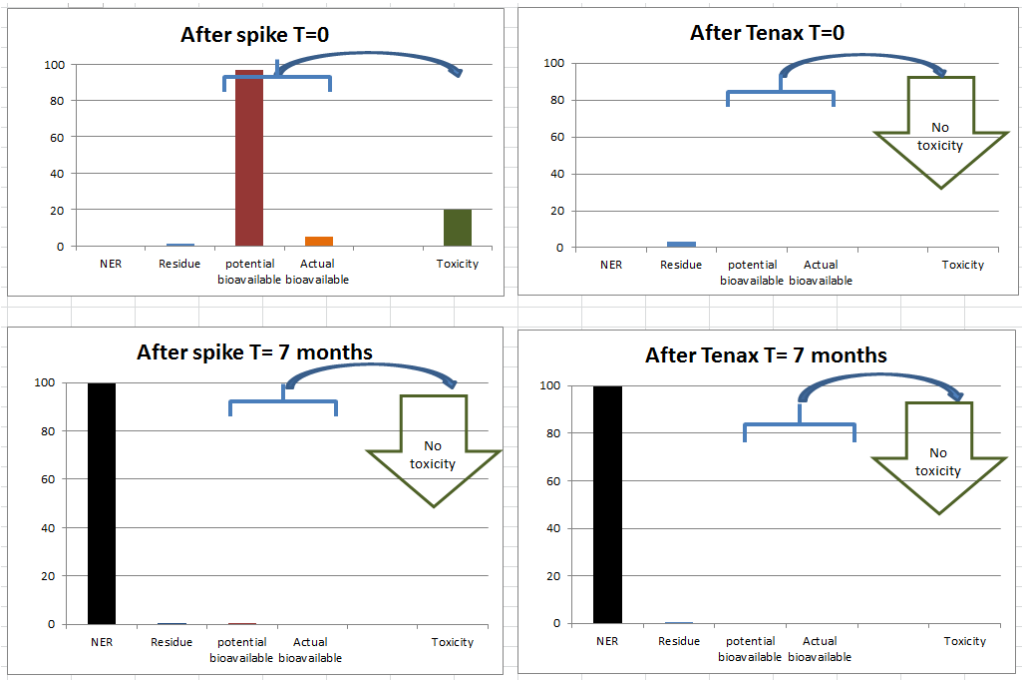


Figure 25 *Results of test with TNT in soil 3. NER, Residue and potential available expressed as percentage of added TNT, Actual available as mg/l TNT and toxicity as 100/EC_{20}. Potential available is calculated as difference of TNT present in soil before and after TENAX-extraction. The same Y-axis can be used for all parameters*

It should be realized that the parameters in the above figures have different dimensions. It is therefore not possible to compare absolute values. It is however possible to correlate parameters. If toxicity is measured, bars for actual and potential bioavailability are also larger. If no toxicity is measured, the values measured for actual and potential bioavailable are low. Removal of available TNT using Tenax, also removed toxicity. After aging, large amounts of TNT were not measured and assumed to be present as NER. The results in all three figures show that toxicity can be explained by the bioavailable part of the TNT. The actually bioavailable concentration and the potentially bioavailable fraction, both measurable, can explain the toxicity. A high portion of NER in absence of bioavailable TNT does not cause toxicity.

5.5.3 Test system applied for cypermethrin

For cypermethrin, the chemical part of the test system used for TNT (see 5.5.2) and described in section 2.6 has been applied. Results are presented in Table 44.

Table 44 *Results of chemical part of the test. Soils were aged during 7 months. Values for soil are presented as percentage of the originally added amount. Concentrations in 0.01M $CaCl_2$ in µg/l*

Soil	Recovery of cyper-methrin from soil [%]		Recovery of cyper-methrin from soil after $Tenax_{20}$ [%]		Available amount of cypermethrin [%]		Cypermethrin in water phase of $Tenax_{20}$ [µg/L]	
	Freshly applied	Aged	Freshly applied	Aged	Freshly applied	Aged	Freshly applied	Aged
01-A	94.0	58.4	55.8	15.5	38.2	42.9		
	97.9	64.8	39.2	28.8	58.7	36.0		
01-A average	**96.0**	**61.6**	**47.5**	**22.2**	**48.5**	**39.4**	**1.2**	**1.8**
02-A	91.3	63.0	20.5	34.5	70.8	28.5		
	102.9	60.7	38.0	32.8	64.9	27.9		
02-A average	**97.1**	**61.9**	**29.3**	**33.7**	**67.9**	**28.2**	**1.3**	**0.7**
03-G	106.3	69.2	33.6	14.7	72.7	54.5		
	97.2	66.4	48.3	43.2	48.9	23.2		
03-G average	**101.7**	**67.8**	**41.0**	**29.0**	**60.8**	**38.9**	**2.7**	**2.3**

Table 44 shows that the amount of extractable cypermethrin decreased during the aging period of 7 months. The reduction was attributed to NER formation or degradation. About half of the amount measured can be classified as bioavailable. In this experiment, the variation in measured values after Tenax extraction is relatively high and needs further investigation.

The concentration of cypermethrin in the 0.01M $CaCl_2$ was low, which means that most of the cypermethrin was adsorbed to the soil and the water phase can be ignored in relation to losses. Two of three values in aged soil (last column in Table 44) were higher than in fresh soils, which was unexpected and could not be explained. Highest values were measured in soil 03-G. This soil has the highest organic carbon and consequently the highest adsorption capacity. We assume that the dissolved organic carbon in this soil may have increased the apparent solubility of cypermethrin. With the centrifuge regime applied (15 min at 2100 rpm = 1218 x g), only solved material was present. In this experiment with a chemical with low solubility or high K_{OC} values, the measurement in the water phase had no additional value.

6 Discussion and conclusions

6.1 Introduction

The objectives of this project were:
- Develop a "soup test" to assess the toxicity of the different fractions of the test chemicals in the soil for different test organisms (plant, soft bodied soil organism, microorganisms) – ECETOC TR118.
- Validate the extraction regime as proposed in ECETOC TR 117.
- Validate the "definition" of bioavailability (as in ECETOC TR117) in toxicity test in relation to exposure scenarios in tests (time scales).

As mentioned, it was necessary to adjust the extraction regime of ECETOC TR 117. Newly developed ideas made it possible to simplify the regime in such a way that it became more understandable for regulation.

In this chapter, we evaluate how far we succeeded. We start with the definition. A lot happened over the last three years that improved the use of bioavailability and NER in regulation. This had consequences for the test to be developed, which could be made more direct and simpler than expected at the start. The development was led by 1) it must be explainable and 2) it must be measurable. However, reality is always more complex and it turned out that the test is not yet applicable for all substances. If the behaviour of a compound can be fully explained, it also becomes measurable as shown for TNT. Uncertainties in behaviour prevents measurability and application of a test to show the relationship between NER and toxicity will become more complex, as shown for cypermethrin and carbendazim.

6.2 Definition of bioavailability and NER

During this project we, as well as members of the monitoring team, were involved in SETAC activities on bioavailability. This has led to a new approach (Ortega-Calvo et all., 2015) in which bioavailability should be explainable and moreover measurable (Figure 4). NER is part of this approach.

In principle, NER is not routinely measurable, because it is not extractable. In experiments where a chemical is added it is however indirectly measurable. NER equals 'the added amount minus the extracted amount' provided no other losses occur. If a chemical is biodegraded or can be lost by, for instance evaporation, the chemical concentration will decrease during the experiment and if not recognized it will lead to an overestimation of NER. If losses can be quantified a proper estimate of NER can be given. If processes cannot be quantified or are very variable, a proper estimate of NER cannot be given. In such a case, NER can only be quantified using labelled compounds. A remaining uncertainty regarding NER is the presence of parent, parent breakdown product, biomass or carbonates.

The distribution of a chemical over different sites, bioavailable, non-bioavailable and NER is not constant in time. The radiolabelled experiments in this project have shown that in the laboratory, it takes about 2 months to create most of the NER and about 6 months to have a stable situation regarding NER. There is also a shift from bioavailable to non-bioavailable sites. In this period, other processes may occur that cause losses (e.g. biodegradation and evaporation). These processes are recognized using radiolabelled chemicals, but will lead to an overestimation of NER if only the extractable chemical can be measured as with non-radiolabelled chemicals. The changes in time have consequences for the test.

The approach finally used in this project is not exactly the same as the extraction regime proposed in ECOTOC TR 117. This regime had played an important role in the discussion leading to Figure 4, which is a simplified and better explainable approach. It was decided within the project to fit the extraction regime within the Ortega-Calvo approach.

6.3 Methodology

The objective of this project was to develop a method that can be used for the assessment of ecotoxicity of non-extractable residues of chemicals in soil. If behaviour is known and all losses are measurable and reproducible it is possible to show that the bioavailable amount is responsible for toxicity. Even high concentrations of NER do not cause toxicity.

The basis of the test is a good description of the behaviour of the chemical. For this research project, the use of radiolabelled chemical is essential, because this is the only direct way to quantify NER. In addition, other losses ($^{14}CO_2$) can be quantified. It was shown (carbendazim test) that an adsorption trap for volatile compounds should be included to obtain a proper mass balance. For the extractable part, it was necessary to identify the compound leading to the radioactive residue. Do we still talk about the parent compound or a degradation product? Based on this research including a proper mass balance combined with specific analysis helps understand the fate and explain observed ecotoxicity effects.

NER is still quantifiable using a non-labelled chemical. It is possible to quantify NER as 'the added amount of chemical minus the extracted amount'. However, this may overestimate the NER unless it is possible to correct for extra losses as identified in the radiolabelled experiments. For TNT, this was possible and using a relatively simple approach with non-labelled TNT the role of NER on toxicity could be estimated.

Having uncertainties in the behaviour, however, prevents the use of a simple approach. The degree of biodegradation for cypermethrin was not reproducible, which created a high uncertainty on the amount of NER-formation. Carbendazim showed an unexpected loss of substance from the test system, presumably via evaporation of an unknown volatile compound, as this is the only way to escape the radio-detection. If NER was defined by amount of test chemical added minus extracted test chemical, it would have resulted in a large overestimation of NER. These high uncertainties are not acceptable in risk assessment.

Because NER-formation was a fast process for the tested chemicals, it was not possible to use ecotoxicological test that last longer than a few days. Therefore, only acute tests were applicable, as chronic tests have usually longer incubation periods. During longer lasting tests, the chemical would change from available to NER and no proper evaluation would have been possible. Consequently, only acute tests were used in the developed method and not chronic tests. Chronic tests can only be applied on aged soils with stable contamination. If these soils are shown to be toxic in chronic tests, the available chemical could be removed by the methods applied in this project and the residual soil containing the non-available portion of the contaminant could be tested.

Organic chemicals in soil residues can be determined using a harsh extraction with organic solvents. The result is assumed to be the total concentration, especially by regulation. Several of these methods are standardized and available as ISO, European (EN) or national standard and have a role in regulation. We also used these methods to have the test developed as part of existing regulation. From a scientific point of view, it can always be proven that if more drastic conditions are used (higher temperature, combination of different solvent, extraction time etc.) more will be extracted. It has been shown in our experiments that using an Accelerated Solvent Extractor (ASE), slightly higher recoveries of added chemical were obtained, but that this does not change the understanding of the behaviour of the chemical. Using the standard extraction instead of ASE will give a slight overestimate of NER.

Use of solvents had a large effect on the toxicity of the soil; special precautions had to be taken to prevent the effect of the small volumes of solvents used with spiking. It will be very difficult to remove the large volumes used with an extraction. Using solvents to remove the bioavailable part and perform ecotoxicological tests with the extracted soil is not possible. Tenax is a solid phase and can be easily separated from water and soil; it did not have an effect on the results of the ecotoxicity tests used in this project. Use of Tenax is also in line with the recommendations in ISO 17402 and the methods suggested in Ortega-Calvo et al. (2015). Tenax is applied in a slurry system. If field moist soil is needed for a subsequent ecotoxicological test, drying of the soil will be necessary. Such a procedure may have effect on a following bioassay, especially if a soil function like ammonium oxidation is measured. In our study after combination of Tenax-extraction and following treatment of the soil, an effect on microbial ammonium oxidation activity was observed. Thus, we cannot recommend this method to establish the toxicity of the spiked and aged soil in this approach. However, it makes clear that it is always necessary to check the effect of using Tenax on the bioassay used.

From a practical standpoint, only limited amounts (<200 g) of soil can be extracted using Tenax, rendering limited availability of soil containing NER for ecotoxicity testing. Especially for some chronical tests (e.g. Earthworm reproduction), larger amounts are necessary.

The amount removed by $Tenax_{20}$ was assumed to represent the "potentially" bioavailable amount. This amount can be established by measuring the amount in Tenax or the concentration of a substance in soil before and after Tenax extraction.

It is possible to define a solvent extraction that will extract the potentially bioavailable amount. Examples are given in Ecetoc Technical report 117. By doing empirical research, it is possible to define the proper conditions, which can be different for different chemicals (e.g. weaker conditions like mixing with water or using shorter extraction times).This approach was not used, because the extracted soils cannot be used, as such for subsequent ecotoxicological tests. According Ortega-Calvo et al., 2015, solvent-based extractions to measure bioavailability have been fruitful in the development of science on bioavailability. In present bioavailability science, they are replaced by methods with a mechanistic base in line with the recommendations mentioned above.

