NER Workshop 17 - 18 February 2021

Chat - Questions relating to extraction methods, technical matters, procedural issues and regulatory aspects (that have arisen during the workshop)

If a CRO does not have a PLE system, is a Soxhlet extraction considered to be an acceptable alternative?

> Direct comparability of Soxhlet extraction with PLE is not tested yet. Thus, the concept currently does not present Soxhlet extraction as an alternative and PLE is mandatory.
>
> According to the UBA proposal, PLE should be the last step, if a stepwise solvent extraction is chosen. Alternatively, solely PLE with a standard mix (methanol/acetone/ water 50/25/25% at 100°C and 100 bar) according to Löffler et al. 2020 [1] can be conducted. Soxhlet extraction can only be considered as an acceptable alternative if PLE is not feasible because the parent compound is not stable and destroyed during the PLE procedure. In case Soxhlet extraction was to be used, equal extraction efficiency would have to be proven and both methods would have to be compared. The use of an alternative extraction procedure will always be on a case-by-case decision.

What can be done if silylation destroys the analyte?

> As with any extraction method, stability of the test substance has to be proven. In the case silylation destroys/modifies the test substance EDTA extraction can be performed. If only one reaction product is formed this product could be considered as parent equivalent as well.

How many solvents should we use in the PLE final extraction step? Only a substance specific solvent or solvents within a range of polarity?

> When searching for a substance specific extraction solvent, various solvents with different polarities have already been tested. In PLE a substance specific solvent should be used or the standard mix methanol/acetone/water (50/25/25) (Löffler et al. 2020[1]).

Hydrolysis procedure: Why is it important to use 105°C? What is the evidence? 100°C should be sufficient for hydrolysis needed for AA.

> According to experience in the Kaestner working group (UFZ) 105 °C resulted in better reproducible hydrolysis.

Regarding the HCl extraction: The procedure may be performed under reflux, not having the system under pressure....

> According to experience in the Kaestner group a closed set-up under N_2 atmosphere results in more stringent hydrolysis and better recoveries.

Open systems are not recommended for hydrolysis -> melanoidin reactions

[1] Loeffler, D., Hatz, A., Albrecht, D., Marvin, F., Hogeback, J., Ternes, T.A., 2020. Determination of non-extractable residues in soils: Towards a standardised approach. Environmental Pollution 259, 113826. https://doi.org/10.1016/j.envpol.2019.113826

See previous comment.

Which specific analysis methods were used to analyse the silylation and EDTA extracts?

Both Radio-TLC and LC-MS-MS methods are applicable and were applied in the project.

In practice, if we make the effort to obtain the Silylation Extract (Step 3b) why would you not then analyse the extract (step 4)? (this question refers to the "worst-case" approach of the project partners)

The silylation extract can be analyzed, see previous comment. However, in a stepwise approach the worst-case assumption is that all released radioactivity is due to type 1 NER. If this is enough to dispel the P-suspicion, chemical analysis is not necessarily required for P assessment.

Reference temperature for OECD tests is 12°C. Is this considered in the MTB model?

Temperature can be taken into account with the MTB model. It would affect the estimation of the microbial growth yield as this is based on the Gibbs free energy.

Generally, the temperature is currently only indirectly considered; lower turnover kinetics result in lower CO_2 development and thus also in the related biomass formation.

What about metabolites DT_{50} evaluation, shouldn't we consider these proposals if the metabolites are part of NER?

The relevant transformation products as part of the remobilisable NER type I should be considered for the derivation of DT_{50} for the specific transformation product.

Why is only the parent considered in the entrapped residues? Transformation products are also expectable here?

Focus of the project is the persistence assessment of the parent compound. However, if relevant transformation products are analysed in type 1 NER, their persistence also has to be assessed. In principle, it should be possible to analyse transformation products in NER type I. A problem with transformation products could be that it might be difficult/impossible to assess if they are transformed by silylation if no reference substance is available. For quantification, the isotope label could be used.

Do you consider it feasible and/or necessary to identify unknown transformation products (usually with several functional groups) in the silylated extracts due to the chemical modifications of the functional groups? The same applies to the EDTA extract.

