Developing a set of reference chemicals for use in biodegradability tests for assessing the persistency of chemicals

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Abstract

This paper describes a project funded by Cefic Long-range Research Initiative (LRi, http://www.cefic-lri.org/) to develop a list of reference chemicals covering a range of environmental persistence and non-persistence. This reference list would be applied to check modified biodegradability test methods and to develop new test methods. The reference set would address concerns that new methods could result in tests becoming too powerful or overly protective. The aim of the research was to establish such a list of chemicals, with an agreed set of properties and characterized set of biodegradability behaviour. A total of 19 chemicals were agreed and assigned to 4 categories of biodegradability behaviour. Two recommendations from the research project are that all research biodegradability tests should run a positive and a negative control from this list when conducting biodegradation research and that the reference chemicals should be subjected to an inter-laboratory ring test to evaluate their biodegradability in both standard RBTs and modified/enhanced biodegradability tests and hence confirm their status with respect to the assigned categories.

Key Words

Biodegradability, ready test, persistence, P/vP

Introduction

The aim of this research, sponsored by the Cefic Long-range Research Initiative (LRi, http://www.cefic-lri.org/) was to develop a list of reference chemicals with an agreed (by regulators and industry) set of properties and characterized set of biodegradability behaviour which cover a range of environmental persistence and non-persistence. This reference set would be available for use to assess the impact of suggested modifications to existing aerobic biodegradability test methods and new test methods. The reference set would also help address concerns that some of the modifications or new methods could result in tests becoming too powerful or overly protective. An Advisory Panel was set-up to discuss and test the proposals, comprising of representatives of Regulators, Industry and Academia.

Information on biodegradability for use in hazard and persistency assessments, or risk in general, is normally based on data obtained in standardised tests described in national and international guidelines (e.g. OECD, US environmental protection agency office of pollution prevention and toxics (OPPTS) and ISO). The OECD methodology, for example, provides a tiered framework for assessing biodegradability. The tests range from simple screening tests, e.g. the OECD 301 ready biodegradability tests (RBTs), to inherent tests and/or relatively complex higher tiered simulation types of tests, e.g. the OECD 308 aerobic and anaerobic transformation in aquatic sediment systems, OECD 309 aerobic and anaerobic transformation in surface water and the OECD 303 aerobic sewage treatment (see Table 1).

The first tier 'ready biodegradability' tests are relatively short term (typically 28 days) stringent tests (see Table 1). Positive results with these type of tests indicate that micro-organisms that generally occur in the environment are able to use the chemical as a carbon source for relatively fast growth and hence any release of the chemical to the environment can be expected to lead to an adaptation of the environmental population and consequently to removal of the chemical. Ready biodegradability tests have provided the basis from which assessments of biodegradability for regulatory purposes have been made for over 20 years. As the regulatory drivers have changed it has become clear that whilst RBTs have been successfully used to identify chemicals that have the ability to undergo rapid degradation in the environment they have limitations when used for assessing persistence. The stringent conditions under which to RBTs are performed may result in a failure to achieve the pass criteria which can lead to false negative assessments. Failing to meet the pass criteria can be as a result of any of a number of the following reasons:

- The chemical is persistent;
- The chemical is only partially biodegraded (i.e. 10-60% mineralisation is observed);

- The chemical has been tested at an initial concentration that is toxic to the microbial inoculum;
- The low level of biomass in the inoculum does not contain competent degraders;
- The artificial test medium is nutritionally unsuitable for growth of competent degraders.

Table 1: Definition of terms from REACH endpoint specific guidance document

Test	Description
Ready biodegradability	Stringent screening tests, conducted under aerobic conditions, in which a high concentration of the test substance (in the range of 2 to 100 mg l^{-1}) is used and ultimate biodegradability is measured by non-specific parameters like Dissolved Organic Carbon (DOC), Biochemical Oxygen Demand (BOD) and CO_2 production. Small amounts of domestic sewage, activated sludge or secondary effluent form the microbial inoculum in tests for ready biodegradability. The inoculum should not have been artificially pre-adapted to the test substance through previous exposure to either the test substance or structurally related chemicals. The test substance is provided as the sole source of carbon for energy and growth. A positive result in a test for ready biodegradability can be considered as indicative of rapid and ultimate biodegradability in most environments including biological STPs.
Inherent biodegradability	Tests are inoculated with a high concentration of micro-organisms and carried out under aerobic conditions in which biodegradation rate and/ or extent are measured. The test procedures offer a higher chance of detecting biodegradation compared to tests for ready biodegradability and therefore if an inherent test is negative this could indicate the potential for environmental persistence.
Simulation	Aerobic and anaerobic tests that provide data on biodegradability under specified environmentally relevant conditions. These tests attempt to simulate degradation in a specific environment by use of indigenous biomass, media, relevant solids (i.e. soil, sediment, activated sludge or fresh or marine surface waters) to allow sorption of the chemical, and a typical temperature that represents the particular environment. A representative and low concentration of test substance is used in tests designed to determine the biodegradation rate constant whereas higher concentrations for analytical reasons are normally used for identification and quantification of major transformation products.

The second tier of consists of tests to assess the inherent biodegradability of chemicals. In these tests the ratio of biomass to food is shifted in favour of the biomass and the potential for adaptation is increased significantly. Currently results from such tests cannot be used to demonstrate a chemical is not persistent. The highest tier of biodegradability testing refers to simulation tests. The OECD 303 series can be considered to adequately represent the fate of a chemical in aerobic sewage treatment. However, there are some concerns over the reprentativeness and interpretation of data generated in the OECD 307, 308 and 309 tests. Indeed, none of these tests has been subject to validation through inter-laboratory ring tests with reference chemicals. There was general consensus at the ECETOC workshop (ECETOC, 2007) that whilst such tests can be considered to have greater relevance than the RBTs there were no soil, sediment or water biodegradability studies that accurately simulate biodegradation in the 'natural' environment and to describe these tests as 'simulations' was misleading.

Whilst they are referred to as standardised tests, the OECD 301 series have not been truly standardised in that variable sources of inoculum (activated sludge, natural waters, effluents etc.) are allowed, preconditioning regimes, media and test apparatus (e.g. volumes, aeration methods etc) differ and different endpoints (CO_2 release, O_2 uptake and dissolved organic carbon removal) are used. Consequently it is understandable that high levels of variability are reported for the results from RBTs including conflicting data between replicates of the same test, different results from the different types of test and conflicting results for the same test but conducted at different times at the same or different locations. Clearly improvements to the current screening tests are needed since they were neither designed to measure 'persistence' nor environmental biodegradation half-lives and were either not subject to any rigorous validation or were only validated with readily biodegradable chemicals.

Under current legislation (e.g. REACH, EC, 2006) biodegradation rates, or environmental half-lives, for the purpose of environmental risk assessment are assigned based on laboratory biodegradability tests. Incomplete and/or slow degradation observed under the conditions of these studies are also compared to national and international criteria for environmental persistence. For example, a biodegradability of <60% in the OECD 301 (OECD, 1992a) screening tests (manometric or carbon dioxide evolution) would result in two separate decisions. Firstly an (initial) assigned environmental half-life of 150 days for the purposes of risk assessment and (as a separate decision) a designation that the substance meets the criteria as a screening "P/vP" substance in a PBT/vPvB assessment. To help industry and regulatory authorities fulfil their duties under REACH, a series of Technical Guidance Documents (TGDs) have been prepared by the Reach Implementation Panels (RIPs). The RIP 3.3.2 EWG 9 (see Appendix A; ECHA 2008a)

discussions led to the development of the TGDs which identified a need for new types of screening tests that can be used to assess whether or not a substance fulfils the P criteria, but are not to be used in Classification and Labelling. These methods could build on the principles of the OECD 301 series of tests for ready biodegradability in such a way that they should lead to fewer chemicals being identified as potentially "persistent" and reducing the need for confirmatory higher tier studies. The TGD identified two types of screening tests beyond the OECD 301 series (OECD, 1992a). These were modified ready biodegradability tests and enhanced biodegradability screening tests.

Modified Ready Biodegradability Tests

Two modifications to the standard OECD 301 tests (OECD, 1992a) for ready biodegradability have been identified:

- Biodegradability testing at low test substance concentrations;
- Biodegradability of poorly water-soluble substances.

Providing all the other conditions in the ready biodegradability tests are fulfilled, these tests are still regarded as ready biodegradability tests and the results can be used directly in Classification and Labelling. Modified ready biodegradability tests, using a lower test substance concentration, are relevant when the test substance is known or expected to exert toxicity to the microbial inoculum. Strategies to assess the biodegradability of poorly water-soluble substances are described in the REACH TGD (Appendix R. 7.9-3, ECHA, 2008a).

Enhanced Biodegradability Screening Tests

The REACH TGD (ECHA, 2008a) states that "A number of potential enhancements to the ready biodegradability test have been identified. These enhancements have been identified to assist in persistency assessments and are not to be used in Classification and Labelling. The enhancements are designed to help improve the environmental relevance of biodegradability assessments without the immediate requirement for simulation level testing."

Test approaches in enhanced biodegradability screening tests include:

- Increased test duration. The test duration for poorly water-soluble substances and substances with extended lag phases is important. Where biodegradation is still occurring in a ready biodegradability test, the duration could be extended up to 60 days.

- Testing in larger vessels. Conducting biodegradability tests using larger volumes of environmental sample increases the total number of micro-organisms introduced into the test. This also increases the number of different types of micro-organisms, without changing the inoculum density. This will increase the probability of introducing a competent degrading microorganism into the test vessel.

- Increasing the biomass concentration. This approach recognises that when conducting biodegradability tests with an environmental water sample, it will not reflect the total number and types of microorganisms that a substance will encounter in the environment. A suitable procedure could be to concentrate the micro-organisms from a larger water volume (e.g. by filtration or centrifugation) and re-suspend the microbial inoculum in a smaller volume of the test medium.

- Low-level pre-adaptation test systems. Adaptation by environmental micro-organisms to degrade particular substances is a natural phenomenon. Low-level pre-adaptation tests could include using a sample from a completed ready biodegradability test to inoculate a subsequent ready biodegradability test. This may reduce the lag period preceding the onset of biodegradation.

- Semi-continuous biodegradability tests. Semi-continuous test systems help maintain the diversity, viability and nutrient status of the biodegradability tests whilst allowing the potential for adaptation to be determined over time such as in the semi-static version of the OECD 309 test (OECD, 2004; EC, 2009a).

The potential enhancements, described above have been published in the ECHA guidance (EC, 2009a) and it was concluded that they would benefit from being ring-tested by appropriate international standards bodies. Although test substances that degrade in these enhanced biodegradability screening tests will not be considered as readily biodegradable, they will be considered as not meeting the screening criteria of persistence.

Inherent Biodegradability Tests

The REACH guidance, on information requirements, (ECHA, 2008a), states that data from inherent biodegradability tests would not normally be used to determine persistence except where a clear lack of biodegradation (<20 % biodegradation in an inherent test) can indicate a lack of environmental biodegradation. Nevertheless, such data can be examined to determine whether the degradation in the test was sufficiently rapid to meet the special criteria detailed elsewhere in the document (ECHA, 2008a). If these conditions are met, then the data can be used at the screening

stage and further testing avoided. Where extensive mineralisation occurs, with bacteria that have not been pre-adapted, in a MITI II study (OECD 302C - pass level 70%, OECD, 2009) within the first 14 days, or in a Zahn-Wellens study (OECD 302B – pass level 70%, OECD, 1992b) in 7 days, these data can be used to conclude that the substance is not persistent.

Following the discussions held during the preparation of the guidance for REACH, ECETOC held a workshop (ECETOC, 2007), to identify the research that was necessary to address many of the issues that had arisen in the development of the guidance. There was a broad consensus at that workshop that an enhanced tier of biodegradability screening studies were required to aid in the prioritisation of PBT (Persistent, Bioaccumulative and Toxic) and vP/vB (very persistent/very bioaccumulative) assessments. Enhancements discussed included extending the test duration, increasing the test volume, enhancing the biomass levels and allowing for acclimation. In general these modifications may result in a better chance for observing biodegradation because the most stringent limitations of the ready tests are reduced. It was envisaged that such enhanced tests could contribute to a weight of evidence approach to decide if a chemical is persistent. To help build confidence that modifications would not lead to overly aggressive methods, it was agreed that a validation set of chemicals was required. The development of a reference set of chemicals was also seen as being essential to all the other projects discussed at the workshop, in that an agreed set of chemicals with a range of biodegradability behaviours would be extremely useful in helping to ensure that methods were "tuned" to their task and not overly protective or too powerful.

Figure 1 shows the relationships between the screening and higher tier tests as described above, together with the potential regulatory impact under REACH. The figure also shows how these studies and the bins (see the following section) align. It is important to realise that these are approximations, due primarily to the variable nature of the environment from which samples for biodegradability testing are taken and the changing nature of that environment with time.

