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Abstract: Consideration of non-extractable residues (NER) in PBT-assessment

The formation of NER in simulation studies on degradation in the environment is an issue considered in all regulations of chemical substances in Europe. Based on efforts in the last years, to differentiate NER into different categories of concern, in September 2018 this project was initiated by UBA in order to harmonise the results with the procedures described in the ECHA discussion paper on NER (Kästner et al., 2018).

In the project, the applicability of a harmonised approach was evaluated experimentally. A set of three different substances was tested in a standard OECD 307 approach. Different procedures for exhaustive extraction were applied in order to compare NER determination approaches. We conducted these experiments using test substances labelled with either ^{13}C or ^{14}C in parallel. Results indicate that data from literature using either label can be reconciled. We showed that NER characterization methods like EDTA extraction and silylation are reproducible and applicable for routine analyses. Experiments were accompanied by the modelling tool Microbial Turnover to Biomass (MTB) (Trapp et al., 2018). This tool relies on the CO_2 measured from the mineralization of test substance as indication of microbial degradation in order to estimate the formation of biogenic NER. Experimental data determined in the current project were used to verify the outcome of this approach. The results of the project were discussed at an international workshop at the German Environment Agency in February 2021. The aim of the workshop was to present and discuss a practicable approach for a harmonised test procedure for NER characterisation. We hereby present two approaches for discussion to determine the relevant fractions of non-extractable residues and to consider them for the derivation of half-lives in the framework of persistency assessment (PBT/ vPvB) of these substances. The approaches are in line with the requirements of ECHA for the handling of non-extractable residues in the assessment of persistence.

Kurzbeschreibung: Berücksichtigung nicht extrahierbarer Rückstände (NER) bei der PBT-Bewertung

Die Bildung von NER bei Simulationstests zum Abbauverhalten in der Umwelt ist ein Thema in allen Bereichen der europäischen Chemikalienregulierung. Auf der Basis von unterschiedlichen Ansätzen der letzten Jahre, NER in verschiedene Besorgniskategorien zu unterteilen, wurde im September 2018 dieses UBA-Projekt initiiert, um die Ergebnisse mit den im Diskussionspapier der ECHA zu NER (Kästner et al., 2018) beschriebenen Verfahren zu harmonisieren.

Im Projekt wurde die praktische Anwendbarkeit eines harmonisierten Ansatzes überprüft. Dabei wurden drei verschiedene Testsubstanzen jeweils ^{13}C- und ^{14}C-Isotopenmarkiert in einem Testansatz nach OECD 307 untersucht. Die Ergebnisse zeigen, dass mit beiden Isotopen-markierungen vergleichbare Ergebnisse erzielt werden. Zur Festlegung der NER Definition wurden verschiedene Verfahren zur erschöpfenden Extraktion, wie EDTA-Extraktion und Silylierung, angewendet. Es konnte gezeigt werden, dass die NER-Charakterisierungsmethoden reproduzierbar und im Routinebetrieb anwendbar sind. Die Experimente wurden durch das Modellierungstool Microbial Turnover to Biomass (MTB) ergänzt (Trapp et al., 2018). Dieses Tool stützt sich auf das beim Abbau der Testsubstanz aufgefangene CO_2, um die Bildung biogener NER abzuschätzen. Diese Ergebnisse wurden mit den aktuellen experimentellen Daten verglichen. Im Rahmen eines internationalen Workshops im Umweltbundesamt im Februar 2021 wurden die Ergebnisse des Projekts diskutiert. Ziel war es, einen praxistauglichen Ansatz für ein harmonisiertes Testverfahren zur NER-Charakterisierung vorzustellen und zu diskutieren. Als Ergebnis wurden zwei Ansätze erarbeitet, anhand derer NER für die Ableitung von Halbwertszeiten im Rahmen der Persistenzbewertung (PBT/ vPvB) dieser Stoffe berücksichtigt werden können. Die Ansätze entsprechen den Vorgaben der ECHA für den Umgang mit Nichtextrahierbaren Rückständen bei der Persistenzbewertung.

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List of abbreviations

Abbreviation	Explanation
°C	Degree Celsius
µg	Microgram
mg	Milligram
g	Gram
kg	Kilogram
µl	Microliter
ml	Milliliter
AAE	Amino acid extract
aR	Applied Radioactivity
BfG	German Federal Institute of Hydrology
bioNER	Biogenic NER
CO_2	Carbon dioxide
Corg	Organic Carbon
d	Day
DegT50	Time taken for 50% of substance to disappear from a compartment due to degradation processes alone
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
Dowex®	Ion Exchange Resins
DT50	Time taken for 50% of substance to disappear from a compartment by dissipation processes
ECHA	European Chemicals Agency
EDTA	Ethylenediaminetetraacetate
g	gravitational acceleration
GLP	Good laboratory practise
H_2O	Water
HPLC-MS	High performance liquid chromatography coupled to mass spectrometry detection
ISI	International Scientific Indexing
kBq	Kilo Becquerel
LSC	Liquid scintillation counting
MASE	microwave assisted solvent extraction
MeOH	Methanol
min	Minute
MTB	Microbial Turnover to Biomass (model to predict formation of bioNER)
NaOH	Sodium hydroxide
NER	Non Extractable Residues
OECD	Organisation for Economic Co-operation and Development

Abbreviation	Explanation
PBT	Persistent, Bioaccumulative, Toxic
PLE	Pressurized Liquid Extraction
Radio-HPLC	High performance liquid chromatography coupled to radio detection
Radio-TLC	Thin layer chromatography with radioactivity detection
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RNA	Ribonucleic acid
rpm	Rotations per minute
SOM	Soil Organic Matter
SPE	Solid Phase Extraction
TMCS	Trimethylchlorosilane
WHCmax	Maximum water holding capacity
Yield	Microbial biomass yield (biomass formation per substrate consumption)

Summary

Introduction

In degradation testing in soils and water/sediment systems according to OECD standard guidelines for regulatory purposes, very often a fraction of the test substance is observed, that cannot be released from the solid matrix with non-destructive extraction methods. These so called Non-Extractable Residues (NER) can be detected only, if isotope labelled test substance is used.

The importance of NER in the assessment of chemicals has been more or less neglected in the past. However, according to new developments, e.g. in REACH guideline revisions (ECHA R.11, 2017), the NER have to be considered in persistence assessment as they may potentially be remobilised as parent or transformation product. One approach is to consider NER as 100% potentially available parent substance if not proven otherwise. This worst-case assumption might be misleading because NER can also represent residues or products of degradation without any environmental relevance as they are irreversibly bound or transformed into biomass. Therefore, the ECHA published in June 2018 the discussion paper "Consultancy services to support ECHA in improving the interpretation of Non-Extractable Residues (NER) in degradation assessment" where guidance is given on how to characterise NER and the different NER types in practical testing. However, the discussion paper clearly states that discussions on NER assessment are still ongoing and the current paper represents a state of the art report only.

In parallel and on behalf of the German Environment Agency (UBA) a project was conducted in order to develop a straightforward extraction procedure for NER characterisation for use in practical testing (FKZ 3713 63 413 1, Loeffler et al., 2020). The procedures developed differ to some extent from the procedures described in the discussion paper and the question came up, how an acceptable method for use in regulatory routine testing can be derived. Thus, in September 2018 the German Environment Agency (UBA) initiated this project in order to develop a harmonised procedure for practical testing taking both current approaches into account. Within the project the applicability of the harmonised approach was proven experimentally using a set of reference substances.

Work program

In a first step, literature reports on NER characterization were compared with focus on extraction procedures, open questions for the subsequent work packages were suggested and a proposal for the experimental assessment of NER with the aim to harmonise the different approaches was drafted.

An experimental comparison of both concepts was conducted with specific focus on:

- Applicability:

The experimental procedure for further NER characterization must not be proven only to deliver information about a specific pre-defined NER fraction, but it must also be practicable in laboratory daily routine. The use of dangerous chemicals like 6 M HCl is not assessed to be problematic as lab staff is trained to work with hazardous substances and usually labs have appropriate protection systems. This does normally not include systems where work under protective atmosphere can be performed, which is needed for silylation. Thus, it was necessary to verify whether silylation is necessary or whether alternative techniques can deliver similar information. Another option was to further develop the silylation procedure to make it fit for laboratory routine. The BfG approach proposes an alternative method with EDTA extraction, which was compared with the silylation and tested for suitability.

The proposed Pressurized Liquid Extraction (PLE) is one more step that had to be checked for practicability. There is no doubt, that PLE works in the laboratory and instruments are commercially available. Nevertheless, so far usually no further analysis, except combustion, is performed with the extracted residue. Further, PLE usually does not work properly with the pure soil matrix but only after mixing of the soil with diatomaceous soil ("Kieselgur") to prevent blocking of the system and/or clumping of the soil (Thermo Scientific, personal communication). That means that for the subsequent steps the diatomaceous soil becomes part of the solid matrix. The influence on subsequent analytical steps was verified.

► Three test compounds with formation of all types of NER:

To cover a wide range of possible interactions with soil, three different test substances were selected for the test, each of them ^{14}C-labelled and ^{13}C-labelled. The substances selected were Isoproturon, a urea herbicide, Bromoxynil, a hydroxybenzonitrile herbicide and Sulfadiazine, a sulfonamide antibiotic. Reasons for the selection are given in section 3.2.

► Comparison of ^{14}C and ^{13}C methods

In general data for either ^{13}C- or ^{14}C-labelled substances were available in literature. In order to compare and reconcile results derived using either labels, the current study was conducted in concurrent experiments using test substances labelled with ^{13}C and test substances labelled with ^{14}C. However, due to the high natural abundancy the applied amount of labelled compound needed to be increased by a factor of ten for the ^{13}C-labelled compounds. The aim was to use data from literature using either label if the study shows a positive correlation of both labels. This improves the transferability of results obtained using the different labels.

► Turnover kinetics, five sampling times

The experiments were conducted with one soil and sampling was performed at start (day 0) plus at least five further sampling dates with NER determination. These samplings were distributed over 120 days. The reported half-lives of the test substances reported in literature were some days only, but provide a significant percentage NER that originally had been counted as "degraded". It was expected that processes in the NER are much slower. Thus, to determine processes occurring in the NER and ideally derive a kinetic information for those processes, the incubation time was extended to the maximum suggested in the OECD 307 technical guideline (120 days). This sampling strategy was accompanied by two sets of sterile samples, which were analysed after two weeks incubation time and at the end of incubation. In sterile soil, no biogenic NER should have formed, however, the abiotic processes should be similar. So, a differentiation between abiotic and biotic NER formation might be possible.

► Estimation of bioNER formation with the MTB method and proposal for tiered approach

The Microbial Turnover to Biomass (MTB) approach uses released CO_2 (as indicator of microbial activity and compound mineralisation) and (measured or theoretical) biomass yield to estimate the biogenic NER formation. The MTB method is therefore in practice an important tool for waiving or triggering further experimental efforts. MTB results can also be used to estimate the fraction of bioNER and, by subtraction from the total NER (NER after PLE extraction), also the fraction of xenoNER formed in existing tests, where total NER was not further discriminated into NER types. In the same way it can be employed to estimate whether further NER differentiation is useful when running a simulation test. However, as Kästner et al. (2018) stated in the discussion paper for the ECHA, "The MTB yield method is quite new …. It has been tested versus available data, but experience is still limited and can thus only be an indicator." The authors list as research needs the application of the new MTB method to more experimental results, preferably from OECD tests relevant for P assessment. For all test compounds, the necessary input data for MTB were collected, the theoretical yield was calculated, and the bioNER formation was estimated. The estimation results were contrasted with the experimental findings.

For practical testing a trigger needs to be defined, which levels of NER, CO_2 and estimated bioNER would call for further experimental testing and under which circumstances the further NER characterisation can be omitted. Once sufficient (positive) experimental data have been gained it may be possible to rely on the calculated bioNER alone. This was finally communicated as a tiered approach proposal, as it is common in regulatory practical testing.

- How to consider NER in the calculation of degradation half-times DegT50

The current P assessment is based on degradation half-lives. Simulation tests described in the OECD test guidelines 307 (OECD 2002), OECD 308 (2002), OECD 309 (2004) or similar tests are required to determine primary or ultimate degradation, the mass balance and other fate parameters (ECHAR.11, 2017). Extractable radioactivity, non-extractable radioactivity and volatile $^{14}CO_2$ are measured at appropriate time points (typically five times plus start). In the extractable fraction substance specific chemical analysis identifies the amount of parent and transformation products. The endpoints determined in the simulation tests are the primary or ultimate degradation rate and degradation half-lives ($DegT_{50}$) (ECHA R.11, 2017). EFSA (2014) released a guidance document for evaluating laboratory and field dissipation studies to obtain $DegT_{50}$ values. This guidance uses the definitions of dissipation and degradation provided in FOCUS (2006), which considers non-extractable residues as degradation products. The concept presented in the ECHA discussion paper (Kästner et al., 2018) suggests to consider only bioNER (NER type III) as degraded substance and NER type II (covalently bound residues) as degraded substance until proven otherwise, while NER type I is considered as potentially remobilisable parent compound. This view is also expressed in the recently released note of ECHA "Options to address non-extractable residues in regulatory persistence assessment" (ECHA Background note, 2019), which is intended to inform duty holders about acceptable approaches to refine assessment of NER in the regulatory context of the REACH and Biocidal Products Regulations, until the guidance documents are updated. There, it is stated that "By default, the residues remaining in the matrix after these extractions (total NER), should be regarded as non-degraded substance". Following those instructions, the degradation rate has to be fitted to the recovered extractable test substance plus NER.

The background note states further that characterisation of the NER may be attempted, and only remobilisable NER (type I) are to be considered as potential concern. If the quantities of the three different types of NER (types I, II, III) are known they can be used for refining the half-life. Nonetheless, half-lives that consider NER type I as parent compound will differ (and be longer) than those obtained from the loss of extractable substance only.

We used the experimental results obtained in this study to test various ways to consider NER in the determination of DT_{50} and $DegT_{50}$ values. We used the methods suggested in FOCUS (2006) (implemented in the tool CAKE) and alternative methods (like direct exponential curve fit) to calculate rates from the measured data. The goal was to find a practical and feasible method for consideration of NER type I or bioNER and NER type II in the $DegT_{50}$ calculation and in the P assessment and to provide respective guidance.

Results of the experimental studies

All degradation tests fulfilled the criteria of OECD 307 with mass balances between 90% and 110% of the applied ^{14}C radioactivity. This is an important parameter for the reliability of the data measured in standard OECD 307 test, which is the basis for all further considerations within this project. The degradation rate of Isoproturon during the study was a bit slower than expected from literature data but no reason could be found. The rate was slightly better in another test soil, but still not as fast as expected. In the end the main objective, to generate samples containing ^{14}C-labelled NER from a standard OECD 307 approach, was achieved for all test substances selected.

Sterile samples that are recommended by OECD 307 proved to be important for process understanding. While for Bromoxynil and Isoproturon mainly biological processes trigger degradation (very low NER formation within 120 days in the sterile samples), Sulfadiazine formed significant amounts of NER also in the sterile samples with more than 81% of the applied radioactivity (aR) after 120 days.

In order to get a better definition of total NER, different extraction procedures were applied in parallel. For the three test substances, a substance specific extraction procedure that routinely has to be developed for each OECD 307 study turned out to be the best option. To get a better standardization, it was agreed to finish extraction with a PLE-extraction using the solvent of the previous extraction. As alternative options only PLE without previous substance specific extraction and PLE with a standard solvent mix in one step (methanol/acetone/water (50/25/25%), BfG approach) were tested. Both worked more or less similar but showed also some outliers depending on the respective test substance. Since substance specific extraction has to be developed anyway within an OECD 307 study, we recommend substance specific extraction (shaking or other technique) followed by PLE with substance specific solvent for definition of total NER. But also, the other options are possible.

Type I NER are of major concern as they represent the NER fraction that might be remobilised over time. Two different methods were tested in the project to release type I NER from the soil matrix and to identify the released fraction: silylation and EDTA -extraction.

Silylation is supposed to be a difficult to apply procedure with dangerous chemicals that require an inert atmosphere for the reaction. However, once established in the laboratory, silylation is a normal procedure in routine chemical analysis, having a long tradition in gas chromatography as derivatization agent. It does not require any precautionary measures that are not already established in a laboratory in a radiation protection area and no special equipment is needed.

As silylation is a harsh chemical treatment, test substances with exchangeable protons of functional groups (carboxyl, amino, hydroxy groups) might be attacked by the procedure. In this case silylation is not suitable to determine type I NER as released parent test substance might react with the silylation reagent and the amount of released parent test substance would be underestimated. Thus, this has to be tested beforehand with a recovery test with blank sample matrix spiked with parent test substance. In the present study Bromoxynil was stable during silylation, Isoproturon showed some degradation (about 20% loss of the spiked parent) and for Sulfadiazine significant losses of up to 65% of the applied parent were observed.

The radioactive mass balance over the silylation of the NER samples in the project was in most cases better than 90%. From the results of 42 samples treated in duplicate by silylation it can be concluded that the reproducibility of the silylation was excellent.

In all cases significant amounts of radioactivity could be releases by silylation, for Sulfadiazine more than 20% of the applied radioactivity (aR), for Bromoxynil and for Isoproturon up to 13%. However, in no case more than 2% aR of parent substance were released from non sterile samples. Sterile samples of Bromoxynil and Isoproturon contained only low amounts of NER (3-9% aR) but the ratio of parent released to total NER was much higher in those sterile samples. Which makes sense as they should consist mostly of entrapped parent substance if degradation is biologically driven. For Sulfadiazine, where NER in sterile and non sterile samples did not differ significantly, this was not observed. Released parent from the sterile samples did not significantly differ from the non sterile samples. Although this statement is not reliable, as the Sulfadiazine was proven not to be stable under the silylation conditions.

The content of released parent by silylation was determined by LC-MS from the ^{13}C-experiments. Comparison with the data obtained from the ^{14}C-experiments (analysis by radio-TLC) showed very similar results, though the start concentration of the ^{13}C-substances were a factor of 10 higher than the start concentration of the ^{14}C-substances.

EDTA-extraction is a very easy to perform aqueous extraction with a chelating chemical used as agent for releasing compounds from organic molecule aggregations in soil (procedures for the use of EDTA to extract proteins, nucleic acids, phospholipids and microorganisms have been reported). It was surprising how much radioactivity could be released from the NER matrix, taking the samples into account that already had been extracted with organic solvent in a harsh PLE procedure.

The radioactive mass balance for the EDTA extraction was also mostly around or above 90%. The extracted radioactivity ranged from 12% aR (Isoproturon) and 17% aR (Bromoxynil) up to 27% aR for Sulfadiazine. However, the sum of parent released from the matrix was similar or lower than for the silylation as discussed above. The comparison of the ^{13}C- and ^{14}C-data again did not show significant differences. Both, silylation and EDTA extraction came to similar results for type I NER determination in both ^{13}C and ^{14}C-experiments. Observed differences are in the range of 1% aR which should not be relevant for P-assessment of the test substances. Chromatographic characterization of NER type I for presence of the parent substance is straight forward with the silylation extract, but trickier with the EDTA extract due to the high salt content in the aqueous solution.

EDTA-extraction is supposed to be a mild extraction procedure. However, recovery experiments showed losses of up to 55% of spiked parent Sulfadiazin. Minor losses of parent substance were also observed for Isoproturon (around 10%) and Bromoxynil (about 5%). Thus, for EDTA-extraction substance stability has to be verified beforehand with a recovery test with blank sample matrix spiked with parent test substance as for silylation.

By definition the amount of NER radioactivity not released by silylation or EDTA extraction are classified type II NER. It will contain still some type III NER (see below) but since both are assessed to be of no concern, the determination of type I NER is most important for risk assessment. Since the radioactive mass balances for determination of type I NER showed values mostly above 90%, the determination of type I NER is in principle also suitable for the estimation of type II NER. This is of course only true for low type III NER contents.

Type III NER or bioNER are assessed to be a safe sink as they represent the turnover of the test substance into biomolecules. The acidic hydrolysis to determine type III NER is in principle a simple acidic digestion with 6M hydrochloric acid at 105°C. However, as this reaction should be performed under inert gas atmosphere in closed vessels it becomes a bit tricky as no commercial digestion system is actually capable to maintain those conditions.

For the hydrolysis, radioactive mass balances were always very good and the amount of released radioactivity ranged from 9% aR (Sulfadiazine) to 13.8% aR (Isoproturon). Chemical analysis of the extract for released ^{14}C-labelled amino acids, as described in Possberg et al. (2016), turned out to be very challenging and not fit for laboratory routine. For practical considerations in this project the recovered radioactivity after the Dowex®-clean-up from the procedure described in Possberg et al. (2016) was used as an indicator for ^{14}C-labelled biomass. This parameter certainly is not more specific than ^{14}C-amino acid analysis by radio TLC but as an indicator for the amount of bioNER it might be useful as well. A good correlation of those data with calculated bioNER (MTB) could be established.

The radioactivity in the cleaned-up Dowex extract (AAE, "amino acid extract") consists mostly of amino acids and can be used as an analytical proxy for the formation of bioNER. Of the three test compounds, the agreement between measurement and MTB bioNER estimation was best for isoproturon. The calculated low MTB, which represents the amino acids, was for all sampling times close to the measured result. Multiplying AAE with a factor of 1.8 gives the total bioNER, and the value was for all sampling times ≥ 29 days within the range of low and high MTB estimate. Moreover, the %aR in AAE of the sterile samples was negligible. Good agreement was also seen for the Bromoxynil MTB estimations. However, there, the measurements also showed a high background in sterile samples. No agreement was achieved for Sulfadiazine, where the measurements gave the highest % aR in AAE of all three substances, while the MTB estimate, due to negligible CO_2-formation, yielded very low estimates for bioNER. For sulfadiazine, sterile samples also had high radioactivity in AAE, which makes the measurements doubtful. In fact, in this comparison of estimates versus measured data, the estimated values seem more reliable than the measurements.

Conclusions

For determination of type I NER, two different extraction procedures were applied. Chemical analysis of the extracts resulted in both cases, silylation and EDTA extract, in very low amounts of released parent substance. These results of LSC and radio-TLC analysis of silylation and EDTA extraction were confirmed by the LC-MS analysis of the ^{13}C experiments.

From the experience of this project we would give the following recommendation for the determination of type I NER:

Table 1: Recommendation for standard type I NER characterisation method

	Silylation	EDTA Extraction
NER type I and II differentiation	suitable (chem. analysis)	suitable (chem. analysis)
Applicability, standardisation	advanced	Easy
Fit for laboratory routine	yes	Yes
Extraction of type III NER	low (non-polar solvent)	Established to extract proteins, nucleic acids, phospholipids
Second extraction required	required for hydrophilic substances	for most substances recommended
Extract processing	standard lab work	difficult due to salt load
Substance stability	proof for stability required	proof for stability required[1]

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[1] Unexpected losses of parent compounds observed in recovery experiments

The results of the experimental determination of type III NER (bioNER) show that it still needs some research.

Special focus of the project was on the applicability of the procedures in routine testing. For the extraction methods for NER definition this is easy to answer since all methods applied are already integral part of standard degradation studies. This includes PLE, which is probably not applied in all current degradation studies but is assessed to be some kind of standard instrumentation in a laboratory dealing with regulatory degradation studies.

The silylation procedure turned out to be easy to apply once established in the lab. Care has to be taken for the use of hazardous chemicals but this is common for other laboratory procedures. The resulting extracts are easy to process for subsequent chemical analyses.

For EDTA extraction it is exactly the other way around: the chemicals are not any critical and the extraction is very simple. But extract work up for subsequent analysis might become more challenging due to the high salt load. Generally, both silylation and EDTA extraction are fit for routine use in the laboratory.

The acidic hydrolysis is more difficult to assess. Generally, rather simple it is limited by the missing standard instrumentation that allows extraction in closed vessels with concentrated acid under pressure conditions for 24 hours. However, it should be possible to solve this problem if necessary. There are more concerns about the complicated and time-consuming clean-up of the extract followed by even more laborious radio-TLC. Such analyses are not considered appropriate for a routine study. In particular as it does not deliver clear data on bioNER but just an indication. Considering our experience, we conclude that the experimental determination of bioNER needs further development and is not assessed to be fit for current lab routine.

Workshop

On February 17 and 18, 2021, the German Environment Agency and the Fraunhofer IME Institute together with the project partners RWTH Aachen (Prof, Dr. Andreas Schäffer), UFZ Leipzig (Prof. Dr. Matthias Kästner) und DTU Lyngby (Prof. Dr. Stefan Trapp) organized an international online workshop entitled 'Proposal to standardize the analysis and persistence assessment of non-extractable residues (NER)'. More than 80 participants from authorities, industry and science, including members of ECHA's PBT Expert Group, discussed the future consideration of NER in persistence assessment. The ECHA discussion paper for NER assessment (Kästner et al., 2018) and the results of the project served as basis for the discussion.

The aim of the workshop was to present and discuss a practical approach for a harmonised test procedure for NER characterisation. In this discussion, industry's point of view was an important indicator for determining the acceptance and practical applicability of the proposed approach. In addition, regarding the effects of NER characterisation on the PBT assessment, the contributions of the regulators were necessary in order to get a reliable statement.

It was concluded that, e.g. PLE should be the final step in a stepwise extraction procedure, to set a defined limit between the extractable fraction and the total NER. Further conclusions were that guidance is needed how to proceed in a stepwise approach for NER characterisation and persistence assessment. Moreover, the practical determination of “bioNER” (type III NER) was found to be only partly fit for practice. Therefore, a modelling approach (MTB) is proposed which should give a good estimate of bioNER.

Based on the results of the discussion, two proposals were made (flow charts in figures 1 and 2) which present ways of how to take NER in the persistence assessment into account. Both proposals represent a stepwise approach with increasing laboratory effort in each step, but at the same time a higher degree of reality or decreasing conservatism.

Proposals for consideration of NER in Persistence assessment

Proposal 1 (figure 1) represents a 'worst-case approach', which focuses mainly on the decision persistent/very persistent (P/vP) or not persistent (not P) with less relevance of the specific calculated DT_{50} values as these are not needed for further risk assessment.

The idea of the proposal is a stepwise approach with increased data requirements at each consecutive step resulting in improved reliability of the assessment. NER characterization is only necessary if Steps 1 and 2 do not result in assessment as non-persistent. In case that after all 4 steps the P/vP-trigger is still exceeded, the substance is finally considered persistent or very persistent.

In order to verify the consequences of the proposed flow charts in terms of calculated degradation half-lives, the experimental data sets obtained during the project (^{14}C-radiolabelled substances) were applied to the flow scheme. CAKE was used for the calculations and SFO kinetics was selected. Table 2 gives an overview on the results determined according to the flow chart.

Table 2: Stepwise estimation of half-lives DT_{50} [days] according to proposal 1

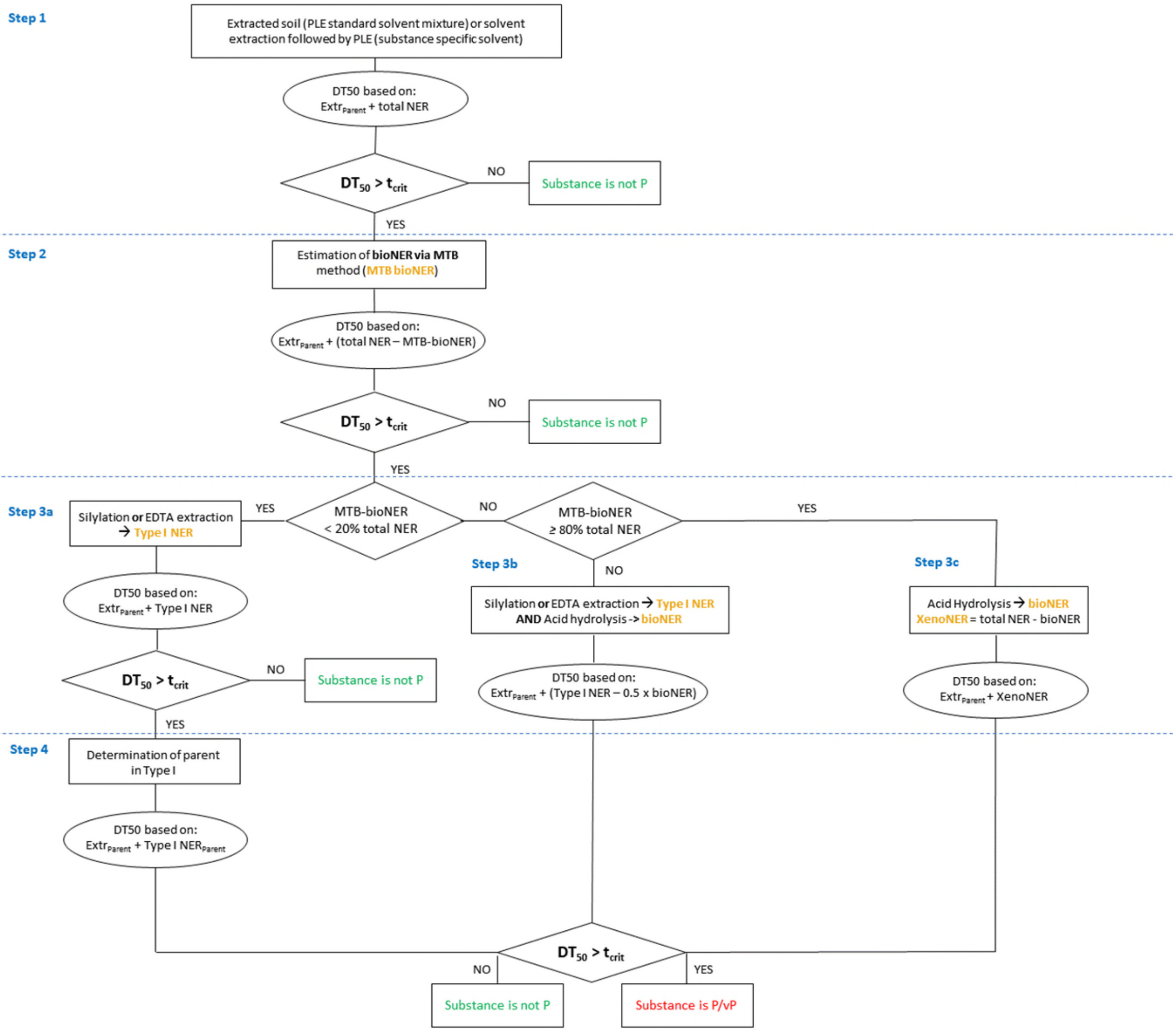
	Description	Bromoxynil	Sulfadiazine	Isoproturon
current	**Extr. $_{Parent}$** (NER considered as sink)	7.0	7.9	53.8
Step 1	**Extr. $_{Parent}$ + total NER**	246	562	250
Step 2	**Extr. $_{Parent}$ + (total NER – MTB bioNER)**	194	528	140
Step 3a	**Extr. $_{Parent}$ + Type I NER**	12.4	39.3	57.9
Step 3b	**Extr. $_{Parent}$ + (Type I NER – 0.5 x bioNER)**	10.3	31.4	49.3
Step 3c	**Extr. $_{Parent}$ + (total NER –bioNER)**	132	365	147
Step 4	**Extr. $_{Parent}$ + Type I NER $_{Parent}$**	8.1	10.4	48.8

Table 3: Stepwise estimation of half-lives DT_{50} [days] according to proposal 2

	Description	Bromoxynil	Sulfadiazine	Isoproturon
Step 1	**Extr. $_{Parent}$** (NER considered as sink)	7.0	7.9	53.8
Step 2a	**Extr. $_{Parent}$ + Type I NER**	12.4	39.3	57.9
Step 2b	**Extr. $_{Parent}$ + (total NER –bioNER)**	132	365	147
Step 3	**Extr. $_{Parent}$ + Type I NER $_{Parent}$**	8.1	10.4	48.8

The results demonstrate that classification of NER as non-degraded parent substance will turn any test substance persistent that tends to form NER in soil. However, a differentiated analysis of the NER shows that the substances tested are far from being persistent.

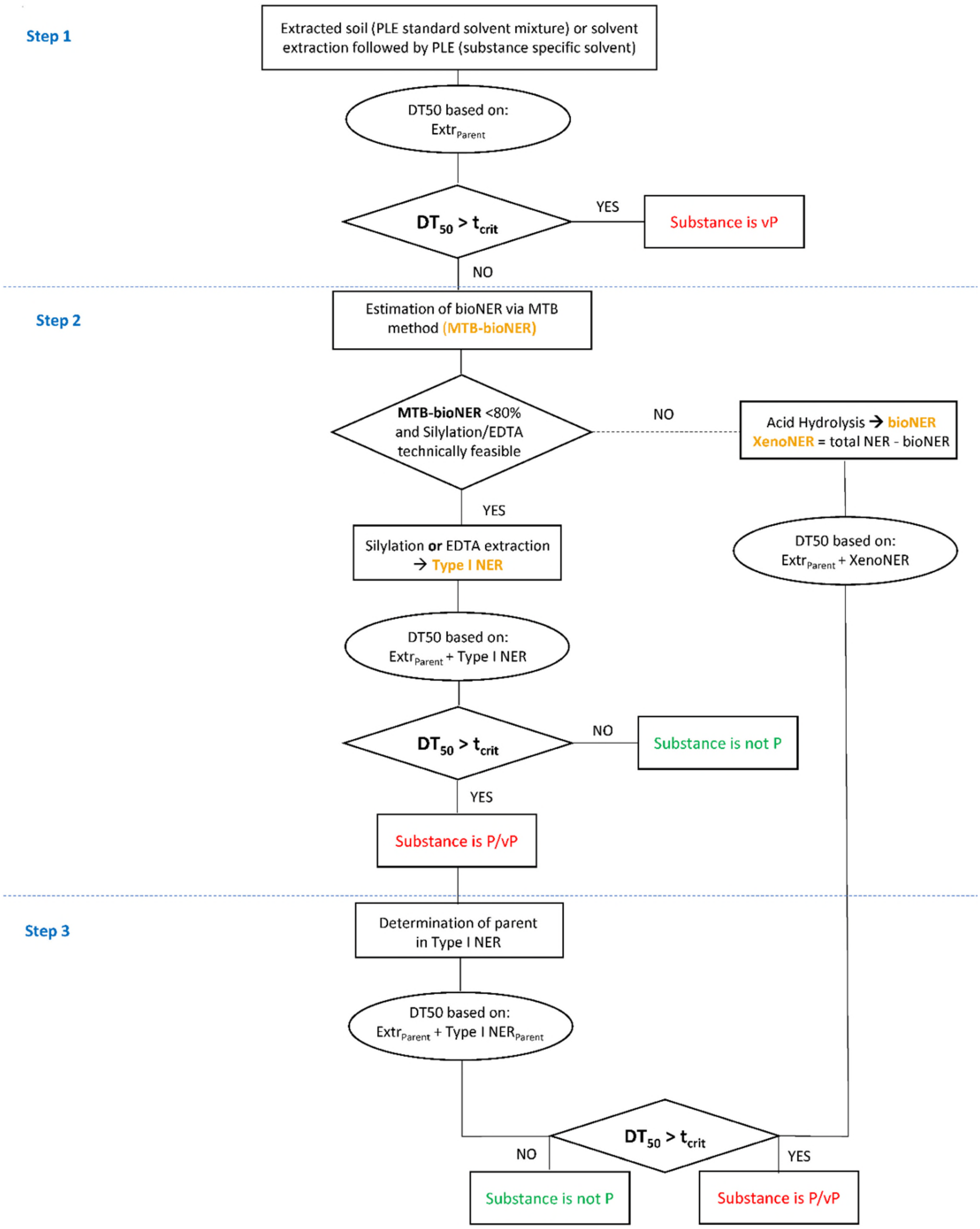
Figure 1: Flow chart for consideration of NER in Persistence assessment, proposal 1



Source: https://www.umweltbundesamt.de/sites/default/files/medien/362/dokumente/proposal_1_final.pdf

Proposal 2 represents a 'realistic-case approach', which sets the focus in trying to derive half-lives of substances as realistic as possible, that can then be used for persistence assessment but also for risk assessment (the latter needs further investigation before implementation). Starting from a 'best-case scenario' in which it is considered that NER are completely assessed as safe sink. If the DT_{50} derived from the data of parent substance determined by chemical analysis of the extracts exceeds the trigger value for vP in soil, then no refinement is necessary (substance is vP also in a best-case scenario, thus persistency would further increase, if fractions of potentially remobilisable NER would also be considered). A refinement and further characterization of the NER in soil should be considered, in case in the first step, the vP trigger value is not reached and for all cases in which the registrant wants to derive a more realistic value for DT_{50} (which considers all the relevant and potentially residues).

Figure 2: Flow chart for consideration of NER in Persistence assessment, proposal 2



Source: https://www.umweltbundesamt.de/sites/default/files/medien/362/dokumente/proposal_2_final.pdf

Zusammenfassung

Einleitung

Bei Abbauuntersuchungen in Böden und Wasser-/Sedimentsystemen gemäß den OECD-Standardrichtlinien zu regulatorischen Zwecken wird sehr häufig ein Anteil der Prüfsubstanz beobachtet, der mit zerstörungsfreien Extraktionsmethoden nicht aus der festen Matrix freigesetzt werden kann. Diese so genannten nicht extrahierbaren Rückstände (NER) können nur nachgewiesen werden, wenn isotopenmarkierte Prüfsubstanz verwendet wird.

Die Bedeutung von NER für die Bewertung von Chemikalien wurde in der Vergangenheit mehr oder weniger vernachlässigt. Nach neuen Entwicklungen, z. B. in den überarbeiteten REACH-Leitlinien (ECHA R.11, 2017), müssen NER jedoch bei der Persistenzbewertung berücksichtigt werden, da sie potenziell als Ausgangsstoff oder Transformationsprodukt remobilisiert werden können. Ein Ansatz besteht darin, NER als 100 % potenziell verfügbare Ausgangssubstanz zu betrachten, sofern nicht das Gegenteil nachgewiesen wird. Diese Worst-Case-Annahme könnte irreführend sein, da NER auch Rückstände oder Abbauprodukte ohne jegliche Umweltrelevanz darstellen können, da sie irreversibel gebunden oder in Biomasse umgewandelt werden. Daher veröffentlichte die ECHA im Juni 2018 das Diskussionspapier "Consultancy services to support ECHA in improving the interpretation of Non-Extractable Residues (NER) in degradation assessment", in dem eine Anleitung zur Charakterisierung von NER und der verschiedenen NER-Typen in praktischen Versuchen gegeben wird. In dem Diskussionspapier wird jedoch deutlich darauf hingewiesen, dass die Diskussionen über die Bewertung von NER noch nicht abgeschlossen sind und das vorliegende Papier lediglich einen Bericht über den Stand der Technik darstellt.

Parallel dazu wurde im Auftrag des Umweltbundesamtes (UBA) ein Projekt durchgeführt, um ein einfaches Extraktionsverfahren zur NER-Charakterisierung für den Einsatz in der Praxis zu entwickeln (FKZ 3713 63 413 1 Loeffler et al., 2020). Die entwickelten Verfahren weichen zum Teil von den im Diskussionspapier beschriebenen Verfahren ab und es stellte sich die Frage, wie ein akzeptables Verfahren für den Einsatz in der behördlichen Routineprüfung abgeleitet werden kann. Daher hat das Umweltbundesamt (UBA) im September 2018 dieses Projekt initiiert, um ein harmonisiertes Verfahren für die praktische Prüfung unter Berücksichtigung der beiden aktuellen Ansätze zu entwickeln. Im Rahmen des Projekts wurde die Anwendbarkeit des harmonisierten Ansatzes anhand eines Sets von Referenzsubstanzen experimentell nachgewiesen.

Arbeitsprogramm

In einem ersten Schritt wurden Literaturberichte zur Charakterisierung von NER mit dem Schwerpunkt auf Extraktionsverfahren ausgewertet, offene Fragen für die nachfolgenden Arbeitspakete vorgeschlagen und ein Vorschlag für die experimentelle Bewertung von NER mit dem Ziel der Harmonisierung der verschiedenen Ansätze erarbeitet. Ein experimenteller Vergleich der beiden Konzepte wurde mit besonderem Augenmerk auf folgende Punkte durchgeführt:

- Anwendbarkeit:

Das experimentelle Verfahren zur weiteren NER-Charakterisierung soll nicht nur Informationen über eine bestimmte vordefinierte NER-Fraktion liefern, sondern muss auch im Laboralltag praktikabel sein. Die Verwendung gefährlicher Chemikalien wie 6 M HCl wird als unproblematisch eingeschätzt, da das Laborpersonal für die Arbeit mit gefährlichen Stoffen geschult ist und die Labore in der Regel über entsprechende Schutzsysteme verfügen. Dazu gehören aber normalerweise keine Systeme, mit denen Arbeiten unter Schutzatmosphäre durchgeführt werden können, was für die Silylierung erforderlich ist. Es musste also geprüft werden, ob eine Silylierung notwendig ist oder ob alternative Techniken ähnliche Informationen liefern können. Eine andere Möglichkeit war, das Silylierungsverfahren weiterzuentwickeln, um es für die Laborroutine tauglich zu machen. Der BfG-Ansatz schlägt eine alternative Methode, die EDTA-Extraktion vor, die mit der Silylierung verglichen und auf ihre Eignung geprüft wurde.