For relatively water-soluble chemicals (e.g. TNT), the concentration measured in the water phase (e.g. 0.01M $CaCl_2$) can also be used to represent bioavailability. Using 0.01M $CaCl_2$, the "actually" bioavailable concentration is measured. This value is less suitable if the compound is only slightly water-soluble (e.g. cypermethrin).

To reduce inherent variation found in bioassays, we did not perform the bioassay immediately after spiking and after aging. Instead, we spiked another portion of the soil after aging and the bioassays and other tests were applied on the aged and freshly spiked soil simultaneously.

6.3.1 TNT

TNT is soluble in water (solubility 130 mg/l (EPA, 2014)) and lower recoveries (65%) were obtained extracting TNT from water using petroleum ether. Solubility was high enough to do direct injections and consequently obtain good recoveries. Tenax is suitable to extract TNT from the water phase (Table 8). In a soil/water system (2 g soil and 100 ml water) most of the TNT was in the water phase (Table 12 and Table 13). It can be concluded that for TNT, bioavailability can be estimated using a 0.01M $CaCl_2$ extract (actual bioavailability) and the amount removed by Tenax (potential bioavailability).

Using radiolabelled TNT good mass balances were obtained and biodegradation (formation of $^{14}CO_2$) was low (Table 20). NER formation was respectively 64; 75 and 77% in 6 months. Most of NER formation was in the first two months and stabilized in the following 4 months. In the subsequent experiment (Table 21), NER formation was respectively 64, 54 and 63% in 6 months. We used a sequential extraction and after spiking, most of the TNT was in the 0.01M $CaCl_2$ and $Tenax_{20}$ extract. After 6 months, bioavailability (0.01M $CaCl_2$ + $Tenax_{20}$) decreased by NER formation and was also a smaller part of the extractable TNT.

In an additional experiment, it was investigated whether the measured radioactivity was caused by the parent compound or whether transformation products are involved. Looking to the total extracted radioactivity, this could be mainly explained by the parent compound just after spiking. In the 6-month period, it reduced to 63, 54 and 48% in the three soils (Table 27). At test start, the identified bioavailable part of radioactivity was the parent compound (Table 28), but radioactivity caused by the stronger adsorbed chemical ($Tenax_{1\ week}$ and organic extract) was only partly caused by the parent compound (Approx. 65 and 30%, respectively) (Table 29, Table 30). During 6 months, the percentages of the parent compound for $Tenax_{20}$ and $Tenax_{1\ week}$ decreased (Table 29), but remained constant for the organic extract (Table 30).

In a normal test, and especially if the soil is further used in bioassays, radiolabelled chemicals are not commonly used. Only the parent compound can be measured. Missing the transformation products and assuming that NER can be defined by the amount added minus the amount extracted using Tenax and organic solvent should not result in a non-acceptable overestimation of NER. At start, most of the TNT is bioavailable and the residue is relatively low. The non-parent compound in the residue is missed and this may give a small overestimation of NER. After 6 months, both bioavailable and extractable residue are low, which may also give a small overestimation of NER. Based on the results reported in 4.1.2 the overestimation of NER will be limited, and values reported can be used.

Most of the NER formation was in the first two months (Figure 17). This confirms that 'fast' bioassays should be used lasting 1 or 2 days when addressing the ecotoxicity of the bioavailable fractions. Using for instance, the earthworm survival test or reproduction test (4-8 weeks) will produce data in a changing system and will create uncertainty. Which measured fractions should be used; measured at t=0 or at t=4-8 weeks. The bioassays used, lasting one or two days, were appropriate (chapter 4.5).

The spiked soil was initially toxic for earthworms and luminescent bacteria. Combining results obtained during the radiolabelled experiments, it was shown that high toxicity was obtained with a high bioavailable fraction (Figure 20). After reaching a high level of NER and consequently a low bioavailable fraction, no toxicity was observed.

The results of the potential ammonium oxidation test were less clear, because handling of the soil had effects on the activity of the soil microflora (chapter 4.5 and Annex 2). Indirectly, it was possible to show that NER did not have inhibitory effects. However, the test has been shown not to be very suitable in further testing.

The behaviour of TNT was explainable; TNT formed high NER with low additional losses due to biodegradation and an adequate mass balance. Uncertainty caused by the presence of transformation products were not expected to impact on the prediction of NER. Initial ecotoxicity could be established using short-term (1-2 days) bioassays.

A system was tested, making use of non-labelled TNT, which was generally applicable in this project. In this system, the actual bioavailability, potential bioavailability, and residual extractable TNT were measured. NER was defined as the added TNT, minus the sum of TNT in extractable fractions. Ecotoxicity was measured using a luminescent bacteria test, which could be performed within one day. Parameters measured were in line with the approach of Ortega-Calvo (2015). It can be concluded that the amount of transformation products (see above) will be small and the estimation of NER is therefore acceptable. Again, the initial ecotoxicity can be explained by the measurable bioavailable chemical. For this water-soluble chemical, both actual and potential bioavailability can be independently used to explain the observed ecotoxicity.

Combining all TNT results it can be concluded that if the behaviour is well understood and the effect of uncertainties (presence of transformation products) is small, both the radiolabelled experiments as well as the simplified final system can be used to explain the toxicity of TNT. The bioavailable TNT (presented as actually available and potentially available) explains the toxicity. High NER, present after aging does not cause toxicity.

6.3.2 Cypermethrin

Cypermethrin, a hydrophobic chemical, is extractable from water (Table 9) and removable from the water phase using Tenax (Table 10). Not all cypermethrin was removed from the Tenax using petroleum ether (Table 11). In a water/soil system most of cypermethrin was present in the soil phase (Table 12). In the following slurry experiment, the recoveries were low and not fully explainable.

In the first radiolabelled experiment, an adequate mass balance was obtained (Table 22). Only a small and ignorable amount (1.9; 2.9 and 4.0%) was lost as $^{14}CO_2$ through biodegradation within 6 months. NER formation was relatively low (17, 22 and 38%) and mostly occurred in the first three months. In the following radiolabelled experiment (Table 20) NER formation was much lower (1.2; 2.7 and 5.4%). It was shown that cypermethrin was moved to stronger adsorption sites in soil during the aging period. There was a shift in extracted fraction from $Tenax_{20}$ (potentially bioavailable) to acetonitrile (non-bioavailable). All extractable cypermethrin was identified as the parent compound (Table 31, Table 32, Table 33 and Table 34).

The amounts measured in the water phase (all cypermethrin) were highly variable. It is assumed that the high centrifuge speed (15 min at 2100 rpm = 1218 x g) did not give solutions with comparable amount of dissolved organic matter and colloids. If one assumes that the dissolved organic matter and colloids associate with cypermethrin, the concentration in the water phase may explain the observed ecotoxicity. Passive sampling (not applied in this project) may be better suited to measure in the water phase and measures the free solved concentration. However, it needs an equilibration period of 28 days and is therefore not suitable for the experiments within this project.

An additional radiolabelled mass balance experiment with a lower applied concentration of cypermethrin was successful regarding the mass balance (Table 24). The $^{14}CO_2$ was much higher (25; 50 and 65%) and NER production was comparable with the first experiment (16; 34 and 32%). With the large amount of cypermethrin biodegraded in this experiment, NER can be expected to have been formed by incorporation of ^{14}C into the biomass. The uncertainty in degradation made it impossible to make a proper estimate of NER in an un-labelled experiment (Table 38).

With the radiolabelled experiments, the behaviour of cypermethrin in individual experiments were explainable. However, experiments were not reproducible, especially regarding biodegradation. This means that in a non-labelled experiment, NER cannot be quantified as 'added test chemical minus extracted amounts of the test chemical'. The potentially bioavailable amount was attributed to the amount removed by $Tenax_{20}$. The water solved concentration measured after centrifugation and extraction was not suitable to indicate actual bioavailability.

The last non-labelled experiment showed that after aging the total amount of cypermethrin that could be extracted reduced to 62, 62 and 68%. The rest was attributed to NER or degraded test chemical. It was not possible to estimate NER based on the earlier experiments. This uncertainty makes it less certain what the role of NER was in the ecotoxicity of the aged soil.

Aging did not have a large effect (Figure 21) on the results of bioassays used (earthworm avoidance and Daphnia immobilization). Probably, the toxicity of cypermethrin at the concentration used was too high in combination with a low percentage of NER formation.

Combining all cypermethrin results, it can be concluded that its behaviour in soil was not reproducible especially regarding *biodegradation (CO_2-formation in labelled experiments). This gives a high uncertainty in the amount of NER and its form (Parent or biomass). Without radiolabelled chemicals, the uncertainty prevents a proper quantification of NER and consequently it may not be possible to relate NER to the absence of ecotoxicity*.

6.3.3 Carbendazim

Carbendazim was extractable from water using petroleum ether (Table 9). Using Tenax to extract it from the water phase, most was extracted, but a residual concentration remained in the water phase (Table 10). Comparable to the results of the other two compounds, the results in Table 9 are difficult to explain. In a water/soil system, a large part of carbendazim was present in the water phase. Therefore, the water phase was used to establish its bioavailability. In a spiking experiment, the extractability quickly dropped, which occurred even faster when a slurry was used. Less than 12% of the added carbendazim was extracted and use of Tenax reduced this to about 3% (Table 15). We could not explain the low recoveries.
In the following first radiolabelled carbendazim experiment, the total recovery was reduced during the 6-month aging period to 38, 56 and 75% (Table 25). NER formation occurred mostly in the first two months (Figure 18) to 27, 32 and 56% after 6 months of aging. Formation of $^{14}CO_2$ was not ignorable (9, 23 and 8%).

In the second radiolabelled experiment, the losses were much higher. Total recovery after 6 months was 7, 15 and 36% (Table 26). Most of it was present as NER (4, 12 and 30% and consequently the extractable fraction was very small (all < 1%). We assume that the high losses in the radiolabelled experiments were caused by volatilisation of a degradation product other than$^{14}CO_2$. No trap for this kind of compound was present in the test system. During aging, the contribution of parent compounds to the extracted and measured radioactivity reduced to 16; 20 and 78% (Table 36, Table 37 and Table 38), which also includes a high uncertainty on formation of NER.

Using the radiolabelled experiments in combination with the toxicity test a decrease of ecotoxicity was shown. After 6 months, mostly NER were present in the soil and almost no bioavailable and extractable carbendazim. With the high uncertainties observed it will not be possible to interpret non-labelled experiments. The extractability will drop to almost zero and it cannot be argued whether NER equals the highest value observed in the radiolabelled experiments of the added amount of 56% or the lowest of 7%.

6.3.4 Benzo(a)pyrene

In the beginning of the project, benzo(a)pyrene was a candidate chemical. This chemical is extractable from the water phase by petroleum ether (Table 9) and Tenax (Table 11). It can be recovered well from Tenax. This is not surprising, because PAH's are used as model compounds in Tenax-research. In a soil/water system most of the very poorly soluble B(a)P is present in the soil phase (Table 12). In a period of 3 days after spiking all B(a)P is extractable and about 45% is measured as bioavailable.

The extractability of B(a)P was as expected, but the chemical was not selected because of its low toxicity.

7 Recommendations

7.1 Method development

This investigation focused on explaining the role of NER in toxicity. NER are not extractable and cannot be measured by usual chemical analysis. Using radiolabelled chemicals the presence of NER can be made visible and measurable. In this report, a method is introduced in which data are produced to determine the toxicity of NER formed by a toxic chemical in soils. Radiolabelled experiments were the basis of the method developed. The method can be applied in any laboratory, which is experienced in fate and ecotox testing of organic chemicals in soils. If the degradation pathway of the chemical in soils is well known, no radiolabelled chemicals are necessary for the test. The most important requisite is that the fate of the chemical is well known and that it is plausible that the amount not extracted can be attributed to NER. Having these data the role of NER regarding toxicity can be explained.

If uncertainties on the fate exist, like non-reproducible degradation or losses, it is not possible to quantify NER as amount added minus amount extracted'. This causes a high uncertainty in the data produced, which makes it impossible to reliably determine the toxicity of NER. To overcome this uncertainty, experiments with ^{14}C-radio-labelled chemicals are needed.

From a practical standpoint, the following has to be taken into consideration:
- Toxicity of NER chemicals is often limited and it is difficult to select a proper test.
- Only limited amounts (<200 g) of soil can be extracted using Tenax, resulting in limited availability of soil containing NER for ecotoxicity testing. This has to be taken into account with the ecotoxicological test to be applied.
- Sample treatment like drying after extraction can have an impact on soil functions and results of tests if these are part of the test; suitability of ecotox-tests has to be proven.
- Use of a solvent in any step (e.g. addition of chemical, extraction) is critical because residual solvent will cause toxicity and interfere with ecotox testing.

Chronic toxicity test were not applied in this project. In the set-up of the project, it was decided that the situation after spiking of the chemical should be compared with the situation after NER-formation. As shown in this report, most of NER is formed in the first 6 weeks after spiking. A period, that also has to be used for chronic toxicity tests. This makes the interpretation of the results of a chronic test uncertain. In following projects, aged soil material can be used for a chronic toxicity tests. However, as the reference (toxicity on day 0 = date of spiking) will be missing, the informative value concerning the reduction of toxicity is limited. Therefore, a combined approach can be useful. Tests with short test duration (usually acute tests) to show the reduction of toxicity may be combined with chronic tests to rebut the long-term exposure concern.

