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Are ready biodegradation tests effective screens for non-persistence in all environmental compartments?

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Abstract

Background Persistence assessment is a cornerstone of chemical hazard and risk assessment in numerous regulatory frameworks, as the longevity of a substance in the environment relates to exposure and ultimately the risk it poses. A chemical that is readily biodegradable is commonly assumed to undergo rapid and ultimate biodegradation under most environmental conditions. Ready biodegradability tests (RBT), such as the OECD 301 test series, are used to quickly screen out non-Persistent substances and focus regulatory scrutiny on the most hazardous substances. The stringency of the RBT as a screen for all environmental compartments is paramount to ensure that there are no readily biodegradable yet Persistent substances. To assess this stringency in practice, we here describe a systematic comparison of substances with both RBT data and biodegradation simulation test data for soil, sediment, or water compartments to see whether there are any substances which are readily biodegradable yet meet EU REACH regulatory Persistence criteria in any specific environmental compartment.

Results A rough assembly of data extracted from the ECHA database showed that, out of 263 substances with both RBT and simulation test data, there were 19 substances that were readily biodegradable but Persistent (based on the most conservative result and after a temperature adjustment to the half-life). However, many of the underpinning simulation study information were either not high-quality guideline studies or the substances were UVCBs. To more accurately compare the RBT and simulation testing outcomes, quality criteria on the RBT and simulation tests were applied, which limited the data set to about one-third.

Conclusions When examining quality-screened, temperature-adjusted simulation testing half-lives for readily biodegradable substances, there were no readily biodegradable substances that were Persistent. A side-by-side comparison of the available data supports the stringency and effectiveness of RBTs to identify non-Persistent chemicals.

Keywords Persistence, Readily biodegradable, Chemical

Background

A chemical which degrades rapidly is generally considered to pose less concern to human health and the environment than a Persistent substance which may remain in the environment for many months or years [12]. This concept is fundamental to many chemical regulations worldwide, for example, the UN Globally Harmonised System of Classification and Labelling of chemicals (GHS), the EU's REACH Regulation, Canada's Chemicals

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Management Plan, and the U.S. EPA's Toxic Substance Control Act. In combination with other properties, such as bioaccumulation and toxicity, Persistence assessment plays a key role in identifying substances that may require regulatory attention and management.

To more effectively focus regulatory attention on potentially Persistent chemicals, it is useful to screen out chemicals which degrade rapidly and are not Persistent. A chemical that is "readily biodegradable" undergoes rapid and ultimate biodegradation under most environmental conditions [13]. Ready biodegradation tests (RBTs), such as the OECD 301 series [14] or the more recent OECD 310 test [15], are stringent tests conducted in aqueous media under aerobic conditions measuring mineralization—the complete breakdown of the organic substance to water, carbon dioxide (CO_2), and other inorganic products. Specifically, a chemical is considered readily biodegradable if it achieves ≥ 70% mineralization measured as dissolved organic carbon removal (OECD TGs 301A, 301E, and 306) or ≥ 60% mineralization measured as theoretical CO_2 evolution (OECD 301B) or theoretical oxygen demand (OECD test guidelines 301C, 301D, 301F, 306, and 310) over the course of a 28-day test; this mineralization may need to occur within a 10-day window for mono-constituent substances but not for multi-constituent substances, depending on the test and use of the data. It is assumed that the remaining 30 to 40% of the carbon is incorporated into biomass, resulting in complete or ultimate biodegradation of the test substance [7]. This means that a readily biodegradable substance does not have any potentially Persistent degradation products.

These screening tests allow for quick and cost-effective assessments of the thousands of chemicals registered and evaluated under global regulatory programmes. Within the context of the REACH standard information requirements (Annex IX), higher tier fate information requirements can be waived if the substance is readily biodegradable [4]. Further, in the context of the REACH Persistent, Bioaccumulative and Toxic (PBT) assessment, information may be gleaned from ready biodegradation tests to reach a conclusion of "not Persistent", such as running an enhanced biodegradation test by extension of the RBT duration to 60 days [5–7].

Under REACH, substances that are not readily biodegradable are considered "potentially Persistent" by the authorities [4]. Further information to derive biodegradation half-lives using more complex biodegradation simulation tests in soil, sediment–water, and water (test guidelines OECD 307, 308, and 309, respectively,) or a weight-of-evidence determination using all available data is needed [16–18]. Degradation half-lives (DegT50) are then compared with single value criteria to allow conclusions on a substance as either not Persistent, Persistent (P), or very Persistent (vP) for the relevant environmental compartment as shown in Table 1 below.