Recognition of differing biodegradabilities and the development of the "bins" for the reference chemicals

The principle goal of this work was to develop a set reference chemicals that could be used to help assess modifications to existing biodegradation methods or the development of new methods, in the context of the discussions described above. Chemicals differ in their susceptibility to biodegrade and it is widely accepted that different chemicals biodegrade at different rates. To help prepare a list of reference chemicals covering the spectrum of easily biodegradable, less easily biodegradable, difficult to biodegrade and persistent, the concept of artificially defined bins has been adopted. The sole purpose of these bins is to group

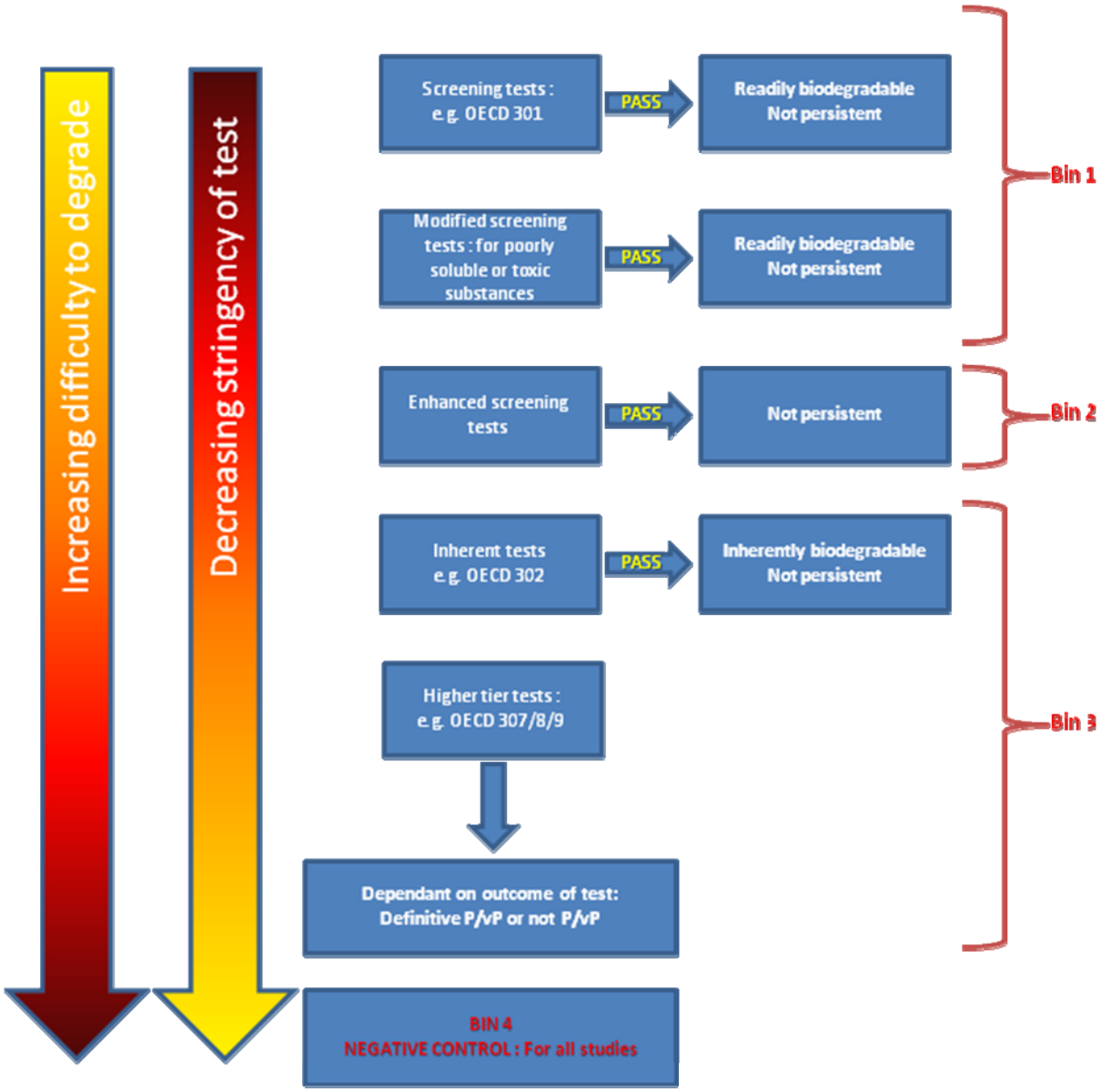


Figure 1: Relationship between screening and higher tier biodegradability tests and the bins

chemicals on their susceptibility to biodegrade and to help identify their usefulness in addressing biodegradability research. The bins should not be considered to be of any use for the Classification and Labelling of chemicals.

A number of publications have suggested the use of bins to differentiate between chemicals that differ in their susceptibility to biodegrade e.g. Blok (2001), Beek *et al*., (2001), SOMS (2002) and

ECETOC (2003). Blok (2001) proposed a system which allocated chemicals to one of 8 different categories based on 3 criteria (rate, probability of competent organisms, and extent). The other three approaches are similar and propose four persistency classes based on the relative power of the test, the result, the endpoint measured, the extent of biodegradation and the potential for bound residues and/or metabolites to exist. In this work, the bins were initially based on those described by ECETOC (which include half-life ranges), which allowed a list of chemicals to be developed. However, the half-lives described by ECETOC are not aligned with REACH regulatory criteria relating to persistency and when developing the final bin descriptions, the likely behaviour in standard tests and known behaviour in the environment were the principle factors considered in assigning the reference chemicals.

Bin 1. These chemicals should usually/regularly pass a ready biodegradability test (RBT) and modified RBT

The comparable bin in the ECETOC (2003) approach is P4, where similar criteria are used. These chemicals ought to pass any of the standard ready tests (OECD 301 series). Chemicals passing these tests can be assumed to have very high potential for biodegradation that will be manifested in all aerobic environmental compartments. In the literature review, chemicals with half-lives of 15 days or less were initially assigned to this bin.

Bin 2. These chemicals should pass an enhanced screening biodegradability test but may fail other more stringent screening tests, for example, an OECD 301 unmodified test. This bin includes other classes of chemicals that are termed “difficult” and for which it can be difficult to demonstrate ready biodegradability and need:

- Increased test duration;
- Testing in larger vessels;
- Increasing biomass concentration;
- Low level pre-adaptation test systems, or
- Semi-continuous biodegradability tests.

In the ECETOC report this was P3, and contained chemicals that could fail the standard or modified ready test, but passed either an aerobic sewage treatment simulation test (e.g. OECD 303 test; OECD 2001a,b); an inherent biodegradability test (OECD 302; according to the criteria

described in Section 2.3.6.4 of the REACH TGD); a ready test using an adapted inoculum (adaptation period of up to 28 days maximum); a standard laboratory soil study (OECD 304, OECD, 1981a); or a standard marine biodegradability study (OECD 306, OECD, 1992c). These results indicate a strong potential for adaptation and growth-linked biodegradation. In the literature review chemicals with median half-lives of between 16 and 40 days were initially assigned to this bin.

Bin 3. These chemicals will normally fail any screening test whether modified RBT or an enhanced screening test.

In describing this bin, a differentiation is made in the extent of adaptation that can occur when using an enhanced screening test. In particular it is recommended that the semi-continuous approach should be of a limited duration. ECETOC described this as P2, which contained chemicals that could pass any of the standard inherent tests (OECD 302 series) without satisfying criteria for demonstration of ready biodegradability. Results from water/sediment tests (e.g. OECD 308 or non standard equivalent studies), anaerobic studies and additional evidence from biodegradability studies (e.g. pure culture studies or co-metabolism studies) may be used to indicate biodegradation might be expected in the environment. When reviewing the literature data, chemicals that were assigned median half-lives of greater than 40 days but less than 60 days were initially placed in this bin.

Bin 4. These chemicals should never pass a modified RBT or an enhanced screening test.

In terms of the ECETOC categories, chemicals in this bin would be assigned P1. These chemicals fail any of the above tests and there is no evidence of biodegradation. The chemicals in bin 4 would act as a negative control and ought never to pass a screening biodegradability test, regardless of the modifications made to the test conditions. In assessing the literature, chemicals with assigned median half-lives in excess of 61 days were initially placed in this bin.

It is important to emphasise that the descriptors associated with the bins and their respective half-lives, are not prescriptive. It refers to observed results under the test conditions and the half-lives are not applicable for environmental modelling. In addition, they should not be considered as inherent chemical properties that can be applied for Classification and Labelling. It should also be recognised that the last two bins could be collapsed into one, as these chemicals would not normally be expected to pass a screening test (and would therefore contain some “false negatives”, as well as chemicals that were probably persistent). However, it was decided to continue with a differentiation as this would help in assessing developments in higher tier tests.

Methods and Materials

The preliminary list of validation chemicals was prepared on the basis of information from five separate and distinct sources:

a) Review of chemicals used as reference chemicals in standard tests (e.g. OECD, ISO);

b) Review of chemicals used in biodegradability test method development studies (e.g. Cefic LRi projects Eco 2a and 11 (www.cefic-lri.org), Torang and Nyholm (2005), EU Nomiracle (www.nomiracle.jrc.ec.europa.eu/.);

c) Review of results from standardised ready biodegradability tests;

d) Review of regulatory priority lists (e.g. UNEP POPs; EU PBT, EU Existing Substances Regulation reports (ESR));

e) Measured half-life data from published literature.

In reviewing the data the same information appeared in more than one database, but these replicated data were not removed from the lists as they were assembled.

Development of preliminary list of possible chemicals

Many, but not all, of the standardised biodegradability test methods (e.g. OECD, ISO, OPPTS) include information on suitable reference chemicals recommended for use as positive control chemicals. These chemicals have usually been recommended either based on the results of inter-laboratory test validation exercises or based on expert judgement. A list of possible reference standards was prepared following a review of the current tests protocols described in national and international guidelines for biodegradability testing (Appendix 1).

The second group of chemicals that were considered for inclusion in the preliminary list was based on a review of the chemicals that have been used, or are currently being used, in biodegradability test method development research, including the results from inter-laboratory ring tests. Many of the publications describing studies to improve testing procedures for biodegradability have used model compounds considered to have different biodegradability behaviour, ranging from easily biodegraded to recalcitrant whilst others use substances considered to be readily biodegradable. This review was not intended to be an exhaustive review but was aimed at identifying chemicals considered by research workers to fall into one of these categories. Examples of such work include O'Malley (2006) who investigated the influence of

test substance concentration and inoculum on the results of the OECD 301F respirometry test using sodium acetate as a model substance. Ingerslev and Nyholm (2000) determined biodegradation rates of ^{14}C-labelled chemicals at low concentrations in surface waters. The chemicals they tested ranged from easily biodegradable to recalcitrant. Aniline was biodegraded after no lag period with half-lives of 10-20 days. Pentachlorophenol and 2,4 dichlorophenoxy acetic acid were biodegraded after lag periods of 0-30 days and exhibited slightly longer half-lives than for aniline. Results with 4-chloroaniline, maleic anhydride and pentachlorophenol indicted that biodegradation sometimes failed and atrazine was not biodegraded at all.

Ahtiainen *et al.*, (2003) used aniline as a readily biodegradable reference and 4-chloroaniline as a more persistant reference chemical to compare degradation rates in standard tests and at realistic environmental concentrations. Thouand *et al.*, (1995) investigated the impact of inoculum density on the probability of 4-nitrophenol biodegradation by activated sludge and river water inocula. Davenport *et al.*, (2008) and Goodhead *et al.*, (2008) used a nitrobenzene diazonium tetrafluoroborate (4-NBTFB) assay with substituted phenols as reference chemicals in their studies on the impact of inoculum density. This work is being further developed under funding from Cefic LRi in Project Eco 11 using 4-hydroxybenzoic, 4-nitrophenol and 4-fluorophenol as progressively more difficult to degrade reference chemicals (Davenport *et al.*, 2009). Hales *et al.* (1996) used aniline, ethanolamine, 1,6-hexanediol and pentaerythritol as rapidly and readily biodegradable reference chemicals when developing a respirometric test with improved sensitivity. Aniline, 1,6-hexanediol and ethanolamine were shown to pass the OECD 301B, D and F and 301C Miti tests, but pentaerythritol was not degraded under 301D conditions and only in seven out of ten vessels under 301B conditions.