Die vorgeschlagene Pressurized liquid extraction (PLE) ist ein weiterer Schritt, der auf seine Praxistauglichkeit geprüft werden musste. Es besteht kein Zweifel daran, dass die PLE im Labor funktioniert und entsprechende Geräte sind im Handel erhältlich. Dennoch wird bisher in der Regel keine weitere Analyse, außer der Verbrennung, mit dem Extraktionsrückstand durchgeführt. Es gibt unterschiedliche Systeme für die Durchführung von PLE am Markt. Es ist zu beachten, dass PLE nicht in allen Geräten mit der reinen Bodenmatrix funktioniert, sondern erst nach Vermischung des Bodens mit Kieselgur, um eine Verstopfung des Systems und/oder eine Verklumpung des Bodens zu verhindern (Thermo Scientific, persönliche Mitteilung). Das bedeutet, dass Kieselgur dann in den nachfolgenden Schritten Teil der festen Matrix wird.

- Drei Referenzverbindungen mit Bildung von NER:

Um ein breites Spektrum möglicher Wechselwirkungen mit dem Boden abzudecken, wurden drei verschiedene Testsubstanzen für den Test ausgewählt, die jeweils mit ^{14}C-Markierung und ^{13}C-Markierung eingesetzt wurden. Bei den ausgewählten Substanzen handelt es sich um Isoproturon, ein Harnstoffherbizid, Bromoxynil, ein Hydroxybenzonitril-Herbizid und Sulfadiazin, ein Sulfonamid-Antibiotikum.

- Vergleich von ^{14}C- und ^{13}C-Markierung

In der Regel waren in der Literatur Daten für entweder ^{13}C- oder ^{14}C-markierte Stoffe verfügbar. Um die mit beiden Markierungen gewonnenen Ergebnisse vergleichen und abstimmen zu können, wurden in der vorliegenden Studie parallel Versuche mit ^{13}C- und ^{14}C-markierten Testsubstanzen durchgeführt. Aufgrund der hohen natürlichen Abundanz musste jedoch die eingesetzte Menge an markierter Substanz für die ^{13}C-markierten Verbindungen um das Zehnfache gegenüber ^{14}C erhöht werden. Ziel der Untersuchungen war die Nutzung von Literaturdaten beider Markierungen, wenn die Experimente eine positive Korrelation beider Markierungen zeigt und damit die Vergleichbarkeit der Ergebnisse beider Markierungen nachweist.

- Umsatzkinetik

Die Experimente wurden mit einem Boden durchgeführt, und die Probenahme erfolgte zu Beginn sowie an mindestens fünf weiteren Probenahmeterminen einschließlich NER-Bestimmung. Diese Probenahmen waren über 120 Tage verteilt, obwohl die in der Literatur angegebenen Halbwertszeiten der ausgewählten Referenzsubstanzen nur einige Tage betragen. Diese Daten wurden aber unter der Voraussetzung ermittelt, dass NER als "abgebaut" bewertet werden. Es wurde erwartet, dass die Prozesse in den NER viel langsamer ablaufen. Um die in den NER ablaufenden Prozesse zu bestimmen und idealerweise kinetische Informationen für diese Prozesse abzuleiten, wurde die Inkubationszeit auf das in der technischen Richtlinie OECD 307 vorgeschlagene Maximum (120 Tage) verlängert. Die Probenahmestrategie wurde durch zwei Sätze steriler Proben ergänzt, die nach zwei Wochen Inkubationszeit und am Ende der Inkubation analysiert wurden. So war eine Unterscheidung zwischen abiotischer und biotischer NER-Bildung möglich.

- Abschätzung der bioNER-Bildung mit der MTB-Methode

Der Microbial Turnover to Biomass (MTB)-Ansatz verwendet freigesetztes CO_2 als Indikator für mikrobielle Aktivität und Mineralisierung von Verbindungen und (gemessenen oder theoretischen) Biomasseertrag, um die biogene NER-Bildung abzuschätzen. Im Entwurf der ECHA R.11-Leitlinien (PBT/vPvP-Bewertung) heißt es: „Wenn sowohl die MTB-Methode als auch andere Informationen anzeigen, dass bioNER gebildet worden sein könnten, sollten diese bioNER quantifiziert werden, z.B. durch Extraktion von Aminosäuren." Die MTB-Methode ist daher in der Praxis ein wichtiges Instrument, um auf weitere experimentelle Bemühungen zu verzichten oder diese auszulösen. MTB-Ergebnisse können auch verwendet werden, um den Anteil an bioNER (und durch Subtraktion von der gesamten NER Menge dann auch den Anteil an xenoNER) zu schätzen, der in bestehenden Tests gebildet wurde, bei denen NER nicht weiter in NER-Typen unterschieden wurde. In gleicher Weise lässt sich damit abschätzen, ob eine weitere NER-Differenzierung bei der Durchführung eines Simulationstests sinnvoll ist. Dazu heißt es im Diskussionspapier der ECHA (Kästner et al., 2018): *„Die MTB-Ertragsmethode ist ziemlich neu …. Sie wurde im Vergleich zu verfügbaren Daten getestet, aber die Erfahrungen sind noch begrenzt und können daher nur ein Indikator sein.*“ Die Autoren nennen als Forschungsbedarf die Anwendung der neuen MTB-Methode auf experimentellere Ergebnisse, vorzugsweise aus OECD-Tests, die für die P-Bewertung relevant sind. Für alle Testverbindungen wurden die notwendigen Inputdaten für MTB erhoben, die theoretische Ausbeute berechnet und die bioNER-Bildung abgeschätzt. Die Ergebnisse wurden den experimentellen Befunden gegenübergestellt.

Für die praktische Erprobung muss ein Schwellenwert definiert werden, bei welchen NER-, CO_2- und geschätzten bioNER-Werten weitere experimentelle Tests erforderlich sind und unter welchen Umständen auf die zusätzlichen NER-Tests verzichtet werden kann. Bei ausreichenden (positiven) experimentellen Daten kann unter Umständen allein auf die berechneten bioNER zurückgegriffen werden. Dies wurde schließlich als gestufter Ansatz kommuniziert, wie er in der regulatorischen Praxis üblich ist.

- Berücksichtigung von NER bei der Bestimmung der Abbaukinetik

Die aktuelle Persistenzbewertung basiert auf der Abbau-Halbwertszeit. Zur Bestimmung des primären oder vollständigen Abbaus, der Massenbilanz und der Abbauwege (ECHA 2017 R.11) sind Simulationstests gemäß OECD-Testrichtlinie 307 oder vergleichbaren Richtlinien erforderlich. Extrahierbare Radioaktivität, nicht-extrahierbare Radioaktivität und flüchtiges $^{14}CO_2$ werden zu geeigneten Zeitpunkten gemessen (typischerweise mindestens fünf plus Start). Die in den Simulationstests ermittelten Endpunkte sind primäre oder endgültige Abbaurate und Abbauhalbwertszeiten ($DegT_{50}$) (ECHA 2017 R.11). Die EFSA (2014) hat einen Leitfaden zur Bewertung von Labor- und Feldabbaustudien zur Ermittlung von $DegT_{50}$-Werten veröffentlicht. Dieser Leitfaden verwendet die Definitionen von Dissipation und Abbau aus FOCUS (2006), die nicht extrahierbare Rückstände als Abbauprodukte betrachtet. Das im Diskussionspapier der ECHA (Kästner et al., 2018) vorgestellte Konzept schlägt vor, bis zum Beweis des Gegenteils nur bioNER (NER Typ III) und NER Typ II (kovalent gebundene Rückstände) als abgebaute Substanz zu betrachten, während NER Typ I als potenziell remobilisierbare Ausgangsverbindung berücksichtigt wird. Diese Ansicht wird auch in der kürzlich veröffentlichten Mitteilung der ECHA „Options to address non-extractable residues in regulatory Persistence assessment“ (ECHA Background note, 2019) im regulatorischen Kontext der REACH- und Biozidprodukte-Verordnung vertreten. Dort heißt es: „Standardmäßig sind die nach diesen Extraktionen in der Matrix verbleibenden Rückstände (Gesamt-NER) als nicht abgebaute Substanz zu betrachten“. Dieser Vorgabe folgend sind für die Berechnung der Abbaukinetik Gesamt NER als Ausgangsverbindung zu berücksichtigen.

In der Hintergrundnotiz heißt es weiter, dass eine Charakterisierung der NER versucht werden kann und nur remobilisierbare NER (Typ I) als potenzielles Problem zu betrachten sind. Wenn die Mengen der drei verschiedenen NER-Typen (Typen I, II, III) bekannt sind, können sie zur Verfeinerung der Halbwertszeit verwendet werden. Nichtsdestotrotz unterscheiden sich die Halbwertszeiten, die NER Typ I als Ausgangssubstanz berücksichtigen, von denen, die durch die Verringerung der Ausgangssubstanz im Extrakt berechnet werden dadurch, dass sie länger sind.

Wir haben die experimentellen Ergebnisse dieser Studie verwendet, um verschiedene Möglichkeiten zur Berücksichtigung von NER bei der Bestimmung von DT_{50}- und $DegT_{50}$-Werten zu testen. Wir haben die in FOCUS (2006) vorgeschlagenen Methoden (implementiert im Tool CAKE) und alternative Methoden (wie der direkte exponentielle Kurvenfit) verwendet, um die Raten aus den gemessenen Daten zu berechnen. Ziel war es, eine praktikable und umsetzbare Methode zur Berücksichtigung von NER Typ I bzw. bioNER und NER Typ II in der $DegT_{50}$-Berechnung und in der P-Bewertung zu entwickeln und eine entsprechende Anleitung zu erstellen.

Ergebnisse

Alle Abbautests erfüllten die Kriterien der OECD 307 mit Massenbilanzen zwischen 90 % und 110 % der eingesetzten ^{14}C-Radioaktivität. Dies ist ein wichtiger Parameter für die Validität des Standardtests OECD 307, der Grundlage für alle weiteren Überlegungen in diesem Projekt ist. Die Abbaurate von Isoproturon während der Studie war etwas langsamer als aus Literaturdaten erwartet, aber es konnte keine Ursache dafür gefunden werden. In einem anderen Testboden war die Abbaurate etwas besser, aber immer noch nicht so schnell wie erwartet. Letztlich wurde das Hauptziel, Proben mit ^{14}C-markiertem NER aus einem Standard-307-Ansatz zu generieren, aber für alle ausgewählten Testsubstanzen erreicht.

Als wichtig für das Prozessverständnis erwiesen sich sterile Proben, die auch gemäß OECD 307 empfohlen werden. Während bei Bromoxynil und Isoproturon hauptsächlich biologische Prozesse für den Abbau verantwortlich waren (sehr geringe NER-Bildung innerhalb von 120 Tagen in den sterilen Proben), bildete Sulfadiazin auch in den sterilen Proben mit mehr als 81 % der applizierten Radioaktivität nach 120 Tagen sehr hohe Mengen an NER.

Um eine bessere Definition von NER zu erhalten, wurden verschiedene Extraktionsverfahren parallel angewendet. Für die drei Testsubstanzen hat sich ein substanzspezifisches Extraktionsverfahren, das ohnehin in jeder OECD 307-Studie zu entwickeln ist, als beste Option herausgestellt. Um eine bessere Standardisierung zu erreichen, wurde vereinbart, die Extraktion mit einer PLE-Extraktion unter Verwendung des Lösungsmittels der vorherigen Extraktion abzuschließen. Diese PLE-Extraktion muss nicht unbedingt signifikante Mengen an Radioaktivität freisetzen. Als Optionen wurden nur PLE ohne vorherige stoffspezifische Extraktion und PLE mit einem Standardlösungsmittelgemisch getestet. Beide wirkten mehr oder weniger ähnlich gut, zeigten aber je nach Testsubstanz auch einige Ausreißer. Da die stoffspezifische Extraktion ohnehin im Rahmen einer OECD 307-Studie entwickelt werden muss, empfehlen wir für die NER-Definition stoffspezifische Extraktion (Schütteln oder andere Technik) gefolgt von PLE mit stoffspezifischem Lösungsmittel.

NER vom Typ I sind von großer Bedeutung, da sie die NER-Fraktion darstellen, die im Laufe der Zeit remobilisiert werden könnte. Im Projekt wurden zwei verschiedene Methoden getestet, um Typ I NER aus der Bodenmatrix freizusetzen und die freigesetzte Fraktion zu identifizieren: Silylierung und EDTA-Extraktion.

Die Silylierung hat den Ruf, ein schwierig anzuwendendes Verfahren mit gefährlichen Chemikalien zu sein, die für die Reaktion eine inerte Atmosphäre benötigen. Einmal im Labor etabliert, kann die Silylierung jedoch als ein normales Verfahren in der chemischen Routineanalytik angesehen werden, das im Übrigen eine lange Tradition in der Gaschromatographie als Derivatisierungsreagenz hat. Sie erfordert keine besonderen Schutzmaßnahmen, die nicht bereits in einem Labor in einem Strahlenschutzbereich etabliert sind, und es ist keine spezielle Ausrüstung erforderlich.

Da es sich bei der Silylierung um eine aggressive chemische Behandlung handelt, können Testsubstanzen mit austauschbaren Protonen entsprechender funktioneller Gruppe (Carboxyl-, Amino-, Hydroxygruppen) durch das Verfahren angegriffen werden. In dem Fall ist die Silylierung zur Bestimmung von Typ I NER nicht geeignet, da die freigesetzte Ausgangsprüfsubstanz mit dem Silylierungsreagenz reagieren könnte und die Menge der freigesetzten Testsubstanz unterschätzt würde. Daher muss dies vorher mit einem Wiederfindungstest mit einer mit Testsubstanz dotierten Blindprobenmatrix überprüft werden. In der vorliegenden Studie war Bromoxynil während der Silylierung stabil, Isoproturon zeigte einen gewissen Abbau (etwa 20 % Verlust der dotierten Ausgangssubstanz) und für Sulfadiazin wurden signifikante Verluste von bis zu 65 % der applizierten Testsubstanz beobachtet.

Die radioaktive Massenbilanz der NER-Proben bei die Silylierung im Projekt war in den meisten Fällen besser als 90 % der eingesetzten Radioaktivität. Aus den Ergebnissen von 42 zweifach silylierten Proben kann geschlossen werden, dass die Reproduzierbarkeit der Silylierung ausgezeichnet war. In allen Fällen konnten durch Silylierung signifikante Mengen an Radioaktivität freigesetzt werden, bei Sulfadiazin mehr als 20 % der applizierten Radioaktivität, bei Bromoxynil und bei Isoproturon bis zu 13 %. Aus nicht sterilen Proben wurden jedoch in keinem Fall mehr als 2 % aR der Muttersubstanz freigesetzt. Sterile Proben von Bromoxynil und Isoproturon enthielten nur geringe Mengen an NER (3-9% aR), aber das Verhältnis von freigesetzter Testsubstanz zu Gesamt-NER war in sterilen Proben viel höher. Was nachvollziehbar ist, da NER in Sterilproben größtenteils aus eingeschlossener Testsubstanz bestehen sollten. Bei Sulfadiazin, bei dem sich die NER in sterilen und nicht sterilen Proben nicht signifikant unterschieden, wurde dies nicht beobachtet. Die freigesetzte Testsubstanz aus den sterilen Proben unterschied sich nicht signifikant von den nicht sterilen Proben. Diese Aussage ist jedoch nicht belastbar, da sich das Sulfadiazin unter den Silylierungsbedingungen als nicht stabil erwies.

Der Gehalt an freigesetzter Testsubstanz durch Silylierung wurde mittels LC-MS auch aus den ^{13}C-Experimenten bestimmt. Der Vergleich mit den Daten aus den ^{14}C-Experimenten (Analyse mittels Radio-DC) ergab sehr ähnliche Ergebnisse, wobei jedoch die Startkonzentration der ^{13}C-Substanzen um den Faktor 10 höher war als die Startkonzentration der ^{14}C-Substanzen.

Die EDTA-Extraktion ist eine wässrige Extraktion mit einer chelatbildenden Chemikalie und damit als Agens zur Auflösung von Molekülaggregationen und zur Freisetzung von `entrapped´ Molekülen aus organischer Bodensubstanz gedacht. Proteine, Nukleinsäuren, Phospholipide und Mikroorganismen werden durch EDTA Behandlung effektiv aus dem Boden extrahiert. Daher war es überraschend, wie viel Radioaktivität aus der NER-Matrix freigesetzt werden konnte, wenn man bedenkt, dass die Proben vorab in einem harschen PLE-Verfahren mit organischem Lösungsmittel extrahiert wurden. Auch die radioaktive Massenbilanz für die EDTA-Extraktion lag meist bei oder über 90 %. Die extrahierte Radioaktivität reichte von 12 % aR (Isoproturon) und 17 % aR (Bromoxynil) bis zu 27 % aR für Sulfadiazin. Die Summe der aus der Matrix freigesetzten Testsubstanz war jedoch ähnlich oder niedriger als bei der oben diskutierten Silylierung. Der Vergleich der ^{13}C- und ^{14}C-Daten zeigte keine signifikanten Unterschiede. Sowohl die Silylierung als auch die EDTA-Extraktion kamen bei der NER Typ I-Bestimmung sowohl in

^{13}C- als auch in ^{14}C-Experimenten zu ähnlichen Ergebnissen. Beobachtete Unterschiede liegen im Bereich von 1 % aR, was für die P-Bewertung der Prüfsubstanzen nicht relevant ist.

Die EDTA-Extraktion gilt als mildes Extraktionsverfahren. Wiederfindungsexperimente zeigten jedoch Verluste von bis zu 55 % des gespikten Sulfadiazin. Auch bei Isoproturon (ca. 10 %) und Bromoxynil (ca. 5 %) wurden geringfügige Verluste an Ausgangssubstanz beobachtet. Daher muss für die EDTA-Extraktion die Stabilität der Testsubstanz vorher mit einem Wiederfindungstest mit Blindprobenmatrix überprüft werden, wie bei der Silylierung beschrieben.

Definitionsgemäß wird die Menge an NER-Radioaktivität, die nicht durch Silylierung oder EDTA-Extraktion freigesetzt wird, als Typ II NER gewertet. Darin enthalten sind noch NER vom Typ III (siehe unten), aber da beide als unbedenklich eingestuft werden, ist die Bestimmung von NER vom Typ I für die Risikobewertung am wichtigsten. Da die radioaktiven Massenbilanzen zur Bestimmung der Typ I NER Werte meist über 90 % aufwiesen, ist die Bestimmung der Typ I NER grundsätzlich auch zur Abschätzung der Typ II NER geeignet. Dies gilt natürlich nur für niedrige NER-Gehalte vom Typ III. Typ III NER oder bioNER werden als sichere Senke bewertet, da sie den Umsatz der Prüfsubstanz in Biomoleküle oder Matrixbestandteile darstellen. Die saure Hydrolyse zur Bestimmung von Typ III NER ist im Prinzip ein einfacher saurer Aufschluss mit 6 m Salzsäure bei 105°C. Da diese Reaktion jedoch unter Inertgasatmosphäre in geschlossenen Gefäßen durchgeführt werden soll, wird es etwas knifflig, da kein kommerzielles Aufschlusssystem tatsächlich in der Lage ist, diese Bedingungen aufrecht zu erhalten.

Die radioaktiven Massenbilanzen der Hydrolyse waren immer zufriedenstellend und die Menge der freigesetzten Radioaktivität reichte von 9% aR (Sulfadiazin) bis 13,8% aR (Isoproturon). Die chemische Analyse des Extrakts auf freigesetzte ^{14}C-markierte Aminosäuren, wie in Possberg et al., 2016 beschrieben, erwies sich aber als sehr anspruchsvoll und nicht für die Laborroutine geeignet. Aus praktischen Erwägungen wurde in diesem Projekt die nach dem Dowex-Clean-up aus der in Possberg et al., 2016 beschriebenen Vorgehensweise zurückgewonnene Radioaktivität als Indikator für ^{14}C-markierte Biomasse verwendet. Dieser Parameter ist sicherlich nicht spezifischer als die ^{14}C-Aminosäure-Analyse durch Radio-DC, aber als Indikator für die Menge an bioNER könnte er nützlich sein. Es konnte eine gute Korrelation dieser Daten mit berechneten bioNER (MTB) festgestellt werden.

Die Radioaktivität im gereinigten Dowex-Extrakt (AAE, „Amino Acid Extract“) besteht größtenteils aus Aminosäuren und kann als analytischer Proxy für die Bildung von bioNER verwendet werden. Von den drei Testsubstanzen war die Übereinstimmung zwischen Messung und MTB bioNER-Schätzung für Isoproturon am besten. Das berechnete niedrige MTB, das die Aminosäuren repräsentiert, lag für alle Messzeitpunkte nahe am Messergebnis. Die Multiplikation von AAE mit einem Faktor von 1,8 ergibt den gesamten bioNER, und der Wert lag für alle Probenahmezeiten ≥ 29 Tage im Bereich der niedrigen und hohen MTB-Schätzung. Darüber hinaus war der %aR Wert in AAE der sterilen Proben vernachlässigbar. Auch bei den Bromoxynil-MTB-Schätzungen wurde eine gute Übereinstimmung festgestellt. Allerdings zeigten die Messungen auch dort einen hohen Hintergrund in den sterilen Proben. Für Sulfadiazin wurde keine Übereinstimmung erzielt, da die Messungen den höchsten % aR Wert in AAE aller drei Substanzen ergaben, während die MTB-Schätzung aufgrund der vernachlässigbaren CO_2-Bildung sehr niedrige Werte für bioNER ergab. Auch für Sulfadiazin wiesen sterile Proben eine hohe Radioaktivität im AAE auf, was die Messungen zweifelhaft macht. Tatsächlich erscheinen bei diesem Vergleich von Schätzungen mit gemessenen Daten die geschätzten Werte zuverlässiger als die Messungen.

Schlussfolgerungen

Zur Bestimmung von Typ I NER wurden zwei unterschiedliche Extraktionsverfahren angewendet. Die chemische Analyse der Extrakte ergab in beiden Fällen, Silylierung und EDTA-Extrakt, sehr geringe Mengen an freigesetzter Ausgangssubstanz. Diese Ergebnisse der LSC- und Radio-DC-Analyse der Silylierungsextrakte und EDTA-Extrakten wurden durch die LC-MS-Analyse der ^{13}C-Extrakte bestätigt. Aus den Erfahrungen dieses Projektes geben wir folgende Empfehlung für die Bestimmung von Typ I NER:

Tabelle 1: Empfehlung für Verfahren zur Bestimmung von Typ I NER

	Silylierung	EDTA Extraktion
NER Typ I und II Unterscheidung	geeignet	geeignet
Anwendbarkeit, Standardisierung	Anspruchsvoll	Einfach
Anwendbar in Routinelabor	Ja	Ja
Erfassung Typ III NER	gering (unpolares Lösungsmittel)	Etabliert zur Extraktion von Proteinen, Nukleinsäuren
Zweiter Extraktionsschritt erforderlich	Notwendig für hydrophile Substanzen	Empfohlen für die meisten Substanzen
Extrakt Aufarbeitung / Anreicherung	Einfach	Schwierig aufgrund der hohen Salzfracht
Substanzstabilität	Nachweis der Stabilität erforderlich	Nachweis der Stabilität erforderlich[2]

Ein besonderer Fokus des Projekts lag auf der Anwendbarkeit der Verfahren in der Routineprüfung. Für die Extraktionsmethoden zur NER-Definition ist dies leicht zu beantworten, da alle angewandten Methoden bereits fester Bestandteil von Standard-Abbaustudien sind. Dies schließt PLE ein, das aber wahrscheinlich nicht in allen aktuellen Abbaustudien angewendet wird. Dennoch kann erwartet werden, dass PLE als Standardgerät in einem Routinelabor, das sich auch mit regulatorischen Abbaustudien befasst, vorhanden ist.

Das Silylierungsverfahren erwies sich als einfach anzuwenden, nachdem es einmal im Labor etabliert war. Bei der Verwendung gefährlicher Chemikalien ist mit besonderer Vorsicht vorzugehen, dies ist jedoch auch bei anderen Laborverfahren üblich. Die resultierenden Extrakte sind für nachfolgende chemische Analysen leicht zu verarbeiten.

Bei der EDTA-Extraktion ist es genau umgekehrt: Die Chemikalien sind unkritisch und die Extraktion ist sehr einfach. Die Aufarbeitung des Extrakts für die anschließende Analyse kann jedoch aufgrund der hohen Salzfracht sehr schwierig werden. Im Allgemeinen sind sowohl die Silylierung als auch die EDTA-Extraktion für den routinemäßigen Einsatz im Labor geeignet.

Schwieriger zu beurteilen ist die saure Hydrolyse. Im Allgemeinen eher einfach, wird es durch die fehlende Standardinstrumentierung eingeschränkt, die eine Extraktion in geschlossenen Gefäßen mit konzentrierter Säure unter Druckbedingungen für 24 Stunden ermöglicht. Dieses Problem sollte jedoch lösbar sein. Es gibt mehr Bedenken wegen der komplizierten und zeitaufwändigen Reinigung des Extrakts, gefolgt von einer noch aufwändigeren Radio-DC. Solche Analysen werden für eine Routinestudie nicht als geeignet erachtet. Zumal es keine eindeutigen Daten zu bioNER liefert, sondern nur einen Anhaltspunkt. Aufgrund dieser Erfahrungen

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[2] Unerwartete Verluste an Testsubstanz wurden in Wiederfindungs-Experimenten beobachtet.

kommen wir zu dem Schluss, dass die experimentelle Bestimmung von bioNER weiterentwickelt werden muss und noch nicht für den Laboralltag geeignet ist.

Workshop

Am 17. und 18. Februar 2021 veranstalteten das Umweltbundesamt und das Fraunhofer IME zusammen mit den Projektpartner RWTH Aachen (Prof, Dr. Andreas Schäffer), UFZ Leipzig (Prof. Dr. Matthias Kästner) und DTU Lyngby (Prof. Dr. Stefan Trapp) einen internationalen Online-Workshop mit dem Titel „Vorschlag zur Standardisierung der Analyse und Persistenzbewertung von nicht extrahierbaren Rückständen (NER). Mehr als 70 Teilnehmer aus Behörden, Industrie und Wissenschaft, darunter Mitglieder der PBT-Expert Group der ECHA, diskutierten die zukünftige Berücksichtigung von NER bei der Persistenzbewertung. Als Diskussionsgrundlage dienten das Diskussionspapier der ECHA zur NER-Bewertung (Kästner et al., 2018) und die Ergebnisse des aktuellen Projekts.

Ziel des Workshops war es, einen praktischen Ansatz für ein harmonisiertes Testverfahren zur NER-Charakterisierung vorzustellen und zu diskutieren. In dieser Diskussion war der Standpunkt der Industrie ein wichtiger Indikator für die Akzeptanz und praktische Anwendbarkeit des vorgeschlagenen Ansatzes. Darüber hinaus waren hinsichtlich der Auswirkungen der NER-Charakterisierung auf die PBT-Bewertung die Beiträge der Aufsichtsbehörden notwendig, um eine belastbare Aussage zur Anwendbarkeit zu erhalten.

Es wurde vorgeschlagen, dass z.B. PLE der letzte Schritt in einem schrittweisen Extraktionsverfahren sein sollte, um eine definierte Grenze zwischen dem extrahierbaren Anteil und den NER (gesamt) festzulegen. Weitere Ergebnisse waren, dass Leitlinien für das schrittweise Vorgehen bei der NER-Charakterisierung und Persistenzbewertung erforderlich sind. Zudem erwies sich die praktische Bestimmung von „bioNER“ (Typ III NER) als nur bedingt praxistauglich. Daher wird ein Modellierungsansatz (MTB) vorgeschlagen, der eine gute Schätzung von bioNER liefern soll.

Basierend auf den Diskussionsergebnissen wurden zwei Flussdiagramme entworfen, die Wege aufzeigen, wie NER bei der Persistenzbewertung berücksichtigt werden können. Beide Flussdiagramme stellen ein stufenweises Vorgehen mit zunehmendem Laboraufwand in jedem Schritt, aber gleichzeitig höherem Realitätsgrad bzw. abnehmender Konservativität dar.

Flussdiagramm zur Berücksichtigung von NER bei der Persistenzbewertung

Vorschlag 1 (Abbildung 1) stellt einen „Worst-Case-Ansatz“ dar, der sich hauptsächlich auf die Entscheidung persistent/sehr persistent (P/vP) oder nicht persistent (nicht P) konzentriert, mit geringerer Relevanz der spezifischen berechneten DT_{50}-Werte, da diese nicht zur weiteren Gefährdungsbeurteilung benötigt werden. Die Idee ist ein schrittweiser Ansatz mit erhöhten Datenanforderungen bei jedem nachfolgenden Schritt, was zu einer Verbesserung der Bewertung führt. Eine NER-Charakterisierung ist nur in den Fällen 1 und 2 erforderlich. Wird der P/vP-Trigger hingegen nach dem 4. Schritt immer noch gerissen, ist der Stoff als persistent oder sehr persistent zu bewerten. Die vorgeschlagenen Flussdiagramme wurden auf die, während des Projekts gewonnenen experimentellen Datensätze (^{14}C-radioaktiv markierte Substanzen) angewendet. Für die Berechnungen wurde CAKE verwendet und die SFO-Kinetik ausgewählt. Die folgenden Tabellen geben einen Überblick über die nach dem Flussdiagramm ermittelten Ergebnisse.

Tabelle 2: Stufenweise Berechnung von Halbwertszeiten gemäß Flussdiagramm 1

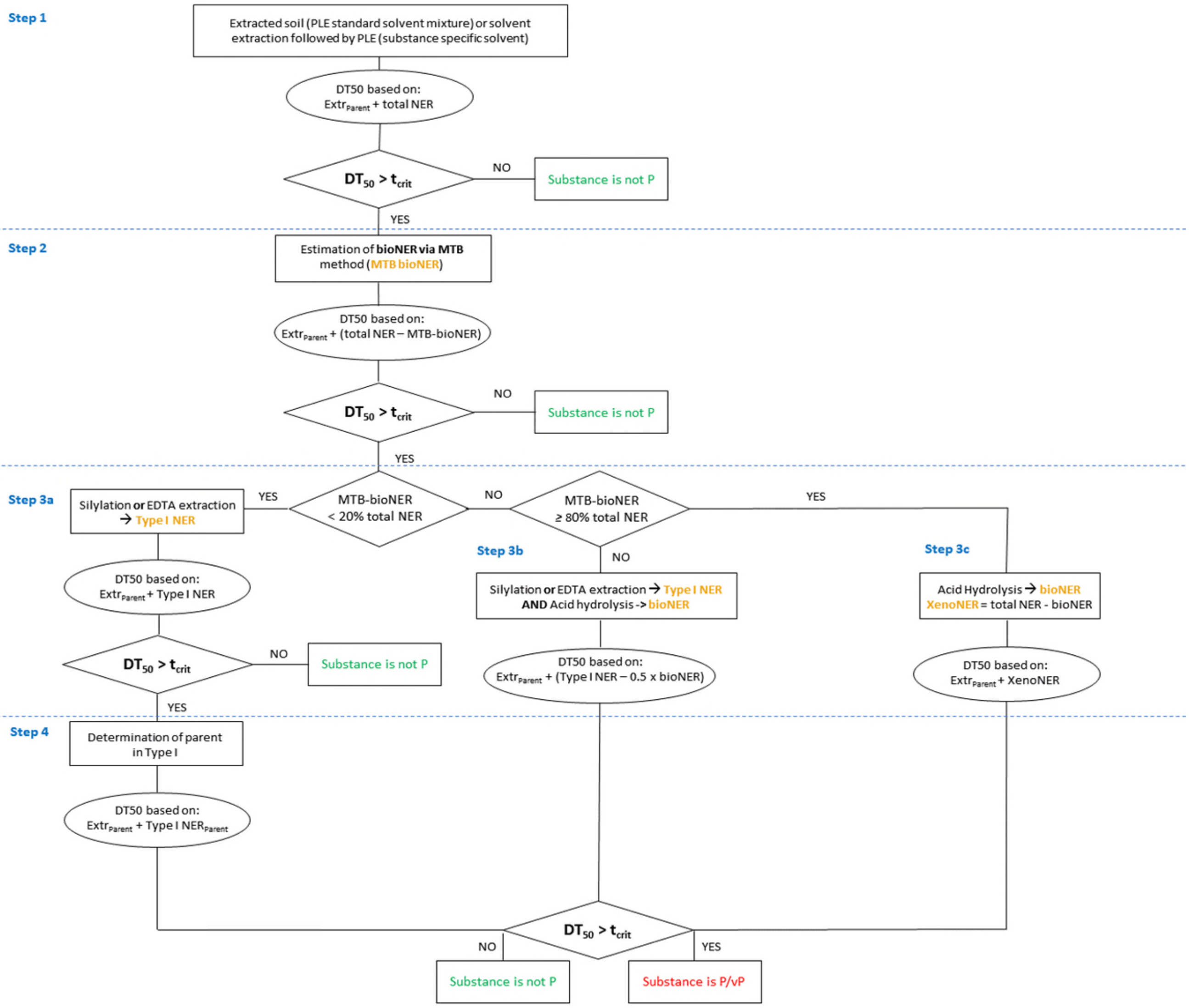
	Beschreibung	Bromoxynil	Sulfadiazin	Isoproturon
aktuell	**Extr.** $_{\text{Parent}}$ (NER als Abbau bewertet)	7.0	7.9	53.8
Step 1	**Extr.** $_{\text{Parent}}$ **+ total NER**	246	562	250
Step 2	**Extr.** $_{\text{Parent}}$ **+ (total NER – MTB bioNER)**	194	528	140
Step 3a	**Extr.** $_{\text{Parent}}$ **+ Type I NER**	12.4	39.3	57.9
Step 3b	**Extr.** $_{\text{Parent}}$ **+ (Type I NER – 0.5 x bioNER)**	10.3	31.4	49.3
Step 3c	**Extr.** $_{\text{Parent}}$ **+ (total NER –bioNER)**	132	365	147
Step 4	**Extr.** $_{\text{Parent}}$ **+ Type I NER** $_{\text{Parent}}$	8.1	10.4	48.8

Tabelle 3: Stufenweise Berechnung von Halbwertszeiten gemäß Flussdiagramm 2

	Beschreibung	Bromoxynil	Sulfadiazin	Isoproturon
Step 1	**Extr.** $_{\text{Parent}}$ (NER als Abbau bewertet)	7.0	7.9	53.8
Step 2a	**Extr.** $_{\text{Parent}}$ **+ Type I NER**	12.4	39.3	57.9
Step 2b	**Extr.** $_{\text{Parent}}$ **+ (total NER –bioNER)**	132	365	147
Step 3	**Extr.** $_{\text{Parent}}$ **+ Type I NER** $_{\text{Parent}}$	8.1	10.4	48.8

Die Ergebnisse zeigen, dass die Einstufung von NER als nicht abgebaute Testsubstanz jede Testsubstanz, die dazu neigt, im Boden NER zu bilden, als persistent erscheinen lässt. Eine differenzierte Analyse der NER zeigen jedoch, dass die getesteten Stoffe alles andere als persistent sind.

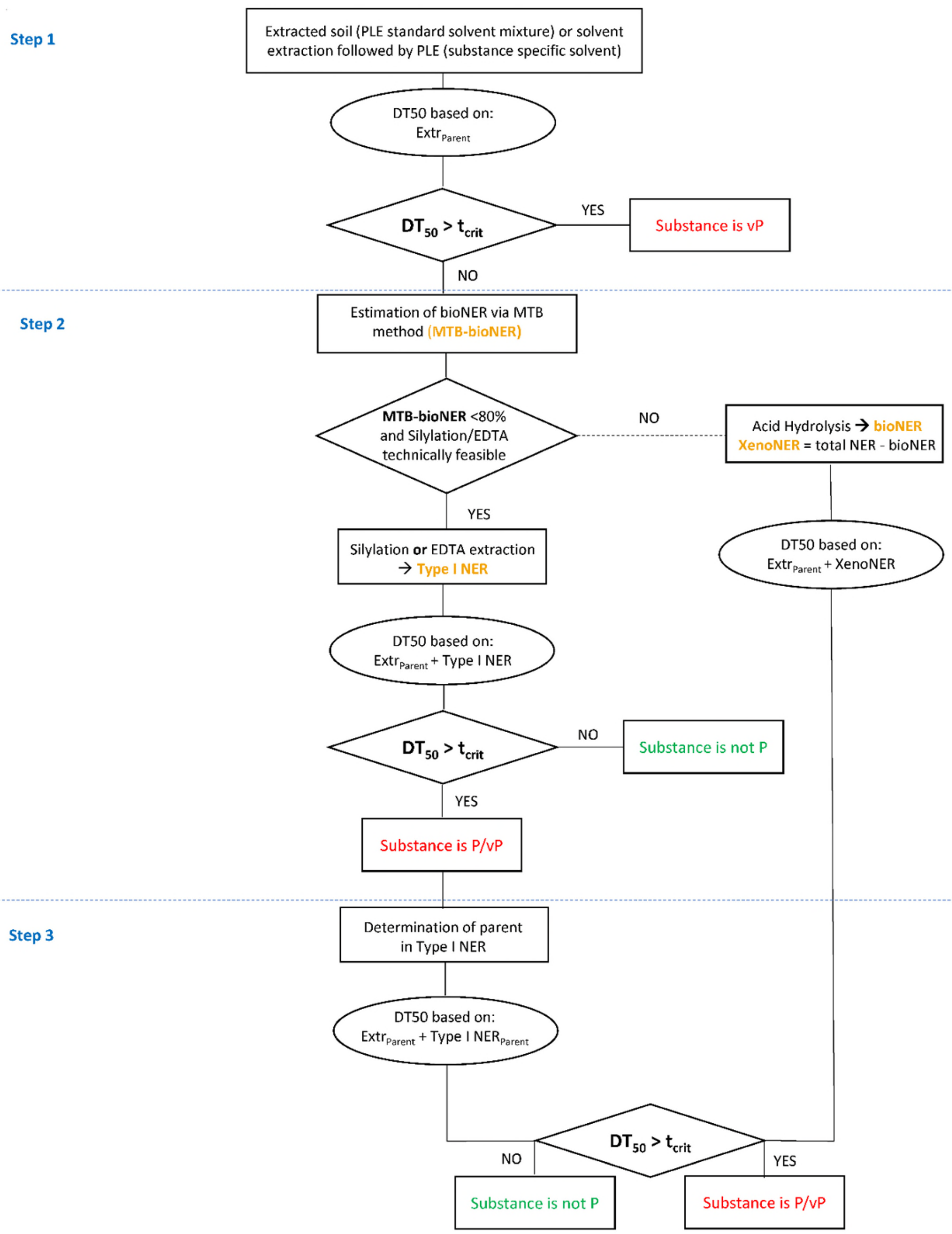
Abbildung 1: Flussdiagramm zu Berücksichtigung von NER in der Persistenzbewertung, Vorschlag 1



Quelle: https://www.umweltbundesamt.de/sites/default/files/medien/362/dokumente/proposal_1_final.pdf

Vorschlag 2 (Abbildung 2) stellt einen „realistischen Ansatz“ dar, der den Schwerpunkt darauf legt, Halbwertszeiten von Stoffen so realistisch wie möglich abzuleiten. Diese könnten dann für die Persistenzbewertung, aber auch für die Expositionsbetrachtung eingesetzt werden. Ausgegangen wird von einem „Best-Case-Szenario“, in dem davon ausgegangen wird, dass NER vollständig als sichere Senke bewertet werden. Überschreitet die aus den durch chemische Analyse der Extrakte ermittelten Daten der Testsubstanz abgeleitete DT$_{50}$ schon den Auslösewert für vP im Boden, ist keine weitere Betrachtung erforderlich. Eine Verfeinerung und weitere Charakterisierung der NER im Boden sollte in Erwägung gezogen werden, falls im ersten Schritt der vP-Triggerwert nicht erreicht wird und für alle Fälle, in denen der Antragsteller einen realistischeren Wert für DT$_{50}$ ableiten möchte.

Abbildung 2: Flussdiagramm zu Berücksichtigung von NER in der Persistenzbewertung, Vorschlag 2



Quelle: https://www.umweltbundesamt.de/sites/default/files/medien/362/dokumente/proposal_2_final.pdf

1 Introduction

1.1 Regulatory assessment of NER

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. Another definition was published by Beulke et al., (2012) (FERA) 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” (Beulke et al., 2012).

The approval and application of pesticides, biocides, REACH chemicals and human and veterinary products in Europe is organized by various legislations: the REACH regulation (EC 1907 2006) for industrial chemicals regulation 528/2012 for biocidal products, EC 1107/2009 for plant protection products as well as EC 726/2004 for human and veterinary medicinal products, respectively. These regulations including PBT assessment are specified in more detail by the guidance documents published by the ECHA, for example for the assessment of PBT properties (ECHA 2017 R.7b; ECHA 2017 R.7c; ECHA 2017 R.11). These documents deliver the general procedures to be followed for assessment of the compounds properties including the quantification of total NER. However, until 2018 no detailed approach to differentiate NER types was available.

NER can be detected only using isotope labelled compounds (e.g. ^{14}C-radiolabel or ^{13}C stable isotope label). Because these residues are non-extractable, they cannot be measured with conventional chemical analytics, which requires liquid samples, and consequently NER appear a black box in environmental monitoring. This makes the assessment in prospective regulation (risk assessment of chemicals) difficult.

Due to the lack of a standardised method allowing the characterisation of NER, they are currently treated incoherently in European regulations. While REACH considers NER as non-degraded substance if not proven otherwise, others, e.g. the plant protection product regulation, up to now consider NER as degraded. This has significant impact on the degradation half-lives determined and thus on the outcome of the persistence assessment.

