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Further development of screening tests for the evaluation of potential PBT substances

Final report

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Further development of screening tests for the evaluation of potential PBT substances

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Abstract: Further development of screening tests for the evaluation of potential PBT substances

Ready biodegradability tests (RBTs) of the OECD 301 series and the OECD 310 are currently used for testing of ready biodegradability and for the identification of potentially persistent substances. The project aimed giving recommendations for further development and standardization of these tests next to improve knowledge about the application of so called "enhanced" ready tests (eRBT), where a longer test duration up to 60 d and larger vessel volumes are allowed. A survey among European laboratories was performed, to identify their experiences with ready biodegradability testing. The results were discussed among experts on an international workshop in April 2019. Further on, a practical testing programme has been realised with five test compounds in 4 testing series under different conditions. Here, Ibuprofen and 4-Fluorophenol were regarded as being non-persistent while the results for Piperonylbutoxide were inconclusive ("potentially P"). Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)-propionate did not meet the 60% pass level in any test series and thus also is "potentially P". Cis-13-Docosenonamide (Erucamide) can be considered as being non-persistent based on results from several tests, but some doubts remain, since a high variability between replicates was observed. The testing experience led to suggestions regarding the test design, the evaluation and the interpretation of eRBTs, which could be used as a starting point for further guidance. The impact of the proposed recommendations for the persistence assessment under REACH are discussed.

Kurzbeschreibung: Titel

Abbautests der OECD 301/310 -Reihe werden derzeit zur Prüfung der leichten biologischen Abbaubarkeit und zur Identifizierung potenziell persistenter Stoffe verwendet. Das Projekt zielte darauf ab, Empfehlungen für die Weiterentwicklung und Standardisierung dieser Tests zu geben und das Wissen über die Anwendung sogenannter „enhanced" Ready-Tests (eRBT) zu verbessern, bei denen eine längere Testdauer bis zu 60 d und größere Behältervolumina zulässig sind. Es wurde eine Umfrage unter europäischen Laboren durchgeführt, um deren Erfahrungen mit Tests zur leichten biologischen Abbaubarkeit zu ermitteln. Die Ergebnisse wurden im April 2019 auf einem internationalen Workshop mit Expertinnen und Experten diskutiert. Darüber hinaus wurde ein praktisches Testprogramm mit fünf Substanzen in 4 Versuchsreihen bei unterschiedlichen Randbedingungen durchgeführt. Dabei wurden Ibuprofen und 4-Fluorphenol als nicht persistent angesehen, während die Ergebnisse für Piperonylbutoxid nicht eindeutig waren („potenziell P"). Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)-propionat erreichte in keiner Versuchsreihe den 60 % Schwellenwert und ist somit ebenfalls „potenziell P". Für Cis-13-Docosenonamid (Erucamid) zeigen die Ergebnisse einiger Tests, dass die Substanz als nicht persistent anzusehen ist. Es bleiben jedoch einige Zweifel bestehen, da eine große Variabilität zwischen Replikaten beobachtet wurde. Die Erfahrung aus dem Untersuchungsprogramm führte zu Vorschlägen bezüglich des Testdesigns, der Bewertung und der Interpretation von eRBTs, die als Ausgangspunkt für weitere Anleitungen verwendet werden könnten. Die Auswirkungen der vorgeschlagenen Empfehlungen für die Persistenzbewertung unter REACH werden diskutiert.

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List of abbreviations

4FP	4-Fluorophenol
ASTM	American Society for Testing and Materials
ATU	Allylthiourea
BOD	Biological oxygen demand; BOD_5 = BOD after 5 days
CAS	Chemical Abstracts Service
CEFIC	European Chemical Industry Council
CHRIP	Chemical Risk Information Platform
CO_2	Carbon dioxide
COD	Chemical oxygen demand
DAR	Draft Assessment Report
DEG	Diethyleneglycol
DOC	Dissolved organic carbon
DoE	Difference of extremes
d.s.	Dry solids
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EU	European Union
eRBT	Enhanced ready biodegradability test
ERU	Erucamide = cis-13-docosenamide
HLC	Henry's Law Constant
HSDB	Hazardous Substances Data Bank
IBU	Ibuprofen
IC	Inorganic carbon
ISO	International Organization for Standardization
ITS	Integrated assessment and testing strategy for persistence assessment
LRI	Long Research Initiative (CEFIC)

MLR	Mass loading rate = biological oxygen demand (BOD_5) per total suspended solids per day (equivalent to the sludge loading rate, SLR)
MITI	Ministry of International Trade and Industry (Japan)
NER	Non-extractable residues
NITE	National Institute of Technology and Evaluation (Japan)
O_2	Oxygen
OBP	Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate
OECD	Organisation for Economic Co-operation and Development
PBT	Persistent, bioaccumulative and toxic
PE	Population equivalent = unit per capita loading
PBO	Piperonylbutoxide
QAC	Quaternary ammonium compounds
RBT	Ready biodegradability test
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals (Regulation (EC) No 1907/2006)
SLR	Sludge loading rate (see MLR)
STP	Sewage treatment plant
SVHC	Substance of very high concern
$ThCO_2$	Theoretical carbon dioxide evolution
ThOD	Theoretical oxygen demand
TNO	Toegepast Natuurwetenschappelijk Onderzoek (The Netherlands Organisation for Applied Scientific Research)
TOC	Total organic carbon
US EPA	United States Environmental Protection Agency
UVCB	Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials
vP/vB	Very persistent, very bioaccumulative
WoE	Weight-of-Evidence
WP	Work package

Summary

Ready biodegradability tests (RBTs) of the OECD 301 series and the OECD 310 are currently used for testing of ready biodegradability and for the identification of potentially persistent substances in the framework of evaluation processes for persistent, bioaccumulating and toxic (PBT) substances. Although these screening tests differ considerably in methodology and historical background, the test results are considered as equivalent. Thus, one goal of the project was to give recommendations for further development and standardization of these tests in order to improve the comparability of the test results.

RBTs are considered stringent, but are based on very artificial test conditions. Therefore, only positive results can be used to show that a substance is non-persistent, whereas a negative result requires the application of higher tier OECD simulation tests, which are carried out under more realistic conditions and allow the derivation of degradation half-lives. However, these tests usually require the use of ^{14}C-radiolabelled test substances and are much more time-consuming and cost-intensive compared to screening tests. In persistency assessment according to ECHA Guidance R.7b biodegradation data derived from so-called enhanced ready biodegradation tests (eRBTs) can be used in an intermediate step to close the gap between screening tests and complex simulation studies (ECHA 2017a). However, only few data from these tests are available and neither guidance on the design and interpretation of eRBTs, nor suitable validity criteria have been established so far. Hence, another objective of the project was to improve the level of knowledge to facilitate the application of eRBTs for persistency assessment.

Literature research and current status of ready biodegradability testing

In WP1 (work package 1), a systematic comparison of the established test guidelines for determining ready biodegradability has been carried out while examining options for their harmonisation. Most RBTs have been developed in the 1970ties and 80ties and have not been adopted since 1992. Due to their historical and technical diversity, they allow some variability and different specifications in terms of the test concentration, source and concentration of the inoculum, and measuring devices. A survey among European laboratories was performed, to identify their experiences with ready biodegradability testing. A detailed questionnaire covering the different options on performing RBTs of the OECD 301/310 series was drafted and sent to highly qualified (mainly GLP certified) laboratories. In total, 16 laboratories contributed to the survey: 10 from Germany, 3 from Switzerland and 3 from the United Kingdom. The outcome of the survey is summarized in the following:

- The two main RBTs applied were the CO_2 Evolution Test (OECD 301 B) and the Manometric Respirometry Test (OECD 301 F), which accounted for at least 80% of all RBTs performed. Activated sludge is the preferred inoculum.
- The CO_2 Headspace (OECD 310) is the third most important test. Most laboratories use activated sludge from municipal STP as inoculum, which is washed in mineral medium and aerated with CO_2 free air for 1-7 days. For the quantification of the CO_2 most laboratories measure the IC in the liquid phase after addition of NaOH.
- The DOC based tests OECD 301 A and E are rarely applied. Abiotic controls (without inoculum) or adsorption controls (with inoculum) are not always used (only on request of the sponsor). The influence of adsorption on DOC-elimination is assessed either by solubility pre-tests (DOC recovery) or by comparing measured DOC values after 0 h, 3 h or 24 h with expected ones

- The MITI (I) test according to OECD 301 C is rarely applied. The main challenge of the test is the inoculum source. Some laboratories use the standard mixed inoculum from at least 10 sites (sewage treatment plants (STP), rivers, lakes, surface soil etc.), which is further cultivated in the laboratory, others use activated sludge.
- With respect to the inoculum source most laboratories supported the use of any of the allowed OECD 301 inoculum sources in any RBT as far as the validity criteria are met. However, the signal-to-noise relation should be considered.
- The main guidance used for test method selection are the general introduction to OECD 301 and Annexes II and III thereof.
- With regard to reduction of the number of different OECD 301 test methods, 7 laboratories suggest to merge OECD 301 A and E (DOC-elimination) and 8 laboratories propose to merge OECD 301 C and F (respirometric methods). In addition, 7 laboratories indicated that OECD 301 C (MITI (I) test) could be withdrawn due to the effort for sampling and cultivating the inoculum and its low potency.
- 6 laboratories apply combination tests with several endpoints (e.g. CO_2 + O_2, CO_2 + DOC, O_2 + DOC, CO_2 + O_2 + DOC).
- Half of the laboratories agree that endpoints referring to mineralization (O_2, CO_2) should be preferred to methods using only DOC-elimination.
- 9 laboratories think that the endpoint CO_2 should be preferred for nitrogen containing substances since oxygen consumption due to nitrification may cause conflicting results.
- Most labs agree that volatile substances should be tested in closed test systems (e.g. OECD 301 C, F or OECD 310).
- For inhibitory substances some laboratories consider OECD 301 D (Closed Bottle test) as best option due to the lowest test concentration, whereas others refer to good results with OECD 301 F (using lower test concentrations) or to OECD 310. Alternatively, adsorbents (e.g. silica gel, humic acids) might be used with OECD 301 B or OECD 301 F to reduce toxicity.
- With regard to the vessel size, some participants suggest to allow any volume as long as the validity criteria are fulfilled.
- In view of the number of replicate vessels needed, most laboratories think that existing requirements are sufficient.
- 10 laboratories propose to use obligatory adsorption controls for DOC-based test methods, whereas 4 laboratories refuse this proposal.
- No concordant opinion exists regarding the question if reference compounds should be selected according to the test item (e.g. microcrystalline cellulose for solid test items, rapeseed oil for non-water soluble lubricants).

- The participants recommend to use inoculum from STP with predominantly domestic sewage and to improve the characterization of the inoculum (i.e. description of origin and source, indication of industrial share and treatment technology). Furthermore, any of the allowed OECD 301 inoculum sources could be used provided that validity criteria are met and the signal-to-noise ratio is considered.
- Measurement of microbiological parameters (e.g. colony counts) are only performed if requested by the sponsor.

The results were presented and discussed at the international workshop on the "Current status of ready biodegradability testing and options for improvements" at the German Environment Agency on April 2nd, 2019.

Selection of test substances for practical testing

In WP2 potential test substances for the practical investigations in WP 3 were searched. In particular, substances with an expected biodegradability of 20-40% in standard screening tests or substances with low water solubility for which valid simulation studies are available should be included. For this purpose, extensive database search has been carried out using the OECD eChemPortal (including ECHA data on registered substances), the EFSA Draft Assessment Reports (EU review of active substances applied in plant protection products), the Japan CHEmical Collaborative Knowledge database (J-CHECK, mainly covering OECD 301C data), as well as scientific reports (e.g. ECETOC) and literature research. The results of the selection process are documented in Annex B of this report.

Practical testing programme

In WP3 five substances selected in WP2 have been tested in order to examine the influence of the changed test conditions in eRBTs. These included an extension of the test duration, the enlargement of the test vessels and the use of alternative inoculum sources. In addition, so-called combination tests with parallel determination of several endpoints (DOC elimination and CO_2 evolution) were performed. The selected test substances were the anti-inflammatory drug Ibuprofen (IBU), the pesticide synergist Piperonylbutoxide (PBO), the antioxidant Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)-propionate (OBP), the intermediate substance 4-Fluorophenol (4FP), and the cosmetic ingredient Cis-13-Docosenamide (Erucamide, ERU). Diethylene glycol was used as reference compound in all experiments. The experiments were performed in 4 test series:

- Test series 1: OECD 301 F test, 28 d and 60 d with typical volume and activated sludge as inoculum
- Test series 2: OECD 301 F test, 28 d and 60 d with 4 times increased volume and activated sludge as inoculum but retaining the substrate/inoculum ratio typical for the test system as defined by the test guideline
- Test series 3: OECD 301 F test, 28 d and 60 d with typical volume and final effluent from STP as inoculum
- Test series 4: OECD 301 A/B combination test with parallel determination of DOC and CO_2 and activated sludge as inoculum

In total, 20 biodegradation tests with the five substances were conducted. Test series 1 and 2 were run in parallel to investigate the influence of the testing volume.

Based on the results of the practical testing programme, the test substances were rated as „not persistent“ or „potentially persistent“ in accordance with ECHA guidance. In these tests the drug IBU and the intermediate 4FP were biodegraded for more than 60% within 60 days and therefore considered as being “not persistent”. A mean mineralisation of 20% - 50% was measured for PBO and OBP (= “potentially P”). The mineralisation of ERU was in the range between 36% and 64% after 60 days with activated sludge, whereas only 21% were obtained with the effluent from the STP. However, the validity criteria were not met due to a high variability between replicates, which may be explained by differing adaptation processes of the inoculum within the tests. Diethylene glycol reached the pass level of 60% mineralisation within 28 days in all test series and thus proved to be a suitable reference substance for eRBTs, the 10-day window was most often fulfilled.

Preliminary guidance for enhanced ready biodegradability testing

In WP4 recommendations for the test design, the evaluation and the interpretation of eRBTs are made, which could be used as a starting point for further guidance. Enhanced screening tests are designed for assessing whether a test substance can be assumed as “not persistent” in the environment. The intention is to avoid the performance of extensive simulation degradation tests for substances failing the criteria for being readily biodegradable. According to ECHA guidance the currently accepted enhancements of RBTs are the prolongation of the test duration beyond 28 days and the use of larger test vessels. The increase of biomass concentration, and the pre-exposure of the inoculum to the test substance at low concentrations are not accepted. Thus, eRBTs are by design ready type tests with a longer test duration for up to 60 days (ECHA 2017a).

The following recommendations reflect the personal view of the authors, resulting from the overall findings of the research project:

- For enhanced testing all respirometric test methods of OECD 301 and OECD 310 for ultimate biodegradability (CO_2 evolution and/or O_2 consumption) can be applied. The OECD 301 A or OECD 301 E are not appropriate, because DOC-elimination might be misinterpreted as degradation, if abiotic processes take place. However, additional DOC-measurements in ultimate biodegradation tests can provide helpful information in combination with CO_2 evolution or O_2 depletion. The OECD 301 A/B combination test is particularly interesting for substances with a limited water solubility, because hydrolysis or other factors influencing water solubility can be detected. Hence, DOC-elimination is considered as a supporting parameter while ultimate biodegradability is referred to CO_2 evolution. For semi-volatile substances closed systems (i.e. OECD 301 F or OECD 310) are preferred and for inhibitory substances the Closed Bottle Test (OECD 301 D) is an option.

- The use of larger test vessels for increasing the initial amount of microorganisms and the biodiversity of the inoculum is recommended. However, the substrate-inoculum ratio should be maintained. The upper limit of the test vessel size is only limited by practical implementation. In practice, the use of only two replicates is standard for OECD 301 B or OECD 301 F tests, but a higher number of replicates increases the accuracy of determined mean values, and allows the exclusion of obvious outliers. It is thus recommended to use at least three flasks or vessels containing the test substance plus inoculum, the same number

containing inoculum only and at least two flasks or vessels containing the reference substance.

- It is suggested that all inoculum sources used for OECD 301 or OECD 310 should also be allowed for eRBTs within the respective inoculum concentrations indicated. When activated sludge is used, it should be sampled from a sewage treatment plant receiving predominantly domestic sewage (with only minor industrial contribution). The location, treatment capacity (preferably the number of inhabitants connected to the STP), main process stages and key parameters (e.g. sludge retention time and sludge concentration) of the sewage treatment plant should be reported. The inoculum sample should be representative and should not be heavily influenced by stormwater from rainfall events. In order to reduce peak or low loads during the course of the week, sampling dates between Tuesday and Thursday may be chosen, when using inocula from sewage treatment plants. The exact sampling point should be described. Pre-conditioning of the inoculum for one or two days (e.g. to reduce the overall activity to comply with the validity criteria) is allowed, but pre-adaption to the test substance not. The use of mixed inocula from different sources (e.g. activated sludge, final effluent, surface water) is allowed, as far as their concentrations do not exceed those specified in OECD 301 (e.g. 30 mg/L dry solids activated sludge or 10 Vol% final effluent) and if the validity criteria for the inoculum blanks are fulfilled.

- In principle, any of the reference substances used for RBTs such as sodium benzoate, sodium acetate or aniline can be used as procedure control. However, for eRBTs the degradation of the positive reference compound should preferably not reach the pass level before day 14 to enable a longer control of inoculum activity during prolonged tests. In the practical testing programmes diethylene glycol has been identified as a suitable reference compound, which is biodegraded by >60% within 28 days under ready type test conditions. The 10-days-window needs not to be applied when using diethylene glycol. The use of additional negative reference compounds, which should fail eRBTs, is recommended, particularly when solvents or inert supports are used for application of the test compound, in order to prevent artefacts in the test design.

- The combination of different endpoints in the combination test according to OECD 301 A/B enables a better understanding of the biodegradation process, particularly for test items, whose water solubility changes within the test period due to hydrolysis or biodegradation, such as IBU or PBO.

The OECD 301 validity criteria for the inoculum blanks of ≤ 60 mg/L oxygen consumption or ≤ 70 mg/L CO_2 evolution were met in all experiments performed in WP3, even after prolongation to 60 days. Obviously, the alternative inoculum (effluent final clarifier) was less potent than activated sludge, despite its higher colony counts compared to activated sludge. This is confirmed by the activity of the inoculum blank values for the effluent final clarifier, which was about a factor of 2 lower compared to that using activated sludge.

The accompanying microbiological analysis of countable colonies in activated sludge revealed highly different values. In addition, the differences observed in the colony counts were not reflected in the degradation extents of the reference substance DEG. Based on these results, the

determination of countable colonies does not provide an accurate estimate of the inoculum activity.

Use of enhanced ready tests for P-assessment

In WP5 the impact of the project results for the persistence assessment under REACH was analysed. According to REACH guidance R.11 all relevant available information on (bio)degradation should be used for persistence assessment, including RBTs, screening information (i.e. inherent tests and eRBTs), further useful information (e.g. QSARs and monitoring data) and simulation tests. Experience shows that results from these diverse test systems can lead to different conclusions. Simulation tests, however, are performed at environmentally realistic conditions. Only from these tests, definitive degradation half-lives that can be compared directly to the persistence criteria of REACH Annex XIII, can be derived. Nevertheless, all test systems have uncertainties and each test system has its own pros and cons that have to be reckoned with.

The role of eRBTs in relation to inherent screening tests needs to be clarified. Enhanced screening tests have a lower inoculum concentration, but a longer test duration compared to inherent tests. The last have a higher inoculum concentration, but are quite restrictive with respect to the test duration (7 or 14 days) when used for persistency evaluation. Our results from the practical testing programme demonstrate that diethylene glycol (DEG) reaches the pass level of >60% within 28 days under (enhanced) ready biodegradation conditions, while it is also used as reference compound in the inherent biodegradability test according to OECD 302 B, where it must reach the pass level of 70% DOC-elimination within 14 days. For persistency evaluation from inherent tests, the pass level has to be reached within 7 days for assessing a test substance as “not P”. It seems that the test duration for reaching the pass level is more conservative for inherent tests than for eRBTs. Biodegradation above 20% in inherent tests might be understood as evidence of inherent, primary biodegradability and suggests that stable degradation products are likely to be formed (ECHA 2017a). Enhanced ready biodegradation tests may be regarded as comparable to inherent tests, but without applying the specific criteria for inherent tests, i.e. lag phase < 3 d, pass-level reached within 7 (OECD 302 B) or 14 days (OECD 302 C). According to ECHA guidelines a lack of biodegradation (<20%) in an inherent test may be considered as evidence that the test item is ”P” without the need of further testing. The authors suggest, that a similar approach may also be used for eRBT. That means that results from eRBTs showing degradation extents ≤ 20% may be used as evidence for “P”, provided that no other evidence of false negative test results such as inhibitory effects have been observed.

With respect to the results from WP3 the following conclusions can be drawn:

- IBU can be regarded as being “non-persistent” (congruent results from test series 1, 2 and 4). In fact, IBU was assessed as being readily biodegradable.
- The results of PBO are inconclusive with respect to persistence. Although, the pass level of 60% was reached in one replicate of test series 4, the validity criterion for the allowed variability between replicates of 20% was failed. Beyond that, results from test series 1, 2 and 3 showed degradation extents clearly below 60% after 60 days.
- OBP did not meet the 60% pass level in any test series. The results are inconclusive with respect to persistency (“potentially P or vP”).
- 4FP is assumed to be “non-persistent” according to data from test series 1, 2 and 4.

- ERU can be assumed as being “non-persistent” based on results of test series 2, even though some doubts remain, since the 20% criterion for the variability between replicates was failed in test series 1 and ultimate biodegradability was clearly below the pass level of 60% in test series 4.
- Neither PBO nor OBP can be assumed as being “persistent” from the results, because some biodegradation above 20% took place, but are “potentially P or vP”, which indicates the continuing need for higher tier biodegradation tests.
- The reference substance DEG reached the pass level for ready biodegradation of 60% in all test series within 28 days (total of 6 independent experiments with 3 replicates each). The 10-day-window criterion was most often met, except in test series 3 with the alternative inoculum. After 60 days the degradation extents reached 79-105%. This is in good accordance with several other results from standard RBTs, reaching 59-98% mineralization based on CO_2 evolution or O_2 consumption as well as 90-100% DOC-elimination (ECHA 2021b, MITI 1992).
- The variability of the degradation extents observed between replicates in the OECD 301 F with larger volume flasks (740 mL) was considerably lower compared to typical volume flasks (164 mL).

The results of the practical testing programme also show, that the added value of enhanced testing compared to standard RBTs was limited for the selected test substances. Only for ERU the prolongation of the test duration in combination with larger test vessels did result in a changed assumption as “non-persistent”, while for IBU, 4FP and the reference compound DEG the pass level of 60% was already reached after 28 days and all were assumed as being readily biodegradable. In addition, the eRBT results for ERU were helpful to evaluate the variable results from other biodegradation data and confirmed the biodegradability of ERU under specific test conditions.

Overall, the test prolongation up to 60 days and the use of larger test vessels proved to be suitable enhancements, resulting in transferable validity criteria and reliable biodegradation data for eRBTs, which can be used to identify non-persistent compounds under REACH according to the integrated assessment and testing strategy for persistence assessment (ECHA 2017b).

Zusammenfassung

Abbaubarkeitstests der OECD 301/310 -Reihe (ready biodegradability tests = RBTs) werden derzeit zur Prüfung der leichten biologischen Abbaubarkeit und zur Identifizierung potenziell persistenter Stoffe im Rahmen der Bewertungsverfahren für persistente, bioakkumulierbare und toxische (PBT) Stoffe verwendet. Obwohl sich diese Screening-Tests in Methodik und historischem Hintergrund erheblich unterscheiden, werden die Testergebnisse als gleichwertig angesehen. Ein Ziel des Projektes war es daher, Empfehlungen zur Weiterentwicklung und Standardisierung dieser Tests zu erarbeiten, um die Vergleichbarkeit der Testergebnisse zu verbessern.

RBTs gelten als stringent, basieren aber auf sehr artifiziellen Testbedingungen. Daher können nur positive Ergebnisse als Hinweis verwendet werden, dass ein Stoff nicht persistent ist. Ein negatives Ergebnis führt hingegen zur Durchführung höherwertiger OECD-Simulationstests, die unter realistischeren Testbedingungen durchgeführt werden und die Ableitung von Abbauhalbwertszeiten ermöglichen. Diese Tests erfordern jedoch in der Regel den Einsatz von ^{14}C-markierten Testsubstanzen und sind im Vergleich zu Screeningtests deutlich zeit- und kostenintensiver. Bei der Persistenzbewertung gemäß ECHA Guidance R.7b können in einem Zwischenschritt Bioabbaudaten aus sogenannten Enhanced Ready Biodegradation Tests (eRBTs) verwendet werden, um die Lücke zwischen Screening-Tests und aufwändigen Simulationsstudien zu schließen (ECHA 2017a). Da nur wenige Daten zu diesen Tests vorliegen und bisher weder Leitlinien für das Design und die Interpretation von eRBTs noch Vorschläge für Validitätskriterien festgelegt wurden, war ein weiteres Ziel des Projektes, den Wissensstand zur Anwendung von eRBTs für die Persistenzbewertung zu verbessern.

Literaturrecherche und aktueller Stand der Prüfung auf leichte biologische Abbaubarkeit

In AP1 (Arbeitspaket 1) wurde ein systematischer Vergleich der etablierten Prüfrichtlinien zur Bestimmung der leichten biologischen Abbaubarkeit durchgeführt und Möglichkeiten zu deren Harmonisierung geprüft. Die meisten RBTs wurden in den 1970er und 80er Jahren entwickelt und seit 1992 nicht mehr überarbeitet. Aufgrund ihrer historischen und technischen Vielfalt lassen sie eine gewisse Variabilität und unterschiedliche Spezifikationen in Bezug auf Testkonzentration, Quelle und Konzentration des Inokulums und Messwerterfassung zu. Es wurde eine Umfrage bei europäischen Labors durchgeführt, um ihre Erfahrungen mit Tests zur leichten biologischen Abbaubarkeit zu ermitteln. Ein detaillierter Fragebogen, der die verschiedenen Optionen zur Durchführung von RBTs der OECD-Reihe 301/310 abdeckt, wurde entworfen und an qualifizierte (hauptsächlich GLP-zertifizierte) Laboratorien versandt. Insgesamt haben 16 Labore an der Umfrage mitgewirkt: 10 aus Deutschland, 3 aus der Schweiz und 3 aus Großbritannien. Das Ergebnis der Umfrage lässt sich wie folgt zusammenfassen:

- Die beiden am häufigsten angewandten RBTs sind der CO_2-Entwicklungstest (OECD 301 B) und der manometrische Respirometertest (OECD 301 F), die mindestens 80% aller durchgeführten RBTs ausmachten. Belebtschlamm ist das bevorzugte Inokulum.
- Der CO_2 Headspace (OECD 310) ist der drittwichtigste Test. Die meisten Labore verwenden Belebtschlamm aus kommunalen Kläranlagen als Inokulum, der in mineralischem Medium gewaschen und 1-7 Tage lang mit CO_2-freier Luft belüftet wird. Zur Quantifizierung des CO_2 messen die meisten Labore den IC in der flüssigen Phase nach Zugabe von NaOH.
- Die DOC-basierten Tests OECD 301 A und E werden selten angewendet. Abiotische Kontrollen (ohne Inokulum) oder Adsorptionskontrollen (mit Inokulum) werden nicht

immer verwendet, sondern nur auf Anfrage des Sponsors. Der Einfluss der Adsorption auf die DOC-Elimination wird entweder durch Löslichkeitsvorversuche (DOC-Wiederfindung) oder durch Vergleich gemessener DOC-Werte nach 0 h, 3 h oder 24 h mit erwarteten bewertet.

- Der MITI (I)-Test nach OECD 301 C wird selten angewendet. Die größte Herausforderung des Tests ist die Inokulumquelle. Einige Labore verwenden das Standard-Mischinokulum von mindestens 10 Standorten (Kläranlagen, Flüsse, Seen, Oberflächenboden etc.), das im Labor weiter kultiviert wird, andere verwenden Belebtschlamm.
- In Bezug auf die Inokulumquelle befürworteten die meisten Laboratorien die Verwendung aller zulässigen OECD 301 Inokuli in jedem RBT, sofern die Gültigkeitskriterien erfüllt sind. Allerdings sollte das Signal-Rausch-Verhältnis berücksichtigt werden.
- Die wichtigsten Leitlinien für die Auswahl der Testmethoden sind die allgemeine Einleitung zu OECD 301 sowie die Anhänge II und III
- Im Hinblick auf die Reduzierung der Anzahl unterschiedlicher Testmethoden nach OECD 301 schlagen 7 Laboratorien vor, die OECD 301 A und E (DOC-Eliminierung) und 8 Laboratorien, die OECD 301 C und F (respirometrische Methoden) zusammenzuführen. Darüber hinaus gaben 7 Labore an, dass OECD 301 C (MITI (I)-Test) aufgrund des Aufwands für die Probenahme und Kultivierung des Inokulums und seiner geringen Wirksamkeit zurückgezogen werden könnte.
- 6 Labore wenden Kombinationstests mit mehreren Endpunkten an (z. B. CO_2 + O_2, CO_2 + DOC, O_2 + DOC, CO_2 + O_2 + DOC).
- Die Hälfte der Labore stimmt der Aussage zu, dass Endpunkte, die sich auf die Mineralisierung (O_2, CO_2) beziehen, Methoden vorgezogen werden sollten, die nur die DOC-Eliminierung erfassen.
- 9 Labore sind der Meinung, dass der Endpunkt CO_2 für stickstoffhaltige Substanzen bevorzugt werden sollte, da der Sauerstoffverbrauch durch Nitrifikation zu widersprüchlichen Ergebnissen führen kann.
- Die meisten Labore stimmen darin überein, dass flüchtige Substanzen in geschlossenen Testsystemen getestet werden sollten (z. B. OECD 301 C, F oder OECD 310).
- Für hemmende Substanzen halten einige Labore OECD 301 D (Closed Bottle Test) aufgrund der niedrigsten Testkonzentration für die beste Option, während andere auf gute Ergebnisse mit OECD 301 F (mit niedrigeren Testkonzentrationen) oder auf OECD 310 verweisen. Alternative Adsorptionsmittel (z.B. Kieselgel, Huminsäuren) können mit OECD 301 B oder OECD 301 F verwendet werden, um die Toxizität zu verringern.
- In Bezug auf die Gefäßgröße schlagen einige Teilnehmer*innen vor, jedes Volumen zuzulassen, solange die Gültigkeitskriterien erfüllt sind.

- In Anbetracht der Anzahl der benötigten Replikate (Parallelansätze der Gefäße) sind die meisten Laboratorien der Meinung, dass die bestehenden Anforderungen ausreichend sind.
- 10 Laboratorien schlagen vor, obligatorische Adsorptionskontrollen für DOC-basierte Testmethoden zu verwenden, während 4 Laboratorien diesen Vorschlag ablehnen.
- Es gab keine übereinstimmende Meinung zu der Frage, ob Referenzverbindungen je nach Prüfgegenstand ausgewählt werden sollten (z. B. mikrokristalline Cellulose für feste Prüfgegenstände, Rapsöl für nicht wasserlösliche Schmierstoffe).
- Die Teilnehmer*innen empfehlen, Inokulum aus Kläranlagen mit überwiegend häuslichem Abwasser zu verwenden und die Charakterisierung des Inokulums (d. h. Beschreibung von Herkunft und Art, Angabe des industriellen Anteils und der Behandlungstechnologie) zu verbessern. Darüber hinaus kann jede der zulässigen OECD 301-Inokulumquellen verwendet werden, vorausgesetzt, dass die Gültigkeitskriterien erfüllt sind und das Signal-Rausch-Verhältnis berücksichtigt wird.
- Messungen mikrobiologischer Parameter (z. B. Koloniezahlen) werden nur auf Anfrage des Sponsors durchgeführt.

Die Ergebnisse wurden am 02. April 2019 auf dem internationalen Workshop „Aktueller Stand der Prüfung der biologischen Abbaubarkeit und Verbesserungsmöglichkeiten“ im Umweltbundesamt vorgestellt und diskutiert.

Auswahl von Testsubstanzen für das praktische Untersuchungsprogramm

In AP2 wurden potentielle Testsubstanzen für die praktischen Untersuchungen in AP3 ausgewählt. Insbesondere sollten Stoffe mit einer erwarteten biologischen Abbaubarkeit von 20-40% in Standard-Screeningtests oder Stoffe mit geringer Wasserlöslichkeit, für die valide Simulationsstudien vorliegen, berücksichtigt werden. Zu diesem Zweck wurde eine umfassende Datenbankrecherche unter Verwendung des OECD eChemPortal (einschließlich ECHA-Daten zu registrierten Stoffen), der EFSA Draft Assessment Reports (EU-Peer-Review von Pflanzenschutzmittelwirkstoffen) und der Japan CHEmical Collaborative Knowledge Database durchgeführt (J-CHECK, das hauptsächlich OECD 301C-Daten abdeckt). Zudem wurden wissenschaftliche Berichte (z. B. ECETOC) ausgewertet und eine Literaturrecherche durchgeführt. Die Ergebnisse des Auswahlverfahrens sind in Anhang B dieses Berichts dokumentiert.

Praktisches Untersuchungsprogramm

In AP3 wurden fünf der in AP2 ausgewählten Substanzen getestet, um den Einfluss der erweiterten („enhanced“) Testbedingungen in eRBTs zu erfassen. Die geänderten Testbedingungen umfassten eine Verlängerung der Testdauer, die Vergrößerung der Testgefäße und die Nutzung alternativer Inokulumquellen. Zusätzlich wurden sogenannte Kombinationstests mit paralleler Bestimmung mehrerer Endpunkte (DOC-Elimination und CO_2-Entwicklung) durchgeführt. Die ausgewählten Testsubstanzen waren der Entzündungshemmer Ibuprofen (IBU), der Pestizid-Synergist Piperonylbutoxid (PBO), das Antioxidans Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionat (OBP), das Zwischenprodukt 4-Fluorphenol (4FP) und der kosmetische Inhaltsstoff Cis-13-Docosenamid (Erucasäureamid, ERU). Als Referenzsubstanz wurde in allen Experimenten Diethylenglykol verwendet. Die Versuche wurden in 4 Versuchsreihen realisiert:

- Versuchsreihe 1: OECD 301 F-Test, 28 d und 60 d mit typischem Volumen und Belebtschlamm als Inokulum
- Versuchsreihe 2: OECD 301 F-Test, 28 d und 60 d mit 4-fach erhöhtem Volumen und Belebtschlamm als Inokulum unter Beibehaltung des Substrat/Inokulum-Verhältnisses wie es für das Testsystem typisch und in der Testrichtlinie definiert ist
- Versuchsreihe 3: OECD 301 F-Test, 28 d und 60 d mit typischem Volumen und dem Ablauf des Nachklärbeckens einer kommunalen Kläranlage als Inokulum
- Versuchsreihe 4: OECD 301 A/B Kombinationstest mit paralleler Bestimmung von DOC und CO2 und Belebtschlamm als Inokulum

Insgesamt wurden 20 biologische Abbautests mit den fünf Substanzen durchgeführt. Die Versuchsreihen 1 und 2 wurden parallel gefahren, um den Einfluss des Flaschenvolumens zu untersuchen.

Basierend auf den Ergebnissen des praktischen Untersuchungsprogramms wurden die Testsubstanzen gemäß den Leitlinien der ECHA als „nicht persistent“ oder „potenziell persistent“ eingestuft. In diesen Tests wurden das Medikament IBU und das Zwischenprodukt 4FP innerhalb von 60 Tagen zu mehr als 60% biologisch abgebaut und daher als „nicht persistent“ eingestuft. Für PBO und OBP (= „potenziell P“) wurde eine mittlere Mineralisierung von 20% - 50% gemessen. Die Mineralisierung von ERU lag nach 60 Tagen mit Belebtschlamm im Bereich zwischen 36% und 64%, während mit dem Ablauf Nachklärbecken als Inokulum nur 21% erhalten wurden. Die Validitätskriterien wurden jedoch aufgrund einer hohen Variabilität zwischen den Replikaten nicht erfüllt, was durch unterschiedliche Anpassungsprozesse des Inokulums innerhalb der Tests erklärt werden kann. Diethylenglykol erreichte in allen Versuchsreihen innerhalb von 28 Tagen das Prüfkriterium (=“pass-level“) von 60% Mineralisierung und erwies sich damit als geeignete Referenzsubstanz für eRBTs, das 10-Tage-Fenster wurde meist eingehalten.

Hinweise zur Durchführung von „enhanced“ Screeningtests

In AP4 werden Empfehlungen für das Testdesign, die Auswertung und die Interpretation von eRBTs gegeben, die als Ausgangspunkt für eine noch zu erarbeitende Leitlinie dienen könnten. Erweiterte Screening-Tests dienen der Beurteilung, ob eine Testsubstanz als „nicht persistent“ in der Umwelt eingestuft werden kann. Damit soll die Notwendigkeit von umfangreichen Abbausimulationstests für Stoffe verringert werden, die die Kriterien für eine leichte biologische Abbaubarkeit nicht erfüllen. Gemäß den ECHA-Leitlinien sind die derzeit akzeptierten Erweiterungen („enhancements“) von RBTs die Verlängerung der Testdauer über 28 Tage hinaus und die Verwendung größerer Testgefäße. Die Erhöhung der Biomassekonzentration und die vorherige Exposition (Adaptation) des Inokulums gegenüber der Testsubstanz in niedrigen Konzentrationen werden nicht akzeptiert. Somit sind eRBTs vom Design her mit Tests auf leichte biologische Abbaubarkeit zu vergleichen, allerdings mit einer längeren Prüfdauer von bis zu 60 Tagen (ECHA 2017a).

Die folgenden Empfehlungen spiegeln die persönliche Sicht der Autoren und Autorinnen wider, die sich aus den Gesamtergebnissen des Forschungsprojekts ergeben:

- Für die erweiterten („enhanced“) Screeningtests können alle respirometrischen Testmethoden der OECD 301 und OECD 310 für die biologische Endabbaubarkeit (CO_2-Entwicklung und/oder O_2-Verbrauch) angewendet werden. Die OECD 301 A oder OECD 301

E sind nicht geeignet, da die DOC-Eliminierung als Abbau fehlinterpretiert werden könnte, wenn abiotische Prozesse ablaufen. Zusätzliche DOC-Messungen in biologischen Endabbautests können jedoch hilfreiche Informationen in Kombination mit der CO_2-Entwicklung oder dem O_2-Verbrauch liefern. Der Kombinationstest OECD 301 A/B ist besonders für Substanzen mit begrenzter Wasserlöslichkeit von Interesse, da Hydrolyse oder andere Faktoren, die die Wasserlöslichkeit beeinflussen, erfasst werden. Dabei wird die DOC-Eliminierung als unterstützender Parameter betrachtet, während die endgültige biologische Abbaubarkeit auf die CO_2-Entwicklung bezogen wird. Für semi-flüchtige Substanzen werden geschlossene Systeme (z. B. OECD 301 F oder OECD 310) bevorzugt, für hemmende Substanzen ist der Closed Bottle Test (OECD 301 D) eine Option.

- Die Verwendung größerer Testgefäße mit dem Ziel einer Erhöhung der Ausgangsmenge an Mikroorganismen und der Biodiversität des Inokulums wird empfohlen. Allerdings sollte hierbei das Substrat-Inokulum-Verhältnis beibehalten werden. Die Obergrenze der Prüfgefäßgröße ist nur aus praktischen Gründen limitiert. Üblicherweise sehen Guidelines für die Durchführung des OECD 301 B- oder des OECD 301 F nur zwei Replikate (Parallelansätze) vor. Eine höhere Anzahl an Replikaten erhöht jedoch die Genauigkeit der ermittelten Mittelwerte und ermöglicht den Ausschluss offensichtlicher Ausreißer. Es wird daher empfohlen, mindestens drei Kolben oder Gefäße mit der Prüfsubstanz plus Inokulum, die gleiche Anzahl mit dem Inokulum (Inokulumblindwert) und mindestens zwei Kolben oder Gefäße mit der Referenzsubstanz zu verwenden.

- Es wird vorgeschlagen, dass alle für OECD 301 oder OECD 310 zulässigen Inokulumquellen innerhalb der jeweils vorgegebenen Inokulumkonzentrationen auch für eRBTs verwendet werden können. Wenn Belebtschlamm verwendet wird, sollte er aus einer Kläranlage entnommen werden, die überwiegend häusliches Abwasser (mit einem geringen industriellen Anteil) behandelt. Der Standort, die Behandlungskapazität (vorzugsweise die Anzahl der an die Kläranlage angeschlossenen Einwohner), die Hauptverfahrensschritte und die wichtigsten Parameter (z. B. Schlammverweilzeit und Schlammkonzentration) der Kläranlage sind anzugeben. Die Inokulumprobe sollte repräsentativ sein und nicht stark durch Regenwasser aus Niederschlagsereignissen beeinflusst werden. Um Spitzen- oder Minderbelastungen im Wochenverlauf zu verringern, sollten bei der Verwendung von Inokula aus Kläranlagen Probenahmetermine zwischen Dienstag und Donnerstag gewählt werden. Der genaue Probenahmeort sollte beschrieben werden. Eine Vorkonditionierung des Inokulums für ein oder zwei Tage (z.B. um die Gesamtaktivität zu reduzieren, um die Gültigkeitskriterien zu erfüllen) ist zulässig, die Präadaptation an die Testsubstanz ist jedoch nicht zulässig. Die Verwendung von gemischten Inokula aus verschiedenen Quellen (z. B. Belebtschlamm, Endabwasser, Oberflächenwasser) ist zulässig, sofern ihre Konzentrationen die in OECD 301 angegebenen nicht überschreiten (z.B. 30 mg/L Trockensubstanz Belebtschlamm oder 10 Vol.% End Abwasser) und die Gültigkeitskriterien für die Inokulum-Blindwerte erfüllt sind.

- Als Verfahrenskontrolle können prinzipiell alle für RBTs verwendete Referenzsubstanzen wie Natriumbenzoat, Natriumacetat oder Anilin verwendet werden. Für eRBTs sollte der Abbau der positiven Referenzverbindung jedoch vorzugsweise nicht vor Tag 14 das „pass

level“ erreichen, um eine Kontrolle der Inokulumaktivität während der verlängerten Testdauer zu ermöglichen. Im praktischen Untersuchungsprogramm erwies sich Diethylenglykol als geeignete Referenzverbindung, die unter üblichen Testbedingungen innerhalb von 28 Tagen zu >60% biologisch abgebaut wird. Das 10-Tage-Fenster entfällt bei Verwendung von Diethylenglykol. Die Verwendung zusätzlicher negativer Referenzsubstanzen, die die eRBTs nicht bestehen sollten, wird insbesondere, wenn Lösungsmittel oder inerte Träger zur Dosierung der Testsubstanz verwendet werden, empfohlen, um Artefakte im Testdesign zu vermeiden.

- Die Kombination verschiedener Endpunkte im Kombinationstest nach OECD 301 A/B ermöglicht ein besseres Verständnis des biologischen Abbauprozesses, insbesondere bei Substanzen, deren Wasserlöslichkeit sich innerhalb des Testzeitraums durch Hydrolyse oder biologischen Abbau verändert, wie z.B. IBU oder PBO.

Die OECD 301-Validierungskriterien für die Inokulum-Blindwerte von ≤ 60 mg/L Sauerstoffverbrauch oder ≤ 70 mg/L CO_2-Entwicklung wurden in allen in Arbeitspaket 3 durchgeführten Experimenten auch nach Verlängerung auf 60 Tage erfüllt. Offensichtlich war das alternative Inokulum (Ablauf Nachklärbecken) trotz seiner höheren Kolonienzahlen im Vergleich zu Belebtschlamm weniger wirksam als Belebtschlamm. Bestätigt wird dies durch die Aktivität der Inokulum-Blindwerte für den Ablauf des Nachklärbeckens, die im Vergleich zum Belebtschlamm um etwa den Faktor 2 geringer waren.