7.2 Communication

This project was presented on the 9th International Workshop on Chemical Bioavailability in the Terrestrial Environment. Warsaw 5th-8th November 2017. It was concluded in this workshop that:
- If chemicals are non-extractable, they are strongly bound and will not cause risks;
- In the past, scientific communications and unclear definitions have caused uncertainty in regulatory context;
- Present scientific "certainties" on bioavailability have to be communicated.

Clear communication is the main message of this discussion, which was also subject of discussions with the monitoring group. The following steps are necessary:
- Make results of this project available for regulators in a proper form;

- Define shortcoming of current approaches (technical and regulatory);
- Show with well-designed experiments, including ^{14}C-experiments the presence and toxicity of NER. Use general accepted definitions;
- If toxicity cannot be shown, define what is necessary to show that NER is really not extractable.

All these steps should be based on the data presented in this report. It should be published separately, because adding it to this report only will not make them available for regulators. As mentioned before, a clear communication of the results is most important.

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Annex 1 Literature review selection of chemicals

The presented selection of plant protection products and personal care products is largely based on findings reported by Barriuso et al. (2008). These authors reported that pesticides and metabolites containing aniline or phenol groups often tend to form larger proportions of non-extractable residues (NER) than compounds not containing these groups. From a practical experimental point of view, compounds of relatively high hydrophobicity are to be preferred in view of the Tenax or HPCD extractions intended to remove compounds from contaminated soil. Moreover, compounds with relatively high toxicity to soil organisms are to prefer over compounds with low toxicity, because of the experimental aim to demonstrate effects or a decrease thereof. Since data on soil toxicity are for most compounds not readily available, the toxicity to Daphnids is used as an alternative indicator for toxicity. An additional requirement is sufficient stability of the compound, i.e. compounds with relatively short half-lives for hydrolysis are not very suitable for use in experimental studies. For pharmaceuticals, these considerations were used for selection of compounds from an elaborate list of compounds provided by Sanderson and Thomsen (2009).

Moreover, some very persistent organochlorine compounds were added as a separate category. The persistence of these compounds has the advantage that any failure to extract them from spiked soil is unlikely to be the result of transformation or metabolism, but is indicative of the extraction potential of the extraction methods used.

Plant Protection Products

For pesticides, the first selection of compounds is based on the percentage of NER as given by Barriuso et al. (2008), focussing on chemicals with a lower limit of NER formation of > 30%; these compounds and their relevant properties (hydrophobicity; whether hydroxyl- and/or amino groups are present within the molecule; toxicity) are summarized in Table 1.

Table 1 *Plant protection products with lower limit of NER > 30% as reported by Barriuso et al. (2008)*

Compound	CAS – number[a]	Log K_{ow}[b]	OH present	NH/NH2 present	LC_{50}[c] (mg/L)
Bentazone	25057-89-0	0.77 (pH=5)	No	Yes	125
Bifenazate	149877-41-8	3.4	No	Yes	0.50
Bromoxynil	1689-84-5	2.8	Yes	No	12.5
Chlorothalonil	1897-45-6	2.92	No	No	0.07
Chlorpropham	101-21-3	3.79 (pH=4)	No	Yes	3.7
Cinidon-ethyl	142891-20-1	4.51	No	No	52.1
Cyazofamil	120116-88-3	3.2	No	No	0.14
Famoxadone	131807-57-3	4.65	No	Yes	0.012
Foramsulfuron	173159-57-4	4.01 (pH=5), 0.17 (pH=7)	No	Yes	>100
Iprodione	36734-19-7	3.0	No	Yes	0.25
Isoproturon	34123-59-6	2.5	No	Yes	507
Maneb	12427-38-2	Ionic	No	Yes	125
Mecoprop-P	16484-77-8	1.43	No	No	>100
Metalaxyl-M	70630-17-0	1.71	No	No	>100
Metiram	9006-42-2	0.3 (pH=7)	No	Yes	0.11
Phenmedipham	13684-63-4	3.59	No	Yes	3.8
Pyraclostrobin	175013-18-0	3.99	No	No	0.006
Pyridate	55512-33-9	4.01	No	No	0.83
Thiophanate-methyl	23564-05-8	1.50	No	Yes	20.2

[a] Chemical Abstracts Service number, taken from Tomlin (2003)

[b] Logarithm of the octanol-water partition coefficient, taken from Tomlin (2003)

[c] Data for Daphnia magna, except pyraclostrobin (rainbow trout) taken from Tomlin (2003)

Compounds with pH-dependent hydrophobicity are not considered for selection, because of the experimental difficulties such a property may cause.

Pesticides with sufficiently high hydrophobicity (log K_{ow} > 3.5) and high toxicity to daphnids are chlorpropham (NER: 54 – 78%), cinidon-ethyl (NER: 48 – 80%), famoxadone (NER: 28 – 54%), phenmedipham (NER: 64 – 74%), pyraclostrobin (NER: 54 – 56%) and pyridate (NER: 54 – 62%). Cinidon-ethyl appears to be less toxic (LC_{50} > 50 mg/L), whereas chlorpropham and phenmedipham appear to be of intermediate toxicity (LC_{50} 1 – 50 mg/L), and famoxadone, pyridate and pyraclostrobin appear to be very toxic (LC_{50} < 1 mg/L) to daphnids. For the chemicals with the most interesting combination of hydrophobicity, toxicity and NER formation the relevant properties are given in Table 2.

Table 2 *Plant protection products from Table 1 of sufficiently high hydrophobicity (log K_{ow} > 3.5) and OH- or NH/NH_2-groups present in the molecule*

Compound	Log K_{ow}[a]	Fraction non-extractable Residue[b] (%)	LC_{50}[c] (mg/L)		
			Algae	Daphnids	Fish
Chlorpropham	3.79	54 – 78	3.3	3.7	10 – 12
Phenmedipham	3.59	64 – 74	0.13	3.8	1.4 – 17
Famoxadone	4.65	30 – 54		0.012	
Pyridate	4.01	54 – 62	82.1	0.83	1 - >100
Pyraclostrobin	3.99	54 – 56	>0.84		0.006

[a] Logarithm of the octanol-water partition coefficient, taken from Tomlin et al. (2003)

[b] Non-extractable residue, taken from Barriuso et al. (2008)

[c] Acute toxicity data, taken from Tomlin et al. (2003)

Chlorpropham slowly hydrolyses in acidic and alkaline solutions but is stable under neutral conditions (Tomlin, 2003). Famoxadone is stable in water with no light at pH=5, but is much less stable at higher pH (DT50 of 41 d, 2 d and 0.06 d at pH=5, 7 and 9 resp.; Tomlin, 2003). Phenmedipham is stable in acidic media, but hydrolysis with a DT_{50} of 14.5 h at pH=7 and a DT_{50} of 10 minutes in alkaline conditions (Tomlin, 2003). Pyraclostrobin is stable to hydrolysis in solution, but is prone to photolysis (Tomlin, 2003). Pyridate is prone to hydrolysis (DT_{50} of 89 h at pH=5; Tomlin, 2003) and is therefore less suitable for longer lasting experiments. Thus, with regard to the stability of the compound during experimental conditions, chlorpropham and pyraclostrobin appear to be the most promising compounds.

Conclusion: the pesticides chlorpropham and pyraclostrobin appear to be the most preferable candidates for preliminary screening.

Pharmaceuticals and Personal Care Products

Similar to the analysis performed for pesticides, the choice of pharmaceuticals and personal care products for use in experimental NER analysis was based on an existing list of compounds which were selected according to pre-defined criteria: log K_{ow} > 3.5, relatively high toxicity to soil organisms (or, alternatively, to Daphnids), and sufficient stability. Since Barriuso et al. (2008) indicated that the presence of OH- or NH/NH2-groups in the molecule is expected to result in increased NER-formation, the presence of such groups is added as a requirement for selection of pharmaceutical and personal care compounds. The starting point was a list provided in a recent article by Sanderson and Thomsen (2009), selecting only compounds with log K_{ow} > 3.5 for further consideration using the other criteria (Table 3).

Table 3 *Pharmaceuticals with log K_{ow} > 3.5 reported by Sanderson and Thomsen (2009)*

Compound	CAS – number[a]	Log K_{ow}[b]	OH present	NH/NH2 present	LC_{50}[c] (mg/L)
2,2'-Dithiobisbenzothiazole	120-78-5	4.5	No	No	23000
Amitriptyline	50-48-6	4.95	No	No	0.78
Atropine sulfate	55-48-1	3.92	Yes	No	661 (F)
Azithromycin	83905-01-5	4.02	Yes	No	120
Benzaldehyde	100-52-7	5	No	No	7.6 (F)
Benztropine mesylate	132-17-2	4.3	No	No	10.6
Capecitabine	154361-50-9	4.5	Yes	Yes	850
Carvedilol	72956-09-3	4.19	Yes	Yes	3
Chloroquine phosphate	50-63-5	4.5	No	Yes	11.7 (F)
Citalopram	59729-33-8	3.74	No	No	3.9
Clofibrate	637-07-0	3.62	No	No	17.7
Diclofenac	15307-86-5	4.02	Yes	Yes	22.4
Dodecanal	112-54-9	5.1	No	No	2.6 (F)
Erythromycin	3521-62-8	5.1	Yes	No	0.94
Estradiol benzoate	50-50-0	5	Yes	No	>100
Ethinylestradiol	57-63-6	4.2	Yes	No	5.7
Ethoxyquin	91-53-2	3.87	No	Yes	2
Fenofibrate	49562-28-9	5.19	No	No	50
Fluoxetine	54910-89-3	4.65	No	Yes	0.51
Flutamide	13311-84-7	3.51	No	Yes	1.38
Gemfibrozil	28512-30-0	4.77	Yes	No	>100
Ibuprofen	15687-27-1	3.97	Yes	No	9.1
Losartan potassium	124750-99-8	4.01	Yes	No	331
Medazepam	2898126	4.43	No	No	1.3 (F)
Mestranol	72-33-3	4.75	Yes	No	>100
Monobenzoate	2211-28-1	4.29	Yes	No	1.3 (F)
Naftopidil	57149-07-2	3.94	Yes	No	>100
Nelfinavir	159989-64-7	4.1	Yes	Yes	>100
Nisolfipine	63675-72-9	3.90	Yes	No	33
NK1 receptor antagonist	290296-68-3	5.4	No	Yes	>100
Octabase H	57849-23-7	4.58	No	Yes	2.3 (F)
Orphenadrine HCl	341-69-5	3.65	No	No	10.6
Paroxetine	61869-08-7	3.95	No	Yes	0.58
Quinacrine HCl	69-05-6	5.15	No	Yes	7.7 (F)
Quinidine sulfate	50-54-4	5.4	Yes	No	60
Spirapril HCl	83647-97-6	3.89	Yes	Yes	930
Testosterone proprionate	57-85-2	4.5	No	No	>100
Tetralin	119-64-2	3.8	No	No	2.4
Thioridazine HCl	50-52-2	5.45	No	No	4.56
Trenbolone acetate	10161-34-9	3.77	No	No	0.9
Triclosan	3380-34-5	4.66	Yes	No	0.13
Trifluoperazine dihydrochloride	440-17-5	5.42	No	No	2.6

[a] Chemical Abstracts Service number, taken from Sanderson and Thomsen (2009)

[b] Logarithm of the octanol-water partition coefficient, taken from Sanderson and Thomsen (2009)

[c] Data for Daphnia magna taken from Sanderson and Thomsen (2009)

Compounds with either an OH- or an NH/NH2-group (or both) in the molecule and with relatively high toxicity to Daphnids (LC50 < 10 mg/L) are given in Table 4. Information concerning the formation of non-extractable residues (NER) with regard to these compounds could not be readily found using SCOPUS. For this reason, this list can only serve as a first guide to chemicals that may undergo preliminary testing for the formation of NER prior to inclusion in the definitive experiment.

Table 4 *Pharmaceuticals from Table 3 with OH- and/or NH/NH_2-groups present in the molecule and relatively high toxicity to daphnids*

Compound	Log K_{ow}[a]	LC_{50}[b] (mg/L)		
		Algae	Daphnids	Fish
Carvedilol	4.19	0.17	3	1
Erythromycin	5.1	0.02	0.94	80
Ethinylestradiol	4.2	0.84	5.7	1.6
Ethoxyquin	3.87		2	18
Fluoxetine	4.65	0.024	0.51	0.7
Flutamide	3.51		1.38	>1000
Ibuprofen	3.97	7.1	9.1	173
Monobenzoate	4.29			1.3
Octabase H	4.58			2.3
Paroxetine	3.95		0.58	2
Quinacrine HCl	5.15			7.7
Triclosan	4.66	0.0045	0.13	0.26

[a] Logarithm of the octanol-water partition coefficient, taken from Sanderson and Thomsen (2009)

[b] Acute toxicity data, taken from Sanderson and Thomsen (2008).