Torang and Nyholm (2005) described a semi-continuous adaptation method (SCAM) to study the effect of adaptation on biodegradability using a different set of model chemicals of known and differing degrees of ease of biodegradation (aniline, 4-nitrophenol, 2,4-dichloroacetic acid and 4-chloroaniline). Following pre-adaptation of between one and five weeks, the lag phases were reduced from 5.2 to <1 day for aniline, 10 days to <1 day for 4-nitrophenol, from 24 days to <1 day for 2,4 dichloroacetic acid and from 88 days to 9 days for 4-chloroaniline in experiments with river water. Unilever (2009) used the method described by Torang and Nyholm (2005) to study the effects of adaptation on the biodegradation of aniline, 4-chloroaniline and 3,4-dichloroaniline at 10 and 100 $\mu g\ l^{-1}$ and potential reduction in the lag phases in surface waters. Lapertot *et al.*, (2006) reported on a study to identify the most suitable method for testing 19 priority list chemicals with a wide range of physico-chemical and toxicological properties. Nyholm (1990) studied biodegradation of poorly water-soluble compounds in the MITI test using anthraquinone and di-isooctylphthalate as reference compounds. When developing the Zahn-Wellens test to allow for continuous O_2 measurements, Norr *et al.*, (2001), used phenol as the readily

biodegradable reference and diethylene glycol as the inherently biodegradable positive control. Aniline was also one of the reference chemicals used in the EU funded Nomiracle project (www.nomiracle.jrc.ec.europa.eu/.) looking at sediment simulation test methodology.

Astrazeneca (2000) proposed six chemicals covering a range of biodegradabilities as the first step in their research programme aimed at understanding and measuring persistence in the marine and terrestrial environments. Their approach to identifying suitable chemicals was based on identifying the criteria which were most likely to influence the degradation of a chemical in the environment (i.e. partitioning parameters, environmental parameters and chemical structure) and the persistence criteria in various priority lists. The preferred source was the US EPA chemical ranking report for the RCRA PBT (Resource Conservation and Recovery Act – Persistent, Bioaccumulative, Toxic Chemicals) list which contains half-life data (predicted or measured) for 1210 chemicals. The chemicals recommended for use in the experimental phase were phenol, caffeine, naphthalene, pentachlorophenol, benzo(a)pyrene and DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane).

A list of all the chemicals identified as being used in biodegradability method development studies is given in Appendix 2.

The third source of data was from standard ready biodegradability studies and the reference substances used therein. Results from standard laboratory tests (particularly OECD ready and inherent biodegradability tests) are available for many more substances than field studies. Most of this data came from industry, including a dataset of mostly unpublished (GLP) data derived from OECD 301F studies on hydrocarbons provide by ExxonMobil, from EU Existing Substance Risk Assessment (ESR) reports (see http://ecb.jrc.ec.europa.eu/existing-chemicals/) and from the database prepared by Biomath at the University of Gent as part of the ERASM (http://www.erasm.org/) funded 10-day window project (Nopens *et al.*, 2000).

The fourth source of possible candidates came from a review of the priority lists from different regulatory regimes. These included:

- United Nations Environment Programme Persistent Organic Pollutants (http://www.chem.unep.ch/pops/);

- EU Technical Committee for New and Existing Substances PBT Working Group (see Appendix 3);

- EU Existing Substances Regulation (ESR) risk assessment reports (http://ecb.jrc.ec.europa.eu/existing-chemicals/).

These sources were used because, in many cases, an extensive review of the biodegradability data had been conducted and agreed with the participating regulatory authorities. These were used with some caution, however, as the purpose of the review was different to the needs of this project. For example under the ESR, the main purpose was to assign the chemicals biodegradable status, ready, inherent or not, to which half-lives were then assigned.

The final step in developing the preliminary list was to identify sources of empirical data. For the data to be included in the measured biodegradation rate list the following quality criteria had to be met:

- The biodegradation occurred under aerobic conditions;
- Single chemicals were tested (including the identity and purity);
- Biodegradation rates and extent were given;
- The origin of the inoculum source including any pre-treatment was available;
- How degradation was measured (primary or ultimate degradation).

The emphasis, in this phase, was put on the identification of existing biodegradability databases. The datasets described by Aronson *et al.,* (2006); Arnot *et al.,* (2005); Syracuse (SRC, 1999); an ExxonMobil hydrocarbons database (personal communication) and the ECETOC marine database (ECETOC, 2009) were evaluated and found to be the most comprehensive and appropriate based on the criteria listed above. During the initial phase, measured rates from standardised and non-standardised laboratory tests and field studies for freshwaters, marine waters, sediments and soils were collated.

The initial list of compounds included in the Aronson *et al.,* (2006) database was developed by querying the BIODEG file of EFDB (www.Syracuse.com) for compounds having data from both screening studies and laboratory grab sample or field studies that used freshwater, seawater, sediments or soils or some combination of these. Additional compounds and data were obtained from Aronson *et al*., (1998), Boethling *et al*., (1995), the US EPA's Office of pesticide programs website (www.Epa.gov/pesticides/registration) and the Japanese Chemicals Inspection Testing Institute database (www.cerij.or.jp/ceri_en/otoiawase). Data were separated into two files (one for

primary and one for ultimate biodegradation). The primary degradation dataset covered 228 compounds and the ultimate degradation dataset contained both screening and environmentally relevant data for 77 compounds.

Arnot *et al*., (2005) compiled primary half-life data for 115 chemicals from environmental handbooks, the Syracuse Research Corporation BIODEG database and from primary literature. The data were largely aqueous aerobic half-lives although some rates were estimated from soil studies.

The Syracuse database 'Aerobic biodegradation of organic chemicals in environmental media: A summary of field and laboratory studies' (SRC, 1999), contains aerobic data and includes biodegradation rate constant information from soil, surface water, sediment, as well as aquifer environments.

The ECETOC marine biodegradation kinetics database (EMBK) (ECETOC, 2009) was prepared specifically to compare measured biodegradation rates in the fresh and seawater environments. It contains 650 kinetic data for 125 different chemicals, mainly from marine environments. Twenty six chemicals had half-lives for freshwater (representing 126 of the data points) and marine waters (381 data points).

The final source of half-life data was provided by ExxonMobil. This dataset is a combination of published (e.g. Prince *et al*., 2007, 2008) and unpublished data derived from freshwater and seawater studies on the primary biodegradation of gasoline and biodiesel hydrocarbon mixtures. Whilst these studies did not meet the single chemical criteria indicated above, the dataset was very comprehensive and considered worthy of inclusion in the weight of evidence approach adopted in this project.

As these databases consisted of either data presented in peer-reviewed literature or were generated in studies carried out under good laboratory practice (GLP), the information was considered to meet the quality criteria. The product of the first phase was a dataset of biodegradation half-lives for over 120 organic chemicals (see Appendix 4).

At this screening stage of the project, measured data for all environmental compartments were included and no effort was made to remove any duplication. The potential candidates were allocated to one of the four bins and are listed therein in order of the number of half-life data available. The half-life descriptors for the 4 bins are derived from regulatory approaches to assessing the risks of chemicals in the environment. In the Existing Substances Regulations Technical Guidance Document for Risk Assessment (EC, 2003), for example, a chemical that

passes the ready biodegradable test, including the 10-day window, is assigned a half-life of 15 days. The approach has been previously described and justified (EC, 2003) and is not further discussed here. Chemicals in bin 1 (half-life <15 days) correspond to those expected to pass a RBT and in bin 4 (>61 days) are considered to fail screening tests. These two bins represent the extremes of readily biodegradable and difficulty to biodegrade. Chemicals which had been assigned half-life ranges of 16-40 days and 41-60 days were initially assigned to bins 2 and 3 and relate to chemicals which are expected to pass or fail the modified or enhanced tests respectively. Finally as part of this review a list of chemicals used, or in use, in CEFIC LRI projects has also been prepared (see Appendix 8). Although useful as a potential source of readily available chemicals upon which research was already being conducted, the chemicals from this list were not considered of value to the selection process, primarily due to the limited data available on their potential to degrade.

Results

Preparation of a refined list of possible chemicals.

Physico-chemical properties

As well as gathering information on the biodegradability of chemicals, as the list was refined, the physico-chemical properties of the chemicals were also gathered. This was restricted to those chemicals that were under consideration for inclusion in the final listing. The properties that were addressed and a brief description of their importance are given below:

Solubility in water – Screening biodegradability tests require test substance concentrations in the range of 2 to 100 mg l^{-1}. Testing above the water-solubility limit for the substance means that both the rate and extent of observed biodegradation would be confounded by bioavailability limitations and dissolution kinetics (Aichinger *et al.*, 1992). In considering whether the reference substances span a range that would be of interest to researchers, three ranges were used to describe the aqueous solubilities. These were >500 mg l^{-1}, 1-500 mg l^{-1} and <1 mg l^{-1}, representing high, medium and low solubilities.

Hydrophobicity (log K_{ow}) – The octanol-water partition coefficient is a useful parameter for assessing the potential ease of which a chemical will partition from water into organic phases. It is also therefore correlated, for non-polar molecules with the potential to partition into organisms and to be adsorbed onto particles and other surfaces. The more hydrophobic a chemical is the more likely that it is that it will adsorb to surfaces and particulates, which will in turn reduce its bioavailability. To ensure that a reasonable range of log K_{ow} values were addressed, the following

ranges were used to describe the chemicals, <2, 2-5 and >5, representing low, medium and high hydrophobicity.

Vapour pressure (VP) and Henry's Law Constant (HLC) – a chemical that is volatile and poorly water-soluble may readily leave aqueous solutions before having the opportunity to biodegrade. Some current protocols are suitable for volatile substances (e.g. OECD 301D and OECD 310, OECD 2006). The guidelines for the OECD 309 simulation test state that the test is applicable to non-volatile or slightly volatile organic substances tested at low concentrations. Using flasks open to the atmosphere (e.g. cotton wool plugged), substances with HLCs of less than about 0.00001 Atm m^3 mol^{-1} can be regarded as non-volatile in practice. Using closed flasks with a headspace, it is possible to test slightly volatile substances (with HLCs <0.001 Atm m^3 mol^{-1}) without losses from the test system. In assessing volatility, where a substance's HLC is >0.1 Atm m^3 mol^{-1} it has been considered volatile and where <0.00001 Atm m^3 mol^{-1}, non-volatile.

The physico-chemical properties quoted in this report have been obtained from SRC EPISUITE programme (http://www.srcinc.com/what-we-do/product.aspx?id=138). Although in some cases experimental data were available, a consistent approach of using the calculated data points was adopted in the project.

Other properties and considerations for choosing reference chemicals

A number of other important criteria were also assessed when drawing up the final list of chemicals. These are briefly discussed below:

- Availability of the chemical – radio-labelled and purity – was the chemical available commercially as a laboratory reagent both as a cold material, but also for low concentration studies (or higher tier studies) was it available as a radio-labelled chemical?

- Hazard relating to handling – were there known hazards relating to the handling of the chemical that might impact the type of study for which it could be used or how the studies might be designed?

A refined list of possible reference chemicals was prepared by cross referencing the chemicals identified by the five approaches detailed above.

The review of the chemicals recommended as positive reference chemicals in standardized biodegradability test guidelines and the chemicals used to demonstrate different ease of biodegradability used in biodegradability test method development studies produced a list of 39 possible candidates for the validation set (Appendix 2).

At this point, the measured data from the individual datasets were combined and duplicate entries were eliminated. A half-life default of 150 days was used in calculations whenever the half-life in the publication was quoted as >150 days to avoid long half-life values derived by extrapolation from having a disproportionate influence on the mean half-life.

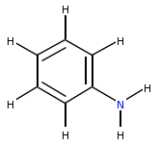
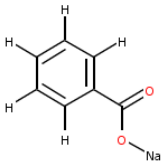
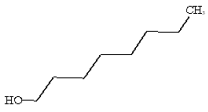
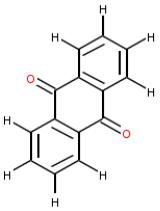
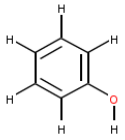
By cross-referencing the chemicals in Appendix 2 with the chemicals for which empirical data were available (Appendix 4), a weight of evidence approach has been used, to identify chemicals which could be considered likely to either pass or fail an enhanced ready biodegradability test. The candidates, identified using this approach, were then allocated to one of the four bins described above. The EU list of possible PBT chemicals (Appendix 3) was used to identify additional candidates for bins 3 and 4 which resulted in 46 possible reference chemicals (see Appendix 5).

A number of other considerations were taken into account before concluding whether a chemical would be suitable for inclusion as a possible reference chemical in the validation set. The physico-chemical properties of the candidate chemicals were assessed to identify chemicals which would be suitable reference chemicals when testing poorly water-soluble or volatile chemicals. In addition to these criteria any hazardous properties relating to handling and the toxicity of the chemical to micro-organisms were identified. Other issues included whether the chemical was readily available, whether a radio-labelled form of the chemical was commercially available and whether there are specific and selective analytical techniques for determining the chemical in test media. The potential for monitoring data being available for the candidate chemicals was also assessed, although an extensive search for such data was not undertaken. Tables 1-4 in Appendix 6 show the possible candidate chemicals, for each of the biodegradability bins, prepared using information from the five distinct approaches.

Reference chemicals

The chemicals detailed below were selected from the lists in Appendix 6. The chemicals represent a range of biodegradabilities, extending from ‘soft’ (readily biodegradable) to ‘hard’ (recalcitrant). They should provide researchers with the means to demonstrate that any modifications or enhancements to existing test methods, or any newly developed tests for assessing persistence, do not result in test conditions that are too favourable, or too stringent, for biodegradation. When developing a new method, positive and negative control reference chemicals should be chosen from the appropriate bins.