Die begleitende mikrobiologische Analyse der Kolonienzahlen im Belebtschlamm ergab sehr unterschiedliche Werte. Außerdem spiegelten sich die beobachteten Unterschiede in den Kolonienzahlen nicht in den Abbaugraden der Referenzsubstanz Diethylenglykol wider. Basierend auf diesen Ergebnissen liefert die Bestimmung der Kolonienzahlen keine genauen Hinweise auf die Inokulumaktivität.

Verwendung von „enhanced“ Screeningtests für die P-Bewertung

In AP5 wurde die Auswirkung der Untersuchungsergebnisse auf die Persistenzbewertung unter REACH analysiert. Gemäß der REACH-Leitlinie R.11 sollten alle relevanten verfügbaren Informationen zum (biologischen) Abbau für die Persistenzbewertung verwendet werden, einschließlich RBTs, Screening-Informationen (d. h. inhärente Tests und eRBTs), weitere Informationsquellen (z.B. QSARs und Überwachungsdaten) und Simulationstests. Die Erfahrung zeigt, dass die Ergebnisse mit diesen verschiedenen Testsystemen zu unterschiedlichen Schlussfolgerungen führen können. Simulationstests werden jedoch unter umweltrealistischen Bedingungen durchgeführt und sind die einzigen Tests, die eine definitive Abbauhalbwertszeit liefern können, die direkt mit den Persistenzkriterien von REACH Anhang XIII verglichen werden kann. Dennoch haben alle Testsysteme Unsicherheiten und jedes Testsystem hat seine eigenen Vor- und Nachteile, die bei der Bewertung berücksichtigt werden sollten.

Die Stellung von eRBTs in Bezug auf inhärente Screeningtests sollte geklärt werden. Erweiterte Screeningtests (eRBT) haben eine geringere Inokulumkonzentration, aber eine längere Testdauer im Vergleich zu inhärenten Tests. Letztere haben eine höhere Inokulumkonzentration, sind aber recht restriktiv in Bezug auf die Testdauer (7 oder 14 Tage), wenn sie für die Persistenzbewertung verwendet werden. Unsere Ergebnisse aus dem praktischen Untersuchungsprogramm zeigen, dass Diethylenglykol innerhalb von 28 Tagen unter („enhanced“) Bedingungen das „pass-level“ von >60% erreicht. Die Substanz wird auch als Referenzverbindung in Tests zur inhärenten biologischen Abbaubarkeit gemäß OECD 302 verwendet, wobei „pass-level“ von 70% DOC-Eliminierung hier innerhalb von 14 Tagen erreicht

werden muss. Für die Persistenzbewertung aus inhärenten Tests muss das „pass-level“ innerhalb von 7 Tagen erreicht werden, um eine Testsubstanz als „nicht P“ einzustufen. Es scheint, dass die Testdauer zum Erreichen des „pass-level“ für inhärente Tests konservativer ist als für eRBTs. Ein biologischer Abbau von über 20% in inhärenten Tests wird als Hinweis auf eine inhärente, primäre biologische Abbaubarkeit gewertet und deutet darauf hin, dass vermutlich stabile Abbauprodukte gebildet werden. „Enhanced“ Screeningtests haben einige Gemeinsamkeiten mit inhärenten Tests, jedoch ohne Anwendung der spezifischen Kriterien für inhärente Tests, d. h. Lag-Phase < 3 d, „pass-level“ innerhalb von 7 (OECD 302 B) oder 14 Tagen (OECD 302 C) erreicht. Gemäß den ECHA-Leitfäden kann ein sehr geringer Abbau (< 20 %) in einem inhärenten Test als Hinweis gewertet werden, dass die Prüfsubstanz „P“ ist, ohne dass weitere Tests erforderlich sind. Die Autoren und Autorinnen schlagen vor, einen ähnlichen Ansatz auch für eRBTs zu verfolgen. Das bedeutet, dass Ergebnisse aus eRBTs, die Abbaugrade von ≤ 20% zeigen, als Hinweis auf „P“ gelten können falls keine anderen Hinweise für falsch negative Testergebnisse, z.B. aufgrund von Hemmwirkungen, beobachtet wurden.

In Bezug auf die Ergebnisse von AP3 können folgende Schlussfolgerungen gezogen werden:

- IBU kann als „nicht persistent“ angesehen werden (kongruente Ergebnisse aus Testserie 1, 2 und 4). Tatsächlich erwies sich IBU in einigen Tests sogar als leicht biologisch abbaubar.
- Die Ergebnisse von PBO sind hinsichtlich der Persistenz nicht schlüssig. Obwohl in einer Wiederholung der Versuchsreihe 4 das „pass-level“ von 60% erreicht wurde, wurde das Gültigkeitskriterium für die zulässige Variabilität zwischen Replikaten von 20% nicht eingehalten. Darüber hinaus zeigten die Ergebnisse der Versuchsreihen 1, 2 und 3 Abbaugrade deutlich unter 60% nach 60 Tagen.
- OBP hat in keiner Versuchsreihe die 60%-pass-level erreicht. Die Ergebnisse sind hinsichtlich der Persistenz („potenziell P oder vP“) nicht eindeutig.
- 4FP wird nach den Daten der Versuchsreihen 1, 2 und 4 als „nicht persistent“ bewertet und ist leicht biologisch abbaubar.
- ERU kann aufgrund der Ergebnisse der Versuchsreihe 2 als „nicht persistent“ bewertet werden, auch wenn einige Zweifel bestehen bleiben, da das 20%-Kriterium für die Variabilität zwischen Wiederholungen in der Versuchsreihe 1 nicht bestanden wurde und die Endabbaubarkeit in der Versuchsreihe 4 deutlich unter dem „pass-level“ lag.
- Weder PBO noch OBP können aufgrund der Ergebnisse als „persistent“ angesehen werden, da ein gewisser biologischer Abbau von über 20% stattfand. Demnach sind sie als „potenziell P oder vP“ einzuschätzen, so dass zur Klärung weiterhin höherwertige Simulationstests gefordert sind.
- Die Referenzsubstanz Diethylenglykol erreichte in allen Versuchsreihen innerhalb von 28 Tagen (insgesamt 6 unabhängige Versuche mit je 3 Wiederholungen) das „pass-level“ für den leichten biologischen Abbau von 60%, wobei das 10-Tage-Fenster-Kriterium in der Versuchsreihe 3 mit alternativem Inokulum nicht eingehalten wurde. Nach 60 Tagen erreichten die Abbaugrade 79-105%. Dies bestätigt mehrere andere Ergebnisse mit Standard-RBTs, in denen eine Mineralisierung von 59–98% basierend auf der CO_2-

Entwicklung oder dem O_2-Verbrauch sowie eine DOC-Eliminierung von 90–100% ermittelt wurden (ECHA 2021b, MITI 1992).

- Die zwischen den Replikaten im OECD 301 F mit Flaschen mit größerem Volumen (740 ml) beobachtete Variabilität der Abbaubarkeit war im Vergleich zu Flaschen mit typischem Volumen (164 ml) erheblich geringer.

Die Ergebnisse des praktischen Untersuchungsprogramms zeigen auch, dass der Mehrwert einer erweiterten Testung als eRBT im Vergleich zu Standard-RBTs für die ausgewählten Testsubstanzen begrenzt war. Lediglich für ERU führte die Verlängerung der Testdauer in Kombination mit größeren Testgefäßen zu einer geänderten Bewertung als „nicht persistent", während für IBU, 4FP und die Referenzsubstanz Diethylenglykol bereits nach 28 Tagen das „pass-level" von 60% erreicht und diese Substanzen als leicht biologisch abbaubar eingestuft wurden. Darüber hinaus waren die eRBT-Ergebnisse für ERU hilfreich, um die variablen Ergebnisse aus anderen Daten zum biologischen Abbau zu bewerten, und bestätigten die biologische Abbaubarkeit von ERU unter bestimmten Testbedingungen.

Insgesamt erwiesen sich die Testverlängerung auf bis zu 60 Tage und die Verwendung größerer Testgefäße als geeignete Erweiterungen („enhancements"), die zu übertragbaren Validitätskriterien und zuverlässigen biologischen Abbaudaten für eRBTs führten. Diese können zur Identifizierung nicht-persistenter Verbindungen unter REACH gemäß der integrierten Bewertungs- und Teststrategie für die Persistenzbewertung verwendet werden.

1 Introduction

Screening tests of the OECD 301 series and the OECD 310 are currently used for testing of ready biodegradability and for the identification of potentially persistent substances in the framework of evaluation processes for persistent, bioaccumulating and toxic substances (PBT substances). Although these tests differ considerably in methodology and historical background, the test results are considered as equivalent. One goal of the project is to provide recommendations for further development and standardization of these tests in order to improve the comparability of the test results. Screening tests are considered stringent, but are based on very artificial test conditions. Therefore, only a positive result can be used to relieve a persistence suspicion. A negative result requires the application of higher tier OECD simulation tests, which are carried out with more realistic substance and inoculum concentrations, and allow the derivation of degradation half-lives. However, these tests usually require the use of ^{14}C-labelled test substances and are much more time- and cost-intensive compared to screening tests. The project aims at contributing to close the existing gap between screening tests and complex studies through so-called "enhanced Ready Biodegradation Tests" (eRBTs).

In WP 1 (work package 1), a systematic comparison of the established test guidelines for determining ready biodegradability has been carried out while examining options for their harmonisation. For this purpose, in particular the experiences of the performing laboratories have been queried based on a survey on the "current status of ready biodegradability testing and options for improvements". The results were presented and discussed in April 2019 at an international one-day workshop at the German Environment Agency. Furthermore, the results of a research on this topic covering literature published mainly in the period from 2015 – 2019 with updates until 2021 are presented. This was done to update the knowledge already gathered in a preceding literature study (Gartiser et al. 2017)

In WP 2, potential test substances intended for the practical investigations in WP 3 have been researched and a data set for 15 preselected substances has been established. The aim was, to verify the conclusions on the persistence assessment from eRBTs for selected test substances. In particular, substances with an expected biodegradability of 20-40% in standard screening tests or substances with low water solubility for which valid simulation studies are available were searched for.

In WP 3, practical laboratory tests with five substances finally selected in WP 2 have been carried out in order to test the influence of the changed test conditions in eRBTs. These include in particular an extension of the test duration, the enlargement of the test vessels and the use of alternative inocula. In addition, so-called combination tests with parallel determination of several endpoints (e.g. DOC elimination combined with O_2 consumption and/or CO_2 development) were performed. In total, 20 biodegradation tests with the five substances were realised.

In WP 4, proposals for the development of a test guideline for eRBTs have been developed.

In WP 5, the impact of the project results for the persistence assessment under REACH was analysed.

The final report covers the results of all WPs. The results of the practical testing programme will be published more detailed in a scientific journal (Gartiser et al. 2022).

2 Data sources and methodology

2.1 Literature research on biodegradability testing

The focus of the literature research was on the scientific literature between January 2015 and March 2019, thus complementing the literature research performed by Gartiser et al (2017). In July 2020 and September 2021 punctual follow-up research was carried out. The literature databases used were Pubmed, Scopus (including e.g. Science direct), and ResearchGate. The main search term combinations used were "biodegradation + OECD" and "biodegradation + enhanced". The names of the specific OECD tests (OECD 307, 308, 303, 314, 301, 310) have also been used as search terms. In addition, internet searches (Google, Google Scholar) were carried out and known sources such as the CEFIC LRI projects on persistence and biodegradation were evaluated. Next to the OECD guidelines also the specifications described in the respective ISO standards have been considered.

2.2 Survey on current status of ready biodegradability testing

Most OECD ready biodegradability tests (RBTs) have been developed in the 70ties and 80ties and have not been adopted since 1992. Due to their historical and technical differences, they allow some variability and specifications in terms of the test concentration, source and concentration of the inoculum, and measuring devices. The objective of the survey was to obtain an overview from laboratories on how these tests are currently applied. For this, a detailed questionnaire covering the different options on performing RBTs of the OECD 301/310 series was drafted and revised after consultation with the experts from the German Environment Agency. A list of qualified European laboratories was gathered from corresponding national EU-lists of laboratories included in the GLP monitoring program and those laboratories certified for GLP category 5 "studies on behaviour in water, soil and air; bioaccumulation" were identified. Not all lists from individual countries (e.g. UK, Finland) indicate the area of expertise in their GLP-lists, UK considers this information as being confidential. The list has been complemented with other well-known laboratories involved in non-GLP biodegradability testing, but with an established quality management system, e.g. according to ISO 17025 (ISO 2017). The contact details of the persons responsible for biodegradability testing were partly known from previous contacts and were gathered from their webpages or via telephone calls. For some laboratories it was very difficult to identify the responsible person and blind requests to central e-mail addresses have not been processed.

From former surveys it was known that detailed questionnaires (23 pages) tend to discourage potential participants. Therefore, a two-stage strategy was followed: a first announcement was sent to about 75 laboratories. Several of them answered that they are not performing biodegradability screening tests. In part, these laboratories perform simulation tests, abiotic degradability tests or bioaccumulation studies. Only few laboratories answered that they are involved in biodegradability tests but do not have the capacity to contribute to the survey. To all labs, which did not respond, an e-mail reminder was sent. In the second stage, the questionnaire was sent to those laboratories, which previously expressed their interest to participate in the survey. Only few laboratories were not able to contribute to the survey within the timeline, which was extended two times.

In total, 16 highly qualified laboratories (mainly GLP certified) contributed to the survey: 10 from Germany, 3 from Switzerland and 3 from the United Kingdom. Thus, there is some bias in the regional allocation of the laboratories contributing to the survey, but this distribution probably reflects the number of available labs in Europe.

2.3 Identification of potential test substances for practical testing

In order to identify potential test substances for upcoming practical testing a search was performed between March 2019 and October 2019 and updated during the whole project covering publicly available reports (e.g. ECETOC technical Reports, EFSA DARs, Cefic), information portals (e.g. OECD eChem Portal, NITE Chemical Risk Information Platform, ECHA registered substances) and databases (e.g. HSDB database). Beyond that, international peer-reviewed journals were searched by means of relevant databases (e.g. Scopus). Furthermore, data from non-confidential experimental studies on ready and inherent biodegradability (e.g. OECD 301 and 302), simulation studies (e.g. OECD 308 and 309) and further development of existing test systems (e.g. compartment-specific screening tests) were considered, which are available at ECT Oekotoxikologie GmbH.

The selection criteria for the test compounds were as follows: They should ideally show a biodegradability of 20-40% in standard RBTs without reaching a plateau within 28 days. Moreover, compounds with a low water solubility (i.e. ≤ 100 mg/L according to OECD 2019a) should be preferred and valid simulation test data for different environmental compartments should be available, preferably for surface water (e.g. OECD 309), alternatively for aquatic sediments (e.g. OECD 308) and/or soil (e.g. OECD 307). If possible, the substances should be registered under REACH and should not be contained in products for household-related applications (e.g. detergents, cleaning agents, personal care products) to avoid a pre-adaptation of the inoculum.

The results of the search and the selection of test substances for practical testing are presented in chapter 5.

2.4 Guidance for the application of eRBTs in P-assessment

The main background information for the application of eRBTs in P-assessment is REACH Guidance on Information Requirements and Chemical Safety Assessment, Chapters R.7b and R.11, describing the approaches and rules currently being accepted by regulatory authorities and industry (ECHA 2017 a,b). Although these documents refer to eRBTs, no specific rules on how to design, perform and assess these tests are described, nor any proposals for validity criteria are given. This opens room for interpretation and uncertainty and makes a harmonized approach more difficult. The objective of the practical testing programme (WP 3) was to put this guidance into practice by the generation of sound biodegradability data for an exemplary set of 5 test substances preselected in WP 2 while applying the enhancements currently being accepted for eRBTs (prolongation of the duration up to 60 days and use of larger test vessels). However, the approaches for other enhancements suggested by industry, mainly referring to the origin and concentration of the inoculum, are also considered in chapter 7 (results of WP 4 “development of guidance for eRBT testing”) and chapter 8 (results of WP 5 “use of eRBTs for P-assessment”).

3 Literature research (WP1)

3.1 Test selection and comparability of results

The introduction to the OECD guidelines for degradation testing of organic chemicals (OECD 2006) states that "Ready biodegradability tests must be designed so that positive results are unequivocal." A positive result in a RBT is interpreted in such a way that "the chemical will undergo rapid and ultimate biodegradation in the environment." Because of "the stringent test conditions, consistent positive test results from valid test(s) generally supersede sporadic negative test results" (see also REACH guidance R.7b; ECHA 2017a). "When conflicting test results are reported, it is recommended to check the origin of the inoculum and whether possible adaptation of the inoculum might be the reason" (OECD 2006). According to regulatory authorities a careful assessment of the available results and study descriptions is necessary when positive and negative tests are occurring in comparable test systems (see also Gartiser et al. 2017).

There have often been complaints that the inoculum variability allowed in the OECD tests is responsible for conflicting results of different test methods (Kowalczyk et al. 2015, Goodhead et al. 2014). The authors concluded, that standard RBTs are not fit for prioritization of chemicals with regard to persistence.

Federle et al. (1997) compared biodegradation results for nine chemicals obtained with the CO_2 Evolution Test (OECD 301 B, 20 mg/L test concentration, 2 mg/L activated sludge solids) with the mineralization rates observed in different compartments at realistic ^{14}C concentrations. The environmental compartments were activated sludge (test concentration 1 mg/L, 2500 mg/L dry solids), river water (test concentration 0.1 mg/L) and soil (test concentration 1 mg/kg). All chemicals were mineralized in the ready test and in all compartments, but no significant statistical correlations between biodegradation rates in the different tests were observed. Mineralization rates in the ready test were on average 8.1, 2.5, and 1.2 times lower than the rates in realistic activated sludge, river water, and soil tests. However, the high variability suggests, that the water solubility and K_{ow} of the chemicals have a decisive influence on the scaling factors from the ready test to environmentally relevant conditions.

In contrast, Boethling et al. (2007) analysed biodegradation data submitted with US pre-manufacture notifications and found that the results from RBTs were consistent with respect to the pass/fail outcome, but not in the absolute percentage of degradation. Of the 640 chemicals, 30 were tested in at least two different RBTs. For these, the average difference between the lowest and the highest biodegradation extent was 21%. This was in line with results from Gerike and Fischer (1979) for 38 existing chemicals studied in up to five different methods similar to the current OECD guidelines. The authors concluded, that the differing results from RBTs are not due to the intra- and inter-laboratory variability. The results were consistent relative to the pass or fail outcome on ready biodegradability for most substances. Some variability was attributed to differences in the potency of the respective RBTs. For seven out of 13 chemicals with differences above 20% between highest and lowest value, the lower degradation extents were derived from a 301 D or 301 E test, which have the lowest inoculum densities (Gerike and Fischer 1979).

Mei et al. (2015) compared the biodegradation of 4,4′-Diaminodiphenylmethane (MDA)[1] in four different tests (OECD 301 A, B, D, F) and found a DOC-elimination of 95% in the OECD 301 A, only 30% in the OECD 301 B, 0% in the 301 D and 100% in the 301 F. The differing results were

[1] 4,4'-methylenedianiline, CAS 101-77-9

explained by the low inoculum concentration and the low test substance concentration of 2 mg/L in the 301 D test.[2] The authors concluded, that the limited source of energy and carbon is disadvantageous for the survival of microorganisms. In the 301 A, B and F systems, oxygen is supplemented through aeration or stirring. A general disadvantage of the 301 B and 301 F tests, using the endpoints O_2 consumption or CO_2 evolution, is seen in the difficulty to distinguish between mineralization and incorporation of the test substance into biomass.

Seyfried et al. (2015) tested the biodegradability of synthetic cyclohexyl- and norbornyl-derived ketones by using the OECD 301 F and 301 D tests. For the 301 D also activated sludge (2 mg/L d.s.) was used. While the degradation extents of cyclohexyl-derived ketones often reached the pass level (60%) after 60 d, significantly less biodegradation (<40%) was observed for the corresponding norbornyl derivatives. The relevance of the accumulation of three identified breakdown products was questioned, because the degradation extent increased, when these compounds were tested individually with fresh inoculum. The authors concluded that both OECD 301 F and 301 D tests underestimate the potential for ultimate biodegradation of norbornyl-derived ketones when using the standard test duration of 28 days. Since for the degradation products non-persistence was demonstrated in extended tests within 60 d, the norbornyl-derived ketones 4–7 should correspondingly be regarded as non-persistent.

Dick et al. (2016) compared different RBTs for determining the biodegradation potential of selected fragrance compounds. The results obtained with 48 predominantly readily biodegradable chemicals in the MITI(I) test (OECD 301 C) demonstrated a significantly higher occurrence of false negatives than with the Manometric Respirometry Test (OECD 301 F), which was explained by the limitations of the MITI inoculum. The impact of the test concentration was studied for two classes of quaternary carbon containing compounds under high (OECD 301 F) and low (OECD 301 D) concentrations and prolonged test durations. Generally, ionones were ultimately or readily biodegradable within 28 days, while damascones reached ultimate biodegradation only in OECD 301 D tests and required a longer test duration of 60 days. As suggested by Kayashima et al. (2014), the MITI(I) data should be “carefully reviewed for their suitability of classifying test compounds as non-readily biodegradable”.

It is known that the differing substrate and inoculum concentrations may have an influence on bacterial growth and therefore biomass production within the test, as identified by the differing plateau phase levels for the readily biodegradable reference substance. Therefore, the Closed Bottle Test (OECD 301 D) is suspected to have the highest biomass growth compared to the mineralization extent (Gartiser et al. 2017). Thus, the different results from RBTs should always be interpreted as readily (or not readily) biodegradable on a fail or pass basis and not in terms of the absolute percentage degradation extents or degradation kinetics.

3.1.1 DOC-based tests OECD 301 A and E

Although DOC-elimination does not distinguish between removal by biodegradation or abiotic elimination (volatilisation, adsorption), OECD 301 considers these tests as also representing “ultimate” biodegradability. The respective ISO 7827 (ISO 2010) standard also refers to “ready ultimate biodegradability testing by analysis of DOC”. Historically, the OECD 301 A DOC Die-Away Test was adopted from the (now replaced) French AFNOR method, while the OECD 301 E Modified OECD Screening Test was derived from the previous OECD Screening Test, which, together with the OECD Confirmatory Test (now OECD 303 A), were widely used for evaluation of biodegradability of surfactants in the 1970ties to 1990ties. The main difference between

[2] Considering the log K_{oc} of 3.8 – 4.0 of MDA reported in the ECHA database (access 20.6.19) the DOC- elimination observed in the OECD 301 A may be attributed to adsorption and not to mineralisation.

OECD 301 A and OECD 301 E is the inoculum concentration allowed. While the OECD 301 A allows up to 10% of secondary effluent or 30 mg/L d.s. activated sludge, the OECD 301 E only allows 0.5 mL/L of secondary effluent.

In the literature research few publications were found, which suggest, that these tests are often used in combination with specific chemical analysis for determining primary biodegradation and the identification of degradation products. For example, Cavalli et al. (1996) analysed the biodegradability of two iso-branched linear alkylbenzene sulphonates (LAS) in the OECD 301 E and the OECD 303 A continuous activated sludge test. Primary and ultimate biodegradation were measured by HPLC and DOC analyses. The authors concluded, that similar to LAS itself, also iso-branched components of LAS are mainly removed and mineralized in STPs.

Szymanski et al. (2002) tested the biodegradability of oxyethylated alcohol, an alcohol ethoxylate, with the OECD 301 A and identified its biotransformation by-products (short chained ethoxylates and polyethylene glycols).

The OECD 301 A is also used to pre-expose/adapt the inoculum for subsequent tests. For example, Docherty et al. (2015) tested the biodegradability of the ionic liquids 1-octyl-3-methylpyridinium bromid (OMP), 1-butyl-3-methylpyridinium bromide (BMP) and 1-butyl-3-methylimidazolium chloride (BMIM) in the OECD 301 A while combining DOC and photometric analysis. The inoculum was derived from two STPs at three dates. Both the location and temporal variation had an influence on the bacterial composition and biodegradability. Neither BMP nor BMIM degraded in any test, while for OMP the results varied considerably depending on STP location and sampling time. When using the inoculum from these tests for preparing an enriched (pre-adapted) inoculum, this was capable of quickly degrading OMP, BMP and BMIM.

3.1.2 Carbon dioxide based tests

CO_2 evolution is an unambiguous proof of mineralisation, which is not influenced by the oxygen consumption due to nitrification. For the CO_2 Evolution Test (OECD 301 B) it has been complained that there is a delay of the CO_2 being produced and being trapped in the absorber.

Larson et al. (1996) determined the kinetics of CO_2 recovery from the reactors (2 L liquid volume, aeration rate 6 mL/min, agitation 140 RPM on a rotary shaker). The time needed for achieving 50% CO_2 absorption in $Ba(OH)_2$ traps was 4-5 hours, and stoichiometric recoveries of CO_2 added as bicarbonate were determined within 24 hours. Thus, when using the recommended aeration rate of 30 – 100 mL/min (for 3 L liquid volume) the delay of recovery should be acceptable, especially if magnetic stirrers (optional recommended by OECD 301 B) are used in addition.

3.1.3 Oxygen based tests

Influence of nitrification

The nitrification of ammonium (NH_4-N) to nitrate (NO_3-N) requires substantial amounts of oxygen, which increases uncertainty when testing nitrogen containing substances in biodegradation tests based on the endpoint oxygen-consumption. By referring the degradation extents to the $ThOD_{NO3}$ as reference point, an overestimation of biodegradability can be avoided. The influence may be corrected by analysing the different nitrogen species NH_4, NO_2 and NO_3 according to Annex IV of OECD 301. An alternative option not described in OECD 301 consists in the addition of a specific nitrification inhibitor. Reuschenbach et al. (2003) used allylthiourea (ATU 10 mg/L) as a powerful inhibitor of nitrification, which did not affect the heterotrophic biodegradation activity.

Liu et al. (2018) determined the optimal dose of ATU for the batch respirometric test with activated sludge and also identified ATU as an effective inhibitor for ammonium oxidiser. However, the authors observed, that the inhibitor itself is also biodegraded below the inhibition threshold after some days, which resulted in overestimation of the heterotrophic respiration. The authors recommended to use e.g. 5 mg/L ATU for this purpose.

Closed Bottle Test OECD 301 D

Seyfried et al. (2015) described an approach of using activated sludge in the Closed Bottle Test (OECD 301 D) for evaluating the biodegradability of cyclohexyl- and norbornyl-derived ketones. The inoculum was diluted to 400 mg/L d.s. and then aerated for 7 days, in order to reduce the endogenous respiration rates. The solution was then diluted to 2 mg/L d.s. in BOD bottles (results see above). A similar approach was used by Ginkel et al. (1995), who analyzed the effect of the amount and origin of the inoculum on degradability extents in the Closed Bottle Test. The inoculum was taken from municipal STPs and preconditioned in the Semi-Continuous Activated Sludge (SCAS) Test (OECD 302 A) test, operated at different sludge retention times (6 – 30 days). This activated sludge (200 mg/L d.s.) was aerated for 7 days to reduce endogenous respiration rates without and with dosage of the test items diethylengylcol and diethylenetriamine (50 mg/L). The inoculum concentration in the test was 2 mg/L d.s., the dilution medium did not include ammonium chloride for preventing nitrification. The lag period prior to biodegradation, which is primarily influenced by acclimation, decreased with increasing numbers of competent microorganisms. The rate of biodegradation was influenced by the sludge retention time. Inoculum from STPs with low sludge retention times resulted in high degradation rates. It should be noted that the use of preadapted inoculum from the SCAS Test is not allowed for ready biodegradability testing according to ECHA guidance R.7b and R.11. The use of low concentrations of activated sludge as inoculum, similar as allowed for the CO_2 Headspace Test (OECD 310), could improve the potency of the Closed Bottle Test. Unfortunately, both publications do not indicate, whether the validity criterion for the inoculum blanks of 1.5 mg/L oxygen consumption was met.

Respirometer tests OECD 301 C and F

Brown et al. (2018) compared the applicability of two different manometric test systems for biodegradability testing of volatile hydrocarbons according to OECD 301 F. With the OxiTop® system, abiotic losses of test substances due to adsorption to plastic material was observed. When using a "plastic-free" test system, based on PreSens optical dissolved oxygen sensors spots attached to the inside of glass bottles, the biodegradation significantly increased, because of reduced adsorption. The authors highlighted the "importance of considering physico-chemical properties of test substances when selecting test methods and equipment" and the "value of incorporating chemical analysis and abiotic controls to improve the interpretation of biodegradation studies."

Reuschenbach et al. (2003) compared two different respirometric systems (OxiTop® and Sapromat®) for performing the OECD 301 F test and found considerable differences for diethylene glycol and 2-ethylhexylacrylate, suggesting that both systems do not always result in similar degradation extents. For acrylic acid, benzoic acid, glycerol, morpholine, NTA, 1.5-pentanediol, and phenol comparable results were obtained. For 2-ethylhexylacrylate and cyclohexanone the authors did not observe different degradation extents when using municipal and industrial inoculum, while cyclohexanone was faster biodegraded with a municipal inoculum.

O´Malley (2006) proposed a modification of the OECD 301 F respiratory test method in respect to the test substance concentration and the inoculum using the OxiTop® equipment. When using

a modified inoculum, isolated from activated sludge and fed with tryptone soya broth, biodegradability of sodium acetate could be accurately measured at test concentrations as low as 2.5 – 20 mg/L ThOD (Standard test concentration 50-100 mg/L ThOD). A comparison with data from the Closed Bottle Test revealed that both methods have a similar sensitivity. The authors concluded, that the 301 F respirometry method can be used as an alternative to the Closed Bottle Test at very low test concentrations. Standard activated sludge was assumed to be an unsuitable inoculum, since "biphasic growth curves were observed at low test concentrations." The tests were also performed with and without addition of 10 mg/L allylthiourea (ATU) as nitrification inhibitor and comparable results were obtained. It should be noted that the biodegradability results obtained with sodium acetate in the respirometer test were between 61% and 65% and thus on the borderline of the pass level for ready biodegradability of 60%, which is a validity criterion for reference substances. The results with the Closed Bottle Test with this inoculum indicated that the test was not valid for test concentrations between 0.5 and 5 mg/L ThOD (pass level 60% not reached for sodium acetate). Thus, it is questioned whether this approach would be suitable in practice.

Takekoshi et al. (2021) compared the biodegradability of 6 compounds with low water solubility in the OECD 301 C and OECD 301 F tests. The MITI(I) test (OECD 301 C) was inoculated with activated sludge derived from a mixed inoculum collected from 10 sites and cultivated with peptone and glucose for about one month. The respirometer test (OECD 301 F) was inoculated with fresh activated sludge from a STP receiving predominately domestic sewage. The biodegradability of Anthraquinone, 2-Hydroxy-4-methoxybenzophenone and Bis(2-ethylhexyl)phthalate was far higher with fresh activated sludge compared to the cultivated one. The remaining compounds were not degraded in both tests. The authors concluded that the cultivation of the activated sludge reduces its microbial diversity and degradation activity. Thus, the artificial OECD 301 C inoculum is considered being very conservative and not reflecting real environmental conditions. According to the authors, since 2018 the Japanese Chemical Substance Control Law accepts also data derived from the OECD 301 F test with fresh activated sludge.

3.1.4 Marine biodegradation tests

According to the REACH guidance biodegradation tests in seawater (OECD 306) can also be used to conclude about the ready biodegradability of a substance as part of a weight-of-evidence approach (ECHA 2017a, 2017b). The OECD 306 describes seawater versions of the Closed Bottle Test (OECD 301 D), the Modified OECD Screening Test (OECD 301 E) and other tests adopted from OECD ready tests. Degradation is determined via DOC, CO_2 evolution or oxygen uptake. The pass level is also set to 60% (ThOD or $ThCO_2$) or 70% for DOC removal. Usually, the degradation of substances in seawater is far slower than in tests using activated sludge or sewage effluent as inoculum. This is partly compensated by a longer test duration of 60 days in seawater tests instead of 28 days as for freshwater tests. However, seawater tests are rarely applied for persistency evaluation despite the importance of marine compartments (water and sediment). There have been several approaches to improve the reliability of seawater biodegradation tests (ECETOC 2017, Ott et al. 2020). In principle, the main suggestions are to increase the marine inoculum by a factor of 100 and to extent the test duration beyond the persistence half-life threshold criteria.

However, this proposal does not correspond to the OECD 306 guideline (adopted:

17.07.92), which refers to "natural seawater both as the aqueous phase and as the source of micro-organisms" while the use of "specific inoculum in addition to the micro-organisms already present in the seawater" is not accepted.

3.2 Combination tests

Strotmann et al. (1995, 2004) described a ready type combination test based on the continuous CO_2 measurement via conductivity measurements in NaOH or KOH absorbers, next to oxygen consumption in a manometric respirometer (Sapromat® or Oxitop®). In parallel experiments, low variabilities of the biodegradation extents were observed. The results obtained with the online CO_2 method were comparable with that of the existing CO_2 Evolution Tests in terms of the maximum growth rates and lag periods. Only the biodegradation extent of aniline was slightly lower. The method can also be combined with additional DOC measurements, thus covering three different endpoints in one test.

A similar test system has been adopted by Norr et al. (2001), who simultaneously measured oxygen consumption and carbon dioxide production in the inherent Zahn-Wellens test.

Campo et al. (2012) tested the biodegradation of canola oil and two synthetic oils in a O_2/CO_2 combination test, using computer-assisted respirometers (Comput-Ox WB Series, N-Con Systems, Crawford, GA, USA). The ultimate oxygen demand was measured respirometrically, the carbon dioxide captured in KOH absorbers and resulting in a pH decrease was determined photometrically via the indicator dye alizarin red, whose colour changes from red to purple in the pH range from 10 to 12. The initial concentration of the oils was 1 g/L, the inoculum consisted of 2.5 mL of a master culture acclimated to canola oil. The biodegradation extents obtained were in the range of 64 – 86% ThOD and 68 – 89% $ThCO_2$. The authors concluded that the three oils are readily biodegradable. It should be noted, that due to the pre-adopted inoculum used no conclusion on ready biodegradability can be drawn.

3.3 Testing of difficult substances

Substances which are poorly or sparingly water-soluble, tending to adsorb on glass or other surfaces, floating to the water surface, volatile or toxic at the testing concentrations may be named “difficult substances”. While for ecotoxicity testing of difficult substances an OECD guidance document exists (OECD 2019a), no such specific guidance exists for biodegradability testing of difficult substances. The general introduction to OECD 301 gives some guidance on the test selection depending on the substance properties. Annex II of OECD 301 refers to testing strategies for substances suspected to be toxic to the inoculum. Annex III of OECD 301 refers to testing of poorly soluble compounds, but ISO 10634 (ISO 2018a) gives far more practical advice in this context. The ECHA Endpoint specific guidance R.7b (ECHA 2017a) gives some general advice for biodegradability testing of difficult substances.

3.3.1 Low water-soluble substances

Annex III of OECD 301 provides some guidance on the evaluation of the biodegradability of poorly water-soluble substances such as homogenisation of the test materials to provide representative samples, use of agitation devices to keep the chemical dispersed, use of a non-biodegradable and non-toxic emulsifier or solvent and use of solid carriers for oily substances. Only respirometric tests (301 B, 301 C, 301 D, 301 F) can be used for poorly water soluble substances and parallel blank vessels should be considered when using auxiliary substances such as emulsifiers, solvents or carriers. The ECHA guidance R.7b states that for poorly water-soluble substances methods using DOC analysis cannot be used, unless biodegradation is measured in addition to another parameter (ECHA 2017a). The ECHA guidance refers to the different options described in Annex III of OECD 301, while stating that the ”use of silica gel matrices is generally the preferred option.” Solid carriers such as silica gel or polyethylene slides are not recommended for solid test substances but may be suitable for oily test items.

Emulsifiers or solvents, which yield a "stable dispersion of the test substance may be used, but it should be verified that they are not toxic to bacteria and must not be biodegraded or cause foaming under test conditions" (ECHA 2017a).

A technical report from ECETOC (1986) provides more detailed guidance, such as the use of ultrasonic or mechanical dispersion, dichloromethane as solvent, and glass filter as inert carrier. Calcium stearate was biodegraded under all these addition conditions while anthraquinone was only biodegraded when being agitated and bee wax was only biodegraded when using emulsifiers and agitation.

The second edition of ISO 10634 (ISO 2018a) describes several methods for preparing poorly water-soluble substances for subsequent biodegradability testing. First, the direct addition by weighing or addition with an inert support such as microscopic slides, polyethylene slides, stainless steel slides or silica gel. Second, the ultrasonic and physical treatment (20 kHz to 35 kHz for 5-30 minutes). Third, the adsorption on an inert support with a volatile solvent removed from the system (test substance dissolved in e.g. trichloromethane and transferred to non-biodegradable inert supports such as silica gel, where the volatile solvent evaporates). The ISO standard notes that the residual carbon content due to unevaporated solvent impurities can affect the test results. Further on, because volatile solvents may mobilize adsorbed organic residues from the equipment, an additional washing step with the solvent before using the equipment is recommended by ISO. The fourth option is addition with a non-biodegradable solvent or emulsifying agent not removed from the system. However, the ISO standard notes that this increases the overall uncertainty of the tests and is generally the least preferred method.

Nyholm (1990) analysed different addition techniques for testing biodegradability of poorly water-soluble compounds in manometric respirometry tests. The author evaluated several dispersion techniques when testing anthraquinone (solid) and di-isooctylphtalate (liquid). Application on silica gel via the solvent dichloromethane was assumed as generally appropriate whereas ultrasonic dispersion was only suitable when a stable emulsion was achieved.

Marcoux et al. (2000) analysed the biodegradability of polycyclic aromatic hydrocarbons (PAH) (pyrene, chrysene, benzo[a]pyrene, perylene) in a two liquid-phase reactor from a process engineering point of view against the background that bioreactors containing a non-aqueous-phase-liquid (NAPL) are applied to improve the biodegradation rate of poorly soluble compounds. The NAPLs tested were assumed as being non-biodegradable and non-toxic and serve as inert carriers for hydrophobic compounds from which the substrate diffuses from to the aqueous phase. The microorganisms biodegrade the substrate at the interface or in the aqueous phase. The authors used two silicone oils (5 centistokes and 20 centistokes), paraffin oil, 2,2,4,4,6,8,8-heptamethylnonane, hexadecane and corn oil as non-water-miscible liquids. When using inoculum derived from a creosote-contaminated soil in a batch test, complete degradation of pyrene was observed via HPLC chemical analysis within 3±17 days with silicon oils whereas a lag-phase of 3 and 12 days was observed for heptamethylnonane and paraffin oil, respectively. Similar results were also obtained for the other PAHs. This principle is also used when applying silicon oil in biodegradability tests as suggested by ISO 10634 (ISO 2018a).

Handley et al. (2002) presented an overview of using inert carriers for biodegradability testing of low density poorly water-soluble substances (cited also in ISO 10634). The authors stated that many compounds with low water solubility fail ready biodegradation tests because the test material has a limited bioavailability. Appropriate methods for adding poorly soluble materials to biodegradability tests are coating the test material inside the test vessels or onto inert supports (i.e., glass cover slide, boiling beads, filter paper, or Teflon stir bar) that are then placed

into the flasks. Volatile solvents are often used to improve this approach. Poorly water-soluble substances with a low density may float on the surface, where they have limited contact to micro-organisms in the test medium. Hence, there is a reduced potential for measuring substantial biodegradability in the test. The following addition techniques were applied according to Handley et al. (2002):

- Direct dispersion in test media with ultra-sonication.
- Adsorption to silica gel (100 mg 230–400 mesh) and dispersion in 100 mL of test medium at 7500 rpm, 10 min prior to dispersal in a final volume of 3 L in the test vessels.
- Addition onto solid supports (microscope slides, glass beads, glass fibre filter).
- Auxiliary solvents (dichloromethane, acetone, chloroform and diethylether), stock solutions were then coated onto the test vessels or added to filter paper strips before allowing the solvent to evaporate.

The CO_2 Evolution Test (OECD 301 B) was used as test system. After 28 days the test substances (two esters) attained 79% and 98% degradation in the OECD 301 B when silica gel was used compared to 22% and 18% degradation when a direct dispersion technique or direct addition was used. At the test end all the studies fulfilled the OECD validation criterion concerning the difference between the replicate vessels (required ≤ 20%). The author proposed to use silica gel as adsorption carrier followed by dispersion into the culture medium. This method results in a better concordance of test results with poorly water-soluble substances with a low density. According to the authors this approach more closely represents the probable transport and fate of these substances in the environment (Handley et al 2002).

Takekoshi et al. (2021) investigated the influence of adding different amounts of silica gel (0.4 – 6 g in 300 mL) to the OECD 301 F test when testing the biodegradability of 2-Ethalanthrachinone (2-EA) and 2-Hydroxyy-4-n-octyloxybenzophenone (OB). The biodegradability of 2-EA decreased from 98% with 0.4 g silica gel to less than 10% with 6 g silica gel. For OB the biodegradability decreased from 80% (0.4 g) to 37% (6 g). In contrast, without adding silica gel no significant biodegradation at all was observed. The authors concluded, that while the bioavailability of both compounds increased with small amounts of silica gel, this effect turned to the contrary at higher amounts of silica gel.

3.3.2 Volatile substances

Volatile substances must be tested in one of the closed test systems with or without headspace (OECD 301 C, D, F, OECD 310). In the Closed Bottle Test the challenge is to dose the volatile substance into the test bottles, in those tests with a headspace, volatile chemicals will partition between water phase and headspace.

Birch et al. (2017) analysed the biodegradation of a mixture of 9 (semi)volatile chemicals, which were added by passive dosing via silicone rods to environmental surface water in the ng/L to µg/L concentration range. After incubation for 2 h up to 28 days, the samples were analysed via Headspace Solid Phase Microextraction (HS-SPME) Gas Chromatography coupled with Mass Spectrometry (GC-MS). The biodegradation was governed by the continuously partition between water phase and headspace. In theory, during degradation the chemical concentration in the water phase will be continuously replenished from the headspace. Primary biodegradation was referred to the abiotic controls. The biodegradation rate constants relating to the concentration of the chemicals in the water phase were up to a factor of 11 higher than those relating to the

total mass of the chemical in the test system. The authors concluded, that the degradation rate constant determined for the water phase only is more suitable as a reference point for risk assessment. The study improves our understanding of the relevance of the partitioning between water and gas phases in tests with headspace but gives no guidance on how to test volatile chemicals in RBTs, due to the simulation character of the test design.

3.3.3 Inhibitory substances

The objective of biodegradability testing is to obtain a result about degradability as an intrinsic substance property. When testing toxic substances in screening tests, a negative result may be influenced by inhibitory effects on the inoculum, thus being assumed as false negative. Annex II of OECD 301 provides some guidance for testing substances suspected to be toxic to the inoculum. These substances should preferably be tested at concentrations corresponding to 1/10 of the EC_{50} values obtained in the Activated sludge respiration inhibition tests (OECD 209). The use of the stringent Closed Bottle Test or the use of ^{14}C-labelled material is suggested. When pre-exposing the inoculum to the test substance, testing at higher test substance concentrations may be possible. However, the specific criteria required for RBT are lost. As a consequence, ECHA Guidance R.7b states that the results may not be used for ready biodegradability or persistency assessment (ECHA 2017a).

The ECHA guidance R.7b states that potentially inhibitory substances should be tested in the test with the lowest test substance concentration, which is the Closed Bottle Test (OECD 301 D). Where necessary, the test substance concentration could be further reduced below the values generally recommended in the test guideline, as long as this still allows the reliable assessment of biodegradability by the endpoints used (CO_2, O_2 or DOC). However, study performance at low concentrations may only be possible, if the test substance is radiolabelled (ECHA 2017a).