Dussault et al. (2008) reported that of the 4 pharmaceuticals and personal care products atorvastatin, carbamazepine, ethinylestradiol and triclosan, the latter was the most toxic to the benthic organisms, establishing LC50 concentrations for survival in water-only acute (10-day) toxicity experiments of 0.4 and 0.2 mg/L for *Chironomus tentans* and *Hyalella azteca* resp. Richardson et al. (2005) also indicate that triclosan is among the most frequently detected compounds in the aquatic environment. Some toxicity data for triclosan for various aquatic organisms are given by Brausch and Rand (2011), indicating that triclosan is indeed quite toxic to a wide range of species including fish, daphnids and algae. Similarly, DeLorenzo and Fleming (2008) reported that of the compounds in Table 4, fluoxetine and triclosan represent some of the most commonly used and/or detected PPCPs in the aquatic environment. 96-Hour EC_{50} values for the marine phytoplankton species *Dunaliella tertiolecta* were 0.17 and 0.0036 mg/L for fluoxetine and triclosan resp., indicating that especially triclosan is very toxic to this species. These two compounds, fluoxetine and especially triclosan, therefore seem preferable for preliminary screening into the possible formation of non-extractable residues.

Schnell et al. (2009) investigated the cytotoxicity of some pharmaceuticals and personal care products to a rainbow trout liver cell line, and concluded that polycyclic musks, followed by anti-depressives showed the highest potential to induce cytotoxicity, whereas nitromusks had the lowest toxicity. When testing synthetic musks in NER testing, polycyclic musks like Tonalide would therefore be preferable over nitromusks like musk xylene (however, neither of these molecules contain OH- or NH/NH_2-groups).

Some information with regard to the formation of NER for specific pharmaceuticals and personal care products was found in Eschenbach (2013), who summarized data on various pharmaceuticals found in open literature. This list was amended with further information found during a quick sweep of literature in Scopus, which resulted in papers by Heise et al. (2006), Kreuzig et al. (2007) and Richter et al. (2007). The resulting information is summarized in Table 5.

Stoob et al. (2006) indicated that the extraction of some sulfonamide antibiotics (sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole and sulfathiazole) from aged soil samples increased substantially when increasing the extraction temperature from 100 to 200oC, whereas no such increase was seen when using short-term spike experiments. Although the authors indicate that the reason for decreased extraction efficiency upon increase of contact time under mild conditions remains unknown, this phenomenon may be indicative of the formation of non-extractable residues, making these compounds interesting as screening candidates. These four molecules all contain two NH/NH_2-groups, increasing the possibility of the formation of non-extractable residues (Barriuso et al., 2008).

Sulfadiazin was found to form over 90% of NER (Heise et al., 2006), but its acute toxicity to Daphnia magna was relatively low (48-h LC_{50}=221 mg/L; Wollenberger et al., 2000) and observed to be dependent on pH (48-h LC_{50} of 27, 188 and 310 mg/L at pH 6.0, 7.5 and 8.5 resp.; Anskjaer et al., 2013).

Sulfamethoxazole was found to form over 70% of NER (Heise et al., 2006), but was not very toxic in acute tests using V. fischeri (EC_{50} =344 mg/L), daphnids (48-h EC_{50}=174 mg/L) and fish (96 LC_{50} > 100 mg/L) (Kim et al., 2006). Chronic toxicity to a species of algae (*Synechococcus leopolensis*) was very high, though, with a NOEC of 0.0059 mg/L (Ferrari et al., 2004).

Table 5 *Data on formation of NER of pharmaceuticals, in part already summarized by Eschenbach (2013)*

Compound	Fraction of NER formed (%)	Reference
Ciprofloxacin	88	Girardi, 2011
Diazepam	75 – 90	Richter et al., 2007
Diflocaxin	74	Junge et al., 2012
Difloxacin	60 – 65	Rosendahl et al., 2012
Fenbendazole	13	Kreuzig et al., 2007
Flubendazole	24	Kreuzig et al., 2007
Ibuprofen	30	Girardi, 2011
Ibuprofen	90 – 100	Richter et al., 2007
Sulfadiazin	>45	Förster et al., 2009
Sulfadiazin	20 – 30	Müller et al., 2013
Sulfadiazin	84 – 88	Junge et al., 2011
Sulfadiazin	92 – 104	Heise et al., 2006
Sulfamethoxazole	73 – 99	Heise et al., 2006

Conclusion: the pharmaceuticals triclosan, and possibly fluoxetine, as well as sulfamethoxazole, and possibly sulfadiazin or any of the other sulfonamide antibiotics, and the polycyclic musk Tonalide appear to be the most preferable candidates for preliminary screening.

Persistent organochlorine compounds

In the CEFIC project proposal by Harmsen (2014), it is indicated that for many 'older' non-degradable or only slowly degradable compounds like perfluorooctane sulfonate (PFOS), dieldrin, DDX and PCB's partitioning and sequestration will be predominant, and the lack of formation of covalent bonds makes the mechanism of sorption for these compounds different from what occurs for more reactive compounds like modern pesticides and pharmaceuticals. Failure to extract such compounds from spiked soil therefore is more likely indicative of the 'strength' of the extraction methods used, and is unlikely to be the result of transformation or metabolism of the parent compound into more reactive substances.

Relevant data for some persistent (mainly organochlorine) compounds are given in Table 6. Only compounds for which Stenzel et al. (2013) reported values of the log K_{ow} > 5 are given.

The PCB compounds are expected to be of very low toxicity to Dahnids and fish. In tests of short duration with aquatic organisms, these compounds exert mostly narcotic effects and are therefore of relatively low toxicity. Dillon and Burton (1991) tested the acute toxicity of 10 PCB's of varying log K_{ow} (PCB – 18 to PCB – 194) using *Daphnia magna* neonates and fry of *Pimephales promelas*. Only the PCB of lowest hydrophobicity, PCB – 18, was somewhat acutely toxic to these organisms, whereas the other PCB congeners did not cause effects at or near concentrations close to their aqueous solubility. These compounds are therefore not expected to be very toxic to soil organisms either, and are therefore expected to be of limited use in the preliminary screening of the formation of soil bound residues.

Heptachlor is known to be transformed relatively fast into heptachlor-epoxide, which is much more water soluble than the parent compound. The epoxide is very stable in soil. In view of the possible transformation into the epoxide, testing of this compound should preferably be done using heptachlor-epoxide. The acute toxicity of the parent compound to chironomids was investigated by Holcombe et al. (1983) and its 48-hour LC_{50} was above 2.5 mg/L in a water-only test.

DDT is transformed in alkaline solutions through hydrolysis into DDE, which is much less toxic to insects and presumably also less toxic to soil organisms than the parent compound. DDT is very stable in soil under temperate conditions (DT_{50} 4 – 30 years; Tomlin, 2003). This should make DDT a favourable compound for preliminary screening.

Dieldrin is an insecticide, which forms by *in vivo* metabolism in insects coming into contact with aldrin. It is a highly persistent organochlorine compound, strongly adhering to soil particles. It is quite toxic to aquatic organisms, according to Ikemoto et al. (1992) the LC50 values for daphnids and fish are below 0.1 mg/L. Similarly, Jarvinen et al. (1988) reported very high toxicity of endrin to fish, with a 96-hour LC50 of less than 0.001 mg/L for fathead minnows. Aldrin seems a less favourable compound for testing, because of experimental difficulties possibly resulting from its tendency to be transformed into dieldrin.

According to the EPA (www.epa.gov/fedfac/pdf/emerging_contaminants_pfos_pfoa.pdf) PFOS has a relatively high solubility in water of 570 mg/L in purified water and 370 mg/L in fresh water, which probably causes this compound to sorb less readily to soil particles than many of the other persistent organic chemicals in this section. For this reason this compound is less preferably for screening.

Table 6 *Persistent compounds and their toxicity to aquatic organisms*

Compound	CAS- number	Log K_{ow}[a]	LC_{50} (mg/L)		
			Other	Daphnids	Fish
Aldrin		6.50			
pp'-DDE		6.74			
pp'-DDT	50-29-3	6.64		0.0045[b]	0.018[b]
Cis-chlordane		6.10	1.44[c]		
Dieldrin		5.30		0.071[b]	0.048[b]
Endrin		5.20			0.0007[d]
Heptachlor-epoxide	1024-57-3	5.19 – 5.1[e]	>2.5[c]		
Hexachlorobenzene		5.52			
PCB-15		5.37	-	-	-
PCB-28		5.67		>Solubility	>Solubility
PCB-29		5.71			
PCB-52		5.91		>Solubility	>Solubility
PCB-101		6.59		>Solubility	>Solubility
PCB-153		7.16		>Solubility	>Solubility
PFOS	2795-39-3	Log K_{oc}=2.57[f]			

[a] Logarithm of the octanol-water partition coefficient, taken from Stenzel et al. (2013)

[b] Acute toxicity data, taken from Ikemoto et al. (1992)

[c] 48-hr LC50 for chlordane and heptachlor for chironomids, taken from Holcombe et al. (1983)

[d] 96-hr LC50 for fathead minnows taken from Jarvinen et al. (1988)

[e] Calculated value of 5.1 taken from: http://whqlibdoc.who.int/publications/2006/9241530707_eng.pdf

[f] Value taken from: http://www.efsa.europa.eu/en/scdocs/doc/653.pdf

Conclusion: The most favourable compounds for preliminary screening appear to be the very lipophilic and stable organochlorine compounds endrin, dieldrin and DDT, all of which appear to be very toxic to fish and aquatic invertebrates. Although quite stable, polychlorinated biphenyl congeners are expected not to be very toxic to soil organisms which makes them less favourable for preliminary screening.

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Annex 2 Results in ecotoxicological tests

The following tables summarize the results. In the first column the bioavailable fraction of the contamination is listed followed by the medium which is used for the test (e.g. soil, eluate). $Tenax_{1week}$ residue means that the soil was extracted with Tenax for one week. The extract was removed and the soil used for testing regarding the potential ammonium oxidation activity (soil microflora). 0.01% $CaCl_2$ is the eluate of the $CaCl_2$ extraction used for aquatic tests with daphnids or luminescent bacteria. $Tenax_{20}$ means that the fraction extracted by Tenax is solubilized in a suitable organic solvent, transferred in water and measured in aquatic ecotox assays. Control refers to the control soil. The next columns refer to the data obtained with the soil or eluate of the set-up with radiolabelled compound or with the unlabelled compound.

Test substance: TNT Test soil: RefeSol 01A

Test start: September 7, 2015

Bioavailability	Medium	Test system	Control	TNT			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total	Soil	Earthworm	10 ± 0% of worms	---	---	0 ± 0	100
availability		Soil microflora	26.0 ± 7.9 ng/gTM/h	18.6 ± 7.8 ng/gTM/h	29	13.3 ± 4.7 ng/gTM/h	49
Remaining availability	$Tenax_{1\ week}$ residue	Soil microflora	100.8 ± 4.6 ng/gTM/h	97.0 ± 11.3 ng/gTM/h	4	---	---
Actual bioavailable	0.01 $CaCl_2$	Luminescent bacteria	No effect even at highest test concentration (80% eluate)	Highest test concentration (80% eluate): EC_{50}: 8.3% eluate	93%	---	---
Potentially bioavailable	$Tenax_{20}$	Luminescent bacteria	Highest test concentration: 100% 6.25% eluate: 64%	Highest test concentration 6.25% eluate	100 78	---	---

Test end: March 9, 2016

Bioavailability	Medium	Test system	Control	TNT			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total	Soil	Earthworm	3.4 ± 1.5 of worms	---	---	6.6 ± 1.5 of worms	0
availability		Soil microflora	23.0 ± 1.9 ng/gTM/h	2.7 ± 1.0	88	0	100
Remaining availability	$Tenax_{1\ week}$ residue	Soil microflora	3.91 ± 0.99 ng/g TM/h (nearly no activity)	0.51 ± 1.02 ng/g TM/h	87	---	---
Actual bioavailability	0.01 $CaCl_2$	Luminescent bacteria	20.5% effect at highest test concentration (80% eluate)	Highest test concentration (80% eluate)	42.5	---	---

Test substance: TNT Test soil: RefeSol 02A

Test start: September 22, 2015

Bioavailability	Medium	Test system	Control	TNT			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total	Soil	Earthworm	10 ± 0% of worms	---	---	0 ± 0	100
availability		Soil microflora	58.7 ± 9.6 ng/gTM/h	3.4 ± 2.4 ng/gTM/h	94	2.4 ± 4,0 ng/gTM/h	94
Remaining availability	$Tenax_{1\ week}$ residue	Soil microflora	134.5 ± 13.2 ng/gTM/h	73.7 ± 4.1 ng/gTM/h	45	---	---
Actual bioavailability	0.01 $CaCl_2$	Luminescent bacteria	No effect even in highest test concentration (80% eluate)	EC_{50}: 12.5% eluate 79% inhibition in highest test concentration (80% eluate)		---	---

Test end: April 04, 2016

Bioavailability	Medium	Test system	Control	TNT			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total	Soil	Earthworm	5.0 ± 3.5 of worms	---	---	5.0 ± 3.5 of worms	0
availability		Soil microflora	165.8 ± 4.3 ng/gTM/h	49.7 ± 5.9 ng/gTM/h	70	102.1 ± 11.4 ng/gTM/h	38
Remaining availability	$Tenax_{1\ week}$ residue	Soil microflora [1]	103.6 ± 2.2 ng/gTM/h	31.9 ± 11.6 ng/gTM/h	69	---	---
Actual bioavailable	0.01 $CaCl_2$	Luminescent bacteria	17% effect in highest test concentration	17% effect in highest test concentration		---	---

[1] It cannot be excluded, that the low microbial activated indicated that TNT inhibited the microflora and that the microflora was not able to recover. Therefore, the low activity would be no indicator for the still available contaminants. To prove this hypothesis an additional experiment was performed in the scope of the set-up with soil 03-G. To prove this hypothesis, the incubated soil was mixed with fresh soil. In these set-ups the microbial activity was increased and no inhibition was observed. Therefore, the hypothesis was proven.