Reference chemicals that would normally pass a RBT and a modified RBT – Bin 1

Aniline	Sodium benzoate	1-octanol	Anthraquinone	Phenol
				

The five reference chemicals recommended as positive reference controls and which should pass any ready test (including modified and enhanced) are aniline, sodium benzoate, 1-octanol, anthraquinone and phenol. Aniline and sodium benzoate are usually very well biodegraded (see comments under each chemical). Anthraquinone (solid) and 1-octanol (liquid) are proposed as positive control standards which would be useful in studies designed to improve test methodologies for poorly water-soluble chemicals and phenol is proposed as a positive control reference chemical especially useful when studying volatile chemicals.

Aniline: Aniline is a reference chemical recommended for use in standardized ready tests including OECD, ISO and OPPTS tests. It has been studied intensively over the past 20 years (e.g. Painter and King, 1985) and shown to be suitable as a positive control in standard tests. Although the biodegradation of aniline normally exceeds 60% and the lag phase is often <4 days, this chemical can sometimes have a longer lag phase and lead to a 'fail' in the test. Measured data (n=36) (see Appendix 4 and 5) in non-standard tests indicate a median half-life of less than 5 days in the freshwater environment and supports the conclusion that this chemical would usually pass any RBT test. Aniline is a liquid at room temperature, with a water solubility of 2 g l^{-1} and a HLC of 0.47 Atm m^3 mol^{-1}.

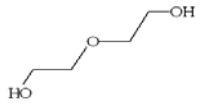
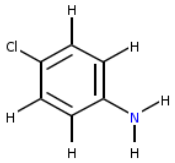
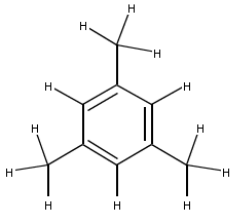
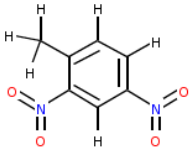
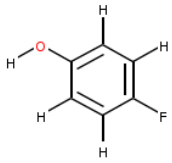
Sodium benzoate: Sodium benzoate is a reference substance recommended for use in standardised ready tests including OECD, ISO and OPPTS tests. It has been studied intensively over the past 20 years (e.g. Painter and King, 1985) and shown to be suitable as a positive control in standard tests. Biodegradation consistently exceeds 60% CO_2 for sodium benzoate and the lag phase is often <1 day. Sodium benzoate is a solid at room temperature, with a water solubility of 56 g l^{-1} and a HLC of 0.0000005 Atm m^3 mol^{-1}.

1-octanol: 1-octanol is recommended as a poorly water-soluble reference chemical in standard tests e.g. OECD 310 and OPPTS 835.3140. The ISO ring test, reported in the OECD 310 test guideline, involving 14 laboratories resulted in a mean biodegradation of 85% (Battersby, 1997). 1-octanol is a liquid at room temperature, with a water solubility of 540 mg l^{-1} and a HLC of 2.1 Atm m^3 mol^{-1}.

Anthraquinone: Anthraquinone is recommended as a reference standard in ISO 10634 (ISO, 1995). de Morsier *et al.,* (1987) reported 75% biodegradation after 24 days (20 mg l^{-1} related to test chemical) in an OECD 301B study and 95% biodegradation after 25 days (100 mg l^{-1} related to test substance) in an OECD 301C. Bayer report >70% biodegradation of anthraquinone after 20 days (0.8 mg l^{-1} related to test substance) in an OECD 301D study (OECD, 1992a). Nyholm (1990) used anthraquinone as a model compound when evaluating the use of various dispersion techniques in the testing of insoluble chemicals. Anthraquinone is a solid at room temperature, with a water solubility of 3.9 mg l^{-1} and a HLC of 0.000005 Atm m^3 mol^{-1} and is recommended as an alternative positive control to 1-octanol when addressing poorly water-soluble chemicals.

Phenol: Phenol was on the first priority list for risk assessment in the EU under the Existing Substances Regulation. The EU Existing Substance Regulation report on phenol concluded from the results of standard biodegradability tests that phenol is readily biodegradable and from available investigations on the biodegradation of phenol in surface waters a rate constant of 0.05 d^{-1} was determined (EU, 2006). Phenol was used as a biodegradable volatile reference chemical by Norr *et al*., (2001) in their studies to improve the Zahn-Wellens test (an inherent test). Phenol is a solid at room temperature, with a water solubility of 2.6 g l^{-1} and a HLC of 0.15 Atm m^3 mol^{-1} and is recommended as a positive control chemical when addressing volatile chemicals.

Reference chemicals that would normally pass an enhanced screening biodegradability test but currently fail any other screening tests – Bin 2.

Diethylene gycol	4-chloroaniline	1,3,5 trimethylbenzene	Di-nitrotoluene	4-fluorophenol
				

The reference chemicals proposed for bin 2 are all reported to behave erratically in RBTs, and give contradictory results but could be expected to normally pass an enhanced ready test. They are:

Diethylene glycol: Diethylene glycol (DEG) is recommended as a reference chemical for the OECD 302A (OECD, 1981b) inherent test. Lapertot *et al.,* (2006) used DEG as the reference chemical in a Zahn-Wellens test and reported 70% degradation in 10 days. Painter and King

(1985) reported lag phases of 1-9 days with a 'pass' being achieved in 9 out of 11 tests. van Ginkel and Stroo (1992) report degradation of 46% after 28 days, 66% after 42 days. Zgajnar Gotvajn and Zagorc-Kocan (2003) reported that DEG failed a 301D test but passed a 301F test. Reushenbach *et al.,* (2003) carried out a critical comparison of respirometric biodegradability tests based on OECD 301 and reported that DEG failed (59%) an OECD 301F using the Sapromat test but passed (89%) an OECD 301F using Oxitop. DEG was used as a biodegradable reference chemical by Norr *et al.,* (2001) in their studies to improve the Zahn-Wellens test (an inherent test). Diethylene glycol is a liquid at room temperature, which is miscible with water and a HLC of 0.00004 Atm m^3 mol^{-1}.

4-chloroaniline: 4-chloroaniline (4-CA) shows erratic behaviour in RBTs. The conclusion in CICAD No 48 (WHO, 2003) is that 4-chloroaniline is considered to fail a ready test (e.g. it failed a closed bottle test in 3 studies referenced, e.g. Rott, 1981a). More than 60% removal was observed in inherent biodegradability tests, however, nearly half of the elimination could be attributed to adsorption (Rott, 1981b; Haltrich, 1983). Ahtiainen *et al.* (2003) report a fail in an ISO 14593 (ISO, 1998) study. Other studies (Roberts, 2009) would indicate a pass with typical results showing a 15-20 day lag phase but 60-70% CO_2 released after 28 days. Torang and Nyholm (2005) described a semi-continuous adaptation method (SCAM) and reported that following an adaptation period of between one and five weeks the lag phase was reduced from 88 days to 9 days. Unilever (2009) used SCAM to study the effects of adaptation on biodegradation and potential reduction in the lag phase of 4-CA using surface water. With no adaptation and a 10 µg l^{-1} test concentration, only 30% biodegradation had occurred after nearly 200 days. At 100 µg l^{-1} only 58% biodegradation occurred with a lag of <44 days. After 22 weeks adaptation, biodegradation of 68.2% and 71.6% was found at test concentrations of 10 µg l^{-1} and 100 µg l^{-1}, respectively. The lag phase was <2 days at both test concentrations. Measured data (n=10) in non-standard tests suggest a median half-life of 133 days with a range from 93-150 days. 4-chloroaniline is a solid at room temperature, with a water solubility of 3900 mg l^{-1} and a HLC of 0.16 Atm m^3 mol^{-1}.

1,3,5-trimethylbenzene: 1,3,5-trimethylbenzene (1,3,5-TMB) is reported to fail the modified 301F test (ExxonMobil, 2009a). In non-acclimated tests the day 28 mean percentage biodegradation was 36%, (standard deviation of 8%). A plateau was reached at 56% biodegradation (standard deviation of 0.4%) by day 53, after which there was no further biodegradation and the test was terminated day after 67 days. With acclimated inoculum (i.e. re-inoculated) biodegradation reached 72.2% biodegradation after 28 days. Prince *et al*., (2008) studied the biodegradation of biodiesel B20 (a complex hydrocarbon mixture) in pond water and calculated a primary biodegradation half-life for C3 substituted benzenes (including 1,3,5-TMB) of 4 days. In studies with gasoline (Prince *et al*., 2007) concluded a median half-life for 1,3,5-

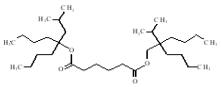
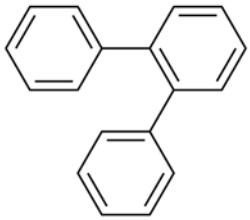
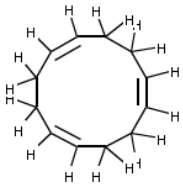
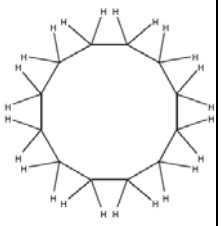
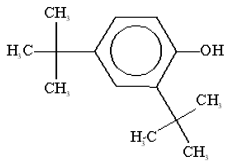
trimethylbenzene of 3.2 days in pond water. The biodegradation of mixtures of substituted aromatic hydrocarbons, including 1,3,5-TMB, using enrichment cultures has been described by Leahy *et al.,* (2003). Theses results suggest that while 1,3,5-TMB can undergo rapid primary biodegradation when tested as part of a mixture of hydrocarbons, it would not be expected to pass a pure substance RBT. Whilst there is limited standard test data available on the biodegradability of this chemical it is considered to be a useful inclusion in the reference list for use in developing methods for volatile and poorly water-soluble substances. 1,3,5-trimethylbenzene is a liquid at room temperature, with a water solubility of 120 mg l^{-1} and a HLC of 0.007 Atm m^3 mol^{-1}.

2,4-dintrotoluene: A SIDS assessment of 2,4 dinitrotoluene (2,4-DNT) was published in 1996 and the conclusion was that 2,4-DNT is biologically inherently biodegradable with adapted inoculum only in aqueous solution (SIDS, 1996). 2,4-dinitrotoluene is on the 4th priority list under Council Regulation (EEC) Nº 793/93 on the control and evaluation of the risks of existing substances. The final Risk Assessment Report was published by the European Chemical Bureau in 2008 (EU RAR, 2008). The last literature research for the RAR was carried out in 2005. 2,4-DNT has also been reviewed in the EU exercise aimed at the identification of a substance as a CMR cat 1 or 2, PBT, vPvB or a substance of an equivalent level of concern (EC, 2009b). The available biodegradability data show that 2,4-dinitrotoluene can undergo primary biodegradation to form several products. Furthermore, in the light of the available information 2,4-dinitrotoluene should be degraded by biological sewage treatment when suitable acclimation is provided to the cultures, so it can be classified as inherent biodegradable with adapted inoculum and not-ready biodegradable.

Biodegradation has been tested under aerobic and anaerobic conditions. In organic soil the time for 50% disappearance for 2,4-DNT was 7 days and for 90% disappearance, a figure of 191 days was determined. IUCLID 3.5 identifies an aerobic ready test that was performed according to a national Japanese standard method comparable to the OECD TG 301C. After two weeks 0 % biodegradation was observed (MITI, 1992). Bausum *et al.,* (1992) studied 2,4-DNT using an enrichment culture from natural surface water downstream from an ammunition plant and found 45-64 % mineralisation after 35 days (dependent on concentration). They also studied 2,4-DNT in natural surface water (4 different sites) and reported no degradation within 6 weeks. Spanggord *et al.,* (1981) examined the biodegradability of several DNT isomers (2,3-,2,4-, 2,5-, 2,6-, 3,4- and 3,5-DNT) but found no mineralisation during a 6 week incubation with natural local waters. From the available studies it has been concluded that 2,4-DNT undergoes primary biodegradation and can also be mineralised by selected adapted microbial cultures under specific conditions. 2,4-DNT is a solid at room temperature, has a water solubility of 450 mg l^{-1} and a HLC of 0.00000005 Atm m^3 mol^{-1}.

4-fluorophenol: 4-fluorophenol is being used by Davenport *et al*., (2009) as a reference substance in their studies to investigate the importance of microbial density and diversity in inocula for use in RBTs. Contrary to their expectations, the initial results showed high probabilities of biodegradation for 4-FP (with reasonably low variation between 6 different locations) when using activated sludge and river water inocula at enhanced microbial densities and extended test duration (60 days). It is considered likely that it would occasionally fail enhanced biodegradability screening tests. 4-fluorophenol is a solid at room temperature, with a water solubility of 30 mg l^{-1} and a HLC of 0.27 Atm m^3 mol^{-1}.