Several concepts for the analysis of NER were developed and evaluated in the last decades, for instance by Eschenbach and Oing (2013) differentiating four NER types. Recently, this approach was further developed by new experimental and modelling techniques (Kästner et al., 2014; Schäffer et al., 2018) resulting in a clear differentiation of three NER types.

In parallel to the approach described in the ECHA discussion paper (Kästner et al., 2018), another concept for NER assessment was developed in the framework of the UBA project FKZ 3713634131 performed by the German Federal Institute of Hydrology, Germany. Overall aim of this project was to validate and refine the approach of Eschenbach & Oing (2013) and to develop a general approach for the quantification of NER in soils (Loeffler et al., 2020, UBA Texte 133/2022).

1.2 Current assessment approaches for NER

Several concepts for the analysis of NER were developed and evaluated in the last decades, for instance by Eschenbach and Oing (2013) differentiating four NER types. Recently, this approach was further developed by new experimental and modelling techniques (Kästner et al., 2018; Schäffer et al., 2018) resulting in a clear differentiation of three NER types described below.

The general approach for investigating non-extractable residues of chemicals in solid matrices such as soil and sediment is the following: after the experimental exposure of isotope labelled compounds – both stable and radioactive labels can be used – to soil or sediment for a certain amount of time, the matrix is treated by liquid extraction methods that are considered not to destroy the nature of the matrix and of the residues contained in it. The remaining isotope label after `exhaustive´ extraction is then considered to constitute the NER.

However, a high diversity of extraction methods was applied by research groups with very variable extraction efficiencies particularly related to the huge variability of the applied parent molecules. The methods applied relied on water-based extractions, extractions with organic solvents up to supercritical fluid extractions aided by thermal or ultrasonic treatments. A thorough review of the applied methods and results that were published in the scientific literature (ISI-ranked) is given in a recent ECHA discussion paper on NER (Kästner et al., 2018).

A general, well-known, and decade-old problem of the assessment of NER is that its definition resulted in misleading science and mismatching regulation and environmental risk assessment. Various definitions, such as from IUPAC (Roberts, 1984) consider only parent active compounds or primary metabolites as constitutes of NER but explicitly exclude natural molecules after biodegradation. However, the experimental method of applying mostly radioactive (often ^{14}C) labelled compounds captures all kinds of molecules in the residues. Thus, previous research targeted on identifying parent molecules or metabolites in the NER matrix by various analytical methods and destructive treatments of the NER containing solid matrix. Many physical and chemical processes were identified that can lead to a more or less irreversible sorption or entrapment of such compounds in the solid matrix and humic molecules in soils and sediments. Unfortunately, in the majority of cases only a few percent of parent compounds or metabolites could be identified (Kästner et al., 2018).

In the last decade upcoming research thus tried to fill the gaps and focused on the conversion of the labelled carbon into natural biomolecules after biodegradation; in fact, the considerable label incorporation into such molecules was detected for various chemicals. Based on these findings a classification approach for NER was developed: according to the extraction steps the total amount of NER is the sum of strongly adsorbed, sequestered or entrapped (type I) and covalently bound residues (type II) both either derived from the parent substance or from transformation or degradation products. A third type (III) refers to natural biogenic NER that are derived from biotic degradation accompanied by the anabolic use of the labelled atoms. These three NER types are formed by competing processes and discriminating analytical methods have been described (Kaestner et al., 2014). This classification will be referred to throughout the present report and takes into account that sequestration – a term describing strongly sorbed and physically entrapped molecules – in the particulate matrix can be identified using current methods, but the processes leading to sequestration so far cannot reliably be evaluated.

The majority of references for extraction procedures and NER determination are already described in the ECHA discussion paper (Kästner et al., 2018). The few additional examples of NER release studies are summarized in the following.

The stability of NER generally depends on their age, as shown in soil experiments with 15-days-old NER and 90-days-old NER derived from 2,4-D (2,4-dichlorophenoxy acetic acid). The addition of fresh soil to "young" NER induced its mineralisation, whereas the aged NER were stable and turned over at rates similar to SOM (Lerch et al., 2009). Soil treated with 14C-parathion contained after 7 weeks of incubation 27% of the applied radiocarbon as NER. After mixing the NER containing soil with fresh soil for 4 weeks 26% of NER was mineralised and 5% became extractable with the parent substance as main component. This indicates that soil microorganisms play an important part in the release and metabolism of NER. Introducing 'fresh' matrix like compost or soil will have both disadvantages and advantages, such as sample dilution, changes in microbial population, potentially increased availability of the extractable fraction, and reduction in potential ongoing toxic affect to biomass associated with a static test system. Promotion of microbial activity by addition of easily degradable substrates such as farmyard manure leads to a partial release of NER (Racke and Lichtenstein, 1985). Plant root exudates can remobilize aged residues as shown with DDE (dichlorodiphenyldichloroethylene) residues and its uptake in squash, pumpkin, cucumber, melon (White, 2002); the presence of DDE residues in the plants was analytically verified but no attempt was made to determine the amount of NER in the soils. ^{14}C-atrazine formed high NER in soil, i.e. more than 50% of the applied amount. After incubation with Pseudomonas species 30-35% of the NER amount was released. The parent substance and its hydroxy- and mono-dealkylated analogues were released into the incubation mixture and were subsequently metabolised by dechlorination and dealkylation (Khan and Behki, 1990). Also physical manipulations like freezing-thawing, pH shifts, and plant growth may lead to the partial release of NER; see references cited in (Kästner et al., 2018).

However, there are reports not published in ISI referenced papers or are published later than mid of 2018 when the ECHA discussion paper was published (Kästner et al., 2018). Such references are described in the following.

In the framework of an UBA review (FKZ 360 01 070: Erarbeitung eines gestuften Extraktionsverfahrens zur Bewertung gebundener Rückstände) (Eschenbach and Oing, 2013) provided a literature review about various concepts for the determination of NER. The authors described in detail different extraction procedures and suggested four types of NER based on their extraction method:

I sorbed compounds that can be extracted by solvents of increasing `harshness´

II entrapped compounds that can be mobilised by matrix destabilising agents, e.g. EDTA-extraction or silylation

III covalently bound compounds that can be evaluated by sequential chemical degradation of different bonds (ester, ether, and C-C bonds) or by immunoassays

IV so-called biogenic fixation that can be evaluated by fatty acid extraction, amino acid or amino sugar extraction, or fumigation-extraction methods; however, this type was considered to be associated with a high bias, since not all biomolecules can be targeted in general.

This Eschenbach and Oing (2013) classification differs from that provided in the ECHA discussion paper (Kästner et al., 2018) where NER I covers both sorbed and entrapped parent substance, because both types are determined in the same extraction step (by silylation). Sorbed and entrapped residues (I and II in the Eschenbach classification) are practically not really distinguishable, since no simple experimental methods are available. The suggested application of immunoassays for NER III is also not really decisive, and the chemical degradation methods are only valid, if they do not also degrade potential metabolites. The authors refer to the methods by Riefer et al. (2011) that are based on cleaving a sequence of defined chemical bonds in SOM: i) alkaline hydrolysis for ester bonds, ii) boron tribromide application for cleavage of ether bonds, iii) ruthenium-tetroxide for the oxidation of aromatic rings and functionalized C atoms, and iv) tetramethyl-ammoniumhydroxid (TMAH) thermochemolysis for the thermal cleavage of C-C bonds and methylation of functional moieties. In addition to the already considered bias of biogenically fixed NER IV, the methods for the determination of dead biomass (necromass) were not fully developed at the time when the review was provided. Hence, the suggested fumigation-extraction methods or fatty acid extraction would always underestimate real bioNER, since necromass would not be captured. Therefore, this classification was not recommended to be applied in the ECHA guidance (Kästner et al., 2018; Schäffer et al., 2018).

Eschenbach and Oing (2013) finally recommended the EDTA based extraction method (Eschenbach et al., 1998), which results in destabilisation and disaggregation of macromolecular structures of soil organic matter (SOM) with a potential release of I (sorbed) and II (entrapped) compounds. The authors considered the silylation method (Haider et al., 2000) as too time consuming but admitted that this method may provide clear differentiation of entrapped and covalently bound residues. Humic matter fractionation was considered not to provide an option for a clear characterisation of NER speciation. The final recommendation by the authors for a NER assessment procedure was:

1) shaking extraction with solvents for NER type I (and production of NER containing materials for procedures 2-4), 2) EDTA extraction or silylation for NER type II, 3) sequential chemical degradation for NER type III, 4) a range of biomethods for NER type IV. Even if 1) is needed to provide the NER containing material, it is clear that NER type I as defined here are no NER in the sense of the term.

Providing real experimental proof for covalently bound NER in general is the most critical issue in NER characterisation and requires severe additional research efforts (Kästner et al., 2018). Single bonds like ester or ether linkages of type II NER are assumed to be rather stable and this type of bonding should be validated in procedures for assessment of potential remobilisation. Currently, no simple methods for the direct assessment of covalently bound NER (Type II) are available but for certain compounds the evidence of covalent bonds was provided. For example, a release of up to 15% ^{14}C from NER in soil containing residues from anthracene and TNT was observed after addition of the complexing agent EDTA, however no degradation products were characterized in the respective study (Eschenbach et al., 1998).

Weiss et al (2004) also applied the EDTA extraction method to extract NER containing soil material derived from ^{14}C-labelled TNT. The authors applied various methods for remobilization of NER, such as treatment by physical methods (freeze and thaw, grinding of soil, and steam extraction), chemical (acid rain and addition of metal complexing agent), and biological methods (addition of compost, white rot fungi, radical-generating enzymes, and germination of plants). The highest mobilisation of NER was found by steam extraction and acid rain extraction, which increased the water extractability by 11 to 29% in the biocomposted material and to 51.6% in the native TNT-contaminated control soil without treatment. The addition of EDTA increased the extractability of the label by 7 to 12% in the control soil but no significant increase was observed for the biotreated material (< 3%). The NER released by these mobilisation treatments were analysed for the presence of the parent substances and known main transformation products but neither anthracene nor TNT or transformation products were found to be released from SOM after EDTA addition, acid rain or hot water treatment. This indicates that the NER in these experiments were either not composed of sequestered parent substances and known transformation product or that they were not released by the methods applied.

Richnow et al. (1994) found the release of labelled PAH metabolites after hydrolysis of residue containing soil in the presence of ^{18}O-labelled NaOH indicating ester bonds of carboxyl group containing metabolites. However, in addition to covalent binding the same transformation products were also found to be strongly sorbed to the soil and sediment; the ratio of both binding types was estimated by comparing the amounts of ^{18}O labelled carboxylic acids and those containing no ^{18}O label. Similarly, 2,2-bis(chlorophenyl)acetic acid, a carboxylic acid transformation product of DDT as well as metalaxyl acid are covalently bound to humic matter in sediments by esterification as shown by $Na^{18}OH$ hydrolysis (Kalathoor et al., 2015).

Another study for differentiating sequestered and covalent NER types was provided by Wang et al. (2017c). After incubation of ^{14}C-phenanthrene in active soil for 28 days, 40% of the initial amount was mineralised and 70.1 ± 1.9% was converted to NER with most of it bound to soil humin. Silylation of the humin-bound residues released 45% of these NER residues, which indicated that they were physically entrapped, whereas the remainder of the residues were chemically bound or biogenic (bioNER were not investigated in this study). By contrast, in sterilised soil, less NER were formed and all of it was completely released upon silylation, which underlines the essential role of microbial activity in NER formation.

Zhu et al., (2019) showed that various specific DDT transformation products could be identified in each fraction after sequential chemical degradation of NER containing soils and river sediments, whereas the parent DDT and primary transformation products were extractable in the initial organic solvent fractions. Chemical derivation used by the authors were alkaline hydrolysis, BBr_3 treatment, RuO_4 oxidation, and TMAH thermochemolysis.

A new and elegant approach for studying covalent binding of chemicals to soil humic matter was recently presented by Matthies et al. (2016). They used a molecule containing an aniline ring and a stable paramagnetic nitroxide spin label (2,5,5-Trimethyl-2-(3-aminophenyl)pyrrolidin-1-oxyl) for incubation with humic acids and recorded electron spin resonance (ESR) spectra. Changes were observed in the ESR spectra comparing the free spin label molecule after incubation with humic acids. ESR line broadening indicated covalent binding of the anilino group to humic acids and thus changes in the microenvironment of the spin label. Half-life for this reaction was ca. 70 min and was not changed when the oxidative enzyme laccase was added but the amount of the reacting anilino-NO species was increased ca. 8-fold. Radical coupling as reaction mechanism was excluded by adding a spin trapping agent. Similar results were obtained with another molecule containing a primary amino group and the nitroxide spin label. This technique provides an important step further in visualizing and understanding covalent binding processes of xenobiotics containing aniline functional groups such as many pesticides, veterinary pharmaceuticals, textile dyes and other classes of industrial chemicals. In addition, chemicals which under reducing conditions form amino groups such as TNT will bind in the same way to soils or sediments. Research is necessary on whether this technique can be applied to complex matrices like soil. However, it has to be noted that the spin label is altering the molecule and thus partly its properties.

Recently, in the frame of the UBA project (FKZ 3715654152: PBT Bewertung Ionischer Stoffe, 2015) NER of ionized chemicals in comparison to structurally similar non-ionized substances have been characterized: NER of ^{14}C-labelled derivatives of 4-n-dodecylphenol, 4-n-dodecylbenzenesulfonic acid and 4-n-dodecylbenzyltrimethylammonium chloride in soil were investigated. After 84 days of incubation, 40.6 %, 21.7 % and 33.5 % of the applied radioactivity of the neutral, the anionic and the cationic substances were converted to NER. Silylation of the respective NERs released varying amounts up to 23 % of the applied radioactivity, equivalent to strongly sorbed and physically entrapped residues (type I NER). Analysis of extracts derived by silylation revealed that the parent of the uncharged chemical but neither those of the anionic and cationic chemicals were released by the silylation procedure. Acid hydrolysis of the NER containing soil and subsequent analysis of released ^{14}C aminoacids indicated that varying amounts up to about 43% represented biogenic residues. Experiments showed that anionic and cationic functional groups affect the amounts of sequestered covalently bound and biogenic residues over time in various soils. The study results have recently been published (Claßen et al., 2021; Holzmann et al., 2021; Holzmann et al., 2021(2); Claßen et al., 2019).

The identification of some labelled amino acids in residues from labelled phenanthrene (Richnow et al., 2000) and TNT ((Richnow et al., 2000; Weiss et al., 2004) indicated that the NER derived from microbial turnover in compost or straw-amended soils may be partly of biogenic origin. For TNT 11% of the NER were identified as potential bioNER and later it was shown that steam or hot water extractions released considerable amounts of bioNER (Kästner, personal communication). The absence of the relevant toxic components after mobilisation of the biotreated material shows that the parent substances were transformed into other compounds during the residue formation process.

1.3 BfG approach (UBA project FKZ 3713634131)

In parallel to the approach described in the ECHA discussion paper (Kästner et al., 2018), for details see below, another concept for NER assessment was developed in the framework of the UBA project FKZ 3713634131 performed by the German Federal Institute of Hydrology (Loeffler et al., 2020, UBA Texte 133/2022). Overall aim of this project was to validate and refine the approach of Eschenbach & Oing (2013) and to develop a general approach for the quantification of NER in soils (the final report has not yet been published). In a pre-screening, 42 organic chemicals with log Kow values of 1.2 to 4.9 were spiked to three German reference soils (Lufa 2.2, 2.3, and 2.4, with increasing pH values from 5.4 to 7.4 and C_{org} contents of 1.6%, 0.7% and 2%) and various extraction solvents and conditions were evaluated for the recovery of the spiked chemicals. The compounds were analysed by means of HPLC-MS/MS calibrated with stable isotope labelled reference substances. The chemicals covered various functional groups of molecules and active compound classes. Overall, 11 extraction methods were applied with seven different solvents and solvent mixtures [isohexane, ethyl acetate, acetone, methanol, methanol/acetone (50/50), methanol/acetone (50/50) plus 1% formic acid, and methanol/acetone/water (50/25/25)]. Five different procedures/conditions (shaking overnight, ultra-sonic extractions, microwave assisted solvent extraction (MASE), 3-step batch extractions, and pressurized liquid extraction (PLE)) have been applied using same extractants. In the screening part of the study methanol/acetone/water (50/25/25) as solvent mixture provided the highest recoveries of the 42 test compounds with recoveries above 90%. Finally, PLE at 100°C and 100 bars using the best-performing solvent mixture was found to be superior to all other methods. However, it has to be noted that the screening experiments were performed with lyophylised soil samples, whereas in the later experiments wet soil samples were employed. The reason for that was not explained.

In the detailed evaluation part of the study nine degradation experiments were performed according to OECD 307 (OECD 307, 2002) in three soils with the three ^{14}C-labelled test compounds: triclosan, fenoxycarb, and acetaminophen (soil moisture conditions of 44-55% WHC_{max}). Sampling dates were 1, 4, 7, 20, 34, 60, and 100 d. The samples were analysed by use of three consecutive shaking extractions steps with increasing `harshness´ in terms of solvent efficiency followed by a last step using PLE compared to the best performing solvent mixture (methanol/acetone/water (50/25/25)) in the PLE in one step under conditions of 100°C and 100 bars. It turned out that only in Lufa 2.4 with triclosan the sequential extraction recovery was higher than the single PLE extraction but all other sequential extractions resulted in lower values. The PLE extracted soils were used to analyse the NER speciation in more detail.

The approach in the BfG study quantifies the remaining amount or label as total NER without suggesting the need for further speciation analysis. The premise of this concept was that these NER should be considered per se non-remobilisable as type II and bioNER. The study also suggested that after the harsh PLE extraction presumably no physically entrapped NER (type I) may be present.

Triclosan was mineralized to $^{14}CO_2$ between 11 % and 27 % of the applied radioactivity within 100 d. NER continuously increased with incubation time, while the extractable fractions decreased for all soils. In Lufa 2.2 the radioactivity in the extracts decreased from 84 % after 7 d to 64 % after 100 d, while it increased in NER at the same time from 16 % to 34 %. In Lufa 2.3 a significantly higher percentage of NER (56 %) was formed. DT_{50}-values based on parent triclosan in the extractable fraction ranged between 43d and < 7 d and DT_{90}-values between 100 and 30 d. The half-life of triclosan was strongly dependent on the extraction method used and the soil type, since triclosan shows very strong sorption towards soil.

Fenoxycarb was rapidly mineralized in all three soils and with a continuous increase of $^{14}CO_2$. After 100 d of incubation between 48 % (Lufa 2.2), 43 % (Lufa 2.3) and 40 % (Lufa 2.4) of the applied label were mineralized. $^{14}CO_2$ was formed rapidly within the first 15 d indicating a rapid biodegradation. Simultaneously the extractable radioactivity decreased rapidly. At day 1, 34% - 65 % of applied radioactivity were extractable, whereas after 100 d only 5 % - 8 % of the applied label was extractable. NER were rapidly formed and did not change significantly during the later incubation. The quantities of NER from fenoxycarb were similar in all three test soils resulting in 45 % NER in Lufa 2.2, 51 % NER in Lufa 2.3 and 55 % in Lufa 2.4. In all soils Fenoxycarb was rapidly transformed with DT_{50}-values < 5d and DT90-values < 11 d.

Acetaminophen was mineralized to $^{14}CO_2$ continuously increasing to 14 % (Lufa 2.2), 18 % (Lufa 2.3) and 11 % (Lufa 2.4) of the applied radioactivity after the shorter incubation times of 35 d. This compound was rapidly bound to the soils and formed NER. A few hours after spiking, only 3 – 5 % of the applied radioactivity was extractable and only 2 % was extractable after 35 d, resulting in 88 – 95 % NER. Subsequent PLE removed 3 – 4 % of the applied radioactivity with 84 – 91 % remaining bound to the soil.

For further speciation of the NER authors of the BfG study suggested to use aqueous EDTA solutions for extractions. They concluded that the extractability of the NER label from all test compounds and soils by EDTA were found to be similar or slightly higher (5-15%) than for the silylation (2-13%) depending on the compound and soil. The study points to a drawback of the silylation: transformation products having polar functional groups such as OH or NH will or may also be silylated, which would increase the challenge of identification of residues after silylation. On the other hand, EDTA extracts will likely contain high amounts of bioNER due to co-extraction of proteins, amino acids, phospholipids, nucleic acids (Tien et al., 1999; Plassart et al., 2012; Rai et al., 2010; Miller, 2005). Also, as a further difficulty for analytical investigation of the EDTA extract is the resulting a high salt content.

Regarding bioNER, the study complained about missing methods for the analysis of ^{14}C labelled amino acids and considers the recently pulished method by Possberg et al. (2016) based on TLC as too laborious for this study. The authors skipped this method and established only the first part of the method using the full HCl hydrolysis of biomaterials. They found a substantial release of label after hydrolysis with 10-20% for triclosan, 25-30 % for fenoxycarb, and 30-35% for acetaminophen. However, it remained unclear how much label was actually released from incorporated labelled C into biomass in comparison to other natural and/or still xenobiotic residues, since the total amount of label will certainly be higher than that for amino acids and microbial cell wall components will also be hydrolysed resulting in a release of amino sugars.

1.4 Philosophy of ECHA approach and BfG approach

Based on the thorough survey of the scientific state of the art in 2017 and 2018 a unified guidance for the differentiation of various NER types was developed and published (Kästner et al., 2018; Schäffer et al., 2018).

Unfortunately, the guidance documents (ECHA R7b and R11) regarding NERs execute a paradigm shift from one extreme position (i), assuming NER as degraded residues of no environmental concern in the regulation of pesticides [(DG SANCO 2012; FOCUS, 2014) at least if the NER are below or the mineralization rate are above certain threshold values to the other extreme of considering them as (ii) potentially bioavailable and non-degraded residues ("parent substance") in the persistence assessment of general industrial chemicals (EC 1907 2006; ECHA 2017 R.7b; ECHA 2017 R.11), if no other specific information is available. This paradigm shift caused severe uncertainties particularly in the assessment of pesticides and biocides.

For a comparison of the approach the NER types of the ECHA approach (Kästner et al., 2018) need to be defined clearly. The following definition is based on the differentiation of (Kästner et al., 2018; Schäffer et al., 2018) and is used in the entire report.

Definition NER in this report

Total NER is the amount of isotope label remaining in the solid extraction residue after PLE as terminal extraction step. Total NER are differentiated in three NER subtypes.

NER type I is strongly sorbed and/or physically entrapped test substance. Binding is reversible and thus, they are considered potentially remobilisable.

NER type II are covalently bound to the solid organic matrix. They became an irreversible part of the soil matrix and there is no concern that they can be released from the matrix.

NER type III (=bioNER) is all isotope label fixed in living biomass and in soil organic matter SOM derived from isotope labelled dead biomass ("necromass"). NER Type III are considered to be of no concern for the environment.

In the new guidance documents the characterization of NER will be embedded in the general PBT assessment of chemicals under REACH based on the suggestions of Kästner et al. (2018) and Schäffer et al. (2018) for differentiating various types of NER. Unknown total NER are considered as remobilisable parent or transformation products, if no additional information is available. Clear indications for bioNER or covalently bound type II NER are considered as `safe sink´, for the latter without remobilisation potential unless indications for such potential are available. In addition, a procedure for the estimation of the conversion potential of chemicals into microbial biomass will be embedded into the guidance document that relates the formation of CO_2 and microbial biomass (bioNER, type III) to the chemical structure, thermodynamic data of biotransformation, and the amount of bioavailable substance (Microbial Turnover to Biomass (MTB) Model) (Brock et al., 2017; Trapp et al., 2018). The knowledge of these estimation results allows a much more focused analysis of the amounts of specific NER.

Although the concepts of exhaustive extractions in the BfG and the ECHA approaches are similar, there are other relevant differences, which could finally lead to different assessments.

First, the ECHA guidance suggests compound specific extraction procedures for assessment of the remobilisation of compounds from the matrix. After exhaustive extractions (case by case decisions on suitable solvents) including PLE extraction, the remaining amount of label is considered as total NER that subsequently undergoes detailed analysis using silylation of the matrix for differentiation of type I and II NER. Extractable parent or primary transformation products after silylation are considered to be type I NER, whereas label remaining in the solid matrix is considered as type II NER. The materials may also contain some bioNER that needs to be corrected by the following approach: another aliquot of the total NER containing sample is fully hydrolysed in 6 N HCL and the label released from proteins will be analysed for label incorporation into biogenic amino acids. This label amount multiplied by a factor of 2 (for incubation times < 30 d or with a decreasing factor down to 1.1 for long term experiments > 120 d) is considered for the total conversion into microbial biomass (type III NER). The ECHA guidance is requesting these determinations particularly for the assessment of the persistence criterion according to REACH but the other regulations require such determinations during general fate assessment. In that respect, parent substance determined as type I NER are considered to increase the degradation half-life (DegT_{50}).

In contrast, the BfG approach (Loeffler et al. 2020, UBA Texte 133/2022) considers the one-step exhaustive quantification of the extractable fraction for all substances with a standard solvent mixture of methanol, acetone and water (50:25:25%, 100 °C, 100 bar, if technically feasible, 3 cycles) as sufficient for quantification of the amount of total NER including the main part of NER I, suggesting that the dominant fraction of such strongly sorbed fractions will be extracted under that conditions. In this approach, the remaining NER are defined as total NER and per se as \`non-remobilisable´. No additional analyses are requested but it was suggested for gaining further information on NER to perform: i) a strong hydrolysis of the solid NER containing sample and taking the released soluble label as bioNER, and for another aliquot of the sample, ii) an EDTA treatment (preferably) or silylation for solubilising trapped/ physically entrapped (sequestered) NER. The latter extraction, however, is somehow contradicting the expectation that initial PLE with standard solvent mixture will remove such residues but may be used as control approach.

The BfG approach provides first options/ ideas for the application of these data for the persistence assessment but does not concretise or validate them. In addition, the approach is not completely consistent with both the ECHA uidance's that requests the determination of the NER speciation and the OECD test guidelines (OECD 307, 2002; OECD 308, 2002) that require anyway compound specific optimisation of the extraction procedure in terms of solvents and physical and chemical treatment procedures but a standard solvent mixture may provide a first approach. NER determined as a result of the extraction procedures as described in the OECD simulation tests will differ from those determined according to the BfG approach. Therefore, the harmonisation of both approaches may be challenging. This is not possible by simple adaptions, and thus requires a general \`political´ decision. Of course, both NER approaches will result in the determination of presumably more or less adequate amounts of total NER. The BfG approach is pragmatic but may lead for certain compounds (those providing less than 90% recovery with the standard solvent mixture) to underestimation of the NER speciation and the associated persistency of the test compound. Harmonization of both approaches seems necessary.

1.5 Conceptual differences, incompatibilities, and detailed comparison of ECHA guidance and the BfG approach

Both concepts for NER determination show conceptual similarities, both with a focus on a better assessment of NER for the approval of chemicals. However, the concepts follow different aims.

The BfG approach can be used as pragmatic, fast, and routine extraction procedure to determine the "maximum extractable" and per definition remobilisable amount of parent substances and transformation products. The remaining amount of label is considered as total NER representing a `safe sink´ which only contains bioNER and/or non-remobilisable type II NER. Further analyses are only considered as option.

Contrary to the BfG approach, the ECHA approach is going beyond the determination of the total NER and focuses on the gain of maximum information by the speciation of NER. It includes an analytical approach to determine type I + II NER after disaggregation of the solid organic matrix by silylation, and to evaluate the bioNER formation by analysing the total amount of label incorporated in microbial proteins and correcting it for total microbial biomass. Some details of the BfG approach have been provided above. With regards to the ECHA approach please refer to Kästner et al. (2018) and Schäffer et al. (2018). However, for chemicals under REACH for which often no fate studies are available, the BfG approach may provide an easy-to-access data set, but again this requires `political´ decisions.

One major problem of both concepts is that the applied extractions steps do not clearly distinguish between the various NER types, since type III (bioNER) will distribute in both type I and II NER containing fractions after silylation or EDTA-extraction. A potential pragmatic approach is to consider the portion of bioNER determined by (separate) strong hydrolysis approximately also for the separated fractions of type I and II NER. A more complex approach would be to apply a ^{14}C-amino acid analysis to NER type I extract and the acidic hydrolysis to the NER type II residue followed by ^{14}C-aminoacid analysis. This methodology, however, needs to be established.

An additional problem for harmonisation of both concepts is derived from their different philosophies. The ECHA guidance documents (ECHA 2017 R.7b; ECHA 2017 R.11) motivated by the persistence assessment consider unknown NER as `hidden hazard´. Non-specified NER after PLE without a far-reaching characterisation cannot simply be classified as `non-remobilisable´. On the other hand, one may argue how reliable a high effort analysis will be, if the preceding silylation procedure possibly may have chemically destroyed the parent compound or the transformation products. This, however, can be tested by corresponding control experiments. The proposed EDTA extraction may thus provide a potential alternative, which needs to be evaluated. The experimental comparison is the basis for the current project.

In addition, general limitations of both approaches are laying in the uncertainties of the NER formation in the biodegradation/simulations tests as already stated in the REACH documents (ECHA 2017 R.7b). Biodegradation and NER formation depend on: i) the chemical properties of the compounds, ii) the applied concentrations and their bioavailability, iii) the microbes in the systems and a potential pre-exposure to the compounds (adaptation), iv) their growth behaviour, and v) the general conditions of the respective test set up, e.g. soil type and organic cabon content. The total amount of NER formed in such experiments generally depends on the extraction efficiency of the applied method. Therefore, weight of evidence evaluations of such results has to be taken with care and expertise.

1.6 Objectives

The aim of the project was to identify similarities and differences between the two approaches (Loeffler et al., 2020 and Kästner et al., 2018) and to test them experimentally.

The aim of the project was also to review the two recommendations for the experimental identification and classification of the overall NER and NER types I, II, III with regard to their consideration in the persistence assessment. Furthermore, the results should be used for a confirmation of the MTB bioNER estimation method.

On the basis of the results, a harmonised procedure for NER characterisation was proposed and compared experimentally with the two approaches available. The harmonised procedure ultimately forms the basis of the evaluation concept to be developed for taking NER into account in the persistence evaluation.

This concept was presented and discussed in an international workshop before the research results are finally published.

2 Working program

2.1 Description of work program and background

In a first step, literature reports on NER characterization were compared with a focus on extraction procedures. Open questions for the subsequent work packages were suggested and a proposal for the experimental assessment of NER drafted with the aim to harmonize the different approaches.

An experimental comparison of both concepts was conducted with specific focus on:

- Applicability:

The experimental procedure for further NER characterization must not be proven only to deliver information about a specific pre-defined NER fraction, but it must also be practicable in laboratory daily routine. The use of dangerous chemicals like 6 M HCl is not assessed to be problematic as lab staff is trained to work with hazardous substances and usually labs have appropriate protection systems. This does normally not include systems where work under protective atmosphere can be performed, which is needed for silylation to avoid hydrolysis of the silylating reagent. Thus, it is necessary to verify whether silylation is necessary or whether alternative techniques can deliver similar information. The BfG approach proposes a comparable method, which will be experimentally verified or falsified.

The PLE is one more step that has to be checked for practicability. There is no doubt, that PLE works in the laboratory and instruments are commercially available. Nevertheless, so far usually no further analysis, except combustion, is performed with the extraction residue. Further, PLE usually does not work properly with the pure soil matrix but only after mixing of the soil with diatomaceous soil (“Kieselgur”) to prevent blocking of the system and/or clumping of the soil (Thermo Scientific, personal communication). That means that for the subsequent steps the diatomaceous soil becomes part of the solid matrix. That might have an influence on subsequent analytical steps.

- Three reference compounds with formation of all types of NER:

To cover a wide range of possible interactions with soil, three different test substances were selected for the test, each of them ^{14}C-labelled and ^{13}C-labelled. The substances selected were Isoproturon, an urea herbicide, Bromoxynil, a hydroxybenzonitrile herbicide and Sulfadiazine, a sulfonamide antibiotic. Details on the reason for the selection are given in section 3.2.

- Comparison of ^{14}C and ^{13}C methods

In general data for either ^{13}C- or ^{14}C-labelled substances are available in literature. In order to compare and reconcile results derived using either labels, the current study was conducted with concurrent experiments using test substances labelled with ^{13}C and test substances labelled with ^{14}C. However, due to the high natural abundancy the applied amount of labelled compound needs to be increased by a factor of ten for the ^{13}C-labelled compounds. In addition, similar setups with ^{12}C and a non-supplemented control are needed. In case of a positive correlation, data from literature using either label can be reconciled. This improves the transferability of results obtained using the different labels.

- Turnover kinetics, five sampling times

The experiments were conducted with one soil and sampling was performed at start plus five further sampling dates with NER determination. These samplings were distributed over 120 days, though the reported half-lives of the reference substances selected were reported to be some days only. However, these half-lives are only valid for the dissipation of the parent substance (DT_{50}). It was expected that NER processes are much slower. Thus, to ideally derive kinetic information for those processes, the incubation time was extended to the maximum suggested in the OECD 307 technical guideline (120 days). This sampling strategy was accompanied by two sets of sterile samples, which were analysed after two weeks incubation time and at the end of incubation. In sterile soil, no bioNER should be formed, however, any abiotic processes should be similar; however, type I or II NER may be prone to biodegradation and thus these types of NER may be lower in the native soil. So, a differentiation into abiotic and biotic NER formation was possible.

- Estimation of bioNER formation with the MTB method and proposal for a tiered approach

The Microbial Turnover to Biomass (MTB) approach uses released CO_2 (as indicator of microbial activity and compound mineralisation) and (measured or theoretical) biomass yield to estimate the biogenic NER formation. The draft ECHA R.11 guidelines (PBT/vPvP assessment) state that "if both the MTB method and other information (e.g., evolved CO_2) indicate that bioNER may be formed and that the quantification of bioNER is needed for half-life calculations, extraction of amino acids is recommended." The MTB method is therefore in practice an important tool for waiving or triggering further experimental efforts. MTB results can also be used to estimate the fraction of bioNER (and, by subtraction, then also the fraction of xenoNER) formed in existing tests, where NER was not further differentiated into NER types. In the same way it can be employed to estimate whether further NER differentiation is useful when running a simulation test. However, as Kästner et al. (2018) stated in the discussion paper for the ECHA, "The MTB yield method is quite new …. It has been tested versus available data, but experience is still limited and can thus only be an indicator." The authors list as research needs the application of the new MTB method to more experimental results, preferably from OECD tests relevant for P assessment. For all test compounds, the necessary input data for MTB were collected, the theoretical yield was calculated, and the bioNER formation was estimated. The estimation results were contrasted with the experimental findings.

For practical testing a trigger needs to be defined, which levels of NER, CO_2 and estimated bioNER would call for further experimental testing, and under which circumstances the additional NER tests can be omitted. Once sufficient (positive) experimental data have been gained it may be possible to rely on the calculated bioNER alone. This was finally communicated as a tiered approach proposal, as it is common in regulatory practical testing.

- How to consider NER in the calculation of degradation half-times $DegT_{50}$

The current P assessment is based on maximum acceptable degradation half-times. Simulation tests described in the OECD test guidelines 307 (OECD 2002) or similar are required to determine primary or complete degradation, the mass balance and other fate parameters (ECHA 2017 R.11). Extractable radioactivity, non-extractable radioactivity and volatile $^{14}CO_2$ are measured at appropriate time points (typically five times). The endpoints determined in the simulation tests are primary or ultimate degradation rate and degradation half-lives (DegT50) (ECHA 2017 R.11). EFSA (2014) released a guidance document for evaluating laboratory and field dissipation studies to obtain $DegT_{50}$ values. This guidance uses the definitions of dissipation and degradation provided in FOCUS (2006), which considers non-extractable residues as degradation products. The concept presented in the ECHA discussion paper (Kästner et al., 2018) suggests to consider only bioNER (NER type III) as degraded substance and NER type II (covalently bound residues) as degraded substance until proven otherwise, while NER type I is considered as potentially remobilisable parent compound. This view is also expressed in the recently released note of ECHA "Options to address non-extractable residues in regulatory persistence assessment" (ECHA Background note, 2019), which is intended to inform duty holders about acceptable approaches to refine assessment of NER in the regulatory context of the REACH and Biocidal Products Regulations, until the guidance documents are updated. There, it is stated that "By default, the residues remaining in the matrix after these extractions (total NER), should be regarded as non-degraded substance". This means also that, if only the fractions "extractable", "non-extractable" and "volatile" radiolabel is quantified and NER are counted as non-degraded, then the degradation rate fitted to the loss of extractable minus total NER will be similar to the mineralisation rate fitted to the formation of volatile CO_2 in most cases. If a significant amount of volatile residues other than CO_2 is formed and trapped, this has to be considered (mineralisation = extractable minus total NER minus further volatiles).

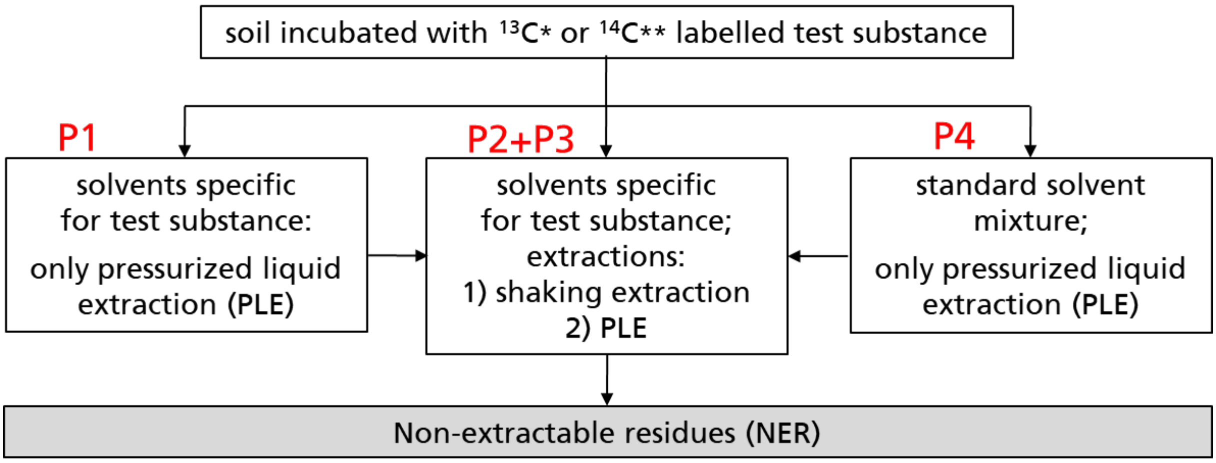
The background note states further that characterisation of the NER may be attempted, and only remobilisable NER (type I) are to be considered as potential concern. If the quantities of the three different types of NER (types I, II, III) are known they can be used for refining the half-life, and it says that "the half-life to be compared to the persistent/very persistent criteria may be calculated using the sum of the concentrations of the parent substance, transformation products and remobilisable NER (NER Type I)". Nonetheless, half-lives that include NER type I as parent compound will differ (and be longer) than those obtained from the loss of extractable substance only.

We used the experimental results obtained in this study to test various ways to consider NER in the determination of DT_{50} and $DegT_{50}$ values. We used the methods suggested in FOCUS (2006) (implemented in the tool CAKE) and alternative methods (like direct exponential curve fit) to calculate rates from the measured data. The goal was to find a practical and feasible method for consideration of bioNER and NER II in the $DegT_{50}$ calculation and in the P assessment and to provide respective guidance.