Degradation data, whether from simulation tests or RBTs, are often highly variable with the outcome subject to both environmental and experimental factors [2, 9, 10, 19–21]. Aspects of the ready biodegradability test design confer significant stringency to its use as a screening test. For example, the high concentration of test substance used in a RBT compared with typical environmental concentrations and low levels of inoculum result in an unfavourably high C/biomass ratio compared to real-life environmental situations. This stringency also results in a high rate of false negative outcomes [3, 11]. For instance, the high concentration of test substance can decrease substance bioavailability. The low levels of inoculum used reduce the chances of inclusion of naturally occurring competent degraders in the test system which can result in variable RBT outcomes [11]. The variability and stringency of RBTs are acknowledged in current regulatory guidance for Persistence assessment under REACH, where guidance advises that where there are multiple RBTs for a substance, a positive test should generally supersede a negative test outcome when the scientific quality of the positive RBT is deemed good, the study is well documented, the test method fits the test substance properties, and the use of non-adapted inoculum is confirmed [5].

To investigate whether the RBT indeed performs well as a conservative screen for Persistence, this work compares biodegradation assessments for REACH registered substances having both ready biodegradation and simulation test data, to determine whether there are substances which are both readily biodegradable and concluded as P/vP in a simulation test according to the REACH Annex XIII criteria. We describe here an exploration of the available evidence on the stringency of standardized RBTs and their use as a screening test in Persistence assessment.

Table 1 EU reach P/vP criteria as defined in annex XIII

Environmental Compartment	P degradation half-life criteria (days)	vP degradation half-life criteria (days)
Fresh, estuarine water	≥ 40	≥ 60
Marine water	≥ 60	≥ 60
Freshwater, estuarine sediment	≥ 120	≥ 180
Marine sediment	≥ 180	≥ 180
Soil	≥ 120	≥ 180

Methodology

The OECD QSAR Toolbox v4.4.1 (referred to hereafter as the Toolbox) was queried in October 2021 for substances with both ready biodegradability and simulation test data. All databases with environmental fate data in the Toolbox were queried simultaneously. Data processing was performed in a data science platform, KNIME v4.2.2, to automate the evaluation of each data point. While the Toolbox lists each study available in its databases under a regulatory relevant endpoint, not every study was performed according to a known guideline, and not all databases give the same amount of detail to enable comparison. All the data referred to below were extracted from the ECHA database in October 2021, as the only source providing a sufficient level of detail for our purposes. While the R.11 guidance is clear that DegT50 is the criteria for P assessment, the OECD QSAR toolbox data are reported as "half-life". An assumption was made that this was equivalent to a DegT50 if the test was performed according to an OECD guidance for simulation testing.

Data processing

Initial processing of raw data from the OECD QSAR Toolbox Only tests for which a guideline was reported in the raw data were extracted into the preliminary data set. For ready biodegradability tests, the required guideline reported was one of the OECD 301 series or their EU test methods regulation equivalent (EC Method C.4). For simulation tests, only data derived from studies which reported the standard OECD test guidelines as 307, 308, or 309 or US EPA equivalents (OPPTS 835.3300 Soil Biodegradation: OPPTS 835.3180: sediment/water microcosm).

Additionally, studies were excluded from the assessment if it was deemed that they did not meet the additional criteria, through the manual evaluation of the publicly available information published by ECHA as Registered Substances Factsheets. Studies were excluded from the preliminary data set if

- major deviations from the standard guideline were reported;
- for the 301 tests, results were reported solely at 60 days, instead of 28 days;
- studies were flagged in the Toolbox as ready biodegradability tests but were actually tested for inherent biodegradability (OECD 302 tests);
- simulation studies results did not report a half-life. Several studies report only the percentage of degradation at a given time, and hence, it was not possible to determine a half-life in order to compare to the P/vP criteria.

The application of the exclusion criteria above resulted in a preliminary data set composed of 316 data points, covering 263 unique substances. Note that for some substances, there are multiple data points as the half-life was measured in multiple compartments. However, due to remaining quality concerns, an additional quality assessment was performed on the preliminary data set, as described below and shown in Fig. 1.

Quality assessment of preliminary data set The quality assurance criteria applied on the preliminary data set were as follows: the test substance name was indicative of a mono-constituent with either EC or CAS number; data available was on the registered substance and no read-across from another substance was used; and the substance was hydrolytically stable. Additionally for simulation tests, data were generated under aerobic conditions; the final mass balance was within 90 to 110%; data were generated according to standard OECD test guidelines 307, 308, or 309 or US EPA equivalents (OPPTS 835.3300 Soil Biodegradation: OPPTS 835.3180: sediment/water microcosm). It should be highlighted that after the quality assessment described above, the guideline reported in the preliminary data set was not always correct, due to misreporting in the QSAR toolbox, and hence, the study was screened out of the final data set. These quality criteria were checked by examining the publicly available information published by ECHA as Registered Substances Factsheets. Since the focus of this work was to identify any readily biodegradable substances which were also Persistent, the simulation test data for the non-readily biodegradable substances were not given rigorous scrutiny by checking the details of the testing in the ECHA Registered Substances Factsheets. The substances meeting all the quality criteria constitute the "final data set".