Reference chemicals that would normally fail any biodegradability screening test whether modified RBT or enhanced screening biodegradability test – Bin 3

Di-isotridecyl adipate	o-Terphenyl	Cyclododeca-1,5,9- triene	Cyclododecane	Dibutylphenol
 **DITA is a mixture of complex isomers of the isotridecyl carbon chain**				

There is little data for substances exhibiting this type of behaviour in screening type biodegradability tests. However, based on the limited studies and on results from non-standard tests the following substances are proposed as substances for bin 3 of the validation set:

Di-isotridecyl adipate: Di-isotridecyl adipate (DITA) is recommended as a reference substance in OECD 302D, OPPTS 835.3215 and CEC L-33-A-93. The OECD 302D guidelines (OECD, 2001c) states that to demonstrate the increased biodegradative power of the test over a ready biodegradability test, di-isotridecyl adipate (DITA) can be used as a more difficult to biodegrade reference substance. These guidelines also quote the results from the CONCAWE 1996/97 ring-test of the method involving 10 laboratories. A mean biodegradation of 65% after 56 days was obtained (Battersby *et al.,* 1999). DITA is typically biodegraded by only around 30% after 28 days with an unexposed inoculum (e.g. in OECD 301 B) but can be mineralised by 40 - 80% in the OECD 302D test. Since DITA is a mixture, it may be an inappropriate reference substance for certain types of study. Di-isotridecyl adipate is a solid at room temperature, with a water solubility of <0.001 mg l^{-1} and a HLC of 0.0007 Atm m^3 mol^{-1}.

o-Terphenyl: Terphenyl is an aromatic hydrocarbon consisting of a chain of three benzene rings. There are three isomers in which the terminal rings are ortho-, meta-, or para-substituents of the central ring. Terphenyl (as a mixture of the three isomers) has recently been assessed under the EU Technical Committee – New and Existing Substances working group on PBT/vPvB substances (ECHA, 2008c). In that assessment the following text appears;

"7-10% biodegradation after 50 days, CO_2 evolution, Acclimated inoculum, Reference: Monsanto report ES-82-SS22.

50 % loss in 16-28 day, River die-away test, comparative study, Reference: Monsanto study MO20020457. In this test with a mixture of the terphenyl isomers, 80% degradation was observed for the o- and m-terphenyl within 45 days, with a half-life of 16-28 days. MITI (1992) ISBN 4-89074-101-1 reports "Not readily biodegradable, 0.5 % after 14 days: MITI I test, 100 mg l^{-1} of terphenyl, 30 mg l^{-1} sludge". ExxonMobil (2009b) reported that the measured primary biodegradation half-live in seawater for m-terphenyl was between 15-32 days in a test lasting 182 days. These data are for primary biodegradation and relate to results in seawater in studies where the terphenyl was introduced into the test media in a complex mixture of hydrocarbons.

While more data are required, the EU Technical Committee concluded that terphenyl is very unlikely to biodegrade rapidly under the normal or enhanced/modified screening tests. When being used as a reference chemical in method development care will need to be taken in interpreting the biodegradation results. It needs to be recognised that, as a mixture of three isomers, there will be some differences in their biodegradability and hence the overall mineralisation. The proposal, based on very limited data, is that the reference chemical for this bin should be the o-terphenyl as based on this data, this is the least likely of the three to pass an enhanced test. o-terphenyl is a solid at room temperature, with a water solubility of 0.6 mg l^{-1} and a HLC of 0.9 Atm m^3 mol^{-1}.

Cyclododeca-1,5,9-triene: Cyclododeca-1,5,9-triene (CDT) is one of the chemicals on the EU PBT list and has been the subject of a TC NES evaluation (ECHA, 2008d). Davis *et al.*, (2006a) tested 1,5,9-cyclododecatriene in an OECD 301F ready biodegradability test at concentrations of 1 and 10 mg l^{-1} at 20±2 °C using activated sludge (final concentration 30 mg SS l^{-1}) as inoculum for 60 days (i.e. an enhanced RBT). The results indicated, that CDT is not readily biodegradable but that primary biodegradation was 60% at 10 mg l^{-1} and complete at 1 mg l^{-1} after 60 days. Davis *et al.*, (2006b) tested CDT in an OECD 301B test at concentrations of 0.2 and 1mg l^{-1}. $^{14}CO_2$ production reached about 50% and 70 % at 0.2 and 1 mg l^{-1} test concentration after 63 and 77 days respectively. In this study, primary degradation of CDT was complete after 42 days at the lower exposure level and 38 % of CDT was remaining after 60 days at the higher exposure level.

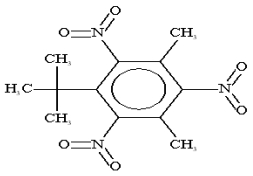
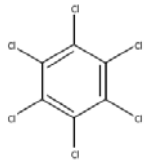
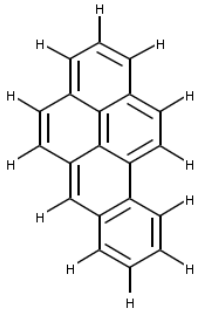
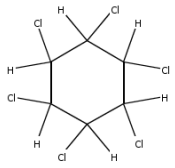
A phase of slow mineralisation was exhibited for about 35 days (44.5 % CDT remaining; 11 % of CDT mineralised at 0.2 mg l^{-1} level). CDT was therefore considered to be not readily biodegradable, but due to the extent of mineralisation reached, the chemical can be expected to be not persistent. MITI (1992) has reported on a ready biodegradability test (modified MITI method), where 0 % was degraded in 14 days. Huels AG (as cited in EC, 2000a) observed that 0 % was degraded in 28 days in an OECD 301D study. In another ready biodegradability test according to OECD 301D, 1 % of CDT was observed to have degraded after 28 days (DuPont Co., 2000 as cited in Degussa, 2002a). Cyclododeca-1,5,9-triene is a solid at room temperature, with a water solubility of 0.39 mg l^{-1} and a HLC of 0.09 Atm m^3 mol^{-1}.

Cyclododecane: ECHA support document (ECHA, 2008e) for cyclododecane reports that very slow or no biodegradation at all was observed in the tests. Whilst significant degradation has been shown by an adapted inocula with a mixed microbial population and by specific strains, cyclododecane is considered not readily biodegradable. Further information is needed to conclude if the chemical is persistent in the environment. It is not expected to hydrolyse abiotically in the environment. According to MITI (1992), 0-12% of the chemical was degraded after 14 days in a ready biodegradability test with a test chemical concentration of 100 mg l^{-1} and a sludge concentration of 30 mg l^{-1}. The following tests, with non-adapted micro-organisms, are cited in the available chemical datasets (EC, 2000b; Degussa AG, 2002b). A closed bottle test according to the OECD 301 D guideline (Huels-Untersuchung, unveröffentlicht) resulted in 3% degradation in 28 days. In a BODIS test according to ISO 10708 (in preparation) degradation of 18% after 28 days was observed (Huels-Untersuchung, unveröffentlicht). In addition, no degradation was detected in 28 days in a modified Sturm test (C.5. of 84/448/EEC; Hüls AG, 1997). Azolay *et al*., (1983) observed that two of five bacterial strains isolated from Mediterranean sediment from a polluted site grew well using cyclododecane as the sole carbon source. In a test employing a mixed bacterial sediment population from the same polluted site, 30% of cyclododecane in a hydrocabon mixture was degraded after 8 days of incubation at 30ºC. Degradation in sediment from an unpolluted site was used as a reference. In addition, Schumacher and Fakoussa (1999) concluded that *Rhodococcus ruber* CD4 was oxidising cyclododecane as the sole carbon source at 28ºC. Cyclododecane was shown to be oxidized to cyclododecanol and cyclododecanone, followed by ring fission. The resulting lactone gives rise to an omegahydroxyalkanoic acid, which is further degraded by common beta-oxidation. Cyclododecane is a solid at room temperature, with a water solubility of 0.11 mg l^{-1} and a HLC of 1.4 Atm m^3 mol^{-1}.

2,4-dibutylphenol: 2,4-dibutylphenol was tested in the ISO 10634 method (ISO, 1995) for poorly water-soluble chemicals, when 2% biodegradation was observed. In an OECD 301C test (at 100 mg/l) no biodegradation was observed. The TC-NES WG on PBTs (ECHA, 2008f) concluded that the chemical was not readily biodegradable, and the data would suggest it is

unlikely to pass an enhanced test. 2,4-dibutylphenol is a solid at room temperature with a water solubility of 0.6 mg l^{-1} and a HLC of 0.00006 Atm m^3 mol^{-1}.

Reference chemicals that should never pass a modified RBT or an enhanced biodegradability screening test – Bin 4

Musk xylene	Hexachlorobenzene	Benzo(a)pyrene	Hexachlorohexane
			

The following chemicals should never pass a screening test and are therefore recommended as negative control reference chemicals.

Musk xylene: Musk xylene (1-tert-butyl-3,5-dimethyl-2,4,6-trinitrobenzene) was tested with activated sludge at concentrations of 10 and 100 mg l^{-1} (in triplicate) in a 28-d test. It was concluded that musk xylene was not biodegradable under the test conditions (Marks and Marks, 1987). Ready biodegradability of musk xylene was tested a concentration of 107 mg l^{-1} musk xylene in the MITI I test (OECD Guideline 301C). Throughout the test, the level of BOD in the sample with musk xylene was identical to the sample without test substance. It was therefore concluded that musk xylene was not readily biodegradable under the test conditions (Calame and Ronchi, 1989). The PBT draft addendum (ECHA, 2008b) to the final report of the risk assessment (2005) concluded that musk xylene is not readily biodegradable. Hanstveit (2006) reported a GLP study on the degradation of radio-labelled musk xylene in both a marine water sediment system (according to OECD guideline 308) and a marine water-only system (according to OECD guideline 309) at 15±2 ºC for 176 and 159 days respectively. It was concluded in the addendum that the half-life for biodegradation in seawater was more than 150 days and that musk xylene should therefore be considered to be very persistent in water. Musk xylene is a solid at room temperature, with a water solubility of 0.8 mg l^{-1} and a HLC of 0.000000008 Atm m^3 mol^{-1}.

Hexachlorobenzene: Hexachlorobenzene (HCB) is considered a persistent chemical and is on a number of national and international priority pollutant lists (e.g. UNEP POP, Ministry of Environment and Enegy (MOEE) Ontario, US EPA Resource Conservation and Recovery Act) and can be considered as very unlikely to pass a ready test. For example, Rott *et al.*, (1982) using

a radio-labelled sample at 50 μg l^{-1} in the GSF (Forschungszentrum für Umwelt und Gesundheit) test found only 1% released as CO_2. The USEPA RCRA database classifies HCB as having a half-life in surface water of between 42 to 208 days. The Euro Chlor dossier on sources, environmental fate and risk characterisation of hexachlorobenzene (Barber *et al.,* 2005), quotes a half-life ranging from 2.7 to 5.7 years in surface water and 5.3 to 11.4 years in groundwater, based on unacclimated aqueous aerobic biodegradation (Mackay *et al.*, 1992 and Howard, 1991). Hexachlorobenzene is a solid at room temperature, with a water solubility of 0.19 mg l^{-1} and a HLC of 0.0024 Atm m^3 mol^{-1}.

Benzo(a)pyrene: Benzo[a]pyrene is a globally distributed five-ring PAH that is considered to be environmentally recalcitrant. Benzo(a)pyrene is on a number of national and international priority pollutant and persistent chemical lists including, for example, the U.S. Environmental Protection Agency's Priority Pollutant List (Kanaly *et al.,* 2000) and the agency's new strategy for controlling persistent, bioaccumulative, and toxic pollutants (Renner, 1999). The USEPA RCRA database classifies benzo(a)pyrene as having a half-life in surface water of between 42-208 days. Measured data (n=7) in non-standard tests suggest a median half-life of 150 days with a range from 85-150 days. Whilst there have been reports in the literature which document benzo[a]pyrene biodegradation by either pure or mixed cultures of bacteria (Barnsley, 1975; Heitkamp and Cerniglia, 1988; Juhasz *et al.*, 1997; Schneider *et al.*, 1996; Trzesicka-Mlynarz and Ward, 1995; Kanaly *et al.*, 2000), benzo(a)pyrene is highly unlikely to pass any modified RBT or enhanced biodegradability screen. Benzo(a)pyrene is a solid at room temperature, with a water solubility of 0.0016 g l^{-1} and a HLC of 0.003 Atm m^3 mol^{-1}.

Hexachlorohexane: Lindane or gamma-hexachlorocyclohexane, (γ-HCH), is considered a persistent substance and is on a number of national and international priority pollutant lists (e.g. UNEP POP, Canadian MOEE, US EPA RCRA). It is more water-soluble than the corresponding aromatic hexachlorobenzene and so may be a more useful reference substance. Whilst it is easier to handle, care should be taken because of its relatively high volatility. Hexachlorohexane is a solid at room temperature, with a water solubility of 4 mg l-1 and a HLC of 4.8 Atm m3 mol-1.