2.2 Detailed work program

In a first step we tested different type of extractions to determine NER and prepare a recommendation on how to proceed in routine testing and thus, a NER definition. Normally in routine OECD 307 tests, which always deliver the matrix where the NER have to be characterized, a (parent-) substance specific extraction method will be developed and applied. In the BfG-approach it is recommended to use only PLE extraction with a standard solvent mixture and NER is according to the definition everything which remains in the soil after PLE. This does not comply with current OECD 307 testing practice. Thus, we prepared 4 replicates per sampling and substance. Two replicates were extracted as is commonly done in OECD 307 tests followed by PLE with the substance optimized extraction solvent. One replicate was extracted by PLE only with the optimized extraction solvent and the fourth replicate was extracted by PLE using the standard solvent mixture as recommended in the BfG-approach. A comparison of the data was conducted to decide on the optimal procedure for a harmonised approach. The following figure summarizes this part of the experimental program:

Figure 3: Comparison of extraction methods to obtain total NER

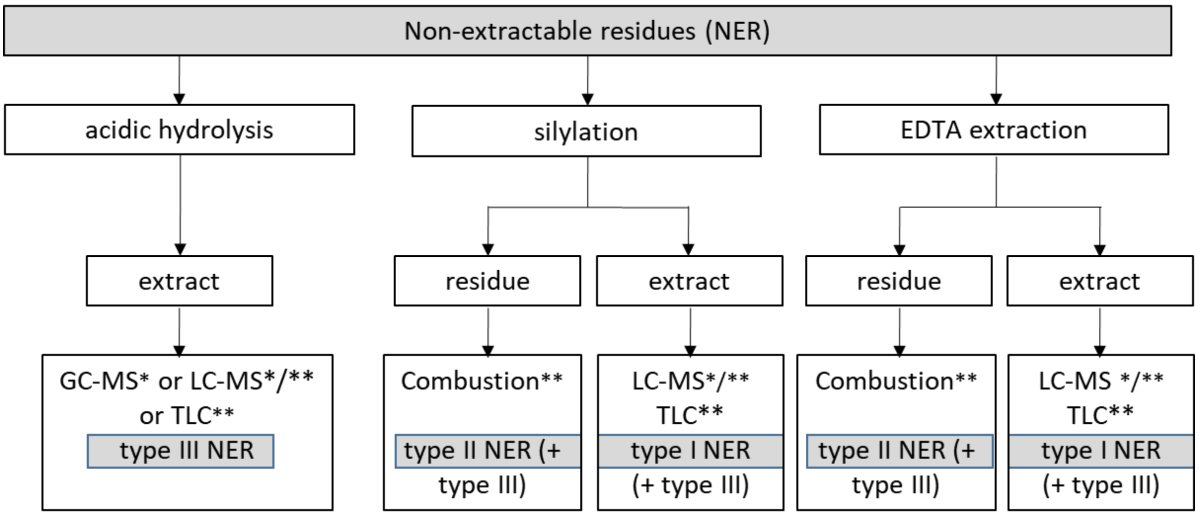


*/** see Figure 4

Source: Fraunhofer IME project proposal

In the second step, the NER were characterised with the different extraction methods. We considered both approaches which includes silylation as well as the EDTA-extraction. In both resulting extracts we applied a substance specific chemical analysis based on the ^{14}C-label in order to learn more about the NER fractions extracted. Based on the results it can be decided if the EDTA-extraction and the silylation deliver similar results. This is an important information for testing practise, as not every chemical resists the harsh conditions of silylation. This is also true for the acidic hydrolysis where we applied methods to quantify ^{14}C-labelled amino acids e.g. as published by Poßberg et al. (2016) in order to identify and quantify bioNER extracted with this procedure. Figure 4 shows this part:

Figure 4: Characterization of NER to obtain a database for further decisions



**analysis with sample from ^{14}C experiment *analysis with sample from ^{13}C experiment

Source: Fraunhofer IME project proposal

Substance specific analysis is necessary to determine if the selected test substances will “survive” the harsh conditions applied for NER characterization. If not, it would not be possible to decide whether parent test substance might have been released by the procedures shown in Figure 4 or whether transformation products are released. For this purpose, the extraction procedures were applied to soil samples freshly spiked with the selected test substances and the extracts were analysed for those parent substances. In case of significant losses, the test substance is considered unstable to the harsh extraction conditions.

As described above, this is the maximum data set that can be determined in current NER characterization and combines both current approaches. For practice, a German proposal for harmonisation needs to consider both `philosophies´ but the remaining question is how much additional characterisation is needed for safely considering non-identified NER as `safe sink´ in routine testing for persistence assessment.

The accuracy and precision of the MTB bioNER estimation method is assessed by comparison with the experimental data. The MTB method can, if successful, be used to waive or trigger experimental testing of the bioNER fraction.

Project results were presented in an international workshop (online) to experts from authorities, industry and science. The aim of the workshop was to present and discuss a practical approach for a harmonised test procedure for NER characterization. For this discussion, the industry's point of view was an important indicator for determining the acceptance and practical applicability of the proposed approach. For further discussions regarding the effects of NER characterization on the persistence assessment, the contributions of the regulators were necessary in order to get a reliable statement on applicability of the proposed approach.

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 is currently the only set of soils officially accepted as reference soils by respective authorities. Soil IME 03-G, which is a silt loam from a greenland site, was used in this project.

As for one test substance (Isoproturon) degradation rates in pre-testing were far below the expectation from literature data, the pre-test was repeated with another soil batch of IME 03-G. However, for some reason degradation still was not comparable to literature data. Thus, finally Lufa 2.4 soil was used for this substance.

Characterisation of the soil IME 03-G is done frequently under GLP conditions (GLP IME-010/7-85) and characteristics are given in Table 4. 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; CECeff: DIN ISO 11260; WHCmax: SOP V3-370. The soil data shown in Table 4 for the Lufa 2.4 soil (clayey loam) were provided by Lufa (see Appendix A).

Table 4: Characteristics of the used soils

soil	according to DIN			according to USDA							
	Sand %	Silt %	Clay %	Sand %	Silt %	Clay %	TOC %	total N g/ kg	pH (CaCl2)	CECeff mmol /kg	WHC g/kg
IME 03-G Silt loam	20.3	55.0	24.6	22.1	52.8	25.1	3.02	4.42	6.03	112.8	697
Lufa 2.4 Clayey loam	26.9	47.1	29.9	32.0	42.0	25.9	1.95	2.2	7.4	212	458

3.2 Selection of test chemicals

According to the original quote experimental testing with two ^{14}C-radiolabelled test substances and one ^{13}C-labelled test substance was planned. This was confirmed in the kick-off meeting in October 2018 but it was already agreed to check for a third ^{14}C-labelled test substance if the substance budget allows. In a project meeting in March 2019 it was discussed to add a third test substance. In an extension proposal, submitted in July 2019, the program was finally extended to three test substances, each in two forms, either ^{14}C-radiolabelled or ^{13}C-labelled.

For the study, it was agreed to select test chemicals with different properties. The choice of proper test chemicals is one of the most important steps for such study. The substances should

- form significant amounts of NER in soils within a period of 120 days
- be available as 14C-radiolabelled and 13C-labelled substance at reasonable cost
- form different types of NER according to literature data

Experimental data with substances from published studies were available. The advantage of these substances was that some of them were available to the project partners as ^{13}C and ^{14}C-labelled test substances and, if selected, could be made available to the project as an own contribution. Most of the data on NER characterization according to the present concepts were available for ^{13}C-labelled substances. Corresponding data for ^{14}C-labelled substances, on the other hand, were mostly lacking or are only available for selected substances. Since existing data are based mostly on ^{13}C-labelled substances, but realistically mainly ^{14}C-labelled substances will be used in a later application of the method, the project partners saw the need to use both labels in parallel in the project in order to be able to compare and validate the results.

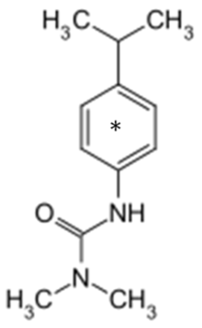
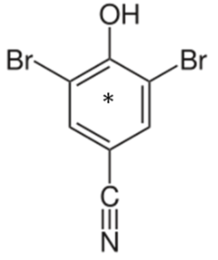
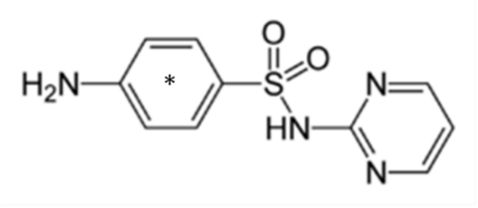
Based on the considerations mentioned above, a list of possible substances was proposed for which data was already available or published and some of which were available to the project partners as isotope-labelled substances.

Table 5: Suggestion of substances for the preselection

Substance	NER Typ I	NER Typ II	NER Typ III
Bentazon (Nowak et al. pers. com.)	+++	+++	
Cypermethrine		++	++
Glyphosate (Nowak et al. 2018)			+++
Bromoxynil (Nowak et al. 2018)	++	+++	+
Isoproturone	+++	+++	
Thiacloprid		++	++
Pendimethaline (Luks et al. 2021)	+	++	+
2,4-D (Nowak et al. 2011, Girardi et al. 2013)			+++
Metamitron (Wang et al. 2016, 2017)		++	++
MCPA			+++
Metalaxyl	+++	+++	

The three test substances selected for the test, each of them ^{14}C-labelled or ^{13}C-labelled, were the urea herbicide Isoproturon, the hydroxybenzonitrile herbicide Bromoxynil and the sulfonamide antibiotic Sulfadiazine.

Table 6: Test substance chemical structures and label positions (^{13}C as well as ^{14}C)

Isoproturon	Bromoxynil	Sulfadiazine
		 * isotope label position

Further details on providers and test substances are shown in Appendix B.

Zhu et al. (2018) quantified the formation of bioNER from Isoproturon by enriching isolated degraders. The experimental time was 46 days. At the end of the experiment the (total) NER contained 35.4% of the applied radioactivity (AR), 55.9% of the applied radioactivity was recovered in CO_2. The ^{14}C in the “apparent NER” (equivalent to bioNER according to the authors) was measured to be 24.7% aR. Using the model developed by Trapp et al. (2018), the formation of bioNER was estimated to be between 17% and 49%.

Poßberg et al. (2016) determined the NER formation of Bromoxynil in soil over 56 days. At the end of the experiment the radioactivity in the (total)NER was 70.8% AR and 19.0% AR was recovered in CO_2. Based on measured ratios of phenylalanine/valine the ^{14}C in amino acids was estimated to be 6.1% aR. Assuming that the amino acids make up 55% of the microbial biomass, the bioNER was estimated to be 11.1% aR (equivalent to 26.4% of the total NER).
Nowak et al. (2018) incubated Bromoxynil in soil for 32 days. At the end of the experiment the radioactivity in the (total)NER was 72.5%, 25% was recovered in CO_2. The ^{13}C in amino acids was measured to be 12% of the applied label (= 24% bioNER) after 32 days. A calculation based on the model developed by Trapp et al. (2018) for the bioNER formation of bromoxynil was estimated to be between 2% and 5% (for 25% CO_2). For this compound significant lower amounts of bioNER are predicted than actually found. This result needs to be examined.

Sulfadiazine was studied mostly applied to soil together with manure as this represents the most relevant entry scenario for antibiotics into soils. In Junge et al. (2011) under these conditions, between 78% and 100% of the label were recovered in the NER following 28 days incubation. However, no differentiation in NER types was performed in that study. The CO_2 formation was negligible and only amounted to 0.7% aR after 28 days. Unpublished data from UBA confirm the results of Junge et al. (2011). Using the method of Trapp et al. (2018), the associated formation of bioNER was calculated to be between 0.2% and 0.5%.

3.3 Chemical analytical methods

3.3.1 Liquid Chromatography with Mass- Spectrometry coupling (LC/MS)

Instrumentation and conditions for LC-MS analysis (^{12}C-substances: given mass -1)

Chromatographic System	Agilent (1290 HSP, Multisampler, MCT)		
Analytical Column*	Phenomenex Luna C18(2) 150 x 3.0 mm, 3.0 µm		
Column Temperature	30°C		
Injection Volume	10 µl		
Mobile Phase A	Mobile Pase B		
Acetonitrile + 0.2 % formic acid	Water : Acetonitrile:, 950:50:, v/v + 0.2 % formic acid		
Flow Rate	1.0 mL/min		
Gradient	Time [min]	Phase A [%]	Phase B [%]
	0.0	90	10
	1.0	90	10
	2.0	5	95
	5.0	5	95
	5.1	90	10
	8.0	90	10
Divert Valve	No		
Detection System**	Sciex Q TRAP 5500		
Ionisation	Electro Spray (ESI)		

Analyte	**Transitions**	**Polarity**	**Retention Time**
^{13}C- Bromoxynil	281.8 → 80.9 281.8 → 78.9	negative	2.6 min
^{13}C- Sulfadiazin	255.9 → 97.1	positive	2.1 min
^{13}C- Isoproturon	213.2 → 72.0 213.2 → 46.0	positive	2.6 min

3.3.2 High performance liquid chromatography (HPLC)

HPLC instrument details

Pump:	Gradientpump Ultimate RS 3000 (Dionex)
Injection system:	WPS-3000 TRS (Dionex)
Column oven:	TCC-3000RS (Dionex)
UV-detector:	UV DAD Detctor Ultimate RS 3000 (Dionex)
Radioactivity monitor:	Flowstar LB 513 (Berthold), cell 100 µL
Software:	Chromeleon 7, Version 7.2.5.9678 (Thermo Fisher Scientific)
Interface:	UCI 50 Universal Chromatography (Dionex)

Table 7: HPLC method for Isoproturon

Column	Column Temp. [°C]	Eluents A	Eluents B	Flow Rate [mL/min]	Gradient Time [min]	Gradient % B
Hypersil ODS C18 5µm (250 x 4.6 mm)	40	Acetonitrile	Amoniumacetate 0.02 m	1.0	0	20
					2	20
					4	98
					8	98
					9	20
					13	20

Retention time of Isoproturon at these conditions: 6.56 min (UV/VIS)

Table 8: HPLC method for Bromoxynil

Column	Column Temp. [°C]	Eluents A	Eluents B	Flow Rate [mL/min]	Gradient Time [min]	Gradient % B
Hypersil ODS C18 5µm (250 x 4.6 mm)	40	Water + Acetonitrile 1% Acetic acid 0.1%	Acetonitrile	1.0	0	10
					1	10
					5	100
					6.5	100
					7	10
					12.5	10

Retention time of Bromoxynil at these conditions: 6.62 min (UV/VIS)

Table 9: HPLC method for Sulfadiazine

Column	Column Temp. [°C]	Eluents A	Eluents B	Flow Rate [mL/min]	Gradient Time [min]	Gradient % B
Hypersil ODS C18 5µm (250 x 4.6 mm)	40	Water + Acetonitrile 1% Acetic acid 0.1%	Acetonitrile	1.0	0	2
					1	2
					4	100
					6.5	100
					7	2
					12.5	2

Retention time of Sulfadiazine at these conditions: 5.55 min (UV/VIS)

Respective HPLC-UV chromatograms are shown in Appendix C.

3.3.3 Radio Thinlayer Chromatography (TLC)

In order to differentiate, in the extractable radioactivity, between the parent test chemicals and potential transformation products, all extracts were subject to radio-TLC. Known amounts of the extracted radioactivity were applied to TLC-plates and 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 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 (see below). A software converts the TLC-plate into a chromatogram and the distribution of the radioactivity is evaluated.

The instrumentation of the TLC system used was as follows:

Sampler	Automatic TLC Sampler 4 (CAMAG)
TLC plates	TLC Silica gel 60 RP18 F254S 20x20 cm (Merck) used for all 3 substances

Software	VisionCats (CAMAG)

TLC separation method:

Isoproturon	Chloroform : Ethanol (90 : 10 /v:v), Rf = 0.61
Bromoxynil	Chloroform : Diethylether : Acetic acid (80 : 18 : 2 / v:v:v), Rf = 0.60
Sulfadiazine	Chloroform : Methanol (90 : 10 / v:v), Rf = 0.47

Plate reading after 1 week exposure time:

Reader	Amersham Typhoon, Software Version 2.0.0.6, Firmware Version 303, FPGA version 10, Serial Number 86110330
Evaluation software	AIDA (Advanced Image Data Analyzer) Version 3.44.035

3.3.4 Radioactivity measurements (LSC)

Radioactivity in liquid samples was determined by Hidex Instruments 300SL liquid scintillation counters (LSC). 1ml of the liquid samples were mixed with 4 ml of an appropriate scintillation cocktail (e.g. Ultima Gold, Pico Fluor Plus, Hi Ionic Fluor or Aquasafe 500). The cocktail selection was dependent on the solvent to be measured.

3.3.5 Combustion analysis

For the determination of NER, the soil residue after ASE-extraction was air dried at room temperature, homogenized in a mortar mill and then combusted in a Zinsser OX700 Oxidizer. In this device, the sample is combusted in an oxygen atmosphere at 900°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 (Oxysolve C400) and quantified by subsequent LSC. Every 12th 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.

3.4 Experimental procedures

3.4.1 Soil degradation experiments

NER are in general a finding in laboratory degradation experiments with soils and sediments. For this study we focussed on NER in soil. For the experimental characterisation of NER first step is to get soil containing NER. To get the test material as realistic as possible regarding routine degradation testing, soil degradation experiments were conducted in accordance to OECD Guideline 307 “Aerobic and Anaerobic Transformation in Soil”.

3.4.1.1 Initial pre-experiments

The experimental work was started on March 5, 2019, after receiving the first ^{14}C- and ^{13}C-labelled substances. After the development of a substance-specific extraction from the selected test soil and the establishment of an HPLC / UV analytical method, which can also be used with radio detection, preliminary tests were carried out to degrade the substances in the selected test soil.

Based on the results of the preliminary tests, laboratory standard working procedures were drawn up to ensure that all samples in the main test were treated similarly for one substance. Based on the results of the preliminary tests, it was also determined which additional sampling times would be carried out in order to achieve a suitable data set for calculating the degradation kinetics of the reference substances, as decided at the meeting on March 27, 2019. Even if the focus of the project was the characterization of NER, it was necessary to perform a guideline-compliant soil extraction test first, since these are the conditions under which the concept to be developed had to prove itself.

3.4.1.2 Main degradation experiments

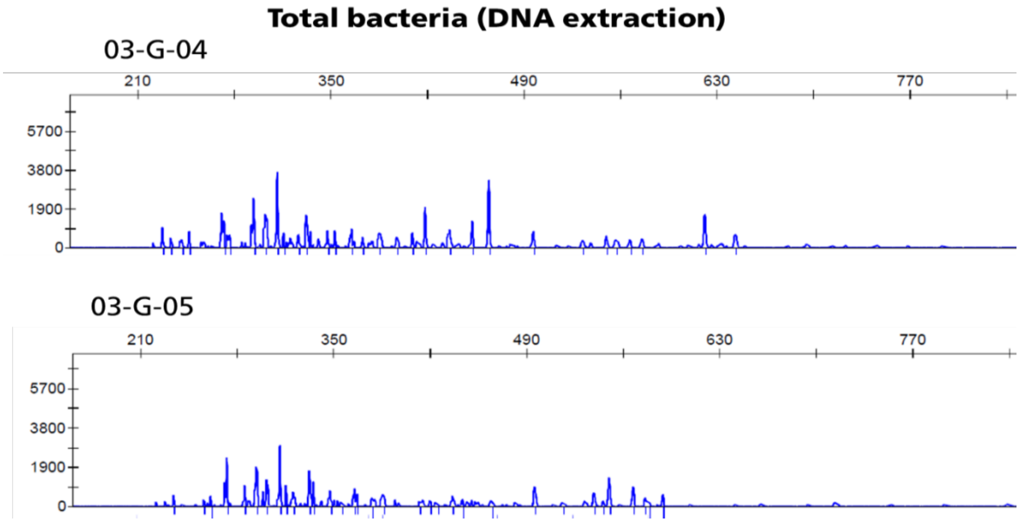
In deviation to the OECD 307 guideline just one soil was used for each test substance: Refesol IME 03-G for Bromoxynil and Sulfadiazine, Lufa 2.4. for Isoproturon.

The main trial with Isoproturon started on July 24, 2019. In contrast to a preliminary test under identical conditions Isoproturone was hardly degraded in IME 03-G. After 14 days, about 90% of the radioactivity was still extractable and about 7% NER was measured. This contradicts the breakdown in the preliminary test and all available literature data. The trial was therefore cancelled on August 23, 2019.

The compulsory tests for microbial biomass (SIR method, ISO 14240-1) showed no abnormalities in the 03-G soil batch used and, at 2.0% [Cmic from Corg], was in the range of the requirements of OECD Guideline 307 (> 1.0% Cmic from Corg).

In order to obtain an indication of the composition of the microflora, the soil batch in which the expected degradation was observed in the preliminary experiment (05) and the soil batch in which no degradation was observed (04) were characterized by means of DNA analysis. As can be seen inFigure 5, the spectrum of the detected DNA does not differ significantly from one another in terms of diversity:

Figure 5: DNA analysis of different IME 03-G soil batches used for isoproturon degradation

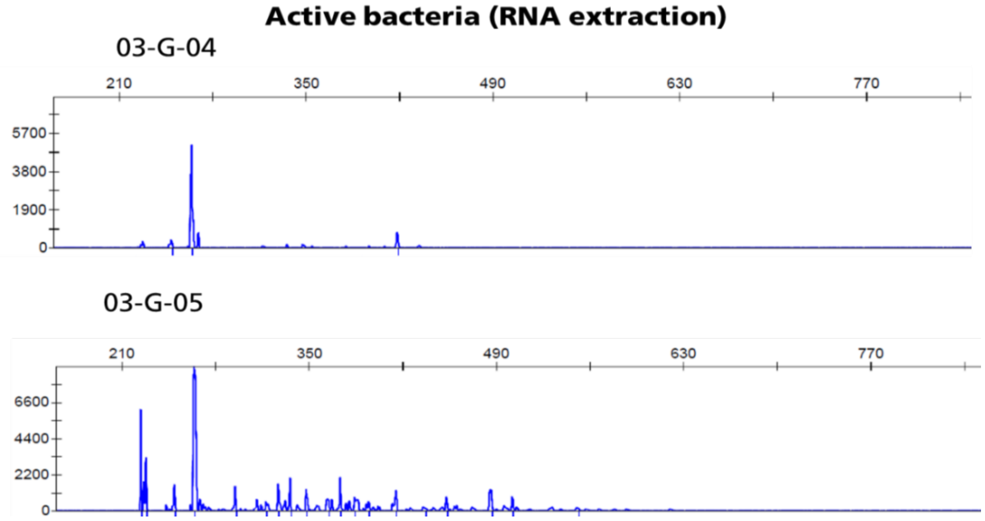


Source: Fraunhofer IME evaluation of project data

However, DNA analysis does not differentiate between active and dead cells. An RNA analysis is necessary for this question. Comparing batch 04 and 05 this RNA analysis showed a significant difference in the diversity of the active microorganisms. It can be speculated that the results shown in Figure 6 might show a relationship between the active microorganism population and the rate of Isoproturon degradation. However, there is no prove for the hypothesis from our experiments.

In further preliminary tests, the degradation rate of the first preliminary test could not be reproduced either with new soil samples IME 03-G fresh from the field or with an alternative soil (IME 01-A). For this reason, further preliminary tests were carried out with Lufa 2.4 standard soil. This soil corresponds to soil 03-G in the essential parameters. The preliminary tests with Lufa 2.4 resulted in a recovery of less than 40% after 22 days, which is still not at the level of the first preliminary test, but suggests a relevant degradation of Isoproturon. The main trial with 14C-isoproturon was therefore restarted on February 17, 2020 with Lufa 2.4. test soil. The non-degradability of Isoproturon in IME 03-G soil was not followed further as this was not the focus of the project.

Figure 6: RNA analysis of different IME 03-G soil batches used for isoproturon degradation



Source: Fraunhofer IME evaluation of project data

Each individual sample contained 50g of soil, calculated on soil dry matter. Soil moisture was 40% of WHCmax. 4 replicates per sampling time were prepared, sacrificial sampling ensured homogenous sampling (potential inhomogeneity of the application is deleted by sacrificial sampling). 6 sampling times are applied at all experiments at day 0, 7, 14, 28, 60 and 120. Due to weekends / holidays sampling time varied by ± 1 day in some cases.

In each experiment 2 sterile samples were prepared with 4 replicates. Heat sterilisation, three times at 121°C in an autoclave, was applied. Sampling times for sterile samples were 14d and 120d.

Depending on the degradation rate observed in the pre-experiments further samples were prepared to determine the degradation rates of the substance properly. However, for these additional samplings just 2 replicates were prepared because the main purpose was the determination of the degradation rate. Consequently, those additional samplings were not subject to further NER characterisation.

Prior to application all soils were pre-incubated at test conditions (dark, 20°C) for around two weeks. Application of the test substances was performed with organic spike solutions. It was agreed between the client and UBA that the application rate of the ^{13}C substance should be a factor of 10 above the ^{14}C-substance. This was necessary because the natural ^{13}C background would otherwise not permit a valid evaluation of the experiments. Final starting concentrations were 4 mg/kg for the ^{14}C-radiolabelled substances and 40 mg/kg for the ^{13}C-labelled substances.

Table 10: Application data overview, application per 50 g soil dry weight

Substance	solvent	applied volume	applied radioactivity	applied substance amount
Bromoxynil ^{14}C	Acetone	94.5 µL	1289 kBq	200 µg
Sulfadiazine ^{14}C	DMSO	88.8 µL	631 kBq	200 µg
Isoproturon ^{14}C	Acetone	45.0 µL	587 kBq	200 µg
Bromoxynil ^{13}C	Acetone	95.8 µL	--	2000 µg
Sulfadiazine ^{13}C	DMSO	121.7 µL	--	2000 µg
Isoproturon ^{13}C	Acetone	98.0 µL	--	2000 µg

After application, the samples were allowed to evaporate solvent for about 30 min in a fume hood. After that samples were connected to a flow through device in a temperature-controlled incubation room. To reduce analytical effort, only a sodium hydroxide trap was connected in order to quantify emitted $^{14}CO_2$ and $^{13}CO_2$. No further volatile transformation products were expected and the good ^{14}C-mass balances confirm this decision. During incubation, a gentle stream of air was passed over the samples.

Sterile samples were prepared by heat sterilization (2 x 20 min at 121°C). Sterile vessels were locked gas tight to keep them sterile during incubation. The closed bottles were placed in the same incubation room as the non-sterile samples. As no biological activity and thus CO_2 production and/or O_2 consumption is expected without biological activity, no gas trapping was conducted.

3.4.1.3 Sampling procedure

At sampling the four / two replicates were removed from the flow through device. The soil from samples P2 and P3 (see Figure 3) was transferred into glass centrifuge vessels and the incubation bottle was rinsed with the respective extraction solvent (see 3.4.2.1). The rinsing solution was then used for further extraction.

The soil from samples P1 and P4 (see Figure 3) was put in glass vessels and around 10 g of diatomaceous earth was added to the soil until the sample was free flowing. The weight of the added diatomaceous earth was documented. It was mixed well with the soil and then the mixture was transferred into the PLE extraction vessel for extraction.

The volume of the respective sodium hydroxide trapping solutions was measured and an aliquot of the solution was measured by LSC for $^{14}CO_2$ radioactivity.

3.4.2 Extraction procedures

3.4.2.1 Shaking extraction

Samples P2 and P3 were extracted first by a substance specific extraction procedure. This was developed in advance and applied during the study. It is optimised for extracting the parent substance only which is common laboratory practise in soil degradation studies.

The following extraction procedures were applied:

Isoproturon:

1. 70 ml MeOH/H_2O (80:20/v:v) 10 min sonication 60 min horizontal shaking
2. 45 ml MeOH/H_2O (80:20/v:v) 10 min sonication 60 min horizontal shaking
3. 45 ml MeOH/H_2O (80:20/v:v) 10 min sonication 60 min horizontal shaking

Centrifugation between extraction steps: 10 min at 2000 rpm (1000 g),

Bromoxynil:

1. 70 ml Acetonitrile 20 min horizontal shaking
2. 45 ml Acetonitrile 20 min horizontal shaking
3. 45 ml Acetonitrile 60 min horizontal shaking

Centrifugation between extraction steps: 10 min at 2000 rpm (1000 g)

Sulfadiazine:

1. 70 ml Methanol 20 min horizontal shaking
2. 45 ml Methanol 20 min horizontal shaking
3. 45 ml Methanol 60 min horizontal shaking

Centrifugation between extraction steps: 10 min at 2000 rpm (1000 g)

All extracts were combined and stored in a freezer in closed bottles at below -18°C until further chemical analysis. Before storage an aliquot of the extract was taken and analysed for extracted radioactivity by LSC.

3.4.2.2 Pressurized liquid extraction

As an agreement prior to the degradation experiments all samples were extracted by pressurized liquid extraction as terminal extraction step. This is at the same time also the decision on the definition of NER for the project: everything of the isotope labelled substance that remains in the soil after PLE is assessed to be NER by definition.

For PLE each sample had to be mixed with diatomaceous earth before the extraction. Also samples after shaking extraction. For practical reasons those samples received the amount of 10 g diatomaceous earth per sample.

The instrumentation used for PLE was an ASE 350 (Dionex). The complete samples were transferred into 100 mL stainless steel extraction vessels and extracted at 100°C in 3 cycles for 10 min per cycle. The rinse volume from the cycles was set to 60%. The following extraction solvents were used:

Sample P1, P2 and P3 (see Figure 3):	Isoproturon	MeOH/H_2O (80:20 / v:v)
	Bromoxynil	Acetonitrile
	Sulfadiazine	Methanol
Sample P4 (standard solvent BfG):	MeOH/Aceton/H_2O (50:25:25 / v:v:v)	

3.4.3 NER determination

The extraction residues were air dried and then homogenized in a mortar mill. This is necessary because subsequent combustion analysis allows sample amounts of only up to 250 mg per combustion. NER were determined by combustion analysis. For details of the combustion procedure, refer to section 3.3.5.

For determination of ^{13}C in the extraction residue, homogenized samples were sent out for isotope analysis to University Göttingen, Büsgen-Institute, Competence Center for stable Isotopes, 37077 Göttingen, https://www.uni-goettingen.de/de/analysen/71362.html. Data processing is shown in Appendix D.

3.5 NER characterisation

3.5.1 Silylation

3.5.1.1 Silylation procedure

First, the air dried ground extraction residue (soil with diatomaceous earth) was weighed in duplicates of 1.5 g each in 250ml Schlenk flasks and an oval magnetic stir bar (20 x 10 mm, VWR) was added. To remove any moisture, the samples were dried for 30 min at 105°C in a drying oven because water would hydrolyze the silylation agent. Afterwards, the samples were placed on a magnetic stirrer and 30 ml of dry chloroform (ChemSolute, p.a., dried with a molecular sieve, 0.3 nm), 1.5 g NaOH micro granulate (ChemSolute, ≥ 99,5%) and 15 ml trimethylchlorosilane (TMCS) (Sigma Aldrich, ≥ 99 %) were added. In order to get a moisture-free inert atmosphere the reaction flask was flushed with argon immediately after the addition of TMCS. A gas bag (Linde PLASTIGAS® bag 5.5 L) filled with argon was connected with a silicon tube to the reaction flask in order to allow a pressure balance for the HCl gas produced and to maintain the protective gas atmosphere (Figure 7).

Figure 7: Schlenk flasks connected with Argon filled gas bags for silylation

Source: Fraunhofer IME

The samples were stirred at room temperature at 100 - 200 rpm to maintain a homogeneous suspension. After three hours further 10 ml TMCS and 1.5 g NaOH were added to each sample. For adding the reagents, plugs were opened only shortly. Slight pressing of the gas bags helped to maintain the inert atmosphere in the reaction flasks during the addition. Then, the plugs were secured with a clamp and the samples were stirred overnight at room temperature. To prevent corrosion of the gas bags they were emptied directly after the reaction was finished. At the slightest suspicion of a leak, the bags were replaced with new ones.

3.5.1.2 Extract separation and work up

The reaction suspension was transferred into a centrifuge vessel (Sarstedt vials, 50 ml) and centrifuged for 10 min at 3000 g. The supernatant was transferred to a 100 ml screw cap glas bottle. The Schlenk flasks and the stir bars were rinsed with 10 ml acetone (ChemSolute ≥ 99.8 %). The washing solution was transferred to the residues in the centrifuge tubes, shaken for 5 min and centrifuged. This washing step was repeated two times with 10 ml acetone and then three times with 10 ml chloroform each. The washing solutions were combined with the initial supernatant and the resulting clear, slightly yellow solution was called "Silylation extract". The silylation extract was stored closed at -20°C in the dark until further analysis.

From recovery experiments with spiked soils (see 3.5.1.4) it was found that sulfadiazine was only partially recovered by the above described procedure due to limited solubility in the solvents used. Thus, the silylation residue was extracted again with a more polar solvent (methanol). Additional extractions were also needed for isoproturon where considerable amounts of radioactivity were extracted from the silylation residue with a methanol:water mixture. Though the recovery in the silylation extract was very good for the spiked samples. For isoproturon the silylation residue was extracted once with 10 ml of methanol:water, (80:20 / v:v), shaking for 60 min. For sulfadiazine the silylation residue was extracted three times with 15 ml methanol for 20 min, 20 min and 60 min.

3.5.1.3 Extract analysis

The silylation extract was analysed either by radio-TLC (^{14}C radioactive labelling) or by LC-MS (^{13}C-labelling). All ^{14}C extracts were initially measured by LSC for total extracted radioactivity.

For subsequent analysis both, the silylation extract and the methanol or methanol:water extract were merged before analysis. For this purpose the silylation extract was transferred into a 50 mL Sarstedt vial and evaporated to less than 15 mL. This ensures that most of the Chloroform phase was removed since the extract contained around 33% of Acetone. To the remaining silylation extract the Methanol / Methanol:water was added and again evaporated by a gentle stream of Nitrogen to around 15 mL final volume. The solution was filled up with Methanol to around 20 ml final volume. The final volume was measured and an aliquot was analysed by LSC. The recovery of radioactivity after extract merge and concentration was mostly between 90% and 100%.

The bromoxynil silylation extract could be applied directly to the TLC-plate without any further treatment. The application volume for radio-TLC was adjusted in a way that each spot contained about 2.5 Bq and 10 Bq, respectively. Exposure time was one week (see section 3.3.4).

3.5.1.4 Stability testing

TMCS will react not only with the soil matrix but might also attack the test substances if they carry corresponding functional groups with exchangeable protons such as hydroxy, carboxy or amino groups. This need to be checked before applying the silylation procedure.

In order to get conditions as realistic as possible, we performed a stability test with the test soil, that was subjected to the same extraction and grinding procedures as the test samples, with the applied test substances. To 1.5 g of the soil material the ^{14}C-parent test substances were applied at amounts of 4.7 kBq to 9.7 kBq. For each substance duplicate samples were prepared. After evaporation of the solvent (2 hours in a fume hood) the samples were subjected to the silylation procedure as described above.

As quality control in our study we also analysed the silylation residue for remaining radioactivity by combustion analysis in order to establish a mass balance for the silylation. First results indicated significant losses of radioactivity but the reason for this finding could be quickly ascertained: the weight of the silylation residue increased because of sodium chloride formed during silylation. This dilutes the solid residue, which was the reason for the apparent loss of radioactivity. Thus, for mass balance determination it is important to determine the exact weight of the silylation residue for each sample.

3.5.2 EDTA Extraction

3.5.2.1 Procedure

An aliquot of 4 g of the air dried ground extraction residue was weight into 50 mL Sarstedt vials and 40 mL of a 0.1 m EDTA solution, adjusted with 10 m NaOH to pH 8, was added. The vial was closed and then it was shaken for 16 hours on a horizontal shaker at 200 rpm. After shaking the vial was centrifuged for 10 min at 3000 g and the supernatant was removed.

40 mL of the substance specific extraction solvent (see section 3.4.2.1) were added to the centrifugation residue. The mixture was shaken again for two hours at 200 rpm. Then the vial was again centrifuged for 10 min at 3000 g and the supernatant was removed and combined with the first supernatant. The combined extracts were called “EDTA-extract”.

After determination of the extract volume, they were analysed by LSC and radio-TLC (^{14}C-label) and LC-MS (^{13}C-label), respectively.

In order to establish a mass balance for EDTA-extraction, the extraction residue was air dried and subject to combustion analysis.

3.5.2.2 Stability testing

EDTA as a chelating agent is supposed not to react chemically with the test substances. However, to prove this assumption, a stability test was conducted also for EDTA extraction.

Test soil, that was subjected to the same extraction and grinding procedures as the test samples, was applied with the ^{14}C-parent test substances. Amounts of 11 kBq to 28.5 kBq were applied to 2g of soil material, each. For each substance duplicate samples were prepared. After evaporation of the solvent (2 hours in a fume hood) the samples were subjected to the EDTA extraction procedure as described above.

3.5.3 Acidic hydrolysis

3.5.3.1 Hydrolysis step

An aliquot of 5 g of the air dried ground extraction residue was weight into a Kjehldahl digestion device and 25 mL of 6 m HCl were added. The mixture was initially cooked under reflux conditions for 22 hours at 110°C. After acidic digestion the reaction mixture was transferred into a 50 mL Sarstedt vial and centrifuged for 10 min at 3000 g. The separated extract was analysed by LSC prior to subsequent work up.

As there was indication that cooking in open vessels might lead to losses of digested radioactive substance, the digestion was later repeated in closed glass bottles with screw caps at 105°C in a drying oven. However, it must be pointed out at this point, that this procedure is not in line with laboratory safety rules since neither the glass bottles are suited for digestion under pressure conditions nor the drying oven should be operated with concentrated acid inside. For safety reasons the bottles were placed in glass beakers to avoid at least spreading of the acid if a bottle should break. This hydrolysis procedure in closed containers is not fit for laboratory routine!

Recovery of both digestion procedures, which was tested by recovery of radioactivity from soil material spiked with a ^{14}C standard amino acid mix (L- Amino acid mixture [14C(U)], Hartmann Analytics, ARC0474, see Appendix B), were not significantly different.

In another experiment, the glass bottle was flooded with Argon to reach an inert atmosphere inside the bottle during heating. The recovery did not improve compared to open vessel hydrolysis.

3.5.3.2 Extract work up

For clean-up with solid phase extraction (SPE) the SPE material had to be conditioned prior to the clean-up. For this purpose, 15 g of Dowex 50WX8 mesh 50-100 were rinsed with 1) 25 ml 2m NaOH, 2) 25 ml 2 m HCl and 3). 40 ml purified water under vacuum. After vacuum drying the sand-like Dowex material can be stored in closed bottles.

For SPE clean-up, 15 g Dowex are slurried with 15 mL of purified water and filled into an appropriate SPE column. After removing the water by vacuum, the column is washed again with further 15 mL of water.

The extract volume for SPE clean-up is selected in a way that it represents 2.5 g of the extracted soil. This extract aliquot is adjusted with 10 m NaOH to pH 2-3. The resulting solution is sucked slowly by vacuum through the SPE column. The following washing steps are applied without letting the column to run dry:

1. 125 mL 0.1 m Oxalic acid (adjusted with 2.5 m NH_4OH to pH 2.5)
2. 25 mL 0.01 m HCl
3. 25 mL purified water

Elution of the SPE after washing is performed by sucking 125 mL of 2.5 m NH_4OH slowly through the SPE column. The eluent is collected and analysed by LSC before radio-TLC analysis.

3.5.3.3 Radio-TLC-analysis for ^{14}C amino acids

For radio-TLC analysis an aliquot corresponding to 2.5 Bq was applied to the TLC plate.

TLC plates	TLC Silica gel KG 60, F254S 20x20 cm (Merck)
TLC separation method:	2-Propanol : water (70 : 30 / v:v)
Exposure time:	1 week exposure time
Analytical standard:	L-Amino acid mixture [14C (U)] with the amino acids in the proportions (%): alanine (8%), arginine (7%), aspartic acid (8%), glutamic acid (12.5%), glycine (4 %), Histidine (1.5%), isoleucine (5%), leucine (14%), lysine (14%), phenylalanine (8%), proline (5%), serine (4%), threonine (5 %), Tyrosine (4%) and valine (8%). Hartmann Analytics, ARC0474

Further details on radio-TLC are shown in section 3.3.3.

3.5.4 Microbial Turnover to Biomass (MTB) approach

The MTB method is based on the relation between released CO_2 (as indicator of microbial activity and mineralization), biomass yield, and biogenic NER formation (Trapp et al. 2018).

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

$$Y = \frac{dX}{dS} \qquad \text{(Eq. 1)}$$

The bacterial growth yield is defined as the mass of microbial biomass formed per mass of substrate consumed (g cells per g substrate, or g C per g C) (Thauer, 1977). Both measured and estimated microbial yields Y can be applied in these equations, but very few measured data of xenobiotics can be found (Brock et al. 2017).

The MTB method is based on the method provided by Diekert (1997), with the main modification that only electron transfers from C-H bonds can be used by microbes to gain catabolic energy (Trapp et al. 2018). In this method, the yield can be limited by the available energy, and by the available carbon.

Once the yield is known, the formation of bioNER is estimated from the carbon balance. When the substrate is mineralised, the carbon of the substrate forms either biomass X (anabolism) or CO_2 (catabolism). Thus, if the unit g labelled C is used, the ratio of microbial biomass X to CO_2 is

$$high\ MTB\ = X - bioNER = \frac{Y}{(1-Y)}[CO_2] \qquad \text{(Eq. 2)}$$

Labelled C fixed in biomass X counts to bioNER, so this relation gives the upper amount of labelled C turning into bioNER, named “high MTB”. Microbes decay with a typical half-life of 14 days (Trapp et al. 2018), and during longer experiments, microbial necromass is digested in the microbial food chain, under formation of non-living soil organic matter SOM, new microbial biomass and more CO2. Empirical data indicate that in longer experiments (in this case 224 days), about 40% of the labelled carbon in biomass X (mainly the protein fraction) turns into SOM, 10% remains within living biomass and f = 50% forms CO_2 (Miltner et al., 2012), formalised (all units g labelled C):

$$low\ MTB - bioNER = \frac{f \times Y}{(1-Y)+(1-f)\times Y} \times [CO_2] \qquad \text{(Eq. 3)}$$

The calculations thus give an upper value (high MTB) representing living biomass, and a lower value (low MTB) representing label turnover in the microbial food chain.

The estimation of microbial yields with the MTB method requires as input data: The chemical reaction equation; Gibbs energy of formation of products and educts at standard conditions (pH 0) ΔG^0_f; the molar mass M; the number of carbon atoms in the molecule; the number of CH bonds.

Unless indicated otherwise, the reaction is always assumed to occur under aerobic conditions, with oxygen O_2 as electron acceptor and CO_2 and H_2O as product. Nitrogen and sulfur moieties are assumed to keep their oxidation status they had in the substrate (e.g., amines are released as NH_3).

Gibbs energies of formation ΔG^0_f for xenobiotics are rarely measured, and the values were estimated by the Weizmann equilibrator (Flamholz et al., 2012) (http://equilibrator.weizmann.ac.il).