Only if the amount of unknown transformation products is ≥ 10% or ≥ 5% AR on two consecutive days or at the end of the study (PSM, Biocides) then the TPs should be identified. Feasibility depends on the respective compounds.

This increases the complexity of the already challenging analyses. So far, non-target analysis in silylation or EDTA extracts seems over-ambitious, but may be easier in EDTA extracts. Exception could be if there is a strong indication of a discrete transformation product in those extracts. In this particular case we expect that identification would make sense to identify this discrete transformation product.

If we are stuck at step 2 (free gibbs energy not available in literature data and data bases), how should we proceed?

Gibbs of the parent substance is not sensitive data and can be estimated with the Weizman Equilibrator [https://equilibrator.weizmann.ac.il/].

Is there a valid recovery check of the silylation and EDTA fractions?

Yes, recovery can be quantified since we use isotope labelled compounds. All procedures have been tested with the pure parent substances and recoveries have been determined. Recoveries in soil spiked with parent compound: Bromoxynil 92,6/ 94.9%, Sulfadiazin 108.3/117%, Isoproturon 103,0/ 103.9%.

Why have measurements of humic acids/fulvic acids and humin acids in residue pellets been completely omitted?

Basis for the project was the ECHA discussion paper [Kaestner et al. 2018[2]] and the approach by Löffler et al. (2020)[1]. The procedures described there were tested with the focus on practicability and significance. No other methods have been assessed at this point. Based on a literature survey humic separation procedures were not considered as appropriate approaches for differentiation of NER types.

Although characterisation of NERs at a formation of ≥10% AR is required by regulations (e.g. pesticides, biocides), there is so far no harmonised approach on how this should be done. Humic fractionation has often been performed to provide the required information, but these results have never been used in persistence or risk assessment as there is no agreed recommendation regarding their interpretation. Unfortunately, humic fractionation distribution varies over different soils and varies over different soils and thus will not provide reproducible and robust data on NER speciation.

The method proposed in this project (NER characterisation through stepwise extraction) is an advance of the characterisation and quantification of NER, where, in contrast to the humic fractionation, clear statements can be made about the type of NER (irreversibly bound or remobilisable) and different types of NER can be quantified. As mentioned before, the scope of the project was to study the practicability and significance of this specific method, and it was not compared to other methods.

The microorganisms are known to act as an isotopic filter! Therefore, their isotopic signature is always different from their environment. They selectively take the ^{12}C instead of ^{14}C which leads in the unique (Delta)^{13}C. This means during the test period they might consume other consumable C-sources and then facing the ^{14}C might go through a starvation period, especially as according to the OECD TG there is no pre-adaptation. This would not only have an effect on the degradation speed, but even on the total feasibility of degradation for the MOs. In such ^{14}C labeled simulation tests how comparable they would be with the behaviour of the ^{12}C in nature?

Isotopic shifts when working with radioactive compounds are negligible because of the very low concentrations used in comparison to ^{12}C. The preferential use of ^{12}C is thus too low to affect mass balances.

Only highly ^{13}C-labelled compounds (the enrichment is measured in the delta nomenclature or as % at higher enrichments) may become difficult in mixtures with non-labelled compounds.

[2] Consultancy services to support ECHA in improving the interpretation of Non-Extractable Residues (NER) in degradation assessment, Discussion paper - final report, Matthias Kaestner, Stefan Trapp, Andreas Schäffer, June 2018

Isotopic fractionation is then of relevance and can be used for turnover assessment. If only highly ^{13}C-labelled compounds are used, fractionation is of minor relevance.

NER can be transported with solid particles. They will ultimately end-up in the marine environment. The protection of the marine ecosystems was initially the purpose of the PBT/vPvB assessment. The degradation potential in the marine environment, is less. So, how can you prove that remobilisable NER are not a concern then?

From our point of view type 1 NER (remobilisable NER) are a matter of concern, also regarding transport to aquatic systems.

Perhaps more insight of NER exposure relevance is needed. Literature reviews are available? Entrapped molecules can be a relevant exposure source?