Discussion

The aim of this work was to develop a list of reference chemicals with an agreed (by regulators and industry) set of properties and characterized set of biodegradability behaviour which cover a range of environmental persistence and non-persistence. This reference set would be available for use to assess the impact of suggested modifications to existing aerobic biodegradability test methods and new test methods. The reference set would also help address concerns that some of the modifications or new methods could result in tests becoming too powerful or overly protective. Whilst some of the OECD biodegradability test guidelines have benefited by being

subjected to interlaboratory validation ring tests (e.g. OECD 301C, 302A, 302D, 310) a number have been published prior to validation or after only limited validation studies had being carried out (e.g. OECD 307, 308, 314). It could be argued that confidence in the suitability and applicability of all standardised biodegradability test methodologies is enhanced by the availability of validation data. The reference set described in this report (once validated) could also be be considered for such a purpose. The study has not addressed anaerobic behaviour of the reference chemicals as this was beyond the remit of this project. However, the reference set could be considered when developing anaerobic test methodology. The final list of 19 chemicals is recommended as a reference set for aerobic biodegradability testing and in assessing persistency. It contains chemicals with a range of structures which can be expected to biodegrade via different pathways and by different microbial populations or can be considered to be recalcitrant. For substances that are poorly soluble in water, volatile or adsorbing OECD concluded that only a subset of the ready biodegradability test guidelines were applicable. Poorly water-soluble substances are defined by OECD (2000) as substances with a limit of solubility <100 mg/l although technical problems are more likely to occur at <1mg/l as defined in TGD (EC, 2003). For poorly water-soluble substances these are the OECD 301B, 301C, 301D and 301F tests and the OECD 310 test. For volatile substances these are the OECD 301C, 301D and 301F tests and the OECD 310 test. For adsorptive substances these are the OECD 301B, 301C, 301D and 301F tests and the OECD 310 test. The techniques to administer poorly water-soluble chemicals in biodegradability testing advocated by OECD (1995) and ISO (1995), including direct addition, ultrasonic dispersion, adsorption onto an inert support and dispersion with an emulsifying agent, have been formalised in REACH (chapter 7b, ECHA 2008a, repeated here as Appendix 9). The chemicals recommended in the reference set cover the range of physico-chemical properties (particularly water solubility, partitioning behaviour and vapour pressure). ISO (1995) recommend the use of two of the reference chemicals in the proposed reference set (anthraquinone and diisooctylphthalate) as positive controls in RBTs. Additional appropriate reference chemicals from bins 2 and 3 should also be included by researchers who intend to develop methods to improve the biodegradability testing of poorly water-soluble and/or volatile chemicals. At least one chemical in each bin is commercially available in ^{14}C form.

Five approaches have been used to identify suitable validation chemicals but it has proved very difficult to obtain large datasets for many chemicals other than those which are considered to be readily biodegradable (i.e. bin 1 chemicals). There is a lack of scientific consensus on how to obtain environmentally realistic estimates of biodegradability rates. The process of deriving half-lives from standardised biodegradability tests is fraught with difficulties but attempts have been made by, for example, Struijs and van den Berg (1995) and Federle *et al.*, (1997) and in REACH (EC, 2006). The reverse process is even more fraught with problems. In proposing the validation chemicals we have sought to avoid placing too much emphasis on half-life data and applied a

weight of evidence to the data from the five separate approaches detailed above. Various inter-laboratory ring test programmes have demonstrated poor reproducibility in the RBTs and also poor comparability between results obtained with different test methods. One of the key sources of variability is the inoculum, in particular the species diversity. It is clear that an inoculum with many different species offers greater potential of including strains that are capable of degrading a wider range of xenobiotics. The inoculum is influenced by the origin and history of the sample. Natural pre-exposure to the test chemical (or structurally related chemicals), as a result of widespread use and release into the environment, might significantly affect the results of an RBT.

It has been observed in conducting this work that (not unexpectedly) the results from standardised biodegradability tests for some chemicals have changed over the last twenty years. Some chemicals that were difficult to biodegrade previously are now being shown to biodegrade in standardised RBTs. The most probable reason for this is related to the use, release pattern and concomitant adaptation of microbial populations to the chemical. Adaptation can be described as a change in the microbial community that increases the rate of biodegradation of a chemical as a result of exposure to that compound. In their natural environment micro-organisms encounter changes in substrate availability, involving either nutrient concentrations or nutrient types. They have to adapt to the new conditions in order to survive. A striking property of many micro-organisms is their enormous metabolic flexibility with respect not only to catabolic and anabolic substrates but also with respect to the continuously changing availability of nutrients. The phenotypic responses to low-nutrient growth conditions involve structural changes in the cellular make-up, changes in the specific capacity of the enzyme system(s) involved in uptake and/or assimilation of the limiting nutrient and changes in the affinity of these enzymes. The mechanisms by which adaptation (i.e. evolution of degradative potential) include gene transfer or mutation, enzyme induction and population changes. The ability to make metabolic changes is important for their survival and the environmental selection pressures driving these changes subsequently determine the acclimation time (lag phase or adaptation period) to xenobiotic substrates - (n.b. in many cases, microbial biocenoses, rather than pure strains are responsible for the elimination of a chemical from the environment). Exposure to xenobiotics can lead to adaptation and therefore to an increased probability of specific degraders being present in test inocula. With advances in modern microbial genetics, knowledge on the evolutionary events that occur during the adaptation process is increasing. In many cases, however, the most relevant mechanism leading to the phenomenon of adaptation being exhibited in standard tests is the relatively simple one caused by environmental selective pressure leading to an increase in the probability of degradation occurring as a result of the multiplication of a low number of specifically degrading cells to a sufficiently high number (Blok, 2001). Despite numerous publications demonstrating the impact of adaptation on biodegradation test results (e.g. Nyholm *et al*., 1984; Ingerslev *et al*., 1998), attempts to include adaptation in standard test methods

continues to be resisted. Nevertheless, the importance of the adaptation phenomena in assessing biodegradability should not be underestimated. A number of substances identified during the initial phase of this work as potential candidates for the reference list were eventually discarded because they showed time related contradictory results (see Appendix 7). For example, twenty years ago there was little evidence of degradation of the herbicide atrazine in RBTs with the result that it was sometimes included as a 'hard' reference substance in method development studies (Ingerslev and Nyholm, 2000). It has recently been shown however to biodegrade in regulatory type studies (Lapertot *et al.*, 2006) and Satsuma (2009) has isolated, from a naturally derived river ecosystem, the microbial community responsible for the complete biodegradation of atrazine. The second possible reason may be due to an increased knowledge of the problems and experimental limitations associated with screening studies, how and why chemicals biodegrade, and hence a better appreciation of how to perform RBTs with difficult chemicals. It may be that the more recent test results reflect biodegradability tests that have been selected based on the physico-chemical properties of the test chemical.

It has been relatively simple to identify chemicals at the extremes of the biodegradation spectrum and therefore suitable for inclusion as positive (readily biodegradable) controls (bin 1) and negative (very difficult to biodegrade) controls (bin 4). However, interpreting the data and assigning chemicals to bins 2 and 3, has been difficult. This difficulty is exacerbated because the development of modified RBTs and enhanced biodegradability screening tests are still in the early stages. The availability of data is therefore limited.

Five chemicals have been chosen as examples of chemicals that can be considered to undergo some biodegradation but which it is concluded would normally fail any new tests developed on the basis of the techniques permitted to meet either the modified RBT or the enhanced biodegradability test criteria (bin 3). DITA is included because of its use as a reference chemical in a number of inherent test guidelines, whilst o-terphenyl, cyclododecane, cyclododeca-1,5,9-triene and 2,4-dintrophenol are chemicals that have been reviewed as part of regulatory programmes addressing PBT type chemicals and have been shown to undergo degradation in inherent type studies.

It has proved much more problematic identifying chemicals which would be expected to fail an RBT but which would be expected to pass, on the majority of occasions, newly developed tests which incorporate 'enhanced' techniques (bin 2). Many of the potential candidates for this bin which were initially identified based their contradictory results and erratic behaviour in RBTs and/or on widely varying 'adaptation' periods or lag phases reported in method development publications were eventually rejected because of difficulties in assessing the likelihood that the lag phases could be reproduced consistently. There is a need to better understand the importance

of different pre-exposure regimes on the response of microbial communities to different chemicals to ensure that any adaptation to a chemical in a laboratory test has relevance to the field. The critical conditions (e.g. inoculum source, organism residence time, chemical concentration) driving or limiting microbial adaptation need to be defined with the goal of establishing acceptable and relevant conditions for pre-exposure. The reference set will be valuable if and when such work is undertaken. The work currently being carried out to develop greater understanding of the importance of inoculum density and diversity (Davenport *et al.*, 2009) will prove very valuable in confirming the suitability of the reference chemicals allocated to bin 2. To ensure greater confidence in the list, given the variability in the results for chemicals tested in both standardised and non-standardised tests, it is recommended that some chemicals require further work to establish a reasonable data set to support their use in the bins to which they have been assigned.

A further recommendation from this research is that studies looking at biodegradability should include both a positive (bin 1) and a negative (bin 3 or 4) chemical, the latter depending upon the type and purpose of the research.

During this research, one topic warranted extensive discussion with the Advisory Panel, this was the interpretation of biodegradation data and assessing whether substances could be expected to be persistent, or not, in the environment (Blok, 2009). Although this was considered not to be directly relevant for the aim of this research project, the discussion was considered potentially interesting for further review by ECETOC/Cefic. The approach describes four categories into which a chemical could fall, namely:

1) Readily biodegradable;

2) Not readily biodegradable but definitely not persistent;

3) Apparently not persistent;

4) Persistent.

It should be noted that these categories are not the same as the bins described throughout this report. Furthermore, persistent does not refer to the criteria that have been defined for this aspect in regulatory frameworks such as REACH.

The readily biodegradable chemicals would be those that can support growth of aerobic micro-organisms as a single carbon source within the criteria of RBT and thus pass the OECD 301/310 screening tests.

The not readily biodegradable but definitely not persistent (NPT) chemicals would be those that can support growth of aerobic microorganisms as a single carbon source but beyond the criteria of RBT. Such growth can be relatively easily demonstrated by modified RBT or enhanced biodegradability screening tests (or non-persistence tests, NPT). The type of modifications would include: pre-adaptation (pre-exposure of natural inoculum samples); extended incubation time; lower test chemical/biomass ratio; improved bioavailability; lower test chemical concentrations to prevent growth-inhibitive effects and larger size of inoculum sampling or concentrated inocula to include the degraders with lower probability numbers. Many of these characteristics are thus those described previously. Growth is evident but slower than in the typical "S" shape curve of readily biodegradable chemicals and the 10-day window concept and the half-life concept is not applicable. Mineralisation may be incomplete after 28 days, but may eventually reach completeness. The prime purpose of such tests would be to demonstrate ultimate biodegradation or extensive primary degradation.

The apparently not persistent category would cover those chemicals which do not support aerobic growth as a single carbon source and therefore would fail in a NPT. This type of chemical may require co-metabolic aerobic or anaerobic processes at low environmental concentrations and/or photodegradation processes and these mechanisms plus further degradation of transformation products might occur at a sufficiently high rate to prevent the occurrence of the chemical in remote areas. This would need to be demonstrated by use of appropriate simulation studies, modelling and/or monitoring studies. For this type of chemical it would need to be demonstrated that the capacity of the degradation systems (sewage treatment, soil, sediment, river water and coastal marine water and sediment) was greater than the possible release rate.

The final category would be of persistent chemicals, which would be those that fail to pass the criteria of categories 1, 2 and 3. These chemicals would need to be shown to be present in remote areas or to be predicted to pass through the degradation systems (sewage treatment, soil, sediment, river water and coastal marine water and sediment). For these chemicals co-metabolic processes or combined photodegradation and biodegradation and anaerobic degradation in sediment layers have no or insufficient capacity to prevent their passage through the littoral zone. This category may also include some POPs if they fail the growth tests (RBT and NPT), are not photodegradable and escape from degradation because of volatility.

It is clear that whilst studies under the category NPT are technically and financially feasible, studies under category 3 could be rather expensive and may still be inconclusive. The main interest would be to show that a not readily biodegradable chemical belongs in the category 2. Then it should be accepted that such a chemical is not persistent. The scientific reasoning is simple: if growth-linked degradation is possible, the degradative power in the environment will always adapt to any release with an appropriate degrading capacity (except some local or extreme release patterns). Therefore, data collection for reference chemicals in category 2 should be given the highest priority. Within this category it should be possible to make a further differentiation based on the mechanism that may have contributed to the fail result in the RBT including:

- availability /solubility;
- molecular size;
- structural hindering of enzymatic attacks in initial phase;
- low probability numbers of specific degraders;
- growth inhibition by test chemicals;
- partially degradable structures.

It is important to demonstrate that these mechanisms do not prevent adaptation under realistic environmental conditions and that these mechanisms are an artefact of the stringent laboratory conditions in RBTs. Therefore chemicals that pass a NPT should always easily pass a category 3 test as well.