Table 11: Postulated chemical reaction and the Gibbs energy for the studied compounds

	ΔG_f^0 kJ/mol	Reaction
Bromoxynil	147.8	$C_7H_3Br_2NO + 6\ O_2 + OH^-$ --> $7\ CO_2 + 2\ Br^- + NH_4^+$
Isoproturon	64.2	$C_{12}H_{18}N_2O$--> $12\ CO_2+7.5\ H_2O+2\ NH_3$
Sulfadiazine	270.3	$C_{10}H_{10}N_4O_2S+ n\ O_2 + 2H_2O$ -> $10\ CO_2 + 4\ NH_3 +HSO_4^- + H^+$

3.6 Data evaluation, calculation of degradation rates

All calculations were performed by computer or electronic calculator with varying degrees of soft- and hardware dependent floating-point precision. Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than were used in the actual calculation. Minor differences in the results obtained with such rounded values in comparison to those obtained with higher precision values are well within the limits of the experimental accuracy and therefore are of no practical concern.

Calculation of the degradation kinetics was performed using the model CAKE for the fitting. CAKE is freely available via the internet. CAKE was developed by Tessella Ltd, Abingdon, Oxfordshire, UK, sponsored by Syngenta.

It is often considered to evaluate the results of degradation studies performed with active compounds (e.g. pesticides, biocides, pharmaceuticals) because the non-linear fitting methodology used by CAKE was recommended by FOCUS degradation kinetics (FOCUS 2014). CAKE considers all recommended kinetics for the fitting:

- SFO (single first order)
- FOMC (first order multi compartment)
- HS (hockey stick)
- DFOP (double first order in parallel)

Recently, EFSA, the European Food Safety Authority, published an opinion where results of CAKE optimisation are presented and the model use is recommended.

The following version of the model was considered: CAKE version 3.3 (Release) running on R version 3.0.0 (2013-04-03).

4 Results

4.1 Pre-experiments and consequences for soil degradation main test

Application of 4 mg/kg on soil IME 03-G, duplicate samplings, incubation at 20°C in the dark

Bromoxynil:	Recovery after 20h incubation:	50.9% / 47.9% of applied substance.
Isoproturon:	Recovery after 20h incubation:	76.5% / 79.5% of applied substance.
	Recovery after 3d incubation:	54.4% / 58.2% of applied substance.
Sulfadiazine:	Recovery after 4d incubation:	44.7% / 48.8% of applied substance.

Recovery of all substances tested were in the range expected from literature data. Based on the data determined the sampling schedule was agreed for the main tests.

However, Isoproturon did unexpectedly show no degradation in the main test. Thus, after further pre-experiments it was decided to change to Lufa 2.4 soil for the Isoproturon main test. For details see section 3.4.1.2.

4.2 Main soil degradation tests

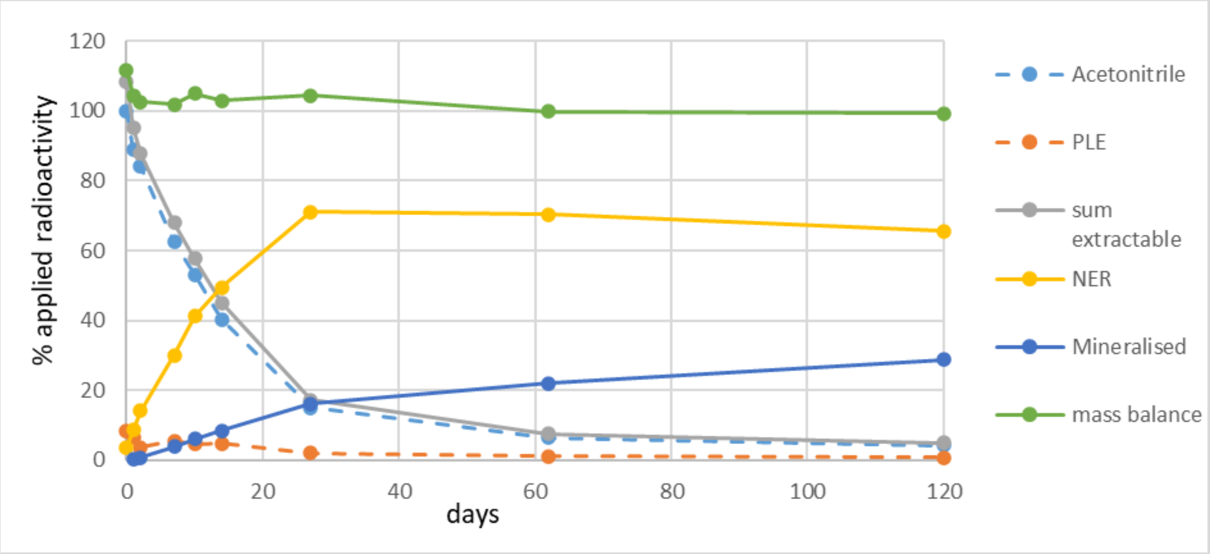
4.2.1 ^{14}C-mass balances

In the following tables and figures the ^{14}C-radioactive mass balances for the three test substances are shown. This is the normal result of an OECD 307 study with one soil but more sampling times.

4.2.1.1 Bromoxynil

On the basis of the preliminary tests, the main tests were started on June 25, 2019 with the application of bromoxynil. On October 23, 2019, the experiment was completed after 120 days of incubation. Two reserve samples were run until day 169 (see Table 12).

Figure 8: Degradation scheme and mass balance of ^{14}C-Bromoxynil.



Source: Fraunhofer IME evaluation of project data with MS Excel

Table 12: Mass balance ^{14}C-Bromoxynil at soil IME 03-G

Day	replicate	Acetonitrile extract [% aR]	PLE [% aR]	sum extractable [% aR]	NER [% aR]	$^{14}CO_2$ [% aR]	Mass Balance [% aR]
0d	1	98.7	9.4	108.1	2.5		110.6
	2	101.4	7.2	108.6	4.4		113.0
1d	1	91.2	7.2	98.4	7.3	0.3	106.0
	2	86.5	5.8	92.3	10.0		102.7
2d	1	83.0	5.8	88.8	15.2	0.7	104.7
	2	85.3	1.4	86.7	12.9		100.3
7d	1	62.1	5.8	67.9	31.2	3.9	103.0
	2	62.9	5.1	68.0	28.9		100.7
10d	1	59.4	5.2	64.6	40.5	6.0	111.1
	2	47.0	3.9	50.9	41.9		98.7
14d	1	37.4	5.4	42.8	50.1	8.5	101.4
	2	43.2	4.0	47.2	48.7		104.5
14d sterile	1	93.4	9.4	102.8	6.6	n.d.	109.4
	2	93.5	7.9	101.4	9.0		110.5
27d	1	16.7	2.3	19.0	67.2	16.1	102.3
	2	13.5	1.9	15.4	75.0		106.5
62d	1	6.4	1.1	7.4	67.5	22.0	97.0
	2	6.5	1.1	7.6	73.0		102.7
119d sterile	1	88.4	11.1	99.6	9.4	n.d.	108.9
	2	56.1	7.5	63.6	33.1		96.8
120d	1	4.1	0.8	4.9	66.4	28.8	100.1
	2	4.2	0.7	4.9	64.6		98.3
169d	1	4.2	0.6	4.8	63.1	31.1	99.0
	2	4.0	0.3	4.3	62.4		97.9

Data show a very good mass balance during the entire study. The extractable radioactivity decreased rapidly and at the same time formation of large amounts of NER was observed. After 27 days reactions slow down significantly.

Mineralisation was detectable until end of the incubation. Though after 60 days the extractable radioactivity decreased by 2.5% only, mineralisation increased in the same time by 10%.

Sterile samples prove that degradation was mainly due to biological processes. The large difference at 119 day duplicate sterile samples indicates that one sample was not entirely sterile. This is confirmed also in later measurements with this sample.

Table 13 shows the recovered parent. Radio-HPLC identifies most of the extractable radioactivity as parent bromoxynil. At 120 days no ^{14}C-Bromoxynil was detectable by radio-HPLC.

Table 13: Parent recovery ^{14}C-Bromoxynil from soil IME 03-G

Day	replicate	sum extractable [% aR]	Parent extractable [% aR]	Acetonitrile extract parent [%aR]	PLE extract (acetonitrile) parent [%aR]
0d	1	108.1	108.1	98.7	9.4
	2	108.6	108.6	101.4	7.2
1d	1	98.4	90.8	83.6	7.2
	2	92.3	86.7	80.9	5.8
2d	1	88.8	77.7	72.9	4.8
	2	86.7	81.3	79.9	1.4
7d	1	67.9	52.5	46.7	5.8
	2	68.0	57.5	52.4	5.1
10d	1	64.6	45.2	41.1	4.1
	2	50.9	39.8	35.9	3.9
14d	1	42.8	32.4	28.3	4.1
	2	47.2	34.3	31.7	2.6
14d sterile	1	102.8	102.8	93.4	9.4
	2	101.4	101.4	93.5	7.9
27d	1	19.0	16.2	13.9	2.3
	2	15.4	11.4	9.5	1.9
62d	1	7.4	6.4	6.4	0.0
	2	7.6	6.5	6.5	0.0
119d sterile	1	99.6	99.5	88.4	11.1
	2	63.6	63.6	56.1	7.5
120d	1	4.9	n.d.	n.d.	n.d.
	2	4.9	n.d.	n.d.	n.d.
169d	1	4.8	n.d.	n.d.	n.d.
	2	4.3	n.d.	n.d.	n.d.

n.d. = not determined

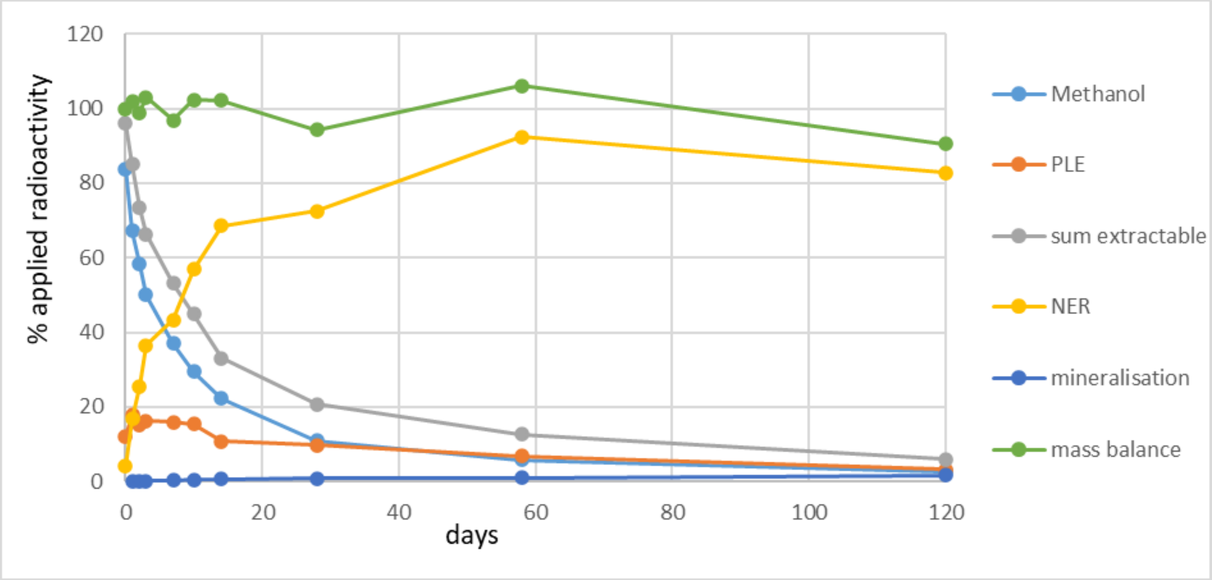
4.2.1.2 Sulfadiazine

On the basis of the preliminary tests, the main tests were started on September 30, 2019 with the application of sulfadiazine. On January 28, 2020, the experiment was completed after 121 days of incubation.

The data show a very good mass balance over the entire test period. The NER formation is very strong and reaches a maximum of over 90% at 58d. Larger deviations between the parallel samples can be observed in some cases. Virtually no mineralization is observed at all.

The sterile samples show that the NER formation is largely due to abiotic processes. This assumption is supported by the very low mineralization.

Figure 9: Degradation scheme and mass balance of ^{14}C-Sulfadiazine.



Source: Fraunhofer IME evaluation of project data with MS Excel

Table 14: Mass balance ^{14}C-Sulfadiazine at soil IME 03-G

Day	replicate	Methanol extract [% aR]	PLE [% aR]	sum extractable [% aR]	NER [% aR]	$^{14}CO_2$ [% aR]	Mass Balance [% aR]
0d	1	84.4	12.9	97.3	3.5		100.8
	2	83.2	11.2	94.5	4.7		99.2
1d	1	68.7	17.5	86.2	15.7	0.0	102.0
	2	65.8	17.9	83.7	17.8		101.5
2d	1	58.2	13.4	71.6	29.4	0.1	101.1
	2	58.4	16.7	75.1	21.6		96.8
3d	1	49.3	15.9	65.2	44.6	0.2	110.0
	2	50.7	16.7	67.4	28.4		96.0
7d	1	37.1	17.1	54.3	47.9	0.3	102.5
	2	37.0	14.9	51.9	38.8		91.0
10d	1	28.8	13.2	42.0	54.6	0.5	97.0
	2	30.3	17.6	47.9	59.2		107.5
14d	1	22.6	7.9	30.5	78.0	0.6	109.1
	2	22.1	13.5	35.6	59.1		95.3
14d sterile	1	42.8	15.8	58.6	41.9	n.d.	100.5
	2	42.1	17.6	59.7	46.8		106.5
28d	1	10.4	9.2	19.6	70.9	0.9	91.4
	2	11.6	10.3	21.9	74.1		96.9
58d	1	5.9	6.3	12.2	90.4	1.0	103.5
	2	5.9	7.3	13.2	94.5		108.6
120d sterile	1	11.2	8.5	19.7	85.6	n.d.	105.3
	2	10.5	5.7	16.2	77.3		93.6
121d	1	2.9	2.0	4.9	88.4	1.7	95.0
	2	2.7	4.4	7.1	77.1		85.9

Table 15: Parent recovery ^{14}C-Sulfadiazin from soil IME 03-G

Day	replicate	sum extractable [% aR]	Parent extractable [% aR]	Methanol extract parent [%aR]	PLE extract (Methanol) parent [%aR]
0d	1	97.3	97.3	84.4	12.9
	2	94.5	94.5	83.2	11.2
1d	1	86.2	86.2	68.7	17.5
	2	83.7	83.7	65.8	17.9
2d	1	71.6	71.6	58.2	13.4
	2	75.1	75.1	58.4	16.7
3d	1	65.2	65.2	49.3	15.9
	2	67.4	67.4	50.7	16.7
7d	1	54.3	54.3	37.1	17.1
	2	51.9	51.9	37.0	14.9
10d	1	42.0	42.0	28.8	13.2
	2	47.9	47.9	30.3	17.6
14d	1	30.5	30.5	22.6	7.9
	2	35.6	35.6	22.1	13.5
14d sterile	1	58.6	58.6	42.8	15.8
	2	59.7	59.7	42.1	17.6
28d	1	19.6	19.6	10.4	9.2
	2	21.9	21.9	11.6	10.3
58d	1	12.2	12.2	5.9	6.3
	2	13.2	13.2	5.9	7.3
120d sterile	1	19.7	19.7	11.2	8.5
	2	16.2	16.2	10.5	5.7
121d	1	4.9	n.d.	n.d.	n.d.
	2	7.1	n.d.	n.d.	n.d.

n.d. = not determined

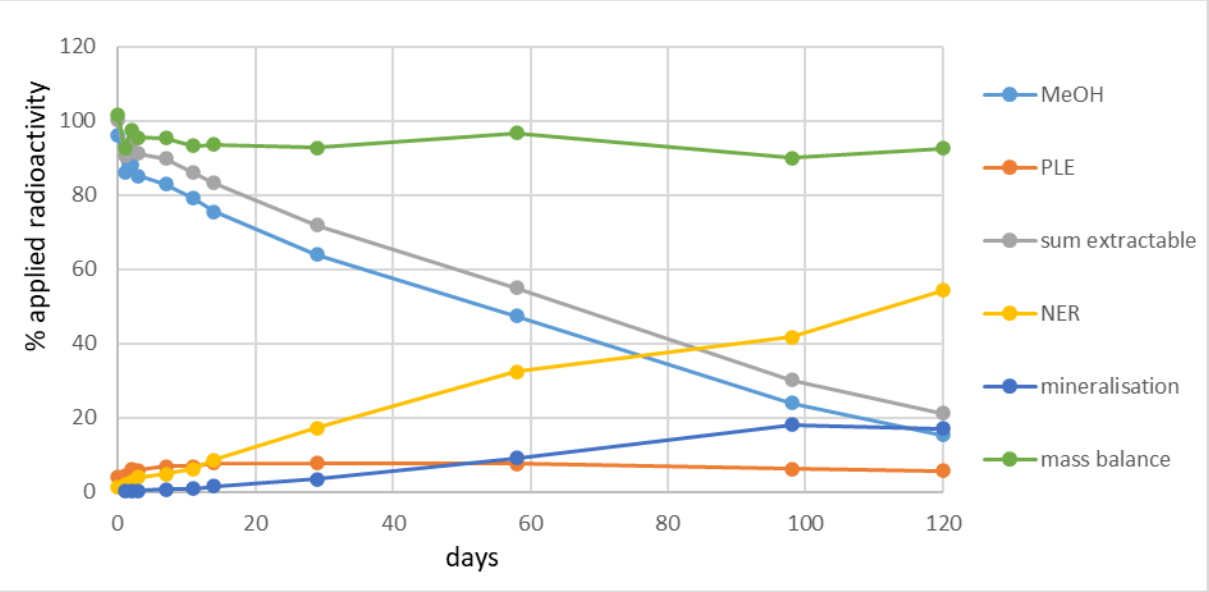
4.2.1.3 Isoproturon

The main experiment with ^{14}C-isoproturon was started on February 17, 2020. Test soil was Lufa 2.4. The experiment was completed with the last sampling on June 17, 2020.

Data show a very good mass balance during the study. The extractable radioactivity decreased slowly compared to the other two substances and at the same time formation of NER was observed also slowly but constantly. The graph indicate that the degradation process is not completely finished at test end of 120 days.

Radio-HPLC identifies most of the extractable radioactivity as parent isoproturon. Mineralisation was also slow and after 100 days no significant further mineralisation was observed. Sterile samples show that degradation was mainly due to biological processes.

Figure 10: Degradation scheme and mass balance of ^{14}C-Isoproturon



Source: Fraunhofer IME evaluation of project data with MS Excel

Table 16: Mass balance ^{14}C-Isoproturon at soil Lufa 2.4

Day	replicate	MeOH/water extract [% aR]	PLE [% aR]	sum extractable [% aR]	NER [% aR]	$^{14}CO_2$ [% aR]	Mass Balance [% aR]
0d	1	94.3	3.7	98.0	1.1		99.1
	2	98.1	4.5	102.6	1.3		103.9
1d	1	85.2	4.6	89.9	1.6	0.2	91.7
	2	87.0	4.2	91.2	2.2		93.6
2d	1	88.3	5.8	94.1	2.8	0.3	97.2
	2	88.1	6.4	94.5	2.7		97.5
3d	1	86.6	5.6	92.2	3.9	0.4	96.5
	2	83.7	6.3	90.0	4.0		94.4
7d	1	82.7	7.0	89.8	5.4	0.7	95.8
	2	83.1	6.9	90.0	4.3		94.9
11d	1	78.7	6.6	85.4	6.9	1.0	93.2
	2	79.4	7.3	86.7	5.6		93.2
14d	1	76.8	7.3	84.1	8.4	1.6	94.1
	2	74.1	8.3	82.3	9.0		92.9
15d sterile	1	93.5	7.5	101.0	2.9	n.d.	103.9
	2	87.9	6.4	94.3	2.6		96.9
29d	1	63.4	8.4	71.8	16.8	3.5	92.2
	2	64.6	7.3	72.0	17.9		93.3
59d	1	42.3	7.7	50.0	36.0	9.2	95.2
	2	52.5	7.5	60.0	29.0		98.2
98d	1	21.3	6.5	27.8	40.3	18.1	86.2
	2	26.5	6.0	32.6	43.1		93.8
120d sterile	1	87.7	0.9	88.6	3.4	n.d.	92.0
	2	88.8	7.6	96.4	2.9		99.3
121d	1	13.2	5.5	18.7	55.1	17.0	90.9
	2	17.6	6.0	23.6	53.5		94.2

Table 17: Parent recovery ^{14}C-Isoproturone from soil Lufa 2.4

Day	replicate	sum extractable [% aR]	Parent extractable [% aR]	MeOH / H2O extract parent [%aR]	PLE extract (MeOH / H2O) parent [%aR]
0d	1	98.0	98.0	94.3	3.7
	2	102.6	102.6	98.1	4.5
1d	1	89.9	89.9	85.2	4.6
	2	91.2	91.2	87.0	4.2
2d	1	94.1	94.1	88.3	5.8
	2	94.5	94.5	88.1	6.4
3d	1	92.2	92.2	86.6	5.6
	2	90.0	90.0	83.7	6.3
7d	1	89.8	89.8	82.7	7.0
	2	90.0	90.0	83.1	6.9
11d	1	85.4	85.4	78.7	6.6
	2	86.7	86.7	79.4	7.3
14d	1	84.1	84.1	76.8	7.3
	2	82.3	82.3	74.1	8.3
15 sterile	1	101.0	101.0	93.5	7.5
	2	94.3	94.3	87.9	6.4
29d	1	71.8	60.5	52.8	8.4
	2	72.0	63.0	55.2	7.3
59d	1	50.0	39.8	32.0	7.7
	2	60.0	50.4	42.9	7.5
98d	1	27.8	27.8	21.3	6.5
	2	32.6	32.6	26.5	6.0
120d sterile	1	88.6	90.7	82.4	8.3
	2	96.4	94.0	86.4	7.6
121d	1	18.7	15.3	9.8	5.5
	2	23.6	22.7	16.6	6.0

4.2.2 Degradation kinetics ^{14}C-test substances (parent dissipation)

Based on the recovery of parent test substances, degradation kinetics were established for each substance. NER fractions were not taken into consideration at this point. The calculation is only used for comparison purposes with existing DT_{50} values from the literature.

The analyses for the parent compounds were based on SFO (single first order), DFOP (Double first order in parallel), HS (Hockey stick), and FOMC (First Order Multi Compartment) kinetics using CAKE (see 3.6). The results of the optimisation are presented in the following three tables. The figures show the graphical presentation of the best fitting. The complete CAKE report is presented in a separate file as it would be too large to attach it to this study report.

4.2.2.1 Bromoxynil

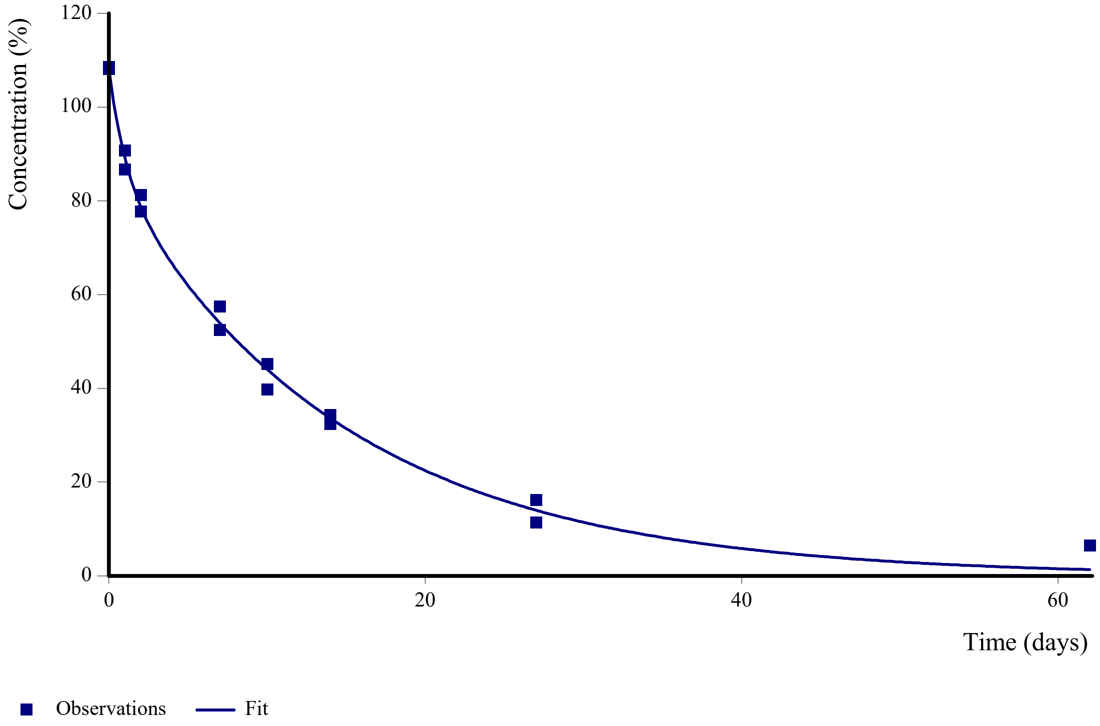
Table 18: Calculated DT_{50} and DT_{90} for ^{14}C-Bromoxynil

	chi^2 (%)	r^2 (-)	Prob. > t k_deg	DT50 (d)	DT90 (d)
SFO	6.63	0.9829	$1.8\ 10^{-10}$	8.29	27.6
DFOP	**3.36**	**0.9946**	**$1.03\ 10^{-7}$***	**6.95****	**30.8**
HS	6.35	0.9853	$1.33\ 10^{-9}$*	8.02**	41.4
FOMC	4.47	0.9902	-	6.93**	41.6

* value related to the dominant rate constant; ** DT50 overall

The best fitting results were obtained when considering DFOP kinetics (lowest chi²-value 3.36%).

Figure 11: **Plot of degradation kinetic (DFOP) of ^{14}C-Bromoxynil calculated using CAKE**



Source: Fraunhofer IME evaluation of project data with CAKE

4.2.2.2 Sulfadiazine

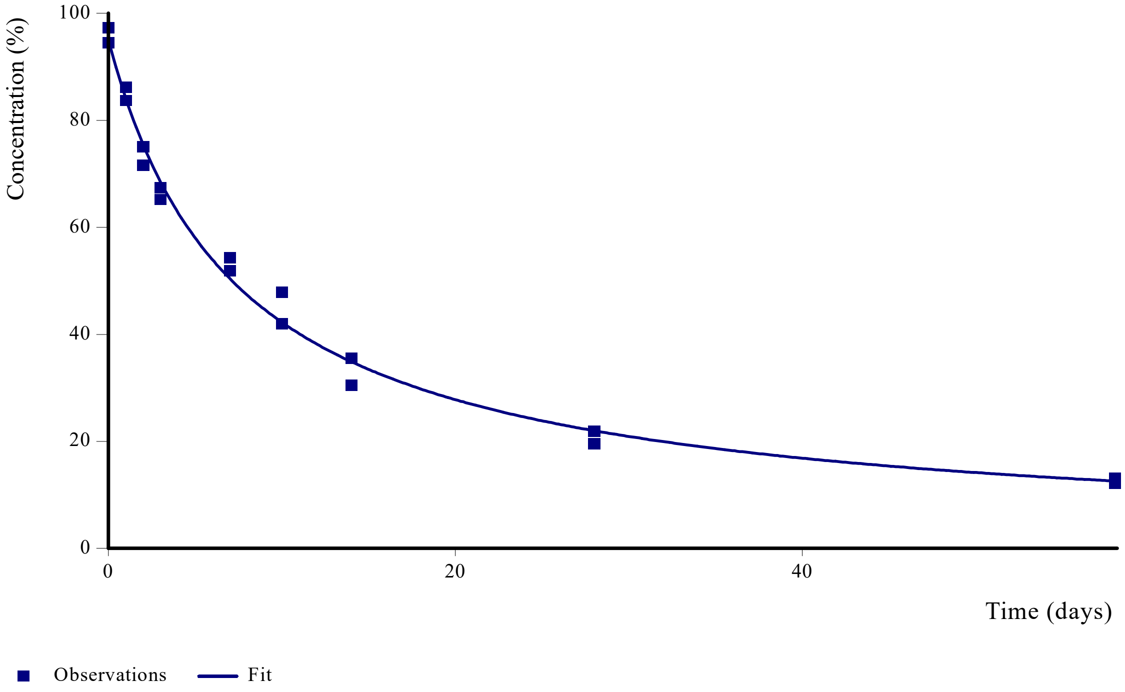
Table 19: **Calculated DT_{50} and DT_{90} for ^{14}C-Sulfadiazine**

	chi² (%)	r² (-)	Prob. > t k_deg	DT50 (d)	DT90 (d)
SFO	8.44	0.9635	4.3E-09	10.5	34.7
DFOP	3.77	0.9891	0.000371	7.94*	64.0
HS	5.71	0.9804	0.0000359	7.67*	57.3
FOMC	**2.88**	**0.9915**	**-**	**7.93***	**81.9**

*DT50 overall

The best fitting results were obtained when considering FOMC kinetics (lowest chi²-value 2.88%).

Figure 12: Plot of degradation kinetic (FOMC) of ^{14}C-Sulfadiazine calculated using CAKE



Source: Fraunhofer IME evaluation of project data with CAKE

4.2.2.3 Isoproturon

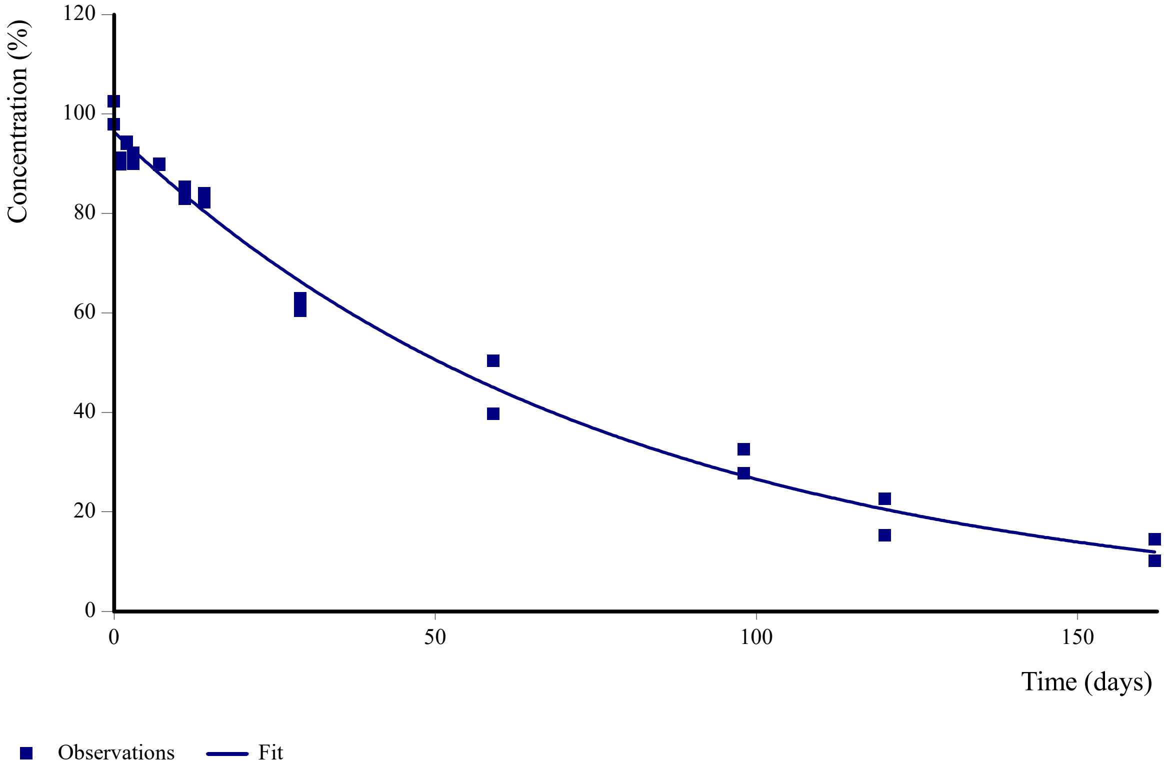
Table 20: Calculated DT_{50} and DT_{90} for ^{14}C-Isoproturon

	chi² (%)	r² (-)	Prob. > t k_deg	DT50 (d)	DT90 (d)
SFO	**3.18**	**0.9871**	**-**	**53.8**	**179**
DFOP	3.42	0.9873	-	52.8*	183
HS	3.44	0.9872	-	54.0*	178
FOMC	3.28	0.9872	-	52.8*	185

*DT50 overall

The best fitting results were obtained when considering SFO kinetics (lowest chi^2-value 2.88%).

Figure 13: Plot of degradation kinetic (SFO) of ^{14}C-Isoproturon calculated using CAKE



Source: Fraunhofer IME evaluation of project data with CAKE

4.2.3 ^{13}C-mass balances and degradation kinetics (parent dissipation)

Due to capacity reasons the ^{13}C-degradation experiments were performed only with the required 6 sampling points. The main purpose was to compare the main findings of ^{14}C and ^{13}C experiments in order to assess if the different isotope labels used lead to different findings. Experimental focus of this project was clearly put to the ^{14}C-radiolabelled test substances.

In the following tables the ^{13}C-label mass balances and degradation kinetics for the three test substances are shown.

Table 21: Recovery of extractable ^{13}C-labelled parent test substance over time

Day	Parent Bromoxynil in extract (HPLC_MS) ^{13}C [% applied] (Start 40 mg/kg)	Parent Sulfadiazine in extract (HPLC_MS) ^{13}C [% applied] (Start 40 mg/kg)	Parent Isoproturon in extract (HPLC_MS) ^{13}C [% applied] (Start 40 mg/kg)
0d	107.5	86.2	n.d.
7d	89.4	73.5	108.0
14d	83.1	69.0	106.9
14d sterile	116.2	71.9	not analysed
28d	43.3	54.4	99.5
60d	28.9	36.7	90.4
120d sterile	106.9	56.7	not analysed
120d	18.9	18.6	76.4

4.2.4 Comparison of degradation kinetics at different starting concentrations

Based on the data obtained, kinetic analyses were performed by CAKE. Table 22 shows the optimal fits for the ^{13}C-label and ^{14}C-label in order to compare the degradation rates in both experiments. The degradation rates of the substances applied at a rate of 4 mg/kg (^{14}C-label) are generally significantly higher than those determined at the 40 mg/kg initial application rate (^{13}C-label).

Table 22: Calculated DT$_{50}$ and DT$_{90}$ for different labels (best fit by CAKE)

		chi^2 (%)	r^2 (-)	DT50 (d)	DT90 (d)
Bromoxynil	HS (^{13}C)	7.87	0.96	22.7	183.0
	DFOP (^{14}C)	3.36	0.9946	6.95	30.8
Sulfadiazine	FOMC (^{13}C)	3.54	0.9842	49.6	246
	FOMC (^{14}C)	2.88	0.9915	7.93	81.9
Isoproturon	SFO (^{13}C)	0.93	0.9550	229	760
	SFO (^{14}C)	3.18	0.9871	53.8	179

The biggest difference lay in the different starting concentration, which was ten times higher for the ^{13}C label than for the ^{14}C label. This might already affect the soil microflora and slow down degradation processes. In OECD 307 tests it is required to quantify the active biomass by substrate induced respiration (SIR method, ISO 14240-1). Minimum requirement is 1% C$_{mic}$ of C$_{org}$ for the test soil. Table 23 shows the development of the microflora activity determined by ISO 14240-1 at different soil treatments and test concentrations.

Table 23: Biomass determination throughout the experiments (ISO 14240-1)

Soil	treatment	test start [Cmic/Corg]		test end [Cmic/Corg]	
		2 mg/kg	40 mg/kg	2 mg/kg	40 mg/kg
IME 03-G Corg: 4.5%	No treatment	1.8	1.4	1.3	1.2
	Only application solvent	1.9	1.5	1.5	1.4
	Solvent plus Bromoxynil	1.9	1.6	1.4	1.4
IME 03-G Corg: 4.5%	No treatment	2.6	1.4	1.7	1.2
	Only application solvent	2.7	1.5	1.7	0.5
	Solvent plus Sulfadiazine	2.7	1.5	1.7	0.6
Lufa 2.4 Corg: 1.95	No treatment	2.4	4.4	2.2	3.9
	Only application solvent	2.5	4.1	2.4	4.9
	Solvent plus Isoproturon	2.4	4.2	2.5	4.7

Interestingly ISO 14240-1 results show no effect of the substance concentration on the biomass. There is a drop of biomass in the Sulfadiazine treatment at test end, but not only at the Sulfadiazine treatment but also in the solvent treatment without Sulfadiazine. Therefore, Sulfadiazine seems not to be the reason for the drop of the biomass.

In addition, as shown in Table 10, the amount of solvent (DMSO) applied to the soil did not differ significantly in the 2 mg/kg and the 40 mg/kg experiment. However, in the 2 mg/kg experiment no drop of biomass was observed at test end. The solvent is thus unlikely the reason for the drop, either. Finally, the data indicate that the biomass represented by the ISO 14240-1 was not too sensitive to the treatments applied to the soil.

However, a lower degradation *rate* does not mean a lower degradation *amount*. At a start concentration of 4 mg/kg at DT_{50} only 2 mg substance are degraded per kg soil within one half-live time. At a concentration of 40 mg/kg, 20 mg substance degraded per kg soil in the same time. Thus, the rate is lower but degradation definitely was observed in the test. Usually in tests according to OECD 307 the starting concentration should not exceed 10 mg/kg but as this was not in line with the analytical sensitivity of the ^{13}C-work this concentration had to be exceeded.

4.2.5 Comparison of total NER in ^{13}C and ^{14}C-experiments

Based on the ^{13}C element analysis (see 3.4.3) ^{13}C-total NER amounts were calculated.

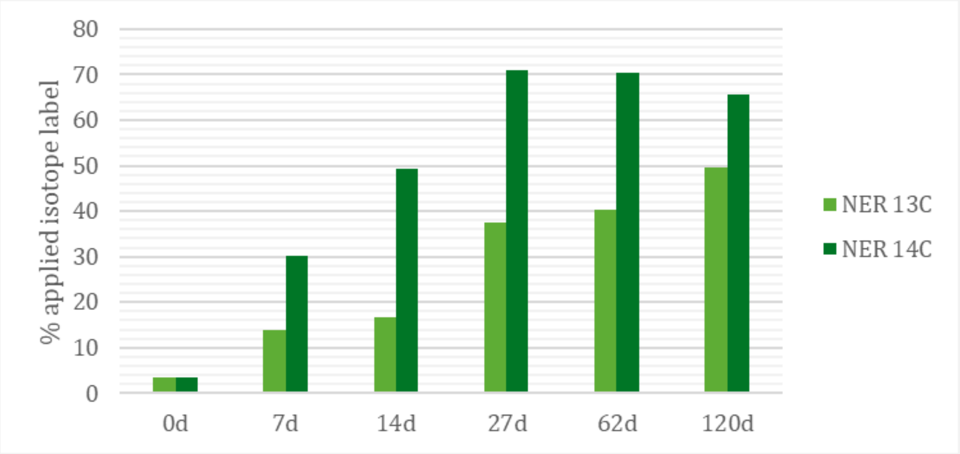
Table 24: total NER at ^{13}C-experiments for Bromoxynil and Sulfadiazine

Day	Bromoxynil		Sulfadiazine	
	NER ^{13}C [%]	NER ^{14}C [%]	NER ^{13}C [%]	NER ^{14}C [%]
0d	3.4	3.5	2.3	4.1
7d	13.8	30.1	18.0	43.4
14d	16.7	49.4	24.0	68.6
28d	37.5	71.1	37.1	72.5
60d	40.2	70.3	70.8	92.5
120d	49.7	65.5	85.7	82.9

All values in % of initially applied parent

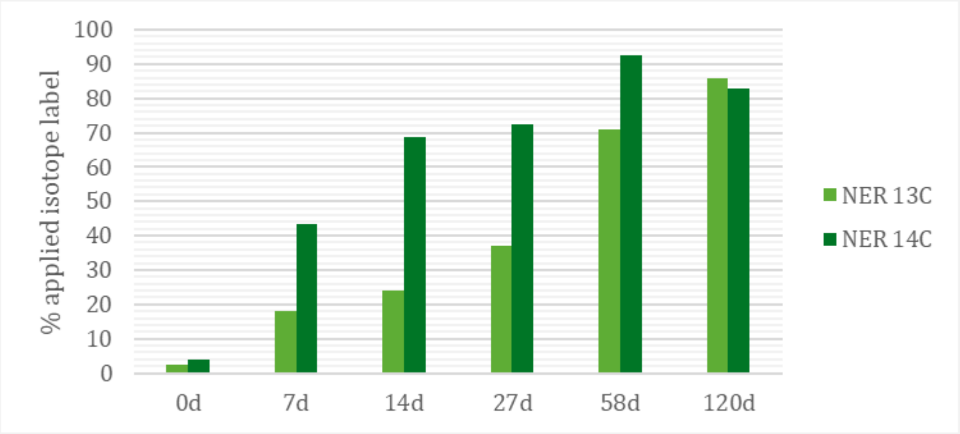
For ^{13}C-Isoproturon no NER determination was conducted since only at the very last sampling the parent recovery falls below 90% (see Table 21). So only in the very last sampling NER could have been formed. This was considered not to be relevant for NER characterisation.