Alexy et al. (2004) assessed the biodegradability of 18 antibiotics using the Closed Bottle Test (OECD 301 D) in the mg/L range and used toxicity controls next to colony forming units (CFUs) for detecting inhibitory effects on the inoculum. None of the antibiotics was readily biodegradable. The results of CFU determinations showed that some of the antibiotics had an inhibitory effect on the inoculum. The best result was observed with benzylpenicillin (27% in 28 d, start concentration 3 mg/L). In contrast, Gartiser et al. (2007) found 78 - 91% mineralisation of benzylpenicillin in an inherent test based on OECD 302 B with additional CO_2 measurements, although the start concentration was 100 mg/L TOC (=174 mg/L benzylpenicillin) and thus was far higher than in the Closed Bottle Test.

Deziel et al. (1999) described options for enhanced degradation of hydrophobic/toxic compounds in two-liquid-phase bioreactors, consisting of a water-immiscible, biocompatible and non-biodegradable solvent and water. These are used in biotechnology for the conversion of hydrophobic or toxic substrates. This technique may also be used to treat pollutants with limited bioavailability by improving their biodegradability. The authors discussed the application of two-liquid-phase bioreactors to enhance the biodegradation of toxic/poorly bioavailable contaminants. The uptake of the substrates by microorganisms occurs both in the aqueous phase and at the interface between the aqueous and the immiscible phases. The approach consists in increasing this interface as far as possible. The application of this technique as an alternative to current bio-treatment technologies is also discussed. Although the objective of this approach is a technical one, it may also open biodegradability testing strategies for toxic substances.

Van Ginkel et al. (2008) describe several options for improving RBTs of fatty amine derivatives. For reducing toxicity and improving bioavailability of alkyl-1,3-diaminopropanes and

octadecyltrimethylammonium chloride the concentration in the aqueous phase was reduced by humic, or lignosulphonic acids or through the addition of silica gel to the test bottles. Positive results on ready biodegradability were obtained from tests with very low initial test substance concentrations and by adding an organic phase. The authors concluded that false negative biodegradability results with fatty amine derivatives can be explained by toxic effects and/or limited bioavailability. This concept is similar to that of ecotoxicity testing by passive dosing (e.g. Butler et al. 2013).

Timmer et al. (2019a, b) performed sorbent-modified biodegradation studies of the biocidal cationic surfactants cetylpyridinium chloride (CPC) and cetyltrimethylammonium bromide (CTAB), whose biodegradability is hindered by inhibitory effects on inoculum at standard test concentrations (10–20 mg organic carbon/L). The authors used ^{14}C-labelled CPC resp. CTAB in the CO_2 Headspace Test (OECD 310) and found that CPC was readily biodegradable (10->60% mineralization within a 10-day window) at test concentrations of 0.006–0.3 mg/L. CPC reduced biodegradation at 1 mg/L and completely suppressed the inoculum activity at 3 mg/L. In a modified study with the inert support silicon dioxide (SiO_2, Davisil Grade 633, particle size 35-75 µm, surface area 480 m^2/g) applied at 0.80, 4.0 and 20 g/L, inhibitory effects of 1 mg/L CPC were diminished, reaching >60% biodegradation within 28 d. At 10 mg/L CPC SiO_2 was still able to reduce inhibitory effects, but bioavailability seemed to be limited, as only 20% biodegradation was reached. By addition of commercial clay powder (illite) as adsorbent a reduced lag phase was observed, but illite limited bioavailability more strongly than SiO_2 and was not able to maintain biodegradation at 10 mg/L CPC. CTAB was evaluated as being readily biodegradable without sorbent. The authors conclude that the balance between toxicity mitigation and bioaccessibility is challenging and suggest combining a sufficiently high adsorbed fraction with adequate desorption kinetics.

Nabeoka et al. (2020) used different carriers in the Manometric Respirometry Test (OECD 301 F) in order to reduce the apparent concentration and therefore inhibitory effects of several QAC. With silica gel, the concentration of all QACs decreased due to adsorption and the biodegradation of octadecyltrimethylammonium chloride, hexadecyltrimethylammonium bromide, and benzyldimethyloctadecylammonium chloride (all with a linear alkyl chain) was 89.9%, 80.6%, and 70.1% on day 28, respectively. In contrast, benzethonium chloride (branched alkyl chain), was not biodegraded. In the tests with activated carbon as carrier no biodegradation of any QAC was observed, indicating that the bioavailability was too much reduced by adsorption. In the tests with sea sand or quartz sand as inert supports the QACs were neither adsorbed nor biodegraded. In conclusion, using an adsorbent carrier for toxic substances may increase biodegradation.

3.3.4 UVCB substances

Substances of Unknown or Variable composition, Complex reaction products or Biological materials (UVCBs) have complex compositions, which may result in a stepwise, sequential adaptation of the microorganisms, which utilise individual compounds with different chain-lengths or fractions. As a consequence, the overlapping biodegradation kinetics of UVCBs often differ from typical shapes (ECHA 2017a). Examples are mixtures of homologue substances of similar structures such as surfactants or petroleum substances with a range of different hydrocarbons. Due to the overlapping biodegradation kinetics, these mixtures may fail the “10-day window” criterion in the ready biodegradation tests. Thus, for UVCBs the “10-day-window” usually is not applied. The differing biodegradation potential and physical-chemical properties of individual UVCB-constituents may cause, “that the biodegradation of a readily biodegradable fraction of a UVCB may mask the presence of persistent constituents, particularly for very

complex substances with different classes of molecules" (Brown et al. 2020). This is a general concern when applying RBTs, which use indirect measures for detecting biodegradation (i.e. O_2, CO_2, DOC) instead of tracking the test substance itself. The authors further state, that, when using RBTs for testing UVCBs, information about their composition is needed, in order to evaluate, whether "the non-specific results of RBTs can be used to draw a conclusion on the biodegradability of all constituents." This approach is based on the "chemical similarity principle", i.e. the assumption that constituents with similar chemical structures and physical-chemical properties behave similarly in biodegradation tests (Brown et al. 2020). The authors argue, that for most UVCBs this is critical, because no complete analytical characterisation of UVCBs is available in the majority of cases. For these UVCBs there is reasonable concern that residues of the test material could represent a less degradable fraction. In contrast, the chemistry of gas-to-liquid hydrocarbons is well characterised and their compositional variability is low. Thus, these UVCBs are considered homologous in terms of their compositional chemical structure and RBTs allow an accurate assessment of their biodegradability.

In this context, it should be noted, that results from RBTs with mixtures of different types of substances generally are difficult to be interpret (OECD 2006). Thus, RBTs with formulations or products often give no meaningful results.

In the context of persistency evaluation Bunke et al. (2020) state, that the REACH concept of PBT/vPvB assessment is designed for pure substances. While for registration purposes all single constituents of UVCBs above 1% w/w have to be identified, single constituents down to 0.1% w/w should also be considered within the PBT assessment. Because simulation tests with radio-labelled substances are hardly practicable, biodegradation screening tests are often performed with the UVCB as such, which do not provide information on the biodegradation of individual constituents. Therefore, it is suggested by the authors to test homologous structures from UVCBs separately, if possible.

For UVCBs originating from petroleum Wassenaar and Verbruggen (2021) presented a case study on alkylated three-ring PAHs, applying the hydrocarbon block concept. This concept groups constituents with similar physicochemical properties and structure under the assumption that they possess comparable environmental fate and hazard properties. The authors concluded that alkylated three-ring PAHs are more persistent, bioaccumulative, and toxic than the parent three-ring PAHs and should also be assumed as PBT/vPvB.

3.4 Test design

3.4.1 Vessel size and number of replicate vessels

Martin et al. (2017a) criticised that RBTs only used one inoculum sample to assess ready biodegradability or persistence in environment while neglecting considerable spatial and temporal microbial variation. The authors developed a colorimetric assay as high throughput biodegradation screening test. The test was performed in 96 well plates (300 µL volume) inoculated with 50 µL activated sludge from a STP and 250 L mineral medium with the test items (10 mg/L). Chemical analysis of structurally diverse aromatic compounds was performed by colorimetric determination after reaction with 4-nitrobenezediazonium tetrafluoroborate (4-NBTFB) to form an azo dye. With this test design, the authors differentiated and prioritized the aromatic compounds based on their relative biodegradability and quantitative structure–activity relationship (QSAR) model outputs. The transferability of these results to standard RBTs is not possible since primary instead of ultimate biodegradation was assessed and because of the very low test volume used, which also limits the occurrence of potential degraders.

3.4.2 Test concentration

Ahtiainen et al. (2003) compared biodegradation kinetics obtained with the CO_2 Headspace Test according to ISO 14593 (ISO 1999a) with those measured in shake batch tests using environmental media from surface water. One important difference of standard biodegradability tests and environmental conditions is the compound concentration. In STPs or environmental samples many chemicals are present at ng/L to µg/L levels while in the standard biodegradation tests the concentrations most often exceed 10 mg/L TOC. This leads to different growth kinetics and therefore differing biodegradation extents. The authors conclude that "at high concentrations, the chemical can serve as a primary substrate and competent microorganisms will grow exponentially, resulting in a sigmoid biodegradation curve. At low environmental concentrations, the chemical does not serve as a primary substrate and does not support significant growth of the degraders", resulting in linear biodegradation rates (Ahtiainen et al. 2003). The authors compared the degradability of aniline and 4-chloroaniline in the Headspace Test (20 mg/L C) with the degradability at low concentrations (2-20 µg/L C), using ^{14}C-labelled substances and various sources of inocula. Biomass growth was monitored by adenosine triphosphate measurement and by determining the residual ^{14}C activity (inorganic carbon) of the particulate matter. According to the authors the hypothesis that low concentrations lead to different biodegradation kinetics compared to the concentrations used in standard biodegradation tests is partly confirmed. However, the source of the inoculum was identified as even more influencing factor for determining biodegradation rates.

Martin et al. (2017b) analysed the impact of two inoculum concentrations on biodegradability, using the CO_2 Evolution Test (OECD 301 B) with 3 mg/L of inoculum (considered as typical RBT concentration) and 300 mg/L (considered as "more environmentally relevant but still an order of magnitude lower than used for inherent tests.") Tests with 300 mg/L were deemed "enhanced" tests. It should be noted, that this is not correct, since the standard activated sludge concentration of most RBTs is 30 mg/L d.s. and thus a factor of ten higher than the lower inoculum concentration used, while the higher inoculum concentration of 300 mg/L d.s. is clearly within the range that should be assigned to an inherent test (OECD 302 B: 200-1000 mg/L d.s.). For inherent tests ECHA guidance R.11 on PBT/vPvB assessment refers to specific criteria (70% degradation in the Zahn-Wellens Test within seven days, degradation-phase < three days, percentage removal before biodegradation occurs <15%) for a substance being assumed as not persistent (ECHA 2017b).

3.4.3 Abiotic elimination

The possible adsorption to activated sludge should be carefully evaluated when using DOC-based tests such as OECD 301 A, especially when using activated sludge as inoculum. No detailed guidance exists on what adsorption extent might be acceptable. OECD 301 only states, that "unless adsorption of the test substance has been ruled out beforehand", DOC-based methods (OECD 301 A, E) "should include an abiotic control, which is inoculated and poisoned." Furthermore, the abiotic degradation of the test substance may be determined in sterile controls containing no inoculum. OECD refers to sterilization by membrane filtration or "by the addition of a suitable toxic substance at an appropriate concentration." However, abiotic controls are not mandatory for all DOC-based tests.

Gartiser et al. (2017) suggested that the elimination through adsorption should be limited by defining a clear criterion, e.g. a maximum of 20% DOC-elimination through adsorption at the test start being acceptable (similar as proposed in ISO 9888, ISO 1999b). The consideration of abiotic controls considerably improves the identification of adsorptive or volatile test substances.

OECD 301 does not indicate suitable biocides for preparing abiotic controls. According to OECD 309 (Surface water simulation test), abiotic degradation or other non-biological removal of the test substance can be analysed be stopping the biological activity by autoclaving (121°C; 20 min) the test water, by adding a toxicant (e.g. sodium azide at 10-20 g/L, mercuric chloride at 100 mg/L or formalin at 100 mg/L), or by gamma irradiation. If $HgCl_2$ is used, the liquids should be disposed of as toxic waste.

3.4.4 Reference substances

According to the OECD 301 introduction suitable reference compounds for RBTs are aniline (freshly distilled), sodium acetate and sodium benzoate. However, these reference compounds all degrade even when no inoculum is deliberately added. Therefore, it was suggested to use a reference compound, which is readily biodegradable but requires the addition of an inoculum. Potassium hydrogen phthalate has been proposed but more evidence needs to be obtained with this chemical before it can be accepted as a reference compound.

When testing potentially volatile substances Comber and Holt (2010) suggested to use 1-octanol as a relatively low water-soluble (540 mg/L) reference chemical with a moderate volatility, which is readily biodegradable.

When testing solid test items with poor water solubility it is generally recommended to use appropriate reference compounds. For example, for degradability testing of plastic material according to ISO 14852 (ISO 2018b) microcrystalline cellulose powder or polyhydroxybutyrate is recommended as reference compound. The ISO standard recommends a maximum particle size of the test and reference species of 250 µm. ASTM D5864, describing a CO_2 Evolution Test for testing the biodegradability of lubricants, recommends rapeseed oil as reference material.

For eRBTs Comber and Holt (2010) suggested to distinguish between reference compounds, which normally pass a RBT (e.g. aniline, sodium acetate, 1-octanol) and those which normally fail a RBT but pass an eRBT (e.g. diethylene glycol, 4-chloroaniline, 1,3,5 trimethylbenzene). Further on, considering a negative reference substance, such as di-isotridecyl adipate, ter-phenyl, or cyclododecane normally failing both standard and enhanced RBT, could prevent artefacts in the test design. Martin (2014) recommended the use of additional non-degradable reference compounds in order to ensure that the test item is not erroneously assessed as not being persistent.

3.5 Inoculum

3.5.1 Inoculum sources and concentrations

Both OECD and ECHA guidance state that the inoculum should be derived from STP predominately treating domestic/municipal wastewater without indicating a definite portion of domestic wastewater. The use of inoculum from industrial STP is not allowed. One reason is that industrial STP are supposed to be potentially adapted to industrial chemicals.

Table 1: Validity criteria for inoculum control vessels

Test	Validity criteria inoculum blanks [1)]	Substance concentration [2)]	Portion allowed inoculum / substrate [3)]
OECD 301 B	≤ 40 mg/L CO_2 or ≤ 11 mg/L IC (up to 70 mg/L CO_2)	10-20 mg/L TOC	55 - 110%
OECD 301 C	20-30 mg O_2 (up to 60 mg/L O_2)	100 mg/L (substrate) (~ 100 - 200 mg/L ThOD)	~ 10 - 35%
OECD 301 D	1.5 mg/L O_2	2-5 mg/L (max. available 9.0-0.5 = 8.5 mg/L O_2)	18%
ISO 10708 (Bodis)	≤ 3 mg/L O_2 1st week, ≤ 1 mg/L O_2 in following weeks ∑ ≤ 6 mg/L O_2	100 mg/L ThOD	6%
OECD 301 F	20-30 mg O_2 (up to 60 mg/L O_2)	50 - 100 mg/L ThOD	30 – 60%
OECD 310	IC < 3 mg/L (independend from inoculum concentration)	10-20 mg/L TOC (2-40 mg/L TOC)	15 - 30%
ISO 14593	IC < 15 % of TOC introduced (< 3 mg/L at 20 mg/L TOC)	10-20 mg/L TOC (2-40 mg/L TOC)	15%

1) Standard inoculum concentration: 30 mg/L d.s. activated sludge, except OECD 301 D: 0.05-5 ml/L secondary effluent and 301 C: 30 mg/L d.s. mixed inoculum and OECD 310: 4-30 mg/L d.s. activated sludge.

2) Standard concentration range, allowed concentration range in brackets.

3) Portion of standard validity criterion inoculum activity (O_2, CO_2 or IC) on theoretical values (ThOD, $ThCO_2$ or TOC) introduced with the test item.

Some methods are quite strict in their inoculum and substrate ranges allowed, other more flexible. The same is true when considering usual oxygen consumption or CO_2 evolution in the inoculum blanks and the highest values allowed for a test being valid. For example, the validity criteria for the CO_2 Headspace Test of IC < 3 mg/L does not make much sense if higher inoculum concentrations (up to 30 mg d.s./L) are used. The criteria of ISO 14593 standard (ISO 1999a) is more suitable because it refers to a standard inoculum blank/substrate ratio of 15% of the TOC introduced.

From table 2 it becomes clear that the OECD 301 B and F are the most often applied tests. The reason probably is, that activated sludge is used as inoculum, which does not need to be starved for several days to comply with the validity criteria.

François et al. (2016) proposed a probability approach on biodegradability assessment by using high-throughput miniaturized system (24-well microplates with oxygen sensors) and different temperature regimes (4-30°C) and inoculum concentrations (10^4-10^8 cells/mL) called "ProbaBio". The concept was tested with sodium benzoate, 4-nitrophenol, diethylene glycol, 2,4,5-trichlorophenol, atrazine, and glyphosate, using 10 mg/L test concentration. A test substance was evaluated as "biodegradable" when the oxygen consumption in the inoculum control was < 0.5 mg/L whereas that of the test wells was greater than 10 times the maximal value observed in the negative control (e.g. > 5 mg/L). The authors complained that RBTs do not consider the quantity and complexity of environmental variables and modelled the probability for a biodegradation test to be positive by fitting logistic functions of the temperature and cell density. The proposed strategy aims to bridge the gap between straightforward experimental

use and probabilistic approach of biological persistence. According to the authors, the ProbaBio microbial degradation profiles allow both the identification and quantification of potential risks of environmental persistence. The results allowed a ranking of the chemicals in terms of their biodegradability, from readily biodegradable to not biodegradable in the environment, sodium benzoate > 4-nitrophenol > diethylene glycol > atrazine > 2,4,5-trichlorophenol. A case study with the herbicide glyphosate revealed poor biodegradation probabilities (maximum 0.48 at 30°C and 10^8 cells/mL). It should be noted that the absolute extent of biodegradation (% ThOD) is not calculated in this approach and that the low volumes also limit the absolute number of potential competent microorganisms present in the test (see 3.5.1).

3.5.2 Characterization of the inoculum

In numerous publications a better characterisation of the inoculum with biological, biochemical or molecular biological parameters is discussed. Madoni (1994) proposed the application of a Sludge Biotic Index (SBI) based on the presence and abundance of key protozoan groups, which is used in wastewater treatment plant control. De Arévalo et al. (2009) applied the SBI for controlling a pilot-scale membrane bioreactor. The authors evaluated the SBI at sludge retention times (SRT) of 25 and 35 days. The protozoan composition of the activated sludge was analysed and results demonstrated a constant predominance of small flagellates, carnivorous ciliates and rotifers in test series with 35-day SRT, independently of effluent quality. In this study the focus was on the protozoic community and not on microorganisms. Bacteria were only described according to their shape (dispersed or filamentous).

Several case studies describing the adaptation of the microorganisms to specific chemicals in laboratory and environmental systems have been performed.

Ertekin et al. (2016) identified the microbial consortia and genes involved in the biodegradation of benzalkonium chlorides (BACs) in different environments with the objective to understand the fate of BACs in the environment and to develop treatment strategies. The authors isolated four microbial communities degrading BACs from sewage, activated sludge, soil and sea sediment. According to 16S rRNA sequencing and shotgun metagenome sequencing analyses, the most abundant species belonged to the genera *Pseudomonas* and *Achromobacter*. BAC biotransformation rates correlated with the abundance of a particular *Pseudomonas* sp. strain, BIOMIG1. Genomes of four BAC degrading and non-degrading BIOMIG1 phenotypes were sequenced and differentially compared with each other. As a result, a gene cluster encoding for the enzymes integrase and ioxygenase, which are involved in BAC biotransformation has been described. The authors concluded that BIOMIG1 is a key species determining the fate of BACs in the environment.

There is also an increasing interest of better describing the temporal and spatial variability of the microbial community in STP with the objective to improve the elimination of micropollutants. Saunders et al. (2016) analyzed the activated sludge ecosystem from 13 Danish STP over 6 years using 16S rRNA gene sequencing and found a core community of abundant organisms that explained 68% of the organisms observed. The same genera were also detected in STPs in China and the USA, suggesting that the factors driving the formation of abundant core communities are common for wastewater treatment plants. Thirty-five percent of the core community observed in the activated sludge were also observed in the wastewater influent to the STP.

Wolff et al. (2018) adopted the methodology of Saunders et al. (2016) and compared the elimination of 33 micropollutants by LC-MS/MS with the microbial community in five pilot-scale reactors operated under different treatment parameters while using 16S rRNA gene sequencing.

The diverse bacteria of the community were assigned to a core and a specialized community. Despite their considerably different operation parameters all treatments shared a core community consisting of 143 genera (9% of the overall community). Only 5.5% of the overall community of all samples (90 of 1625 genera with the highest variability in relative abundance) explained 70% of the dissimilarity between the communities in the different treatments. The specialized community correlated with the removal of certain micropollutants. The authors concluded, that comparing the specialized community with the elimination of micropollutants and operating conditions via correlation analysis is a useful tool for an in-depth assessment of common process conditions.

Zhang et al. (2018) also applied 16S rRNA gene sequencing to describe the microbiologic community of three municipal STP and two industrial STP, treating also textile dyeing and fine chemicals wastewater. Although considerable differences in microbial community composition were observed, the metabolic potential (attribution to hierarchical metabolic categories) of the different activated sludge showed no significant differences.

The use of 16S rRNA gene sequencing offers an additional instrument sequencing for better describing the microbiologic communities of the inoculum sources. So far, this instrument has been applied in research and development projects with the aim to improve the performance of STP and the degradation tests used were mostly non-standardized and only covered primary degradation.

Muñoz-Palazon et al. (2018) analysed the degradation performance and the microbial community of three aerobic granular sludge reactors inoculated with different inocula (activated sludge from Finland, Spain, and a mixture of both) adapted at mild and low temperatures. After 90 days of operation similar physico-chemical parameters were observed in all reactors. Real-time PCR showed that Archaea diminished from inoculum to granular biomass, while numbers of bacteria and fungi remained stable. The reactors behaved similar regardless of the origin of inocula used. The authors concluded that independent from the inocula used, the microorganisms growing in aerobic granular sludge systems operated under the same conditions were similar and were not affected by the initial temperature.

3.5.3 Influence of pre-exposure and adaptation

The pre-exposure (pre-adaptation)of the test item to the inoculum is generally not accepted by OECD and ECHA Guidance, neither for RBTs nor for eRBTs (OECD 301, OECD 310, ECHA 2017a). From part of the concerned industry it has been proposed that pre-exposure of the inoculum to the test item could also reduce its background activity and allow higher inoculum densities, while maintaining the signal-to-noise relation of lower densities also for higher densities (Battersby et al. 1999, ECETOC 2007). For test substances with inhibitory effects to the inoculum Annex II of OECD 301 (1992a) allows that the inoculum may be pre-exposed to the test substance in order to permit higher test substance concentrations after adaptation. However, the results cannot be used for classification of a substance as being readily biodegradable or for the purpose of persistency assessment. The different proposals on how to implement pre-adapted inoculum in biodegradability testing have been described in the preceding literature study (Gartiser et al. 2017). In the following years further results have been published:

Birch et al. (2017) investigated the influence of inocula derived from different surface waters varying in the degree of urbanisation and thus pre-exposure to hydrocarbons on the biodegradation of hydrocarbons at environmentally relevant levels (ng-µg/L). Aqueous solutions of 9 hydrocarbons were prepared by passive dosing, diluted with surface water and analysed by Solid Phase Microextraction coupled with GC-MS. The results showed that the

source (location) of the inoculum influences biodegradation kinetics such as the duration of the lag phase and the biodegradation rate constants. Similarly, Birch et al. (2018) used the same approach to determine primary biodegradation kinetics of 53 hydrocarbons in the ng/L - µg/L range and different orders of magnitude in hydrophobicity and volatility. Different inoculum sources (activated sludge filtrate, seawater, lake water) were used. The lowest half-lives were obtained with activated sludge, followed by lake water and seawater.

The transferability of these results on RBTs is not given since low test concentrations (ng-µg/L instead of mg/L) and low test volumes (15 mL instead of 100 – 2000 mg/L) have been used and primary biodegradability instead of ultimate biodegradability has been assessed. In fact, the test design used corresponds to a simulation test.

Boonnorat et al. (2017) analysed the elimination of different toxic compounds (bisphenol A, 2,6-di-tert-butyl-phenol, di-butyl-phthalate, di-(ethylhexyl)-phthalate, carbamazepine, diclofenac and N,N-diethyl-m-toluamide (DEET) under different nutrient ratios in activated sludge membrane bioreactors. The bioreactor was run for 106 days at a C/N ratio of 14 and subsequently for further 214 days at a C/N ratio of 6. The elimination of the toxic substances, as determined by chemical analysis, increased from 73-96% at C/B = 14 to 87-100% at C/N = 6. The authors concluded, that the treatment could be enhanced by increasing the nitrogen concentration, which supports the bacterial growth of nitrifying bacteria, which produce the enzymes crucial to degrade the toxic compounds. The improved biodegradation also resulted in lower effluent fish toxicities. However, the influence of the C/N ratio might have been overlapped by general adaptation processes.

Itrich et al. (2015) analysed the microbial adaptation of activated sludge to l-Glutamate-N,N-diacetate (L-GLDA) following its market introduction as a phosphate replacement in automatic dishwashing detergents (consumer product) in the USA. Prior to introduction, L-GLDA was hardly biodegraded in OECD 301 B ready tests inoculated with activated sludge. However, in the laboratory activated sludge simulation test according to OECD 303A after a lag period of 40−50 days a significant biodegradation (>80% DOC-elimination) was observed, indicating increasing biodegradability after adaptation. In the following 22 month after market introduction different OECD 301 B tests with fresh activated sludge were performed. At the beginning, all sludge inocula had a limited biodegradation capacity, but as introduction on the market proceeded, both the rate and extent of degradation increased significantly. After 22 months, L-GLDA was readily biodegradable using inocula from 12 STPs. In an OECD 303A study repeated 18 months after the commercial launch, significant and sustained carbon removal (>94%) was observed after a 29-day acclimation period. According to the authors “this study systematically documented field adaptation of a new consumer product chemical across a large geographic region and confirmed the ability of laboratory simulation studies to predict field adaptation.”

Within the CEFIC Long-range Initiative project ECO29 the implications of microbial adaptation for P-assessment were analysed by Poursat et al. (2019a). The authors state that including adaptation strategies in biodegradability testing would allow a better prediction of persistency of compounds, especially those being present at very low concentrations levels or new chemicals. The adaptation process is linked to the environmental concentration of a chemical. The review refers to several studies which concluded that adaptation occurs only when a minimum threshold concentration is reached. At lower concentrations, adaptation does occur even after long term exposure. For example, Spain et al (1983) tested the biodegradation of p-nitrophenol and 2, 4-Dichlorophenoxyacetic acid (2, 4-D) in sediment/water cores pre-exposed to different test concentrations and found a threshold level of 10 µg/L below which no adaptation was observed. Similarly, Toräng et al (2003) analysed the biodegradation kinetics of the herbicides Mecoprop and 2,4-D at low concentrations in aquifers. Only above concentrations

of 5 µg/L and after a lag phase between 9 and 14 d degradation accelerated due to adaptation. This observation was also linked with theoretical considerations , which predict a threshold for growth-linked degradation at a substrate concentration of ca. 0.2 µg/L. According to the authors "the thresholds can be interpreted as the concentration levels where the net growth of specific biomass is zero (loss equals total growth)."

Within the ECO29 project the effect of adaptation on the biodegradation of metformin and its transformation product guanylurea has been studied by Poursat et al. (2019b), who compared the capability of activated sludge from the STP of Amsterdam to degrade metformin and guanylurea, before and after long-term exposure in a chemostat system. The original activated sludge was able to completely biodegrade both substances within 28 d using the OECD 310 CO_2 Headspace Test. After pre-exposure in the chemostat fed with 1.5 mg/L metformin a partial loss of the biodegradation capacity was observed after 34, 95 and 280 days of exposure. Cultivation in the reference chemostat without metformin quickly led to a complete loss of the biodegradation capacity as well as a shift in the community composition and abundance compared to the exposed community. The authors concluded that "adaptation to this compound is not only driven by its presence in wastewater, but also by various unknown environmental parameters, that will be difficult to mimic in laboratory test systems. Finally, the results of this study showed that pre-exposure to a chemical already present at high concentrations in the environment does not enhance biodegradation in RBT´s as activated sludge microbial communities are probably already adapted to such chemicals."

Aelion et al. (1989) studied the adaptation of microbial communities in aquifers to contaminants and the influence of substrate concentration and preexposure on adaptation. While for ethylene dibromide, aniline and m-nitrophenol biodegradation and adaptation were not determined by chemical concentration, biodegradation of p-chlorophenol increased with concentration while for p-nitrophenol a gradual adaptation and biodegradation was observed up to a threshold concentration of around 30 ng/g (referred to suspended sediment).

Nyholm et al. (1992) analysed the biodegradability of the contaminants, 2,4-dichlorophenoxy acetic, acid (2,4-D); 2,4,6-trichloropbenol (TCP), pentachlorophenol (PCP); 4-nitrophenol (4-NP) and lindane, continuously spiked to synthetic sewage at µg/L levels (5-1000 µg/L) in laboratory sewage treatment reactors. In most experiments, a gradual adaptation took place resulting in increased biodegradation rates. The adaptation times varied between chemicals and experiments and was in the range of 2-5 days for 4-NP and up to 1-2 months for 2.4-D and lindane. No concentration thresholds required for adaptation were observed.

The influence of the test concentration on adaptation and growth has also been explained as a specific microbial strategy to survive in nutrient-poor environments (Egli 2010). The author states that "most free-living microbes were in a dormant state, and only those attached or in close vicinity to algal cells or particulate matter were metabolically active and growing." At very low concentrations no or only very slow growth occurs, suggesting that there is a threshold concentration for bacterial growth in the range of 1-100 µg carbon/L, depending on organism and carbon source. The basic work on this microbial strategy has already been done in the 1940ties by Monod, who postulated, that utilisation of a "better" carbon source is preferred over a "worse" substrate, which makes "ecological sense because the regulation of catabolic enzyme synthesis avoids wasting resources on a low-quality substrate as long as a better one is available."

Helbling (2015) analysed the influence of pesticide concentration in contaminated water resources for optimizing bioremediation. The phenomenon of threshold concentrations which, according to the author are often reported in the range of 1–100 µg/L, may be triggered by

catabolic gene induction. In low concentration environments, mixed substrate utilization has been widely reported, however, the specific activity of a pesticide degrader was suppressed in the presence of easily degradable substrate.

In a more recent reflection paper evolution of biodegradation capacities and pathways in bacteria Kolvenbach et al. (2014) stated that evolution is driven by an individual's gain in fitness. If substrates are not bioaccessible or bioavailable, or their structural modifications reduce degradability, the evolutionary development of degrading enzymes may be restrained. Another prerequisite is the substrate concentration. Below certain concentrations potential sources of nutrition may not be worth acquiring. Emerging pollutants are often present as traces in the environment. Thus, enzymes dedicated to degrading them may not evolve. On the other hand enzymes that catalyse unspecific reactions and are capable of transforming a variety of structural similar substances with their primary substrate (so called promiscuous biocatalysts) often have a drastically lower enzyme activity.

Kundu et al. (2019) described the limits of biodegradation on the example of the herbicide atrazine from a natural attenuation perspective. Although atrazine was banned in Germany 30 years ago, "it is still the most frequently detected contaminant in German groundwater." Several studies suggest that atrazine in principle can be degraded. The question why a compound that is biodegradable persists at low concentrations (µg/L range) remains unresolved. The authors suggest that most laboratory biodegradation tests have been performed at higher concentrations (mg/L range) while in groundwater microorganisms face "concentrations that are of similar magnitude as average concentrations of dissolved readily degradable organic carbon, or amino acids (10 and 1 µg/L respectively)." For investigating the influence of the concentration range and mass transfer limitations the authors performed chemostat experiments with *Arthrobacter aurescens*, a bacterium known to grown on atrazine. Chemostat-based growth was determined by the limited mass transfer at residual concentrations of 30 µg/L of atrazine, which corresponded to a doubling time of the bacterial population of 14 days. Growth kinetics indicated that atrazine can be effectively biodegraded to concentrations as low as 12 µg/L. The mass transfer limitation was assumed to determine the start of the adaptation process. It was stated that at low concentrations and depending on the energy available, microorganisms exist in three different physiological states: "growth phase," "maintenance phase", and "survival phase". The authors hypothesized that the threshold concentration for long-term atrazine degradation corresponds to the concentration where the bacteria enter the "survival phase" with minimal energy supply.

Summarising one could conclude that the test design of the screening tests (relative high test and inoculum concentrations which allow growth kinetics) promotes adaptation of the inoculum within the test, while in simulation tests with environmental more realistic test and inoculum concentrations adaptation is not enhanced. Thus, the demand for allowing pre-adapted inoculum in screening tests seems not being justified.

4 Current status of ready biodegradability testing (WP1)

4.1 Contributing laboratories

In total 16 highly qualified laboratories (mainly GLP certified) contributed to the survey: 10 from Germany (BASF, Currenta, Eurofins, Hydrotox, Ibacon, IDUS Biologisch Analytisches Umweltlabor, Fraunhofer Institut für Molekularbiologie und angewandte Ökologie - IME, Noack Laboratorien, SGS Fresenius, German Environment Agency), 3 from Switzerland (Arcadis, Firmenich, Innovative Environmental Services – IES), and 3 from the United Kingdom (Agromex, Envigo, Scymaris). Thus, there is some bias in the regional allocation of the laboratories contributing to the survey, but this distribution probably reflects the number of available labs in Europe. The 23 pages questionnaire can be made available on request.

4.2 Test selection

The laboratories were asked, which share of tests has been performed in 2017. With respect to the RBTs, thus excluding inherent and simulation tests. The distribution of tests indicated by laboratories is presented in table 2.

Table 2: Ready biodegradability tests perform in 2017 in %

OECD	RBT	No. of labs	Median	Mean	Max
301 A	DOC Die-Away	10	0	3	20
301 B	CO_2 evolution	12	47	45	100
301 C	MITI (I)	7	0	1	4
301 D	Closed bottle	16	0	4	40
301 E	Modified OECD Screening	8	0	1	6
301 F	Manometric respirometry	16	43	46	100
306	Biodegradation in seawater	3	0	2	7
310	CO_2 Headspace	13	3	5	18

Survey among 16 European laboratories, inherent and simulation tests excluded from evaluation.

The two main RBTs currently applied are the CO_2 Evolution Test (OECD 301 B) and the Manometric Respirometry Test (OECD 301 F), which account for at least 80% of all RBTs performed, followed by the CO_2 Headspace Test (OECD 310). The Closed Bottle Test (OECD 301 D) is rarely applied with the exception of one laboratory, which has a preference on this test. The DOC-based tests (OECD A and E), the MITI (I) test (OECD 301 C) and the marine tests (OECD 306) have a minor importance.

The laboratories were also asked, whether they have noticed changes in the distribution of performed tests compared to previous years: Each two laboratories indicated a tendency that the DOC based methods and the MITI (I) tests decreased. Four laboratories observed an increase of the CO_2 Evolution Test. For the CO_2 Headspace Test and the Closed bottle Test both increase or decrease were indicated by single laboratories.

With respect to the main guidance used for the test selection the laboratories answered that the general introduction to OECD 301 next to the Annex II (on testing of inhibitory substances) and

Annex III (on testing of poorly water-soluble substance) are main guidance used. The ECHA Guidance R.7b (especially the Appendix R.7.9-3 on testing poorly water-soluble substances) is also considered while ISO 10634 (ISO 2018a; "Preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability") is only partly known. The ISO/TR 15462 (ISO 2006; "Selection of tests for biodegradability") is out of date and not used.

Another question regarded the general testing strategy applied by the laboratories depending on the substance properties. Here, no consistent answers on the preferred test methods were obtained. The laboratories use all their established and running tests. A potential selection of appropriate test methods could e.g. be, to prefer tests with few parallel vessels for low water soluble test items, which are directly added by weighing, considering that the Closed Bottle Test and CO_2 Headspace Test require numerous flasks, which are sacrificed for each measurement.

With respect to volatile substances the laboratories mainly apply the closed systems OECD 301 C, F and D next to OECD 310. For inhibitory substances most laboratories refer to the Closed Bottle Test (OECD 301 D) while some also refer to the CO_2 Evolution Test (301 B) but using half of the standard test concentration (10 mg/L instead of 20 mg/L TOC).

4.3 Test specifications

4.3.1 DOC-based tests OECD 301 A and E

The DOC based tests OECD 301 A and E are not often applied. The range of the vessel size used varies from 600 to 2000 mL. A typical test consists of 2 test- and 2 control vessels and 1-2 reference vessels while abiotic controls (without inoculum) or adsorption controls (with inoculum) are not always used, but only on request of the sponsor.

With respect to the question how to exclude any influence of adsorption on DOC-elimination, the laboratories follow distinct strategies: some perform solubility pre-tests with DOC for describing the recovery, others also consider the measured values after 0 h, 3 h or 24 h in the test and compare the expected and measured values (similar to 3 h-values in Zahn-Wellens Test OECD 302 B). The biocides applied in the abiotic and/or adsorption controls are mercury chloride ($HgCl_2$: 10-50 mg/L) or sodium azide (NaN_3: 100 - 3750 mg/L). Some laboratories additionally autoclave the inoculum for the adsorption control prior to use.

The main inoculum source used by laboratories for the OECD 301 A test is activated sludge (30 mg/L d.s., solids not removed) followed by activated sludge supernatant (solids removed by filtration/centrifugation) or secondary effluent (100 mL/L). The main inoculum source applied for the OECD 301 E test is the supernatant of activated sludge (solids removed) at 0.5 mL/L. Surface water is not used for the OECD 301 A, but for the OECD 301 E (up to 10 mL/L river or lake water). Usually, no pre-treatment of the inoculum is foreseen, but some laboratories wash the activated sludge with tap water or mineral medium, followed by aeration for 1 to 3 or 5 to 7 days.

It should be noted that in the general introduction to the OECD 301 (section 19) it is stated that *"unless adsorption of the test substance has been ruled out beforehand, tests which measure biodegradation as the removal of DOC, especially with activated sludge inocula, should include an abiotic control which is inoculated and poisoned."* The ISO 7827 (ISO 2010; "*Evaluation of the 'ready', 'ultimate' aerobic biodegradability ... - Method by analysis of ... DOC*") gives further guidance on how to detect an influence of abiotic, physicochemical mechanisms like stripping or adsorption on DOC-elimination by comparing the percentage elimination in test flasks and abiotic elimination. It is stated that "*if a significant loss of organic carbon is observed, no*

differentiation between biotic and abiotic elimination is possible." Further on, only *"if the test substance is not significantly eliminated abiotically (e.g. by adsorption) and the elimination curve has a typical shape with a lag and degradation phase"* the measured DOC elimination should be assigned to biodegradation.

4.3.2 CO_2 Evolution Test OECD 301 B

The CO_2 Evolution Test according to OECD 301 B is one of the most often applied methods. The range of the flask size used varies from 1500 to 5000 mL (average 3000 mL). A typical test consists of 2-3 test- and control flasks and 1-2 reference flasks. Normally no abiotic controls are foreseen (only on request of the sponsor). All laboratories use activated sludge (30 mg/L d.s.), some additionally use surface water or soil as alternative inocula. Usually, the activated sludge is washed with tap water or mineral medium, followed by aeration (with CO_2-free air) for 1-7 d. The preferred test concentration is 10-20 mg/L TOC (corresponding to the guideline) while some institutes also apply minimum concentrations as low as 5 mg/L TOC.

With respect to the measuring device used for CO_2 quantification 7 from 12 laboratories apply IC-measurement (with or without exchange of absorber vessels) while 4 laboratories use the titration method with barium carbonate with exchange of absorber vessels and 1 laboratory applies conductivity measurements in sodium or potassium hydroxide.

The main challenges of the test indicated by the laboratories are air tightness, air flow, and compliance with validity criteria.

4.3.3 MITI (I) test OECD 301 C

The MITI (I) test according to OECD 301 C is rarely applied. The usual test flask size is 500 mL but some laboratories use flasks up to 1000 mL. A typical test consists of 2-3 test- and control flasks and 1-2 reference flasks. As equipment both manometric and electrolytic respirometers are used. The main challenge of the test is the inoculum source. In total 4 from 7 laboratories use the standard mixed inoculum from at least 10 sites (STP, rivers, lakes, surface soil etc.), which is further cultivated in the laboratory with synthetic sewage (glucose, peptone and potassium orthophosphate) for several months as described in the 301 C. However, 4 laboratories use activated sludge instead, as used for the other RBTs. Rarely surface water or soil or seawater are also applied.

Most laboratories complained the labour-intensive inoculum preparation and cultivation of the inoculum and its weak potency. Many clients are not willing to cover these costs and thus the test is often run with "normal" activated sludge (not guideline conform but usually accepted by authorities according to the laboratories). Some laboratories also referred to the similarity of the MITI (I) test to the Manometric Respirometry Test OECD 301 F.

For nitrogen containing substances, as for all oxygen based RBTs, the test results may be influenced by the oxygen consumption needed for nitrification. This may optionally be considered by additional nitrite and nitrate measurements and the correction procedure described in Annex V of OECD 301. Some laboratories optionally follow this approach on request of the sponsor, others first perform a routine test without nitrite and nitrate measurements and subsequently compare the results with both reference points ($ThOD_{NO3}$ and $ThOD_{NH3}$) with and without considering complete nitrification. Only if conflicting overall results (< 60% $ThOD_{NO3}$ but > 60% $ThOD_{NH4}$) are obtained a repetition of the test with additional nitrite and nitrate analytics is envisaged.

4.3.4 Closed Bottle Test OECD 301 D

The Closed Bottle Test according to OECD 301 D is not often applied, but preferred by some laboratories for volatile or inhibitory substances. The flask size usually is about 250 mL but may be increased for eRBTs up to 1000 mL. A typical test consists of 16-20 test- and control-flasks (2-3 for each time point) and 10-20 reference flasks. A main challenge of this test is the validity criterion on inoculum blanks of less or equal 1.5 mg/L oxygen consumption, which may require a specific cleaning procedure of the flaks. In this context some laboratories indicated that they use conventional dishwasher with an additional acid rinse or conventional dishwasher followed by a 6 h drying period at 180°C. Others use an iodine/acid mixture or hot tap water followed by an ethanol/acid mixture, tap water and ultrapure water.

The preferred inoculum is secondary effluent from municipal STP from which solids are removed via a coarse filter, followed by an aeration period of 0-7 days. Alternatively, the filtrate or centrifugate of activated sludge is used and aerated for 7 days (which essentially should be similar to the secondary effluent). Some laboratories sometimes also use surface water as inoculum. One laboratory also uses activated sludge (2 mg/L d.s.) as inoculum (not guideline conform).

With respect to the oxygen measuring devices most laboratories use galvanic or optical oxygen sensors, but one laboratory applies the Winkler methods (precipitation as manganese hydroxide followed by titration with thiosulfate).

For nitrogen containing substances the oxygen consumption for nitrification may cause conflicting results (see remarks for the MITI (I) test above). Some laboratories optionally perform nitrite and nitrate measurements on request of the sponsor, others only consider a repetition of the test with nitrogen measurements in case of conflicting results in a previous test. One laboratory indicated that nitrogen measurements are only considered if the nitrogen content of the molecular weight of the test item is higher than 10%. Another strategy is to always refer the results to the $ThOD_{NO3}$, thus assuming nitrification (worst case assumption). One laboratory indicated that they avoid the addition of ammonium chloride (NH_4Cl) with the mineral medium, while assuming that the endogenous nitrogen-supply is sufficient.