It is implicit in these discussions that the categories are linked to certain tests. Although these are not described in detail is this report.

Conclusions and recommendations

19 chemicals have been chosen and assigned to 4 bins of biodegradability behaviour. These are;

- Bin 1: Reference compounds that would normally pass a RBT and a modified RBT; Aniline, Sodium benzoate, 1-octanol, Anthraquinone, Phenol

- Bin 2: Reference compounds that would normally pass an enhanced screening biodegradability test but currently fail any other screening tests; Diethylene glycol, 4-chloroaniline, 1,3,5-trimethylbenzene, 2,4-dintrotoluene, 4-fluorophenol
- Bin 3: Reference compounds that would normally fail any screening test whether modified RBT or enhanced screening biodegradability test; Di-isotridecyl adipate, o-terphenyl, Cyclododeca-1,5,9-triene, Cyclododecane, 2,4-dibutylphenol
- Bin 4: Reference compounds that should never pass a modified RBT or an enhanced screening test; Musk xylene, Hexachlorobenzene, Benzo(a)pyrene, Hexachlorohexane

It is a recommendation that research studies addressing biodegradability should include both a positive (bin 1) and a negative (bin 3 or 4) chemical, the latter depending upon the type and purpose of the research.

Finally, it is recognised by the authors that many of the reference chemicals were selected based on limited data, frequently derived from non-standard biodegradability tests. It is recommended that these chemicals be subjected to an inter-laboratory ring test to evaluate their biodegradability in both standard RBTs and modified/enhanced biodegradability tests.

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Appendix 1 Reference substances and summary of the characteristics of biodegradability test methods

Method	Test duration	Inoculum	Measurements	Reference chemical
OPPTS 835.3220 (UK Porous Pot Method, Painter and King, 1978)	At least 21 days	Activated sludge mixed liquor from a domestic plant	Primary biodegradation determined by test chemical removal, DOC analysis provides measure of ultimate biodegradation	Sodium benzoate
OECD 301A (DOC die-away test) ISO 7827	Up to 28 days	Micro-organisms (~10^7 - 10^8 cells/ml) in surface waters, unchlorinated sewage treatment works effluents or activated sludge	DOC removal	Aniline Sodium acetate Sodium benzoate
OECD 301B (CO_2 evolution test) ISO 9439 OPPTS 835.3120	Up to 28 days	Micro-organisms (~10^7 - 10^8 cells/ml) in surface waters, unchlorinated sewage treatment works effluents or activated sludge	CO_2 production	Aniline Sodium acetate Sodium benzoate Sodium stearate (low solubility ref)
OECD 301C (Modified MITI test)	Up to 28 days	Micro-organisms (~10^7 - 10^8 cells/ml) in surface waters, unchlorinated sewage treatment works or industrial effluents or activated sludge	O_2 uptake	Aniline Sodium acetate Sodium benzoate
OECD 301D (Closed bottle test) ISO 10707	Up to 28 days	Micro-organisms (~10^5 cells/ml) in surface waters or unchlorinated sewage treatment works effluents	O_2 uptake	Aniline Sodium acetate Sodium benzoate

Method	Test duration	Inoculum	Measurements	Reference chemical
OECD 301E (Modified OECD screening test) ISO 7827	Up to 28 days	Micro-organisms (~10^7 – 10^8 cells/ml) in unchlorinated sewage treatment works effluents	Dissolved organic carbon (DOC)	Aniline Sodium acetate Sodium benzoate
OECD 301F (Manometric respirometry test) ISO 9408	Up to 28 days	Micro-organisms (~10^7 - 10^8 cells/ml) in surface waters, unchlorinated sewage treatment works effluents or activated sludge	O_2 uptake	Aniline Sodium acetate Sodium benzoate
OECD 310 (Headspace test)	Up to 28 days	Activated sludge 4-30mg l^{-1} or 10% v/v secondary effluent or surface water or soil	CO_2 production giving % degradation	Aniline Sodium benzoate Ethylene glycol 1-octanol
ISO 14593	Up to 28 days	Inoculum of aerobic mixed micro-organisms (approx 10^7-10^8 cells/l)	CO_2 production giving % degradation	
OECD 302A (Modified SCAS test) OPPTS 835.3210	Months (often 120 days)	Settled domestic sewage and activated sludge.	DOC Potentially ^{14}C	4-acetyl aminobenzene sulphonate 4-nitrophenol Diethylene glycol Aniline

Method	Test duration	Inoculum	Measurements	Reference chemical
OPPTS 835.5045 (Modified SCAS for insoluble and volatile chemicals)	40-120 days	Settled domestic sewage and activated sludge Unadapted or pre-adapted inoculum	DOC	No reference chemical yet recommended
OECD 302B (Zahn Wellens test) OPPTS 835.3200 ISO CD 9888	28 days	Inoculum of 200 - 1000 mg l^{-1} (TSS) of activated sludge	DOC or COD or Specific analysis for primary transformations	Ethylene glycol Diethylene glycol Aniline Lauryl sulphate
OECD 302C MITI (II)	14-28 days	Aerobic mixed, specially grown, unadapted micro-organisms at 100 mg l^{-1} (TSS, or approx. 3×10^7 - 3×10^8)	O_2 demand and possibly specific chemical analysis	Aniline Sodium acetate Sodium benzoate
OECD 304A Soil OPPTS 835.3300 ISO 14239 (Biometer system)	Up to 64 days	Soil (50g)	$^{14}CO_2$	No reference chemical yet recommended
OECD 307 (Aerobic and anaerobic transformation in soil)	Up to 120 days Longer under some circumstances	Soil (50 to 200 g) samples (a sandy loam or silty loam or loam or loamy sand) are treated with the test substance and incubated in the dark, in biometer-type flasks or in flow-through systems under controlled laboratory conditions.	CO_2	No reference chemical yet recommended

Method	Test duration	Inoculum	Measurements	Reference chemical
OPPTS 835.3100 (Aerobic aquatic biodegradation)	28 days after pre-adaptation	Pre-adapted inoculum	DOC removal and hydroxide trapped CO_2 ^{14}C provides mass balance and phase distribution data	Aniline Sodium citrate Dextrose Trimellitic acid
OECD 302D CONCAWE Inherent biodegradation of oil products	56 days or until biodegradation plateau is reached	Activated sludge inoculum (approx 10^7 - 10^8 cells l^{-1}) pre-exposed to the test chemical for up to 14 days	CO_2 evolution giving % degradation	n-hexadecane Di-isotridecyl adipate (DITA) n-octadecane
OPPTS 835.5045 (Modified SCAS test for insoluble and volatile chemicals)	40 to 120 days	Settled domestic sewage and activated sludge	DOC. Specific analysis can provide primary transformation data. Kinetic data and half-life determination available. >20% removal of DOC =inherent biodegradation. >70% removal of DOC =ultimate biodegradation.	4-acetyl aminobenzene sulphonate 4-nitrophenol Diethylene glycol Aniline
ISO 14592-1 OPPTS 835.3170	No fixed duration	Micro-organisms in surface water samples filtered through 100 um filter for a 'pelagic test' which may be amended with an aerobic sediment slurry from the study site for a 'suspended sediment test'	Specific chemical or radio-chemical analysis (and DOC or TOC if possible) giving 1^{st} order rate const.	No reference chemicals recommended
ISO 14592-2	No fixed duration but less than 60 days	Micro-organisms in surface water	Specific chemical or radio-chemical analysis giving 1^{st} order rate constant	

Method	Test duration	Inoculum	Measurements	Reference chemical
OPPTS 835.3180 Sediment/ water microcosm	Less than 60 days	Natural microbial assemblage	Chemical analysis of transformation products or $^{14}CO_2$ analysis where labelling used	Methyl parathion Linear alkylbenzene sulphonate
OECD 308 Aerobic and anaerobic transformation in aquatic sediment systems	Less than 100 days	Natural microbial assemblage	Chemical analysis of transformation products or $^{14}CO_2$ analysis where labelling used	No reference chemicals recommended
OECD 309 Aerobic mineralisation in surface water	Up to 90 days	Micro-organisms in surface water May include suspended sediment and/or semi-continuous operation	Residual ^{14}C or residual parent concentration	Aniline Sodium benzoate
OECD 306 Simulation test for marine waters ISO 7827, 10707 OPPTS 835.3160	Up to 60 days	Micro-organisms in test seawater Not pre-adapted inoculum	DOC	Aniline Sodium acetate Sodium benzoate

Method	Test duration	Inoculum	Measurements	Reference chemical
OECD 314 314A - Sewer Systems 314B - Activated sludge 314C - Anaerobic digester 314D - Treated effluent- surface water mixing zone 314E - Untreated wastewater – surface water mixing zone	 Variable 28 days - but can be extended Up to 60 days 28 days - but can be extended 28 days - but can be extended	This guideline is designed to provide a comprehensive strategy to assess biodegradation of chemicals which are discharged to water and consists of five simulation tests that address biodegradation in critical scenarios relevant for chemicals released to wastewater. Inocula can be activated sludge, anaerobic sludge or alternative inocula	Primary degradation by specific analysis of parent substance Ultimate degradation measured by $^{14}CO_2$ or $^{14}CH_4$	No reference chemicals yet recommended

Appendix 2: Summary table of studies and chemicals used in biodegradability test method development

Chemical	Test inoculum	Purpose	Reference
Aniline Sodium benzoate Sodium stearate Diethylene glycol Pentaerythritol Sulphanilic acid Benzene-1,3-disulphonilic acid 2-chloroanilne	Ready test	Ring test to validate a respirometric method for the assessment of biodegradability Results from 12 laboratories with 8 chemicals covering a range of biodegradabilities	Painter HA, King EF. 1985
Aniline 4-chlorophenol Hexadecane 4-nitrophenol Pentaerythritol Phenanthrene	Ready tests	Studies to assess the impact of source and concentration of test inoculum on the extent and rate of biodegradation and to develop guidelines for testing of poorly water-soluble and volatile chemicals	Snape JR, Aldington RWJ, Roberts GC, Evans MR. 1997

Toluene			
Aniline Ethanolamine Hexanediol Pentaerythritol Linear alcohol ethoxylate Linear alkylbenzene sulphonate	Ready tests	Development of improved sensitivity of OECD 301 B, C, D and F using a selection of reference chemicals considered to be readily biodegradable	Hales SG, Philpotts CJ, Gillard C. 1996
Diethylene glycol 2-ethylhexylacrylate Cyclohexanone Phenol Nitrilotriacetic acid (NTA)	Ready tests	A critical comparison of respirometric biodegradability tests based on OECD 301 using 10 model reference chemicals. Differences observed with diethylene gycol and 2-ethylhexylacrylate	Reushenbach P, Pagga U, Strotmann U. 2003
4-hydroxybenzoate 4-nitrophenol 4-fluorophenol	Ready tests	Investigating the effect of biomass and biodiversity on ready test. Chemicals considered to cover the range readily degradable to difficult to degrade were used	Davenport RJ, Snape J, Ericson J, Madsen T, Pedersen A. 2009
Phenol Hydroxybenzoic acid Hydroquinone 4-nitrophenol	Ready tests	Investigating the effect of biomass and biodiversity on ready test. Chemicals considered to cover the range readily degradable to difficult to degrade were used. The chemicals were assigned the following evaluation codes described in Biodeg 5 database: phenol (BF), hydroxybenzoic acid (BF), hydroquinone (BF) 4 nitrophenol (BS/BF), 4-chlorophenol (BS/BF), 2-naphthol (BF), 1-naphthol 4 sulphonic acid (BS)	Goodhead AK, Snape JR, Head IM, Davenport RJ. 2008

4-chlorophenol 2-naphthol 1-naphthol-4- sulphonic acid 1,5-n disulphonic acid 1-n-6 sulphonic acid		1,5-n disulphonoc acid (BS), 1-n-6 sulphonic acid (BS). (BF - biodegrades fast, BS - biodegrades slow)	
Diethylene glycol Diethylenetriamine Dodecylbenzene sulphonate	Ready tests	Study on the effect of sludge retention time by which the inocula are maintained on the rate of biodegradation in closed bottle test	Van Ginkel CG, Haan A, Luijten MLGC, Stroo CA. 1995
Aniline 4-chloroaniline	Surface waters	Comparison of rates in standard tests and realistic environmental concentrations	Ahtaiainen J, Aalto M, Pessala. 2003
2,4-dinitrophenol Naphthalene-1-sulphonic acid Sulphanilic acid (4-amino benzene sulphonic acid	River water simulation	Development of a cascade test system and batch shake flask test for non-volatile and non-sorbing chemicals using three readily degradable compounds as reference chemicals at low concentrations	Koziollek P, Knackmuss H-J, Taeger K, Pagga U. 1996
Aniline, 4-nitrophenol (4-NP), 2,4 dichlorophenoxy acetic acid (2,4-D),	Surface waters	Lag phases following adaption periods were reduced from 5.2 to <1 day for aniline, 10 days to < 1 day for 4-NP, 88 days to 9 days for 4-ClA	Torang L, Nyholm N. 2005