Figure 14: Comparison total NER from ^{14}C and ^{13}C-Bromoxynil degradation experiments



Source: Fraunhofer IME evaluation of project data with MS Excel

Figure 15: **Comparison total NER from ^{14}C and ^{13}C-Sulfadiazine degradation experiments**



Source: Fraunhofer IME evaluation of project data with MS Excel

The figures above show an increasing NER formation until 120 days in both cases. Considering the lower degradation rate, the lower NER-formation rate in the ^{13}C-experiment compared to the ^{14}C-experiment is assessed to be reasonable provided that NERs are mainly the result of degradation processes. In case of major fractions of parent substance in NER by physical entrapment processes, NER formation must not necessarily follow the degradation rates. However, as shown later (see chapter 4.4 ff) no significant amounts of parent were found in NER, which supports the assumption formulated above. Both ^{13}C-experiments apparently did not reach the maximum NER formation within the 120 days incubation period as could be seen in the ^{14}C-experiments.

4.3 Comparison of extraction procedures for NER definition

Bromoxynil

Table 25 shows the extraction efficiency of the different solvents / extraction methods used. The most effective solvent per sampling is highlighted in bold numbers.

Table 25: Comparison of extraction efficiency for different NER definitions for Bromoxynil.

Day	ACN followed by PLE [%aR]	PLE (with ACN) only [%aR]	PLE standard solvent mix [%aR]
0	**108.3**	85.2	107.6
7	68.0	62.5	**72.0**
14	45.0	**48.3**	42.7
27	17.2	**22.9**	19.0
62	7.5	1.1	**10.2**
120	4.9	5.2	**6.2**
14d sterile	102.1	103.9	**105.8**
119d sterile	**99.6**	82.8	99.0

The even distribution of the bold numbers in the table demonstrates that there were no significant differences between the applied extraction procedures regarding recovery of radioactivity from ^{14}C-Bromoxynil applied soils.

Sulfadiazine

Table 26: Comparison of extraction efficiency for different NER definitions for Sulfadiazine.

Day	MeOH followed by PLE [%aR]	PLE (with MeOH) only [%aR]	PLE standard solvent mix [%ar]
0	**95.9**	53.6	**95.9**
7	53.1	53.3	**63.4**
14	33.0	3.8	**52.1**
28	20.7	24.9	**34.2**
58	12.7	13.6	**25.6**
121	6.0	2.3	**18.8**
14d sterile	59.2	63.1	**67.1**
120d sterile	18.0	5.7	**35.3**

For Sulfadiazine the standard solvent mix clearly works best. Generally, it must be stated that Sulfadiazine was difficult to extract from soil as the substance is not soluble in g/L amounts in any solvent. After application in DMSO the extraction with methanol worked well. However, at later samplings the standard solvent mix worked better and extracts around 10% to 14% more radioactivity than methanol.

Isoproturon

Table 27: **Comparison of extraction efficiency for different NER definitions for Isoproturon.**

Day	MeOH/H_2O followed by PLE, [%aR]	PLE (with MeOH/H_2O) only, [%aR]	PLE standard solvent mix [%ar]
0	**100.3**	92.4	69.4
7	89.9	84.1	**92.8**
14	**83.2**	78.6	17.6
29	71.9	63.5	**75.4**
59	**55.0**	42.8	46.9
120	**21.2**	8.4	17.2
15d sterile	97.7	**99.8**	99.5
120d sterile	**92.5**	88.7	85.8

The most effective extraction for Isoproturon is shaking with substance specific solvent (methanol/water, 80/20, v/v) followed by PLE with this solvent. The standard solvent mix shows very strong variations which makes it unsuitable, even if it has the best efficiency in two samplings.

As a result of the different recoveries for the three test substances the shaking extraction followed by PLE provides the most stable extraction efficiency without any outliers followed by the PLE standard solvent mix with methanol/ acetone/ water (50/25/25, v/v/v) at 100°C, 100 bar, three cycles (Loeffler et al, 2020, UBA Texte 133/2022), where outliers at 0d and 14d were observed in our experiments. As it is anyway integral part of an OECD 307 study to develop an appropriate extraction method for the parent test substance from soil, it seems most straight-forward to use this solvent also for NER definition. Though for difficult to extract substances the standard solvent mix can work better as demonstrated for Sulfadiazin, it might also produce huge variations as shown for Isoproturon which cannot be predicted easily. To avoid these unpredictable variations the recommendation from the study is to use the solvent that gives the best recovery in initial experiments. For standardisation, PLE should be used as final extraction step for NER determination.

4.4 NER characterisation: silylation

4.4.1 Silylation stability

Results of the test-silylation (extracted soil matrix, spiked with parent substance followed by silylation, see 3.5.1.4) show generally a good recovery of the applied radioactivity. As for parent Isoproturon the recovery was already 100% in the silylation extract, no MeOH/water extraction was conducted for the test silylation.

Table 28: **Overview recovery and stability testing for silylation (radio-TLC).**

Substance	Silylation extract	Parent in extract	MeOH-extract	Parent in extract
Bromoxynil	92.6 [% aR]	100 %	--	--
	94.9 [% aR]	100 %	--	--
Sulfadiazine	13.1 [% aR]	0 %	83.1 [% aR]	34.1 %
	13.6 [% aR]	0 %	78.0 [% aR]	40.8 %
Isoproturon	103.0 [% aR]	82.7 %	--	--
	103.9 [% aR]	82.5 %	--	--

For Bromoxynil recovery and stability could be proven and also for Isoproturon only minor losses by degradation were observed. Sulfadiazine is proven to be sensitive to the silylation procedure. However, the substance does not entirely react during silylation, in the methanol extract more than 34% of the applied parent substance could be recovered. Recovery of radioactivity was also sufficient for Sulfadiazine.

4.4.2 Silylation procedural recovery

Another question about the silylation procedure is the procedural recovery. In routine this might not be necessary to determine, but for method development it is an important parameter. The tables below show that in most cases the mass balance over the silylation procedure was close to 100%. So, the risk to lose potentially released type I NER during silylation is assessed to be very low. Care has to be taken for combustion analysis because during silylation considerable amounts of salt (NaCl) are formed and "dilutes" the residue. This needs to be considered in the evaluation.

Table 29: Procedural recovery at silylation of samples containing NER from Bromoxynil.

Day	total NER [%aR]	Silylation extract [%aR]	Silylation residue [%aR]	mass balance	
				[%aR]	[% of total NER]
0	3.5	--			
7	30.1	11.6	22.6	34.4	114.6
14	49.4	12.3	35.2	47.5	96.2
27	71.1	13.5	49.8	63.3	89.3
62	70.3	11.5	51.7	63.2	90.0
120	65.5	10.5	53.5	64.0	97.8
14d sterile	7.8	6.2	0.3	6.5	83.0
119d sterile	9.4	9.0	4.0	13.0	131.6

Table 30: Procedural recovery at silylation of samples containing NER from Sulfadiazine.

Day	total NER [%aR]	Silylation extracts [%aR]	Silylation residue [%aR]	mass balance	
				[%aR]	[% of total NER]
0	4.1	--			
7	43.5	12.1	27.3	39.4	92.1
14	68.6	17.2	47.2	64.4	95.4
28	72.5	21.6	64.2	85.8	118.6
58	92.4	23.1	59.3	82.4	89.3
121	82.8	22.7	57.5	80.2	97.2
Sterile 14d	44.4	10.8	44.5	55.3	124.6
Sterile 120d	81.4	20.9	64.8	85.7	105.6

Table 31: Procedural recovery at silylation of samples containing NER from Isoproturon.

Day	total NER [%aR]	Silylation extracts [%aR]	Silylation residue [%aR]	mass balance	
				[%aR]	[% of total NER]
0	1.2	--			
7	4.9	1.6	4.2	5.8	120.3
14	8.7	2.1	6.3	8.4	96.8
29	17.4	3.9	13.4	17.3	99.8
59	35.5	9.4	37.7	47.1	145.1
120	54.3	13.1	59.5	72.6	133.8
15d sterile	2.8	0.9	1.3	2.2	79.8
120d sterile	3.2	1.4	4.6	6.0	193.9

4.4.3 Silylation of samples from degradation tests

4.4.3.1 ^{14}C-experiments

While considerable amounts of NER radioactivity were released by silylation, radio-TLC analysis showed only minor amounts of parent Bromoxynil in the silylation extract. However, in sterile samples with low NER the part of parent substance was very high. This could be expected since degradation of Bromoxynil seems to be driven by microbial activity as demonstrated by the sterile samples. Silylation confirms this hypothesis.

Table 32: Mass balance of the silylation of samples containing NER from Bromoxynil.

Day	total NER [%aR]	Silylation extract [%aR]	Parent release [%aR]	Parent release [% of NER]
0	3.5	--		
7	30.1	11.6	1.3	4.4
14	49.4	12.3	0.9	1.9
27	71.1	13.5	0.3	0.5
62	70.3	11.5	0.2	0.3
120	65.5	10.5	0.1	0.2
14d sterile	7.8	6.2	4.3	55.0
119d sterile	9.4	9.0	4.7	30.9

For Sulfadiazine the silylation was conducted anyway just to compare the results with EDTA-extraction, which is supposed to be the extraction of choice when a substance proves to be instable during silylation. As shown in Table 33, the extraction with methanol after silylation recovers the most radioactivity from the soil sample. Parent can still be detected but in minor amounts only. Due to the instability of the substance no further conclusions should be drawn from the data.

Table 33: Mass balance of the silylation of samples containing NER from Sulfadiazine.

Day	total NER [%aR]	Silylation Extract [%aR]	MeOH after Silylation [%aR]	Parent release, sum, [%aR]	Parent release [% of total NER]
0	4.1	--			
7	43.5	3.2	8.9	1.2	2.8
14	68.6	4.4	12.8	1.2	1.7
28	72.5	5.6	16.1	1.2	1.7
58	92.4	3.1	20.0	1.8	1.9
121	82.8	5.0	17.8	1.2	1.4
Sterile 14d	44.4	2.2	8.6	1.5	3.4
Sterile 120d	81.4	5.0	15.9	3.3	4.1

From Isoproturon amounts of radioactivity released by silylation were still significant but the parent content in the extracts was very low. However, as observed already for Bromoxynil the parent amount released from the sterile samples was much higher in terms of a release rate. But this is only because the NER in the sterile samples are very low and thus, the total amount released was not higher than in all other Isoproturon samples.

Table 34: Mass balance of the silylation of samples containing NER from Isoproturon.

Day	total NER [%aR]	Silylation Extract [%aR]	MeOH/H_2O after Silylation [%aR]	Parent release, sum, [%aR]	Parent release [% of total NER]
0	1.2	--			
7	4.9	1.2	0.4	0.5	11.2
14	8.7	2.9	1.0	0.5	5.4
29	17.4	2.9	1.0	0.6	3.4
59	35.5	5.4	4.0	0.3	0.9
120	54.3	6.7	6.4	0.0	0.0
15d sterile	2.8	0.8	0.1	0.5	17.3
120d sterile	3.2	1.2	0.3	0.7	22.1

4.4.3.2 ^{13}C-experiments and comparison with ^{14}C-experiment

Residues from ^{13}C-experiments were subject to silylation as well. However, since at IME no ^{13}C-isotope element analysis equipment is available, the chemical analysis was limited to compound-specific LC-MS analysis of the silylation extracts for extracted parent. These data can be compared directly with the parent substance recovered in the ^{14}C-experiments. In addition, analysis proved that silylation extracts can be measured by LC-MS like any other extract. No extreme effects of the extract matrix were observed. This is important if this silylation is considered to become part of the routine characterisation of NER. Though NER will be determined with isotope labelled test substances only, LC-MS can serve as confirmatory analytical method.

Table 35: Parent substance release from NER Typ I by silylation of ^{13}C and ^{14}C-test samples.

Day	Bromoxynil		Sulfadiazine		Isoproturon	
	^{13}C silylation, release of parent in % applied	^{14}C silylation, release of parent in % applied	^{13}C silylation, release of parent in % applied	^{14}C silylation, release of parent in % applied	^{13}C silylation, release of parent in % applied	^{14}C silylation, release of parent in % applied
7d	2.0	1.3	1.4	1.2	0.1	0.5
14d	2.4	0.9	1.2	1.2	0.1	0.5
28d	1.3	0.3	1.7	1.2	0.2	0.6
60d	1.5	0.2	2.1	1.8	0.2	0.3
120d	1.5	0.1	4.6	1.2	0.1	0.0
14d sterile	1.4	4.3	2.2	1.5	n.d.	n.d.
120d sterile	1.7	4.7	2.1	3.5	n.d.	n.d.

Data as presented in Table 35 do not show considerable differences in the different experiments. In no case really significant amounts of NER were released as parent test substance. However, still keeping in mind that samples from Sulfadiazine might degrade during silylation.

4.5 NER characterisation: EDTA Extraction

4.5.1 EDTA extraction stability

Similar to the test silylation (see 4.4.1) also test EDTA extractions were conducted with freshly spiked blank samples in order to test stability of the substances against EDTA extraction conditions. Table 36 shows the result of test extraction and subsequent radio-TLC analyses of the EDTA extracts.

Table 36: Overview recovery and stability testing for EDTA extraction (radio-TLC).

Substance	EDTA extract	Organic solvent extract	Parent in extract
Bromoxynil	99.1 [% aR]	14.7 [% aR] (acetonitrile)	92.2 %
	99.1 [% aR]	14.6 [% aR] (acetonitrile)	94.7 %
Sulfadiazine	81.4 [% aR]	6.9 [% aR] (MeOH/H_2O)	44.8 %
	81.5 [% aR]	7.1 [% aR] (MeOH/H_2O)	48.4 %
Isoproturon	118.9 [% aR]	31.6 [% aR] (MeOH/H_2O)	89.3 %
	122.5 [% aR]	31.6[% aR] (MeOH/H_2O)	82.9 %

The recovery of radioactivity by EDTA extraction and subsequent extraction with an organic solvent was very good, but radio-TLC showed in all cases losses of the applied parent. Repeating the experiment with Sulfadiazine showed no change of the results.

In order to prove that this is no chromatographic effect due to the high salt load, the extracts were analysed by LC-MS. A sulfadiazine standard in solvent, sulfadiazine standard spiked to an EDTA blank extract and the EDTA extract of a Sulfadiazine spiked soil were analysed. To be able to compare the data, at each injection exactly the same amount of radioactivity was injected for each sample. While the spiked EDTA blank extract and the Sulfadiazine standard showed identical amounts of parent Sulfadiazine, in the EDTA-Extract of the Sulfadiazine spiked soil only about 70% of parent were found.

From the results there is no doubt that Sulfadiazine is sensitive to the EDTA-extraction. This is unexpected and it seems to be necessary to check stability of the test substance also against EDTA-extraction to be sure to receive valid data from the extraction.

4.5.2 EDTA extraction procedural recovery

In a first step the soils containing NER were extracted with an aqueous EDTA solution. After extraction with EDTA solution, a further extraction with either acetonitrile (Bromoxynil), methanol (Sulfadiazine) or methanol/water (Isoproturon) was carried out to avoid that limited water solubility of the released substances will pretend low release. The following tables represent the sum of radioactivity recovered by both extractions plus the not releasable radioactivity.

Table 37: Procedural recovery at EDTA extractions of samples containing NER from Bromoxynil.

Day	total NER [%aR]	EDTA extract, sum [%aR]	extraction residue [%aR]	mass balance	
				[%aR]	[% of total NER]
0	3.5	2.8	0.7	3.5	102.6
7	30.1	12.0	19.2	31.2	104.0
14	49.4	15.1	34.1	49.2	99.6
27	71.1	17.3	45.8	63.1	88.8
62	70.3	16.8	53.8	70.6	100.6
120	65.5	15.8	47.0	62.8	95.9
14d sterile	7.8	7.0	2.0	9.0	116.9
119d sterile	9.4	8.5	2.1	10.6	112.6

Table 38: Procedural recovery at EDTA extractions of samples containing NER from Sulfadiazine.

Day	total NER [%aR]	EDTA extract, sum [%aR]	extraction residue [%aR]	mass balance	
				[%aR]	[% of total NER]
0	4.1	2.1	1.8	3.9	96.4
7	43.5	18.2	30.4	48.6	113.0
14	68.6	25.0	44.4	69.4	102.6
28	72.5	27.0	52.3	79.3	109.5
58	92.4	27.2	55.0	82.2	89.0
121	82.8	26.3	55.2	81.5	98.8
Sterile 14d	44.4	15.9	28.7	44.6	100.8
Sterile 120d	81.4	23.6	48.7	72.3	89.1

Table 39: Procedural recovery at EDTA extractions of samples containing NER from Isoproturon.

Day	total NER [%aR]	EDTA extract, sum [%aR]	extraction residue [%aR]	mass balance	
				[%aR]	[% of total NER]
0	1.2	--			
7	4.9	1.6	2.5	4.1	84.2
14	8.7	2.3	5.5	7.8	90.2
29	17.4	4.9	9.8	14.7	84.8
59	35.5	9.0	20.0	29.0	88.7
120	54.3	12.2	37.7	49.9	92.1
15d sterile	2.8	0.9	1.0	1.9	71.0
120d sterile	3.2	1.3	2.0	3.3	123.6

As already demonstrated for the silylation, procedural recoveries for EDTA extraction were also mostly close to 100%. Only for Isoproturon recovery was slightly lower. As an overall result it can be stated that the mass balance over the EDTA extraction does not indicate significant systematic losses of radioactivity.

4.5.3 EDTA extraction of samples from degradation tests

4.5.3.1 ^{14}C-experiments

EDTA extraction released significant amounts of radioactivity. However, radio-TLC analysis showed that only very low amounts of parent Bromoxynil were released. Except the sterile samples where similar to silylation larger amounts of the NER were recovered as parent test substance by EDTA extraction.

Table 40: Mass balance of the EDTA extraction of samples containing NER from Bromoxynil.

Day	total NER [%aR]	EDTA extract [%aR]	Acetonitrile extract [%aR]	Parent release Sum [%aR]	Parent release [% of NER]
0	3.5	2.3	0.5	0.0	0
7	30.1	10.0	2.0	1.2	4.0
14	49.4	12.5	2.7	0.0	0
27	71.1	14.1	3.2	0.0	0
62	70.3	13.7	3.1	0.0	0
120	65.5	12.8	3.0	0.0	0
14d sterile	7.8	5.8	1.2	1.8	23.1
119d sterile	9.4	11.2	1.5	3.6	38.3

EDTA extraction results are of specific importance since silylation was considered to be invalid because of the sensitivity of Sulfadiazine against silylation. However, the recovered parent Sulfadiazine was still very low. The distribution of radioactivity in the aqueous and the methanol extract indicate that the main reason for the distribution, also in the silylation, is the solvent polarity. Most of the recovered radioactivity consist of hydrophilic polar substances.

Table 41: Mass balance of the EDTA extraction of samples containing NER from Sulfadiazine.

Day	total NER [%aR]	EDTA Extract [%aR]	MeOH after EDTA [%aR]	Parent release, sum, [%aR]	Parent release [% of NER]
0	4.1	1.8	0.3	0.0	0
7	43.5	16.5	1.7	0.8	1.8
14	68.6	22.7	2.3	1.1	1.6
28	72.5	24.6	2.4	0.6	0.8
58	92.4	24.7	2.6	0.6	0.6
121	82.8	23.6	2.7	0.4	0.5
Sterile 14d	44.4	14.4	1.5	3.4	7.7
Sterile 120d	81.4	21.4	2.3	2.2	2.7

As already seen for silylation the parent content in the EDTA extracts were very low. Related to total NER, the parent content released decreased from 6% to zero within the 120 days. Thus, data make sense because there is also a permanent decrease of parent substance in the sample (see Table 16) but all at a very low level.

Table 42: Mass balance of the EDTA extraction of samples containing NER from Isoproturon.

Day	total NER [%aR]	EDTA Extract [%aR]	MeOH/H_2O after EDTA [%aR]	Parent release, sum, [%aR]	Parent release [% of NER]
0	1.2			--	
7	4.9	1.2	0.3	0.3	6.1
14	8.7	1.3	0.4	0.2	2.3
29	17.4	4.2	0.7	0.1	0.6
59	35.5	7.8	1.2	0.1	0.3
120	54.3	10.7	1.4	0.0	0
15d sterile	2.8	0.7	0.3	0.2	7.1
120d sterile	3.2	1.0	0.3	0.5	15.6

4.5.3.2 ^{13}C-experiments and comparison with ^{14}C-experiment

Residues from ^{13}C-experiments were subject to EDTA extraction as well. Again, the chemical analysis was limited to LC-MS analysis of the EDTA extracts for extracted parent. These data can be compared directly with the parent substance recovered in the ^{14}C-experiments.

Table 43: Parent substance release from NER by EDTA extraction of ^{13}C and ^{14}C-test samples.

Day	Bromoxynil		Sulfadiazine		Isoproturon	
	^{13}C EDTA, release of parent in % applied	^{14}C EDTA, release of parent in % applied	^{13}C EDTA, release of parent in % applied	^{14}C EDTA, release of parent in % applied	^{13}C EDTA, release of parent in % applied	^{14}C EDTA, release of parent in % applied
7d	0.4	1.2	1.3	0.8	0.1	0.3
14d	0.5	0.0	1.3	1.1	0.1	0.2
28d	0.4	0.0	1.4	0.6	0.2	0.1
60d	0.2	0.0	1.2	0.6	0.1	0.1
120d	0.2	0.0	1.4	0.4	0.1	0.0
14d sterile	0.3	1.8	2.4	3.4	n.d.	0.2
120d sterile	0.8	3.6	2.5	2.2	n.d.	0.5

Data presented in Table 43 do not show considerable differences. In no case really significant amounts of NER were released as parent test substance. Again, the low amounts of released parent Sulfadiazine is confirmed by the ^{13}C-experiment.

4.5.4 Silylation versus EDTA extraction

The direct comparison of data from silylation and EDTA extraction is based on the ^{14}C-experiments because ^{13}C and ^{14}C experiments show only minor differences as pointed out in detail above. Data from the ^{13}C-experiments are presented in the tables above so it would be easy to compare if required.

In the following two tables the total extracted radioactivity and the total extracted parent substance is compared across the different extraction procedures.

The data show very clear that there are only minor differences between the very different procedures. This is in particular surprising for Sulfadiazine which degrades during silylation. Nevertheless, the amounts of parent Sulfadiazine found in the silylation extracts are higher than in the EDTA extracts. Though everything in the range between 0.1 and 1%, which is assessed to be not relevant for an overall degradation study.

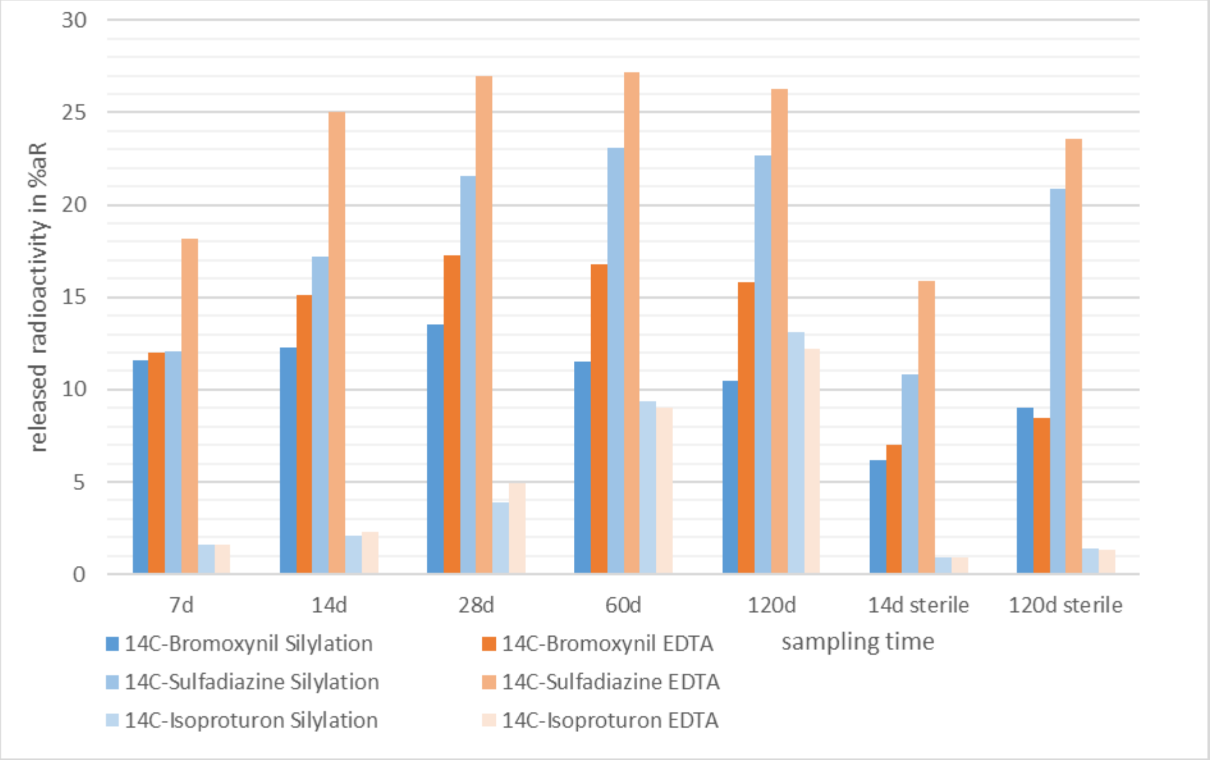
Table 44: Comparison of radioactivity released by silylation and EDTA extraction.

Day	^{14}C-Bromoxynil		^{14}C-Sulfadiazine		^{14}C-Isoproturon	
	Silylation, release of radioactivity [% aR]	EDTA, release of radioactivity [% aR]	Silylation, release of radioactivity [% aR]	EDTA, release of radioactivity [% aR]	Silylation, release of radioactivity [% aR]	EDTA, release of radioactivity [% aR]
7d	11.6	12.0	12.1	18.2	1.6	1.6
14d	12.3	15.1	17.2	25.0	2.1	2.3
28d	13.5	17.3	21.6	27.0	3.9	4.9
60d	11.5	16.8	23.1	27.2	9.4	9.0
120d	10.5	15.8	22.7	26.3	13.1	12.2
14d sterile	6.2	7.0	10.8	15.9	0.9	0.9
120d sterile	9.0	8.5	20.9	23.6	1.4	1.3

Table 45: Comparison of Parent substance released by silylation and EDTA extraction.

Day	^{14}C-Bromoxynil		^{14}C-Sulfadiazine		^{14}C-Isoproturon	
	Silylation, release of parent in % applied	EDTA, release of parent in % applied	Silylation, release of parent in % applied	EDTA, release of parent in % applied	Silylation, release of parent in % applied	EDTA, release of parent in % applied
7d	1.3	1.2	1.2	0.8	0.5	0.3
14d	0.9	0.0	1.2	1.1	0.5	0.2
28d	0.3	0.0	1.2	0.6	0.6	0.1
60d	0.2	0.0	1.8	0.6	0.3	0.1
120d	0.1	0.0	1.2	0.4	0.0	0.0
14d sterile	4.3	1.8	1.5	3.4	n.d.	0.2
120d sterile	4.7	3.6	3.5	2.2	n.d.	0.5

Figure 16: Extracted Radioactivity, Silylation versus EDTA Extraction.



Source: Fraunhofer IME evaluation of project data with MS Excel

The total extracted radioactivity shows a trend that EDTA extraction recovers more radioactivity from most samples as shown in Figure 16. This is in particular observed for Sulfadiazine. However, this trend cannot be confirmed for the extracted parent substances. Again, parent Sulfadiazine, which is proven to be attacked chemically by silylation, is found in slightly higher concentrations in the silylation extract compared to the EDTA extract (see Table 45).

4.6 NER characterisation: acidic hydrolysis

4.6.1 Release of radioactivity by hydrolysis

Hydrolysis also released significant amounts of radiolabels from NER in soils. The trend follows the trend of NER formation, which means that if NER formation slows down release of radioactivity from those NER also slowed down.

Table 46: Radioactivity released by acidic hydrolysis.

Day	^{14}C-Bromoxynil		^{14}C-Sulfadiazine		^{14}C-Isoproturon	
	total NER [%aR]	Hydrolysis extract [% aR]	total NER [%aR]	Hydrolysis extract [% aR]	total NER [%aR]	Hydrolysis extract [% aR]
0d	3.4	1.5	4.1	0.5	1.2	
7d	30.1	6.8	43.5	5.2	4.9	1.3
14d	49.4	9.1	68.6	7.0	8.7	2.2
28d	71.1	11.1	72.5	8.6	17.4	4.3
60d	70.3	11.8	92.4	8.7	35.5	8.5
120d	65.5	11.7	82.8	9.3	54.3	13.8
14d sterile	7.8	3.3	44.4	5.0	2.8	0.6
120d sterile	9.4	4.0	81.4	9.5	3.2	1.1

In order to determine the mass balance for the acidic hydrolysis, all hydrolysis residues were subject to combustion analysis. The mass balance showed a very good recovery in all samples, except Isoproturon 14d sterile. This indicates that at least no massive losses of radioactivity occurred during acidic hydrolysis.

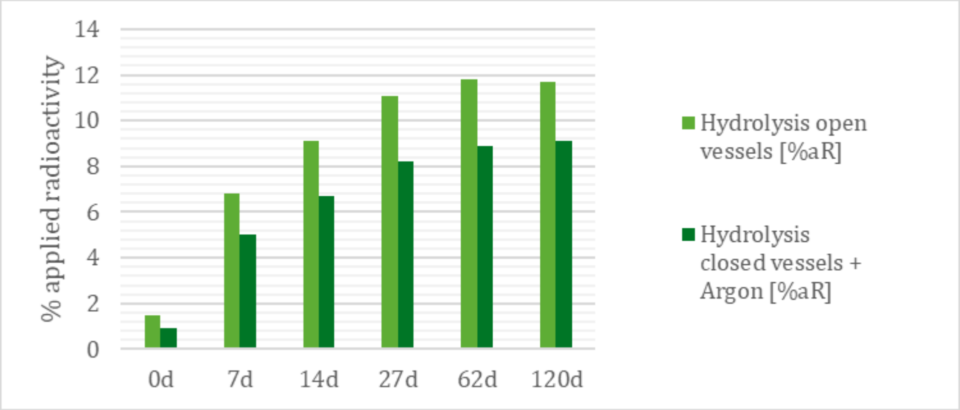
Table 47: Mass balance acidic hydrolyses (procedural recovery).

Day	Bromoxynil [%aR]	Sulfadiazine [%aR]	Isoproturon [%aR]
0d	120.9	98.0	
7d	117.3	116.7	105.3
14d	106.1	105.4	101.4
28d	118.5	121.0	99.0
60d	121.7	98.7	97.8
120d	119.8	117.4	101.7
14d sterile	101.9	99.9	77.3
120d sterile	169.3	109.4	128.3

In order to compare values from hydrolysis in open and closed test vessels under Argon, the hydrolysis of Bromoxynil was repeated in closed vessels. The initial hydrolysis of Bromoxynil was conducted in open vessels because it was communicated only later that this could influence hydrolysis results. All other hydrolyses were conducted in closed vessels with Argon as inert gas. As shown in Figure 17 the amount of radioactivity released is higher in open vessels hydrolysis then in closed vessel hydrolysis under Argon.

Figure 17: Comparison hydrolysis in open vessels and in closed vessels

Source: Fraunhofer IME evaluation of project data with MS Excel



Though the quantity of released radioactivity did not improve by closed vessel hydrolysis, question was to answer if the inert gas has an advantage in terms of recovery of amino acids (avoiding oxidation reactions), which are the final target for the hydrolysis procedure.

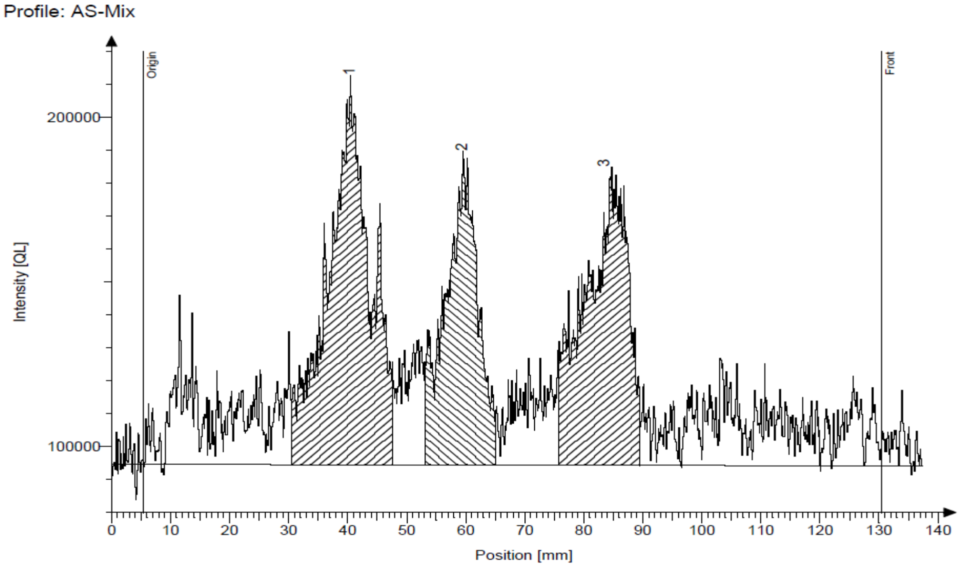
Trials with 2.5g soil spiked with 39.2 kBq of a ^{14}C-labelled amino acid standard mix were hydrolysed in closed vessel at 110°C for 22 hours with and without Argon atmosphere. Recovery of radioactivity was 32.6% without Argon atmosphere but 53.1% with Argon.

4.6.2 Quantification of ^{14}C-amino acids by radio-TLC

Analysis showed very low signals on radio-TLC. Only for Bromoxynil samples any ^{14}C-amino acids could be quantified by radio-TLC, but all below 0.5% aR. In none of the samples of the other substances any amounts above 0.1%aR, which represent the limit of determination, were found.

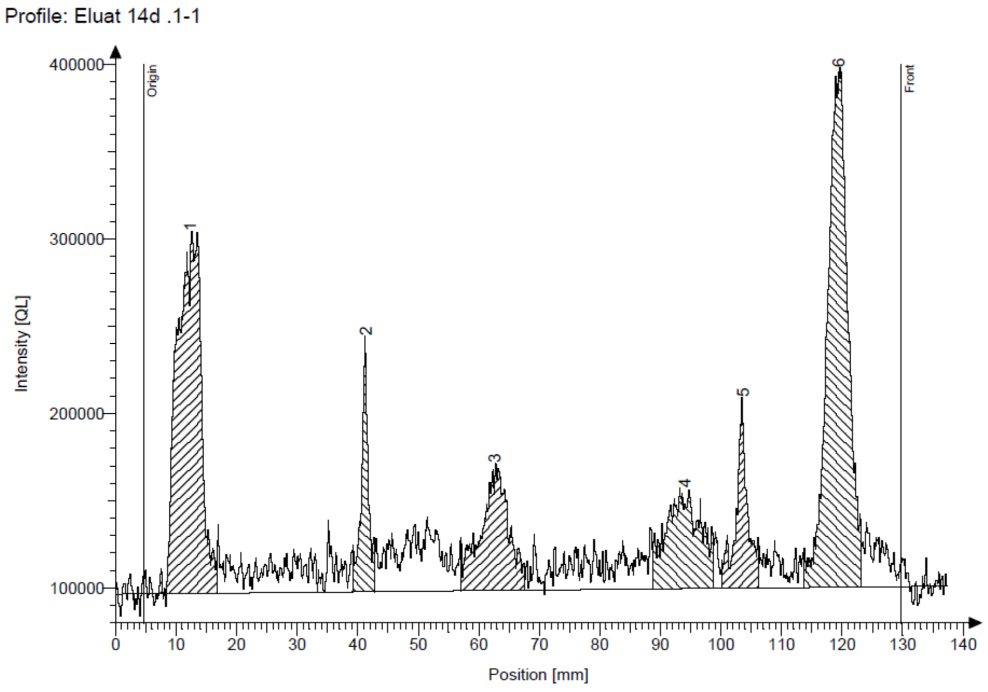
Figure 18 shows a radio-TLC-chromatogram of the ^{14}C-amino acid standard mix (ARC0474). The 15 different amino acids of the mixture form three isolated signals. In the chromatogram of the sample (Bromoxynil, 14d) after SPE clean-up at least two of the signals detected (peak No 2 and 3) can be assigned to the amino acid mixture.

Figure 18: Radio-TLC chromatogram of ^{14}C-L amino acid standard mix.



Source: Fraunhofer IME evaluation of project data with AIDA software

Figure 19: Radio-TLC chromatogram of 14d sample eluent after work up.



Source: Fraunhofer IME evaluation of project data with AIDA software

However, as the results of this analysis were much lower than expected, there was some doubt about the reliability of the analysis by one-dimensional TLC. In addition, the clean-up procedure and the subsequent two-dimensional TLC are quite laborious and time consuming (see Possberg et al., 2016) and is not justified in relation to the results obtained which were at 0.5% aR far below the precision of a common NER determination. Thus, it was decided to skip the final radio-TLC analysis and take the values from the Dowex eluent as indicator for ^{14}C-labelled biomass. Later those data were compared with the results of the MTB model calculation and based on the results a factor was derived to estimate the type III bioNER based on the radioactivity determined in the Dowex eluent of the hydrolysis extract. Finally, the data of the Dowex eluent shown in Table 48 more or less represent the released radioactivity presented in Table 46.

Table 48: Radioactivity in Dowex eluent of acidic extract.

Day	Bromoxynil [%aR]	Sulfadiazine [%aR]	Isoproturon [%aR]
7d	2.4	3.0	0.5
14d	3.5	4.4	0.8
28d	3.4	4.9	1.5
60d	3.6	5.4	3.7
120d	3.1	7.0	5.3
14d sterile	2.0	2.2	0.2
120d sterile	2.7	3.6	0.4

4.7 Calculation of bioNER (type III NER) using the MTB model

Table 49 shows the calculated yields and the measured CO_2-release used as input data to the MTB-bioNER estimation. Moreover, it shows the calculated high MTB-bioNER (eq. 2) and the low MTB-bioNER (eq. 3). The CO_2 corresponds to the measured data at day 120.

Table 49: Results of MTB calculations

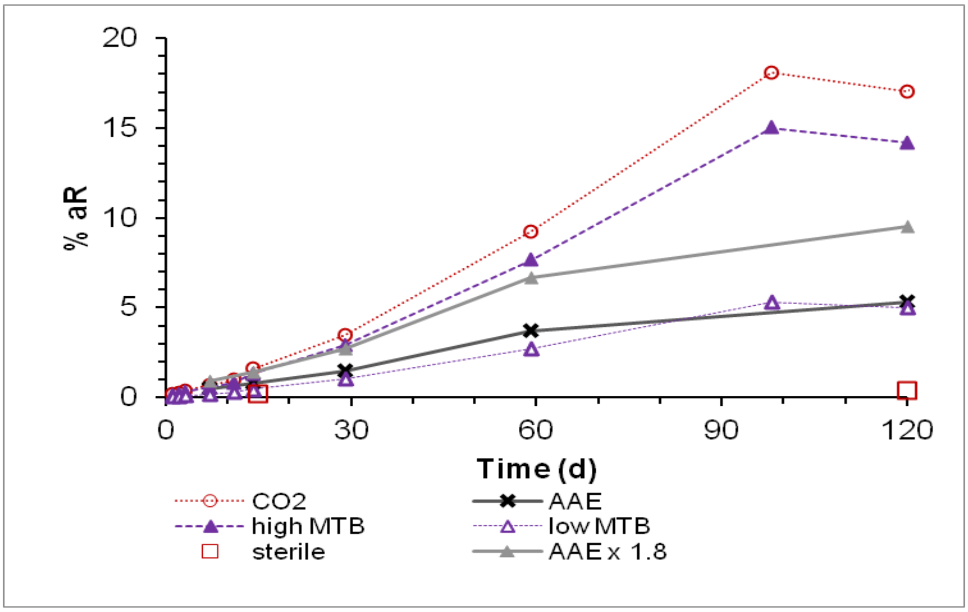
	day	CO_2 %aR	Yield gC/gC	low MTB-bioNER %aR	high MTB-bioNER %aR	measured in Dowex eluent %aR
Bromoxynil	120	28.8	0.164	2.6	5.7	3.1
Sulfadiazine	121	1.7	0.36	0.4	0.9	7.0
Isoproturon	120	17.0	0.46	5	14	5.3

BioNER formation can be calculated for each sampling time from the released ^{14}C. The relation between measured and predicted bioNER over time is shown in Figure 20 for the substance Isoproturon. All data is given in the unit % of applied radioactivity (% aR). The hydrolysis extract after Dowex column clean-up is named "amino acid extract" AAE. Biomass consists only to 50 - 55% of amino acids, and the measured %aR in AAE was multiplied with a factor b = 1.8 to derive a proxy for the total measured bioNER. Moreover, figure 18 shows the calculation result for low and high MTB, along with measured CO_2 and the measured AAE in sterile samples at five consecutive sampling times (7, 14, 29, 59 and 120 days) for the degradation study with ^{14}C-isoproturon. It can be seen that the ratio of measured CO_2 to AAE is continuously increasing over time, from 1.4 at day 7 to 3.2 at day 120. This is consistent with the process of biomass turnover leading to up-concentration of amino acids and release of new CO_2. In sterile samples, very little AAE was found (≤ 0.4% aR). The calculated low MTB-bioNER is very close to the measured AAE for all five samples. Multiplying AAE by a factor 1.8 gives values above but close to the high MTB until day 14, later on approaching low MTB. Assuming that high MTB describes the living biomass without any decay or death, it makes sense to have an initial factor close to 2 on amino acids to derive the real bioNER. Later on, a lower factor is more realistic. The value of AAE x 1.8 lies most of the time between low and high MTB and can thus be considered a “reasonable average factor on tAA to derive real bioNER for most of the time” (Trapp et al., 2022) in the case of this isoproturon degradation experiment.