Data processing of final data set

Data from ready biodegradation tests Ready biodegradation test (RBT) results were recorded as "readily biodegradable", if the relevant pass level for the test $\geq 70\%$ biodegradation measured as dissolved organic carbon (DOC) removal (OECD TGs 301A, 301E, and 306) or $\geq 60\%$ biodegradation when measured as Theoretical Carbon Dioxide ($ThCO_2$) (OECD TG 301B) or Theoretical Oxygen Demand (ThOD) (OECD TGs 301C, 301D, 301F, 306, and 310) was achieved at 28 days. In line with Persistence assessment as described in REACH guidance R.11 [4], the 10-day window for "readily biodegradability" was not considered for this assessment, so a substance was concluded to be not Persistent and marked as

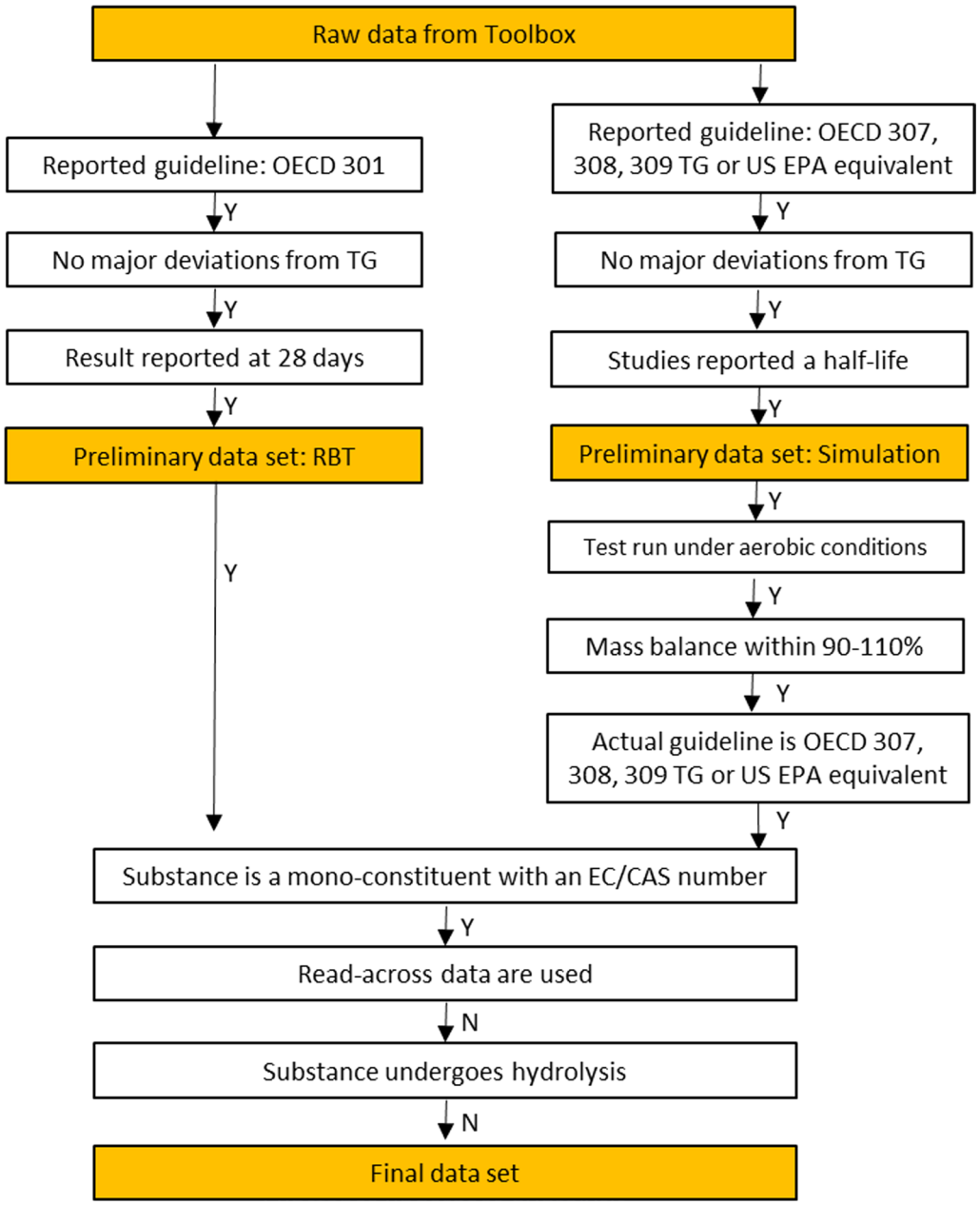

Fig. 1 Process for screening RBT and simulation test data

readily biodegradable solely based on the pass level reach at 28 days.

Where results were reported as a percent degradation range, the substance was only recorded as readily biodegradable if the whole range of values was greater than the pass level. If multiple valid ready biodegradation tests with both positive and negative results were reported, the substance was concluded to be readily biodegradable in line with REACH guidance R7b, which states that a positive test result should generally supersede negative test results [5].