We have only the review of the ECHA discussion paper[2] at hand, but we see this as follows: Partitioning into pores of the soil or sediment matrix is concentration-dependent and driven by diffusion towards an equilibrium. If the concentration in the pore water is high, residues will reach the pores from which residues cannot readily be extracted. If concentration in the pore water becomes lower because of degradation, molecules are slowly released from the pores and enter pore water, to reach equilibrium. Also, if humic matter in soil or sediment is microbially degraded, entrapped molecules may be slowly released. Both phenomena may lead to a slow release of type 1 NER.

In the case where release kinetics for type 1 NER could be determined, this could contribute accordingly to exposure. But to our knowledge there are no harmonized concepts for consideration of relevant NER types in exposure assessment at that time. However, if NER containing particles are taken up by organisms, e.g. by soil or sediment feeding organisms, they may be of relevance.

Studies like that of Riedo et al. (2021)[3] document that pesticides appear in the environment a long time after they were banned. This may be due to slow release of NER. If so, NER become relevant for exposure.

…Can erosion release entrapped NER?

Yes, see comment before.

…But erosion is simulated by first extractions in the methodology?

Erosion often contains small soil particles and thus they will be dominantly transferred and not necessarily released. Particles < 0;45 µm may then be considered as `dissolved´. Erosion is partly simulated by silylation/ EDTA extraction.

I would be in favor of silylation reaction if the workflows not only for parent compounds but also for transformation products is straight forward. How is the experience for analysing transformation products?

In principle possible, but so far focus was on analysis of parent and its persistence assessment. Problem might be to prove the stability of the transformation products against silylation. No

[3] Riedo, J., Wettstein, F.E., Rösch, A., Herzog, C., Banerjee, S., Büchi, L., Charles, R., Wächter, D., Martin-Laurent, f., Bucheli, T.D., Walder, F., and van der Heijden, M.G.A.,2021, Environ. Sci. Technol, 55, 5, 2919-2928 https://dx.doi.org/10.1021/acs.est.0c06405

problem if reference substances for transformation products are available (see comment above).

If we look from the chemical point of view, the affinity of the absorption of the substance to the soil particle depends on the cation exchange capacity of the soil and also the electrophilic/nucleophilic behavior of the substance. Are these factors even considered before performing the test? It can cause different results for testing the same compound on different soils. I assume this might be one of the main reasons why a compound like sulfadiazin showed a very different behavior in the presented results. This is due to the pyridinic Ns which can make it have physical/chemical bounding to the soil particles (a phenomenon similar to ORSs observed in crude oil which is full of pyridinic N compounds). That would directly affect the lack of bioavailability of the substance and also introduce extraction problems. Are different N-solvents like Dimethylformamid (DMF) or pyridine used for extraction of this substance from soil particles?

In studies according to OECD 307 four different soils are used to consider the variations. Also, for each study different solvents are used to discover which gives the best recovery for the respective test substance from all soils. DMF or Pyridine are rather unusual extraction solvents as they will make subsequent chemical analysis more complicated. Substances with pyridinic Ns might bind chemically to the soil matrix. Associated research is for example conducted by the group of M. Matthies, University Osnabrück. Chemically bound compounds are actually assessed as being unable to be mobilized again and should be assigned to NER type II.

Could you estimate the additional effort (e.g., % time) required to perform silylation/EDTA as well as HCl extraction in a standard OECD 307 study?

To do the silylation is not super time consuming because you can store NER samples in the freezer (-20°C). They should be stable as they are extracted several times before. Silylation including analysis I would estimate one week if you do silylation of samples all at once in parallel. EDTA depends on how good chemical analysis works.

For persistence assessment we look at parent and/or persistent metabolites. Isn't it then not sufficient enough if we do a silylation provided that we can measure parent/metabolites in the extract? I really need more information on the analysis of the silylation and or EDTA extracts. The presented results of the project showed very low amounts of parent in these extracts. Is that realistic?

Yes, the analyses relied on well-established methods (TLC, LC-MS-MS) and are reliable. Recoveries with parent substance have been performed in parallel in the same matrices.

In the current project only three substances have been investigated. However, there is evidence from literature that significantly higher amounts of type I NER including the parent compound as well as transformation products can be formed (e.g. Cao et al. (2020)[4])

To me it was not clear if completely labelled (^{13}C or ^{15}N) are used for experiments. From a biochemical point of view a fully labelled compound should be used. From a practical point of view a partially labelled compound may be sufficient.