4.3.5 Manometric Respirometry Test OECD 301 F

The Manometric Respirometry Test according to OECD 301 F is often applied. The usual test flask size is 500 mL but some laboratories use flasks up to 1000 mL. A typical test consists of 2-3 test- and control flasks and 1-2 reference flasks. The used equipment includes both manometric and electrolytic respirometers.

Most laboratories use activated sludge from municipal STP as inoculum, which is washed with mineral medium and aerated for 1 to 7 days in order to reduce the blank respiration. Each one laboratory occasionally also uses secondary effluent or surface water (undiluted, suspended solids removed by a coarse filter). The preferred test concentration is 50-100 mg/L ThOD (as described in the guideline).

The biocides applied in the abiotic controls are mercury chloride ($HgCl_2$: 1-10 mg/L), sodium azide (NaN_3: 100 - 1500 mg/L) or copper sulphate ($CuSO_4$: 20.5 mg/L).

For nitrogen containing substances the oxygen consumption for nitrification may cause conflicting results (see remarks for the MITI (I) test above). Some laboratories optionally perform nitrite and nitrate measurements on request of the sponsor, others only consider a repetition of the test with nitrogen measurements in case of conflicting results in a previous test or consider a nitrogen content of 10% (w/w) as unproblematic (see remarks for the Closed

Bottle Test above). Another strategy is to always refer to the $ThOD_{NO3}$, thus assuming nitrification (worst case assumption). One laboratory indicated that they use the specific nitrification inhibitor allythiourea (ATU: 2 mg/L) for avoiding nitrification.

The main challenge of the test is the air tightness and constancy of temperature, next to uncertainties for determining the COD or for calculating the ThOD from the sum formula or elementary analysis.

4.3.6 CO_2 Headspace Test OECD 310

The CO_2 Headspace Test according to OECD 310 is one of the most important tests (after the OECD 301 B and F). The typical flask size is between 160 and 310 mL. A typical test consists of 15-35 test- and control-flasks (2-3 each time point and 5 at day 28) and 8-20 reference flasks. A main challenge of this test is the validity criterion on inoculum blanks of less or equal 3 mg/L inorganic carbon (IC), which may require a specific cleaning procedure of the flasks. In this context, some laboratories indicated that they use conventional dishwasher with an additional acid rinse or an ethanol/acid mixture, tap water and ultrapure water. One laboratory uses as ethanol/acid mixture, followed by the solvents n-hexane and acetone, tap water and ultrapure water.

Most laboratories use activated sludge from municipal STP as inoculum, which is washed in mineral medium and aerated with CO_2 free air for 1-7 days. One laboratory indicated that occasionally also secondary effluent from municipal STP or soil are used.

The preferred test concentration is 10-25 mg/L TOC, the minimum possible 2-5 mg/L TOC (range indicated in the guideline is 4-20 mg/L).

For the quantification of the CO_2 most laboratories measure the IC in the liquid phase after addition of NaOH. Two laboratories measure the CO_2 in the headspace after acidification (both options are described in the guideline).

The biocides applied in the abiotic and/or adsorption controls are mercury chloride ($HgCl_2$: 10 mg/L) or sodium azide (NaN_3: 100 - 4000 mg/L). One laboratory adds formaldehyde (4 mL 37% per bottle) for this purpose.

4.4 Improvement of testing principles

4.4.1 Potentially obsolete tests

The laboratories were asked whether certain test procedures have become unnecessary. In total 3 from 16 laboratories answered with “no”. About half of the laboratories suggested omitting the DOC-based tests OECD 301 A and E because of the unspecific endpoint DOC. The remaining 7 laboratories suggested to merge the OECD 301 A and E into one method. About half of the laboratories also indicated that the MITI (I) test OECD 301 C could be withdrawn due to the effort for cultivating the inoculum and its low inoculum potency. The remaining 8 laboratories suggested to merge the OECD 301 C and F, both being respirometric methods. Two laboratories proposed to avoid the Closed Bottle Test 301 D due to the low potency of the inoculum.

4.4.2 Reduction of the number of tests

In this context, the experts were also asked whether the number of tests should be reduced. Again, about half of the laboratories suggested to merge the OECD 301 A and E as well as the OECD 301 C and F. Six out of 16 laboratories were also open to merge different testing principles by establishing combination tests with several endpoints, such as CO_2 evolution combined with

oxygen consumption, or CO_2 combined with DOC, next to oxygen consumption combined with DOC or all three different endpoints. However, 2 laboratories were against obligatory multi-end-point testing due to the equipment needed and the different substrate/inoculum ratios required for accurate testing. One expert also referred to the difficulty to handle differences between results obtained from the different monitored parameters.

4.4.3 Ranking of proposed tests

Another question to the laboratories was whether they would support a ranking of the proposed test methods according to the endpoints measured and characterisation of the test substances. Eight laboratories agreed that those endpoints referring to mineralization (O_2, CO_2) should be preferred against methods using only DOC-elimination due to its susceptibility to abiotic elimination through adsorption or volatility. Further on, 9 laboratories suggested that for nitrogen containing substances the endpoint CO_2 should be preferred against O_2 because of uncertainties of oxygen consumption due to nitrification.

4.4.4 Testing of volatile substances

Most laboratories agreed that volatile substances should be tested in closed test systems OECD 301 C or OECD F or OECD 310 while one expert did to see risks from using open test systems or those with a headspace as long as O_2 or CO_2 are measured, since biodegradation results would be underestimated. One lab referred to OECD 309 (surface water simulation test), where a Henry's law constant < 1 $Pa \cdot m^3 \cdot mol^{-1}$ ($\sim 10^{-5}$ $atm \cdot m^3 \cdot mol^{-1}$) is regarded as non-volatile and <100 $Pa \cdot m^3 \cdot mol^{-1}$ as slightly volatile. The last could be tested in closed systems with headspace. Likewise in the CO_2 Headspace Test guideline OECD 310 it is mentioned, that when using the recommended headspace to liquid volume ratio of 1:2, volatile substances with a Henry's law constant of up to 50 $Pa \cdot m^3 \cdot mol^{-1}$ can be tested. For these, the amount of the test substance in the headspace is assumed to remain below 1%.

4.4.5 Testing of inhibitory substances

Considering the best testing strategy for inhibitory substances 6 laboratories of 16 considered the Closed Bottle Test (OECD 301 D) as best option, due to its lowest test concentration from all RBTs of 2 mg/L while two experts doubt whether the 301 D is the best option due to its low inoculum potency. One laboratory reported good experience when using activated sludge 2 mg/L d.s. as inoculum in the Closed Bottle Test. Five labs referred to good results with inhibitory substances when tested in the OECD 301 F (with lower test concentrations) or to the OECD 310. Four laboratories apply adsorbents (silica gel, humic acids) with the 301 B or 301 F to reduce toxicity.

4.5 Test design

4.5.1 Vessel size and number of replicate vessels

Another question to the laboratories was whether the vessel size of the different RBTs should be modified or prescribed. Most laboratories did not consider this proposal as useful. Some suggested that any size of the vessels should be allowed as long as the validity criteria are fulfilled and that the volume should be adopted to the specific needs.

Most laboratories also regarded the number of replicate vessels as sufficient for accurate testing. The existing rules were considered as sufficient. Some laboratories suggested to increase the number of replicate vessels of the OECD 301 B and F from 2 to 3 for improving the data basis but also for allowing to exclude outliers. Some experts suggest adopting the number of sacrificed

flasks of the Closed Bottle Test (301 D) to that of the CO_2 Headspace Test (OECD 310) to a minimum of 3 flasks per measurement point and 5 at the end of the test (day 28).

4.5.2 Identification of abiotic elimination

The DOC-based methods OECD 301 A and E have lost much of their former importance and have the drawback that they strictly speaking cannot distinguish between abiotic DOC-elimination and mineralisation. The laboratories were therefore asked whether the DOC based methods should be accompanied by obligatory adsorption controls as suggested in the general introduction to OECD 301. 10 of 16 laboratories suggested this as useful, while 4 labs were against such a proposal. Two laboratories suggested omitting the 301 A or E anyway.

Another expert suggested to introduce a 3 h value when using the DOC based OECD 301 A or E, similar as in the inherent Zahn-Wellens Test OECD 302 B where the DOC-elimination after 3 h is considered as a surrogate for adsorption. Another laboratory also referred to the need of biocides to be added to the abiotic and adsorption controls such as mercury chlorid ($HgCl_2$) which is a substance of very high concern (SVHC). This implicates occupational and environmental concerns next to the generation of hazardous waste.

4.5.3 Selection of reference substances

In the general introduction to OECD 301 (section 11) it is mentioned that the most often used reference substances sodium acetate, sodium benzoate and aniline all degrade in RBTs even when no inoculum is deliberately added. It was suggested that a reference compound that is readily biodegradable but requires the addition of an inoculum should be looked for. Potassium hydrogen phthalate has been proposed but more evidence needs to be obtained before it can be accepted as a reference compound. This was one of the reasons why laboratories were asked whether the reference compounds should be selected according to the characteristics to the test item in terms of water solubility physical state (water soluble/non-water-soluble liquid or solid). Half of the laboratories were in favour to such a proposal, half against it. Reference was given to microcrystalline cellulose for solid test items, rapeseed oil for non-water-soluble lubricants and di-isooctylphthalate. However, it was noted that further experience and research is needed before one could decide on supplemental reference substances.

4.6 Inoculum

4.6.1 Allowed inoculum sources

The OECD 301 (section 17) requires, that inocula derived from STP should be restricted to those STP which receive predominantly domestic sewage. However, considering that most municipal STP also treat indirectly discharged wastewater from manufacturing industry, OECD 301 does not give guidance on how “predominately domestic wastewater” could be distinguished from industrial STP. For this, no clear criteria have been established. One option would be to compare the capacity of the STP in population equivalents (PEs = unit per capita loading) with the real inhabitants connected to this STP. One PE equals to 54 grams of BOD per 24 hours discharged by one person, the organic load from industrial indirect dischargers is also described by their number of PEs. When the population equivalents are far higher than expected from the inhabitants connected to the STP this could be attributed to industrial dischargers.

Laboratories were therefore asked, which suggestions they have for selecting the inoculum and for excluding STP with a high industrial share. However, none of the expert provided a proposal on exclusion of STP with a “high” industrial share. One expert noted that as long as the OECD 301 allows such a wide range of inocula (from STP to soil), the type of STP was irrelevant. Other

experts considered the current guidance on inoculum as reasonable. Only those STP assigned as "industrial STP" should be excluded.

One laboratory suggested that if there is a concern regarding the contribution of industrial discharges around the sludge collection area, a microbial community analysis could be performed and compared with two other domestic sources. If at least 80% similarities are found, the inoculum from the first STP should be acceptable.

Generally, the high variability of the inoculum over time and location was mentioned. Thus, most laboratories see no benefit in further specifying the inoculum composition. Another comment was that also older test chemicals are on the market to which STPs are adapted, thus reflecting the real situation. This is also in line with the ECHA Guidance on information requirements R.7b (section R.7.9.4.1), which states that inoculum from municipal STPs is acceptable although being "pre-exposed to substances, which are generally continuously emitted to municipal STPs" (ECHA 2017a).

4.6.2 Characterization of the inoculum

The general introduction to the OECD 301 (section 27) requires that the test report must include information about nature and sampling site(s) of the inoculum, its concentration and any pre-conditioning treatment, the proportion and nature of industrial waste water in sewage, if known. Regulatory authorities complain that this information is not regularly provided with the (qualified) study summaries being part of a REACH registration. Thus, the laboratories were asked whether and which information should be documented in order to better characterize the inoculum. Most laboratories were in favour of describing the origin and source, some supported also to indicate the industrial share and treatment technology if available. However, the last information may not be known or made available from all STPs. Most laboratories did not support to determine additional microbiological parameters such as colony forming units (CFU) for a better characterisation of the inoculum. It was doubted how these data could be interpreted within the framework of a single test on one substance only. Instead, it was suggested that additional measurements should better be implemented by testing different inocula in the same test.

4.6.3 Allowed inoculum origin and concentrations

In the general introduction to OECD 301 (section 17) a variety of inoculum sources from activated sludge, sewage effluents, surface waters and soils or from a mixture of these sources is mentioned. The different OECD 301 RBTs partly allow a wide range of inocula (OECD 301 A, B) while others such as the Closed Bottle Test (OECD 301 D) are quite strict by only mentioning secondary effluent and surface water. The MITI (I) test (OECD 301 C) is a specific case because it requires a mixture of inocula from at least 10 sources, which are subsequently cultured under artificial laboratory conditions with synthetic wastewater. Obviously, several laboratories use other standard inocula such as activated sludge in the MITI (I) test. The laboratories were therefore asked whether they would support changes in the allowed inoculum sources and concentrations. Currently, the maximum allowed inoculum concentration allowed are as follows:

- Activated sludge 30 mg/L d.s. (OECD 301 A, B, C, F, 310)
- Secondary effluent 0.5 Vol. % (OECD 301 E) - 10 Vol. % (OECD 301 A, B, F, 310)
- Surface water 10% (OECD 310) - 100% (OECD 301 A, B, F)

Most laboratories support the use of any of the allowed OECD 301 inoculum sources in any RBT as far as the validity criteria are met. However, the signal-to-noise ratio should be considered.

One expert also proposed routine testing with 3 different inocula (STP, surface water, soil) and to compare the results. Another expert insisted that the current inoculum sources of the RBT should be maintained because these different study types were developed using the inoculum that is stated in respective RBT. It was also stated that the results from tests with activated sludge and tests with lower density of bacteria (surface water, sewage plant effluent) may differ significantly and are therefore difficult to compare. It was suggested to differentiate between tests with or without activated sludge or to perform tests with two different inocula (e.g. activated sludge and surface water).

In case of tests with sewage sludge, the inoculum concentration could be specified more precisely. The definition of substrate-to-inoculum ratio could be helpful to improve the signal-to-noise ratio.

4.6.4 Influence of new treatment technologies

The treatment technology of municipal STPs has changed considerably in the last decades. Formerly, a typical STP consisted of a primary treatment (settlement of solids), the secondary treatment (usually activated sludge) and the final clarifier (settlement of activated sludge flocs). In the last decades further treatment steps have been included for removal of nutrients (nitrogen, phosphorus). These are attributed as tertiary treatment. Moreover, the effluent may be further treated with sand filter or ultraviolet light (UV) or Ozone for the improvement of hygienic conditions. Currently, the fourth treatment step for the removal of micropollutants is being established. Therefore, the laboratories were asked whether they expect an influence of these new treatment technologies on the inoculum derived from STP and on the test results.

Most experts do not expect any influence. The activated sludge process still represents the most important treatment step. The quality of final effluent may have been improved in the last decades, but there still exist enough smaller municipal STPs representing the standard technology. One laboratory observed reduction of inoculum quality after changes of the aeration procedure. Another laboratory faced reduced inoculum quality of secondary effluent behind final sand filter and after addition of iron salts for phosphorus-precipitation. Some laboratories circumvent uncertainties of the quality of the secondary effluent by using the supernatant of settled activated sludge inoculum for this purpose.

5 Selection of test substances for practical testing

In the following subchapters the results of the search performed to identify potential test substances for upcoming practical testing are presented and discussed. Screening test results for the candidates are presented in Appendices B.1-B.6, physico-chemical parameters are summarised in Appendix B.7 and molecular formulas are shown in Appendix B.8.

5.1 OECD eChem Portal

5.1.1 Compounds with water solubility < 100 mg/L

An extensive search was performed within the OECD eChemPortal (OECD, 2019b). More than 400 hits (i.e. compounds with different CAS No.) were obtained when using the following query criteria (combined query blocks): biodegradation in water: screening tests (OECD 301 and 310 as well as respective US EPA test guidelines and EU Methods); 20-40% degradation; aerobic; experimental study; reliability 1 (reliable without restriction) and 2 (reliable with restriction); water solubility ≤ 100 mg/L.

Therefore, the search was refined by only considering compounds with results from simulation tests for water (e.g. OECD 309), aquatic sediment (e.g. OECD 308) and/or soil (e.g. OECD 307). 16 compounds were found with simulation test data for soil, 16 compounds were found with simulation test data for aquatic sediment systems and 5 compounds were identified with simulation test data for both compartments. Only 4 compounds were available with simulation test data for surface water (e.g. OECD 309).

In total, 17 potential test compounds could be identified. In addition, six further potential test compounds without information on simulation test data were identified in a subsequent search with restrictions made for physico-chemical parameters (i.e. Henry's law constant, hydrolysis, toxicity to microorganisms). Test results are as listed in Appendix B.1.

5.1.2 Compounds with water solubility > 100 mg/L

A subsequent search was performed using the same query criteria as described in chapter 5.1.1 with the following exceptions: no restrictions were made for water solubility but restrictions were made for physico-chemical data on volatility (Henry's law constant < 1Pa*m³/mol), hydrolysis (dissipation half-life of parent compound > 28 days) and toxicity to microorganisms (EC_{50} > 300 mg/L).

Nine further potential test compounds could be identified as presented in Appendix B.2.

5.2 EFSA Draft Assessment Reports

About two hundred EFSA Draft Assessment Reports (DARs; Rapporteur Member State assessment reports submitted for the EU peer review of active substances used in plant protection products) published before 2015 were consulted for suitable compounds based on reported results from RBTs. Seven candidates could be identified (see Appendix B.3). For all these compounds simulation test data are available for aquatic sediment systems and soil.

5.3 NITE Chemical Risk Information Platform / J-CHECK Database

The Japan CHEmical Collaborative Knowledge database (J-CHECK)[3] of the National Institute of Technology and Evaluation (NITE, Japan) was searched for biodegradation data (mainly screening test data from OECD 301C) with 20-40% biodegradation obtained within a test period of 28 days. In a first step, 50 compounds were identified. When excluding compounds with a water solubility clearly above 100 mg/L, 22 potential test compounds could be determined (see Appendix B.4).

5.4 Scientific reports and Literature

Potential test compounds were also searched within scientific reports (e.g. ECETOC, Cefic) and literature (i.e. international peer-reviewed journals). Based on this search, three additional test compounds were identified (see Appendix B.5).

Supplementary, phenanthrene could be identified as potential test substance, although biodegradation rates >40% could be observed in two RBTs according to OECD 301 C (see Appendix B.6). Phenanthrene might be an interesting test compound since biodegradation rates are extremely dependent on the (a)biotic conditions. Furthermore, simulation test data are available for surface water (OECD 309), aquatic sediment (OECD 308) and soil (OECD 307). Phenanthrene has recently been proposed as a substance of very high concern (SVHC) due to a proposed attribution as vP based on a failed RBT (with regard to 54% mineralization in 28 d) and very slow degradation in soil (half-lives >180 d) (ECHA 2018a). The proposal was intensely discussed in consideration of further biodegradation data from RBTs (67.2% mineralization, Junker et al. 2016) and simulation studies for sediment and soil (half-lives <120 d) (ECHA 2018b, Hughes et al. 2020).

5.4.1 Reference chemicals identified by Comber & Holt

The report published on reference chemicals for use in biodegradability tests for assessing the persistency of chemicals (Comber and Holt 2010) was worked through in view of suitable test compounds. The authors proposed to distinguish between reference chemicals that would

- pass a RBT and a modified[4] RBT (Bin 1),
- pass an enhanced screening biodegradability test but fail other screening tests (Bin 2),
- normally fail any modified RBT or enhanced screening biodegradability test but could pass standard inherent tests (OECD 302 series) (Bin 3),
- never pass a modified RBT or an enhanced screening biodegradability test and there is no evidence of biodegradation (Bin 4).

[3] The Japan CHEmical Collaborative Knowledge database (J-CHECK) was developed to provide the public with the safety information on chemical substances related to the Act on the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc. (hereinafter "the Chemical Substances Control Law") by the Ministry of Health, Labour and Welfare (MHLW), the Ministry of Economy, Trade and Industry (METI), and the Ministry of the Environment (MOE).

[4] ECHA guidance differentiates between enhanced RBTs and modified RBTs. Two modifications are identified: Testing at low test substance concentrations due to inoculum toxicity and the application of specific dosing methods (e.g. emulsifiers, solvents, carriers, ultrasonication) for biodegradability assessment of poorly water-soluble substances. Provided that all other conditions of RBTs remain unchanged, modified RBTs are considered as equivalent to regular RBTs and the results can be used accordingly (ECHA 2017a), i.e. to assess ready biodegradability. This is not true for enhanced RBTs which are exclusively used as a tool of low time and effort to identify non-persistence.

The search was focused on compounds belonging to Bin 2 (i.e. reference chemicals that would normally pass an enhanced screening biodegradability test but currently fail any other screening tests; n = 13) and Bin 3 (i.e. reference chemicals that would normally fail any biodegradability screening test whether modified RBT or enhanced screening biodegradability test). Compounds of Bin 2 and Bin 3 are considered to have the highest potential to meet the selection criteria (i.e. showing a biodegradability of 20-40% in standard RBTs without reaching a plateau within 28 days), whereas Bin 1 and Bin 4 might contain suitable positive and negative reference compounds, respectively (see chapter 5.4.1.3). For all candidates, further screening test results were collected from information portals and databases (ECHA, J-CHECK, HSDB) and from literature. The data are presented and discussed in the following. For test results see also Table 3.

5.4.1.1 Substances of Bin 2

Diethylene glycol (111-46-6): Variable results are reported by Comber and Holt. The test substance failed tests according to OECD 301 D and biodegradation rates between 59% and 89% were observed in tests based on OECD 301 F, depending on the measurement method. However, 70-80% biodegradation based on CO_2 evolution (OECD 301 B) are reported by ECHA (2019) and the substance is therefore classified as readily biodegradable.

4-chloroaniline (106-47-8): According to Comber and Holt, 4-chloroaniline failed a Closed Bottle Test (OECD 301 D). This result is confirmed by ECHA (2019) for another OECD 301 D test. The substance also failed an ISO 14593 test (ISO 1999a; equivalent to OECD 310; Ahtiainen et al. 2003). In addition, long lag phases (e.g. 15-20 d) were observed before start of biodegradation. Martin et al. (2017b) investigated the biodegradation of 4-chloroaniline in studies based on OECD 301 B. In the experiments, the test duration was prolonged to 42 days and two different inoculum concentrations (3 mg/L and 300 mg/L) were used. At the end of the studies, 56.6% biodegradation was observed when using the low inoculum concentration (variability was high showing results in the range 25.2-72.4%) and 74.0% (range: 71.5-75.2%) CO_2 evolution were measured when using the high inoculum concentration. After 28 days, mean biodegradation was approximately 40% and 60% for the low and the high inoculum concentration, respectively.

1,3,5-trimmethylbenzene (108-67-8): The substance failed the modified OECD 301 F test, when using non-acclimated inoculum (36% biodegradation at day 28 and 56% biodegradation at day 53), but passed the test, when using acclimated (i.e. re-inoculated) inoculum. ECHA (2019) reported 61% biodegradation in an OECD 301 F test. The substance is considered to be readily biodegradable but failing the 10-day window.

2,4-dinitrotoluene (121-14-2): For this compound only one experimental result from an RBT is available for dinitrotoluene (CAS No. 25321-14-6). No degradation was observed in an MITI test (OECD 301C) within 14 days.

4-fluorophenol (371-41-5): On the ECHA website this compound is considered to be readily biodegradable based on a non-guideline study, where 4-fluorophenol (50 to 125 mg/L) was completely biodegraded (primary degradation based on parent analysis) within 3.5 to 7 hours (ECHA 2019). Martin et al. (2017b) tested 4-fluorophenol in a modified RBT test design (i.e. OECD 301 B, prolonged to 60 days). When using 3 mg/L inoculum concentration, biodegradation rates in the range between 46.2 and 52.8% (mean: 50.2%) were observed. However, when using a higher inoculum concentration (300 mg/L) 61.8-63.1% (mean: 62.4%) biodegradation were achieved. After 28 days, mean biodegradation was approximately 40% and 60% for the low and the high inoculum concentration, respectively.

The following substances were initially considered as potential candidates by Comber and Holt, but were excluded afterwards, because biodegradability could not be assessed with sufficient confidence.

Benzene (71-43-2): Variable RBT results are reported by Comber and Holt for benzene. The substance is classified as readily biodegradable by ECHA (2019) based on results from OECD 301 F. Further RBT results are available for OECD 301 D (Closed Bottle Test) from six different laboratories. The results show a high variability with biodegradation rates ranging from 4% to 88% within 28 days. However, insufficient details are provided to determine whether the validity criteria of the test were met. Nevertheless, ECHA states that the results indicate that benzene can biodegrade rapidly.

Hexadecane (544-76-3): Variable RBT results are reported by Comber and Holt (2010). Only one biodegradation study in seawater according to OECD 306 is available, resulting in 74% biodegradation within 28 days. The substance is therefore considered as readily biodegradable by ECHA (2019).

Di-isooctylphthalate (27554-26-3): Comber and Holt (2010) reported a typical mineralization rate of 50-60% within 28 days. Further biodegradation rates reported in literature are in the same range. Nyholm (1990) studied the biodegradation of di-isooctylphthalate by means of manometric respirometry using different methods of test substance application. After direct addition, 65% biodegradation was measured, whereas 46-60% biodegradation was observed when using application on silica gel and glass fiber filters, sonication and emulsification (with Tween 80 and Ufasan). 57% CO_2 evolution were observed in a test according to guideline EPA 560/6-82-003 (US EPA 1982), reported by Sugatt et al. (1984).

1,3,5-dichlorobenzene (108-70-3): Based on Comber and Holt (2010) the substance is reported to fail RBTs. This classification is corroborated by a test result for OECD 301 C, showing no degradation within two weeks reported by NITE (2019).

4-nitrophenol (100-02-7): For 4-nitrophenol screening test results show a strong variation of results with degradation rates of 1-97% depending on the type of test and the inoculum used. The compound is therefore considered to be inherently biodegradable (ECHA 2019). Martin et al. (2017b) observed 83.8-91.1% biodegradation (mean: 87%) in a modified RBT (OECD 301 B, prolonged to 60 days) when using an inoculum concentration of 3 mg/L and 92.4-94.6% biodegradation (mean: 93.5%) when using a 100-fold inoculum concentration (i.e. 300 mg/L). After 28 days, mean biodegradation was approximately 80% and 90% for the low and the high inoculum concentration, respectively.

4-nonylphenol (84852-15-3): Variable RBT results are reported by Comber and Holt (2010). The substance did not pass an RBT according to OECD 301 B, showing 47.5% degradation. However, in a test according to OECD 301 F, 57.4%-68.4% (mean: 62%) biodegradation were achieved, but the 10d-window was failed. The substance is classified as inherently biodegradable by ECHA (2019).

Octylphenol (140-66-9): Variable RBT results are reported by Comber and Holt (2010). Based on the ECHA registration dossier (ECHA 2019), octylphenol showed no degradation within 28 days in a modified MITI test (OECD 302 C). In contrast, the substance showed 70% CO_2 evolution in an OECD 301 B test after 35 days. Overall, octylphenol is considered to be inherently biodegradable based on a BOD test for insoluble substances using Octylphenol (CAS No. 27193-28-8), resulting in 20% mineralization (O_2 consumption) within 28 days. Furthermore, the substance meets the criteria for ready biodegradability except for the 10-day window based on OECD 301 B.

Pentaerythritol (115-77-5): Several RBT results are reported by ECHA (2019), showing biodegradation rates >60%. The compound is therefore considered to be readily biodegradable. Contrarily, only 13.2% biodegradation (O_2 consumption) within 25 days is reported by NITE (2019). Further data reported by Comber and Holt (2010) show variable test results, e.g. 32.7% degradation within 28 days in an OECD 301 F and 13-97% in OECD 301 E studies. The authors explain that the compound was excluded from the list of candidates since results indicate that pentaerythritol passes a RBT more easily than expected.

5.4.1.2 Substances of Bin 3

Di-isotridecyl adipate (26401-35-4): Di-isotridecyl adipate is recommended as reference substance in the CONCAWE test on inherent biodegradability to demonstrate the increased power of this test over a RBT. The CONCAWE test has been submitted as proposal for a new OECD 302 D guideline in 1999, but was not accepted by OECD. According to the CONCAWE test, the substance "is typically biodegraded by approximately 30% ThIC (=theoretical inorganic carbon, similar to $ThCO_2$) after 28 days with an unexposed inoculum (e.g. in OECD 301 B) but can be mineralised by 40 - 80% ThIC in this test." Ring test results are reported showing 65 ± 21% biodegradation within 56 days. However, the substance is considered to be readily biodegradable based on studies according to OECD 301 F and OECD 301 B studies according to ECHA (2019).

Diethylhexylphthalate (117-81-7) is considered to be readily biodegradable based on an OECD 301 B test (ECHA 2019) For all other potential reference compounds of Bin 3, available data on RBT show no degradation according to ECHA (2019) and/or NITE (2019).

Table 3: Potential reference compounds of Bin 2 according to Comber and Holt (2010)

Substance name	CAS No.	RBT Result [%]	Parameter	Test Duration [d]	Test Guideline	Reference
Final recommendations for reference set						
Diethylene glycol	111-46-6	90-100 70-80 82-98 65 59 89	DOC removal CO_2 evolution O_2 consumption O_2 consumption O_2 consumption O_2 consumption	28 28 28 28 28 28	301 A 301 B 301 C 301 D (acclimated sludge) 301 F (Sapromat) 301 F (OxiTop)	ECHA (2021) ECHA (2019) NITE (2021) SIDS (2021) SIDS (2021) SIDS (2021)
4-chloroaniline	106-47-8	0[2)] 25.2-72.4 71.5-75.2	O_2 consumption CO_2 evolution CO_2 evolution	30 42 42	301 D 301 B (low inoculum)[5)] 301 B (high inoculum)[6)]	ECHA (2019) Martin et al. (2017b) Martin et al. (2017b)
1,3,5-trimethylbenzene	108-67-8	61[3)]	O_2 consumption	28	301 F	ECHA (2019)
2,4-dinitrotoluene	121-14-2	0[4)]	O_2 consumption	14	301 C	NITE (2019)
4-fluorophenol	371-41-5	100 46.2-52.8 61.8-63.1	Parent analysis CO_2 evolution CO_2 evolution	3.5 – 7.0 h 60 60	Non-guideline study 301 B (low inoculum)[5)] 301 B (high inoculum)[6)]	ECHA (2019) Martin et al. (2017b) Martin et al. (2017b)
Di-isotridecyl adipate[1)]	26401-35-4	59-69 57-81 65	O_2 consumption CO_2 evolution CO_2 evolution	28 28 56	301 F (n=2) 301 B (n=3) 302 D (ISO 14593)	ECHA (2019) ECHA (2019) ECHA (2019)
Compounds that were initially included but excluded afterwards						
Benzene	71-43-2	63-96 4-88 40 69	O_2 consumption O_2 consumption O_2 consumption Parent analysis	28 28 14 14	301 F 301 D 301 C 301 C	ECHA (2019) ECHA (2019) NITE (2019) NITE (2019)
Hexadecane	544-76-3	74	O_2 consumption	28	306 (seawater)[7)]	ECHA (2019)

Substance name	CAS No.	RBT Result [%]	Parameter	Test Duration [d]	Test Guideline	Reference
Di-isooctyl phthalate	27554-26-3	57	CO_2 evolution	28	EPA 560/6-82-003	Sugatt et al. (1984)
1,3,5-trichlorobenzene	108-70-3	0	O_2 consumption	14	301 C	NITE (2019)
4-nitrophenol	100-02-7	1-97 83.8-91.1 92.4-94.6	Various CO_2 evolution CO_2 evolution	Various 60 60	Various 301 B (low inoculum)[5)] 301 B (high inoculum)[6)]	ECHA (2019) Martin et al. (2017b) Martin et al. (2017b)
4-nonylphenol	84852-15-3	47.5 57.4-68.4	CO_2 evolution O_2 consumption	28 28	301 B 301 F	ECHA (2019) ECHA (2019)
Octylphenol	140-66-9	70	CO_2 evolution	35	301 B	ECHA (2019)
Pentaerythritol	115-77-5	84 >95 64 13	CO_2 evolution DOC removal O_2 consumption O_2 consumption	28 28 28 25	310 301 E 301 F 301 C	ECHA (2019) ECHA (2019) ECHA (2019) NITE (2019)

[1)] The substance belongs to Bin 3 (compounds that should normally fail any screening test whether modified RBT or enhanced screening test. [2)] Tested concentrations were in the range of measured toxicity for bacteria. [3)] failing 10-d window. [4)] Result for dinitrotoluene (CAS No. 25321-14-6). [5)] Inoculum concentration: 3 mg/L. [6)] inoculum concentration: 300 mg/L. [7)] "Hydrocarbons, C14-C20, aliphatics (≤2% aromatics)" were found to be readily biodegradable (biodegradation > 60% ThOD) in an OECD 306 ready biodegradability test. Taking into account that all those substances have been found biodegradable in seawater test OECD 306, this is taking over all screening tests results and lead to the conclusion that all the substances belonging to this category with the same properties and are therefore considered readily biodegradable (ECHA 2019).

Based on these data, particularly 4-chloroaniline (water solubility: 3900 mg/L) and 4-fluorophenol (water solubility: 5500 mg/L) might be used as test compounds within the biodegradation experiments, i.a. when considering the biodegradation rates reported by Martin et al. (2017b) after 28 days and after a prolonged test period of 42-60 days. In addition, diethylene glycol (water solubility: 1000 g/L), 1,3,5-trimethylbenzene (water solubility: 48 mg/L) and di-isooctyl phthalate (water solubility: 0.09 mg/L) might be suitable when taking into account all available information on biodegradation and the physico-chemical properties. For physico-chemical parameters see Appendix B.7 (Substance No. 66-70) for molecular formulas see Appendix B.8 (Substance No. 66-70).

5.4.1.3 Potential positive and negative reference compounds

Compounds of Bin 1 (i.e. reference chemicals that would normally pass a RBT and a modified RBT) and Bin 4 (i.e. reference chemicals that should never pass a modified RBT or eRBT can be considered as potential positive and negative controls in an eRBT, respectively.

Aniline (CAS No. 62-53-3) and sodium benzoate (CAS No 532-32-1) are recommended as positive reference chemicals that are usually very well biodegraded in water and are used as reference chemicals in standard biodegradation tests (e.g. OECD 301, OECD 310). However, aniline sometimes shows long lag-phases, which might result in a failed RBT (Comber and Holt 2010). Aniline did not reach the pass level of 60% biodegradation in a standard RBT according to OECD 301 B (54.3-62.5%; mean: 58.2%) performed by Martin et al. (2017b), even if prolonged to 42 days. However, 89.1-92.1% (mean: 90.9%) biodegradation were obtained within the test period when using a clearly higher inoculum concentration (300 mg/L). In addition, mineralization rates below 60% were observed in compartment-specific screening tools (i.e. 62.0% ± 3.7% in the water-sediment screening tool and 25.6% ± 6.3% in the soil screening tool; Junker et al. 2016). Sodium benzoate reliably exceeds the trigger value of 60% biodegradation in RBTs with a lag-phase < 1 day. 1-octanol (CAS No 111-87-5) is recommended by Comber and Holt (2010) and in OECD 310 (OECD 2014) as positive control when addressing poorly water-soluble substances.

Musk xylene (CAS No. 81-15-2), Hexachlorobenzene (CAS No 118-74-1) and Benzo(a)pyrene (CAS No. 50-32-8) are recommended as non-volatile negative control reference chemicals by Comber and Holt (2010) that should never pass a screening test.

5.5 Selection of compounds

One compound (CAS No. 2082-79-3; compound No. 12 and 50) was identified from both the OECD eChem Portal as well as from NITE Chemical Risk Information Platform/J-check database. Thus, overall 69 different potential test substances could be identified. Additional relevant substance properties (e.g. physico-chemical properties, toxicity to microorganisms, additional abiotic and biotic degradation data) are summarized in Appendix B.8, structural formulas are shown in Appendix B.9.

5.5.1 Physico-chemical properties

For all identified potential test substances relevant information on physico-chemical properties, biodegradability and stability were checked on the ECHA website for registered substances (ECHA 2019) originating from registration dossiers submitted to ECHA. Missing information was obtained from TOXNET databases ChemID*plus* (now merged to PubChem of the National Library of Medcine; https://pubchem.ncbi.nlm.nih.gov/ and HSDB (Hazardous Substances Data Bank; also now included in PubChem).

To evaluate the suitability of test substances based on their physico-chemical properties, the following categorizations might be useful.

- Solubility in water: According to ISO 10634 (ISO 2018a) and OECD (OECD 2019a) substances with a solubility in water < 100 mg/L are considered as poorly water-soluble. Therefore, compounds with a reported water solubility clearly > 100 mg/L were not considered (for exceptions see chapter 5.1.2). For further information on the testing of substances with low water solubility see chapter 3.3.1.
- Volatility: According to Thomas (1990) substances can be classified based on their Henry's law constant (HLC) as follows: < 1 Pa*m^3/mol = volatilizes slowly; 1-100 Pa*m^3/mol = medium volatilization; > 100 Pa*m^3/mol = rapid volatilization. According to OECD (OECD 2019a) the losses due to volatilization may become significant for test chemicals with a HLC of 1-10 Pa*m^3/mol under vigorous mixing conditions. For compounds with a HLC of 100 Pa*m^3/mol, more than 50% of the test chemical could be lost from the water phase within 3-4 hours. For further information on the testing of volatile substances see chapter 3.3.2.
- Toxicity to microorganisms: According to Annex II of OECD 301, compounds with an EC_{50} value > 300 mg/L in an activated sludge respiration inhibition test (e.g. OECD 209 or ISO 8192) are not likely to have toxic effects on microorganisms in RBTs, whereas compounds with an EC_{50} < 20 mg/L would cause serious problems for testing. For further information on the testing of inhibitory substances see chapter 3.3.3.

5.5.2 Nitrogen containing compounds

In respirometric methods based on the endpoint oxygen-consumption (e.g. OECD 301 C, 301 F) nitrogen containing chemicals may affect the oxygen uptake due to the nitrification of ammonium (NH_4-N) to nitrate (NO_3-N). This could result in erroneous evaluations, if the oxygen uptake is not corrected for the amount of oxygen needed for oxidising ammonium to nitrite and nitrate (see also chapter 3.1.3). Therefore, test compounds without nitrogen should be preferred. From the 69 potential test compounds 26 candidates contain nitrogen.

5.5.3 Pre-selected substances for the practical testing programme

The analysis of physico-chemical data and biodegradation behavior of the potential test substances revealed that the requested properties (low water solubility, degradation extent in ready tests 20-40%, data from simulation tests available) most often do not fit all together.

Beyond that, substances without nitrogen, chlorine, fluorine and phosphorus were preferred. Inhibitory substances such as many pesticide active ingredients are difficult to be tested at high concentrations usually applied in RBTs. For them, other application methods exist, such as the addition via inert carriers such as silica gel or silicone oil. However, this would result in an even more complex testing strategy (i.e. prolongation, larger test vessels, and alternative inoculum sources).

In total 15 substances have been preselected as suitable for biodegradation testing in the practical testing program, based on their physico-chemical properties, their toxicity to microorganisms and available (bio)degradation data. Beyond that, compounds that might function as positive or negative controls in an eRBT were proposed. The proposal for suitable test compounds is presented in Table 4.

From this pool of substances, 5 compounds were selected for practical testing at a project meeting in January 2020 at the German Environment Agency. In addition, five further compounds were identified as potential alternatives, and one compound was proposed as positive control. The agreed substances are highlighted in bold and indicated as selected test compound in Table 4 as well.

Table 4: Preliminary proposal for suitable test compounds

Substance name [No.]	CAS No.	Accordance with selection criteria	Remarks / Critical aspects / Missing information
Diethylene glycol [68]	111-46-6	– standard reference substance for inherent tests – ambiguous results in ready tests (failed OECD 301 D using acclimated sludge but passed OECD 301 B); 59-89% degradation observed in OECD 301F depending on measurement method – non-volatile; not toxic to microorganisms; no hydrolysis expected	– classified as "readily biodegradable" by ECHA – no simulation test data available – high water solubility (1000 g/L) – Selected as positive reference substance
4-chloroaniline [66]	106-47-8	– wide range of biodegradation rates in OECD 301 – not volatile – test data available with higher inoculum concentration and prolonged test period	– high water solubility (3900 mg/L) – contains Cl and N – no simulation test data available
Cis-13-Docosenamide (Erucamide) [1] [61]	112-84-5	– wide range (15-88%) of biodegradation rates in OECD 301 – very low water solubility; not volatile; microbial toxicity and hydrolysis not expected	– contains N – no simulation test data available – Selected as test compound – classified as "readily biodegradable" by ECHA
1,3,5-trimethylbenzene [69]	108-67-8	– not readily biodegradable in OECD 301 F with non-acclimated inoculum but 61% degradation with re-inoculated inoculum (failing 10 d window) – water solubility < 100 mg/L; microbial toxicity not expected	– volatile (HLC = 781 Pa*m^3/mol) – no simulation test data available
Isodecyl neopentanoate [62]	60209-82-7	– ~ 35% biodegradation in OECD 301 B (higher degradation observed, when bioavailability was improved) – water solubility < 100 mg/L; not toxic to microorganisms; no hydrolysis	– Volatile (HLC = 532 Pa*m^3/mol) – no simulation test data available
Di-isooctyl phthalate [70]	27554-26-3	– Typical mineralization rate of 50-60% in OECD 301 within 28 days – very low water solubility; not volatile	– no simulation test data available – no data on toxicity to microorganisms and hydrolysis

Substance name [No.]	CAS No.	Accordance with selection criteria	Remarks / Critical aspects / Missing information
Phenanthrene [65]	85-01-8	– Currently assumed as vP but ready test results with 54-67% degradation available – simulation test data available for OECD 307, 308 and 309 – water solubility < 100 mg/L; not volatile; no hydrolysis expected	– no data on toxicity to microorganisms – photolysis half-life = 6.3 hours
Piperonyl Butoxide [6]	51-03-6	– 24-48% degradation in OECD 301 B; no degradation in OECD 301 D – water solubility < 100 mg/L; not volatile; no hydrolysis – simulation studies available for soil and aquatic sediment systems	– microbial toxicity: only EC_{10} = 2 mg/L from OECD 301 D available – Selected as test compound
tributyl(dodecanoyloxy)stannane [53]	3090-36-6	– 35% degradation in OECD 301 C – water solubility < 100 mg/L	– Volatile (HLC = $1.0*10^5$ Pa*m^3/mol) – no simulation test data available – no data on toxicity to microorganisms and hydrolysis
Ibuprofen [4]	15687-27-1	– 31% degradation in OECD 301D (range: 20-60%) – aquatic sediment simulation study available – water solubility < 100 mg/L; not volatile; not toxic to microorganisms; hydrolysis not expected	– Selected as test compound
Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate [12]	2082-79-3	– 21-39% degradation in OECD 301 C and 301 B – soil simulation study available (non-guideline) – water solubility < 100 mg/L; not volatile; not toxic to microorganisms; no hydrolysis	– Selected as test compound – 34% degradation in inherent test (OECD 302 B; range: 21-47%)
Propylidynetrimethyl trimethacrylate [20]	3290-92-4	– 29-53% degradation in OECD 301 B – water solubility < 100 mg/L; not volatile; not toxic to microorganisms; no hydrolysis	– no simulation test data available
Diisobutyl hexahydrophthalate [23]	70969-58-3	– 8-23% degradation in different RBTs – 99% degradation when using adapted inoculum – water solubility < 100 mg/L; not volatile; not toxic to microorganisms; no hydrolysis	– no simulation test data available

Substance name [No.]	CAS No.	Accordance with selection criteria	Remarks / Critical aspects / Missing information
Acrylic acid, monoester with propane-1,2-diol (mixture of isomers) [27]	25584-83-2	– 35% degradation in OECD 301 D, 90-100% DOC removal in OECD 301 A – not volatile; not toxic to microorganisms; no hydrolysis	– no simulation test data available – water solubility >1000 g/L – mixture of isomers
4-fluorophenol [67]	371-41-5	– 46-53% degradation in OECD 301 B within 60 d (3 mg/L inoculum) – >60% degradation OECD 301 B within 60 d (300 mg/L inoculum) – test data available with higher inoculum concentration and prolonged test period – not volatile	– predicted hydrolysis half-life = 11.3 hours – no data on toxicity to microorganisms – no simulation test data available – Selected as test compound

[1] The compound was selected as a replacement for the pre-selected similar substance Docosenamide (CAS No. 112-84-5) due to commercial availability for an acceptable price.