Data from simulation tests If a substance had biodegradation data from several valid simulation tests, a conservative approach was adopted, and the longest half-life value was used per REACH guidance R.11 [5]. A substance was deemed to be Persistent if the highest available degradation half-life met the EU REACH criteria in Table 1. No distinction was made between the Persistent

(P) and very Persistent (vP) conclusion, since a RBT cannot be used to discriminate those two categories.

Since 2017, regulatory guidance from ECHA on Persistence assessment has required that simulation studies are carried out at the average European surface water temperature of 12 ℃ [4, 5]. However, most studies retrieved from the Toolbox were performed at temperatures between 20 and 25 ℃. If half-lives were measured at several temperatures, the half-life closest to 20 ℃ was selected; if the study was conducted at 12 ℃, then that value was used. If studies were not performed at 12 ℃, the data were examined with and without temperature correction to 12 ℃ using the Arrhenius equation, per ECHA guidance R.7b with an E_a of 65.5 kJ/mol [4, 5].

Characterization of physico-chemical space

To define the physico-chemical space represented by the data set, key chemical properties for environmental fate using EPISUITE v4.1 were calculated (see Additional file 1: Table S1). The octanol–water partition coefficient (log K_{ow}), water solubility, organic carbon partition coefficient (log K_{oc}), and vapour pressure were calculated using CAS numbers as input in KOWWIN™, WATERNT™, KOCWIN™ using the MCI model, and MPBPWIN™ using the "selected value option", respectively. For some substances, predictions were not generated as there was either no CAS number, EPISUITE gave an error, or the substance was a UVCB.

Results

The preliminary data set of substances with both RBT and simulation test data comprised 316 data points related to 263 unique substances. The full list of substances, CAS numbers, and associated data are provided in the supporting information (Additional file 1: Table S1). For most substances, only one data point was available, *i.e.* the test substance was only tested in one environmental compartment; however, data from multiple compartments were available for 43 substances. The 263 substances included in the preliminary data set have a wide range of physico-chemical properties, including substances with difficult-to-test properties, such as poorly soluble, volatile, and sorptive substances (Additional file 1: Table S1). Water solubility values vary between 0.4 ng/l to 1000 g/l; vapour pressure values vary between 1.2×10^{-22} to 1590 mmHg; octanol–water partition coefficient (log K_{ow}) ranged from − 10 to 15.5; and organic carbon partition coefficients (log K_{oc}) values vary between 0 and 9.16.

Some of the studies which were screened into the preliminary data set as being equivalent or similar to an OECD test guideline were deemed to be too dissimilar to the original test guideline and hence were excluded from the final data set. Due to additional quality concerns for data interpretation in the preliminary data set, an additional quality screen was needed. Such concerns included that the test substance was a UVCB and not a mono-constituent, or that the test conditions for the simulation were anaerobic.

The final data set comprised 105 data points for 74 unique substances, capturing 48 data points for soils, 37 for sediment, and 20 for freshwater. Of the 74 substances, eight are readily biodegradable substances (Table 2), and 66 are for non-readily biodegradable substances (Additional file 1: Table S2). For the 11 readily biodegradable substances, the water solubility values vary between 300 ng/l to 1000 g/l; vapour pressure values vary between 8×10^{-10} to 479 mmHg; octanol–water partition coefficient (log K_{ow}) ranged from -10 to 7.9; and organic carbon partition coefficients (log K_{oc}) values vary between 0.28 and 5.32. This stringent application of quality criteria to selection of simulation studies was applied to minimize sources of variability and ensure that a reasonable comparison of studies could be performed.

Figure 2 illustrates the degradation half-lives reported for freshwater, soil, and sediment, respectively, for the

Table 2 Number of readily biodegradable substances with simulation test data per compartment. The right-hand side of the table shows how many of those data points met all of the data quality criteria defined and were included in the final data set. A similar assessment of the non-readily biodegradable substances is available in Additional file 1: Table S2

Readily biodegradable substances	Preliminary data set			Final data set (data meeting all quality criteria)		
Simulation study compartment	Water	Soil	Sediment	Water	Soil	Sediment
No. of simulation studies	*17*	*64*	*17*	2	5	4
No. of substances meeting P criteria before temperature correction (in percent)	*3 (18%)*	*2 (3%)*	*4 (24%)*	0	0	0
No. of substances meeting P criteria after temperature correction (in percent)	*4 (24%)*	*8 (13%)*	*7 (41%)*	0	0	0

The italicized values are for the preliminary data set

Fig. 2 Distribution of simulation half-lives found in reach registration dossiers for readily biodegradable substances in freshwater, soil, and sediment compartments

readily biodegradable substances in the preliminary data set and final data set. For each environmental compartment, the "x" depicts the half-lives of substances as reported in the Toolbox in the preliminary data set, the "Δ" represents the temperature-adjusted half-lives for the substances in the preliminary data set, and the "●" shows the temperature-adjusted half-lives for the substances in the final data set.