Test substance: TNT Test soil: RefeSol 03G

Test start: October 07, 2015

Bioavailability	Medium	Test system	Control	TNT			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total availability	Soil	Earthworm	10 ± 0% of worms	---	---	0 ± 0	100
		Soil microflora	76.4 ± 17.6 ng/gTM/h	9.3 ± 10.6 ng/gTM/h	88	33.7 ± 30 ng/gTM/h	55
Remaining availability	$Tenax_{1\ week}$ residue	Soil microflora	154.5 ± 13.5 ng/gTM/h	176.3 ± 10.2 ng/gTM/h	-14		
Water soluble bioavailability	0.01 $CaCl_2$	Luminescent bacteria	No effect even in highest test concentration (80% eluate)	EC_{50}: 25% eluate 79% inhibition in highest test concentration (80% eluate)		---	---

Test end: April 18, 2016

Bioavailability	Medium	Test system	Control	TNT			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total availability	Soil	Earthworm	5.8 ± 1.5 of worms	---	---	4.2 ± 1.5 of worms	16
		Soil microflora [1]	365.1 ± 9.6 ng/gTM/h Experiment 2 - mixture of 50% fresh control soil + 50% treated control soil: 538.7 ± 31.3 ng/gTM/h mixture of 25% fresh control soil + 75% treated ^{14}C soil: 408.9 ± 39.4 ng/gTM/h	180.3 ± 27.2 ng/gTM/h Experiment 2 - mixture of 50% fresh control soil + 50% treated ^{14}C soil 433.3 ± 39.1 ng/gTM/h mixture of 25% fresh control soil + 75% treated ^{14}C soil: 331.8 ± 43.5 ng/gTM/h	51 13 19	203.1 ± 36.9 ng/gTM/h	44
Remaining availability	$Tenax_{1\ week}$ residue	Soil microflora	196.4 ± 10.3 ng/gTM/h	47.4 ± 12.5 ng/gTM/h	76	---	---
Water soluble bioavailability	0.01 $CaCl_2$	Luminescent bacteria	14% inhibition	17% inhibition (comparable to control)		---	---

[1] It was assumed, that the low microbial activated indicated that TNT inhibited the microflora and that the microflora was not able to recover. Therefore, the low activity would be no indicator for the still available contaminants. To prove this hypothesis, the incubated soil was mixed with fresh soil. In these set-ups the microbial activity was increased and no inhibition was observed. Therefore, the hypothesis was proven.

Conclusion – TNT

There is an obvious reduction in toxicity during the incubation period.

Test substance: **Cypermethrin** **Test soil:** **RefeSol 01A**

Test start: November 2015

Bioavailability	Medium	Test system	Control	Cypermethrin ^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total	Soil	Earthworm	9.2 ± 0.8	---	---	0.6 ± 0.5	94
availability		Soil microflora [1]	2.14 ± 6.1 ng/gTM/h	2.79 ± 6.6 ng/gTM/h	-30	2.74 ± 2.38 ng/gTM/h	-28
Water soluble bioavailability	0.01 $CaCl_2$	Daphnids [2]	0% inhibition	EC_{50}: 17.8% eluate [11.8 - 25.0]		---	---

Test end: July 2016

Bioavailability	Medium	Test system	Control	Cypermethrin ^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total availability	Soil	Earthworm	8.6 ± 1.4	---	---	1.4 ± 1.4	72
Water soluble bioavailability	0.01 $CaCl_2$	Daphnids	0% inhibition	EC_{50}: < 6.25% eluate (6.25% eluate: 70% inhibition)		---	---

Test substance: Cypermethrin Test soil: RefeSol 02A

Test start: November 2015

Bioavailability	Medium	Test system	Control	Cypermethrin ^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total	Soil	Earthworm		---	---	0.8 ± 0.8 worms in test soil	84
availability		Soil microflora [1]	0 ng/g dm/h	7.4± 11.2 ng/g dm/h	-	60.0± 5.8 ng/g dm/h	-
Water soluble bioavailability	0.01 $CaCl_2$	Daphnids	No effect	Highest test concentration (100% eluate): EC_{50}: 38.2% eluate [11.1 - 1064.9]	95%	---	---

Test end: August 2016

Bioavailability	Medium	Test system	Control	Cypermethrin ^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total availability	Soil	Earthworm	0.6 ±0.09 Repetition: 8.0 ± 1.0 [1]	---	---	9.4 ± 0.9 (contaminated soil pH: 5.5 Control: pH 4.5) Repeated experiment (three replicates) with adjusted pH (contaminated soil pH: 5.5 Control: pH 6.0): 2.0 ± 1.0	0 Repeated experiment: 60%
Water soluble bioavailability	0.01 $CaCl_2$	Daphnids	100% eluate: 100% inhibition; 50% eluate: no inhibition	EC_{50}: < 6.25% eluate[2] 50% eluate	(6.25%: 65% inhibition) 95%	---	---
Remaining availability	0.01 $CaCl_2$ of Tenax 2 extracted soil	Daphnids	No effect	100% eluate: 50% eluate EC_{50}: 28.6% eluate [14.0 – 67.8]	70% 55%	---	---

[1] It was assumed that the avoidance of the control soil was based on the low pH. Therefore, the test was repeated with an adapted pH. In the repetition the contaminated soil was avoided. This result corresponds to the results determined with soil 03-G.

[2] It was assumed that the toxicity of Cypermethrin on daphnids is so high that the remaining concentration in the soil pore water causes the toxicity. Therefore, the Tenax 2 extracted soil was eluated again using $CaCl_2$ solution; it was expected that no water soluble Cypermethrin was present. Toxicity was lower, however, still present.

Test substance: **Cypermethrin** **Test soil:** **RefeSol 03G**

Test start: February 2016

Bioavailability	Medium	Test system	Control	Cypermethrin ^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total	Soil	Earthworm	9.6 ± 0.5 of worms			0.4 ± 0.5 of worms	92
availability		Soil microflora [1]	211.8 ± 16.6 ng/g dm/h	197.8 ± 37.5 ng/gTM/h	7	39.0 ± 5.3 ng/g dm/h	82
			684.1 ± 34.6 ng/g dm/h	256.8 ± 31.1	62	724.6 ± 21.1 ng/gTM/h	-6
Remaining availability	Tenax 2 residue	Soil microflora	281.2 ± 39.4 ng/g dm/h	381.8 ± 18.6 ng/gTM/h	-36 (=stimulation)	---	---
Decreasing bioavailability	0.01 $CaCl_2$	Daphnids	No effect	Highest test concentration (100% eluate): EC_{50}: 17.3% eluate [7.2 – 29.3]	100%	---	---

Test end: August, 2016

Bioavailability	Medium	Test system	Control	Cypermethrin ^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total availability	Soil	Earthworm [1]	9.8 ± 0.4 of worms	---	---	0.2 ± 0.4	96
Decreased bioavailability	0.01 $CaCl_2$	Daphnids	25% effect in highest test concentration	Highest test concentration (100% eluate): EC_{50}: < 6.25% eluate (6.25% lowest test concentration)	90%	---	---

[1] pH of control soil and treated soil comparable (control soil: 5.7; Cypermethrin: 5.5)

Conclusion - Cypermethrin:

Cypermethrin has a very low water solubility; the results indicate that this amount is highly toxic. At test end, a high toxicity is still observed. The amount of remaining test substance is sufficiently high to exceed the water solubility and to cause the toxicity.

Test substance: Carbendazim Test soil: RefeSol 01A

Test start: June 2016

Bioavailability	Medium	Test system	Control	Carbendazim			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total availability	Soil	Earthworm	6.0 ± 1.4 worms in soil	---	---	4.0 ± 1.4 worms in soil	20
Water soluble bioavailability	0.01 $CaCl_2$	Daphnids [1]	0% immobilization	EC_{50}: 7.4% [4.5 – 9.8] [1]		---	---

Test end: December 2016

Bioavailability	Medium	Test system	Control	Carbendazim			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total availability	Soil	Earthworm	5.4 ± 0,9 worms in soil	---		4.6 ± 0,9 worms in soil	8
Water soluble bioavailability	0.01 $CaCl_2$	Daphnids	0% immobilization	EC_{50} > 100% eluate; 35% inhibition in highest test concentration (100% eluate)		---	---

Test substance: Carbendazim Test soil: RefeSol 02A

Test start: June 2016

Bioavailability	Medium	Test system	Control	Carbendazim			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total availability	Soil	Earthworm	5.2 ± 1.5 One outlier – without this outlier: 5.7 ± 1.0	---	---	4.8 ± 1.5 One outlier – without this outlier: 4.3 ± 1.0	4 12
Water soluble bioavailability	0.01 $CaCl_2$	Daphnids	10% inhibition (2 animals dead; validity criterium fulfilled; immobilization in control ≤ 10%)	EC_{50} < 6.25% (6.25% eluate = lowest test concentration; 75% inhibition)		---	---

Test end: January 2017

Bioavailability	Medium	Test system	Control	Carbendazim			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total availability	Soil	Earthworm	3.8 ± 1.1			6.2 ± 1.1	
Water soluble bioavailability	0.01 $CaCl_2$	Daphnids [1]	40% inhibition in highest test concentration (100% eluate); validity criterium fulfilled; immobilization in control ≤ 10%)	EC_{50} > highest test concentration; 20% inhibition in highest test concentration (100% eluate); validity criterium fulfilled; immobilization in control ≤ 10%)		---	---

Test substance: Carbendazim Test soil: RefeSol 03G

Test start: July 2016

Bioavailability	Medium	Test system	Control	Carbendazim			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total availability	Soil	Earthworm	6.8 ± 2.2 worms in test soil One outlier – without this outlier: 7.5 ± 1.7	---	---	3.2 ± 2.2 worms in test soil One outlier – without this outlier: 2.5 ± 1.7	28 32%
Water soluble bioavailability	0.01 $CaCl_2$	Daphnids	10% inhibition (2 animals dead; validity criterium fulfilled; immobilization in control ≤ 10%)	EC_{50} < 6.25% (6.25% eluate; 65% inhibition)		---	---

Test end: February 2017

Bioavailability	Medium	Test system	Control	Carbendazim			
				^{14}C		cold	
			Mean ± SD	Mean ± SD	% inhibition / avoidance	Mean ± SD	% inhibition / avoidance
Total availability	Soil	Earthworm	5.2 ± 0.8 worms in test soil	---	---	4.8 ± 0.8 worms in test soil	
Water soluble bioavailability	0.01 $CaCl_2$	Daphnids [1]	Max 35% inhibition; validity criterium fulfilled; immobilization in control ≤ 10%)	EC_{50} > 100% eluate; max 25% inhibition; validity criterium fulfilled; immobilization in control ≤ 10%)		---	---

Conclusion - Carbendazim:

The concentration of bioavailable contaminants detected by daphnids decreased during the incubation period. Carbendazim is the recommended reference substance in the earthworm reproduction test. However, the earthworms have no sensors for Carbendazim or Carbendazim is not the toxic compound but a metabolite and therefore, the avoidance test is less suitable for the present subject.

Annex 3 Potential organic solvents for extraction of bioavailable residues of TNT, benzo(a)pyrene, carbendazim and cypermethrin from soils/plants

Overview was provided by Charles Eadsforth.