6 Practical testing program

6.1 Selection of test compounds and testing strategy

The test compounds for the practical testing program have been pre-selected in a literature and data bank research (see chapter 5). The origin of the 5 test compounds selected and the reference compound Diethylene glycol are given in table 5).

Table 5: Purchase of the test compounds

Test compound	Acronym	CAS	Supplier	Lot number
Ibuprofen	IBU	15687-27-1	Sigma Aldrich	SLBX6584
Piperonylbutoxide	PBO	51-03-6	Merck	S27776
Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)-propionate	OBP	2082-79-3	J&K Scientific GmbH	L740Q63
4-Fluorophenol	4FP	371-41-5	J&K Scientific GmbH	LF60R06
Cis-13-Docosenamide (Erucamide)	ERU	112-84-5	Sigma-Aldrich	STBH9859
Diethylene glycol (reference compound)	DEG	111-46-6	Chemsolute	P5D037045E

For assessing the influence of enhancements on the overall test results the test compounds were tested in 4 test series:

- Test series 1: OECD 301 F test, 28 d and 60 d with typical volume and activated sludge as inoculum
- Test series 2: OECD 301 F test, 28 d and 60 d with 4 times increased volume and activated sludge as inoculum
- Test series 3: OECD 301 F test, 28 d and 60 d with typical volume and final effluent from STP as inoculum
- Test series 4: OECD 301 A/B combination test with parallel determination of DOC and CO_2 and activated sludge as inoculum

For comparing the influence of the testing volume, test series 1 and 2 were run in parallel.

All test items were directly dosed into the test vessels by weighting (solids) or micro-pipetting (liquids). No optimization methods for dosage of poorly water-soluble substances as described in ISO 10634 (ISO 2018) were applied (e.g. ultrasonic dispersion, adsorption to inert supports, etc.). All tests were run in triplicate (each 3 vessels for test, blank, and reference).

6.2 Test performance

6.2.1 Enhanced respirometer tests (OECD 301 F)

The Manometric Respirometry test investigates the ready biodegradability of the test item in closed flasks at a constant temperature within 28 days. A solution or suspension of the test item

in a mineral medium, corresponding to 100 mg test item/L giving at least 50 – 100 mg/L of theoretical oxygen demand (ThOD), is inoculated with activated sludge.

The ultimate biodegradation (mineralisation) is determined by measuring the negative pressure in the closed system that occurs, when oxygen is taken up by the microbial population (biological oxygen demand, BOD) to transform organic carbon into CO_2, which is subsequently absorbed by 0.2M sodium hydroxide (NaOH). After correction for the oxygen uptake by blank inoculum run in parallel, the BOD is expressed as the percentage of the ThOD or the chemical oxygen demand (COD) according to the test guideline. The pass level for ready biodegradability is 60% of ThOD and must be reached within a 10-d window, which begins when the degree of biodegradation reaches 10%. However, the concept of the 10-day-window is not applied for substances of unknown or variable composition, complex reaction products or biological materials (UVCB substances) or eRBTs

The systems OxiTop® (WTW/Xylem) and Sensomat (AQUALYTIC®) were used as test systems. The tests were performed with two different flask volumes in parallel:

- Narrow-necked 500 mL flasks (total volume 515 mL, liquid volume 164 mL) with stirrer platform OxiTop® IS 12 (Xylem Analytics, Weilheim, Germany) (test series 1 + 3).
- 2000 mL Duran glass flasks (total volume 2300 mL, liquid volume 740 mL, Schott, Mainz, Germany) with adapters for OxiTop®-C pressure measuring heads with OxiTop™ AD adapter (Xylem-Analytics, Weilheim) and magnetic stirrers MONO direct (H+P Labortechnik AG, Oberschleißheim, Gemany) (test series 2).

In total, three bottles containing the test item, only inoculum (blank) or the reference compound were set up, respectively. One bottle containing test item and reference compound served as inhibition control.

6.2.2 CO_2/DOC Combination Test (OECD 301 A/B)

The CO_2/DOC Combination Test (test series 4) was performed using the apparatus of the CO_2 Evolution Test (OECD 301 B). In total, 20 gas wash bottles (2000 mL volume each) equipped with lateral connecting pieces with butyl rubber septa for DOC sampling were used as reactors and aerated with CO_2-free air. The liquid volume was fixed at 1.500 mL each. Mixing was performed by a magnetic stirrer (MONO direct, H+P Labortechnik AG, Oberschleißheim) with 2 cm stir bars. The activated sludge was used directly after sampling and washed twice with mineral medium according to OECD 301 B. In total 30 mg d.s./L activated sludge were added into the reactors and the whole system was pre-aerated for 24 h with CO_2-free air before adding the test compounds (20 mg TOC/L each). Ultimate degradation was followed according to OECD 301 B (OECD 1992a) by determining the CO_2 produced and absorbed to NaOH via IC-measurements. The amount of CO_2 produced by mineralisation of the test compound less the amount of CO_2 produced by the blank inoculum was expressed as percentage of $ThCO_2$ (theoretical amount of CO_2) introduced with the test compound. For the inhibition controls the sum of the $ThCO_2$ of both test compound and reference compound DEG was used as reference point. The dissolved organic carbon (DOC) was determined from 12 mL samples withdrawn from the reactors through the butyl rubber septum after centrifugation of the samples at 4000 *g* for 15 minutes (Hettich Universal 32 R, Tuttlingen, Germany) using the NPOC (Non-Purgeable Organic Carbon) method. Both IC and DOC measurements were performed using a total carbon analyser (TOC-L, Shimadzu Deutschland, Duisburg) with autosampler.

DOC-elimination was calculated from the DOC in the respective test flask less the mean DOC in inoculum blanks in relation to the TOC introduced with the test compound, in order to assess total elimination by sorption and biodegradation. For the evaluation of the CO_2/DOC Combination Test the TOC removed from the reactor and the IC removed from the absorber flaks for measurements were considered.

6.2.3 Inoculum sources

The standard inoculum from the outflow of the activated sludge basin or final clarifier effluent was sampled from the municipal sewage treatment plant (STP) "Staufener Bucht", Grezhausen, 20 km southwest of Freiburg, receiving predominantly domestic sewage and thus can be assumed to be non-adapted to industrial chemicals. The treatment steps consist of a primary sedimentation basin (1,100 m^3), three activated sludge basins run in parallel (total volume 10,800 m^3) with alternating anoxic denitrification and aerobic biodegradation sectors, and three final clarifier circular basins (diameter 40-49 m) also run in parallel. Iron(II) sulfate is dosed at the inlet to the activated sludge basis for phosphorous precipitation. The sludge retention time of the STP is run between 9 and 12 days. Inoculum sampling points were the aerated outflow of the 1st activated sludge basis and of the 3rd final clarifier basin (no sand-filter or other treatment step used).

In order to minimize week course fluctuations as far as possible, sampling took place on Thursday or Wednesday. The total population equivalent of the STP corresponds to 114,000 inhabitants of which 63,000 residence inhabitants are connected. The dry solids (d.s.) content of the activated sludge was determined by weight measurements after drying at 105°C for about 3-5 hours (mean of triplicate measurements). In all tests using activated sludge this was washed twice with tap water (no treatment such as disinfection) by settling the sludge, decanting the supernatant and resuspending the sludge. The inoculum was immediately used or incubated under the test conditions for 1-2 days. The inoculum was added separately from a continuously stirred Erlenmeyer flask via pipettes into each individual flask. The activity of the inoculum was checked by testing the degradability of the reference substance diethylene glycol (DEG).

Furthermore, the total colony counts of all inoculum batches were determined according to ISO 6222 (ISO 1999c) at GIU Gewerbliches Institut für Umweltanalytik GmbH, Teningen by counting the colonies formed in a nutrient agar culture medium after aerobic incubation at 36 °C for 44 h and at 22 °C for 68 h. Although the main application areas of this method are the drinking water supply, cooling water treatment or surface water, it is also applicable to wastewater samples. For this purpose, an additional blank flask was inoculated with the inoculum concentration used in the test at the start of each test series and this flask was sacrificed immediately after the test start for determining the total culturable colonies. Before plating, the inoculum samples as used in the tests were thoroughly homogenized without removing the activated sludge.

6.2.4 Preliminary validity criteria

The OECD 301 describes several validity criteria. For the inoculum blanks a maximum O_2 consumption ≤ 60 mg L/L or a maximum CO_2 evolution ≤ 70 mg/L should be measured within 28 days. So far, no corresponding validity criterion exists for the prolonged test duration of 60 days, but as a first assumption the respiration of the inoculum blanks should also remain within these ranges for eRBTs. Moreover, the validity criterion demanding that the difference of extremes of replicate biodegradation values should be less than 20% should also be applied for enhanced testing. In addition, OECD 301 requires that the percentage degradation of the reference compound must have reached the pass levels of 60% ThOD or $ThCO_2$, respectively, by

day 14. Although no corresponding validity criterion is available for eRBTs, it was suggested that the reference compound DEG should reach these pass levels within 60 days.

6.3 Results

6.3.1 Test series and inoculum sources and quality

The practical testing programme was organised in 4 test series with in total 6 experiments. An overview including the inoculum used and results of colony counts is presented in table 6.

Table 6: Overview of test series with inoculum sources and colony counts

Test Series	Test design	Exp. No.	Inoculum[1)]	Samling date inoculum (water temp. °C)	Countable colonies 22°C / 36°C [10^3 CFU/ mL] [2)]	Test compounds
1 + 2	OECD 301 F (different flask volumes)	1	AS	30 Jun 20 (19.8)	3.4 / 2.5	IBU, PBO
1 + 2	OECD 301 F (different flask volumes)	2	AS	16 Sep 20 (23.6)	3.8 / 19.3	4FP, OBP
1 + 2	OECD 301 F (different flask volumes)	3	AS	16 Dez 20 (14.4)	8.3 / 4.8	ERU
3	OECD 301 F (effluent as inoculum)	4	EFC	09 Feb 21 (8.7)	290 /245	IBU, PBO, OBP, 4FP, ERU
4	OECD 301 A/B (combi test DOC, CO_2)	5	AS	08 Sept 20 (20.9)	2.3 / 4.0	IBU, PBO, OBP
4	OECD 301 A/B (combi test DOC, CO_2)	6	AS	03 Nov 20 (17.1)	1.5 / 4.1	4FP, ERU

1) AS: Activated Sludge (30 ppm dry solids), EFC: Effluent final clarifier (10 Vol. %)

2) CFU: colony forming units

The considerably higher amount of CFU in the effluent inoculum may be explained by the higher volume (10 Vol. %) used in series 3 compared to activated sludge (0.75 Vol. % or 7.5 mL/L to obtain 30 mg/L dry solids used in the other series. Other possible reasons are that the floc structure of activated sludge impedes the separation of individual bacteria besides the fact that the yeast and peptone extract agar is not optimized for activated sludge.

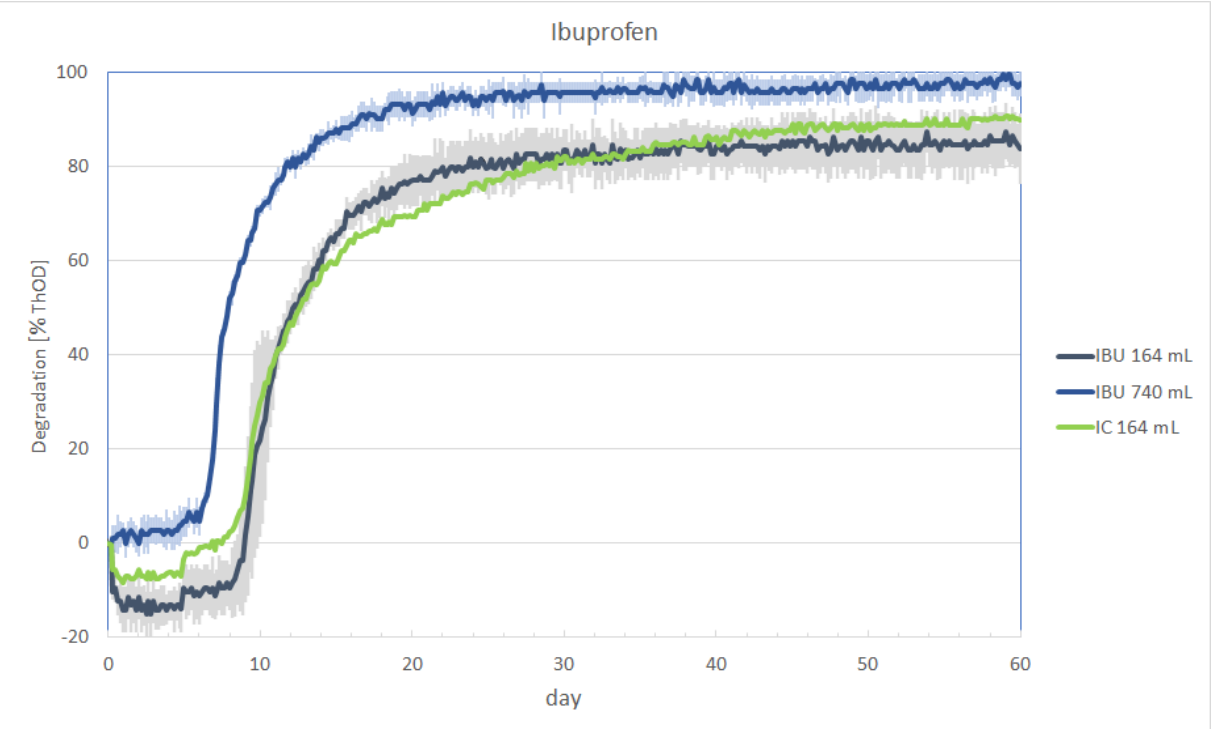
6.3.2 Enhanced respirometer tests with different volumes (test series 1 + 2)

Test series 1 and 2 were started in parallel, in order to analyse the influence of different flask volumes on the degradation kinetics (mean of three replicates) and variability of parallel vessels (standard deviation, SD).

IBU was readily biodegradable after a lag-phase of 9 days, while fulfilling the criterion of the 10-day window. The degradation extent was slightly higher in the larger reactor volume (94-98%

ThOD within 28 d) compared to the typical volume (78-89% ThOD within 28 d, see figure 1). However, this did not change the conclusion of IBU as being "not persistent".

Figure 1: Influence of flask volume on biodegradability of Ibuprofen (OECD 301 F)



Black and blue lines indicate mean values from three replicates. Grey and blue shadings represent SD, green lines the respective inhibition controls (=IC, 1 replicate)

PBO showed a moderate biodegradation in the typical vessels (31-45% ThOD within 60 d) and in the larger vessels (33-42% ThOD within 60 d), but clearly failed the pass level of 60%.

OBP was moderately biodegradable with a slightly higher degradation extent in the larger reactor volume (41-49% ThOD) than in the typical flasks (27-32% ThOD) after 60 days. The lag phase was about 5 days in both reactor volumes.

The degradation of 4FP started after a lag-phase of 10-12 days and reached 68-79% ThOD (typical flasks) and 94-98% ThOD (large flasks) after 60 days, respectively. The 10-days window was met and 4FP was considered as being readily biodegradable.

The first test with ERU resulted in degradation extends of 32 to 104% ThOD in the typical volume flasks and 25 to 90% ThOD in the larger volume flasks (data not shown). Due to the high variability of the results the test was repeated. However, a considerable variability of the degradation extends was observed again. After 60 days the degradation extends reached 47-92% ThOD (typical flaks) and 59-71% ThOD (large flaks).

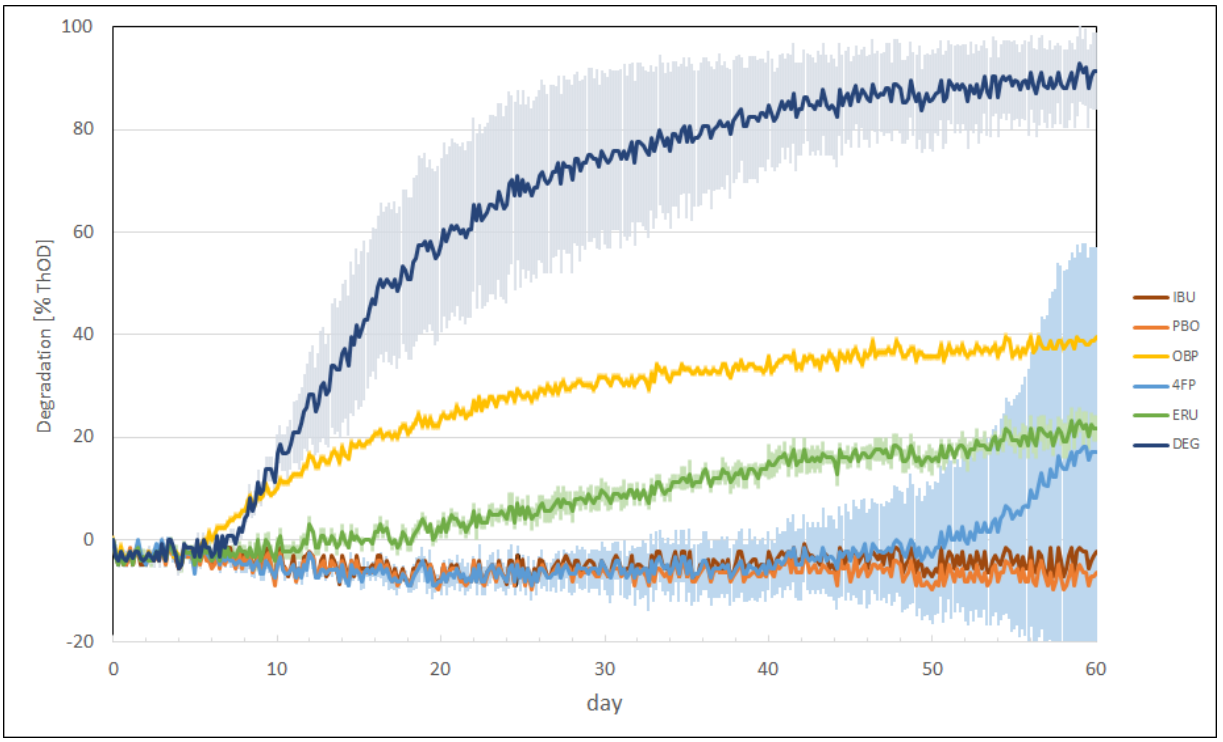
The reference compound DEG reached the pass level of 60% within 28 days in all replicates of the three experiments. The lag-phase differed between 5 and 9 days. In 8 out of 9 replicates the 10-day-window criterion was met.

In test series 1 and 2 all validity criteria of OECD 301 F were met. According to the OECD criteria for inhibitory effects (OECD 1992a), none of the substances was toxic against the inoculum (i.e. degradation in the inhibition control ≤ 25% ThOD by day 14), although degradation in the inhibition control of PBO was only slightly higher (27% ThOD at day 14). The figures with the detailed results are presented in Gartiser et al. (2022, submitted).

6.3.3 Respirometer test with alternative inoculum (test series 3)

In test series 3 the outflow of a municipal sewage treatment plant (effluent final clarifier) was used as inoculum, using the maximum allowed volume (10 Vol. %) in OECD 301 A. In test series 3 all five test compounds were tested in parallel. The results demonstrate, that the alternative inoculum (effluent final clarifier) is less potent than activated sludge, despite it's about a factor of 50 - 200 higher amount of countable colonies. The biodegradation graphs are given in figure 2.

Figure 2: Biodegradability with final clarifier effluent from STP (test series 3)



The shadings represent the respective SDs.

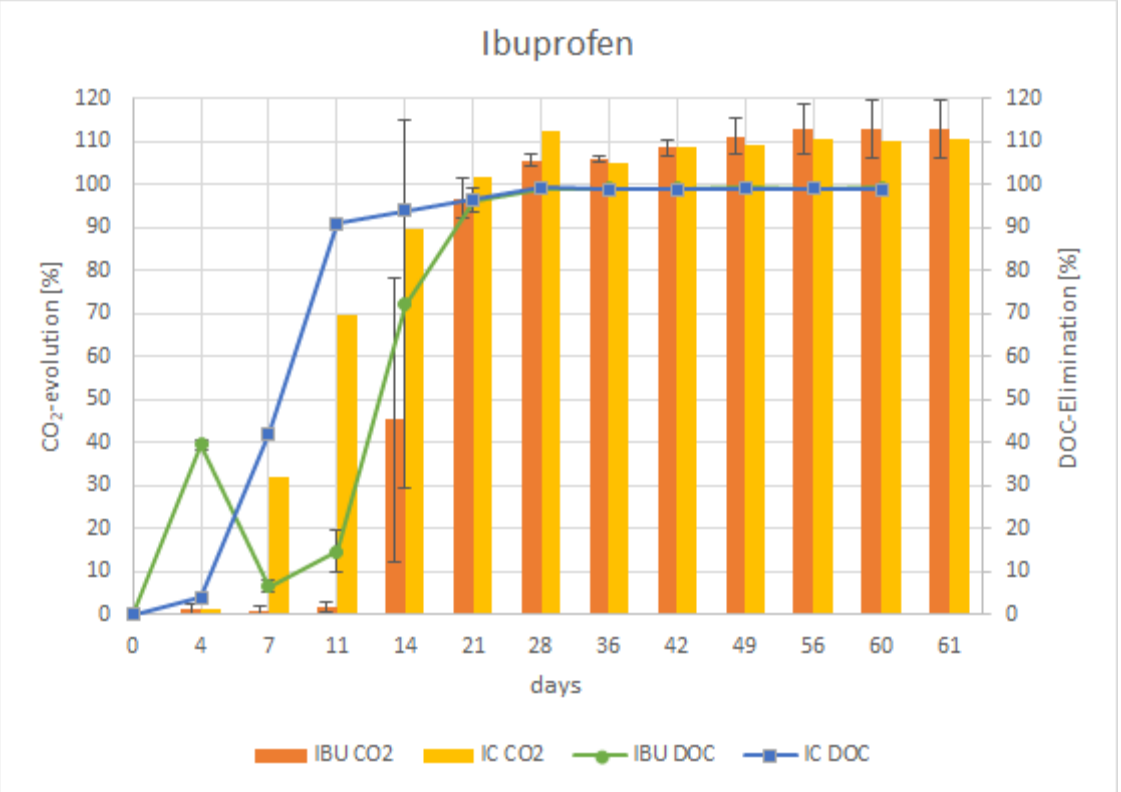
No degradation was observed for IBU and PBO and only 17% degradation was found for 4FP, which showed a clearly higher biodegradation when using activated sludge. The biodegradation of 4FP could only be observed in one out of three replicates after a very long lag phase of 48 days. For this replicate, the pass level of 60% ThOD was reached within 60 days, while no degradation was detected in the remaining two flaks. This explains the very high variability for 4FP at the end of the test (light blue shading). In contrast, the biodegradability of the reference substance DEG, OBP and ERU was comparable to that of testing series 1 and 2 using activated sludge. Again, the lag-phase of DEG was about 9 days, but afterwards biodegradation kinetics differed considerably between replicates until reaching the plateau phase at 83-93% ThOD. Only one out of three replicates with DEG met the 10-day window criterion for ready biodegradability. The slightly negative degradation extends observed for IBU, PBO and 4FP within the first 28 days might be explained by inhibitory effects. However, no inhibition controls were included in test series 3.

6.3.4 CO_2/DOC Combination Test OECD 301 A/B (test series 4)

In test series 4 two endpoints, DOC-elimination and CO_2 evolution, were combined for evaluation.

The DOC-elimination of IBU after 4 days was 40% due to its limited water solubility, and subsequently declined from day 7-10, probably because of hydrolysis. Afterwards, DOC-elimination reached almost 100% as a result of the mineralization of the test compound. The CO_2 evolution started after a lag-time of 10 days and reached 108-121% after 60 days (see figure 3).

Figure 3: Biodegradation of Ibuprofen in the combination test OECD 301 A/B (test series 4)



Mean value and SD represent data from 3 replicates.

The DOC-elimination of PBO after 4 days was 69-82%, declined afterwards and reached a plateau of approximately 40% after 28 days. Only in one out of three vessels a significant mineralization was observed based on both DOC-elimination and CO_2 evolution. The congruence of both endpoints confirms that this result is not a measuring artefact.

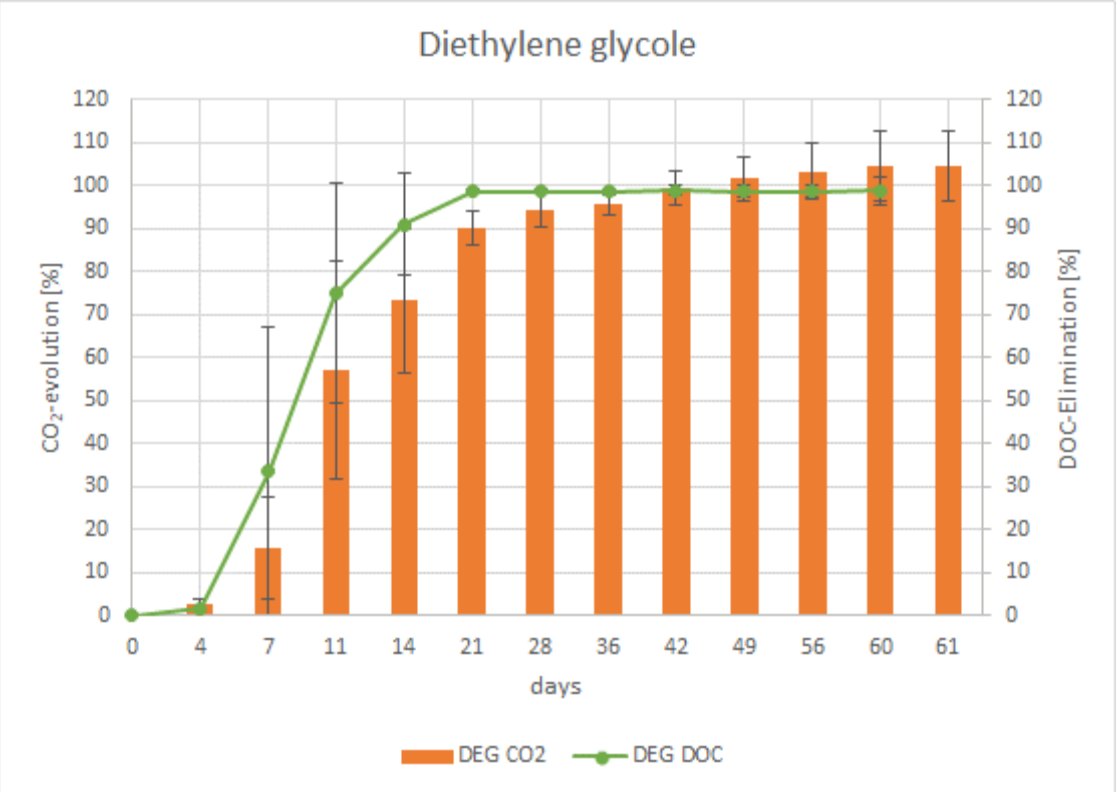
DOC-elimination of OBP remained on a high level (75-99%) throughout the test period until the end of the test (60 d). The CO_2 evolution starts after a lag-phase of about 7 days and reaches 43-48% after 60 days.

After a lag phase of 11–14 days the mean DOC-elimination of 4FP was 86% (81.8 – 88.2%) within 14 days and 98% (98 – 99%) within 60 days. The CO_2 evolution started after a lag-time of 7 -11 days and reached 86-101% $ThCO_2$ after 60 days. The biodegradation of the inhibition control of 4FP lagged behind that of DEG and reached only 25% by day 14, which may be interpreted as a slight inhibitory effect.

Due to its low water solubility the DOC-elimination of ERU stayed on a high level throughout the whole test duration of 60 days. The CO_2 evolution increased continuously up to 32 – 40% $ThCO_2$ at day 60. The plateau of degradation was not reached until the end of the test. The inhibition control reached only 26% $ThCO_2$ by day 14, which is slightly above the 25% criterion for assuming toxic effects against the inoculum.

The reference compound DEG was readily biodegradable in both experiments of test series 4. The degradation started after a lag-phase of 4-7 days and reached degradation extents >70% after 14 days. The degradation in one vessel from the first experiment was a bit slower but reached an extent of >80% within 28 days (see figure 4). All validity criteria were met in both experiments.

Figure 4: Biodegradation of Diethylene glycole in the combination test OECD 301 A/B (test series 4)



Mean value and SD represent data from two experiments (6 replicates in total)

6.3.5 Assessment of biodegradability from all test results

Four test series have been performed for assessing the biodegradability of five selected test compounds, covering the Manometric Respirometry Test (OECD 301 F) with typical and higher flask volume and the combination test (OECD 301 A/B) with parallel determination of DOC-elimination and CO_2 evolution. While for these experiments activated sludge was used as standard inoculum, final clarifier effluent from a STP was applied as an alternative inoculum in another test series. All tests were carried out for a duration of 60 days. The overall results of all test series are shown in table 7.

Table 7: Biodegradation extends of all tests after 28 and 60 days (mean of 3 replicates)

Test Compound	Day	Test Series 1	Test Series 2	Test Series 3	Test Series 4
Test design		OECD 301 F	OECD 301 F	OECD 301 F	OECD 301 A/B
End point		O_2	O_2	O_2	CO_2
Results		[%] (SD) DoE	[%] (SD) DoE	[%] (SD) DoE	[%] (SD) DoE
IBU	28	82.6 *(5.6) +*	95.8 *(2.3) ++*	-6.3 *(2.4) ++*	105.7 *(1.5) ++*
IBU	60	83.7 *(7.5) ++*	97.7 *(2.7) ++*	-2.4 *(4.9) ++*	112.8 *(6.7) +*
PBO	28	31.2 *(4.3) ++*	35.8 *(5.9) ++*	-8.1 (3.7) ++	31.9 ***(39.9) -***
PBO	60	36.0 *(8.1) +*	37.6 *(5.9) ++*	-6.5 (2.4) ++	36.4 ***(42.8) -***
OBP	28	24.4 *(2.0) ++*	35.5 *(7.4) +*	30.2 *(1.3) ++*	33.0 *(3.1) ++*
OBP	60	29.0 *(2.6) ++*	46.7 *(4.9) ++*	39.6 *(4.9) ++*	44.2 *(3.6) ++*
4FP	28	67.2 *(11.5) +*	67.8 *(4.7) ++*	-6.5 *(4.3) ++*	82.7 *(3.4) ++*
4FP	60	73.5 *(5.7) +*	95.6 *(2.1) ++*	17.3 ***(39.8) -***	94.6 *(8.1) ++*
ERU	28	52.0 ***(32.9) -***	34.7 *(16.2) -*	5.6 *(2.3) ++*	18.7 *(0.4) ++*
ERU	60	62.6 ***(25.5) -***	64.3 *(6.0) +*	21.6 *(2.5) ++*	36.7 *(4.2) ++*
DEG	28	73.7 *(11.6) +*	81.5 *(2.7) ++*	70.3 *(18.3) ++*	96.8 *(2.2) ++*
DEG	60	79.3 *(12.4) +*	89.3 *(5.5) +*	91.4 *(7.6) ++*	104.7 *(9.3) +*

Mean biodegradation in % from 3 replicates. Numbers in brackets indicate the respective standard deviation SD. Data in bold show a high SD, with degradation extends >60% in 1 out of 3 replicates.
DoE: Validity criterium difference of extreme values: + < 20%, ++ < 10%, - ≥ 20%.
DOC-Elimination in the OECD 301 A/B not reported here.

The test item **IBU** showed high degradation extents of 84-113% within 60 d in all tests with activated sludge. The CO_2 evolution reached values >100%, which cannot be explained by impurities of the test item. One explanation could be, that the antibacterial had a negative impact on the inoculum (negative degradation extents observed in the OECD 301 F up to day 9). This impact could be aligned with the decomposition of the activated sludge, thus delivering additional degradable carbon sources. When using the alternative inoculum (effluent final clarifier) no degradation at all was detected.

The results for **PBO** using activated sludge as inoculum are consistent, with degradation extents of 36-41% within 60 days. Again, no degradation was observed when using the alternative inoculum (effluent final clarifier).

The mineralization extents of the test item **OBP** were comparable in all test series, including test series 3 with alternative inoculum (29-47%). The higher DOC-elimination of up to 78% does not reflect mineralization, but can be explained by adsorption to activated sludge due to physical-chemical properties of OBP (e.g. water solubility of 0.003 mg/L and Log K_{ow} of 13.5).

The results with **4FP** using activated sludge as inoculum were consistent and reached 73-98% degradation. In test series 3 with alternative inoculum, the degradation started after a lag phase of about 50 days in one of three replicates and reached 63% within 60 days, while no degradation at all was observed in the other two test vessels.

The mineralization of **ERU** reached 36 – 64% degradation after 60 days with activated sludge and 21% with the alternative inoculum. A clearly higher degradation was observed compared to the standard test duration 28 days (19 – 52% with activated sludge, 6% with secondary clarifier effluent (see table 2). The complete DOC-elimination (near 100%) does not reflect mineralization, but is due to adsorption to activated sludge. Thus, the choice of the test conditions has a wide influence on the results.

The reference substance **DEG** reached the pass level for ready biodegradation of 60% in all test series within 28 days (total of 6 independent experiments with 3 replicates each), but the 10-day-window criterion was not always met, especially in test series 3 with alternative inoculum. After 60 days the degradation extents reached 79-105%.

The variability of the degradation extents observed between replicates in the OECD 301 F with larger volume flasks (740 mL) was considerably lower compared to typical volume flasks (164 mL).

The combination of different endpoints in the combination test according to OECD 301 A/B clearly enables a better understanding of the biodegradation process, particularly for test items, whose water solubility changes within the test period due to hydrolysis or biodegradation, such as IBU or PBO.

The OECD 301 validity criteria for the inoculum blanks of ≤ 60 mg/L oxygen consumption or ≤ 70 mg/L CO_2 evolution were met in all experiments, even after prolongation to 60 days (table 8). Obviously, the alternative inoculum (effluent final clarifier) was less potent than activated sludge, despite its higher colony counts compared to activated sludge. This is confirmed by the activity of the inoculum blank values for the effluent final clarifier, which was about a factor of 2 lower compared to that using activated sludge (table 8).

Table 8: Inoculum blank values of all tests after 28 and 60 days

Test Series	Test Duration / Volume	Endpoint	No. of Experi-ments/vessels	O_2 consumption Mean [mg/L]	O_2 consumption SD [mg/L]
1+2	28 d / 164 mL	O_2	3/9	23.9	5.1
1+2	60 d / 164 mL	O_2	3/9	29.9	6.8
1+2	28 d / 740 mL	O_2	3/9	22.6	5.3
1+2	60 d / 740 mL	O_2	3/9	26.7	5.1
3	28 d / 164 mL	O_2	1/3	11.3	1.4
3	60 d / 164 mL	O_2	1/3	13.8	1.4
4	28 d / 1500 mL	CO_2	2/6	25.6	2.1
4	60 d / 1500 mL	CO_2	2/6	36.1	4.8

The accompanying microbiological analysis of countable colonies in activated sludge at 22°C and 36°C revealed highly differing values, especially for the colony counts at 36°C (table 1). The colony counts at 36°C are not environmentally relevant but are documented for completeness. The differences observed in the colony counts are not reflected in the degradation extents of the reference substance DEG. The absolute values of countable colonies are a factor of 10 below the inoculum concentration indicated in OECD 301 of 10^4-10^5 cells/mL. This may be explained by

the fact, that the countable colonies were determined after processing the activated sludge by washing and pre-incubation for one day. In contrast, the colony counts in the alternative inoculum were about a factor of 50-200 higher than in active sludge, but resulted in lower degradation extents. Based on these results, the countable colonies do not provide an accurate estimate of the inoculum activity.

6.4 Discussion

Enhanced ready screening tests are designed for assessing whether a test substance can be assumed as "not persistent" in the environment. The intention is to avoid the performance of extensive simulation degradation tests for substances failing the criteria for being readily biodegradable. According to ECHA Guidance (ECHA 2017a) the currently accepted enhancements of RBTs are the prolongation of the test duration beyond 28 days and the use of larger test vessels. Other approaches like increasing the biomass concentration and pre-exposure of the inoculum to the test substance are currently not accepted. Thus, eRBTs are by design ready type tests with a longer test duration for up to 60 days.

For eRBTs the endpoints O_2 consumption (OECD 301 F) or CO_2 evolution (OECD 301 B) proved to be suitable and the existing validity criteria for inoculum blanks from the 28 day test can be applied. DOC-based methods (OECD 301 A, OECD 301 E) are not recommended, because DOC-elimination may be influenced by abiotic factors such as adsorption, volatility or precipitation.

The DOC/CO_2 Combination Test (OECD 301 A/B) provides data for two different endpoints, which improves the interpretability of test results, especially when the variability between replicates is high. Furthermore, the combination test is particularly appropriate for substances with a limited water solubility, because hydrolysis or other factors influencing water solubility can be observed. One example is PBO, where a high DOC-elimination was observed at the beginning, followed by a decreased DOC-elimination until day 11 probably due to hydrolysis. Afterwards, DOC-elimination increased again in parallel to mineralization and was slightly higher than mineralization at the end of the test. However, DOC-elimination should only be considered as a supporting endpoint, whereas ultimate biodegradability is referred to CO_2 evolution.

In principle, any of the reference substances used for RBTs such as aniline, sodium benzoate or sodium acetate can be used. However, for eRBTs the degradation of the reference compound should preferably not reach the pass level until day 14 and the 10-day-window is not applied. Diethylene glycol (DEG), which is also used as reference compound in the inherent biodegradability test according to OECD 302 B (OECD 1992b), where it must reach the pass level of 70% DOC-elimination within 14 days, has been identified as a suitable reference compound, which is biodegraded by >60% within 28 days under RBT conditions.

If in a inhibition control less than 25% degradation (based on ThOD or$ThCO_2$) is measured within 14 days, the test substance can be assumed as inhibitory to the microorganisms and the test should be repeated, using a lower concentration of test substance and/or a higher concentration of microbial inoculum. To date, no corresponding criteria exist for eRBTs, but the presented data suggest that this criterion is also applicable when using DEG as reference compound, despite the database is still scarce and different criteria (e.g. ≤ 25% by day 28) might also be reasonable.

Only few publications dealing with eRBTs according to the ECHA strategy are currently available. Gartiser et al. (2017) reviewed the applicability of established and eRBTs for assessing persistence in a theoretical approach based on literature research. Kowalczyk et al. (2015) reviewed limitations of current OECD biodegradation tests including RBTs resulting in high

levels of variability and often false negative results. The authors further discussed options for enhanced and modified screening tests like extended test duration, source, concentration and preparation of microbial inoculum and test volume. Martin et al. (2017b) focused on increased test duration (60 days) and inoculum concentrations. The biodegradation of five test compounds was investigated in experiments according to OECD 301 B with 3 mg inoculum/L (corresponding to typical RBT concentrations of 10^5 cells/mL) and with 100-fold increased inoculum concentration of 300 mg inoculum/L, which is slightly higher than the lower level for inherent tests (e.g. 200 mg/L according to OECD 302 B). They found that higher cell densities resulted in higher degradation rates, shorter lag-periods and reduced inter-replicate variation.

Takekoshi et al. (2021) investigated the influence of different test volume in the OECD 301 F test (i.e. 300, 900, and 3900 mL) on the biodegradability of 6 compounds. With increasing test medium volume, they observed a reduction of the lag-phase and of the coefficient of variation between replicates next to an increase of the biodegradation percentages. They thereby confirmed comparable results obtained by Ingerslev et al. (2000) and Kowalczyk et al. (2015). This can be explained by the decisive influence of the higher initial population of competent degraders (see also Gartiser et al. 2017).

A recent publication by Whale et al. (2021), summarises the discussions and the outcome of a workshop on recent developments in science supportive to the persistence/biodegradation assessment held in Helsinki in 2018, but lacks to present controversial opinions given. Some of the suggestions raised during the workshop sessions were the need for robust (enhanced) RBTs allowing longer test periods, improved inocula (e.g. higher concentrations and diverse sources) and adaptation (pre-exposure) of the microorganisms to the specific test substance, but no consensus was reached on several points according to participants.

Up to now results from eRBTs are not used to rate a substance as being persistent (“P”), but only for excluding persistence. In contrast, results from inherent biodegradability tests (OECD 302 B or C) showing degradation extents ≤ 20% can be used as evidence for persistence according to ECHA guidelines, provided that no other indications of false negative test results such as inhibitory effects have been observed (ECHA 2017b). However, the role of eRBTs (lower inoculum concentration and longer test duration) in relation to inherent tests (higher inoculum concentration, but shorter test duration) in the context of persistency evaluation needs to be clarified. Apparently, the reference compound DEG is biodegraded by ≥ 70% in inherent biodegradability tests within 7 days (own experimental data not presented here), while in RBTs or eRBTs about 14-28 days are needed to reach the pass level. Thus, considering the behaviour of the reference compound DEG, results from eRBTs with degradation extents above 60% in 60 days may be interpreted as evidence for inherent biodegradability while results < 20% in eRBT may be considered as an indication that the substance is “P”.

With respect to the assessment of persistence the following overall conclusions can be drawn from all eRBTs performed within this work when applying the criteria according to ECHA (ECHA 2017a, 2017b):

- IBU can be regarded as being non-persistent (congruent results from test series 1, 2 and 4). In fact, IBU was assessed as readily biodegradable in test series 1, 2 and 4 after lag phases of 6.5 to 12 days while fulfilling the 10 day window criterion.
- The results of PBO are inconclusive with respect to persistence. Although, the pass level of 60% was reached in one replicate of test series 4, the validity criterion for the allowed

variability between replicates of 20% was failed. Beyond that, results from test series 1, 2 and 3 showed degradation extents clearly below 60% after 60 days.

- OBP did not meet the 60% pass level in any test series. The results are inconclusive with respect to persistency (potentially P or vP).
- 4FP is assumed to be non-persistent according to data from test series 1, 2 and 4. In test series 1 and 4 the substance was assessed as being readily biodegradable after a lag phase of 10 respectively 9 days, while fulfilling the 10-day window.
- ERU can be assumed as being non-persistent based on results of test series 2, even though some doubts remain, since the 20% criterion for the variability between replicates was failed in test series 1 and ultimate biodegradability was clearly below the pass level of 60% in test series 4.
- Neither PBO nor OBP can be assumed as being "P" from the results, because some biodegradation above 20% took place, but are potentially P or vP.
- In general, the variability of the degradation extents observed between replicates was low and fulfilled the validity criterion on the difference of extreme values of 20%, although in some tests performed with PBO (test series 4), 4FP (test series 3) and ERU (test series 1 and 2) this criterion was failed.
- The higher variability observed for ERU and PBO may indicate that specific test conditions and/or adaptation to specialised microorganisms in conjunction with a relatively long lag-phase plays an important role for these compounds. Thus, the results show that the variability of biodegradation test outcomes may be dependent on both the quantity (i.e. total amount used) and the quality (i.e. presence and viability of specific degraders) of the microbial inoculum, as recently discussed by Davenport et al. (2022).
- Only for ERU the prolongation of the test duration in combination with larger test vessels did result in a changed conclusion as non-persistent, while for IBU and 4FP the pass level of 60% was already reached after 28 days. Overall, the prolongation of RBTs according to OECD 301 B, F up to 60 days is a suitable approach, resulting in transferable validity criteria for eRBTs.