Biodegradation testing in freshwater

Data on biodegradation in freshwater from OECD 309 or equivalent studies on 58 substances were available in the preliminary data set; 17 are readily biodegradable (Table 2) and 41 substances are not readily biodegradable (Additional file 1: Table S2). Three substances, namely, phenol; sodium O-isobutyl dithiocarbonate; and sodium O-isopentyl dithiocarbonate were identified as readily biodegradable but have degradation half-lives in water greater than the Persistence criteria (40 days). On temperature adjusting the degradation half-life, an additional substance—2,2′-iminodiethylamine—would be concluded as Persistent (Table 2). As described in Table 3, while these substances passed the more automated screening, upon closer inspection, the higher tier degradation studies for these three substances do not meet all quality/selection criteria and have been excluded from the final data set.

Table 3 Substances identified as readily biodegradable and Persistent in water in the preliminary data set but excluded from the final data set and the rationale for the quality screen failure

Substance CAS (EC)	Reason for elimination from final data set
Phenol 108-95-2 (203-632-7)	The simulation study was incorrectly reported as a guideline simulation study. This study was designed to assess anaerobic biodegradation of several compounds, including phenol, under stringent conditions using secondary activated sludge. No half-life was derived
Sodium o-isobutyl dithiocarbonate 25,306-75-6 (246-805-2) **Sodium o-isopentyl dithiocarbonate** 2540-36-5 (807-374-1)	For both substances, the simulation study was incorrectly reported as following a guideline for simulation test and refers to hydrolysis (abiotic degradation); hence, it is not truly a simulation test. The half-life of xanthates has been reported in literature as 2–8 days in water; therefore, these substances are most likely not Persistent [1]
2,2′-Iminodiethylamine 111-40-0 (203-865-4)	Test conducted in 1987 and incorrectly reported as a guideline simulation test. Limited details indicate it may have been a modified US EPA test method but no further details available to confirm

After applying the quality/selection criteria, 38 out of the 58 substances in the preliminary data set were excluded. Of the 20 substances in the final data set, only two substances (1,1-biphenyl and trisodium nitriloacetate) with simulation test data in water were readily biodegradable (Table 3). In both cases, before and after temperature correction to 12 ℃ (Fig. 2), the reported DegT50 values were below the 40-day threshold for Persistence in water. Nine (50%) of the substances that were not readily biodegradable had degradation half-lives in water less than 40 d and thus did not meet the criteria for Persistence either, demonstrating the conservatism of the RBT as a Persistence screening test (Additional file 1: Table S2).

Biodegradation testing in soil

Data on biodegradation in soil from OECD 307 or equivalent studies on 193 substances were available in the preliminary data set, of which 64 were concluded to be readily biodegradable (Table 2). Two substances—bis(2-ethylhexyl) phthalate and dichloromethane—had conflicting Persistence conclusions in the preliminary data set by being both readily biodegradable and having a biodegradation half-life greater than 120 d. Temperature adjustment of degradation half-lives to 12 ℃, resulted in an additional six readily biodegradable substances with half-lives in soil exceeding the Persistence criteria (Table 2), specifically triethyl citrate, tridecane, hexadecanamide, didecyldimethylammonium chloride, C9–C11 alkanes and 4-nonylphenol (branched). Upon applying the quality/selection criteria, these substances were excluded from the final data set as described in Table 4.

Upon further evaluation of the studies in the preliminary data set following the method in Fig. 1, the number of substances with simulation test data in soil was reduced to 48 in the final data set, of which only five were readily biodegradable. All readily biodegradable substances had soil degradation half-lives below the regulatory threshold for Persistence, even after the biodegradation half-life was adjusted to 12 ℃ (Table 2). Of the 43 substances in the final data set concluded as not readily biodegradable, 18 (43%) had a degradation half-life that exceeded the criteria for Persistence in soil (120 days) (Additional file 1: Table S2). The remaining twenty-five (57%) substances were not readily biodegradable and had half-lives below the Persistence threshold for soil, again demonstrating the conservatism of the RBT as a Persistence screening test (see Additional file 1: Table S2).