It´s all a matter of money to allow for fully labelled or partially labelled substances.
From our experience it is already very seldom to have more than one isotope label in REACH regulatory studies. Would be great to have more but is unrealistic from our point of view.

[4] Cao, s.; Wang, S., Zhao, Y., Wang, L., Ma, Y., Schäffer, A., Ji, R.; 2020, Environ. Intern. 143, 105908, DOI: 10.1016/j.envint.2020.105908

Studies with co-labelled compounds are very seldom, since these compounds are very expensive.

Should the Xeno-NER be targeted due to hypothesis and knowledge of degradation pathway or non-targeted?

Focus is on the parent compound, i.e. analysis is targeted.

Chat-Questions on regulatory aspects (that have arisen during the workshop)

From the previous talk it was not clear that different sets of DT_{50} (based on different NER concepts) are needed for the exposure modelling and the persistence assessment.

The proposal of a flowchart for P assessment of NER-forming substances is a result of the project. It represents a stepwise approach with an increased level of detail in the experimental data required. In principle one DT_{50} calculation should be fine for P assessment if it represents the highest level of NER characterization. But if already the most conservative approach works (NERtotal = parent, Proposal 1), cost and time intensive laboratory work can be omitted. This was the idea.

The flow charts lack an important aspect: once you can robustly assess the substance as P/vP you should also be able to stop.

You are partly right. If a substance is assessed to be **vP** based on the parent compound in solvent and PLE extracts, further extractions of the NER containing soil to determine relevant type I NER are not necessary (see UBA approach). Otherwise silylation or EDTA extraction should be performed. Whatever the choice, it is necessary to show that the extraction method chosen was adequate for the purpose. If the substance is assessed to be only **P** further extractions are necessary as the vP criterion has to be clarified.

On the other hand, if a substance is assessed to be **not P** based on the DT50 calculated by including parent compound in solvent and PLE extracts plus total NER (see approach project partners), no further extraction steps are necessary to refine the P assessment. The latter only applies to persistence assessment (decision not P, P or vP). If the determined DT_{50} is relevant for persistence and risk assessment, the DT_{50} should be as realistic as possible and further extraction steps are required as only type I NER (*MTB-NER< 80% total NER*) or XenoNER (*MTB-NER ≥ 80% total NER*) should considered for DT_{50} determination. For details please see the corresponding extraction approaches. Although there has not been a discussion or a decision regarding the consideration of NER in exposure assessment so far, we recommend collecting this information because it cannot be excluded that type I NER will be taken into account somehow in the future.

Will the half-lives cut-off remain the same once this proposal comes into effect, especially for sediment?

Yes, as the calculated DT_{50} reflects the potentially remobilisable fraction which might be relevant in the environment.

I assume the cut-off values were set at the time where NER was assessed as a safe sink. We now know that this is not true. So, there is no need to adjust the cut-off but the need is to consider NER in the P assessment.

We agree.

I understand your assumption. But I think a sound examination of the data used at the time when P cut-offs were derived would be preferable so as to integrate NERs in an appropriate way in the regulation.

P trigger values are defined as degradation half-lives, which describe the time it takes for half of a substance to degrade in an environmental compartment. For P assessment, only the potentially remobilisable NER fraction is considered. However, this was neglected for a long time simply because there was no harmonised approach to identify and assess NER. A discussion of P trigger values is not needed from our point of view, as only the potentially remobilisable NER fraction has to be considered for the P assessment.

I agree that we need a better understanding of the cut-off values and the protection goal which we want to achieve with these cut-off values. A cut-off of 180 d is nearly equal to a degradation of > 90% in 2 years. For this reason, if we define this > 90% as recovery time after emission is stopped, is this the protection goal we want to have, that substances after emissions stopped is removed from the compartment within 2 years? We have to define why a substance > 2 years has a higher hazard, or is a value of for example > 5 years more appropriate. Here we need a better understanding and agreement.