7 Recommendations for eRBT testing

7.1 Introduction and Scope

Enhanced screening tests are designed for assessing whether a test substance can be assumed as "not persistent" in the environment. The intention is to avoid the performance of extensive simulation degradation tests for substances failing the criteria for being readily biodegradable. According to ECHA Guidance the currently accepted enhancements of RBTs are the prolongation of the test duration beyond 28 days and the use of larger test vessels. The increase of biomass concentration, and the pre-exposure of the inoculum to the test substance at low concentrations are not accepted. Thus, eRBTs are by design ready type tests with a longer test duration for up to 60 days (ECHA 2017a). The following recommendations reflect the personal view of the authors, resulting from the overall results of the research project.

7.2 Acceptable test methods for enhanced biodegradability testing

The current ECHA guidance differentiates between "enhanced" RBTs and "modified" RBTs, whereof the latter ones include deviations from the standard test protocol such as testing at lower test substance concentrations due to inoculum toxicity or the application of specific dosage methods for testing biodegradability of poorly water-soluble substances (e.g. use of emulsifiers, solvents, carriers, ultrasonication). Provided that all other conditions of RBTs remain unchanged, "modified" RBTs are considered as equivalent to regular RBTs and the results can be used accordingly (ECHA 2017a), i.e. to assess ready biodegradability. This is not true for "enhanced" RBTs, which are exclusively used as a time-saving and cost-effective tool to identify non-persistence.

Enhanced ready biodegradation tests are derived from the standard ready biodegradation tests regularly applied in chemical legislation. For enhanced testing all test methods of OECD 301 and OECD 310 for ultimate biodegradability (CO_2 evolution and/or O_2 consumption) can be applied. The OECD 301 A or OECD 301 E are not appropriate, because DOC-elimination is prone to misinterpretation as degradation, if there are abiotic processes such as adsorption, volatilisation or precipitation, which are difficult to control. However, additional DOC-measurements in ultimate biodegradation tests can provide additional information in combination with CO_2 evolution or O_2 depletion. As the only remaining enhancement currently accepted by ECHA is the test prolongation up to 60 days, an eRBT starts as a regular RBT and only turns into an eRBT in case of prolongation. Thus, the data can also be used to assess a substance in terms of ready biodegradability (pass level reached within 28 days, 10-day-window fulfilled).

It is recommended to exclude tests according to OECD 301 A and E that are merely based on DOC as parameter for degradation. This recommendation is not covered by ECHA R.7b (2017) where DOC based tests are not excluded and 70% DOC-elimination is referred to as pass level. However, DOC based tests are more susceptible to false positive tests results than CO_2 or O_2 based tests. At present the ECHA guidance R.7b (ECHA 2017a) states that "*careful interpretation of data must be performed when considering the use of DOC removal as a degradation sum parameter to ensure that elimination did not occur due to adsorption or volatilisation (both of which are physical removal processes which should not be misinterpreted as transformation or biodegradation)*."

7.3 Selection of applicable test methods for assessing degradation behaviour

OECD301 B: CO_2 Evolution Test

OECD 301 D: Closed Bottle Test (no experience with 60 d test duration so far)

OECD 301 F: Manometric Respirometry Test

OECD 310: CO_2 Headspace Test

OECD 301 A/B: DOC-elimination/CO_2 Evolution Test (= combination test). The OECD 301 A/B combination test is especially interesting for substances with a limited water solubility, because hydrolysis or other factors influencing water solubility is noticed. However, DOC-elimination is considered as a supporting parameter while ultimate biodegradability is referred to CO_2 evolution.

For semi-volatile substances closed systems (OECD 301 F or OECD 310) are preferred.

For inhibitory substances the Closed Bottle Test (OECD 301 D) is an option.

For nitrogen containing substances CO_2 based methods (OECD 301 B, OECD 310) are preferred.

7.4 Test design

The use of larger test vessels for increasing the initial amount of microorganisms and the biodiversity of the inoculum is recommended. The upper limit of the test vessel size is only limited by practical constraints.

In practice, use of only two replicates is standard for OECD 301 B or OECD 301 F tests, but a higher number of replicates increases the accuracy in determining mean values and variability of results and allows the exclusion of obvious outliers. It is thus recommended to use at least three flasks or vessels containing the test substance plus inoculum, the same number containing inoculum only and at least two containing the reference substance.

The test concentration corresponds to that indicated for the standard OECD tests:

- 10-20 mg TOC/L (OECDs 301 B and 310) or
- 100 mg ThOD/L (OECD 301 F) or
- 2-3 mg/L (OECD 301 D).

The application of specific dosage methods for testing poorly water-soluble substances are also allowed for enhanced ready tests as far as they are generally accepted for standard ready tests. Guidance is given in Annex III of OECD 301 (OECD 1992a), ECHA (2017a) or ISO 10634 (ISO 2018a).

7.5 Inoculum

All inoculum sources used for OECD 301 or OECD 310 testing are allowed within the respective inoculum concentrations indicated. When activated sludge is used, it should be sampled from a sewage treatment plant receiving predominantly domestic sewage (with only minor industrial contribution). The location, treatment capacity (preferably the number of inhabitants connected to the STP), main process stages and parameters (e.g. sludge retention time and sludge concentration) of the sewage treatment plant should be reported.

The inoculum sample should be representative and should not be heavily influenced by stormwater from rainfall events. In order to minimize peak or low loads during the weekly course, sampling dates between Tuesday and Thursday may be chosen, when using inocula from sewage treatment plants. The exact sampling point should be described.

Pre-conditioning of the inoculum for one or two days (e.g. to reduce the overall activity to comply with the validity criteria) is allowed, but pre-adaption to the test substance is prohibited.

The use of mixed inocula from different sources (e.g. activated sludge, final effluent, surface water) may be acceptable, as far as its concentrations do not exceed those indicated by OECD 301 (e.g. 30 mg/L dry solids activated sludge or 10 Vol% final effluent) and when the validity criteria for the inoculum blanks are fulfilled.

7.6 Reference compounds

In principle, any of the reference substances used for RBTs such as sodium benzoate, sodium acetate or aniline can be used as procedure control. However, for eRBTs the degradation of the positive reference compound should preferably not reach the pass level before day 14 to enable a longer control of inoculum activity during prolonged tests. Diethylene glycol has been identified as a suitable reference compound, which is biodegraded by >60% within 28-60 days under ready type test conditions. The 10-days-window is not applied when using diethylene glycol.

Remark: Diethylene glycol is also used as reference compound in the inherent test according to OECD 302 B, where it must reach the pass level of 70% DOC-elimination within 14 d.

The use of additional negative reference compounds, which should fail eRBTs, is recommended, particularly when solvents or inert supports are used for the dosage of the test compound, in order to prevent artefacts in the test design. Di-isotridecyl adipate, terphenyl, or cyclododecane normally fail both standard and enhanced screening tests (Martin 2014). The same applies for musk xylene, hexachlorobenzene, benzo(a)pyrene, hexachlorohexane (Comber and Hold 2010).

7.7 Methods of adding the test and reference substances

The method for addition of test and reference substances into the test system depends on the nature of the chemical, especially its water solubility. For substances of adequate solubility, the test solution could be prepared using stock solutions. For less water-soluble substances, direct addition to the mineral medium may be an option. For those, reference is given to Annex III of OECD 301 and to ISO 10634 (ISO 2018a), both dealing with appropriate dosing methods of poorly water-soluble compounds, intended for biodegradability testing. When using inert supports such as silica gel, solubilisers or volatile solvents additional controls are necessary that enable to observe any effects on the inoculum.

7.8 Test duration

The standard test duration is 28 days for ready biodegradability testing and 60 days for the conclusion as non-persistent. In principle, the test can be designed as an eRBT and terminated ahead of schedule, if the pass level is already reached after 28 days.

7.9 Pass levels

28 days

The pass levels for ready biodegradability are 60% of ThOD or $ThCO_2$ to be reached within a 10-day window (10-day window not applied for UVCB substances).

If the pass level is reached, but the 10-day-window criterion is not met, the test substance is still considered as being “readily biodegradable, 10-day-window not fulfilled” and being “not persistent” under environmental conditions. However, the (non-)fulfilment of the 10-day-

window in a standard test on ready biodegradation has further impacts for the assessment of environmental exposure and fate.

60 days

The pass levels for not being persistent (not P) are 60% of ThOD or $ThCO_2$ (except UVCB substances) at the end of the prolonged test period. The 10-day-window criterion is not applied.

Only endpoints for unambiguous mineralisation (CO_2 and/or O_2) are used for evaluation. When DOC-elimination is determined in parallel (e.g. in combinations tests according to OECD 301 A/B), these data are used as supporting information, but not for persistency assessment.

A backward conclusion, that a substance that exceeds the pass level of 60% mineralisation in an eRBT after 60 days also qualifies as "readily biodegradable" is not admissible. Results from an eRBT can also not be used for the purpose of environmental exposure and fate assessment.

A negative result in an enhanced test is an indication that the test substance is still "potentially persistent", but represents no proof for persistency (ECHA 2017b). For these substances, the final conclusion with regard to persistency can only be drawn based on the results of simulation tests, representing different environmental compartments. Options to consider negative results from eRBTs as evidence for persistency, similar to results from inherent tests, are discussed in chapter 8.

7.10 Validity criteria

28 days

The existing validity criteria of OECD 301 and 310 tests can be applied:

- Standard reference compounds reach the pass levels of 60% of $ThCO_2$ or ThOD within 14 days.
- The difference of extremes (DoE) of biodegradation replicate values at the end of the test is less than 20%.
- Inoculum blank activity must be within the range indicated in the test guidelines:
  - OECD 301 B: CO_2 evolution in control vessels should not exceed 40 mg/L and must not exceed 70 mg CO_2/L after 28 days.
  - OECD 301 F: Oxygen uptake is normally 20-30 mg 0_2/L and should not exceed 60 mg O_2/L after 28 days.
  - OECD 301 D: Oxygen depletion should not exceed 1.5 mg dissolved oxygen/L after 28 days.
  - OECD 310: Mean amount of inorganic carbon at the end of the test is <3 mg C/L (=11 mg CO_2/L)

60 days

For eRBTs with a test duration up to 60 days the following validity criteria should be applied:

- Enhanced testing reference compound (e.g. diethylene glycol) reached the pass levels of 60% of $ThCO_2$ or ThOD within 28 or 60 days (dependent on reference compound).

- The DoE at the end of the test is less than 20%.
- The difference of replicate values in inoculum controls at the end of the test correspond to a coefficient of variation (CV) of less than 50%. Remark: The accuracy and therefore the CV depend on the number of replicate vessels (3-5 replicates are recommended).
- Inoculum blank activity must be within the following rages:
  - OECD 301 B: CO_2 evolution in control vessels must not exceed 40 mg/L after 28 days and 70 mg CO_2/L after 60 days.
  - OECD 301 F: Oxygen uptake must not exceed 40 mg O_2/L after 28 days and 60 mg O_2/L after 60 days.
  - Inoculum blank criteria for eRBTs using OECD 301 D or OECD 310 should be defined, when data from prolonged tests are available.
  - One from three test vessels may be regarded as outlier when all other validity criteria are fulfilled. In this case the test results (degradation extents) are presented for each single test vessel and not as mean value.

7.11 Inhibitory effects

If in a toxicity test less than 25% degradation (based on total ThOD or $ThCO_2$) occurred within 14 days (using standard reference substances) or 28 days (using diethylene glycol), respectively, the test substance can be assumed as inhibitory to the inoculum and the test series should be repeated.

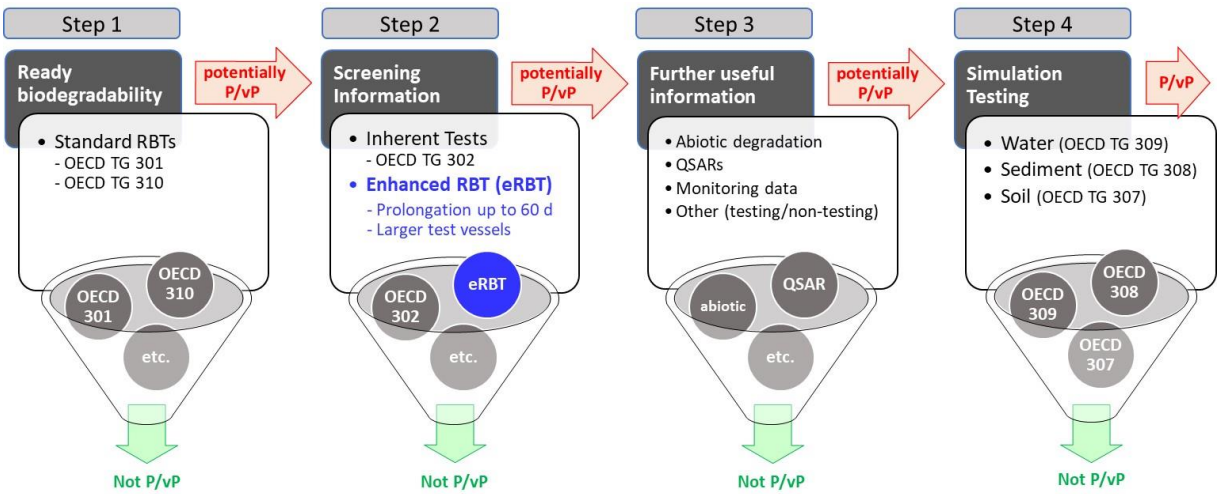
Remark: The time point (14 or 28 days) depends on the reference compound chosen. For other reference substances the time point may be adopted to the time when the reference compound reached the pass level (60% ThOD or $ThCO_2$).

8 Use of enhanced ready tests for P-assessment

8.1 Existing ECHA guidance

In general, all relevant available information on biodegradation should be considered for the evaluation of persistence. However, in practice persistence is mainly assessed based on screening criteria (i.e. "not P" according to (enhanced) ready or inherent screening tests) or compartment-specific half-lives from time-consuming and cost intensive simulation tests. ECHA guidance R.11 describes an integrated assessment and testing strategy (ITS) for persistence assessment with the objective to derive a conclusion on persistence for all three environmental compartments (or five, with marine compartments) with the least possible testing effort.

Figure 5: Simplified ITS Scheme for persistence assessment based on ECHA (2017b)



In the tiered approach (see Figure 5) RBTs are used in **Step 1** to decide if a compound can be assumed as "not P/vP". Accordingly, positive results from RBTs (i.e. ≥60% ThOD consumption or $ThCO_2$ evolution or ≥70% DOC-reduction, without the 10-day window) can be used as proof for non-persistence (ECHA 2017b). In contrast, a negative result does not allow for a definite conclusion on persistence and the substance has to be considered as "potentially P/vP". Consequently, further testing under less stringent test conditions is needed and other screening information are consulted in **Step 2**, namely inherent biodegradation tests and eRBTs. In practice the importance of inherent tests for persistency evaluation is limited due to the restrictions in terms of the test duration (7 or 14 days) and the shortcoming of inherent tests with standard inoculum and unambiguous endpoints for mineralisation (CO_2, O_2). This gap could be filled by RBTs.

Beyond that, ECHA guidance R.7b states, that when results of RBTs (i.e. OECD 301 series or OECD 310) "indicate that the pass level criterion is almost fulfilled (i.e. ThOD or DOC slightly below 60% or 70%, respectively) such results can be used as evidence for inherent biodegradability" (ECHA 2017a). However, this inference is only relevant for exposure assessment of inherently biodegradable compounds, but not for P-assessment, where the specific criteria described in the following apply for test results from inherent tests (ECHA 2017b).

Tests on inherent biodegradation according to OECD 302 series use more favourable conditions than RBTs (e.g. increased biomass to test substance ratio). Thus, they are conducted to show if

there is any potential for degradation and only substances, which reach the pass level of 70% degradation within 7 days (OECD 302 B) or 14 days (OECD 302 C) and for which a log-phase of less than 3 days is observed, are assumed as "not P/vP". If either of these criteria is not met, the substance is still assigned as "potentially P or vP". In contrast, a lack of degradation (<20%) provides proof of slow degradation in the environment and therefore can be used to draw a conclusion on persistence ("P"). Thus, further simulation testing for the purpose of PBT/vPvB assessment is not required. In addition, it might be possible to conclude from inherent biodegradation tests, that the vP-criteria are fulfilled, if there is additional specific information supporting this conclusion (e.g. specific stability of the chemical bonds).

So far, such reasonings do not exist for eRBTs. According to ECHA (2017b) eRBTs 'offer a cost-effective intermediate screening test in those cases where persistence in the environment is not expected although standard RBTs give the result "not readily biodegradable"'. Hence, a substance can be assumed as "not P/vP" if the pass levels for RBTs are reached, provided that only an extended test duration of maximum 60 days and/or larger test vessels are used as enhancements and the test was performed with non-pre-adapted inoculum. Substances which fail the pass levels in eRBTs are considered as being "potentially P or vP" and further simulation testing is needed to draw a conclusion on persistence (Step 4).

In certain cases, a Weight-of-Evidence (WoE) approach might be used to provide evidence of (non-)persistence and to conclude the P/vP assessment (**Step 3**). For this purpose, all available information on degradation, including abiotic degradation data, QSARs, monitoring data, field studies and other testing or non-testing information are considered for evaluation.

Quite recently, Redman et al. (2021) illustrated that the existing P-assessment strategy would benefit from the development of more flexible and holistic evaluation schemes including multimedia fate and transport models to predict overall persistence (P_{ov}) and a transparent WoE approach to combine all available information. The authors propose to use P_{ov} (i.e. the residence time of a contaminant within a defined environment) as an integrated metric for environmental persistence, since this parameter represents an appropriate and advantageous alternative to compartment-specific half-lives that integrates single-media half-lives and phase partitioning of substances in the environment.

Although models like the EPI Suite Level III fugacity model (US EPA 2017) or the OECD P_{ov} & LRTP Screening Tool (overall persistence threshold & long-range transport potential, Wegmann et al. 2009) are widely accepted in the scientific community to estimate overall environmental persistence, there is currently no (single) overall persistence threshold (P_{ov} in days) that can be used for persistence assessment in the regulatory context. This is because specific combinations of compartment-based criteria, emission scenario, and physicochemical properties might result in a range of acceptable P_{ov} values (Redman et al. 2021). Scheringer et al. (2009) suggested to use a threshold half-life of 90 days as an alternative, which means that a substance would be removed from the environment within one year. However, according to ECHA guidance (ECHA 2017a,b) the results of multimedia fate and transport models should be interpreted with care, since the predictions are strongly dependent on the default assumptions used by the model (e.g. size of the environmental compartments, emission pattern, partitioning and transformation parameters). The results should therefore be regarded as qualitative or semi-quantitative and a case-by-case evaluation is required.

If no conclusion is possible, further simulation testing is also needed. In general, simulation tests (**Step 4**) are performed for relevant compartments (e.g. (marine) water, (marine) sediment and soil) using natural environmental media (e.g. surface water, aquatic sediment, soil) with

radiolabelled test substances at low (environmentally realistic) concentrations. These are the only test systems that result in primary or ultimate degradation half-lives (i.e. $DegT_{50}$), which can be compared directly to the persistence (P/vP) criteria of REACH Annex XIII. It is not always necessary to perform simulation tests for each compartment, i.e. if persistence is already proved for one compartment no further higher tier data needs to be generated. However, if the substance is concluded to be non-persistent in one compartment it should be demonstrated that there is no concern in remaining compartments.

ECHA Guidance for the marine environment

According to ECHA guidance R.11, the allowed enhancements can also be applied to marine biodegradability tests like OECD 306 (OECD 1992c), but the guidance does not define specific conditions for enhanced ready biodegradability in such tests. As described in chapter 3.1.4, OECD 306 consists of variants of the 28-day Closed Bottle Test (OECD 301 D) and the 60-day Modified OECD Screening Test (OECD 301 E). Strictly speaking, the tests cannot be considered as RBTs since no inoculum is added in addition to the natural seawater micro-organisms and the tests do not simulate the marine environment due to the addition of nutrients and the high test substance concentration. However, a positive result (>70% DOC removal; >60% ThOD) indicates the potential for ultimate biodegradation in the marine environment. Since the degradation of substances in seawater is known to be slower than in freshwater, the ECHA guidance (ECHA 2017a, b) permits its use to describe ready biodegradability in seawater and consequently the assumption as "not P/vP". Furthermore, a degradation half-life in marine water > 60 days, e.g. derived in a marine surface water simulation test according to OECD 309 (OECD 2014b), leads to the conclusion, that the compound is persistent (ECHA 2017b).

ECHA Guidance for microplastics

In 2019, ECHA https://echa.europa.eu/registry-of-restriction-intentions/-/dislist/details/0b0236e18244cd73CHA 2019b). ECHA's Committee for Risk Assessment (RAC) supported the proposal while recommending more stringent criteria for derogating biodegradable polymers (ECHA 2020a). Details on this topic and on persistence assessment are presented in a final background document to the RAC opinion (ECHA 2020b). Based on the current available information non-biodegradable microplastics will likely meet the criteria for (very) persistent substances according to REACH Annex XIII with half-lives of several hundred years or more (ECHA 2020b), while biodegradable microplastics might be derogated from the restriction. Although several uncertainties exist regarding the suitability of the specified established standard biodegradation test methods, and modifications or even new test methods might be necessary to assess the biodegradability of microplastics, the existing methods can provide useful information. In this context eRBTs are also referred to (next to ready biodegradation tests, inherent tests, simulation tests and respective ISO test methods on the biodegradability of plastic materials). Accordingly, biodegradable particles reaching 60% mineralisation ($ThCO_2$ or ThOD) within 60 days in an eRBT are considered as biodegradable and exempted from the definition and restriction of microplastics.

8.2 Consequences for P-assessment

The use of all relevant available information on (bio)degradation for persistence assessment, including RBTs, screening information (i.e. inherent tests and eRBTs), further useful information (e.g. QSARs and monitoring data) and simulation tests might result in differing conclusions on biodegradability and persistence from different test results.

For example, phenanthrene was shown to be readily biodegradable in several RBTs, but was assumend as persistent (half-live > 120 days) in some (but not all) higher-tier simulation tests

with soil (OECD 307) and aquatic sediment (OECD 308). In contrast, phenanthrene did not fulfill the criterion for persistence in water (half-life > 40 days) in non-guideline studies. This discrepancy was explained by the reduced bioavailability of phenanthrene due to its hydrophobicity as well as considerable losses due to its volatility (ECHA 2018a,b; Hughes et al 2020).

This leads to the controversial discussion on the significance and interpretation of non-extractable residues (NER) from simulation tests with soil or sediment. The ECHA guidance R.11 states that NERs should ideally be differentiated in remobilisable and irreversibly bound fractions. Procedures to measure and classify different types of NER according to their binding status and concept proposals to be used in persistency assessment are currently discussed, but no standard concept to measure different fractions of the residue is agreed to date[5]. According to the ECHA guidance, NERs should be regarded as non-degraded substance, unless, on a case-by-case basis, it can reasonably be justified or analytically demonstrated that a certain part of the residues can be considered to be irreversibly bound and thus non-critical (ECHA 2017b).

Although simulation tests are performed at environmentally realistic conditions and are the only tests that can provide a definitive degradation half-life that can be compared directly to the persistence criteria of REACH Annex XIII, several uncertainties and shortcomings have been identified. For example, the outcome of aquatic sediment simulations studies according to OECD 308 can be affected by the geometry of the test vessels and the associated water-sediment interface size (ECHA 2017b). Hence, several modifications of the OECD 308 test system haven been discussed. Recently, Seller et al. (2021) proposed to use a modified OECD 308-type simulation test similar to that introduced by Shrestha et al. (2016) to study the biodegradability of compounds at the water-sediment interface in order to improve observability and interpretability of biotransformation. With the standard sediment–water ratio of 1:3 or 1:4 according to OECD 308, even moderately adsorptive substances are often excessively shifted into the sediment layer where mixed redox conditions prevail. By reducing the sediment–water ratio to 1:10 (v/v), and ensuring an aerobic water phase (e.g. by gentle stirring or aeration) a thicker aerobic sediment layer is obtained and the mass transfer is more shifted towards the water column. This resulted in higher biodegradation rates compared to the standard system. Reliability and interpretability of results could be improved and it was shown that the results are less dependent on differing sediment and inoculum properties compared to standard OECD 308 tests (Seller et al. 2021). However, Shrestha et al. (2016) observed that 'although standard OECD 308 was initially criticized for high NER formation due to its high sediment–water ratio, NER formation was even higher in modified OECD 308 tests', which might complicate the evaluation and interpretation of results. Thus, it needs to be further discussed, which test system can be considered as more realistic in view of the scenario, e.g. depending on whether stagnant (e.g. lakes and ponds) or flowing (e.g. streams and rivers) water bodies are examined. On the one hand, the anaerobic zone in the sediment might be underrepresented in the modified test system, resulting in an overestimation of the degradation in the environment. On the other hand, Bunke et al. (2020) argue that the aerobic water phase in the environment is much bigger than the anaerobic part of the sediment phase when compared to test systems. Hence, the degradation of substances which are degraded more rapidly under anaerobic than under aerobic

[5] In February 2021, an international online workshop entitled "Proposal to standardize the analysis and persistence assessment of non-extractable residues (NER)" took place. More than 70 participants from authorities, industry and science, including members of ECHA's PBT Expert Group, discussed the future consideration of NER in persistence assessment. The workshop documents (incl. two concept proposals to be used in persistency assessment) are available at: https://www.umweltbundesamt.de/en/topics/chemicals/reach-what-is-it/non-extractable-residues-in-persistence-assessment#hidden-hazard-or-a-safe-sink

conditions might be overestimated in the standard OECD 308 system and wrong conclusions on persistence may be drawn (ECHA 2017b).

The role of eRBTs in relation to inherent screening tests needs to be clarified. Enhanced screening tests have a lower inoculum concentration, but a longer test duration compared to inherent tests. Diethylene glycol (DEG) is suggested as suitable reference substance for eRBT and is also used as reference compound in the inherent biodegradability test. Our results from the practical testing programme demonstrate that DEG reaches the pass level of >60% within 28-60 days under (enhanced) ready biodegradation conditions, while it must reach the pass level of 70% DOC-elimination within 14 days in inherent tests. In practice, DEG is biodegraded by ≥ 70% in inherent biodegradability tests within 7 days (unpublished inhouse study results of consultants).

Inherent tests of the OECD 302 series have a higher inoculum concentration than eRBT and are mainly used for assessing inherent biodegradability of substances. Biodegradation above 20% in inherent tests is understood as evidence of inherent, primary biodegradability and suggests that stable degradation products are likely to be formed. Biodegradation above 70% of theoretical (ThOD, $ThCO_2$ or TOC) is regarded as evidence of inherent, ultimate, biodegradability. However, the pass level has to be reached within 7 days (OECD 302 B) or 14 days (OECD 302 C) and there are further specific criteria such as a maximum lag phase of 3 days (ECHA 2017a). For persistency evaluation from inherent tests, only test substances fulfilling all these criteria are considered as being "not P". Thus, it seems that the test duration for reaching the pass level is more conservative for inherent tests than for eRBTs. Further on, according to the ECHA guidance, a lack of degradation (< 20%) in an inherent test may provide sufficient information to confirm that the P-criteria are fulfilled without the need for further simulation testing for the purpose of PBT/vPvB assessment (ECHA 2017b).

Enhanced ready biodegradation tests may be regarded as similar to inherent tests, but are only used for excluding persistence when the pass levels are reached (not "P"). Substances which fail the pass levels in eRBTs remain in suspect of being "potentially P or vP" and further simulation testing is needed to draw a conclusion on persistence. A bottom line, similar to that for inherent tests (e.g. < 20%), suggesting that the substance may be considered as "P" without further testing does not exist for eRBTs at the moment. ECHA (2017b) refers to eRBTs only as a cost-effective intermediate screening test in those cases where persistence in the environment is not expected' although the substance fails the criteria for being "readily biodegradable". The authors suggest, that results from eRBTs indicating degradation extents ≤ 20% may also be used as evidence for "P", provided that no other evidence of false negative test results such as inhibitory effects have been observed.

Further arguments for using such a criterium for a substance being evaluated as "persistent" are as follows: The test duration of eRBTs is similar to that of OECD 309 ("Aerobic Mineralisation in Surface Water – Simulation Biodegradation Test"), which states, that the duration of the test should normally not exceed 60 days. Parameters of eRBTs, such as the test substances concentration (inducing growth conditions) and the test temperature are more favourable in eRBTs than in the OECD 309 simulation test. Degradation rates generally increase with increasing temperature. Although, the recommended temperature range of OECD 301 and OECD 309 is identical (20-25°C), it should be noted that under REACH the preferred testing temperature for new simulation studies was changed to 12°C[6] and that results obtained from already existing tests performed at 20-25°C are transformed to 12°C using the Arrhenius

[6] Result discussions during the 32nd meeting of the Member State Committee (MSC-32, November 2013); https://echa.europa.eu/documents/10162/13578/meet_minutes_msc_32_en.pdf/6d0f441f-05fe-46ac-ae96-46097d33283a; applied in the subsequent revisions of the REACH Guidances R.7b and R.11 (ECHA 2017 a,b)

equation. However, testing at 20°C might be appropriate to monitor the formation of metabolites. Degradation and transformation products of ≥10% of the applied concentration of the parent substance in simulation tests are considered as being sufficiently relevant to perform specific risk assessments for these compounds. In contrast, enhanced ready biodegradation tests are not designed to identify relevant degradation and transformation products and the low pass levels of 60% ThOD or $ThCO_2$, derived from the assumption of biomass growth, do not allow a conclusion on persistency of these transformation products. Bacterial biomass concentrations of eRBTs and the pelagic OECD 309 are in the same order of magnitude: the natural waters used in an ISO ring-test of the OECD 309 method were reported to have a bacterial biomass of 10^3 to 10^4 CFU/mL (OECD 2014b), total colony counts at 22°C with the activated sludge concentrations used in the eRBTs resulted in 1.5-8.3 * 10^3 CFU/mL (see Table 6). In contrast, bacterial cell densities in natural freshwater bodies are typically higher and range from 10^4 to 10^7 cells/mL (Seller et al. 2021).

8.3 Overall persistence assessment for selected test substances

In the following, the results of a simplified persistence assessment will be presented and discussed according to the different steps of the ITS in ECHA guidance R.11 by using the available degradation data for the selected test substances.

In addition, QSAR predictions using the Level III fugacity model integrated in EPI Suite (US EPA 2017) were used as further information in step 3. The model provides information on the distribution of the chemical between water, sediment and soil, respective half-lives for each compartment and the overall persistence time. Besides substance properties, overall persistence depends on the model used and the applied emission scenario. In the default EPI Suite scenario the substance is emitted to all relevant compartments (air, soil, water) in equal amounts. The results of the model predictions are summarised in Appendix C.1. Half-lives ≥ REACH Annex XIII persistence criteria were mainly predicted for the sediment compartment. However, except for ERU the percentage release into the sediment predicted by the QSAR model is less than 1% and thus can be neglected.

The results of the simplified persistence assessment are summarised in table 9, details on the underlying degradation data can be found in Appendix C.2.

The results from the eRBTs of the practical testing programme are in good accordance with other available (bio)degradation data. Beyond that, the results are also reflective of the QSAR predicted overall persistence time with half-lives clearly below 60 days for IBU, 4FP and DEG, an intermediate half-life for ERU and half-lives above 60 days for PBO and OBP.

With respect to the persistence assessment the following overall conclusions can be drawn from all eRBTs performed within this work when applying the criteria according to ECHA (ECHA 2017a, 2017b):

- Ibuprofen (IBU) can be regarded as being non-persistent (congruent results from test series 1, 2 and 4).
- This is in accordance with the ECHA registration dossier (ECHA 2021)[7], where IBU is rated as not readily but moderately/partly biodegradable based on a RBT according to OECD 301 D where a degradation extent of 31% (O_2 consumption) within 28 days was measured. Furthermore, the high mineralisation rates observed in the eRBTs are consistent with results

[7] The reliability and validity of biodegradation data, their evaluation and resulting regulatory conclusions published by third parties (e.g. on the ECHA website) could not be verified by the authors of the present report. Thus, no legal responsibility is taken for the correctness of this information.

from a water-sediment simulation study, showing 77% mineralisation within 100 days and DT_{50} values of 10 days (water) and < 6 days (total system), respectively (Löffler et al. 2005). Further experiments with water-sediment systems and fortified lake water resulted in half-lives of about 20 days and lower (Buser et al. 1999, Radke and Maier 2014).

- The results of Piperonylbutoxide (PBO) are inconclusive with respect to persistence. Although, the pass level of 60% was reached in one replicate of test series 4, the validity criterion for the allowed variability between replicates of 20% was failed. Beyond that, results from test series 1, 2 and 3 showed degradation extents clearly below 60% after 60 days. Thus, PBO has to be rated as "potentially P".

- The low degradation conforms to data reported for other RBTs showing 24-48% degradation (OECD 301 B) and <0 – 4% degradation (OECD 301 D) within 28 days (ECHA 2021). For a water-sediment simulation study with two aquatic sediment systems from a creek and a pond according to OECD 308, consistent degradation half-lives for the whole system ($DegT_{50,\,system,12°C}$) of 102-104 days were calculated (ECHA 2021), whereas the dissipation half-lives for water ($DT_{50,\,water,\,12°C}$) differed considerably with 51 and 313 days for the pond and the creek system, respectively. From two aerobic simulation studies with four different soils a geometric mean $DT_{50,12°C}$ of 58 days was derived, under anaerobic conditions, reduced degradation was observed resulting in a $DT_{50,25°C}$ of 144 days. Based on the data from the water-sediment study, PBO is assumed as very persistent (vP) compound by authorities (ECHA 2021).

- Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)-propionate (OBP) did not meet the 60% pass level in any test series, but high DOC-elimination > 70% was observed in the combination test (OECD 301 A/B). However, the DOC-elimination does not reflect mineralization, but can be explained by adsorption to activated sludge. Therefore, the results are inconclusive with respect to persistency.

- The results are in good accordance with standard 28-day RBTs, resulting in mean degradation rates of 34% (OECD 301 B; range 32-35%) and 31% (OECD 301 C; range 21-39%), whereas fast primary degradation of 86% within 10 days based on substance-specific analysis was observed in a ready type OECD 301 A study (ECHA 2021). 21-47% degradation based on IC analysis were observed in an OECD 302 B test within 35 days (ECHA 2021). In a degradation experiment in soil with radiolabelled OBP a mineralization (CO_2 evolution) of 8% was observed within 14 days (ECHA 2021). Consequently, OBP is rated as not readily biodegradable but is assumed as not P/vP by ECHA (ECHA 2021) due to the rapid primary degradation, despite the formation of the stable main metabolite metilox acid (CAS 20170-32-5).

- 4-Fluorophenol (4FP) is assumed to be non-persistent according to data from test series 1, 2 and 4.

- This confirms the results of the ECHA registration dossier, where 4FP is reported to be biodegradable based on a QSAR predicted half-life of 38 days in water (ECHA 2021). In addition, 4FP was found to be biodegradable within 3.5 to 7 hours at concentrations of 50-

125 mg/L in adapted activated sludge (ECHA 2021). However, the use of adapted inoculum does not allow any conclusion on ready biodegradability. In contrast, Martin et al. (2017) determined mean degradation extents of 50.2% (range 46.2-52.8%) after 60 days in experiments according to OECD 301 B when using a standard RBT inoculum concentration of 3 mg/L. An improved degradation of 62.4% (range 61.8-63.1%) was observed at a substantially higher inoculum concentration of 300 mg/L.

- Cis-13-Docosenonamide (Erucamide, ERU) can be assumed as being non-persistent based on results of test series 2, even though some doubts remain, since the 20% criterion for the variability between replicates was failed in test series 1 and ultimate biodegradability was clearly below the pass level of 60% in test series 4.
- The eRBT results show a high variability (i.e. 34.5% to 64.3%) depending on the test conditions, which is corroborated by the data from other standard RBTs, with degradation extents in the range between 15% (OECD 301 B, D; ECHA 2021) and 88% (OECD 301 C; MITI 1992). Overall, ERU is considered as readily biodegradable and thus "not P" according to ECHA (2021), based on 64% CO_2 evolution observed in an OECD 301 B study despite failing the 10-day window.
- The reference substance **DEG** reached the pass level for ready biodegradation of 60% in all test series within 28 days and thus is concluded to be non-persistent. This is in good accordance with several other results from standard RBTs, reaching 59-98% mineralization based on CO_2 evolution or O_2 consumption as well as 90-100% DOC-elimination (ECHA 2021b, MITI 1992).

The results of the practical testing programme also show, that the added value of enhanced testing compared to standard RBTs was limited for the selected test substances. Only for ERU the prolongation of the test duration in combination with larger test vessels did result in a changed conclusion as being non-persistent, while for IBU, 4FP and the reference compound DEG the pass level of 60% was already reached after 28 days and both were assessed as being "readily biodegradable" because the 10-day-window was reached. Nevertheless, the eRBT results for ERU were helpful to evaluate the variable results from other biodegradation data and confirmed the biodegradability of ERU under specific test conditions.

Overall, the test prolongation up to 60 days and the use of larger test vessels proved to be suitable enhancements from a technical point of view, resulting in transferable validity criteria and reliable biodegradation data for eRBTs, which can be used to identify non-persistent compounds under REACH according to the ITS for persistence assessment. Furthermore, results from eRBTs might be used in the context of an integrated persistence assessment framework that combines multimedia approaches and a clear WoE approach as recently proposed by Redman et al. (2021).

However, the results and conclusions obtained in this research project should be verified on a broader database, i.e. by testing further substances in enhanced ready biodegradability tests while considering the suggestions made in chapter 7. Future research should also consider other potential enhancements such as improving the quality and amount of the inoculum next to other modifications such as the optimisation of the dosage method of low water-soluble test substances. In this context also the role of eRBTs in relation to inherent biodegradation screening tests in the context of persistency evaluation should be clarified.

Table 9: Persistence Assessment for selected test substances

Test substance	**Step 1** RBTs (this study)	**Step 1** RBTs (other data)	**Step 2** Screening information (this study)	**Step 2** Screening information (other data)	**Step 3** Other information /WoE	**Step 4** Simulation Studies	**Conclusion**
IBU	Not P	Not P	Not P	Not P	Not P	Not P	Not P
PBO	potentially P	potentially P	potentially P	potentially P	potentially P [1, 2]	P [4]	P/vP
OBP	potentially P	Not P [5]	potentially P	Not P [5]	potentially P [1, 2, 3]	n.a.	Not P
4FP	Not P	Not P	Not P	Not P	Not P [1]	n.a.	Not P
ERU	potentially P	Not P [6]	Not P	Not P	Not P [1]	n.a.	Not P

n.a. = not available

1 = QSAR predicted half-life for sediment >120 d, but percentage release into sediment is <1%

2 = QSAR predicted half-life for soil =120 d

3 = QSAR predicted overall persistence < 90 d but > 60 d

4 = variable results; $DT_{50,\ 12°C,\ water}$ and $DT_{50,\ 12°C,\ soil}$ >120 d; $DT_{50,\ 12°C,\ system}$ > 100 d

5 = Based on fast primary degradation of 86% within 10 days according to OECD 301 A. However, loss of the parent compound might be attributed to sorption to the glass walls and the main degradation product (metilox acid; CAS No. 20170-32-5) was determined to be not readily biodegradable according to OECD 301 B.

6 = Based on 64% CO_2 evolution observed in an OECD 301 B study but failing the 10-day window and on 88% O_2 consumption observed within 21 days in an OECD 301 C study.

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A Workshop protocol: Status of ready biodegradability testing

Workshop: Current status of ready biodegradability testing and options for improvements

German Environment Agency, Dessau-Roßlau

April 2nd, 2019

Welcome by Jan Moltmann

Johann Moltmann (UBA REACH section) welcomed the participants and remembered, that REACH has a focus on PBT-assessment. The question is, whether we can make a statement on persistency in the environment based on ready biodegradability tests (RBTs) and what this statement tells us with respect to the disappearance of a substance? Mr. Moltmann had to leave the workshop because there is a discussion round on waste water charges for direct dischargers of trace contaminants. This reflects the high (political) relevance of biodegradability tests also in this area.

Presentation 1 – Overview on the project

Stefan Gartiser (Hydrotox GmbH) presented a short introduction on the UBA-project "Further development of screening tests for the evaluation of potential PBT substances" which started in autumn 2018 and will run till September 2021. One objective of the project is to provide proposals, how the gap between screening tests and more complex simulation tests could be closed.

The expert workshop aims contributing to the work package 1 (WP 1) "Comparison of existing test guidelines" where proposals for the harmonization and further development of the OECD 301/310 tests will be elaborated to allow better comparability of the results. These standard screening tests also provide the basis for "enhanced screening tests", which allow some modifications in terms of the test duration and vessel size. A positive result in a screening or enhanced test indicates that a substance is not persistent while a negative result is inconclusive. A first analysis about the inconsistencies between different ready type screening tests has been elaborated in a preceding literature survey (UBA-Texte 10/2017).

In WP 2 suitable test items for practical testing in (enhanced) screening tests will be selected based on a literature and data bank survey. An ideal substance is one which does not pass a standard RBT, has a low water solubility and for which valid simulation tests are available.

In WP 3 in total 5 test items will be tested in standard and enhanced RBT with extended test duration (60 d), larger test vessel, an alternative inoculum (e.g. surface water) and parallel determination of different endpoints such as O_2 and CO_2 (in total 25 tests).

In WP 4 the results obtained from WP 1-3 will be used to develop a first draft proposal for an OECD guideline on "enhanced ready tests". Here, the testing principles (endpoints) and boundary conditions, suitable reference substances, proposals for validity criteria and guidance for evaluation and reporting shall be described. The draft aims providing a first bases for further handling in OECD boards.

In WP 5 a guidance with recommendations for the use of enhanced tests for persistency evaluation under REACH will be elaborated. Here, the interpretation of test results from enhanced ready tests next to proposal of threshold values and the use of test results for P-assessment (only for "Non-P" or also for "P"?) will be discussed. A further discussion with workshop participants and other experts also for WP 2-5 would be very valuable.

Discussion

Selection of the test items:

- From part of industry, it was stated that the selection of only 5 test items is no sufficient database for a statistical evaluation and for developing a new guideline. It was suggested that the selection of appropriate test items to be included WP 3 is a critical step especially and should be carefully justified. One consultant referred to a CEFIC LRi-funded Eco12 project where the method validation of enhanced tests has been performed with a similar number of test items.[1]
- A representative of authorities answered that it is not an easy step to develop or change a guideline. However, now it is more important to see, which guidelines are used and which test conditions can be adapted. The aim is to obtain a better understanding what the current status is. This project is only a first step. Based on the results, it will be discussed, whether further data (e.g. additional reference compounds or compounds for which typically a long lag phase can be observed in extended OECD screening tests") will be needed.