2,4,6-trinitrotoluene (TNT)

Solvent used	Method	Recovery	Study type	Reference
Acetonitrile	Samples (~40 g) were placed into each thimble and extracted for 5 h with 175 ml of acetonitrile. The acetonitrile was removed and replaced with fresh acetonitrile for two subsequent stages of extraction of 4 h. Analysis was via HPLC. "Use of a solvent aggregate ratio of 4–5 ml/g insures efficient one-stage extraction of over 90% for either Soxhlet or ultrasonic methods."	Unspecified (but presumably good) "Use of a solvent aggregate ratio of 4–5 ml/g insures efficient one-stage extraction of over 90% for either Soxhlet or ultrasonic methods."	Soxhlet extraction	Williford and Bricka, 1999
	A three stage ultrasonic extraction was first performed with 50 g of aggregate and 250 ml of acetonitrile in each of two, 1 L centrifuge bottles. The bottles were sonicated for 18 h, centrifuged, and decanted of solvent. Two subsequent extractions were performed for 2–4 h periods, and the extracts for all three stages were analyzed separately. HPLC results indicated that the TNT was effectively removed in the first two stages, and the third stage extraction was eliminated for subsequent samples. "Use of a solvent aggregate ratio of 4–5 ml/g insures efficient one-stage extraction of over 90% for either Soxhlet or ultrasonic methods."	1st extraction: 94.5% 2nd extraction: 5.1% 3rd extraction: 1.1%	Ultrasonic extraction	
	Both acetonitrile and deionized water (DW) were used for extraction and quantified by HPLC. 10 ml of acetonitrile or DW was added to moist soil (equivalent to 2 g dry wt.) and was vortexed for 1 min at full speed. Then the sample was sonicated for 18 h in the dark at 21°Cn then allowed to settle for 30 min. 5 ml supernatant was removed and combined with 5 ml of 0.5% (w/v) CaCl2. The sample was filtered through 0.45 mm after vortexing (a few seconds) and settling for another 30 min. Concentrations of nitroaromatics in both water and acetonitrile extracts were measured using HPLC. Acetonitrile was considered a "mild extractant"	TNT (acetonitrile extractable) recovered after 1 week varied from 4% at 25 mg TNT/kg to 82% at 1,000 mg TNT/kg. If both TNT and its metabolites Were summated, the	U.S. EPA Method 8330	Gong et al., 1999

Solvent used	Method	Recovery	Study type	Reference
		total recovery was between 18% and 86%, but most recoveries were below 60%.		
	TNT was extracted from soil with acetonitrile (>95% efficiency) in an ultrasonic bath. The supernatant was passed through 0.2-µm filters and analyzed by HPLC using EPA method 9330.	">95% efficiency"	Ultrasonic extraction	Robertson and Jjemba, 2005
	Plant samples were heated at 100°C to remove water, and extracted in 10-mL acetonitrile by an 18-h sonication in a water-cooled bath at 15°C. The extracts were freed from particles by centrifugation for 10 min at 2,000 g. HPLC analysis of the extracts was performed after cutting the supernatants 1:1 with Millipore-filtered RO water, recentrifugation, and clean-up over a 0.5-g Florisil column. For soil, 2 g (fresh weight) samples was extracted in 10 mL of acetonitrile by 18-h sonication at 15 °C, clean-up over a Florisil column, and 10x concentration.	Plant recovery: 46.4 ± 6.7% *Soil recovery*: 10.4 ± 0.2%	Sonication extraction	Best et al., 2004
	TNT quantification used modifications of the U.S. EPA method 8330B for soils. Extractions in soil were determined by extracting a 2-g fresh weight aliquot in 10 ml of acetonitrile by 18-h sonication at 15°C, cleanup over a Florisil column, and 10 x concentration. Analysis used HPLC.	Recovery of 4NT ranged from 65 to 95%	Sonication extraction	Best et al., 2008
	Soil samples are air dried (at room temperature or colder, away from direct sunlight). Dried samples are grinded and homogenised thoroughly in an acetonitrile-rinsed mortar to pass a 30 mesh sieve. 2 g subsamples of each soil sample are placed in a 15 ml glass vial, and 10 ml acetonitrile added. Vial is capped and vortex swirled for 1 min, and placed in a cooled ultrasonic bath for 18 h. After sonication, sample is allowed to settle for 30 min. 5 ml of supernatant is removed and combined with 5 ml calcium chloride solution in a 20 ml vial. Shake and let stand for 15 min. Supernatant is placed in a disposable syringe and filtered through a 0.45 µm Teflon filter. Discard first 3 ml and retain remainder in a Teflon-capped vial for RP-HPLC analysis.	N/A	Sonication extraction	US EPA, 1997
Hexane:Acetone (1:1)	Approximately 10 g of sample was placed in a pre-weighed cellulose thimble with a glass wool plug on top of the contents in each extraction thimble. Fifty ml of a 1:1 (v/v) mixture of hexane:acetone was poured into pre-weighed extraction cups and heated to 140°C. Solvent was allowed to boil and then thimbles were immersed into boiling solvent for 60 min and then rinsed for an additional 60 min. The solvent was allowed to evaporate and collect in the condenser. Extraction cups containing the sample extracts were removed and allowed to air dry. Sample residue was transferred with a glass pipet and several volumes of solvent into a pre-weighed culture tube. The culture tube was placed under a stream of nitrogen gas, weighed, and then the extract was redissolved in DMSO. Extraction efficiencies assume a 100% extraction rate from the solvent extracts.	Soils spiked with B(a)P and TNT resulted in a recovery of 29% TNT for both soil types at pH2, and 23 and 17% for clay soil and sandy soil at pH7, respectively. Thus, the recovery of the various chemicals appeared to be influenced greatly by the physical characteristics of the soil.	Soxhlet extraction	Bordelon et al., 1989

Solvent used	Method	Recovery	Study type	Reference
Acetone	Solvent usage: 17 to 50 ml per sample (2.5 to 30 g) Extraction time: 14 min Data demonstrated that ASE is equivalent or superior to sonication and Soxhlet extraction for the determination of explosives and their metabolites in soils. ASE provides these results in a short period of time (less than 15 min per sample) and with minimum solvent usage (less than 15 ml for a 10 g sample).	98.3% recovery (3.5% RSD)	Accelerated solvent extraction (ASE) and sonication	Dionex, 2011
Methanol	Solvent: 10 ml methanol, Sample size: 10 g soil samples, Extraction time: 15 min.	Unspecified	Sonication	
	Solvent: 150 ml methanol, Sample size: 50 g soil samples, Extraction time: 2 h	Unspecified	Sonication	
	Solvent usage: 17 to 50 ml per sample (2.5 to 30 g) Extraction time: 14 min Data demonstrated that ASE is equivalent or superior to sonication and Soxhlet extraction for the determination of explosives and their metabolites in soils. ASE provides these results in a short period of time (less than 15 min per sample) and with minimum solvent usage (less than 15 ml for a 10 g sample).	98.3% recovery (3.5% RSD)	Accelerated solvent extraction (ASE) and sonication	
	200 ml methanol used for 10 g (w/w) soil sample extractions. Soil extracts were filtered through a 0.22-µm Nylon 66 filter, and the volume was then reduced to approximately 20 ml by rotary evaporation. After the concentrated extract was again filtered through a 0.22-µm filter, the final volume was adjusted to a total of 25 ml. Extracted soils were dried at **105°C** overnight, cooled in a desiccator, and weighed to obtain an accurate oven-dry weight. Portions of the extracted soils were further analyzed by total combustion in a Packard Model 306 oxidizer to determine the amount of irreversibly bound TNT residues not removed by Soxhlet extraction. The extraction efficiency of the procedure was determined by comparing the amount of radiolabel contained in the final methanol extract with the amount originally added to the soil (both values were determined by liquid scintillation spectrometry). The methanol extract was subsequently analyzed for TNT and transformation products by HPLC.	0 days: 89 to 101% radiolabelled in methanol extract 10 days: 48 to 71% radiolabelled in methanol extract 61 days: 37 to 60% radiolabelled in methanol extract	Soxhlet extraction	Cataldo et al., 1989
Methanol : Acetonitrile (1:1)	An ASE 200 extractor was used. Extraction began with the filling of extraction cells (containing the sample) used methanol–acetonitrile (1:1, v/v), then a 5-min preheating time, followed by a 5-min static extraction. The extract was flushed out of the system with a 60-s nitrogen purge into a glass collection vial. The sample extract (~ 30 mL) was concentrated with a rotary evaporator and brought to 4–5 mL for GC–MS or LC–MS determination.	1 day samples: 74 ±5% 2 months samples: 47 ±2% 6 months samples: 54 ±1%	Accelerated solvent extraction (ASE)	Campbell et al., 2003

Benzo(a)pyrene

Solvent used	Method	Recovery	Study type	Reference
Dichloromethane (DCM)	Soxhlet extractions used 10 g soil samples to which 10 g of anhydrous sodium sulfate was added. The mixture was transferred into a cellulose extraction thimble and inserted into a Soxhlet assembly with a 250 ml flask. 150 ml of dichloromethane was added and the whole assembly heated for 24 h using an isomantle. The extracts were concentrated to 10 ml using a rotary evaporator and then diluted two fold before the addition of the internal standards.	Soxhlet extraction mean: 39 (23% RSD)	Soxhlet extraction	Saim et al. 1997
	70 ml DCM were added to 2 g soil samples. Extraction was done using 99% of the microwave's power (maximum power: 300 W) for 20 min. The extract was then quantitatively transferred through GF/A glass microbore filter. Extracts were then concentrated to 5 ml using a rotary evaporator before the addition of internal standards.	Atmospheric microwave-assisted extraction mean: 49 (10% RSD)	Atmospheric microwave-assisted extraction (MAE)	
	Soil extraction (12.5 g dry weight) with methylene chloride (200 ml) was performed by first drying the soil with anhydrous sodium sulfate, followed by extraction in a Soxhlet apparatus for 8 h (70 cycles). Extracts were analyzed by liquid scintillation counting.	Unspecified	Soxhlet extraction	Kanaly and Bartha, 1999
	Soils were extracted by adding 100 g of sodium sulfate (drying agent) and 100 ml of methylene chloride to the respective 250 ml Quarpak glass jars and by rolling them for 12 h at ≈100 rpm.	Unspecified	-	Huesemann, et al., 2004
	Extractions used ASE 200 accelerated solvent extractor. Samples (7 g) were accurately weighed into the 11 mL cells. The sample cells were then closed to finger tightness and placed into the carousel of the ASE 200 system. Solvent was added. Extracted analytes were purged from the sample cell using pressurized nitrogen. The extracts were concentrated to 5 mL using a rotary evaporator and then diluted 2-fold before the addition of internal standards.	45.1 mg kg^{-1} recovered	Accelerated solvent extraction (ASE)	Saim et al. 1998
Acetone	40 ml of acetone were added to 2 g soil samples. New rupture membranes were fitted into each cap which screwed onto the vessels. Vessels were then placed symmetrically on the microwave turntable together with the control containing the temperature and pressure sensory equipment. The magnetron power was set at 30%, with a constant temperature of 120°C for an extraction time of 20 min. After a cooling period, extracts were concentrated to 5 ml using a rotary evaporator before the addition of internal standards.	Pressurised Microwave-assisted extraction mean: 32 (17% RSD)	Pressurised MAE	Saim et al. 1997
	Extractions used ASE 200 accelerated solvent extractor. Samples (7 g) were accurately weighed into the 11 mL cells. The sample cells were then closed to finger tightness and placed into the carousel of the ASE 200 system. Solvent was added. Extracted analytes were purged from the sample cell using pressurized nitrogen. The extracts were concentrated to 5 mL using a rotary evaporator and then diluted 2-fold before the addition of internal standards.	33.1 mg kg^{-1} recovered	ASE	Saim et al. 1998
	50 g soil samples inoculated with 5 mL of stock solution B[a]P in acetone (1000 mg/l) to yield a soil concentration of 100 mg/kg. After complete evaporation of the acetone (~12 h) in a chemical hood, 100 ml of solvent were added to the soil. Soil slurries were shaken overnight on a rotary shaker at 150 rpm, in the dark, at room temperature. The soil-solvent slurry was then passed through filter paper containing 5 g sodium sulfate, washed with an additional 2 x 50 mL aliquots of the solvent, and pooled solvent extracts were dried in a chemical hood in the dark and re-suspended in 10 ml acetone.	58% recovery	Standard solvent extraction	Numergut et al. 2000

Solvent used	Method	Recovery	Study type	Reference
Methanol	1 g soil samples were used, with 20% concentration of methanol. (The following conditions were used: pressure: 250 kg cm^{-2}, temperature:70°C, 30 min dynamic extraction time preceded by a 5 min static period, flow-rate: 2 ml min^{-1}). Two portions of the 1 g extracts were combined and then concentrated to 5 ml using a rotary evaporator before the addition of internal standards.	Supercritical fluid extraction mean: 45 (10% RSD)	Supercritical fluid extraction (SFE)	Saim et al. 1997
	Extractions used ASE 200 accelerated solvent extractor. Samples (7 g) were accurately weighed into the 11 mL cells. The sample cells were then closed to finger tightness and placed into the carousel of the ASE 200 system. Solvent was added. Extracted analytes were purged from the sample cell using pressurized nitrogen. The extracts were concentrated to 5 mL using a rotary evaporator and then diluted 2-fold before the addition of internal standards.	24.2 mg kg^{-1} recovery	ASE	Saim et al. 1998
	50 g soil samples inoculated with 5 mL of stock solution B[a]P in acetone (1000 mg/l) to yield a soil concentration of 100 mg/kg. After complete evaporation of the acetone (~12 h) in a chemical hood, 100 ml of solvent were added to the soil. Soil slurries were shaken overnight on a rotary shaker at 150 rpm, in the dark, at room temperature. The soil-solvent slurry was then passed through filter paper containing 5 g sodium sulfate, washed with an additional 2 x 50 mL aliquots of the solvent, and pooled solvent extracts were dried in a chemical hood in the dark and re-suspended in 10 mL acetone.	11% recovery	Standard solvent extraction	Numergut et al. 2000
Dichloromethane-Acetone (1:1)	Samples (7 g) were placed into the ASE system. Extractions used dichloromethane-Acetone (1:1). Extracted analytes were purged from the sample cell using pressurised nitrogen (125 - 150 psi). Extracts were concentrated to 5 ml using a rotary evaporator and the diluted two-fold before the addition of internal standards.	Accelerated solvent extraction mean: 33 (18% RSD)	ASE	Saim et al. 1997
	Extractions used ASE 200 accelerated solvent extractor. Samples (7 g) were accurately weighed into the 11 mL cells. The sample cells were then closed to finger tightness and placed into the carousel of the ASE 200 system. Solvent was added. Extracted analytes were purged from the sample cell using pressurized nitrogen. The extracts were concentrated to 5 mL using a rotary evaporator and then diluted 2-fold before the addition of internal standards.	41.3 mg kg^{-1} recovered		Saim et al. 1998
	10 g soil sample. Extraction was performed with 1:1 mixture of DCM and acetone at 140°C and 1500 psi for 6 min heat-up followed by a 5 min static extraction. The vessel was then rinsed with 17 mL of the same solvent and the extracted analytes were purged from the sample cell using pressurized N_2 at 1500 psi. The whole procedure for one sample needed ~30 min. After extraction, the sample went through the clean-up procedure.	110.39% recovery (10.19% RSD)	ASE	Wang et al., 2007
	50 g soil samples inoculated with 5 mL of stock solution B[a]P in acetone (1000 mg/l) to yield a soil concentration of 100 mg/kg. After complete evaporation of the acetone (~12 h) in a chemical hood, 100 ml of solvent were added to the soil. Soil slurries were shaken overnight on a rotary shaker at 150 rpm, in the dark, at room temperature. The soil-solvent slurry was then passed through filter paper containing 5 g sodium sulfate, washed with an additional 2 x 50 mL aliquots of the solvent, and pooled solvent extracts were dried in a chemical hood in the dark and re-suspended in 10 mL acetone.	64% recovery	Standard solvent extraction	Numergut et al. 2000