Biodegradation testing in sediment

Data on biodegradation in sediment from OECD 308 or equivalent studies on 65 substances were available in the preliminary data set, of which 17 were concluded to be readily biodegradable (Table 2). Four substances in

Table 4 Substances identified as readily biodegradable and Persistent in soil in the preliminary data set but excluded from the final data set and the rationale for the quality screen failure

Substance CAS (EC)	Reason for elimination from final data set
Bis(2-ethylhexyl) phthalate 117-81-7 (204-211-0)	Several soil degradation studies conducted but incorrectly reported as following a standard guideline. Data are highly variable, being both below and above the P threshold. Reported half-lives in decisive study are based on mineralisation and not directly comparable with P criteria
Dichloromethane 75-09-2 (200-838-9)	Several soil degradation studies conducted but incorrectly reported as following a standard guideline. Data are highly variable, being both below and above the P threshold
Triethyl citrate (1,2,3-Propanetricarboxylic acid, 2-hydroxy-, 1,2,3-triethyl ester) 77-93-0 (201-070-7)	Test was incorrectly reported as following a standard guideline
Tridecane 629-50-5 (211-093-4)	Data reported are for a read-across study from a modified OECD 304 test (inherent biodegradability in soil) conducted on a UVCB substance
Hexadecanamide 629-59-4 (211-096-0)	Data reported are for a read-across study from a modified OECD 304 test (inherent biodegradability in soil) conducted on a UVCB substance
Didecyldimethylammonium chloride (1-Decanaminium, N-decyl-N,N-dimethyl-, chloride) 7173-51-5 (230-525-2)	Test was incorrectly reported as following standard guideline and test substances was not solely a mono-constituent
C9–C11 alkanes 64,742-48-9 (919-857-5)	Substance is a UVCB
Phenol, 4-nonyl-, branched 84,852-15-3 (284-325-5)	Substance is a UVCB

the preliminary data set—bis(2-ethylhexyl) phthalate; 5,5-dimethyl-2,4-imidazolidinedione; alkanes, C14-17, chloro; and alkanes, C10-13, chloro—were readily biodegradable but have reported degradation half-lives that exceed Persistence criteria in sediment (120 d). For these substances, a review of the simulation studies available in the REACH disseminated dossiers indicated shortcomings in the conduct of the test, and hence, test outcomes were deemed less reliable (Table 5). For instance, the initial concentration of DEHP in the sediment test was 834 mg/kg sediment dry weight; this is a very high dose for a simulation test. The two substances, alkanes, C10-13, chloro (MCCP) and alkanes, C14-17, chloro (SCCP), were also initially identified as having conflicting Persistence data. The RBTs for the chlorinated paraffins have been conducted on substances using different constituents with varying alkyl chain length and degrees of chlorination. The simulation study was also not performed on a mono-constituent, hence complicating the comparison between the RBT and the sediment simulation test.

When DegT50 values were extrapolated to a temperature of 12 ℃, there were an additional three readily biodegradable substances with conflicting Persistence conclusions: ammonium dodecylbenzene sulphonate, benzenesulphonic acid, mono-C10-13-alkyl derivatives, and phenol, 4-nonyl, branched (Table 5). However, after further evaluation of the registration dossier, these were identified as UVCBs, so they did not meet the specified quality criteria (Fig. 1) and were not included in the final data set.

Of the 17 readily biodegradable substances in the preliminary data set, only four substances were included in the final data set after quality screening. In all cases the degradation half-lives were below the Persistence threshold, and the substances were concluded to be not Persistent, even after the degradation half-life was adjusted for temperature (Table 2). For those substances in the final data set concluded as non-readily biodegradable (33 substances), only 12 substances (36%) had half-lives exceeding Persistence criteria, again demonstrating the

Table 5 Substances identified as readily biodegradable and persistent in sediment in the preliminary data set but excluded from the final data set and the rationale for quality screen failure

Substance CAS (EC)	Reason for elimination from final data set
Bis(2-ethylhexyl) phthalate 117-81-7 (204-211-0)	Several sediment degradation studies available but were incorrectly reported as following a standard OECD 308 test guideline. Variable degradation rates reported
5,5-dimethyl-2,4-imidazolidinedione 77-71-4 (201-051-3)	Two sediment simulation studies available. One is conducted according to a non-standard test guideline, only partially aerobic conditions maintained. More recent OECD 308 study in the publicly available REACH registration dossier reports DegT50 of 23.6d, supporting a conclusion of not Persistent
Alkanes, C10-13, chloro 85,535-84-8 (287-476-5)	The ready biodegradation test was a modified study. The substances are UVCBs
Alkanes, C14-17, chloro 85,535-85-9 (287-477-0)	The ready biodegradation test was a modified study. The substance is a UVCB
Ammonium dodecylbenzene sulphonate 1331-61-9 (215-559-8)	Substance is a UVCB; simulation test was conducted on one constituent while RBT was on the whole UVCB substance
Benzenesulphonic acid, mono-C10-13-alkyl derivatives 85,480-55-3 (287-335-8)	Substance is a UVCB
Phenol, 4-nonyl-, branched 84,852-15-3 (284-325-5)	Substance is a UVCB

Table 6 Ready biodegradability and simulation test conditions related to the relevance and stringency of the outcome

	RBT	Simulation test (for half-life determination)
Test temperature	20–25 ℃	12 ℃
Inoculum source	Activated sludge/sewage effluent/mixture of soil and surface water	Environmental samples of soil, sediment, or water
Inoculum concentration	10^4–10^8 cells/l	Highly variable (based on environmental sample)
Test substance concentration	2 to > 100 mg/l	< 1–10 µg/l (for OECD 309)
Test substance metabolism	Sole carbon source	Not the primary substrate, allowing for competing substrates and co-metabolism
Kinetics	Growth linked	First order or biphasic

conservatism of the RBT (see Additional file 1: Table S2). Considering only the final data set of reliable data, there were no substances which were both readily biodegradable and met the Persistence criteria in sediment (Table 6).