A discussion of the adjustment of the P cut-off value goes beyond the purpose of this project and, in our opinion, should not be correlated to the consideration of remobilisable NER (as parent and transformation products in type I NER) in the P assessment.

Perhaps NER characterisation and interpretation is appropriate for those chemicals which can be mobilized again and which have DT_{50}s greater than the P cutoff criteria?

This approach would be appropriate if the total NER would also be considered for the DT_{50} calculation as a worst-case assumption, then a further NER characterisation would decrease the DT_{50} value (see project partners approach). Instead, if the DT_{50} values are higher than the vP cut-off criteria and only the parent identified in the solvent and PLE extracts, then further characterization of NER seems unnecessary (see UBA approach).

For chemicals which do not exceed the P-criteria (without consideration of NER), then there is little expectation that they would be persistent when/if remobilised again.

We disagree, as type I NER which are reversibly bound, might contain high amounts of parent compound or transformation products (see e.g. Cao et al., 2020) which have to be considered for the P assessment/ calculation of the DT_{50} values.

In the persistence assessment, the primary degradation half-lives and the P criteria of annex XIII of REACH have to be compared. Taking into account type-I NERs, how do you calculate the half-life? Do you simply add extractable parent + NER-type altogether and calculate as if the whole were the same? If yes, is that to say that a substance with >50% NER-type I would be considered infinitely persistent? (Question was addressed during the meeting to Vincent Bonnomet, ECHA)

This question is addressed by this presentation [2021_02_17_15_50_NER-Projects_Kinetic_Zur_Veröffentlichung]. One possible option is indeed to sum the amount of parent substance extracted with the amount of type I NER. Another option, is to sum the

amount of parent substance extracted with the amount of parent substance analysed from type I NER extracts. In both cases, data for different times should be available in order to get a kinetic, to calculate a degradation rate and a half-life. Furthermore, in any case, if degradation products are identified, a PBT/vPvB assessment should be conducted for them as well**. (Answer from Vincent Bonnomet, ECHA)**

What is the implication of this scheme for the future use of non-radiolabelled biodegradation data from simulation tests and other non-standard experiments (e.g. from academic studies) in the weight of evidence for persistence assessments? And what is the implication of this for the assessment of complex substances (UVCBs - ~20% of REACH registered substances) which are generally not amendable to radiolabelling? At present it is envisaged that P assessment of UVCBs could be conducted based on a whole substance approach, but this scheme seems to necessitate a constituent-based approach by default? (Question was addressed during the meeting to Vincent Bonnomet, ECHA)

This question goes beyond the scope of the workshop. UVCB substances present specific challenges that are not restricted to the assessment of NER. Obviously, if one does not have a sensitive enough analytical method for UVCB, then the whole PBT/vPvB assessment becomes challenging. It may be possible to work from homogenous fractions (e.g. so-called blocks) or from worst-case or representative constituents. One can refer to guidance R.11 for further details on the PBT/vPvB assessment of UVCB substances. **(Answer from Vincent Bonnomet, ECHA)**

In addition, I would also like to clarify another point which I realize may have been misunderstood. (Statement from Vincent Bonnomet, ECHA, valid only for REACH substances)

The QUANTIFICATION of total NER is in general mandatory in ECHA Decisions. The reason is because the mass balance has to be proven.

The CHARACTERISATION of NER (i.e. into Type I, II and III) is optional. It can be conducted by the registrants to try to demonstrate that their substance is not P/vP, if they believe that a large fraction of NER are actually immobilized NER or bioNER.

General Questions

What are the next steps? We talked about consensus but is there going to be an opportunity for participants to discuss with our policy makers? Not every regulatory body that is affected by this topic has been able to attend today and therefore many of us would like to return to our teams to obtain opinions from our colleagues

Next step is this public consultation on the proposed concepts for consideration of NER in the P assessment, so that all stakeholders have the opportunity to comment.

Based on the comments received within the framework of this consultation one proposal for a harmonised concept for consideration of the relevant NER in persistence assessment will be developed. The concept will then be discussed in the ECHA PBT Expert group, in ECHA and possibly in other regulatory authorities (e.g. EFSA for pesticides, EMA for pharmaceuticals). Further steps should then be initiated by ECHA/ COM and possibly further competent authorities.