Solvent used	Method	Recovery	Study type	Reference
Hexane:Acetone (1:1)	Approximately 10 g of sample was placed in a pre-weighed cellulose thimble with a glass wool plug on top of the contents in each extraction thimble. Fifty ml of a 1:1 (v/v) mixture of hexane:acetone was poured into pre-weighed extraction cups and heated to 140°C. Solvent was allowed to boil and then thimbles were immersed into boiling solvent for 60 min and then rinsed for an additional 60 min. The solvent was allowed to evaporate and collect in the condenser. Extraction cups containing the sample extracts were removed and allowed to air dry. Sample residue was transferred with a glass pipet and several volumes of solvent into a pre-weighed culture tube. The culture tube was placed under a stream of nitrogen gas, weighed, and then the extract was redissolved in DMSO. Extraction efficiencies assume a 100% extraction rate from the solvent extracts.	Samples spiked with B(a)P showed a recovery of 24 and 13% for pH7 and pH2, respectively.	Soxhlet extraction	Bordelon et al., 1989
	Soil (10 g dry weight equivalent) was mixed with anhydrous sodium sulfate (40 g), placed in a Soxhlet apparatus and refluxed for 14 h with solvent (150 ml acetone+150 ml hexane). After cooling, the solvent was shaken for 30 s with dw (300 ml). After 5 min, the lower aqueous layer was discarded and the extract washed twice with dw (300 ml). The washed organic phase was dried using anhydrous sodium sulfate (10 g) and was reduced under nitrogen to give a column load of 50-100 mg. The extract was fractionated using a glass column, wet-packed with 2 g silica gel 60 as the lower layer and 1 g alumina as the upper layer in hexane. The column was washed with hexane (20 ml) before adding the extract.	78.91 to 84.96% recovery		Smith et al., 1999
	Soxhlet extraction was performed using 5g portion of soil to which 5g of anhydrous sodium sulfate was added. The mixture was transferred into a clean filter paper and inserted intoa Soxhlet assembly. A 100mL portion of n-hexane and acetone (1:1, v/v) was added and the whole assembly was heated for 15h. After the extraction, the sample went through the clean-up procedure.	84.83% recovery (11.55% RSD)	Soxhlet extraction	Wang et al., 2007
	Portions of 5g soil were used, and 25 ml of solvent was used. Extraction temperature was 110°C, microwave power was 1200 W (100%). After the extraction completed, soil and solvent were separated by filtration and the solvent was decanted into a pear-shaped flask. The sample went through the clean-up procedure.	126.11% recovery (3.73% RSD)	MAE	
	Extractions used ASE 200 accelerated solvent extractor. Samples (7 g) were accurately weighed into the 11 mL cells. The sample cells were then closed to finger tightness and placed into the carousel of the ASE 200 system. Solvent was added. Extracted analytes were purged from the sample cell using pressurized nitrogen. The extracts were concentrated to 5 mL using a rotary evaporator and then diluted 2-fold before the addition of internal standards.	40.7 mg kg^{-1} recovered	ASE	Saim et al. 1998
	A 0.1–2 g aliquot of soil was weighed and 10 ml of hexane:acetone (1:1) added. The extraction vessel was closed, after ensuring that a new rupture membrane was used for each extraction. 3–6 samples were extracted simultaneously at 115°C for 20 min at 100% power. After cooling, the supernatant was filtered through a 45 µm nylon disk and made to a known volume. In the case of low analyte concentration, concentration by nitrogen blow-down was necessary before analysis.	not specified (however it was concluded that ASE was a better method)	Closed-vessel MAP™ extraction procedure	Li et al. 2003
	A 0.1–2 g aliquot of soil was weighed. Samples were extracted with 5 ml aliquots of hexane:acetone (1:1) for 20 min and the process was repeated two more times with fresh solvent (3×5 ml total). The combined	not specified (however it was concluded that	Ultrasonic extraction	

Solvent used	Method	Recovery	Study type	Reference
	extract was filtered and analysed without any clean-up. In some cases concentration by rotary evaporation and nitrogen blow-down was necessary.	ASE was a better method)		
	A 0.1–2 g aliquot of soil was weighed. For most of the extractions, a polar solvent as a modifier was added just before extraction. consisted of 15 min static extraction at 100 atm and 50–150°C. The solvent used was hexane (however, adding acetone as a polar modifier resulted in higher recoveries). After extraction, the raw extract was collected as noted above The extract was analysed directly or after concentration by nitrogen blow-down.	80 to 116% recovery ASE was concluded to be a better method that MAP or sonication methods	ASE (recoveries found to be comparable to MAP™ extraction, and superior to sonication)	
Acetonitrile	Extractions used ASE 200 accelerated solvent extractor. Samples (7 g) were accurately weighed into the 11 mL cells. The sample cells were then closed to finger tightness and placed into the carousel of the ASE 200 system. Solvent was added. Extracted analytes were purged from the sample cell using pressurized nitrogen. The extracts were concentrated to 5 mL using a rotary evaporator and then diluted 2-fold before the addition of internal standards.	32.9 mg kg^{-1} recovered	ASE	Saim et al. 1998
	50 g soil samples inoculated with 5 mL of stock solution B[a]P in acetone (1000 mg/l) to yield a soil concentration of 100 mg/kg. After complete evaporation of the acetone (~12 h) in a chemical hood, 100 ml of solvent were added to the soil. Soil slurries were shaken overnight on a rotary shaker at 150 rpm, in the dark, at room temperature. The soil-solvent slurry was then passed through filter paper containing 5 g sodium sulfate, washed with an additional 2 x 50 mL aliquots of the solvent, and pooled solvent extracts were dried in a chemical hood in the dark and re-suspended in 10 mL acetone. (Acetonitrile : H_2O (1:2) was used in this study)	54% recovery	Standard solvent extraction	Numergut et al. 2000
Hexane	Extractions used ASE 200 accelerated solvent extractor. Samples (7 g) were accurately weighed into the 11 mL cells. The sample cells were then closed to finger tightness and placed into the carousel of the ASE 200 system. Solvent was added. Extracted analytes were purged from the sample cell using pressurized nitrogen. The extracts were concentrated to 5 mL using a rotary evaporator and then diluted 2-fold before the addition of internal standards.	34.3 mg kg^{-1} recovered The poor recoveries obtained for hexane are attributed to its lower polarity.	ASE	Saim et al. 1998
	A 0.1–2 g aliquot of soil was weighed. For most of the extractions, a polar solvent as a modifier was added just before extraction. consisted of 15 min static extraction at 100 atm and 50–150°C. The solvent used was hexane. After extraction, the raw extract was collected as noted above The extract was analysed directly or after concentration by nitrogen blow-down.	Adding acetone as a polar modifier resulted in higher recoveries. ASE was concluded to be a better method that MAP or sonication methods	ASE (recoveries found to be comparable to MAP™ extraction, and superior to sonication)	Li et al. 2003

Solvent used	Method	Recovery	Study type	Reference
Ethyle acetate	50 g soil samples inoculated with 5 mL of stock solution B[a]P in acetone (1000 mg/l) to yield a soil concentration of 100 mg/kg. After complete evaporation of the acetone (~12 h) in a chemical hood, 100 ml of solvent were added to the soil. Soil slurries were shaken overnight on a rotary shaker at 150 rpm, in the dark, at room temperature. The soil-solvent slurry was then passed through filter paper containing 5 g sodium sulfate, washed with an additional 2 x 50 mL aliquots of the solvent, and pooled solvent extracts were dried in a chemical hood in the dark and re-suspended in 10 mL acetone.	84% recovery	Standard solvent extraction	Numergut et al. 2000
Subcritical water (alternative to organic solvent)	"An environmentally friendly solvent, a shorter time for analysis and a higher content of BaP extraction (to 96%) as compared to that extracted by organic solvents."	Optimum conditions: 250°C and 100 atm for 30 min.	Subcritical water extraction	Sushkova et al. 2013

Carbendazim

Solvent used	Method	Recovery	Study type	Reference
Methanol	Soil samples were exhaustively extracted with methanol using a Soxhlet apparatus, and radioactivity analyzed by liquid scintillation counting. The concentrated methanol extracts from the soil and plant samples were also analyzed by TLC. Most of the fungicide residues were retained in plant roots, whereas small amounts were carried to the above-ground parts.	Unspecified	Soxhlet extraction	Lewandowska and Walorczyk, 2010
	10 g dw soil sample extracted with 50 mL methanol by ultrasonication for 30 min. The mixture was filtered through a Buchner funnel, and the filter cake was rinsed successively three times with 20 mL of methanol. The filtrates were collected in a 250 mL flat bottom flask and concentrated to approximately 2 mL with a rotary evaporator at 60 °C. The concentrated product was transferred into a 10 mL volumetric flask and adjusted to 10 mL with methanol prior to analysis.	Unspecified	Ultrasonication	Yan et al. 2011
	10 g soil samples combined with 5 mL Carbendazim. Soil samples were then dried at 105°C for 2 hours, then powdered, and 1 g was extracted with 30 mL of the extracting solvent for 16 hours. Soil sample was then centrifuged at 6000 rpm for 10 min and 25 mL of the supernatants were extracted with 15 mL of chloroform twice time, then vacuum evaporated. Dry extracts were re-dissolved in methanol. (Methanol : water (8:2) was used in this study)	88.9 – 98.7% recovered (depending on soil type)	Standard solvent extraction	Virág and Kiss, 2009
Ethyl acetate	Moist soil was extracted by shaking for 1 hour with 50 cm^3 ethyl acetate. A volume of 100 mm^3 of the extract was transferred to an HPLC-vial and the solvent was evaporated. The drying residue was redissolved in 1 cm^3 HPLC-water by repeated shaking and ultrasonic vibration.	88.1% average recovery	Standard solvent extraction / ultrasonication	Matser and Leistra, 2000
	10 **g** of spiked clay soil samples were extracted with 20 ml of solvent during 10, 20 and 30 min. It appeared that an extraction time of at least 20 min provided highest recoveries.	Recoveries were highest when ethyl	Ultrasonication extraction	Hogendoorn et al., 2000

Solvent used	Method	Recovery	Study type	Reference
		acetate was mixed with acetone (60-80%).		
Chloroform	10 g soil samples combined with 5 mL Carbendazim. Soil samples were then dried at 105°C for 2 hours, then powdered, and 1 g was extracted with 30 mL of the extracting solvent for 16 hours. Soil sample was then centrifuged at 6000 rpm for 10 min and 25 mL of the supernatants were extracted with 15 mL of chloroform twice time, then vacuum evaporated. Dry extracts were re-dissolved in methanol.	7 – 42.1% recovered (depending on soil type)	Standard solvent extraction	Virág and Kiss, 2009
Na-acetate – acetic acid buffer (pH 5.6)	10 g soil samples combined with 5 mL Carbendazim. Soil samples were then dried at 105°C for 2 hours, then powdered, and 1 g was extracted with 30 mL of the extracting solvent for 16 hours. Soil sample was then centrifuged at 6000 rpm for 10 min and 25 mL of the supernatants were extracted with 15 mL of chloroform twice time, then vacuum evaporated. Dry extracts were re-dissolved in methanol.	24.4 – 63.9% recovered (depending on soil type)	Standard solvent extraction	Virág and Kiss, 2009
$CaCl_2$ solution (0.01 M)	10 g soil samples combined with 5 mL Carbendazim. Soil samples were then dried at 105°C for 2 hours, then powdered, and 1 g was extracted with 30 mL of the extracting solvent for 16 hours. Soil sample was then centrifuged at 6000 rpm for 10 min and 25 mL of the supernatants were extracted with 15 mL of chloroform twice time, then vacuum evaporated. Dry extracts were re-dissolved in methanol.	37 – 46.2% recovered (depending on soil type)	Standard solvent extraction	Virág and Kiss, 2009
Humic acid solution (0.5 $^{v}/_{v}$% SERA© humic acid solution)	10 g soil samples combined with 5 mL Carbendazim. Soil samples were then dried at 105°C for 2 hours, then powdered, and 1 g was extracted with 30 mL of the extracting solvent for 16 hours. Soil sample was then centrifuged at 6000 rpm for 10 min and 25 mL of the supernatants were extracted with 15 mL of chloroform twice time, then vacuum evaporated. Dry extracts were re-dissolved in methanol.	32.9 – 49.7% recovered (depending on soil type)	Standard solvent extraction	Virág and Kiss, 2009
NH_4 Cl	Samples ***(5*** g) were extracted with solvent (10 ml) by shaking for 2 h, then centrifuged to separate the soil. Aliquots of the supernatant removed for scintillation counting.	12.9% (2-[^{14}C]-Carbendazim radioactivity extracted)	Scintillation counting of ^{14}C-labelled Carbendazim	Austin and Briggs, 1976
Acetone : calcium chloride (1:1)	Samples ***(5*** g) were extracted with solvent (10 ml) by shaking for 2 h, then centrifuged to separate the soil. Aliquots of the supernatant removed for scintillation counting.	61.9% (2-[^{14}C]-Carbendazim radioactivity extracted)	Scintillation counting of ^{14}C-labelled Carbendazim	Austin and Briggs, 1976
Acetone : ammonium chloride (1:1)	Samples ***(5*** g) were extracted with solvent (10 ml) by shaking for 2 h, then centrifuged to separate the soil. Aliquots of the supernatant removed for scintillation counting.	71.3% (2-[^{14}C]-Carbendazim radioactivity extracted) Considered "a suitable carbendazim extractant"	Scintillation counting of ^{14}C-labelled Carbendazim	Austin and Briggs, 1976
Ethanol : ammonium chloride (1:1)	Samples ***(5*** g) were extracted with solvent (10 ml) by shaking for 2 h, then centrifuged to separate the soil. Aliquots of the supernatant removed for scintillation counting.	68% (2-[^{14}C]-Carbendazim radioactivity extracted)	Scintillation counting of ^{14}C-labelled Carbendazim	Austin and Briggs, 1976
Methanol : ammonium	Samples ***(5*** g) were extracted with solvent (10 ml) by shaking for 2 h, then centrifuged to separate the	64.2% (2-[^{14}C]-	Scintillation counting of	Austin and Briggs, 1976