Discussion

Our aim in this study was to examine evidence on the rigour and conservatism of the RBT as a screening test for potentially Persistent chemicals. A data set comprising substances with existing degradation half-life data for soil, sediment, and water compartments, and RBT outcomes have been compiled and evaluated using Persistence criteria specified in the EU REACH regulation. Inspection of the preliminary data set identified nine substances that were potentially both readily biodegradable and met REACH Persistence criteria when looking at the most conservative simulation test outcomes without temperature adjustment and 19 substances with temperature adjustment (Table 2, Fig. 2). However, further scrutiny of the conduct of the simulation tests for these 19 substances indicated the data did not meet the authors' quality and selection criteria for inclusion in the final data set; this meant that it was not possible to make a clear comparison between the outcome of that simulation test and the RBT. In the final data set, there were no substances which were found to be both readily biodegradable and P/vP. It is noted that no effort was made in this work to perform a weight-of-evidence assessment to arrive at a Persistence conclusion; rather substances were deemed Persistent based on the most conservative available simulation testing data.

While the number of substances was limited by the strict data quality criteria employed in this study, such quality criteria were necessary to maintain the comparability of the outcomes. This resulted in a marked reduction in the size of the data set, most notably for the soil data set where the number of soil degradation simulation studies was reduced from 194 to 49. The quality criteria imposed in this paper are not meant as a criticism of the available data, but are necessary to be able to consistently compare simulation test outcomes. Degradation data that have been eliminated from the data set may still be used for regulatory Persistence assessment, since some data were rejected from the data set for non-quality-related issues, like being read-across or tests on UVCBs. The number of substances that had both RBT and simulation test data was also limited by the fact that there is seldom need for simulation test data for a readily biodegradable substance.

Conservatism of the RBT

As mentioned in the introduction, several aspects of test design in a RBT not only confer conservatism and stringency but also lead to variable test outcomes, resulting in a high rate of false negatives (meaning a readily biodegradable substance can fail an RBT). Kowalczyk, et al. [11] discuss more thoroughly specific aspects of RBT test design leading to the stringency, including inoculum source/concentration/preparation, test chemical concentrations, test volume, and test duration [11].

While RBT data and simulation test data are not directly comparable since they provide different measures of Persistence, *i.e.* an RBT indicates intrinsic propensity to mineralize entirely and quickly in most environments while simulation tests provide situation-specific half-lives as a reflection of Persistence potential, it is still of interest to compare the conclusions from the two tests.

In this work, a significant proportion of substances that do not meet the criteria for ready biodegradability have measured degradation half-lives in soil, sediment, or water indicating that they are not Persistent. This reinforces the conservatism of the RBT as a screen for Persistence. In fact, our examination of the data herein suggests that a substance failing the ready biodegradability criteria is still likely not to meet the P/vP criteria of REACH in water, soil, and sediment. Analysis of substances included in the final data set shows that, according to REACH criteria, 50%, 57%, and 64% of the substances which are not readily biodegradable are also not Persistent in water, soil, and sediment, respectively (Additional file 1: Table S2). This is consistent with observations reported in the wider scientific literature where incidences of "false negatives" are reported to range between 20 to 80% [3, 11]. This is to be expected, since the RBT is not intended to identify Persistent substances but is applied in a tiered Persistence assessment to screen for non-Persistence. Under REACH, sediment and soil are recommended compartments for simulation testing and subsequent P/vP assessment when the substances have high adsorption properties (log Koc > 4).

Limitations of this data review

There are several limitations in this study which should be highlighted. First, not all of the "false negative" substances have been tested for the three compartments (water, sediment, and soil). For this reason, a definitive not-P conclusion cannot be reached with certainty for most of the substances. Second, ECHA recently introduced the requirement to account for "non-extractable residues", which are test substance entrapped in the soil or sediment matrices (NER-type I); these were formerly considered as degraded but are now considered as non-degraded for the purpose of the P/vP assessment. It is very likely that none or few of the wealth of half-life data derived from the existing studies used in our analysis included NER-type I as non-degraded. Therefore, highly