Figure 21 shows the measured radiolabel (% aR) in the cleaned column extract and CO_2 and column extract in sterile samples (%aR) of **bromoxynil** at five consecutive sampling times (7, 14, 27, 62 and 120 days). Already in the first sample at t = 7d, measured AAE is rather high (2.4% and 2.5% of aR). At the second sample, t = 14 d, 3.4% and 3.6% were found in AAE. Also, in sterile controls, a similar amount (1.7% and 2.4% aR) is located in AAE. The label in AAE remains at this level over all sampling times, and is similar in sterile probes at t = 120 d. This pattern is different from the measured CO_2 and the calculated MTB-bioNER, which increase with time. Thus, even though there is a good numerical agreement between calculated MTB-bioNER and measured AAE at t = 120 d, with measured AAE in between lower and higher MTB, there seems to be a disturbance of the measurement that leads to a high background.

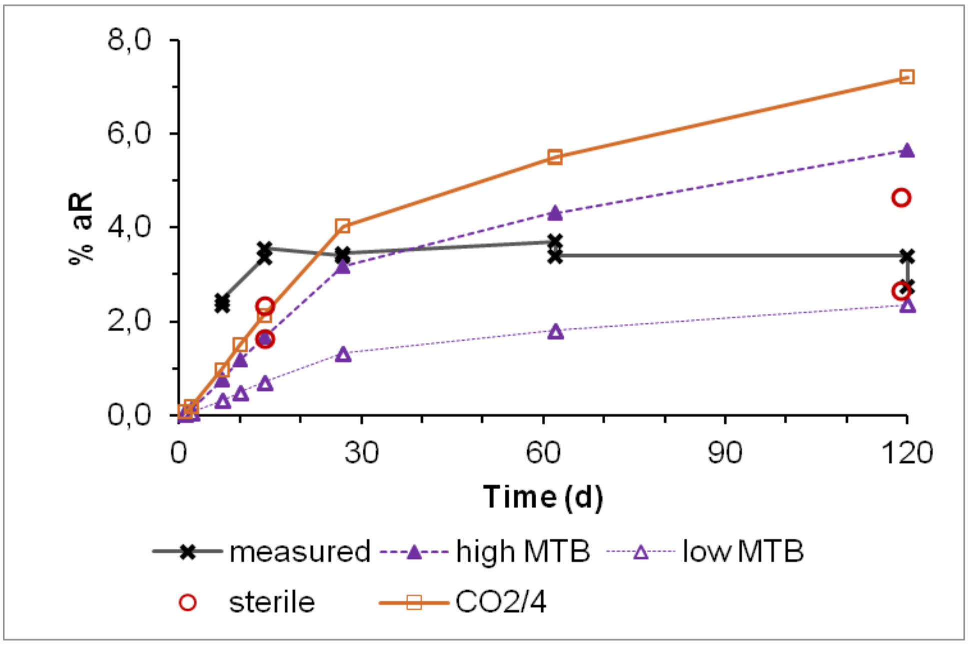
A similar pattern occurred for the compound sulfadiazine. Here, measured AAE was consistently high, up to 7.0% aR at t = 120 d, and also in sterile controls (3.6% at t = 120 d), despite very low CO_2-development (< 2% at t = 120 d). The result cannot be considered valid (not shown).

Figure 20: **Measured %aR in cleaned-up column extract (“amino acid extract AAE”) and CO_2 (%aR), AAE in sterile samples (%aR), AAE times factor b = 1.8, in comparison to lower and higher calculated MTB-bioNER for isoproturon at five consecutive sampling times (7, 14, 29, 59 and 120 days).**



Source: DTU Copenhagen, evaluation of project data

Figure 21: **Measured label in cleaned-up column extract (measured %aR) and CO_2 (%aR, divided by 4 for scaling), column extract of sterile samples (sterile, %aR) in comparison to lower and higher calculated MTB-bioNER for bromoxynil at five consecutive sampling times (7, 14, 27, 62 and 120 days). Replicate samples are shown (n=2).**



Source: DTU Copenhagen, evaluation of project data

5 Discussions and conclusion

5.1 Results of degradation tests and NER characterisation

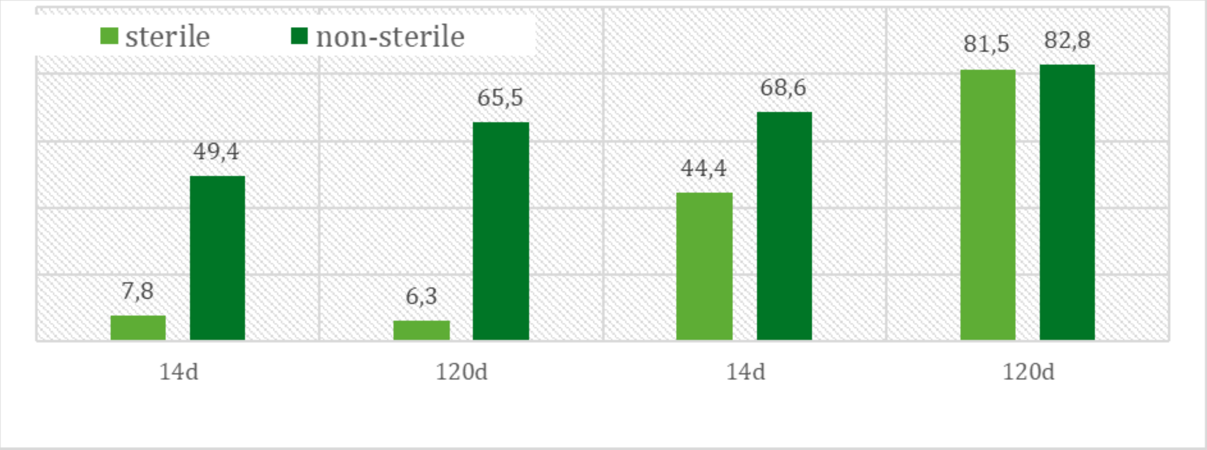
5.1.1 Mass balance and degradation rates

From the results of the degradation test as presented in section 4.2.1 it can be stated that all degradation tests fulfil the criteria of OECD 307 with mass balances between 90% and 110% of the applied ^{14}C radioactivity.

Nevertheless, the degradation rate obtained for Isoproturon was not in line with data from literature (EU dossier lab studies, reporting range of DT_{50} 7.2-18.2 days, DT_{90} = 23.8-111.1 days in lab experiments, and in field studies DT_{50} 12-33 days, DT_{90} range 34-68 days; PPDB: Pesticide Properties DataBase, (Lewis, et al., 2016)). A reason for that finding could not be found but was not on the focus of the project, either. It might be speculated that the high initial dosing of 4 mg/kg slowed the degradation. Usually the doses in OECD 307 tests are at 1 mg/kg or below and in the ^{13}C-experiment at 40 mg/kg initial dose degradation was significantly slower than at 4 mg/kg. But that cannot be scientifically proven on the basis of the data collected. In the end the main objective of the degradation test, to generate samples containing ^{14}C-labelled NER from a standardised approach, was achieved for all test substances selected.

For process understanding, it was important to also run sterile samples. As Figure 22 shows, NER formation processes were very different e.g. for Bromoxynil and Sulfadiazine. While for Bromoxynil NER formation was mainly driven by biological processes, NER were formed abiotically in Sulfadiazine experiments. Another indication for that conclusion is the very low mineralisation observed in the Sulfadiazine experiments, which is in line with literature data.

Figure 22: **NER formation in sterile and non-sterile samples in [%aR].**



Source: Fraunhofer IME evaluation of project data with MS Excel

5.1.2 Proposed NER definition

The three extraction procedures applied did not give a definite answer on the best extraction method. In fact, the standard solvent mix performed very good and for Sulfadiazine much better than the other procedures. Also, the substance specific shaking extraction followed by PLE with the same solvent produced good results. As it is anyway integral part of an OECD 307 study to develop an appropriate extraction method for the parent test substance from soil, it seems most straightforward to use this approach also for NER definition.

Though for difficult to extract substances, the standard solvent mix can work better as demonstrated for Sulfadiazin, it might also show huge variations, as shown here for Isoproturon. This cannot be predicted and as it is not possible to change the extraction method from sampling to sampling, we recommend substance specific extraction (shaking or other technique) followed by PLE with the substance specific solvent or solvent mixture. Recoveries from this method did not show significant outliers over the three test substances.

5.1.3 Type I NER determination

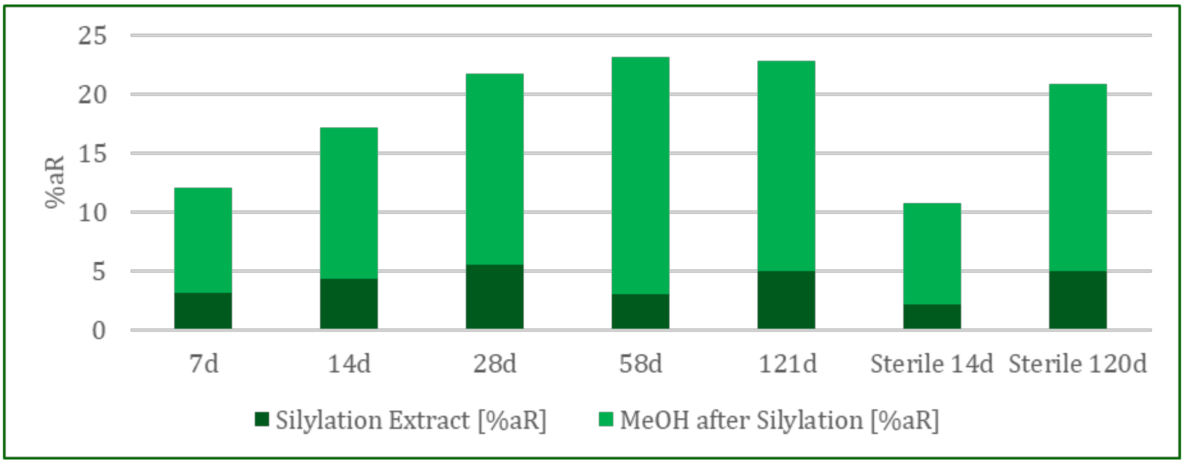
From all NER types the strongly sorbed and physically entrapped type I NER, which represent the potentially remobilisable NER fraction, are of most concern (Kästner et al., 2018, Loeffler et al., 2020, UBA Texte 133/2022). Two different methods were tested in the project to estimate type I NER.

Silylation is supposed to be a difficult to apply procedure with dangerous chemicals that require an inert atmosphere for the reaction. However, once the technical staff got familiar with the procedure, silylation can be assessed as a normal procedure in routine chemical analysis. For trained technical staff this is no challenge, the special equipment needed is very minor (see 3.5.1).

To analyse the reproducibility of the method, the recoveries of radioactivity of both replicate samples are compared using the Mann-Whitney-U-Test (also known as Wilcoxon rank-sum test). Calculation was performed using the R function wilcox.test (R-Core-Team, 2020). There is no significant difference between both replicate samples (p-value 0.78 > 0.05). Thus, the two distributions are stochastically equivalent and reproducibility of the method is confirmed. In addition the variation of both replicate samples is similar (Levene test, F(1, 82) = 0.014, p = 0.91 > 0.05) (Fox, 2019). The data used for this analysis and results of the statistical evaluation are summerized in Appendix E.

A clear disadvantage of silylation is that it is not applicable for every test substance as the reagent may react chemically with the test substance. Thus, stability of the parent test substance against silylation must be checked first. The best way for checking stability is to silylate soil that has been spiked with the parent test substance before. This pre-experiment will also show, if the test substance, even if it will be released from the matrix, will be dissolved by the silylation solvent chloroform. If not, it might be necessary to do a second extraction of the silylation residue. In the project this was necessary e.g. for Sulfadiazine as shown in Figure 23.

Figure 23: Radioactivity released by silylation of sulfadiazine samples.



Source: Fraunhofer IME evaluation of project data with MS Excel

The silylation extract (chloroform) itself is easy to handle and to process for further analysis like e.g. radio-TLC. Details on the silylation procedure applicable under routine laboratory conditions are published as a result of this project (Hennecke et al., 2023).

The EDTA-extraction is easy applicable, does not need any special equipment and is supposed not to attack the test substance chemically. So, generally fit for routine testing. It was impressive how much NER could be released by an aqueous extraction only with the addition of a chelating agent, considering which extraction process the samples had previously been subjected to. Figure 16 shows that the extracted radioactivity by EDTA was higher than by silylation.

Surprisingly, the stability check with spiked blank soil showed that the substances, in particular Sulfadiazine were by no means stable during EDTA extraction. So, this has to be verified before applying EDTA extraction. Otherwise the released parent might be underestimated.

However, like for the silylation also the EDTA solvent has to be checked for solubility of the test substance, because it is an aqueous solution and substances like Bromoxynil will not dissolve in that solvent even if it is released from the matrix.

If the test substance is hydrophilic like Sulfadiazine, problems might occur with the high salt load in subsequent analysis. The salt is not easy to remove and it will limit the volume of extract that can be applied on radio-TLC, but also in LC-MS high salt loads are difficult to deal with, as the salt may accumulate in the detector.

Another concern is that EDTA is known for co-extraction of biological material (proteins, amino acids, phospholipids, nucleic acids). Thus, increased levels of type III NER (bioNER) could be extracted. Differentiation of type I NER and type III NER required advanced chemical analysis. However, since none of the selected test substances showed formation of significant amounts of type III NER, this was no subject of concern in the project. Differentiation of bioNER as part of NER type I is a matter of further research.

Chemical analysis of the extracts resulted in both cases, silylation and EDTA extract, in very low amounts of released parent substance. As shown in

Table 45 the amount of parent substance was very slightly higher in the silylation extract. These results of LSC and radio-TLC analysis of silylation and EDTA extraction were confirmed by the LC-MS analysis of the ^{13}C experiments.

From the experience of this project we would give the following recommendation for the determination of type I NER:

Table 50: Recommendation for standard type I NER characterisation method.

	Silylation	EDTA Extraction
NER type I and III differentiation	suitable (chem. analysis)	suitable (chem. analysis)
Applicability, standardisation	advanced	easy
Fit for laboratory routine	yes	yes
Extraction of type III NER	low (non-polar solvent)	established to extract proteins, nucleic acids
Second extraction required	required for hydrophilic substances	for most substances recommended
Extract processing	standard lab work	difficult due to salt load
Substance stability	proof for stability required	proof for stability required

5.1.4 Type III NER (bioNER) determination

The acidic hydrolysis with 6 M HCl is in principle easy to perform. The problem in the procedure is that we do not know a specific laboratory device for hydrolysis at 105°C with 6 M HCl in closed vessels over 24 hours. Microwave digestion systems allow in principle heating in closed containers, but the time limit is below 24 hours in our systems. Heating in closed Schott bottles without a containment in a drying oven does not comply with laboratory safety rules. If a bottle breaks under those conditions, the hot strong acid might damage the oven and is a risk for the lab staff. This can be avoided by using a safety containment, for example a stainless-steel box. Heating in an autoclave seemed to be the safest procedure because there would be no pressure difference inside and outside the bottles. But the producers of the autoclaves strongly recommend not to use the instrument with concentrated acid inside. At this point we do not want to encourage to use drying oven or autoclave to perform the acidic hydrolysis in closed vessels and we strongly recommend to follow the instructions of the instrument providers who clearly stated that those instruments are not suitable for work with mineral acids.

Following this recommendation, there is still the question in what kind of device the hydrolysis should be carried out. We used in parallel a Kjehldahl digestion system with open vessels for hydrolysis to compare with the closed bottles digestion. Figure 17 demonstrate that the recovered radioactivity in open vessels were even better than in closed vessels and the system is well known in laboratory routine. However, there is still an argument for the closed vessels with inert atmosphere, as the recovery of a spiked ^{14}C-aminoacid mixture was significantly better in the closed vessel procedure. Recovery of radioactivity was 32.6% without Argon atmosphere but 53.1% with Argon (see 4.6). However, this can be recommended only, if a respective system is available that allows performance of the hydrolysis in a safe way.

In general, as shown in Table 47, the mass balance does not indicate significant losses of radioactivity during the hydrolysis step.

The most challenging task is the chemical analysis of the ^{14}C-amino acids as described in Possberg et al., 2016. As we worked with ^{14}C-radiolabelled compounds, radio-TLC was the analytical method of choice. For optimisation of the sample work up and the following radio-TLC analysis, a bachelor thesis was prepared in addition to this project (Kleine-Birkenheuer, 2020). Hydrolysis extracts are highly loaded with matrix and direct LC-MS analysis without sample clean-up is impossible. In fact, as the extract will also contain non-radiolabelled amino acids released from the soil matrix, a high resolution LC-MS would be necessary, to analyse for ^{14}C-amino acids. For routine laboratory work this seems not proportionate.

The radio-TLC was in principle capable to detect ^{14}C-amino acids but the levels of ^{14}C-amino acids detected were very low. In no extract more than 0.5% aR can be assigned to ^{14}C-amino acids. And even these 0.5% are not proven to be amino acids since radio-TLC is rather unspecific in this case. Two-dimensional TLC will provide a better resolution of the individual amino acids than the here performed 1D-TLC (Possberg et al., 2016). Thus, it was decided to skip the final radio-TLC analysis because it means high work effort and the identity of the ^{14}C-amino acids spots (in 2D-TLC) would have to be proven by GC-MS / LC-MS. Instead the values from the Dowex eluent as indicator for ^{14}C-labelled biomass was used. We are aware that this parameter is not more specific than amino acids by radio TLC, but as an indicator for the amount of bioNER it might be useful as well. A good correlation of those data with calculated bioNER by the MTB model could be established.

On the other hand, it was surprising that Sulfadiazine samples showed the highest amount of radioactivity in the Dowex eluent. Theoretically, Sulfadiazine samples should show the lowest amounts of bioNER, if mineralisation correlates with bioNER formation. For Sulfadiazine almost no mineralisation was observed and NER formation in the sterile samples was in the same magnitude as in the non-sterile samples. This is reflected somehow in the total released radioactivity relative to the NER formed. This is lowest for Sulfadiazine among the three test substances. However, in the Dowex eluent (Table 48) this is not reflected any more, which is not in line with MTB estimations (see 5.4), either.

The results show that the experimental determination of type III NER (bioNER) still needs some research. It might work properly in research projects using highly sophisticated analytical methods (Possberg et al., 2016). But this is not applicable in laboratory routine and alternative parameter as described above are not yet fit for laboratory routine. Meanwhile the MTB estimation could be used as easy to apply alternative to estimate bioNER.

5.2 Comparison of experiments with ^{14}C- and ^{13}C-labelled test substances

For the ^{13}C-experiments no full mass balances were obtained as this requires a specific instrumentation that is not available at Fraunhofer IME. Nevertheless, from chemical analysis of the parent test substances, experiments can be compared.

The degradation kinetics for the ^{13}C-test substances was significantly lower than for the ^{14}C-test substances as shown in Table 22. It is obvious that this is due to the 10-fold higher starting concentrations of 40 mg/kg for the ^{13}C test substances that might slow down the biological activity of the test soils and thus the degradation rate. In absolute numbers more substance has been degraded during the same time in the ^{13}C-experiment compared to the ^{14}C-experiment.

NER formation, which was investigated for Bromoxynil and Sulfadiazine only due to the very low disappearance of ^{13}C-Isoproturon, followed the same trend in ^{13}C- and ^{14}C-experiments. However, since in the ^{13}C-experiments degradation rate was lower, NER formation of Isoproturon did not reach the maximum within 120 days of incubation. In ^{14}C-experiments the maximum was reached within the 120 days window and after that NER slightly decreased again (see Figure 14 and Figure 15).

NER characterisation was based on LC-MS analyses of the silylation and EDTA extracts. Compared to the ^{14}C-data no significant differences were obtained.

It can be concluded that a degradation experiment with an increase of the starting concentration by one order of magnitude might lead to a lower degradation rate. This is in line with literature data on Bromoxynil (Novak et al, 2018). However, from our results there is no indication that the basic processes are significantly different from degradation at lower starting concentrations with regard to NER formation and NER characteristics (see Table 43). Transfer of research results from either label should be possible.

5.3 Applicability of the experimental procedures in routine testing

As described above, special focus of the project was on the applicability of the procedures in routine testing. For the extraction methods for NER definition this is easy to answer since all methods applied are already integral part of standard degradation studies. This includes PLE which is probably not applied in all current degradation studies but nowadays can be considered as standard instrumentation in a laboratory dealing with regulatory degradation studies.

The silylation process also lost its initial scare in the course of the project and turned out to be easy to apply once the respective work place is built and the technical staff gets some experience. Care has to be taken for the use of hazardous chemicals but this is common for other laboratory procedures and there are no increased concerns about work safety conducting silylation in a standard laboratory. The resulting extracts are clean solutions which are easy to prepare for subsequent chemical analyses.

For EDTA extraction it is exactly the other way around: the chemicals are not critical at all and the extraction is very simple. But extract work up for subsequent analysis might become more challenging due to the high salt load. Generally, both silylation and EDTA extraction are fit for routine use in the laboratory.

A suggestion for a further alternative method (pyrophosphate extraction) was discussed during the workshop (see 5.5) This is currently investigated on the present sample set as part of a Bachelor thesis at Fraunhofer IME.

The acidic hydrolysis is more difficult to assess. Generally, it is limited by the missing standard instrument that allows extraction in closed vessels with concentrated acid under pressure conditions for 24 hours. However, it should be possible to solve this problem if necessary. There are more concerns about the complicated and time-consuming clean-up of the extract followed by radio-TLC which is one further step taking weeks depending on the radioactivity recovered. Such time-consuming analysis are not considered appropriate for a routine study. In particular as it does not deliver clear data on bioNER but just an “indication”. An option, HR-LC-MS analytics, is not available in all laboratories. Considering our experiences, we conclude that the experimental determination of bioNER needs further development and is not assessed to be fit for current lab routine.

5.4 Estimation of bioNER with the MTB method

The radioactivity in the cleaned-up Dowex extract (AAE, "amino acid extract") consists mostly of amino acids and can be used as an analytical proxy for the formation of bioNER. Of the three test compounds, the agreement between measurement and MTB bioNER estimation was best for Isoproturon. The calculated low MTB, which represents the amino acids, was for all sampling times close to the measured result (Figure 20). Multiplying AAE with a factor of 1.8 gives the total bioNER, and the value was for all sampling times ≥ 29 days within the range of low and high MTB estimate. Moreover, the %aR in AAE of the sterile samples was negligible. Good agreement was also seen for the bromoxynil MTB estimations. However, there, the measurements also showed a high background in sterile probes. No agreement was achieved for sulfadiazine, where the measurements gave the highest %aR in AAE of all three substances, while the MTB estimate, due to negligible CO_2-formation, yielded very low estimates for bioNER. For sulfadiazine, sterile samples also had high radioactivity in AAE, which makes the measurements doubtful. In fact, in this comparison of estimates versus measured data, the estimated values seem more reliable than the measurements. More efforts should be directed into the comparison of bioNER estimates with empirical data before final conclusions are drawn.

Principally, several effects can lead to deviations between theoretical, estimated bioNER, and real bioNER formation in experiments. Some of those have been listed in Kästner et al (2018), and we repeat here the potential shortcomings:

Deviations from the predicted range of bioNER may occur

- if the degradation is incomplete, i.e., transformation/degradation products accumulate and are not considered in the calculation.
- if the degradation is (partly or fully) anaerobic, and methane (CH_4) is formed instead of CO_2; for nitrate and sulfate as the electron acceptors, the yield is lower due to lower Gibbs energy of the reaction (Brock et al., 2017).
- if there is significant storage of carbon within microbes, e.g., in form of carbohydrates, poly-ß-hydroxy acids, or polyphosphates. In this case, the release of CO_2 by mineralisation is delayed. The equations for the bioNER assume, however, immediate release of CO_2. In consequence, the true bioNER may be higher than predicted from released CO_2 and potential yields.
- if the natural inoculum does not contain microbes with enzymes for efficient and complete mineralisation of the substrate, the resulting experimental data may differ from the theoretical result.
- If the substrate is toxic to microbes, or inhibits enzyme reactions (McCarty 2007, Rein et al., 2016), the yield can be lower than expected.
- if the substrate contains more N or P than typically present in microbial biomass, the microorganisms may shift they degradation pathways from productive degradation with growth towards prevention of N or P overflow as found for glyphosate with the AMPA formation (Brock et al., 2019).

In degradation experiments, any of these limitations may occur, but it may not be noticed and it is difficult to proof that it occurred. Nonetheless, if there are large deviations between estimated yield or bioNER and experimentally determined values, it may be due to unrealistic assumptions, but may also indicate problems of the experimental set-up.

5.5 International workshop

On February 17 and 18, 2021, an international online workshop entitled “Proposal to standardize the analysis and persistence assessment of non-extractable residues (NER)” took place. It was organized by the German Environment Agency and the Fraunhofer IME.

More than 70 participants (Annex 1 'Participants') from authorities, industry and science, including members of ECHA's PBT Expert Group, discussed the future consideration of NER in persistence assessment. As a basis for the discussion, the ECHA discussion paper for NER assessment (Kästner et al., 2018) was presented by the authors, as well as the results of the current UBA research project "Consideration of non-extractable residues (NER) in the PBT assessment" (FKZ 3718 65 407 0).

The aim of the workshop was to present and discuss a practical approach for a harmonised test procedure for NER characterisation. In this discussion, the industry's point of view was an important indicator for determining the acceptance and practical applicability of the proposed approach. In addition, regarding the effects of NER characterisation on the PBT assessment, the contributions of the regulators were necessary in order to get a reliable statement.

Questions raised during the workshop related to details of the practical laboratory procedure and to suggestions regarding additions or modifications to the presented procedure. Moreover, the regulatory context was discussed. On the second day, the questions focused more on the chemical analysis of silylation extracts. Most laboratories have no experience with this extraction method and the extracts have the reputation of being difficult to analyse. This was rebutted by the results of the current research project. In cases where the test substance is sensitive to silylation, EDTA extraction offers an alternative procedure. A suggestion for a further alternative method (pyrophosphate extraction) was discussed during the workshop.

The two main topics "laboratory practice" and "NER in persistency assessment" were discussed in more detail in breakout groups.

Based on the results of the first day, two flow charts were drafted which present ways of how to consider NER in the persistence assessment (see 5.6). This was explained using a practical example with data from the research project. Both flow charts represent a stepped approach with increasing laboratory effort in each step, but at the same time a higher degree of reality or decreasing conservatism.

It was concluded during the workshop that, e.g. PLE should be the final step in a stepwise extraction procedure. Further conclusions were that guidance is needed how to proceed in a stepwise approach for NER characterisation and persistence assessment. In addition, the practical determination of "bioNER" (type III NER) was found to be not fit for routine practice. However, a modelling approach (MTB) is proposed which should give a good estimate of bioNER. For more details on the workshop see https://www.umweltbundesamt.de/en/topics/chemicals/reach-what-is-it/non-extractable-residues-in-persistence-assessment#what-are-non-extractable-residues

5.6 Proposals for consideration of NER in Persistence assessment

We presented a method on how to experimentally characterise NER fractions that are formed during a soil degradation study. What is still missing is an instruction how to use the information obtained by those methods for persistency assessment.

We hereby present those two proposals, which also have been presented and discussed on the mentioned international NER workshop, for further discussion to determine the relevant fractions of non-extractable residues and to consider them for the derivation of half-lives in the framework of persistency assessment (PBT/ vPvB) of these substances. The approaches are in line with the expectation of ECHA for the handling of NER in the assessment of persistence.

Nevertheless, the final regulatory decision about persistency of a substance or non-persistency is based on the PBT/ vPvB criteria according to Annex XIII of the REACH regulation (ECHA R.11, 2017).

5.6.1 Proposal 1

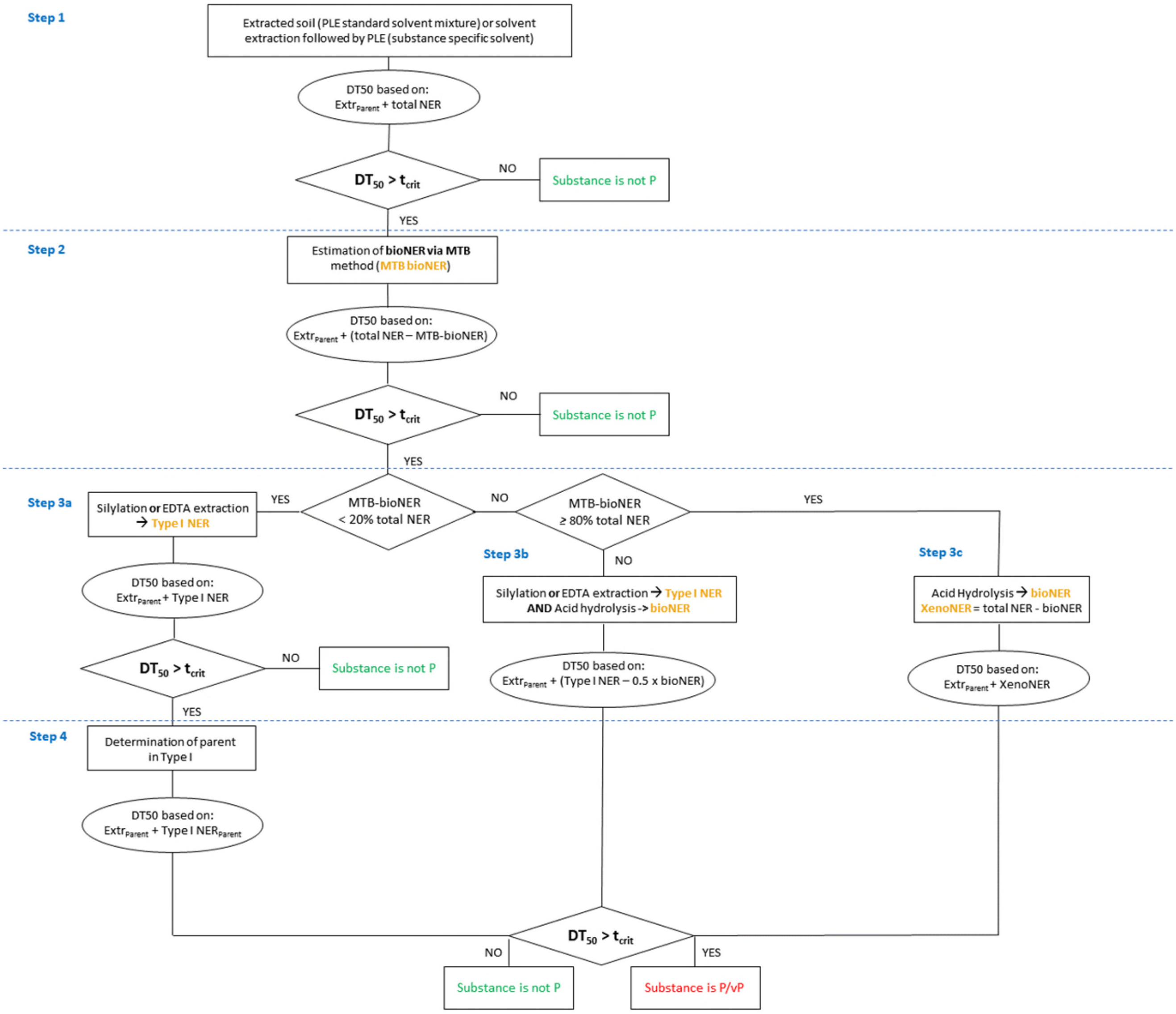
Both proposals show a common procedure for the first extraction steps for total NER quantification using either pressurized liquid extraction (PLE) with a standard solvent mixture (MeOH, acetone, water 50/25/25 at 100°C and 100 bars if the instrumentation allows) or conducting a solvent extraction followed by PLE with a substance specific solvent or solvent mixture (depending on the efficiency of the two extraction procedures). This first step is able to distinguish between extractable residues („Solvent+PLE extractable (Extr.)") and total non-extractable residues („Total NER") by definition.

After this first step, the two proposed approaches differ in their strategy to refine the persistence assessment with respect to the relevant NER fraction.

Proposal 1 (Figure 24) represents a 'worst-case approach', which focuses mainly on the decision persistent/very persistent (P/vP) or not persistent (not P) with less relevance of the specific calculated DT_{50} values as these are not needed for a further risk assessment.

The idea of the flowchart is a stepwise approach with increased data requirements at each consecutive step resulting in improvement of the reliability of the assessment. NER characterization is only necessary in case steps 1 and 2 do not result in classification as non-persistent.

Figure 24: Flow chart for consideration of NER in Persistence assessment, proposal 1.



Source: https://www.umweltbundesamt.de/sites/default/files/medien/362/dokumente/proposal_1_final.pdf

In the first most conservative step (**Step 1**) it is assumed that all non-extractable residues (total NER) consist of unchanged parent test substance, which can be released over time. In this way, the worst-case half-life of the substance is evaluated by considering extracted and total non-extracted residues in the derivation of half-lives through kinetic evaluation ([DT_{50} based on: Extr. + Total NER)]. If at this stage it can already be proven that a substance is not persistent, then the persistence assessment can be stopped and no further extraction steps are needed. If proven otherwise a refinement is needed, i.e. if half-life is higher than the trigger value for persistency ($DT_{50} > t_{crit}$) which is the case when soil t_{crit} = 120 days (P) and 180 days (vP), then a refinement is needed.

As a first refinement **(Step 2**), the presented proposal considers the fact that the degraded test substance is used for growth of biomass and forms biogenic NER (bioNER). The latter can be considered as a safe sink as it poses no risk to the environment and should not be considered in the derivation of the DT_{50} of test substances. bioNER can be estimated by the MTB Model („Microbial Turnover to Biomass"-Model, Trapp et al., 2022) using, amongst others, the experimental mineralisation data of the soil degradation test. In this step, no additional experimental work on NER is required but only a calculation is conducted. In fact, to the experimentally determined total NER measured in the first step, the bioNER fraction can be subtracted. The remaining NER fraction is still assumed to consist of 100% potentially available parent test substance. The sum of extractable parent and total NER reduced by the amount of bioNER estimated by the MTB model is used for DT_{50} evaluation ([DT_{50} based on extr. + (Total NER – MTB-bioNER)]. If the calculated DT50 under this assumption does not exceed the P-trigger ($DT_{50} < t_{crit}$), the test substance is considered not persistent and no further refinement is needed.

If, after bioNER estimation by the MTB calculation, the DT_{50} of the test substance still exceeds the P trigger value, then in Step 3 experimental characterisation of NER is required. The soil containing NER after the first extraction (Extr.) should then be further extracted by silylation or EDTA extraction (depending on the substance properties and the feasibility for the respective substance) in order to obtain a fraction of potentially remobilisable NER (type I NER) (Step 3a). This fraction can be summed up as the extractable parent obtained in the first step for deriving a refined DT_{50} (DT_{50} based on extr. + Type I NER). The strongly adsorbed, covalently bound NER (type II NER) can instead be omitted when deriving the half-lives, since this fraction is assumed to be irreversibly bound and will not become available, even after many years. If still after this step DT_{50} is above t_{crit}, it is strongly recommended to conduct a chemical analysis of the extracted type I NER (Step 4), to differentiate between released parent test substance and other potential molecules carrying the isotope label which were released from the soil matrix. This is the least conservative step but also needs the maximum amount of additional laboratory effort. The sum of released parent substance by silylation together with the extracted parent will be used for the DT_{50} calculation (DT_{50} based on extr. + Type I NER Parent). Depending on the amount of estimated bioNER with the MTB Model (% of MTB-bioNER in the total NER) the choice whether to conduct silylation/EDTA extraction can be conducted a priori.

Step 3c deals with the rare case that the amount of MTB-bioNER is equal to or more than 80% of total NER. Instead of refinement through silylation/EDTA a different refinement is possible in which the potentially harmful fraction of NER (the XenoNER) is considered, which is the fraction of total NER subtracted by the biogenic NER. bioNER can be estimated experimentally by conducting an acid hydrolysis of the soil containing total NER (Step 3c). The DT_{50} would then be calculated by considering the parent from the solvent extracts (from Step 1) and the total NER minus the solid-phase (Dowex) eluate from the HCl extracts (DT_{50} based on extr. + (total NER – bioNER) = extr. + XenoNER).

As seen before, if the estimated MTB-bioNER are ≥ 80% of total NER, and thus the major portion of NER can be considered biogenic, the acid hydrolysis is recommended (Step 3c). If MTB-bioNER is < 20% of total NER, then the silylation/EDTA extraction is recommended (Step 3a). If MTB-bioNER is ≥ 20% of total NER and < 80% total NER then it is recommended to derive the DT_{50} by considering the fact that also silylation could release bioNER and this part should also be subtracted (Step 3b). Since the amount of bioNER in the silylation extract is unknown, a default value of 50% of the bioNER will be subtracted from the NER in the silylation extract. In case there is any indication of another ratio of bioNER to be released by silylation, it should replace the default of 50%. Thus, the DT_{50} will be calculated by considering Extracted Residues + Type I

NER – 0.5 x bioNER (DT_{50} based on extr. + (Typ I NER – 0.5 x bioNER). If after all these steps, the P/vP-trigger is still exceeded, the substance is finally to be considered persistent or very persistent.

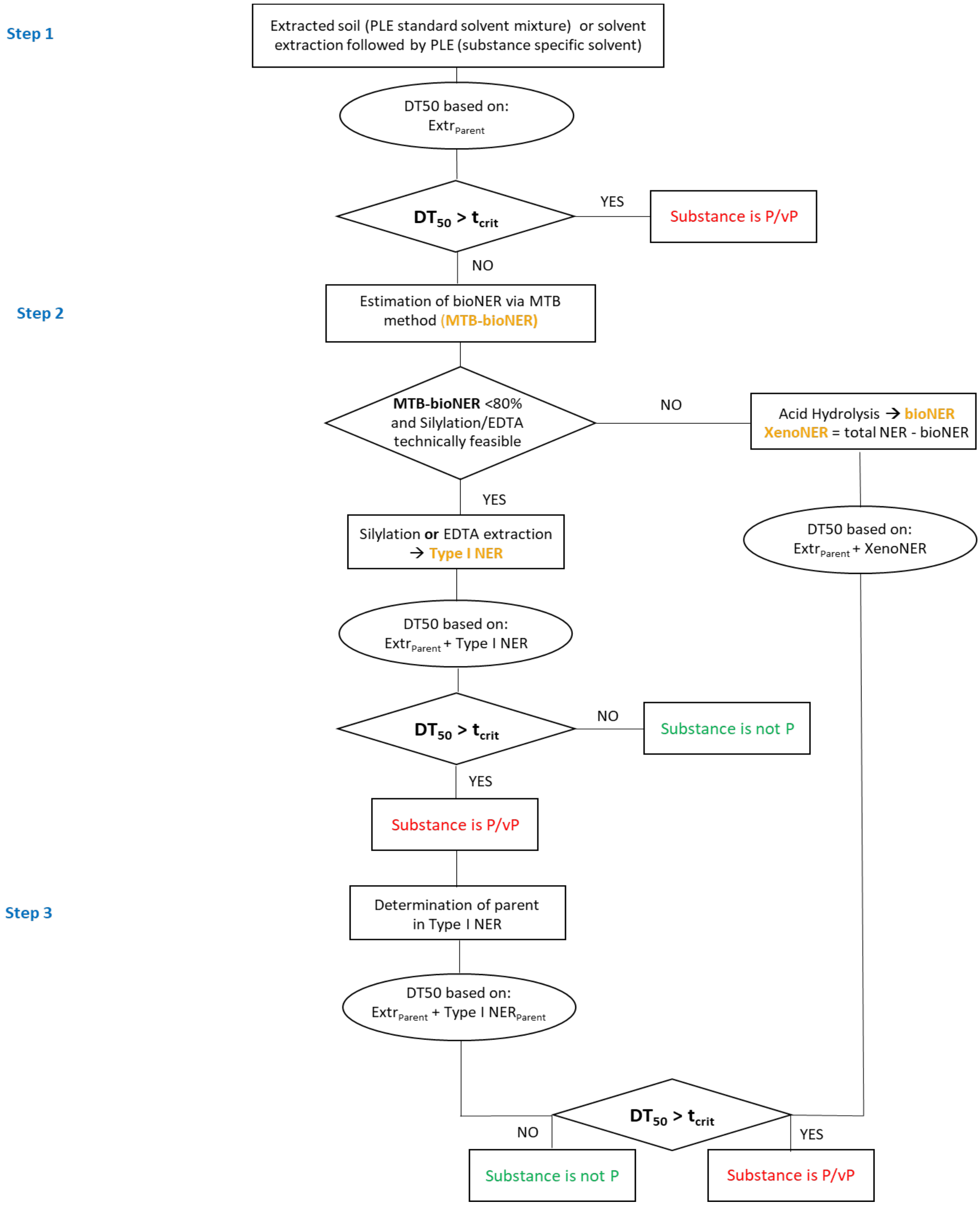
5.6.2 Proposal 2

Proposal 2 (Figure 25) represents a 'realistic-case approach', which sets the focus in trying to derive half-lives of substances as realistic as possible, that can then be used for persistence assessment but also for risk assessment (the latter needs further exposure investigation before implementation will be possible). Starting from a 'best-case scenario' in which it is considered that no fraction of non-extractable residues will become mobilised again, even after many years, and after conducting a chemical analysis of the extracts to determine the parent substance in the extracts (Step 1), the DT_{50} is derived [DT_{50} based on: $Extr_{parent}$]. When this exceeds the trigger value for vP in soil, then no refinement is necessary (substance is vP also in a best-case scenario, thus persistency would further increase, if fractions of potentially remobilisable NER would also be considered). A refinement and further characterization of the NER in soil should be considered, in case that in the first step, the vP trigger value is not reached and for all cases in which the registrant wants to derive a more realistic value for DT_{50} (which considers all the relevant and potentially residues). The following step (Step 2) consists in extraction of the soil containing total NER through silylation or EDTA depending on the technical feasibility of the two methods for the test substance. After extraction the remaining soil contains type II NER (and type III/ bioNER), which are considered irreversibly immobilized unless experimental (remobilization) data prove the contrary. The silylation or EDTA extracts (EDTA/Silylation) contain type I NER, which is deemed to be remobilisable and thus of relevance from a regulatory point of view. To clarify if the type I NER may contain physically entrapped parent compound (EDTA/silylation parent), the silylation or EDTA extracts have to be chemically analysed (Step 3). For the calculation of the DT_{50} with respect to type I NER, two options are possible: if chemical analysis of silylation or EDTA extracts is possible, DT_{50} should be calculated on the basis of $Extr_{parent}$ and $EDTA/silylation_{parent}$ [DT_{50} based on $Extr_{parent}$ + type I Parent]. If it is shown that the chemical analysis of silylation or EDTA extracts is technically not feasible, DT_{50} should be calculated on the basis of $Extr_{parent}$ and EDTA/silylation (i.e. the whole extract) [DT_{50} based on $Extr_{parent}$ + Type I NER].