Solvent used	Method	Recovery	Study type	Reference
chloride (1:1)	soil. Aliquots of the supernatant removed for scintillation counting.	Carbendazim radioactivity extracted)	^{14}C-labelled Carbendazim	
Methanol : HCl (4:1)	Samples ***(5*** g) were extracted with solvent (10 ml) by shaking for 2 h, then centrifuged to separate the soil. Aliquots of the supernatant removed for scintillation counting.	22.2% (2-[^{14}C]-Carbendazim radioactivity extracted)	Scintillation counting of ^{14}C-labelled Carbendazim	Austin and Briggs, 1976
Methanol : ammonium chloride : HCl (2:1:1)	Samples ***(5*** g) were extracted with solvent (10 ml) by shaking for 2 h, then centrifuged to separate the soil. Aliquots of the supernatant removed for scintillation counting.	44% (2-[^{14}C]-Carbendazim radioactivity extracted)	Scintillation counting of ^{14}C-labelled Carbendazim	Austin and Briggs, 1976
Acetone	Samples ***(5*** g) were extracted with solvent (10 ml) by shaking for 2 h, then centrifuged to separate the soil. Aliquots of the supernatant removed for scintillation counting.	15.4% (2-[^{14}C]-Carbendazim radioactivity extracted)	Scintillation counting of ^{14}C-labelled Carbendazim	Austin and Briggs, 1976
	10 **g** of spiked clay soil samples were extracted with 20 ml of solvent during 10, 20 and 30 min. It appeared that an extraction time of at least 20 min provided highest recoveries.	Recoveries were highest when acetone was mixed with ethyl acetate (60-80%).	Ultrasonication extraction	Hogendoorn et al., 2000
Acetone : Ethyl acetate (75:25)	10 g soil samples were used. 25 ml solvent were added, the vessel was closed, shaken and placed in an ultrasonic bath for 30 min. The organic extract was filtered over a funnel containing ~10 g sodium sulfate and 5 ml of extract were transferred to a rotavapor flask. After solvent evaporation using **a** water bath temperature of 40°C and a gentle stream of nitrogen, the residue was redissolved in 1 ml of acetonitrile-0.01% TFA in water (10-90; v/v).	60-80% recovered	Ultrasonication extraction	Hogendoorn et al., 2000
	MASE was performed at 100% power, 20 min extraction time and temperature (recovery was highest at 70°C) In comparison to ultrasonic extraction, MASE did not substantially improve the recovery, the time of sample pretreatment and solvent consumption.	60-80% recovered	Microwave-assistant solvent extraction (MASE)	

Cypermethrin

Solvent used	Method	Recovery	Study type	Reference
n-hexane : acetone (1:1)	25 *g* of chopped plant sample transferred into a vortex beaker containing 20 g of anhydrous sodium sulphate. 100 ml of solvent added, and mixture homogenized for 5 min. Solvent was decanted through filter-paper, and a further 100 ml hexane-acetone added to the vortex beaker and homogenized for a further 5 min. Mixture was filtered and the vortex beaker washed with two 15 ml portions of hexane, and the washings used to rinse the residue. Filtrate was transferred to a 500 ml separating funnel and the flask washed with two 50 ml portions of distilled water. The water was added to the separating funnel and shaked gently to avoid emulsions. Phases allowed to separate, discard the lower aqueous acetone layer and then dry the hexane phase by passing it through 15 g of anhydrous sodium sulphate in a 15 mm diameter glass column. Concentrate the hexane extract to about 5 ml using a rotary evaporator under reduced pressure with the water-bath at 40°C.	95 to 109% recovery (from apples, pears, cabbage, potato)	Standard solvent extraction	Baker and Bottomley, 1982
	Residues were extracted with acetone-hexane and partitioned into hexane using aqueous sodium chloriode solution. After concentrating hexane extracts, column chromatography cleanup was performed on Pasteur pipette micro columns containing Florisil, using benzene as the elution solvent.	Average recoveries: 82 to 98% (from wheat and milled fractions fortified at 0.41 to 3.80 mg/kg. No interference was observed from co-extractives)	Standard solvent extraction	Joia et al. 1985
	50 g soil sample was extracted with acetone followed by n-hexane. Each sample was allowed to soak in the solvent for 1 hour with intermittent shaking. Samples were then centrifuged and decanted into a separatory funnel containing sufficient de-ionized distilled water (100-150 mL) to facilitate partitioning of residues into hexane portion. The hexane was washed twice with de-ionized distilled water. After separation the organic portion was concentrated to appropriate volume (10 mL) using rotary evaporator. For cleanup purpose the sample was then passed through a Florisil mini-column topped with anhydrous sodium sulfate, using 12 mL n-hexane followed by 12 mL of 1% methanol in n-hexane.	Unspecified	Standard solvent extraction	Nafees and Jan, 2009
Acetone	Soil samples were extracted on the sampling day by mixing 100 g soil with 100 ml acetone and tumbling for 1 h. The mixture was filtered and soil on the filter paper were rinsed with an additional 25 ml acetone. Acetone extracts were each mixed with 50 ml hexane in a 500 ml separatory funnel, distilled water and 25 ml saturated sodium sulfate solution were added, and the mixture was shaken. The hexane phase was collected and the aqueous phase was extracted again with 50 ml of hexane. The two hexane extracts were combined and dried by adding anhydrous sodium sulfate.	>95% recovery (from sample concentrations of 0.01 to 10 ppm)	Standard solvent extraction	Harris et al. 1981
	10 g soil samples were sonicated 15 min with 20 ml of solvent in an ultrasonic bath. Extracts were filtered and filtrates evaporated on a rotary evaporator at 40°C to dryness and the residues were dissolved in 1 ml	97.2 ±4.4% recovery	Ultrasonic solvent extraction	Babić et al. 1998

Solvent used	Method	Recovery	Study type	Reference
	methanol. Amounts extracted was determined by thin layer chromatography.			
	4 g soil samples were weighed, then 10mL acetone was added. The flask was vigorously shaken for 30 min at 250rpm on amechanical shaker. The supernate was decanted into a 10mL test tube. For the HLLE process: an aliquot of 5mL purified water was placed in a 10mL screw cap glass centrifuge tube with conical bottom. 40.0 µL CCl4 was added to 1.00mL acetone extract used as the extraction solvent. Then, 1mL of acetone solution was transferred to the centrifuge tube rapidly. The mixture was gently shaken. The homogeneous solution was broken by the addition of 0.3 g NaCl and a cloudy solution was formed. The cloudy mixture was centrifuged for 4min at 3000 rpm. After centrifugation, the fine droplets of the extraction phase were sedimented at the bottom of the conical test tube.	77.1 – 91.6% recovered (depending on soil type)	Homogeneous liquid–liquid extraction (HLLE)	Wang et al. 2008
Diethyl ether	10 g soil samples were sonicated 15 min with 20 ml of solvent in an ultrasonic bath. Extracts were filtered and filtrates evaporated on a rotary evaporator at 40°C to dryness and the residues were dissolved in 1 ml methanol. Amounts extracted was determined by thin layer chromatography.	75.9 ±3.2% recovery	Ultrasonic solvent extraction	Babić et al. 1998
Chloroform	10 g soil samples were sonicated 15 min with 20 ml of solvent in an ultrasonic bath. Extracts were filtered and filtrates evaporated on a rotary evaporator at 40°C to dryness and the residues were dissolved in 1 ml methanol. Amounts extracted was determined by thin layer chromatography.	83.2 ±3.5% recovery	Ultrasonic solvent extraction	Babić et al. 1998
Hexane	10 g soil samples were sonicated 15 min with 20 ml of solvent in an ultrasonic bath. Extracts were filtered and filtrates evaporated on a rotary evaporator at 40°C to dryness and the residues were dissolved in 1 ml methanol. Amounts extracted was determined by thin layer chromatography.	66.7 ±2.7% recovery	Ultrasonic solvent extraction	Babić et al. 1998
Benzene	10 g soil samples were sonicated 15 min with 20 ml of solvent in an ultrasonic bath. Extracts were filtered and filtrates evaporated on a rotary evaporator at 40°C to dryness and the residues were dissolved in 1 ml methanol. Amounts extracted was determined by thin layer chromatography.	77 ±3.4% recovery	Ultrasonic solvent extraction	Babić et al. 1998
Acetonitrile	10 g soil samples were sonicated 15 min with 20 ml of solvent in an ultrasonic bath. Extracts were filtered and filtrates evaporated on a rotary evaporator at 40°C to dryness and the residues were dissolved in 1 ml methanol. Amounts extracted was determined by thin layer chromatography.	69.4 ±2.9% recovery	Ultrasonic solvent extraction	Babić et al. 1998
	Extraction used end-over-end tumbling with 2 ml solvent (7:3 v/v, acetonitrile : water) for each 1 g of soil followed by filtration through sintered glass disks. Soil residues were rinsed on the filter with acetonitrile (2 x 50 ml) and the combined filtrate was radiocounted. Aliquots **(10-40** ml) of the extracts were diluted with water **(150** ml) containing sodium sulphate ***(5*** g) and shaken twice with 50 ml volumes of either ethyl acetate or chloroform. The combined organic solutions were dried and concentrated, radiocounted and examined by t.1.c.		End-over-end tumbling and radiocounting	Roberts and Standen, 1977
	Cypermethrin was extracted from celery using acetonitrile, partitioned into hexane, and cleaned up on Florisil for quantitation by electron capture gas chromatography. Minimum detectable levels of < 5 ng/g were readily attainable.	Recoveries averaged 94 to 103% (from celery)	AOAC multiresidue method	Braun and Stanek, 1982
Dichloromethane	10 g soil samples were sonicated 15 min with 20 ml of solvent in an ultrasonic bath. Extracts were filtered and filtrates evaporated on a rotary evaporator at 40°C to dryness and the residues were dissolved in 1 ml	76.8 ±3.3% recovery	Ultrasonic solvent extraction	Babić et al. 1998

Solvent used	Method	Recovery	Study type	Reference
	methanol. Amounts extracted was determined by thin layer chromatography.			
Toluene	2 g soil samples were introduced in a PTFE reactor of 100 ml internal volume. 10 ml toluene and 1 ml water were added and the reactor closed and irradiated at 700 W for 9 min. After cooling, the reactor was opened and 8 g of Na_2SO_4 were added in order to dry the extract. Extracts were then evaporated and reconstituted in 2 ml hexane and copper wires were added to remove sulphur. After 8 h, the hexane solution was passed through a 2 g florisil activated column and eluted with 20 ml of an ethyl acetate in hexane 33% (v/v) solution. The fraction recollected was evaporated and dissolved in 200 µl isooctane.	103 ±4% recovery	Microwave-assisted extraction	Esteve-Turrillas et al. 2004
Methanol	The ball-mill extraction method was used to extract the residues of cypermethrin and metabolites. Homogenized air-dried soil (15 g), ground elm bark (2.5 g), or ground air-dried litter (2.5 g) was weighed into a stainless steel centrifuge tube containing two stainless steel balls. Methanol (25 mL) and 0.6 mol/L HCl (5 mL) were added and the tightly sealed capped tube was mounted horizontally on the wristaction shaker and shaken for 30 min. The closed tube was centrifuged for 20 min at 2900 rpm.	Cypermethrin isomer pairs: 82 to 112% Cypermethrin metabolites: 83 to 107%	Ball-mill extraction method	Jin and Webster, 1998

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Wageningen Environmental Research
P.O. Box 47
6700 AA Wageningen
The Netherlands
T +31 (0)317 48 07 00
www.wur.nl/environmental-research

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Wageningen Environmental Research
P.O. Box 47
6700 AB Wageningen
The Netherlands
T +31 (0) 317 48 07 00
www.wur.eu/environmental-research

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