adsorptive substances which were concluded not-P in soil or sediment may need to be retested or re-evaluated. It should be, however, noted that the REACH P/vP criteria were elaborated in the early 2000, probably on the basis of a set of degradation half-life data that had also considered NER as degraded parent. This means that it is scientifically more relevant to derive experimental half-life excluding NERs from the calculation. A third limitation is that the simulation data for non-readily biodegradable substances was not given the same scrutiny as the simulation data for the readily biodegradable substances, since the non-readily biodegradable data were of less interest for the purposes of this exercise. Therefore, it may appear that the quality of simulation test studies for non-readily biodegradable substances is higher, based on the data shown in Table 2 and Additional file 1: Table S2. This could indeed be the case, since there is little reason to perform a simulation test on a readily biodegradable substance, so the data available could either be older or less likely to follow standard guidelines. A fourth limitation is that no re-analysis of the raw data used to generate the simulation test half-lives or the percent biodegradation in the RBTs was undertaken; all data were taken as reported in the OECD Toolbox or the ECHA Registered Substances Factsheets. Fifth, and probably most important, it is clear that the size of the data set for the comparison of RB substances and their simulation testing outcome is extremely limited. Despite starting with 316 data points, at the end, there were only eight readily biodegradable substances with quality-screened simulation testing data.

Variability in simulation testing outcome

While the use of standardized test protocols such as the OECD test guidelines (OECD 307, 308, 309) provides a route for benchmarking the Persistence of substances, half-lives determined from simulation tests will still result in variable outcomes [9, 21, 22], with half-lives differing by one to two orders of magnitude observed in OECD 308 and 309 studies for some compounds [20]. Some of this variability can be attributed to differences in environmental parameters such as microbial community and organic matter [8]. Other sources of variability and confounding factors are experimental factors, like temperature, light, concentration of the substance, and geometry of the test system. For example, since biodegradation occurs at an interface, the influence of test system geometry in an OECD 308 on phase transfer affects the biodegradation of the test substance [9]. A useful broader discussion on sources of variability in degradation data is provided by Hughes et al. [10]. Modifications to the test systems, such as use of lower sediment–water ratios in OECD 308 test systems to significantly reduce variability, have recently been successfully demonstrated by Seller et al. [21]. Given the stringent nature of the RBT and the difficulties in performing simulation testing, a repeat of a well-conducted RBT or the use of an enhanced RBT could offer a more reliable, pragmatic, and efficient step before a simulation test [6].

As expected, significant variability in degradation half-lives for a substance can result in ambiguous outcomes in Persistence assessment, most notably for those substances with degradation half-lives closer to the cut-off criteria for Persistence/non-Persistence [19, 20]. The variable outcomes in degradation testing, even when conducted according to standardized test guidelines, combined with the large body of degradation data generated from non-standardized tests highlight the value of transparent and systematic weight-of-evidence determination to account for all available evidence in Persistence assessment.

Concluding statements

The objective of this work was to understand whether the RBT is performing as designed, i.e. as a stringent screening test that would obviate the need for further compartment-specific simulation tests. The biodegradation data collated and examined in this study suggest that RBTs are a conservative screening tool for Persistence assessment applicable to all environmental compartments. In the data set examined, there were no readily biodegradable substances which could be concluded as Persistent based on the most conservative half-life derived from a higher tier simulation test.

Abbreviations

DegT50	Degradation half-life
ECHA	European Chemicals Agency
NRB	Not readily biodegradable
OECD	Organization for Economic Cooperation and Development
PBT/vPvB	Persistent Bioaccumulative and Toxic/very Persistent and very Bioaccumulative
RB	Readily biodegradable
RBT	Ready biodegradability test
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
UVCB	Substance of Unknown, Variable composition, Complex reaction product, or Biological origin

Supplementary Information

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Additional file 1: Table S1. The list of substances and biodegradation conclusions as retrieved from the ECHA database via the OECD QSAR Toolbox v4.4.1 in October 2021. In order to conform with the Intellectual Property Rights Notice in ECHA's legal notice, specifically on the subject of systematic data collection, only the outcomes of the testing are here provided, with no specific values underpinning those conclusions. For the specific data, the reader is directed to the ECHA database at https://echa.europa.eu. The phys-chem parameters (log *K*ow, vapour pressure,

water solubility, and log *Koc*) were predicted by entering the CAS number into the US EPA EpiSuite model, as described in the methods. It is noted that for substances without a CAS or for UVCBs, it was not possible to generate the data (marked as #NA). **Table S2.** Number of non-readily biodegradable substances with simulation test data per compartment. The right-hand side of the table shows how many of those data points met all of the data quality criteria defined and were included in the final study set. A similar assessment of the readily biodegradable substances is available in Table 2.

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AMA performed the primary data gathering and analysis and wrote the methods and results sections as well as provided manuscript review; LC assisted with writing, data interpretation and manuscript review; CH assisted with data interpretation and manuscript review; EP reviewed the data gathering and analysis and assisted with data interpretation and manuscript revision; DS assisted with data interpretation and manuscript review; NW assisted with data gathering, data interpretation, and manuscript review; and DYL wrote the background and discussion sections as well as assisted with data interpretation.

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