An alternative way in considering the environmental relevant parts of NER in the half-live calculation is using the acid hydrolysis method instead of silylation or EDTA extraction. The microbial turnover to biomass (MTB) approach as a tool for the estimation of type III NER (bioNER) should be used to decide which method, acid hydrolysis or silylation/EDTA, should be performed. If the type III NER is predicted to be high (>80% of total NER) based on the MTB approach, acid hydrolysis of total NER is recommended. In the next step (Step 2b), type III NER can be experimentally quantified with the help of the Dowex purified HCl extract after acid hydrolysis as proxy for the amount of amino acids, amino sugars and other biomolecules. Finally, the experimentally quantified type III NER is deducted from total NER to obtain the xenobiotic derived NER (XenoNER), which in principle includes both type I and type II NER. The DT_{50} with respect to the XenoNER should be calculated on the basis of $Extr_{parent}$ and XenoNER [DT_{50} based on Extr. + (Total NER – bioNER) = Extr. + XenoNER]. However, the use of the XenoNER for half-life modelling can lead to an overestimation of the persistence, since XenoNER consists of type I NER and type II NER, the latter having a low potential of being released from soil/sediment. Furthermore, the XenoNER evaluation does not allow for analysis of the parent only, which means that in the case of high % of transformation products in the XenoNER, these would not be subtracted to obtain DT_{50} only for the parent.

Nevertheless, the DT_{50} values obtained from EDTA/silylation extraction but also from acid hydrolysis represent the most realistic scenarios. If DT_{50} does not exceed the trigger value for P/vP at this stage, then persistence of the test substance can be excluded.

Figure 25: Flow chart for consideration of NER in Persistence assessment, proposal 2.



Source: https://www.umweltbundesamt.de/sites/default/files/medien/362/dokumente/proposal_2_final.pdf

5.6.3 Application of proposal 1 on the current data sets

In order to verify the consequences of the proposed flow charts in terms of calculated degradation half-lives, the experimental data sets obtained during the project (^{14}C-radiolabelled substances) were applied to the flow scheme. CAKE was used for the calculations and SFO kinetics was selected. Table 51 gives an overview on the results determined according to the flow chart.

Table 51: Stepwise estimation of half-lives DT_{50} [in days] according to proposal 1.

	Description	Bromoxynil	Sulfadiazine	Isoproturon
current	**Extr. Parent** (NER considered as sink)	7.0	7.9	53.8
Step 1	**Extr. $_{Parent}$ + total NER**	246	562	250
Step 2	**Extr. $_{Parent}$ + (total NER – MTB bioNER)**	194	528	140
Step 3a	**Extr. $_{Parent}$ + Type I NER**	**12.4**	**39.3**	57.9
Step 3b	**Extr. $_{Parent}$ + (Type I NER – 0.5 x bioNER)**	10.3	31.4	**49.3**
Step 3c	**Extr. $_{Parent}$ + (total NER –bioNER)**	132	365	147
Step 4	**Extr. $_{Parent}$ + Type I NER $_{Parent}$**	8.1	10.4	48.8

The numbers in bold indicate which of the three options should be applied to the respective substance according to flow chart 1.

The results demonstrate that classification of NER as non-degraded parent substance will turn any test substance that forms NER in soil persistent. However, a differentiated analysis of the NER shows that the substances tested are far from being persistent.

5.6.4 Application of proposal 2 on the current data sets

The following Table shows the results of proposal 2 applied to the current data sets according to the flow chart shown in Figure 25. DT_{50}-values calculated are basically the same as in Table 51 but the order of steps is different.

Table 52: Stepwise estimation of half-lives DT_{50} [in days] according to proposal 2.

	Description	Bromoxynil	Sulfadiazine	Isoproturon
Step 1	**Extr. Parent** (NER considered as sink)	7.0	7.9	53.8
Step 2a	**Extr. $_{Parent}$ + Type I NER**	12.4	39.3	57.9
Step 2b	**Extr. $_{Parent}$ + (total NER –bioNER)**	132	365	147
Step 3	**Extr. $_{Parent}$ + Type I NER $_{Parent}$**	8.1	10.4	48.8

6 Conclusions

In this project, we proposed a harmonised concept for NER based on corresponding experiments with three reference substances. An evaluation concept was developed for taking NER into account in the persistence evaluation and the experimental data served as input parameter to verify the suitability of the concept for high NER forming test substances.

In a first step we proposed a harmonised procedure for the characterisation of NER, taking into account the BfG approach (Loeffler et al., 2020) and the approach presented in the ECHA discussion paper (Kästner et al., 2018, Schäffer et al., 2018). In order to compare the approaches experimentally, soil degradation tests with three selected test substances were performed.

Initially the methods to derive only NER containing matrix (total NER definition) were checked using various extraction methods (substance specific solvents vs a standard solvent mixture) and their extraction yields were compared. PLE extraction was specified as the final extraction step to have at least some standardization in terms of extraction harshness (see Figure 3).

For practical reasons the parent substance specific extraction procedure which has been developed anyway for the soil degradation study, taking different substance properties into account, followed by final PLE with the same solvent or solvent mixture was selected as the preferred extraction procedure. Within all substances tested in this project, no huge variations or outliers regarding extraction yield were observed for this method. A PLE extraction using a standard solvent mix (MeOH, acetone, water 50/25/25 v/v/v at 100°C and 100 bar) as proposed by BfG (Loeffler et al., 2020, UBA Texte 133/2022) showed an even better extraction yield than the substance specific extraction for one of the substances tested but for one other substance significant variations were observed. Thus, PLE with the proposed standard solvent mix might work as well but care should be taken since variations may occur. Further, it will mean additional analytical effort if the parent test substance and known transformation products have to be analysed from two different extracts (using substance specific solvents or the standard solvent mix). Thus, from a practical point of view the substance specific extraction followed by PLE with the same solvent will be the better choice in most cases.

For NER characterisation again different experimental methods have been applied. For the harmonised procedure it was agreed to focus on three NER types: strongly sorbed and physically entrapped (type I, potentially remobilisable), covalently bond (type II) and included in biological matrices (type III, “bioNER”).

For determination of type I NER silylation and EDTA extraction turned out to provide similar results for the three test substances. The procedural recovery and reproducibility for both methods was good and thus in principle both are proven to be suitable for routine testing. Comparing the performance of both procedures, lab work needs protective measures for the silylation procedure, but is manageable without problem for an experienced technician in a typical lab environment. Final silylation extracts can be analysed for the presence of parent and metabolites by conventional methods like TLC and LC-MS. If a test compound is derivatized by the silylation reagent, which can be tested in a pre-experiment with the parent compound, the EDTA method can be performed, which is fit for experimental standard work with aqueous solutions. Two shortcomings for the EDTA method are the co-extraction of high amounts of bioNER, rendering the differentiation of types 1 NER and type 2 NER difficult, and the presence of high salt concentrations in the EDTA extract which might interfere with standard TLC and LC analyses. In addition, care has to be taken regarding solubility of the test substance in the respective extraction solvent and stability of the test substance against extraction conditions.

Both can lead to significant lower recoveries even if high amounts of type I NER should be released from the soil matrix.

Experiments demonstrated further that both extraction procedures extract also huge amounts of unknown decomposition products, which carry the ^{14}C-radiolabel but are neither parent test substance nor relevant transformation products. This could be degradation products associated to the soil matrix, but also type III NER. It will not be possible to identify those ^{14}C-radioactive residues. Therefore, the focus should be placed on the determination of the parent test substance and known released relevant transformation products. However, this requires a chemical analysis of the extracts. This might become challenging for substance specific reasons but turned out to be possible without disproportionate effort. For a stepwise assessment (see Figure 24) chemical analysis of the extracts is an indispensable integral part.

The experimental determination of type II NER still needs further development. This can be concluded from the results of the project. Regarding type III NER, the acidic hydrolysis is an easy applicable method though actually appropriate specific containment instrumentation needs to be used, which is not available as standard device. In addition, as the harsh conditions (strong acid, high temperature) produce matrix extracts that are highly loaded, complex purification is necessary. During this purification, losses of amino acids, which are the target molecules for bioNER determination, cannot be avoided. Experiments with a standard amino acid mixture resulted in significant losses of about 50% just by hydrolysis, even before extract purification. It must be concluded that this method is not yet fit for routine laboratory work, though in research projects it has been often successfully applied (Nowak et al, 2018).

In contrast, the MTB determination, based on, amongst others, mineralisation detected during the degradation experiment, allows a good estimate of the bioNER formed. The module is simple to use and does not require data (except Gibbs energy) in addition to the parameters that have already been collected during the degradation study. The comparison to the measured data gave good agreement for Isoproturon and Bromoxynil. For Sulfadiazine, however, there are indications that the molecule is perhaps degraded without the formation of CO_2 as described in a recent paper for the conversion of ^{13}C from Sulfamethoxazole into microbial proteins (Ouyang et al., 2020).

Two proposals were developed as a stepwise assessment concept to be used in persistence assessment. Fed with the experimental data determined in this project they gave reasonable results. And they demonstrate the relevance of the classification of NER (*safe sink* versus *hidden hazard*) for persistence assessment of chemicals.

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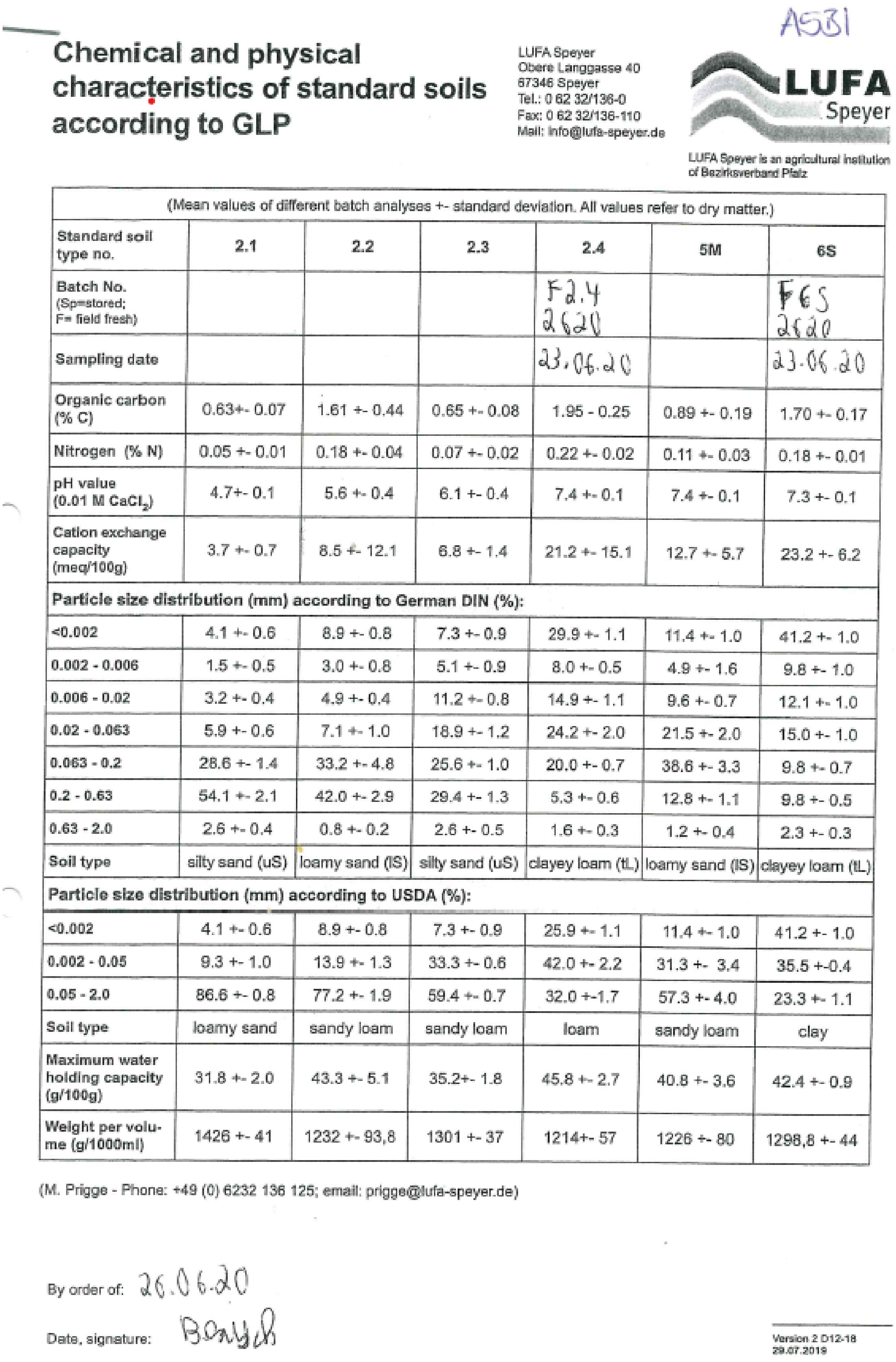
A Soil characterisation Lufa 2.4

A.1 Page 1 original certificate



A531

Chemical and physical characteristics of standard soils according to GLP

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(Mean values of different batch analyses +- standard deviation. All values refer to dry matter.)

Standard soil type no.	2.1	2.2	2.3	2.4	5M	6S
Batch No. (Sp=stored; F= field fresh)				F2.4 2620		F6S 2620
Sampling date				23.06.20		23.06.20
Organic carbon (% C)	0.63+- 0.07	1.61 +- 0.44	0.65 +- 0.08	1.95 - 0.25	0.89 +- 0.19	1.70 +- 0.17
Nitrogen (% N)	0.05 +- 0.01	0.18 +- 0.04	0.07 +- 0.02	0.22 +- 0.02	0.11 +- 0.03	0.18 +- 0.01
pH value (0.01 M $CaCl_2$)	4.7+- 0.1	5.6 +- 0.4	6.1 +- 0.4	7.4 +- 0.1	7.4 +- 0.1	7.3 +- 0.1
Cation exchange capacity (meq/100g)	3.7 +- 0.7	8.5 +- 12.1	6.8 +- 1.4	21.2 +- 15.1	12.7 +- 5.7	23.2 +- 6.2
Particle size distribution (mm) according to German DIN (%):						
<0.002	4.1 +- 0.6	8.9 +- 0.8	7.3 +- 0.9	29.9 +- 1.1	11.4 +- 1.0	41.2 +- 1.0
0.002 - 0.006	1.5 +- 0.5	3.0 +- 0.8	5.1 +- 0.9	8.0 +- 0.5	4.9 +- 1.6	9.8 +- 1.0
0.006 - 0.02	3.2 +- 0.4	4.9 +- 0.4	11.2 +- 0.8	14.9 +- 1.1	9.6 +- 0.7	12.1 +- 1.0
0.02 - 0.063	5.9 +- 0.6	7.1 +- 1.0	18.9 +- 1.2	24.2 +- 2.0	21.5 +- 2.0	15.0 +- 1.0
0.063 - 0.2	28.6 +- 1.4	33.2 +- 4.8	25.6 +- 1.0	20.0 +- 0.7	38.6 +- 3.3	9.8 +- 0.7
0.2 - 0.63	54.1 +- 2.1	42.0 +- 2.9	29.4 +- 1.3	5.3 +- 0.6	12.8 +- 1.1	9.8 +- 0.5
0.63 - 2.0	2.6 +- 0.4	0.8 +- 0.2	2.6 +- 0.5	1.6 +- 0.3	1.2 +- 0.4	2.3 +- 0.3
Soil type	silty sand (uS)	loamy sand (lS)	silty sand (uS)	clayey loam (tL)	loamy sand (lS)	clayey loam (tL)
Particle size distribution (mm) according to USDA (%):						
<0.002	4.1 +- 0.6	8.9 +- 0.8	7.3 +- 0.9	25.9 +- 1.1	11.4 +- 1.0	41.2 +- 1.0
0.002 - 0.05	9.3 +- 1.0	13.9 +- 1.3	33.3 +- 0.6	42.0 +- 2.2	31.3 +- 3.4	35.5 +-0.4
0.05 - 2.0	86.6 +- 0.8	77.2 +- 1.9	59.4 +- 0.7	32.0 +-1.7	57.3 +- 4.0	23.3 +- 1.1
Soil type	loamy sand	sandy loam	sandy loam	loam	sandy loam	clay
Maximum water holding capacity (g/100g)	31.8 +- 2.0	43.3 +- 5.1	35.2+- 1.8	45.8 +- 2.7	40.8 +- 3.6	42.4 +- 0.9
Weight per volume (g/1000ml)	1426 +- 41	1232 +- 93,8	1301 +- 37	1214+- 57	1226 +- 80	1298,8 +- 44

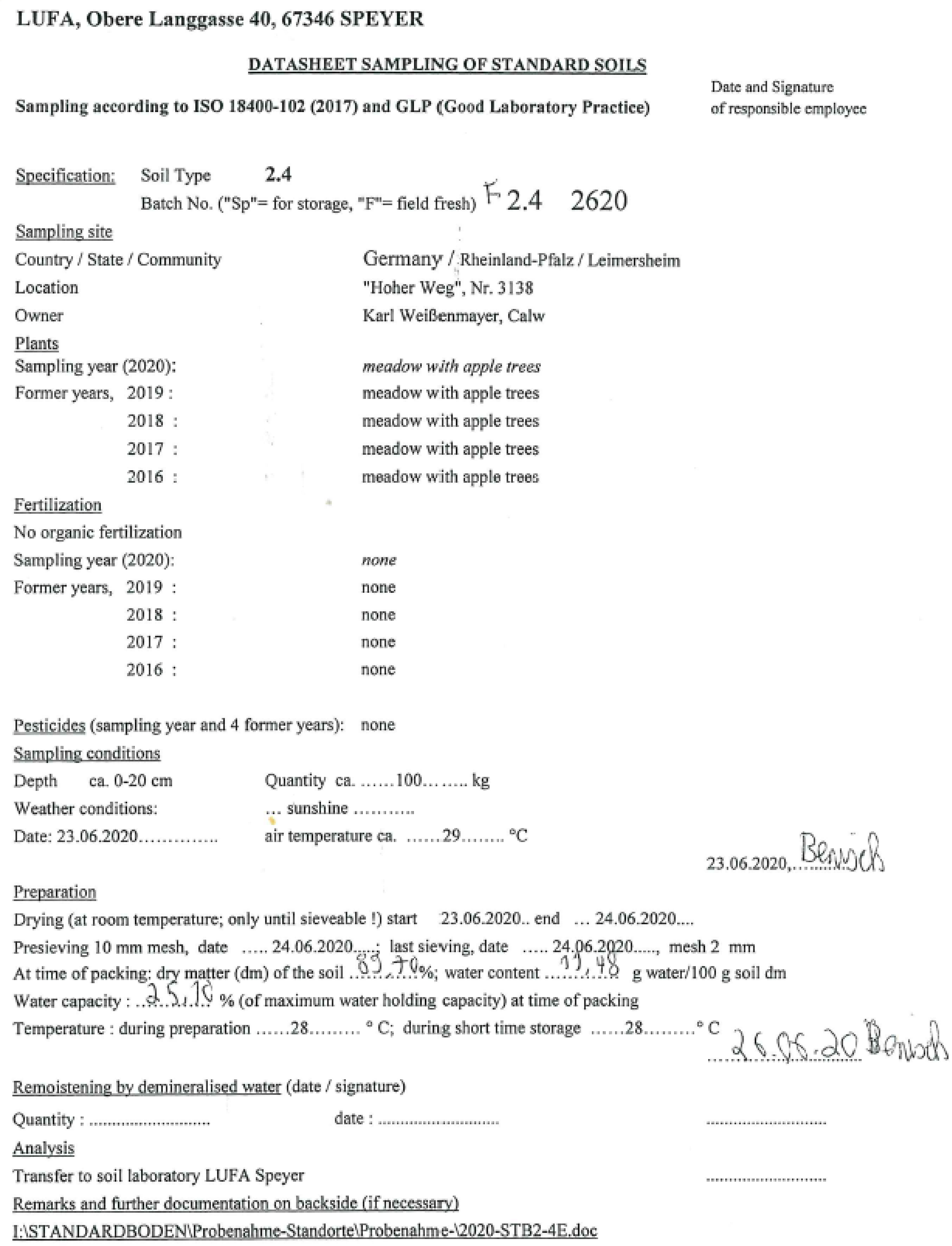
(M. Prigge - Phone: +49 (0) 6232 136 125; email: prigge@lufa-speyer.de)

By order of: 26.06.20

Date, signature:

Version 2 D12-18
29.07.2019

A.2 Page 2 original certificate



LUFA, Obere Langgasse 40, 67346 SPEYER

DATASHEET SAMPLING OF STANDARD SOILS

Sampling according to ISO 18400-102 (2017) and GLP (Good Laboratory Practice)

Date and Signature
of responsible employee

Specification: Soil Type **2.4**
Batch No. ("Sp"= for storage, "F"= field fresh) F 2.4 2620

Sampling site

Country / State / Community	Germany / Rheinland-Pfalz / Leimersheim
Location	"Hoher Weg", Nr. 3138
Owner	Karl Weißenmayer, Calw

Plants

Sampling year (2020):	*meadow with apple trees*
Former years, 2019 :	meadow with apple trees
2018 :	meadow with apple trees
2017 :	meadow with apple trees
2016 :	meadow with apple trees

Fertilization

No organic fertilization

Sampling year (2020):	*none*
Former years, 2019 :	none
2018 :	none
2017 :	none
2016 :	none

Pesticides (sampling year and 4 former years): none

Sampling conditions

Depth ca. 0-20 cm Quantity ca. ……100…….. kg
Weather conditions: … sunshine ……….
Date: 23.06.2020…………. air temperature ca. ……29……. °C

23.06.2020,……… Bensch

Preparation

Drying (at room temperature; only until sieveable !) start 23.06.2020.. end … 24.06.2020….
Presieving 10 mm mesh, date ….. 24.06.2020…..; last sieving, date ….. 24.06.2020….., mesh 2 mm
At time of packing: dry matter (dm) of the soil …89,70…%; water content ……11,48 g water/100 g soil dm
Water capacity : …25,10… % (of maximum water holding capacity) at time of packing
Temperature : during preparation ……28……… ° C; during short time storage ……28………° C

……26.06.20…… Bensch

Remoistening by demineralised water (date / signature)

Quantity : ……………………… date : ……………………… ………………………

Analysis

Transfer to soil laboratory LUFA Speyer ………………………

Remarks and further documentation on backside (if necessary)

I:\STANDARDBODEN\Probenahme-Standorte\Probenahme-\2020-STB2-4E.doc

B Test substances

Table B1: test substances ^{14}C

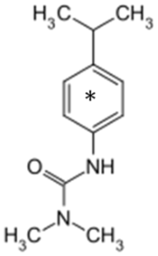
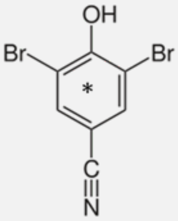
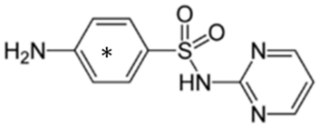
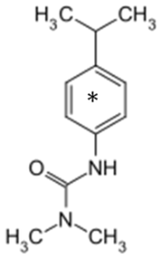
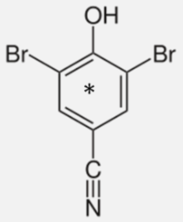
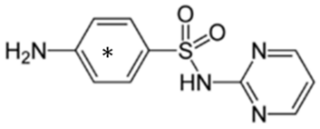
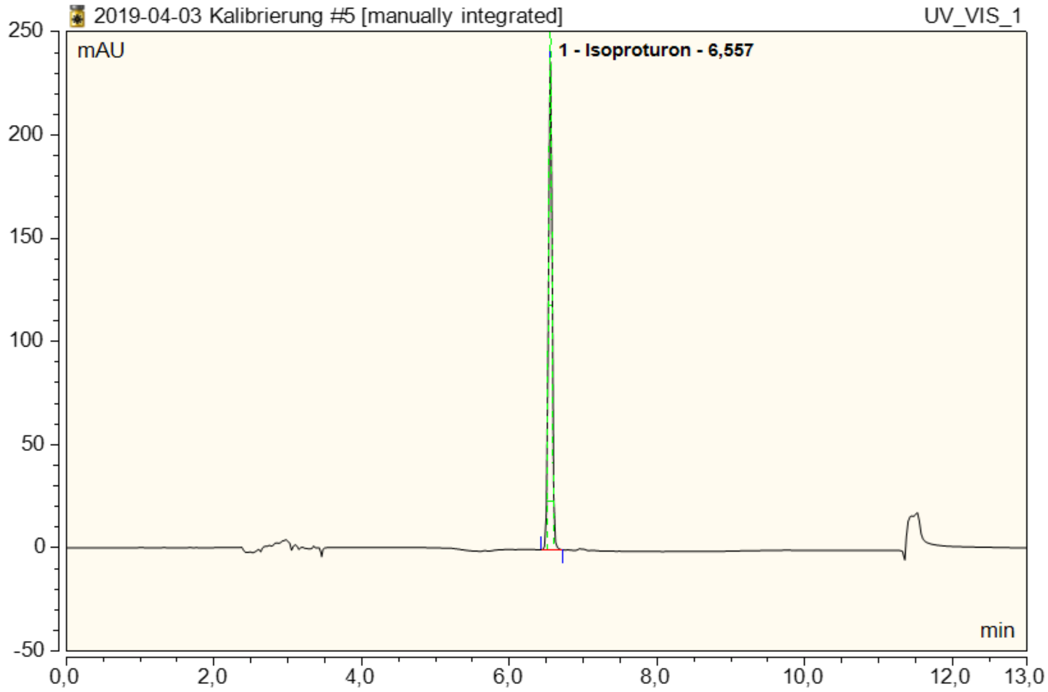
Substance name	label position (*) denotes ^{14}C labelling position	supplier	specific radioactivity
Isoproturon IUPAC: (4-propan-2-ylphenyl)urea CAS-No 56046-17-4		IZOTOP (Hungary) Code:CC-337	9.46 MBq/mg Chemical Purity: 97.4 %
Bromoxynil IUPAC: 3,5-dibromo-4-hydroxybenzonitrile CAS-No 1689-84-5		Bayer AG (Germany) Code: KML-10686	6.49 MBq/mg Chemical Purity: 97.0 %
Sulfadiazine IUPAC: 4-amino-N-pyrimidin-2-ylbenzenesulfonamide CAS-No 68-35-9		IZOTOP (Hungary) Code: CC-523	3.32 MBq/mg Chemical Purity: 99.4 %
L-Amino acid mixture [14C(U)] ARC0474	Composition: [14C(U)] with Amino acids in the following amounts: Alanin (8 %), Arginin (7 %), Asparaginacid (8 %), Glutamineacid (12,5 %), Glycin (4 %), Histidine (1,5 %), Isoleucine (5 %), Leucine (14 %), Lysine (14 %), Phenylalanin (8 %), Proline (5 %), Serin (4 %), Threonin (5 %), Tyrosin (4 %) und Valin (8 %).	Hartmann Analytical (Germany)	

Table B2: test substances ^{13}C

Substance name	label position (*) denotes ^{13}C labelling position	supplier	Purity
Isoproturon [Ring-13C6] IUPAC: (4-propan-2-ylphenyl)urea CAS-No 56046-17-4		IZOTOP (Hungary) Code:CS-337	Chemical Purity: 97.1 %
Bromoxynil [Phenyl-UL-13C6] IUPAC: 3,5-dibromo-4-hydroxybenzonitrile CAS-No 1689-84-5		Bayer AG (Germany) Code: K-1482	Chemical Purity: 99.7 %
Sulfadiazine [Phenyl Ring-U-13C6] IUPAC: 4-amino-N-pyrimidin-2-ylbenzenesulfonamide CAS-No 68-35-9		IZOTOP (Hungary) Code: CS-523	Chemical Purity: 98.6 %

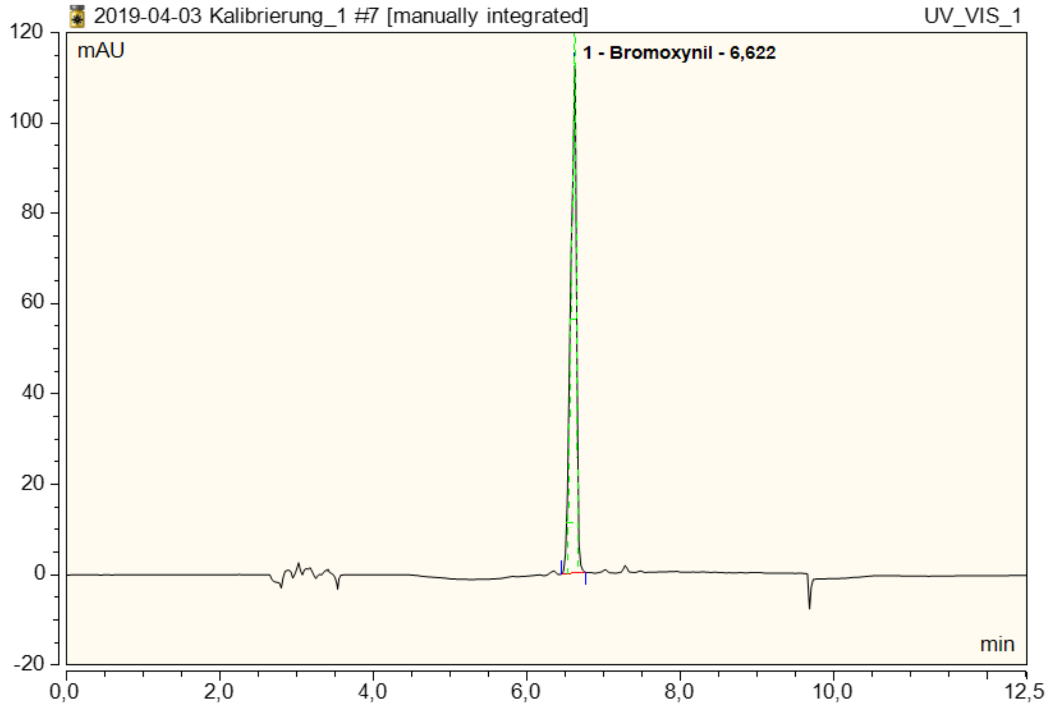
C HPLC/UV-chromatograms of test substances

C.1 Isoproturon



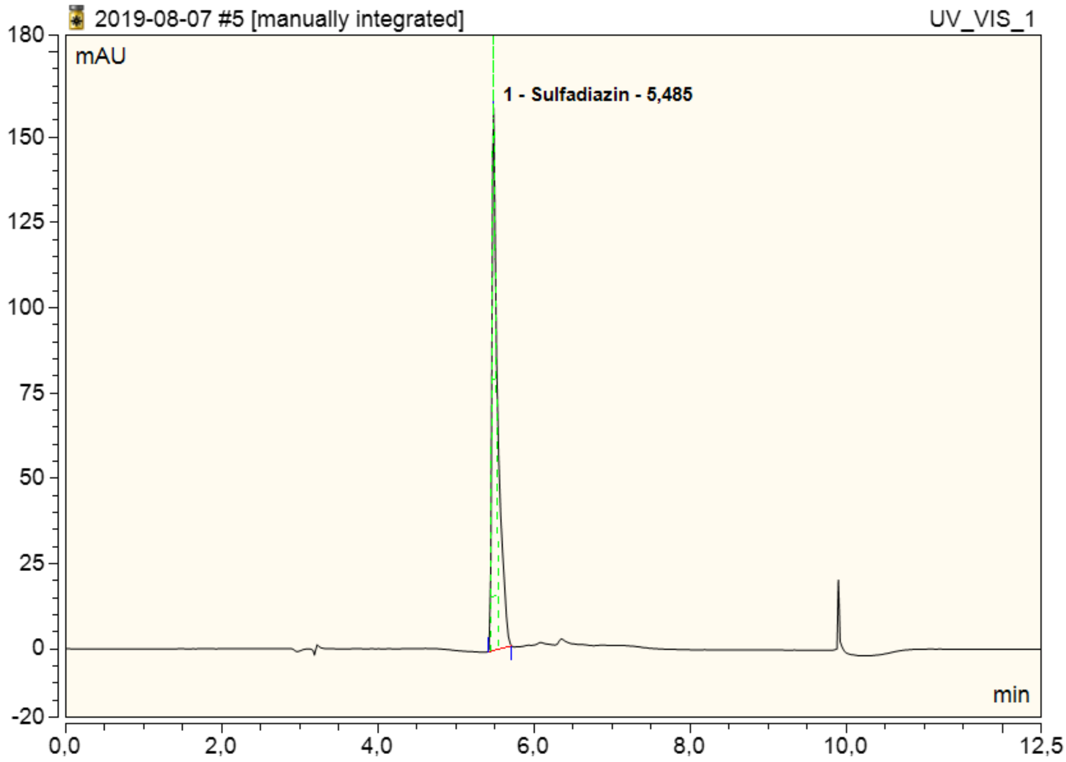
Source: Fraunhofer IME evaluation of project data with Chromeleon

C.2 Bromoxynil



Source: Fraunhofer IME evaluation of project data with Chromeleon

C.3 Sulfadiazine



Source: Fraunhofer IME evaluation of project data with Chromeleon

D ^{13}C data processing

D.1 Bromoxynil

Soil Background	1.07414	[% ^{13}C of total C]
Start after application	1.09257	[% ^{13}C of total C]
Bromoxynil ^{13}C applied	0.01843	[% ^{13}C of total C]

^{13}C in soil sample after application with ^{13}C-Bromoxynil

	mass [mg]	total C measured [µg]	^{13}C of total C measured [%]	Mean ^{13}C [%]
Applied soil before extraction	5.13	225.30	1.09254	1.09257
	3.26	143.70	1.09243	
	5.83	245.19	1.09276	

Evaluation of extraction residues of samples from ^{13}C-Bromoxynil experiment

Sampling	mass [mg]	total C measured [µg]	^{13}C of total C measured [%]	Mean ^{13}C [%]	Difference from Background [^{13}C of total C in %]	NER [%]
0d	3.20	127.36	1.07480	1.07477	0.00062	3.4
	3.43	147.91	1.07463			
	3.59	152.57	1.07487			
7d	3.63	169.88	1.07640	1.07668	0.00254	13.8
	3.29	142.22	1.07689			
	3.48	158.08	1.07675			
14d	3.85	152.72	1.07743	1.07723	0.00308	16.7
	4.03	209.00	1.07692			
	3.25	141.87	1.07733			
29d	3.08	148.18	1.08164	1.08105	0.00691	37.5
	3.45	149.85	1.08255			
	3.66	167.56	1.07897			
59d	3.01	120.68	1.08143	1.08155	0.00740	40.2
	3.08	133.03	1.08171			
	3.27	133.87	1.08150			
119d	3.17	148.21	1.08542	1.08330	0.00916	49.7
	3.38	138.00	1.08288			
	3.69	178.89	1.08161			
14d steril	3.52	155.68	1.07477	1.07471	0.00057	3.1
	3.30	142.97	1.07461			
	3.03	125.77	1.07476			
119d steril	3.47	165.28	1.07531	1.07533	0.00118	6.4
	3.45	146.71	1.07537			
	3.30	145.88	1.07531			

D.2 Sulfadiazine

Soil Background	1.07421	[% ^{13}C of total C]
Start after application	1.09314	[% ^{13}C of total C]
Sulfadiazine ^{13}C applied	0.01893	[% ^{13}C of total C]

^{13}C in soil sample after application with ^{13}C-Sulfadiazine

	mass [mg]	total C measured [µg]	^{13}C of total C measured [%]	Mean ^{13}C [%]
Applied soil before extraction	3.648	170.87	1.09347	1.09314
	4.025	190.51	1.09281	

Evaluation of extraction residues of samples from ^{13}C-Bromoxynil experiment

Sampling	mass [mg]	total C measured [µg]	^{13}C of total C measured [%]	Mean ^{13}C [%]	Difference from Background [^{13}C of total C in %]	NER [%]
0d	3.661	162.26	1.07450	1.07465	0.00044	2.3
	4.085	191.37	1.07475			
	3.079	149.29	1.07469			
7d	3.310	150.85	1.07762	1.07762	0.00341	18.0
	3.222	146.42	1.07731			
	3.981	172.25	1.07793			
14d	3.315	143.46	1.07872	1.07875	0.00454	24.0
	3.981	176.71	1.07835			
	3.221	137.51	1.07918			
27d	3.271	137.59	1.08109	1.08123	0.00702	37.1
	3.313	159.25	1.08238			
	3.935	166.41	1.08022			
58d	3.255	153.02	1.08179	1.08761	0.01340	70.8
	3.118	120.38	1.08457			
	3.773	169.15	1.09648			
120d	3.093	130.89	1.08871	1.09043	0.01622	85.7
	3.799	165.13	1.08863			
	3.296	140.48	1.09395			
14d steril	3.429	140.94	1.07717	1.07677	0.00256	13.5
	3.229	139.25	1.07678			
	3.622	154.85	1.07636			
119d steril	3.114	131.27	1.08045	1.08069	0.00649	34.3
	3.265	144.35	1.08203			
	3.364	146.00	1.07961			

E Data set for analysis of silylation method reproducibility

E.1 Results of recovered radioactivity in %aR of samples for statistical evaluation

Sulfadiazine

Sample	**7d-1**	**7d-2**	**14d-1**	**14d-2**	**14ds-1**	**14ds-2**	**28d-1**	**28d-2**	**58d-1**	**58d-2**	**120ds-1**	**120ds-2**	**121d-1**	**121d-2**
replicate 1	30.0	30.0	24.4	29.7	29.3	27.8	31.3	29.7	28.3	27.5	31.4	29.0	28.0	27.5
replicate 2	28.9	32.8	25.9	38.3	29.0	27.6	31.9	33.2	28.3	28.3	29.9	29.6	27.1	26.8

Isoproturon

Sample	**7d-1**	**7d-2**	**14d-1**	**14d-2**	**15ds-1**	**15ds-2**	**29d-1**	**29d-2**	**59d-1**	**59d-2**	**120ds-1**	**120ds-2**	**120d-1**	**120d-2**
replicate 1	30.9	35.8	26.7	20.4	43.4	27.4	23.2	23.0	30.1	28.0	24.3	23.8	44.7	54.4
replicate 2	35.6	28.9	28.1	22.4	29.5	30.8	23.0	20.5	29.0	28.3	23.8	24.6	34.3	52.7

Bromoxynil

Sample	**7d-1**	**7d-2**	**14d-1**	**14d-2**	**14ds-1**	**14ds-2**	**27d-1**	**27d-2**	**62d-1**	**62d-2**	**119ds-1**	**119ds-2**	**120d-1**	**120d-2**
replicate 1	31.0	45.4	21.9	27.3	74.0	81.1	20.5	18.0	16.9	16.6	78.1	26.0	15.3	16.8
replicate 2	36.7	42.3	22.2	28.1	76.8	82.0	20.8	16.8	16.3	15.6	98.7	33.2	15.7	16.5

E.2 Result of statistical functions LeveneTest and Wilcox.test (R output)

```
Levene's Test for Homogeneity of Variance (center = median)
Df F value Pr(>F)
group  1  0.0142 0.9055
82
```

```
        Wilcoxon rank sum test

data:  data2$Value by data2$Replicate
W = 851.5, p-value = 0.7849
alternative hypothesis: true location shift is not equal to 0
```