Characterization of the inoculum:

- Authorities: Different inocula are often the main reason of differing results. Perhaps, more consistent results/interpretations can be achieved, if the inoculum is better characterized.
- Laboratories: Availability of 5 further references substances allowed in the ready tests would be helpful.
- Laboratories with respect to the 20% validity criteria (difference between degradation replicate values allowed in ready tests): What difference can be expected for enhanced tests? From part of authorities is was answered that nowadays better measuring techniques are available than at the time the guidelines were developed. The guidelines are old and maybe the criteria can be lowered down.
- Considering the high variability in results from OECD 301 tests, ranging from 0% to 100% with different test methods, one expert asked whether there is a database, which compares results for one compound from the various (all) ready tests? One consultant refered to literature data with comparative data in different OECD 301 tests.[2]
- Industry: One substance tested in different OECD 301 tests will show different biodegradation results. First different OECD 301 tests should be prepared with the same inoculum, to compare the test results.
- Laboratories: Seasonal changes of inoculum are reflected in the test results. High variability of inoculum and it is often difficult to characterize the inoculum. Descriptive characterization might be useful but no added value of microbiological analysis.
- Laboratories: Determination of CFU needs 3 days, this is a knock out criterion if it´s intended to determine the inoculum concentration from this, because the start of the test would be delayed by these 3 days. However, a retrospective description would be possible and could provide valuable information. If molecular biological methods are needed for a better

description of the inoculum the price for this characterization is 3 times higher as the costs for the biodegradation test.

- Laboratories: He doubts good test results will be obtained, when activated sludge will be replaced by surface water.

Standardization of the inoculum:

- Industry: Proposal to use a harmonized/standardized artificial inoculum. UBA does not consider this as meaningful because use of a standardized inoculum poses the question how much this affects the informative value even though the MITI test is accepted as equivalent to other tests on ready biodegradability. Are such results still appropriate for real world predictions? From part of consultants the MITI-inoculum was referred to, which is highly standardized, but loses its high microbial diversity and potency during pre-culturing in the laboratory prior to testing.
- Consultant: The preceding literature study revealed, that standardized or artificial inocula or those with extended pre-treatments lose some of their quality. Inoculum potency depends also on the operational performance and thus quality of the STP (e.g. sludge load). The lower the sludge load (the worse the STP) the higher the inoculum variability/potency.[3]
- Consultant: MITI inoculum has to be fed with artificial sewage mainly containing peptone and glucose for at least four weeks, which is not representative for what you find in a STP.
- Laboratories: The determination of dry weight of inoculum is not described in the test guideline(s) at all, so every lab does it in a different way (e.g. filtration and overnight drying or drying only for a few hours, …). Thus, the use of different methods for determination of dry-weight is another source of variability. This should be clarified in the guideline.
- Laboratories: Sampling and transport of sludge has also high influence. It is often difficult to take representative samples from a STP.
- Laboratories: Do not pre-treat the inoculum too much, but apply/use it as fast as possible ➔ Determination of CFU is not meaningful. Realistic results are needed. If the substance is degraded ➔ "not P". There is no reason to standardize the inoculum.

Use of test results for P-assessment:

- Industry: The authorities probably will not accept the results from enhanced ready tests for a definitive conclusion on "P" and "not P", because they often decide in a conservative way.
- Industry: One positive result in a degradation test supersedes all negative results, which is the opposite of the approach in the ecotoxicity tests. Shouldn't there be more than one result needed for the registration? One representative of the UBA did not agree that one positive result can supersede several negative results in RBTs.
- Authorities: The proposed aim to use enhanced tests also to define "P" (not only "potential P") is challenging and not realistic. Keep it as a screening test, keep expectations a bit lower. Enhanced tests give interim results from a screening test, i.e. biodegradable (yes/no) but not

"P"/"not P". Keep it as it is. Simulation tests are very important for the assessment of P and vP and cannot be replaced by screening tests.

- Consultant: But from inherent tests there already exists a proposal in the ECHA guideline, for defining "P" if < 20% degradation is achieved without the need of performing simulation tests.
- Authorities: Disagrees, because inherent tests belong to another test category. UBA mentions that, although this option is given in the ECHA guidance, this approach is not used in practice.
- Laboratories: Proposal to perform parallel tests with different inoculum sources (e.g. activated sludge, surface water). Other experts referred to the difficulty to derive a consistent overall result (mean value or best value)?
- Industry: Refers to the different rules applied for ecotoxicity and biodegradability testing. While for ecotoxicity assessment the lowest value is used in biodegradability testing one positive result (pass levels reached) skips all negative test results. UBA does not agree, in accordance with the technical guidelines for REACH this is only true for purpose of using data from screening tests to make decisions for harmonized classification and labelling, but not for P-assessment. For decision making UBA considers all available data and thus also screening tests with negative result in their decision on persistency. UBA also states, that the validity of the tests and plausibility of the results is also take into account in the decision-making process.

<u>Testing of difficult substances:</u>

- Laboratories: Solubility and bioavailability are important. Main goal of the project should be to transfer rules from one to another test system, i.e. to bring all test methods on the same level. For example, the silica gel method is only described in the OECD 310, but not for the OECD 301 F ➔ Same options should be available for all screening test methods. One authority did not agree to accept 301 F tests with silica gel although its use is considered in ECHA guidance R.7b.
- UBA refers to OECD Guidance document on difficult substances (OECD Series on testing and assessment No. 23), which is only used for ecotoxicity test but could also be adopted for biodegradation testing.
- A representative of authorities mentioned the ISO-guideline 10634, where additional methods are described.[4]
- Laboratories: Uses activated sludge in the Closed Bottle Test. This is not in accordance with the current guideline method, but has been used and validated for a long time (van Ginkel and Stroo, 1992, Ecotoxicol. Environ. Saf. 24:319).
- Industry: The bioavailability, especially from volatile substances should be regarded more. For example, OECD 301F is used for volatile compounds but often is not suitable because of lack of bioavailability in the aqueous phase (e.g. Brown et al. 2018). Modified test systems

proved to be more suitable but are not in accordance with the guideline. Nonetheless, lack of bioavailability can only result in underestimation of bio-degradability.

- Consultants: The Annex of OECD 301 also contains information how to deal with substances with low water solubility.

Presentation 2 – Results from the survey on current status of RBTs

Stefan Gartiser (Hydrotox GmbH) presented the results of a survey on the current status of RBTs which was based on a detailed questionnaire distributed to GLP and other experienced laboratories performing RBTs. In total 16 laboratories from Germany, Switzerland and the United Kingdom contributed to the survey.

Currently the OECD 301 B and F are the most often applied test methods, which contribute to more than 80% of all tests performed. The OECD 310 has also gained some importance while the DOC-based methods OECD 301 A and E are rarely applied. The OECD 301 D is preferred by some laboratories for inhibitory or volatile substances while others consider its inoculum as too weak (if effluent inoculum suggested by the guideline is used). Thus, methods based on CO_2 evolution or oxygen consumption are preferred against methods following DOC-elimination. The OECD 301 C MITI(I) test is not often applied due to the highly artificial and weak inoculum.

According to the laboratories about half of them suggest to omit the 301 A and/or E because of the end-point DOC and half suggest to merge the OECD 301 A and E. About half of the laboratories indicated that the MIT(I) test 301 C could be withdrawn due to the effort for cultivating the inoculum and its low potency and half suggested to merge the OECD 301 C and F (respirometric methods).

In total 6 from 16 laboratories were open to apply combination tests with several endpoints (CO_2 + O_2, CO_2 + DOC, O_2 + DOC, CO_2, O_2 and DOC) while others were against obligatory multi-end-point testing due to the equipment needed and the different substrate/inoculum ratios required for accurate testing. Further on it was referred to the difficulty to handle differences between results obtained from the different monitored parameters.

With respect to the testing strategy for inhibitory substances 6 of 16 laboratories consider the Closed Bottle Test 301 D as best option due to the lowest test concentration, while other doubt that this would be the best option due to its low inoculum potency. One lab has used sludge as inoculum (up to 2 mg/L) for years which remedies the potency problem while delivering reliable results (see publications, e.g. van Ginkel and Stroo, 1992). Other laboratories refer to good results with the 301 F (with lower test concentrations) or to the OECD 310, while 4 laboratories apply adsorbents (silica gel, humic acids) with the 301 B or 301 F to reduce toxicity.

Most laboratories do not regularly assess microbiological parameters such as colony forming units (CFU), but only on request of the sponsor. Instead, they agree to better describe the inoculum source as long as no microbiological parameters must be assessed. Further on, most laboratories suggest that all inoculum sources/concentrations allowed in any OECD 301/310 test should be allowed in other tests as far as validity criteria are fulfilled. Considering the specifications described in the different tests both old (as described in 1992 guidelines) and adopted test designs (with minor modifications such as measuring devices or parallel vessels) are being applied in practical test performance.

Discussion

<u>Merging or withdrawal of test guidelines:</u>

- Industry: Refers to a study where 600 tests have been performed with low water solubility substances. Here the OECD 301 D gave good results (highest degradation) ➔ we should not disregard this test.
- Another expert from laboratories also referred the 301 D test performance. In 2017 (as requested in the questionnaire) there have been few, but in former years far more. He thinks, that the survey would look different, if a wider time-range would have been regarded.
- An expert from industry referred to the test 301 C [MITI (I)]: No one likes this test, but for registrations in Japan you need it. Recently though, even the Japanese authorities allow using the 301F test with sludge inoculum for registration in Japan.
- Industry: In the survey, the discussion on feasibility to reduce test concentration in our study, the focus being on test items with low water solubility, is missing. For example: is it possible in OECD 301 D to test below 2 mg/L with useful results? In a recent list of test substances 150 substances (= 25%) had a water solubility below 1 mg/L. Substances of low water solubility should be covered by enhanced tests. After the workshop more details were provided from Dr. Essers, who referred to his ongoing thesis for a postgraduate studies course in ecotoxicology (SETAC GLB and GDCh) where a set of substances from the OECD eChemPortal for which data on biodegradation and water solubility are available was aggregated and filtered regarding several qualitative and quantitative criteria. One objective of the thesis is to investigate a correlation of results obtained from biodegradation studies (OECD 301, 310) with intrinsic properties of the test substances like water solubility. The resulting set of 608 substances was divided into three water solubility classes of which 24% had a water solubility < 1mg/L.
- UBA asked whether DOC methods 301 A/E and MITI (I) could be skipped.
- Laboratories: DOC-methods may be useful for substances with a good water solubility or substance mixtures. Test should be selected according to the properties of the test items. The 301 B also has its drawbacks, because part of the IC is bound to the matrix and only recovered at the end of the test, which may be critical for the 10-day-window. The DOC-based tests are easy to perform for mixtures. Why should we disregard this test guideline? You always can apply another test guideline. Merging 301 A/E is o.k. Merging C+F only if DOC measurement is also included in the OECD 301 F. Question: Will chemical analysis become obligatory, when OECD 301 C/F will be merged? There was no consensus to include chemical analytics if the oxygen depletion already gives a definite result.
- Laboratories: Have only experience with the MITI (II) tests (OECD 302 C). Here authorities accepted MITI II tests with only one inoculum. One representative of the UBA also would agree to such an approach, because the MITI test has its origins in the manometric test method.

<u>Test concentration</u>:

- Laboratories: Low test concentrations would be desirable, especially for inhibitory substances. But at low test concentrations there might be problems with the signal-to-noise ratio, i.e. risk arises that the background noise of the inoculum on the test result might be too high. Thus, the inoculum-substrate ratio should be maintained.
- UBA: Flexibility is needed with regard to substance properties.
- An expert from authorities asked whether it would be useful to have a common set of ranges for the key parametres (such as test substance concentration, inoculum concentration, and test substance/inoculum ratio), that would be applied for each of the RBT (instead of the current situation where different protocols have different ranges of allowed parameters). According to one expert from laboratories the test concentration in 301 F of 100 mg/L (resulting in 50-100 mg ThOD) has historical reasons, but now there are better measuring devices. Why not perform a 301 F with 10 mg/L, e.g. also with a lower inoculum concentration? It depends on the measurement method. If the method is sensitive enough, there is nothing to be said against testing below 2 mg/L. The test concentration should not be fixed. Instead, we need quality information about the background noise.
- UBA is not in favor of such a proposal, because it offers too much flexibility. The inoculum: test substance ratio must not be changed and the ratio should not be arbitrary. A freely selectable concentration range would be a fundamental encroachment on the guideline and is not desirable. There is a certain tolerance in choosing different methods of addition.
- An expert from authorities suggested that min/max ranges for test substance concentration and substance: inoculum ratio should be given for each test. When considering the use of lower test concentrations, it has to take into account that we should maintain the difference between simulation tests and screening tests. Screening tests are based on the ability of microorganisms to utilise the chemical as a sole carbon and energy source.
- Laboratories: Often they perform tests with two test concentrations (e.g. 50 and 100 mg/L ThOD in the OECD 301 F) and often observe better results (i.e. higher biodegradation) with the lower one. It would be more environmentally realistic to use lower concentrations.
- Authorities: It is critical to use very low test concentrations since the differences in the test design (next to test concentration also inoculum source and concentration or test duration) compared to simulation tests are still high and should be kept.

<u>Validity criterion in parallel vessels</u>:

- Laboratories: with respect to the variability of parallel vessels of inoculum blanks: If you have only a low variation in parallel vessels (by testing for example with many more replicates) you could reduce the test concentration.
- An expert from part of laboratories asked who has done 301 D with solid test items and what was the variability? Another laboratory expert answered that the number of 301 D tests performed per year does not allow a statistically evaluation.

- From part of the laboratories, one referred to the possibility that degradation products might be insoluble while other experts assume this risk being low, except if precipitation reactions occur.

Presentation 3 - Outlook on enhanced testing strategies and consequences for PBT assessment

Stefan Gartiser (Hydrotox GmbH) gave an outlook on enhanced testing strategies. Here, the answers from the questionnaire on current status of ready tests should also be considered, because some suggestions for RBTs also describe the borderlines for enhanced tests. According to the ECHA guidance R.7b from June 2017 the enhancements are designed to improve environmental relevance without the need of extensive simulation tests. Results from enhanced tests must not be used for „ready biodegradability", but only for „P"-assessment. So far, the ECHA refers to two enhancements:

- Prolongation up to 60 days (especially with poorly water-soluble substances with low bioavailability and slow but steady biodegradation without reaching plateau phase in 28 d).
- Use of larger test vessels (no upper limit defied, only practical constrains).

The same pass level as for ready tests (60% or 70% degradation) apply, but without the 10-day window. Further enhancements not deemed being acceptable are the increase of biomass concentration, the use of pre-adapted inoculum, semi-continuous assessments or the addition of co-substrate (test substance should be the only carbon source).

A negative result in an enhanced ready biodegradation test has an inconclusive conclusion as "potentially P and vP".

It has been suggested that the variability of parallel vessels is expected to increases with the lag-phase and the test duration. Thus the 20% validity criterion might be difficult to achieve. By increasing the number of replicate vessels the statistically evaluation could be improved and outliers could be excluded.

There would be an option to allow only respiratory methods using O_2 and/or CO_2 (OECD 301 B, C, D, F, 310) for enhanced testing due to the difficulty to interpret DOC-elimination methods of the 301 A and E. If the last are also allowed, an obligatory abiotic and/or adsorption control may be foreseen?

With respect to the inoculum both the ECHA guidance and OECD guidelines demand that the inoculum used should be from STP with predominantly domestic sewage. The question is what is "predominantly domestic" or what is considered a STP with a "high" industrial share except those attributed as "industrial STP". One proposal is to compare the population equivalent load of the STP with the inhabitants connected to the sewer. Most laboratories were against such a strict criteria for a "high industrial share" and refer the high variability of inoculum over time and location. Municipal STPs are adapted to continuously emitted "old" substances.

Most laboratories support the use of any of the allowed OECD 301 inoculum sources in any test as far as the validity criteria are met. However, the signal-to-noise relation should be considered. One suggestion was to routinely perform tests with 3 different inocula: STP, surface water and soil and to compare all results.

Currently, for the evaluation of the enhanced tests the same pass level as for RBTs should be applied (60% $ThCO_2$/ThO_2 or 70% DOC). The validity criteria inoculum blanks for prolonged tests need to be defined or should be referred to the 28 d. Further on, the validity criteria of 20% difference of extremes of replicate values (of degradation at the time point used for evaluation)

could be changed with CV < 20%. The result of the P-assessment is a "yes" or "no" decision as "(potentially) persistent" (for decision "yes") or not.

Discussion

Can results be used for P-Assessment?

- Industry: Only "potential P" or "not P" can be derived from an enhanced test. P or vP needs more testing than enhanced tests.
- One of the consultants agreed as long as there is no agreed value for being P without the need of simulation tests similar to the inherent tests.

Description of inoculum source:

- Industry: It may be difficult to describe the catchment area. Often it is not known, which industries discharge which amount of chemicals/waste water.
- From part of the consultants and the UBA: Treatment capacity (inhabitant equivalents) of the STP and total number of inhabitants connected are normally known at the STP. Every STP is designed for the specific local conditions. In cases where treatment capacity clearly exceeds the number of connected inhabitants, impacts of industrial sites with regard to possible pre-adaption of the inoculum to the test substances are more likely. It was also stated that classifying surface water in terms of (absence of) industrial influence would be more difficult.
- UBA: Surface water is even harder to describe than activated sludge.
- Industry: inoculum should be described as good as possible.

Should DOC methods be excluded?

- Laboratories: It depends on test item, possibly accompanying chemical analyses needed.
- Industry: DOC can give valuable additional information.

Differentiation domestic vs. industrial sewage:

- Industry: No borderline for STP suggested. You never know, if there wasn't any pre-treatment of the industrial part of the waste water beforehand. There should be no borderline between domestic and industrial share of a STP.
- Laboratories: What's the probability of a pre-adaptation? You will never know, whether due to the industrial waste water there is an adaptation for your respective test item. Only exclude STP from those companies producing the test item. But this information is normally not available. Would a definition of allowed industrial share help us? No, because not only the volume or load, but also quality of the inoculum is important. It is impossible to use inoculum only from domestic STP, because there always is an industrial part.
- UBA: Normally this information is not available. Thus, a possible adaptation is assumed, if industrial wastewater is used.

- Laboratories: A statement like “adaptation is not allowed” would be sufficient.
- Laboratories: It would also not be fair to restrict the inoculum source for those labs which don’t have the chance to select between different STP.
- Laboratories: The OECD wording of using inoculum from STP with predominantly domestic wastewater and excluding the use of adapted inoculum, is sufficient.
- Authorities: Not sure of the origin of OECD wording of using inoculum from STP with predominantly domestic wastewater. However, variability of inocula from different municipal STPs could be considered to be lower than variability between inocula from municipal and industrial STPs, or between different industrial STPs.

Do we always start with an enhanced test?

- No, a preceding ready biodegradation test might be available or a ready test might be prolonged.

Is it possible to go for an enhanced test while the standard RBT is running?

- Often not possible based on measuring method (e.g. prolongation when using OxiTop) or test design (e.g. test vessel size).
- Laboratories: Only the use of larger test vessels must be decided beforehand or the number of flasks in the 301 D/310 tests with sacrificed vessels must be considered before starting the test.
- Consultant: Refers to the potentially limited oxygen supply for prolonged tests.
- Laboratories: For the 301 F the oxygen is only limited if manometric tests are used, but not for the electrolytic methods.
- Laboratories: has good experience with the Closed Bottle Test when using OECD 310 flasks, into which the volatile substances are injected.
- Authorities: For the evaluation of ultimate biodegradability CO_2 is the best option, since it can be ensured that biodegradation is based on mineralization of the test compound. This can be important, e.g. if a test substance is a multi-constituent or a UVCB substance and if there is a need to determine mineralisation of a specific constituent, which may also need the determination of primary degradation and transformation products, in addition to CO_2 measurement.

Maximum inoculum concentration:

- Industry: Refers to the report of the preceding project, where there has been the proposal to allow 100 mg/L d.s., which is below the inoculum concentration in inherent tests (starting at 200 mg/L d.s.). Will this be discussed here again?
- Consultant: Formerly the increased inoculum concentration was mentioned in the ECHA guidance. This proposal should avoid the use of inoculum concentrations which are more

related to inherent tests (200-1000 mg/L d.s.) than to ready tests (30 mg/L d.s.). This proposal was not accepted at the workshop in February 2016 by authorities. The discussion is documented in the workshop report in the annex of the UBA-Text.

What about OECD 314?

- Laboratories: Refers to the OECD 314 simulation test ("Simulation Tests to Assess the Biodegradability of Chemicals Discharged in Wastewater") where 28 days or longer are allowed with activated sludge of 3000-4000 mg/L d.s.
- Industry: Refers to REACH discussions. The guidance is clear: test results from these test cannot be used for P-assessment.
- Authorities: Yes, must not be used for P-assessment (comparison with Annex XIII criteria), but can be used within a weight-of-evidence approach.

Closing remarks

Daniel Sättler (UBA) thanked all participants for coming and the fruitful discussion and gave an outlook on the next steps. The draft minutes of the workshop will be sent to participants for comments and the results of the workshop will be published. Any suggestions for suitable test compounds are welcome, particularly further data, which are not freely available.

Stefan Gartiser (Hydrotox) asked whether the participants would be interested in a 2nd workshop, when the results will be available. Many participants are interested in being kept in contact and in second workshop when all results and a first proposal of the OECD guideline on enhanced testing are available.

From part of industrial laboratories, it was offered to perform enhanced tests with more test items to get a better data base.

Protocol by

Stefan Gartiser

Thomas Junker

Andrea Brunswik-Titze

The presentations can be sent as pdf documents via e-mail on request.

Further remarks:

1. Poster Davenport et al. (2013). The Rational Analysis and Design of Enhanced Biodegradation Tests for Persistence Assessments, download http://cefic-lri.org
2. E.g. Mei et al (2015). A comparative study of biodegradability of a carcinogenic aromatic amine (4,4'-diaminodiphenylmethane) with OECD 301 test methods. Ecotoxicol Environ Saf. 111:123-30; Seyfried et al. (2015). Persistence assessment of cyclohexyl- and norbornyl-derived ketones and their degradation products in different OECD screening tests. Chemosphere 131: 63–70; Dick et al. (2016). Current limitations of biodegradation screening tests and prediction of biodegradability: A focus on fragrance substances. Environmental Technology & Innovation 5: 208-224; Kayashima et al. (2014). Comparison of biodegradation performance of OECD test guideline 301 C with that of other ready biodegradability tests. Environmental Toxicology and Chemistry 33: 328-333
3. E.g. Vázquez-Rodríguez et al. (2007). Inocula from activated sludge for ready biodegradability testing: Homogenization by preconditioning. Chemosphere 68, 1447-1454

4. ISO 10634 (ISO, 2018a): Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium.
5. Remark from consultants: Some guidance is given in Brown et al. (2018): Assessing the suitability of a manometric test system for determining the biodegradability of volatile hydrocarbons. Chemosphere 195:381-389.

B Selection of test substances for practical testing

B.1 Results of the search for potential test substances - OECD eChem Portal (compounds with water solubility < 100 mg/L)

Table 10: Results of the search for potential test substances from OECD eChem Portal (water solubility < 100 mg/L)

No	Substance name	CAS No.	Water solubility [mg/L]	Year	Result [%]	Parameter	Test duration [d]	Concen-tration [mg/L]	Guide-line	Available simulation test data
1	Cloquintocet-mexyl	99607-70-2	0.59	1993	38	CO_2 evolution	29	23.9	301B	2x OECD 308 5x OECD 307
2	1,4-Dichlorobenzene	106-46-7	82.9	1982	30 67	O_2 consumption parent analysis	28 28	40 2	301C 301D	flooded soil and sediment
3	AHTN, Tonalide	1506-02-1	1.25	1994	21	O_2 consumption	21	27	CBT	CAS-Test, water, river water
4	Ibuprofen	15687-27-1	11.4	1992	31	O_2 consumption	28	2	301D	W/S
5	Triclosan	3380-34-5	6.5	1989	18 37	O_2 consumption O_2 consumption	28 28	20 10	301B 301B	OECD 308, OECD 307
6	Piperonyl Butoxide	51-03-6	29	2002	24-48	CO_2 evolution	28	15	301B	Soil (US EPA)
7	DCBS, N,N-Dicyclohexylbenzothiazol-2-sulfenamid	4979-32-2	0.002	1989 2013 2013	2 55 29	O_2 consumption O_2 consumption O_2 consumption	28 60 28	100 10 10	301C 301F 301F	OECD 307 (vP); OECD 308 waived
8	N,N'-Dithiodi-o-phenylendibenzamid	135-57-9	0.048	2014 2014	27 10	O_2 consumption CO_2 evolution	42 28	0.5 20 mg TOC/L	301D 301B	OECD 309; soil only QSAR
9	Thiram	137-26-8	17.1	1985 1985	20 40	O_2 consumption O_2 consumption	28 28	2 10	301D 301D	2x W/S (BBA-Guideline) 2x soil (EU C.23)

No	Substance name	CAS No.	Water solubility [mg/L]	Year	Result [%]	Parameter	Test duration [d]	Concen-tration [mg/L]	Guide-line	Available simulation test data
10	Hexabromcyclododecan	25637-99-4	0.066	2006 2006	45 21	parent analysis parent analysis	28 28	1 10	301F 301F	4x OECD 307, 4x OECD 308
11	Pyrithione zinc	13463-41-7	6.3	2002	18-39	CO_2 evolution	28	13.2	301B	several (e.g. 303A, soil)
12	Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate	2082-79-3	0.003 (20°C, ECHA)	1997 1984	31 (21-39) 34 (32-35)	O_2 consumption inorg. C analysis	28 28	100 10-20	301C 301B	1x Soil
13	3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctan-1-ol	647-42-7	18.8	2000 2010	0-21 0	CO_2 evolution O_2 consumption	28 28	40 100	301B 301D	OECD 307
14	ethyl 2-({[(4-chloro-6-methoxypyrimidin-2-yl)carbamoyl]amino}sulfonyl)benzoate	90982-32-4	8.55 (HPLC water); 381 (well water)	1992 1992	34.8 17.5	CO_2 evolution CO_2 evolution	28 28	10 20	301B EU C.5	several soil studies (e.g. OECD 307)
15	methyl 2-[[4-ethoxy-6-(methylamino)-1,3,5-triazin-2-yl]carbamoylsulfamoyl]benzoate	97780-06-8	0.56 (pH 7, 20°C)	2009	31	CO_2 evolution	28	33.33	301B	several soil studies (e.g. OECD 307)
16	1,3-dihydro-4(or 5)-methyl-2H-benzimidazole-2-thione, zinc salt	61617-00-3	32 (pH 5.9-7; 20°C)	2003 1992	27 0	CO_2 evolution O_2 consumption	28 28	10.3 100	301B 301F	OECD 309
17	3a,4,5,6,7,7a-hexahydro-4,7-methano-1H-indenyl propionate	68912-13-0	57 (23°C)	1996 2010	15 22	O_2 consumption O_2 consumption	28 28	100 100	301F 301F	OECD 309
18	(2R,3S)-3-amino-S-(4-aminophenyl)-N-tert-butyl-2-hydroxy-4-phenylbutane-1-sulfonamido	169280-56-2	46.1	2002	30	CO_2 evolution	28	16.3	301B	n.a.
19	Copolymer of hexahydro-2H-azepin-2-one and 1,6-diisocyanatehexane	26776-30-7	0.37	2002 2007	0 22.3	O_2 consumption O_2 consumption	28 28	70 100	301F 301F	n.a.

No	Substance name	CAS No.	Water solubility [mg/L]	Year	Result [%]	Parameter	Test duration [d]	Concen-tration [mg/L]	Guide-line	Available simulation test data
20	Propylidynetrimethyl trimethacrylate	3290-92-4	20.1	1992 1992	29 53	CO_2 evolution CO_2 evolution	28 28	20 10	301B 301B	n.a.
21	Estrone	53-16-7	0.76	1997 1997	37 64	CO_2 evolution CO_2 evolution	28 44[8]	10 mg DOC/L 10 mg DOC/L	301B 301B	n.a.
22	1,2,3-Propanetriol, oligomers, docosanoate	64366-79-6	3-4	2000 2001 2001	30-40 20-30 30-40	O_2 consumption CO_2 evolution CO_2 evolution	28 28 49	100 27 27	301F ISO 14593 ISO 14593	n.a.
23	Diisobutyl hexahydrophthalate	70969-58-3	18.31	2007 2007 2007 2007 2007 2007 2007	99[9] 18 16 21 21 23 8	CO_2 evolution CO_2 evolution CO_2 evolution CO_2 evolution TOC removal TOC removal O_2 consumption	28 28 28 60 28 56 28	15 30 30 30 30 30 2	301B 301B 301B 301B ISO 14539 ISO 14539 301D	n.a.

W/S = water-sediment; CBT = 2-phase closed bottle test

[8] The experiment was extended to 44 days because the plateau was not reached by day 28

[9] using an adapted sewage sludge

B.2 Results of the search for potential test substances - OECD eChem Portal (compounds with water solubility > 100 mg/L)

Table 11: Results of the search for potential test substances from OECD eChem Portal (water solubility > 100 mg/L)

No	Substance name	CAS No.	Water solubility [mg/L]	Year	Result [%]	Parameter	Test duration [d]	Concen-tration [mg/L]	Guideline
24	4-({N'-[(2-hydroxyphenyl)methylidene]hydrazinecarbonyl}methyl)-4-methylmorpholin-4-ium chloride	1254469-57-2	181000	2011	20-30	CO_2 evolution	28	34	301B
25	Disodium 4,4'-bis[(4-anilino-6-morpholino-1,3,5-triazin-2-yl)amino]stilbene-2,2'-disulphonate	16090-02-1	1900	2015	10-20	DOC removal	28	956.9	301A
26	1-vinylhexahydro-2H-azepin-2-one	2235-00-9	49300	2011	30-40	DOC removal	28	30	301A
27	Acrylic acid, monoester with propane-1,2-diol (mixture of isomers)	25584-83-2	>1000000	1996 1992	34.9 90-100	O_2 consumption DOC removal	28 14	3 35	301D 301A
28	1,4-Benzenedisulfonic acid, 2,2'-[1,2-ethenediylbis[(3-sulfo-4,1-phenylene)imino[6-[bis(2-hydroxypropyl)amino]-1,3,5-triazine-4,2-diyl]imino]]bis-, hexasodium salt	371756-75-1	500000	2000 2005 1994	23 39 17	O_2 consumption parent analysis O_2 consumption	28 28 28	8 100 not reported	301D 301F 301D
29	N,N'-bis(2,2,6,6-tetramethylpiperidin-4-yl)hexane-1,6-diamine	61260-55-7	1760	1992 1993 1993	3 13 23	CO_2 evolution DOC removal CO_2 evolution	28 28 28	11.2+21.7 10.6 mg DOC/L 10.1	84/449/EEC C.5 (301B) 301A 301B
30	trisodium (2R)-2-[(1S)-1,2-dihydroxyethyl]-5-oxo-4-(phosphonooxy)-2,5-dihydrofuran-3-olate	66170-10-3	789000	1997	20-30	O_2 consumption	28	100	301F

31	Dimethyl sulfoxide (DMSO)	67-68-5	1000000	2009	31	O_2 consumption	28	2	301D[10]
32	Ethanol, 2,2'-oxybis-, reaction products with ammonia, morpholine derivs. residues	68909-77-3	100000	2010	21	CO_2 evolution	28	18.5	301B
				2010	25	DOC removal	28	18.5	301B
				2010	15	CO_2 evolution	28	18.5	301B[11]
				2010	18	CO_2 evolution	42	18.5	301B[7]

[10] OECD 303A available showing 90% O_2 consumption within 32 d (65 mg DMSO/L); Soil biodegradation study available showing >60% degradation (DMSO reduction to DMS)

[11] Enhanced test using longer test period (up to 60 d), larger test vessels (4 L instead of 3 L) and higher and increased biomass (60 mg SS/L)

B.3 Results of the search for potential test substances - EFSA DARs

Table 12: Results of the search for potential test substances from EFSA DARs

No.	Substance name	CAS No.	Water solubility [mg/L]	Year	Result [%]	Parameter	Test duration [d]	Concentration [mg/L]	Guideline	Reference
33	Clodinafop-propargyl	105512-06-9	4	1993	25	CO_2 evolution	28	22	301B	EFSA (2005a)
34	Fipronil	120068-37-3	1.9	1997	47	CO_2 evolution	28	Not available	301B	EFSA (2005b)
35	Carbosulfan	55285-14-8	insoluble	1989	28	O_2 consumption	28	2	301D	EFSA (2005c)
36	Acequinocyl	57960-19-7	0.007	2000	20-28	CO_2 evolution	28	20	301B	EFSA (2008)
37	Bifenox	42576-02-3	< 0.1	1989 1989	14[a] 12[a]	CO_2 evolution CO_2 evolution	28 28	10 20	301B 301B	EFSA (2013)
38	Thiobencarb	28249-77-6	16.7	2003	29[b]	O_2 consumption	28	3	301D	EFSA (2006)
39	Fluometuron	2164-17-2	111	2002	39[c]	O_2 consumption	18	4	301D	EFSA (2007)

[a] The test solution was acidified on day 28 and the released CO_2 was determined on day 29, resulting in 25.0% (10 mg/L) and 21.5% (20 mg/L) biodegradation; [b] A biodegradation plateau was not reached by the end of the test; [c] Plateau reached on day 18

B.4 Results of the search for potential test substances - NITE Chemical Risk Information Platform / J-CHECK Database

Table 13: Results of the search for potential test substances from NITE Chemical Risk Information Platform and J-CHECK Database

No.	Substance name	CAS No.	Water solubility [mg/L]	Year	Result [%]	Parameter	Test duration [d]	Concentration [mg/L]	Guide-line
40	n-(3,4-dichlorophenyl)propanamide / Propanil	709-98-8	152	1989	21	O_2 consumption	28	100	301C
41	malathion	121-75-5	143	2002	22	O_2 consumption	28	100	301C
42	hexachlorobutadiene	87-68-3	3.2	2002	24	O_2 consumption	28	100	301C
43	tripentyl phosphate	2528-38-3	0.332 (25°C, EPISuite); 2.865 (EPISuite);	1996	25	O_2 consumption	28	100	301C
44	1,2-benzisothiazole, 3-(2-propen-1-yloxy)-, 1,1-dioxide / Probenazole	27605-76-1	150	2002	26	O_2 consumption	28	100	301C
45	Phosphoric acid, [1,1'-biphenyl]-2-yl diphenyl ester	132-29-6	0.009 (25°C, EPISuite); 0.022 (EPISuite);	1993	27	O_2 consumption	28	100	301C
46	benzenesulfonic acid, 2-methyl-, sodium salt	15046-75-0	1,000,000 (25°C, EPISuite); 118.9 (EPISuite);	1998	27	O_2 consumption	28	100	301C
47	Propane, 1,3-dibromo-2,2-bis(bromomethyl)-	3229-00-3	1.6	1985	29	O_2 consumption	28	100	301C
48	Benzene, (1,1-dimethylpropyl)-	2049-95-8	4.68 (20°C, ECHA)	2003 2008	29 4	O_2 consumption O_2 consumption	28 28	100 8.5	301C 301D
49	Poly(oxy-1,2-ethanediyl), .alpha.-sulfo-.omega.-(nonylphenoxy)-, sodium salt (1:1)	9014-90-8	21.44 (25°C, EPISuite); 0.006 (EPISuite);	1989	31	O_2 consumption	28	100	301C

No.	Substance name	CAS No.	Water solubility [mg/L]	Year	Result [%]	Parameter	Test duration [d]	Concentration [mg/L]	Guide-line
			100,000 (EPISuite,EXP)						
50	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester	2082-79-3	see No. 12	see No. 12	see No. 12	see No. 12	see No. 12	see No. 12	see No. 12
51	Acetic acid, 2-mercapto-, 2-ethylhexyl ester	7659-86-1	4.73 (20°C, ECHA)	2001 2009	32 82	O_2 consumption O_2 consumption	28 28	100 102 mg ThOD/L	301C 301F
52	Dodecanoic acid, 2,3,4,5,6-pentachlorophenyl ester	3772-94-9	0 (EPISuite)	1983	35	O_2 consumption	28	100	301C
53	tributyl(dodecanoyloxy)stannane	3090-36-6	0 (25°C, EPISuite); 1.2 (EPISuite,EXP)	1985	35	O_2 consumption	28	100	301C
54	Pentane, 1-bromo-	110-53-2	127 (25°C)	2008 1995	36 1	O_2 consumption O_2 consumption	28 28	100 2/4	301C 301D
55	Tetracosane, 2,6,10,15,19,23-hexamethyl- / Squalane	111-01-3	0 (25°C, ECHA)	2008 2011 2012	36 65 77	O_2 consumption CO_2 evolution CO_2 evolution	28 28 28	100 10 mg C/L 28 mg C/L	301C 301B 301B
56	Phosphoric acid, tris(3-methylphenyl) ester / Tri-m-cresyl phosphate	563-04-2	0.1 (25°C)	1977	37	O_2 consumption	28	100	301C
57	2,5-pyridinedicarboxylic acid, dipropyl ester	136-45-8	20.8 – 75.0 (25°C, EPISuite)	1986	37	O_2 consumption	28	100	301C
58	Stannane, tributyl[[[(1R,4aR,4bR,10aR)-1,2,3,4,4a,4b,5,6,10,10a-decahydro-1,4a-dimethyl-7-(1-methylethyl)-1-	26239-64-5	0 (EPISuite)	1990	38	O_2 consumption	28	100	301C

No.	Substance name	CAS No.	Water solubility [mg/L]	Year	Result [%]	Parameter	Test duration [d]	Concentration [mg/L]	Guide-line
	phenanthrenyl]carbonyl]oxy]- / Tributyltin abietate								
59	18-Pentatriacontanone / Diheptadecyl ketone	504-53-0	0.02 (20°C, ECHA)	1997	39	O_2 consumption	28	100	301C
60	1-Naphthalenecarboxylic acid / 1-Naphthoic acid	86-55-5	86 (25°C)	1980	40	O_2 consumption	28	100	301C
61	Cis-13-Docosenamide (Erucamide)	112-84-5	0.000738 (20°C, ECHA)	2001 1991 1991 1993 1993 1985	64 28 15 15 43 88	CO_2 evolution CO_2 evolution CO_2 evolution O_2 consumption O_2 consumption O_2 consumption	28 28 28 28 140 21	10 mg DOC/L 10 mg DOC/L 10 mg DOC/L 2 mg/L 2 mg/L 100 mg/L	301B 301B 301B 301D 301D 301C

B.5 Results of the search for potential test substances – Scientific Reports and Literature

Table 14: Results of the search for potential test substances from scientific reports and literature

No.	Substance name	CAS No.	Water solubility [mg/L]	Year	Result [%]	Parameter	Test duration [d]	Concentration	Guideline	Reference
62	Isodecyl neopentanoate	60209-82-7	0.05 mg/L	2016 1998	36[b] 34	CO_2 evolution CO_2 evolution	28 28	20 mg C/L 20 mg TOC/L	301B 301B	Sweetlove et al. (2016) ECHA (2019)
63	Isooctyl-3-mercaptopropionate	30374-01-7	7.7 mg/L	2018 2018 2008	32 0 55	O_2 consumption O_2 consumption CO_2 evolution	28 28 28	5 mg ThOD/L 30 mg ThOD/L 10 mg C/L	301D 301F 301B	Rücker et al. (2018) Rücker et al. (2018) ECHA (2019)

No.	Substance name	CAS No.	Water solubility [mg/L]	Year	Result [%]	Parameter	Test duration [d]	Concentration	Guideline	Reference
64	Dibutyl Methylenebis(thioglycolate)	14338-82-0	14 mg/L	2018	59	O_2 consumption	28	30 mg ThOD/L	301F	Rücker et al. (2018)
				2018	49	O_2 consumption	28	5 mg ThOD/L	301D	Rücker et al. (2018)
				2000	25[a]	O_2 consumption	28	3.4 mg/L	301D	ECHA (2019)

[a] The test substance was found to be inherently biodegradable (78% biodegradation based on O_2 consumption after 28 days; OECD 302C); [b] higher biodegradation was observed, when using bioavailability improvement methods.

B.6 Results of the search for potential test substances – Scientific Reports and Literature (supplement)

Table 15: Results of the search for potential test substances from scientific reports and literature (supplement)

No.	Substance name	CAS No.	Water solubility [mg/L]	Year	Result [%]	Parameter	Test duration [d]	Concentration [mg/L]	Guideline	Reference
65	Phenanthrene[12]	85-01-8	1 mg/L	1978 2016	54 67	O_2 consumption O_2 consumption	28 28	100 mg/L 100 mg/L	301 C 301 C	NITE (2019) Junker et al. (2016)

B.7 Results of the search for potential test substances – Further physico-chemical properties

Table 16: Further physico-chemical properties of potential test substances

No.	Substance name	CAS No.	Sum formula	Log K_{ow}	Henry's Law Constant [Pa·m³/mol]	Microbial Toxicity	Hydrolysis half-life	Photolysis half-life in water
1	Cloquintocet-mexyl	99607-70-2	$C_{18}H_{22}ClNO_3$	5.2 (25°C)	0.003 (25°C)	NOEC: 1000 mg/L (OECD 209)	134 d	0.62 d
2	1,4-Dichlorobenzene	106-46-7	$C_6H_4Cl_2$	3.37 (20°C)	262.4 (20°C)	$IC_{50\ (12h)}$: 330 mg/L (ISO 8192)	stable	33 d
3	AHTN, Tonalide	1506-02-1	$C_{18}H_{26}O$	5.4 (25°C)	37.1 (25°C)	NOEC: > 30 mg/L	1 year (25°C)	4 h
4	Ibuprofen	15687-27-1	$C_{13}H_{18}O_2$	3.87 (25°C)	0.015 (25°C)	IC_{50} (15 min): 800 mg/L	not expected	not expected

[12] Simulation studies according to OECD 307, 308 and 314B available

No.	Substance name	CAS No.	Sum formula	Log K_{ow}	Henry's Law Constant [Pa·m³/mol]	Microbial Toxicity	Hydrolysis half-life	Photolysis half-life in water
5	Triclosan	3380-34-5	$C_{12}H_7Cl_3O_2$	4.9 (20°C)	0.000505 (25°C)	EC_{50} (3h): 11 mg/L (OECD 209)	> 1 year (25°C; pH 4, 7, 9)	41 min
6	Piperonyl Butoxide	51-03-6	$C_{19}H_{30}O_5$	5.0 (25°C)	0.889 (25°C)	EC_{10}: 2 mg/L (OECD 301D)	stable (25°C; pH 5, 7, 9)	n.a.
7	DCBS, N,N-Dicyclohexylbenzothiazol-2-sulfenamid	4979-32-2	$C_{19}H_{26}N_2S_2$	5.5 (20°C)	0.069 (ambient Temp.)	EC_{50}: 10000 mg/L (ISO 8192)	57, 53, 48 h (pH 4, 7, 8) (OECD 111)	n.a.
8	N,N'-Dithiodi-o-phenylendibenzamid	135-57-9	$C_{26}H_{20}N_2O_2S_2$	4.0 (20°C)	0 (25°C)	EC_{50}: > 1000 mg/L (OECD 209)	1 d (12°C)	n.a.
9	Thiram	137-26-8	$C_6H_{12}N_2S_4$	1.8-2.1	0.033 (25°C)	EC_{50} (3h): 3.11 mg/L (OECD 209)	68.5 d, 3.5 d, 6.9 h (pH 5, 7, 8)	4.1 h (25°C)
10	Hexabromcyclododecan	25637-99-4	$C_{12}H_{18}Br_6$	5.625 (25°C)	0.174 (25°C)	EC_{50}: 15 mg/L (OECD 209)	not expected	-
11	Pyrithione zinc	13463-41-7	$C_{10}H_8N_2O_2S_2Zn$	0.9 (25°C)	$< 5 * 10^{-5}$	EC_{50} (3h): 2.4 mg/L (OECD 209)	108 d (25°C)	< 30 min
12	Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate	2082-79-3	$C_{35}H_{62}O_3$	13.5 (25°C)	0.163 (25°C)	EC_{50} (3h): >100 mg/L (OECD 209)	> 7 years (pH 7) 264 d (pH 8; calculated)	n.a.
13	3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctan-1-ol	647-42-7	$C_8H_5F_{13}O$	4.54	n.a.	n.a.	n.a.	n.a.

No.	Substance name	CAS No.	Sum formula	Log K_{ow}	Henry's Law Constant [Pa·m³/mol]	Microbial Toxicity	Hydrolysis half-life	Photolysis half-life in water
14	ethyl 2-({[(4-chloro-6-methoxypyrimidin-2-yl)carbamoyl]amino}sulfonyl)benzoate	90982-32-4	$C_{15}H_{15}ClN_4O_6S$	1.3 (25°C)	n.a.	MEC-90: 10000 mg/L (*Spirillum volutans*)	stable for 30 d (pH 7, 9; 25°C)	n.a.
15	methyl 2-[[4-ethoxy-6-(methylamino)-1,3,5-triazin-2-yl]carbamoylsulfamoyl]benzoate	97780-06-8	$C_{15}H_{18}N_6O_6S$	-0.28 (20°C)	0	EC_{50} (3h): >100 mg/L (OECD 209)	stable (pH 7)	stable (pH 7, sterile)
16	1,3-dihydro-4(or 5)-methyl-2H-benzimidazole-2-thione, zinc salt	61617-00-3	$C_{16}H_{14}N_4S_2Zn$	3.07 (20°C)	$8.8*10^{-10}$ (25°C)	EC_{50} (3h): 6620 mg/L (ISO 8192)[13];	> 1 year (25°C, pH 7)	n.a.
17	3a,4,5,6,7,7a-hexahydro-4,7-methano-1H-indenyl propionate	68912-13-0	$C_{13}H_{18}O_2$	4.4 (30°C)	2.4 (25°C)	EC_{50}: 245 mg/L (ISO 8192)	> 1 year (25°C, pH 4, 7); 13 d (pH 9)	n.a.
18	(2R,3S)-3-amino-S-(4-aminophenyl)-N-tert-butyl-2-hydroxy-4-phenylbutane-1-sulfonamido	169280-56-2	$C_{20}H_{29}N_3O_3S$	2.3 (pH 10.5, 40°C)	0.001 (25°C)	EC_{50} (3 h): 96-410 mg/L (OECD 209)	> 1 year (pH 4, 7, 9, 25°C)	n.a.
19	Copolymer of hexahydro-2H-azepin-2-one and 1,6- diisocyanatehexane	26776-30-7	Exact identification is not feasible	6.5 (23.3°C)	0.001 (20°C)	EC_{50} (3 h): >1000 mg/L (OECD 209)	> 1 year (pH 4, 7, 9, 25°C)	n.a.
20	Propylidynetrimethyl trimethacrylate	3290-92-4	$C_{18}H_{26}O_6$	4.19 (25°C)	0 (25°C)	EC_{50} (3 h): >1000 mg/L (OECD 209)	481 d (pH 7, 25°C)	n.a.
21	Estrone	53-16-7	$C_{18}H_{22}O_2$	2.6 (25°C, pH 7)	0 (25°C)	EC_{50} (44 d): >12.5 mg/L (OECD 301B)	> 1 year (pH 5, 7, 9, 25°C)	n.a.

[13] 46% respiration inhibition observed within 3h

No.	Substance name	CAS No.	Sum formula	Log K_{ow}	Henry's Law Constant [Pa·m³/mol]	Microbial Toxicity	Hydrolysis half-life	Photolysis half-life in water
22	1,2,3-Propanetriol, oligomers, docosanoate	64366-79-6	$C_{22}H_{44}O_2$ *x $(C_3H_8O_3)x$	6.5 (23°C, pH 6.2)	0 (25°C)	EC_{50} (3 h): >1000 mg/L[14] (OECD 209)	Not expected	n.a.
23	Diisobutyl hexahydrophthalate	70969-58-3	$C_{16}H_{28}O_4$	4.83 (pH7.1, 40°C)	0.248 (25°C)	EC_{50} (3 h): >1000 mg/L (OECD 209)	4 years (pH 7, 25°C) 148 d (pH 8, 25°C)	n.a.
24	4-({N'-[(2-hydroxyphenyl)methylidene]hydrazinecarbonyl}methyl)-4-methylmorpholin-4-ium chloride	1254469-57-2	$C_{14}H_{20}ClN_3O_3$	-2.4 (23°C)	0 (25°C)	EC_{50} (3 h): >520 mg/L (OECD 209)	28 d (pH 7, 25°C) 50 d (pH 7, 20°C)	n.a.
25	Disodium 4,4'-bis[(4-anilino-6-morpholino-1,3,5-triazin-2-yl)amino]stilbene-2,2'-disulphonate	16090-02-1	$C_{40}H_{40}N_{12}O_8S_2$ 2Na	-1.22 (25°C)	0 (25°C)	EC_{50} (3 h): >100 mg/L (OECD 209)	> 1 year (pH 4, 7, 9, 25°C)	4.1 h (pH 8.3, 25°C)
26	1-vinylhexahydro-2H-azepin-2-one	2235-00-9	$C_8H_{13}NO$	1.2 (25°C, pH 7.2)	0.009 (20°C)	EC_{50} (17 h): 622 mg/L (DIN 38412, Part 8)	> 1 year (pH 7, 9, 25°C) 6.5 h (pH 4, 25°C)	n.a.
27	Acrylic acid, monoester with propane-1,2-diol (mixture of isomers)	25584-83-2	$C_6H_{10}O_3$	0.2 (25°C)	0.001 (25°C)	EC_{50} (0.5 h): >1000 mg/L (OECD 209)	>230 d (pH 7, 25°C)	n.a.
28	1,4-Benzenedisulfonic acid, 2,2'-[1,2-ethenediylbis[(3-sulfo-4,1-phenylene)imino[6-[bis(2-hydroxypropyl)amino]-1,3,5-triazine-4,2-diyl]imino]]bis-, hexasodium salt	371756-75-1	$C_{44}H_{46}N_{12}Na_6O_{22}S_6$	-18 (20°C)	0 (25°C)	EC_{50} (3 h): >100->1000 mg/L[15] (OECD 209)	> 1 year (pH 4, 7, 9, 25°C)	5.5 h[6]

[14] read-across from supporting substance (structural analogue or surrogate)

[15] read-across from supporting substance (structural analogue or surrogate)

No.	Substance name	CAS No.	Sum formula	Log K_{ow}	Henry's Law Constant [Pa·m³/mol]	Microbial Toxicity	Hydrolysis half-life	Photolysis half-life in water
29	N,N'-bis(2,2,6,6-tetramethylpiperidin-4-yl)hexane-1,6-diamine	61260-55-7	$C_{24}H_{50}N_4$	-1.3 (21°C)	0 (25°C)	EC_{50} (16.7 h): 39 mg/L (DIN 38412-8)	> 1 year (pH 4, 7, 9, 25°C)	n.a.
30	trisodium (2R)-2-[(1S)-1,2-dihydroxyethyl]-5-oxo-4-(phosphonooxy)-2,5-dihydrofuran-3-olate	66170-10-3	$C_6H_9O_9P$ 3Na	<-4 (25°C)	0 (25°C)	EC_{50} (16 h): 7700 mg/L (ISO 10712)	> 42 d (pH 4, 7, 9, 50°C)	n.a.
31	Dimethyl sulfoxide (DMSO)	67-68-5	C_2H_6OS	-1.35 (20°C)	0.001 (21°C)	EC_{50} (0.5 h): 10-100 mg/L (ISO 8192)	variable[16]	n.a.
32	Ethanol, 2,2'-oxybis-, reaction products with ammonia, morpholine derivs. residues	68909-77-3	$C_4H_{10}O_3.C_4H_9NO.NH_3$	0.565 (20°C)	0 (25°C)[17]	EC_{50} (3 h): >1000 mg/L (OECD 209)	1 year (pH 4, 7, 9, 25°C)	n.a.
33	Clodinafop-propargyl	105512-06-9	$C_{17}H_{13}ClFNO_4$	3.9 (25°C)	$2.8*10^{-4}$ (25°C)	EC_{50}: > 94 mg/L (OECD 209)	4.8 d (25°C)	24 d (pH 5, 25°C)
34	Fipronil	120068-37-3	$C_{12}H_4Cl_2F_6N_4OS$	3.5-4.0 (20°C)	$2.3*10^{-4}$ (25°C)	EC_{50} (3 h): >1000 mg/L (OECD 209)	stable (pH 7)	8 h (pH 5, 25°C)
35	Carbosulfan	55285-14-8	$C_{20}H_{32}N_2O_3S$	5.6 (EST)	$5.1*10^{-2}$ (25°C)	EC_{50} (3 h): >1015 mg/L (OECD 209)	11.4 h (pH 7, 25°C)	n.a.

[16] DMSO hydrolytic stability may vary depending on aqueous phase composition. The more OH radicals occur, the less DMSO is stable.

[17] read-across from supporting substance (structural analogue or surrogate)

No.	Substance name	CAS No.	Sum formula	Log K_{ow}	Henry's Law Constant [Pa·m³/mol]	Microbial Toxicity	Hydrolysis half-life	Photolysis half-life in water
36	Acequinocyl	57960-19-7	$C_{24}H_{32}O_4$	> 6.2 (25°C)	0.097	EC_{50} (3 h): >1000 mg/L (OECD 209)	53 h (pH 7)	14 min (pH 5)
37	Bifenox	42576-02-3	$C_{14}H_9Cl_2NO_5$	3.64 (20-25°C)	$>1.62*10^{-4}$ (20°C)	EC_{50} (3 h): >1000 mg/L (OECD 209)	265 d (pH 7, 25°C)	24 h (pH 5, 20°C)
38	Thiobencarb	28249-77-6	$C_{12}H_{16}ClNOS$	4.23 (20°C)	0.0368 (25°C)	EC_{50} (3 h): >500 mg/L (OECD 209)	7900 d (pH 7, 25°C)	190 d (pH 7, 25°C)
39	Fluometuron	2164-17-2	$C_{10}H_{11}F_3N_2O$	2.38 (25°C)	$2.6*^{10-4}$ (22-25°C);	EC_{50} (3 h): >100 mg/L (OECD 209)	stable	stable
40	n-(3,4-dichlorophenyl)propanamide / Propanil	709-98-8	$C_9H_9Cl_2NO$	3.07	$1.73*10^{-4}$ (25°C)	n.a.	n.a.	n.a.
41	malathion	121-75-5	$C_{10}H_{19}O_6PS_2$	2.36	$4.95*10^{-4}$ (25°C)	n.a.	n.a.	n.a.
42	hexachlorobutadiene	87-68-3	C_4Cl_6	4.78	1040 (25°C)	n.a.	n.a.	n.a.
43	tripentyl phosphate	2528-38-3	$C_{15}H_{33}O_4P$	5.29	0.757 (25°C)	n.a.	n.a.	n.a.
44	1,2-benzisothiazole, 3-(2-propen-1-yloxy)-, 1,1-dioxide / Probenazole	27605-76-1	$C_{10}H_9NO_3S$	1.4	0.154 (25°C)	n.a.	n.a.	n.a.
45	Phosphoric acid, [1,1'-biphenyl]-2-yl diphenyl ester	132-29-6	$C_{24}H_{19}O_4P$	6.46	$1.73*10^{-4}$ (25°)C	n.a.	n.a.	n.a.

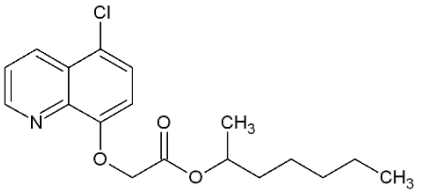
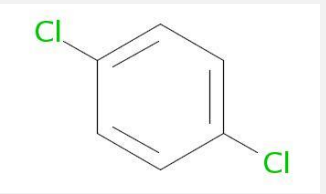
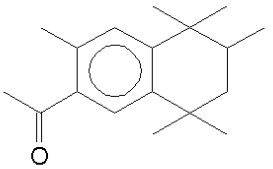
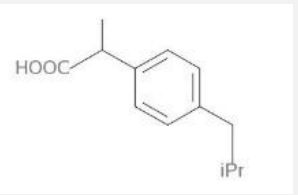
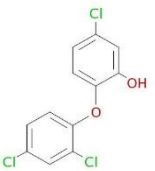
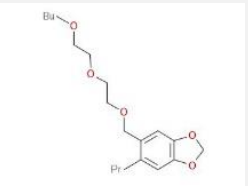
No.	Substance name	CAS No.	Sum formula	Log K_{ow}	Henry's Law Constant [Pa·m³/mol]	Microbial Toxicity	Hydrolysis half-life	Photolysis half-life in water
46	benzenesulfonic acid, 2-methyl-, sodium salt	15046-75-0	$C_7H_8O_3S.Na$	-2.4	$2.83*10^{-4}$ (25°C)	n.a.	n.a.	n.a.
47	Propane, 1,3-dibromo-2,2-bis(bromomethyl)-	3229-00-3	$C_5H_8Br_4$	4	4.09 (25°C)	n.a.	n.a.	n.a.
48	Benzene, (1,1-dimethylpropyl)-	2049-95-8	C11H16	4.95 (20°C)	1870 (25°C)	n.a.	n.a.	n.a.
49	Poly(oxy-1,2-ethanediyl), .alpha.-sulfo-.omega.-(nonylphenoxy)-, sodium salt (1:1)	9014-90-8	$(C_2H_4O)_nC_{15}H_{24}O_4S.Na$	1.13	$4.59*10^{-14}$ (25°C)	n.a.	n.a.	n.a.
50	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester	2082-79-3	$C_{35}H_{62}O_3$	13.4 (25°C)	0.163 (25°C)	EC_{50} (3 h): >100 mg/L (OECD 209)	>7 years (pH 7) 264 d (pH 8	n.a.
51	Acetic acid, 2-mercapto-, 2-ethylhexyl ester	7659-86-1	$C_{10}H_{20}O_2S$	4.7 (20°C)	4.58 (25°C)	EC_{50} (3 h): >100 mg/L (OECD 209)	12-16 h (25°C, pH 4-9)	n.a.
52	Dodecanoic acid, 2,3,4,5,6-pentachlorophenyl ester	3772-94-9	$C_{18}H_{23}Cl_5O_2$	9.72	24.9 (25°C)	n.a.	n.a.	n.a.
53	tributyl(dodecanoyloxy)stannane	3090-36-6	$C_{24}H_{50}O_2Sn$	8.15	$1*10^5$ (25°C)	n.a.	n.a.	n.a.
54	Pentane, 1-bromo-	110-53-2	$C_5H_{11}Br$	3.37	2000 (25°C)	n.a.	n.a.	n.a.
55	Tetracosane, 2,6,10,15,19,23-hexamethyl- / Squalane	111-01-3	$C_{30}H_{62}$	17 (25°C)	$1.55*10^8$ (25°C)	EC_{50} (3 h): > solubility (OECD 209)	not expected	n.a.

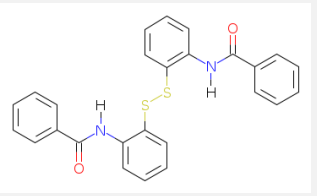
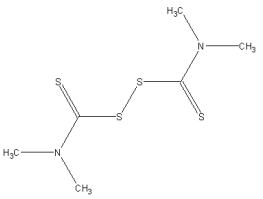
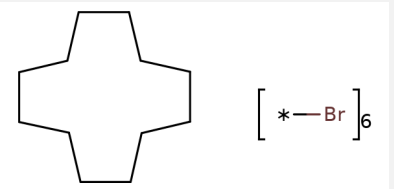
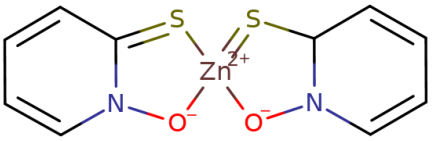
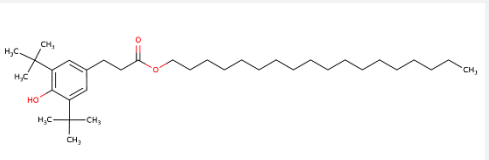
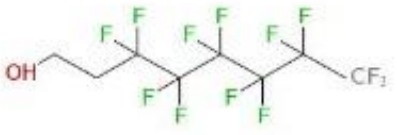
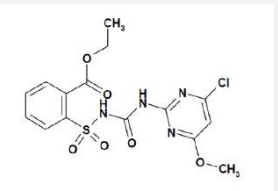
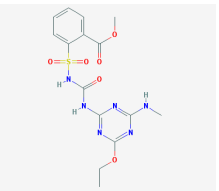
No.	Substance name	CAS No.	Sum formula	Log K_{ow}	Henry's Law Constant [Pa·m³/mol]	Microbial Toxicity	Hydrolysis half-life	Photolysis half-life in water
56	Phosphoric acid, tris(3-methylphenyl) ester / Tri-m-cresyl phosphate	563-04-2	$C_{21}H_{21}O_4P$	6.34	8.41 (25°C)	n.a.	n.a.	n.a.
57	2,5-pyridinedicarboxylic acid, dipropyl ester	136-45-8	$C_{13}H_{17}NO_4$	3.57	$9.21*10^{-5}$ (25°C)	n.a.	n.a.	n.a.
58	Stannane, tributyl[[[(1R,4aR,4bR,10aR)-1,2,3,4,4a,4b,5,6,10,10a-decahydro-1,4a-dimethyl-7-(1-methylethyl)-1-phenanthrenyl]carbonyl]oxy]- / Tributyltin abietate	26239-64-5	$C_{32}H_{56}O_2Sn$	9.61	$7.26*10^{4}$ (25°C)	n.a.	n.a.	n.a.
59	18-Pentatriacontanone / Diheptadecyl ketone	504-53-0	$C_{35}H_{70}O$	15.48	$4.35*10^{4}$ (25°C)	n.a.	n.a.	n.a.
60	1-Naphthalenecarboxylic acid / 1-Naphthoic acid	86-55-5	$C_{11}H_8O_2$	3.1	$1.07*10^{-3}$ (25°C)	n.a.	n.a.	n.a.
61	Cis-13-Docosenamide (Erucamide)	112-84-5	$C_{22}H_{43}NO$	8	0	not expected based on biodeg. studies	not expected	n.a.
62	Isodecyl neopentanoate	60209-82-7	$C_{15}H_{30}O_2$	3.54	532 (25°C)	EC_{50} (3 h): >1000 mg/L (OECD 209)	> 1 year (pH 7)	n.a.
63	Isooctyl-3-mercaptopropionate	30374-01-7	$C_{11}H_{22}O_2S$	4.6-5.3 (30°C)	6.08 (25°C)	n.a.	8.6 d (pH 7, 25°C)	n.a.
64	Dibutyl Methylenebis(thioglycolate)	14338-82-0	$C_{13}H_{24}O_4S_2$	4.27 (30°C)	$2.64*10^{-5}$ (25°C)	EC_{50} (3 h): 9690 mg/L (OECD 209)	15.3 d (25°C)	n.a.

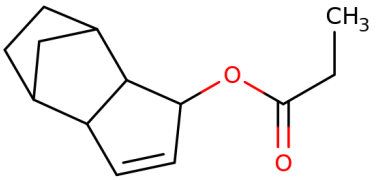
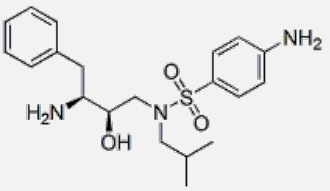
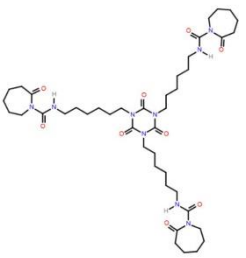
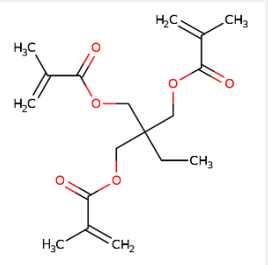
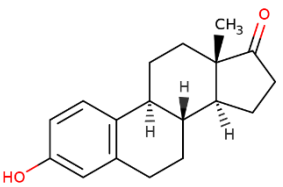
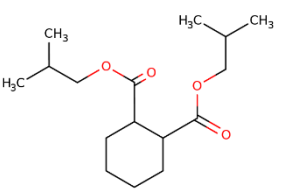
No.	Substance name	CAS No.	Sum formula	Log K_{ow}	Henry's Law Constant [Pa·m³/mol]	Microbial Toxicity	Hydrolysis half-life	Photolysis half-life in water
65	Phenanthrene	85-01-8	$C_{14}H_{10}$	4.57 (25°C)	3.7 (25°C)	n.a.	not expected	half-life = 6.3 h
66	4-chloroaniline	106-47-8	C_6H_6ClN	1.83	$1.18*10^{-1}$ (25°C)	n.a.	n.a.	n.a.
67	4-fluorophenol	371-41-5	C_6H_5FO	3.6 (27°C)	0.066 (25°C)	n.a.	11.3 h (calculated)	n.a.
68	Diethylene glycol	111-46-6	$C_4H_{10}O_3$	-1.98 (20°C)	0.0002 (25°C)	EC_{50} (0.5 h): >1995 mg/L (ISO 8192)	not expected	n.a.
69	1,3,5-trimethylbenzene	108-67-8	C_9H_{12}	3.42 (20°C)	781	No toxicity in tox-control (OECD 301)	n.a.	n.a.
70	Di-isooctyl phthalate	27554-26-3	$C_{24}H_{38}O_4$	8.4	3.18 (25°C)	n.a.	n.a.	n.a.

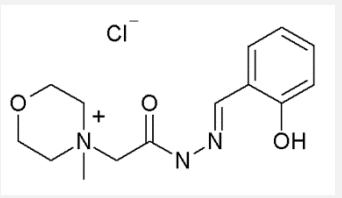
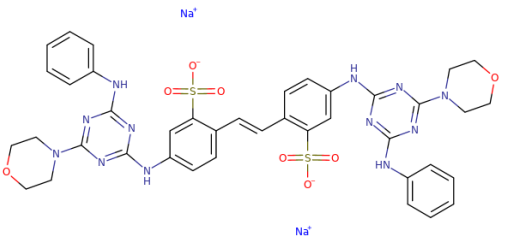
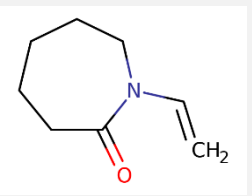
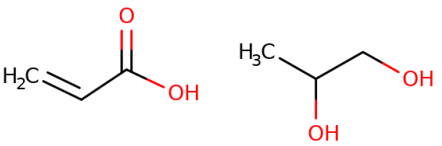
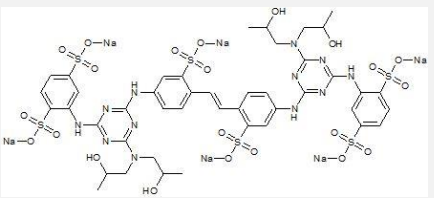
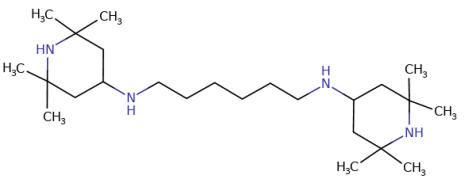
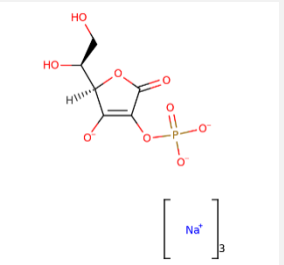
B.8 Results of the search for potential test substances – Structural formulas

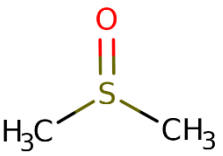
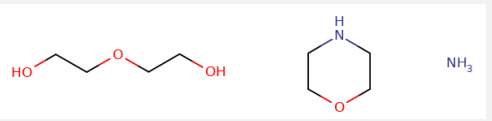
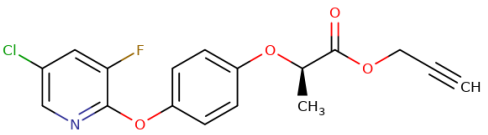
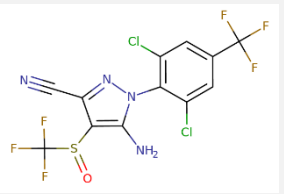
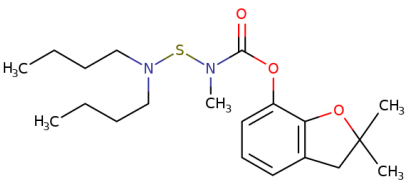
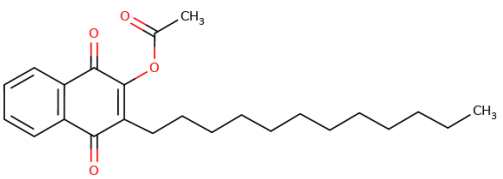
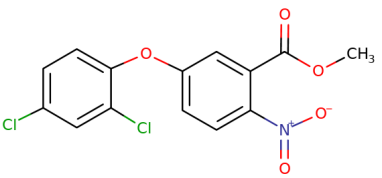
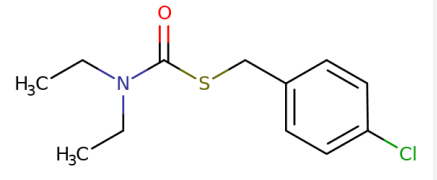
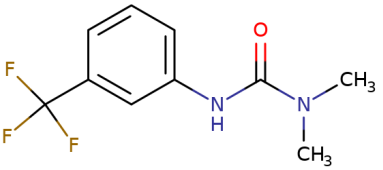
Table 17: Structural formulas of potential test substances

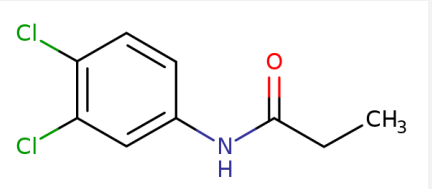
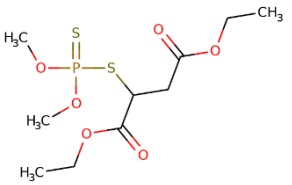
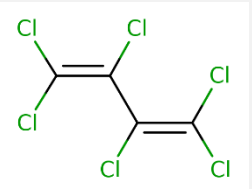
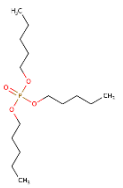
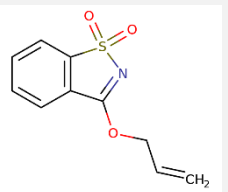
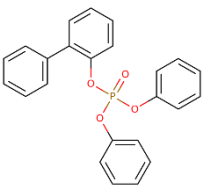
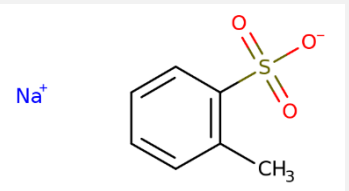
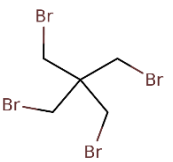
No.	Substance name	CAS No.	Structural formula
1	Cloquintocet-mexyl	99607-70-2	
2	1,4-Dichlorobenzene	106-46-7	
3	AHTN, Tonalide	1506-02-1	
4	Ibuprofen	15687-27-1	
5	Triclosan	3380-34-5	
6	Piperonyl Butoxide	51-03-6	
7	DCBS, N,N-Dicyclohexylbenzothiazol-2-sulfenamid	4979-32-2	

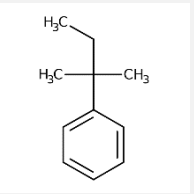
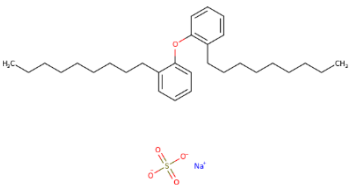
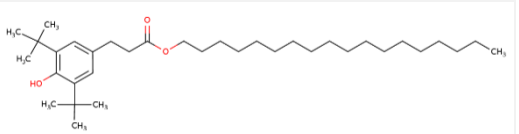
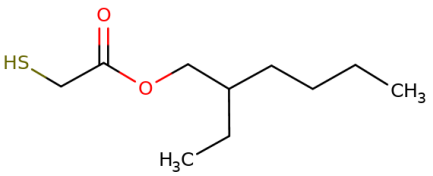
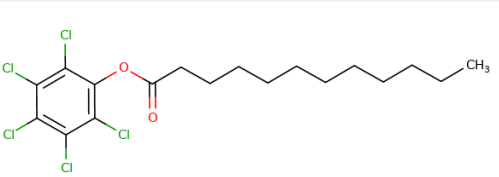
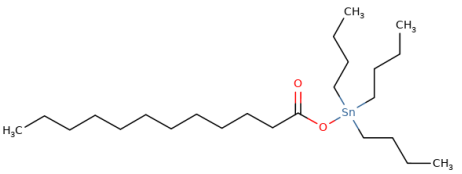
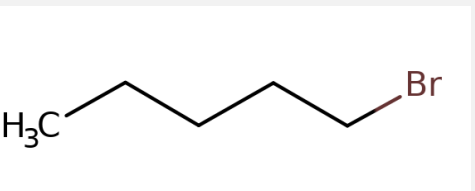
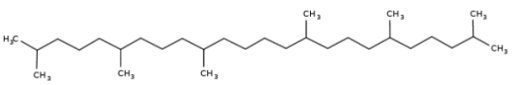
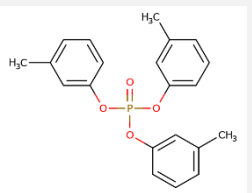
No.	Substance name	CAS No.	Structural formula
8	N,N'-Dithiodi-o-phenylendibenzamid	135-57-9	
9	Thiram	137-26-8	
10	Hexabromcyclododecan	25637-99-4	
11	Pyrithione zinc	13463-41-7	
12	Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate	2082-79-3	
13	3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctan-1-ol	647-42-7	
14	ethyl 2-({[(4-chloro-6-methoxypyrimidin-2-yl)carbamoyl]amino}sulfonyl)benzoate	90982-32-4	
15	methyl 2-[[4-ethoxy-6-(methylamino)-1,3,5-triazin-2-yl]carbamoylsulfamoyl]benzoate	97780-06-8	

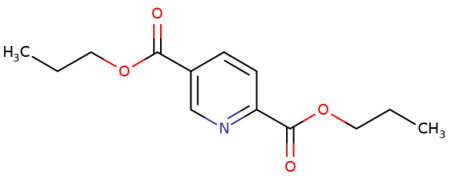
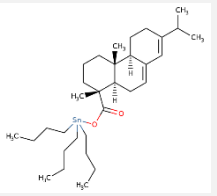
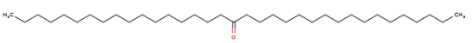
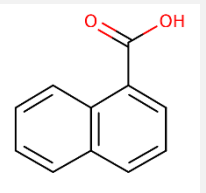
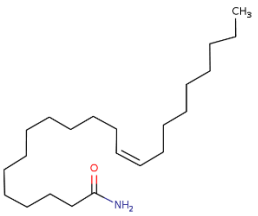
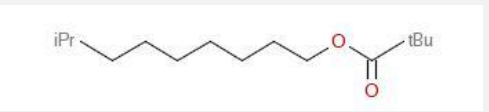
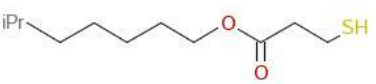
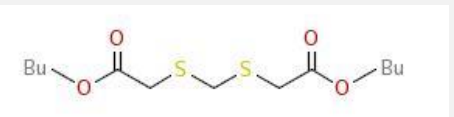
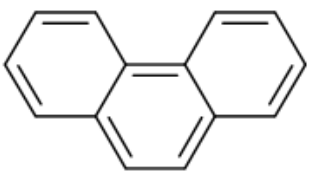
No.	Substance name	CAS No.	Structural formula
16	1,3-dihydro-4(or 5)-methyl-2H-benzimidazole-2-thione, zinc salt	61617-00-3	
17	3a,4,5,6,7,7a-hexahydro-4,7-methano-1H-indenyl propionate	68912-13-0	
18	(2R,3S)-3-amino-S-(4-aminophenyl)-N-tert-butyl-2-hydroxy-4-phenylbutane-1-sulfonamido	169280-56-2	
19	Copolymer of hexahydro-2H-azepin-2-one and 1,6- diisocyanatehexane	26776-30-7	
20	Propylidynetrimethyl trimethacrylate	3290-92-4	
21	Estrone	53-16-7	
22	1,2,3-Propanetriol, oligomers, docosanoate	64366-79-6	n.a.
23	Diisobutyl hexahydrophthalate	70969-58-3	

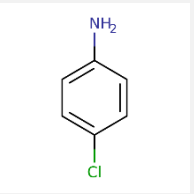
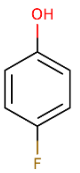
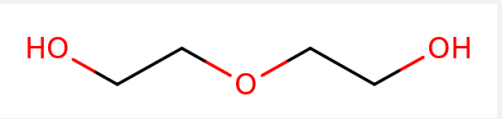
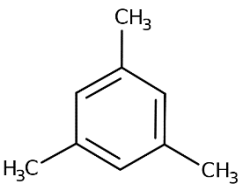
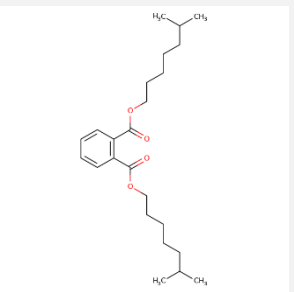
No.	Substance name	CAS No.	Structural formula
24	4-({N'-[(2-hydroxyphenyl)methylidene]hydrazinecarbonyl}methyl)-4-methylmorpholin-4-ium chloride	1254469-57-2	
25	Disodium 4,4'-bis[(4-anilino-6-morpholino-1,3,5-triazin-2-yl)amino]stilbene-2,2'-disulphonate	16090-02-1	
26	1-vinylhexahydro-2H-azepin-2-one	2235-00-9	
27	Acrylic acid, monoester with propane-1,2-diol (mixture of isomers)	25584-83-2	
28	1,4-Benzenedisulfonic acid, 2,2'-[1,2-ethenediylbis[(3-sulfo-4,1-phenylene)imino[6-[bis(2-hydroxypropyl)amino]-1,3,5-triazine-4,2-diyl]imino]]bis-, hexasodium salt	371756-75-1	
29	N,N'-bis(2,2,6,6-tetramethylpiperidin-4-yl)hexane-1,6-diamine	61260-55-7	
30	trisodium (2R)-2-[(1S)-1,2-dihydroxyethyl]-5-oxo-4-(phosphonooxy)-2,5-dihydrofuran-3-olate	66170-10-3	

No.	Substance name	CAS No.	Structural formula
31	Dimethyl sulfoxide (DMSO)	67-68-5	
32	Ethanol, 2,2'-oxybis-, reaction products with ammonia, morpholine derivs. residues	68909-77-3	
33	Clodinafop-propargyl	105512-06-9	
34	Fipronil	120068-37-3	
35	Carbosulfan	55285-14-8	
36	Acequinocyl	57960-19-7	
37	Bifenox	42576-02-3	
38	Thiobencarb	28249-77-6	
39	Fluometuron	2164-17-2	

No.	Substance name	CAS No.	Structural formula
40	n-(3,4-dichlorophenyl)propanamide / Propanil	709-98-8	
41	malathion	121-75-5	
42	hexachlorobutadiene	87-68-3	
43	tripentyl phosphate	2528-38-3	
44	1,2-benzisothiazole, 3-(2-propen-1-yloxy)-, 1,1-dioxide / Probenazole	27605-76-1	
45	Phosphoric acid, [1,1'-biphenyl]-2-yl diphenyl ester	132-29-6	
46	benzenesulfonic acid, 2-methyl-, sodium salt	15046-75-0	
47	Propane, 1,3-dibromo-2,2-bis(bromomethyl)-	3229-00-3	

No.	Substance name	CAS No.	Structural formula
48	Benzene, (1,1-dimethylpropyl)-	2049-95-8	
49	Poly(oxy-1,2-ethanediyl), .alpha.-sulfo-.omega.-(nonylphenoxy)-, sodium salt (1:1)	9014-90-8	
50	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester = substance No. 12	2082-79-3	
51	Acetic acid, 2-mercapto-, 2-ethylhexyl ester	7659-86-1	
52	Dodecanoic acid, 2,3,4,5,6-pentachlorophenyl ester	3772-94-9	
53	tributyl(dodecanoyloxy)stannane	3090-36-6	
54	Pentane, 1-bromo-	110-53-2	
55	Tetracosane, 2,6,10,15,19,23-hexamethyl- / Squalane	111-01-3	
56	Phosphoric acid, tris(3-methylphenyl) ester / Tri-m-cresyl phosphate	563-04-2	

No.	Substance name	CAS No.	Structural formula
57	2,5-pyridinedicarboxylic acid, dipropyl ester	136-45-8	
58	Stannane, tributyl[[[(1R,4aR,4bR,10aR)-1,2,3,4,4a,4b,5,6,10,10a-decahydro-1,4a-dimethyl-7-(1-methylethyl)-1-phenanthrenyl]carbonyl]oxy]- / Tributyltin abietate	26239-64-5	
59	18-Pentatriacontanone / Diheptadecyl ketone	504-53-0	
60	1-Naphthalenecarboxylic acid / 1-Naphthoic acid	86-55-5	
61	Cis-13-Docosenamide (Erucamide)	112-84-5	
62	Isodecyl neopentanoate	60209-82-7	
63	Isooctyl-3-mercaptopropionate	30374-01-7	
64	Dibutyl Methylenebis(thioglycolate)	14338-82-0	
65	Phenanthrene	85-01-8	

No.	Substance name	CAS No.	Structural formula
66	4-chloroaniline	106-47-8	
67	4-fluorophenol	371-41-5	
68	Diethylene glycol	111-46-6	
69	1,3,5-trimethylbenzene	108-67-8	
70	Di-isooctyl phthalate	27554-26-3	

C Persistence Assessment for selected test substances

C.1 Results of the Mackay Level III fugacity model (EPI Suite) for the selected test substances

Table 18: Results of the Mackay Level III fugacity model (EPI Suite) for the selected test substances

Test substance	Water % release	Water half-life* [d]	Soil % release	Soil half-life* [d]	Sediment % release	Sediment half-life* [d]	Overall persistence time [d]
IBU	22	15	77	30	< 1	**135**	23
PBO	13	38	86	75	< 1	**338**	68
OBP	12	**60**	87	**120**	< 1	**542**	78
4FP	20	38	80	75	< 1	**338**	44
ERU	12	38	64	75	24	**338**	58
DEG	34	9	66	17	< 1	78	16

* half-lives ≥ REACH Annex XIII P-criteria displayed in bold

C.2 Degradation data used for persistence assessment

Table 19: Degradation data used for persistence assessment

ITS Step	Data	Test method	Remarks	IBU	PBO	OBP	4FP	ERU	Reference
Step 1	RBT	OECD 301 A	% DOC-elimination	-	-	86 (10 d)	-	-	ECHA
	RBT	OECD 301 B	% Mineralisation	-	36 (24-48)	34 32-35)	40-60	15-64	ECHA
	RBT	OECD 301 C	% Mineralisation	-	-	31 (21-39)	-	88	ECHA
	RBT	OECD 301 D	% Mineralisation	31 (20-60)	< 0 - 4	-	-	15	ECHA
	RBT	OECD 301 F	% Mineralisation	82.6	31.2	24.4	67.2	52	this study
Step 2	Inherent	OECD 302 B	% IC analysis	-	-	34 (21-47; 35d)	-	-	ECHA
	eRBT	OECD 301 B	% Mineralisation; 60 d, 3 mg/L d.s. inoculum[18]	-	-	-	46-53	-	Martin et al. (2017)
	eRBT	OECD 301 B	% Mineralisation; 60 d, 300 mg/L d.s. inoculum[19]	-	-	-	62-63	-	Martin et al. (2017)
	eRBT	OECD 301 F	% Mineralisation; 164 mL, 60 d, AS	83.7	36	29	73.5	62.5	this study
	eRBT	OECD 301 F	% Mineralisation; 164 mL, 60 d, EF	-2.4	-6.5	39.6	17.3	21.6	this study

[18] It has to be noted that the experiments performed by Martin et al. (2017) are strickly speaking no eRBTs since the standard activated sludge concentration of most RBTs is 30 mg/L d.s. and thus a factor of ten higher than the lower inoculum concentration used, while the higher inoculum concentration of 300 mg/L d.s. is clearly within the range that should be assigned to an inherent test (OECD 302 B 200-1000 mg/L d.s.).

[19] Ibid.

ITS Step	Data	Test method	Remarks	IBU	PBO	OBP	4FP	ERU	Reference
	eRBT	OECD 301 F	% Mineralisation; 740 mL, 28 d	95.8	35.8	35.5	67.8	34.5	this study
	eRBT	OECD 301 F	% Mineralisation; 740 mL, 60 d	97.7	37.6	46.7	95.6	64.3	this study
	eRBT	OECD 301 A/B	% Mineralisation; CO_2, 28 d	105.7	31.9	33	82.7	18.7	this study
	eRBT	OECD 301 A/B	% Mineralisation; CO_2, 60 d	112.8	36.4	44.2	94.6	36.7	this study
	eRBT	OECD 301 A/B	% DOC elimination, 28 d	99.1	38.7	78.3	94.7	100	this study
	eRBT	OECD 301 A/B	% DOC elimination, 60 d	99.4	40.6	72.4	98.2	99.5	this study
Step 3	QSAR	Level III fugacity	See Appendix C.1						US EPA (2017)
	RBT	OECD 301	% degradation, parent analysis, 3.5-7.0 h	-	-	-	100	-	ECHA
	Simulation	Similar to OECD 308	$DT_{50,\ water,\ 15°C}$ [d]	2.7-8.1	-	-	-	-	Radke & Maier (2014)
	Simulation	Non-guideline	$DT_{50,\ fortified\ lake\ water}$ [d]	~ 20	-	-	-	-	Buser et al. (1999)
Step 4	Soil simulation	US EPA 162-1 /162-2	$DT_{50,\ aerobic,\ 20\text{-}25°C}$ [d]	-	10-14	-	-	-	ECHA
	Soil simulation	US EPA 162-1 /162-2	$DT_{50,\ aerobic,\ 12°C}$ [d]	-	40	-	-	-	ECHA
	Soil simulation	US EPA 162-1 /162-2	$DT_{50,\ anaerobic\ 25°C}$ [d]	-	144	-	-	-	ECHA

ITS Step	Data	Test method	Remarks	IBU	PBO	OBP	4FP	ERU	Reference
	Soil simulation	OECD 307	$DT_{50,\ aerobic,\ 20°C}$ [d]	-	23-64	-	-	-	ECHA
	Soil simulation	OECD 307	$DT_{50,\ aerobic,\ 12°C}$ [d]	-	44-121	-	-	-	ECHA
	Soil simulation	OECD 307	Geometric mean $DT_{50,\ aerobic,\ 12°C}$ [d]	-	58	-	-	-	ECHA
	Soil simulation	Non-guideline	% Mineralisation, 14 d	-	-	8.3	-	-	ECHA
	Water-sediment simulation	OECD 308	$DT_{50,\ 20°C,\ water}$ [d]	10	27-165	-	-	-	ECHA, Löffler et al. (2005)
	Water-sediment simulation	OECD 308	$DT_{50,\ 12°C,\ water}$ [d]	-	51-313	-	-	-	ECHA
	Water-sediment simulation	OECD 308	$DT_{50,\ 20°C,\ sediment}$ [d]	-	36-81	-	-	-	ECHA
	Water-sediment simulation	OECD 308	$DT_{50,\ 20°C,\ system}$ [d]	<6	54-55	-	-	-	ECHA, Löffler et al. (2005)
	Water-sediment simulation	OECD 308	$DT_{50,\ 12°C,\ system}$ [d]	-	103.4	-	-	-	ECHA
	Water-sediment simulation	OECD 308	% Mineralisation, 100 d	77	-	-	-	-	ECHA, Löffler et al. (2005)