4-chloroaniline (4-ClA)			
Anthraquinone di-iso octylphthalate	Freshwater	Various dispersion techniques were evaluated with anthraquinone and di-iso-octylphthalate	Nyholm N. 1990
2,4-dichlorophenoxy acetic acid (2,4-D), 2,4,6 trichlorophenol (TCP), pentachlorophenol (PCP) 4-nitrophenol (4-NP) lindane	Activated sludge	A gradual adaptation took place resulting in increases in biodegradation rates by an order of magnitude or more compared to initial rates. Times for adaptation ranged from 2-5 days for 4NP to 1-2 months for 2,4-D and lindane	Nyholm N, Jacobsen BN, Pedersen BM, Poulsen O, Damborg A, Schultz B. 2003
Sodium acetate Aniline 4-chloroaniline Pentachlorophenol	Activated sludge	Rate constants of 0.003, 0.03, 0.2 2.9 d^{-1} were estimated for acetate, aniline, 4-chloroaniline and pentachlorophenol respectively	Nyholm N, Ingerslev F, Berg UT, Pedersen JP, Frimer-Larsen H. 1996
2,4-dichlorophenoxy acetic acid (2,4-D) 4-nitrophenol (4-NP)		Study on the impact of total test medium volume in biodegradability shake flask tests using 2,4-D and 4-NP, chemicals known to be readily biodegradable after variable lag phases	Ingerslev F, Torang L, Nyholm N. 2000
Di-isotridecyl adipate Hexadecane	Surface waters	30% after 28 days with unexposed inoculum. 40-80% after 28 days in 7	Concawe test for inherent biodegradability. 2001
4-nitrophenol	Surface waters and activated	Investigated the impact of inoculum density on the probability of biodegradation by activated sludge and river water inocula	Thouand G, Friant P, Bois F, Cartier A. 1995

	sludge		
Sodium acetate	Surface waters	Modification of the OECD 301F with regard to test chemical and inocula evaluated with sodium acetate	O'Malley LP. 2006
Aniline Sodium benzoate Diethylene glycol Pentaerythritol 4-nitrophenol (4-NP)	Seawater	Screening methods for assessment of biodegradability of chemicals in seawater – results of a ring test Sodium benzoate and aniline were considered to rank equally with respect to biodegradability. Diethylene glycol and pentaerythritol had lag times of 10-14 days and were concluded to rank equally (but slower than aniline and benzoate). Five compounds could be ranked sodium benzoate and aniline > diethylene glycol and pentaerythritol > 4-NP (long adaptation may be needed	Nyholm N, Kristensen P. 1992
Aniline Sodium benzoate Diethylene glycol Pentaerythritol 4-nitrophenol (4-NP) 4-chloroaniline (4-ClA) Malein hydrazide	Seawater	A comparative study on biodegradability of chemicals in seawater. All chemicals that passes the screening test were also degraded in the simulation test, but negative screening test results did not exclude positive results in simulation tests	Nyholm N, Damborg A, Lindgaard-Jorgensen P. 1992
Benzo(a)pyrene Caffeine DDT Naphthalene Pentachlorophenol	Seawater	Review of factors that are most likely to influence biodegradation in the environment and of the various national and international priority lists. Six chemicals were identified as suitable test chemicals for method development work.	Astrazeneca. 2000

Phenol			
Glucose	Seawater	Studies to develop new or adapted methods which can be used to measure the biodegradability of chemicals in the marine environment	Snape JR. 2005
Aniline Naphthalene 4-chloroaniline 4-nitrophenol	Seawater	Studies to develop new or adapted methods which can be used to measure the biodegradability of chemicals in the marine environment	Snape JR. 2006
Phenol Octadecanoic acid-2-ethylhexyl ester Diethylene glycol	Inherent tests	Modifiied the Zahn-Wellens test for the determination of biodegradability of poorly water-soluble (octadecanoic acid-2- ethylhexyl ester), adsorbing and volatile (phenol)	Norr C, Meinecke S, Brackemann H. 2001

Appendix 3: EU TC-NES PBT Working Group - Status for (potential) PBT and vPvB substances meeting the screening criteria (plus specific comments re biodegradation data)

(Substances have been deleted from this table that were considered inappropriate for assessment as a reference chemical, e.g. mixtures or metalo-organics or that the data were not reviewed as the substance was not an HPVC)

No.	CAS	Name	PBT TC-NES RESULT EVALUATION	COMMENTS RE P	POSSIBLE BIN
1	1506-02-1	1-(5,6,7,8-tetrahydro-3,5,5,6,8,8-hexamethyl-2-naphthyl)ethan-1-one	Deleted (not B and T, but potentially P)	Limited experimental data	-
2	1222-05-5	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylindeno[5,6-c]pyran	Deleted See also previous substance	Quoted ½ life of 2 days	-
3	87-61-6	1,2,3-trichlorobenzene	PBT Due to high potential for long range transport	Assessed as P - screening data shows not readily biodegradable unless adapted sludge used	3
4	120-82-1	1,2,4-trichlorobenzene	PBT Due to high potential for long range transport. Extensively discussed in context of ESR risk assessment and WFD	Ditto	3
5	118-82-1	2,2',6,6'-tetra-tert-butyl-4,4'-methylenediphenol	Further testing needed	0% in OECD 301B – but at concentration 1000 x approx solubility	3/4
10	88-06-2	2,4,6-trichlorophenol	Deleted (not P)	No RBT data River die-away test approx ½ life <10 days	2/3
11	121-14-2	2,4-dinitrotoluene	Deleted (not B)	RBT <10% But substance degrades with adapted innocula – inherently biodegradable	2/3
12	96-76-4	2,4-di-tert-butylphenol	Deleted B and T not fulfilled (e.g. based on a METI test)	RBT <10%	2/3
13	128-39-2	2,6-di-tert-butylphenol		RBT <10%	2/3
14	497-39-2	4,6-di-tert-butyl-m-cresol		Limited biodegradation	2/3
17	84989-41-3	2-oxetanone, 3-C12-16-alkyl-4-C13-17-alkylidene derivs.	Deleted (not P)	Mixture, biodegradable but very insoluble	2
18	90552-07-1	2-propenoic acid, 2-methyl-, C9-11-isoalkyl esters, C10-rich	Deleted (not P)	Mixture, inherently biodegradable >80 in OECD 301B	2
19	5208-93-5	3-methyl-1-(2,6,6-trimethylcyclohex-1-en-1-yl)penta-1,4-dien-3-ol	Deleted (not P)	50 – 60% in RBT	1/2
20	5124-30-1	4,4'-methylenedicyclohexyl diisocyanate	Deleted (not P)	Inherently biodegradable if using adapted sludge	3

No.	CAS	Name	PBT TC-NES RESULT EVALUATION	COMMENTS RE P	POSSIBLE BIN
22	50849-47-3	5-nonylsalicylaldehyde oxime	Further testing/ evaluation needed	No biodegradation in RBT or inherent tests	4
25	5216-25-1	Alpha,alpha,alpha,4-tetrachlorotoluene	Deleted (not P – fast hydrolysis and reaction products not PBT)	Rapidly hydrolysable – no further assessment	
39	4904-61-4	Cyclododeca-1,5,9-triene	Further evaluation/ testing needed	Not readily biodegradable, well reviewed by WG	3
40	294-62-2	Cyclododecane	Potential PBT	Not readily biodegradable, well reviewed by WG	3
41	11138-60-6	Decanoic acid, ester with 2-ethyl-2-(hydroxymethyl)-1,3-propanediol octanoate	Deleted (not P)	18 – 76% in RBTs; biodegradable but may not be readily biodegradable	2
43	26898-17-9	Dibenzyltoluene	Deleted Parent compound and its metabolites are not P	Not readily biodegradable, but does degrade, although may not be complete	2 or 3
48	1163-19-5	Bis(pentabromophenyl) ether	Further evaluation under 793/93 – Stage II –conclusion (i) regarding the PBT properties	Mixture - not evaluated	
49	32536-52-0	Diphenyl ether, octabromo derivative	PBT	Not biodegradable	4
55	27193-86-8	Dodecylphenol	Deleted (Not B, vB)	20% biodegradation in OECD 301B and 10% in ISO 14593 tests	3
58	25637-99-4	Hexabromocyclododecane	Further testing needed.	Complex – not readily biodegradable but with apparently very short measured half-lives. Extensive biodegradation work also addressing metabolites	
59	118-74-1	Hexachlorobenzene	POP (under Stockholm Convention)	Not biodegradable	4
60	87-68-3	Hexachlorobuta-1,3-diene	PBT & vPvB, UNECE POP candidate	Very volatile, not degradable in 1 study but extensive loss	
64	51338-27-3	Methyl 2-(4-(2,4-dichlorophenoxy)phenoxy)propionate	Deleted (not B)	Data not reviewed	
66	4979-32-2	N,N-dicyclohexylbenzothiazole-2-sulphenamide	Deleted (not P)	Rapidly hydrolysable – no further assessment	
67	14861-17-7	4-(2,4-dichlorophenoxy)aniline	Deleted (not B)	1 test in a RBT <20%	3
68	1836-75-5	Nitrofen	PBT (Based on the available exposure information the WG does not consider further action needed)	Not biodegradable in standard studies – but limited assessment	
69	25154-52-3	Nonylphenol	Deleted (P and B not fulfilled)	Biodegradable in RBT studies varying from 10 – 70%	2 or 3
73	1843-05-6	Octabenzone	Deleted (not B)	1 test – 5% in a RBT	3
74	2082-79-3	Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate	Deleted (not B, degradation products not PBT	Primary biodegradation – but not readily biodegradable	3

No.	CAS	Name	PBT TC-NES RESULT EVALUATION	COMMENTS RE P	POSSIBLE BIN
77	6683-19-8	Pentaerythritol tetrakis(3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate)	Further testing needed	Not biodegradable	3 or 4
79	61788-44-1	Phenol, styrenated	Further testing needed.	Not readily biodegradable, mixture – not further assessed	
86	26140-60-3	Terphenyl	Further discussion needed	Not biodegradable	3
87	61788-32-7	Terphenyl, hydrogenated	Further discussion needed	The weight of evidence over all the tests performed shows that hydrogenated terphenyls are inherently biodegradable, although they are not readily biodegradable	3
90	117-08-8	Tetrachlorophthalic anhydride	Deleted (not P)	Rapidly hydrolysable – no further assessment	
94	603-35-0	Triphenylphosphine	Deleted (not P and B)	Rapidly hydrolysable – no further assessment	
96	693-36-7	Dioctadecyl 3,3'-thiodipropionate	Provisionally delisted	Biodegradable, but not meeting 10 day window	2
97	793-24-8	N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD)	Deleted (not P – due to fast hydrolyse, degradation products are not B)	Rapidly hydrolysable – no further assessment	
98	25103-58-6	Tert.dodecanethiol	Further testing needed	30 – 40% in OECD 301B study, substance still under evaluation	2 or 3
100	31570-04-4	Tris(2,4-di-tert-butylphenyl) phosphite	Further testing needed	No data	
101	32588-76-4	Ethylene-bistetrabromophthalimide	Deleted (Not B)	Not readily biodegradable, limited data	3 or 4
120	51000-52-3	Vinyl neodecanoate	Further testing needed	Not biodegradable, but limited data	
125	38640-62-9	Di-iso-propyl-naphthalene (DIPN)	Further testing/evaluation needed	Probably readily biodegradable	2

Appendix 4: List of potential candidates prepared from empirical databases (includes half-lives for all environmental compartments unless specifically stated and no attempt has been made to check for duplication of data between the datasets)

Table 4.1 Median half- life <15 days

Chemical	Cas No	Source of data	No. of Data Points	Median Half-life (d)
Naphthalene	91-20-3	EMBK (FW)	15	13.9
		EMBK (SW)	81	12.6
		Aronson	5	40
		Arnot	16	
		Syracuse (FW+SW)	36	
Nitriloacetic acid	0139-13-9	Aronson	20	12.2
Aniline	62-53-3	EMBK (FW)	20	
		EMBK (SW)	15	
		Arnot	4	
		Aronson	3	
4-nitrophenol	0100-02-7	Aronson	14	9
Acetone		Aronson	13	
Benzoate (sodium)	532-32-1	EMBK	13	
Benzoic acid	65-85-0	Arnot	3	2
m-cresol	0108-39-4	Aronson	10	
		Syracuse (FW+SW)	16	
Dibutylphthalate	84-74-2	Arnot	9	8
		Aronson	5	
Phenol	108-95-2	Arnot	6	5
		Aronson	18	1.2
		Syracuse	96	
Diethylphthalate	84-66-2	Arnot	7	11
Butylbenzenephthalate	85-68-7	Arnot	6	4

Ethylbenzene	100-41-4	Arnot Syracuse	6	14
p-cresol	106-44-5	Arnot Syracuse Aronson	5 - 1	2
Dimethylphthalate	131-11-3	Arnot	5	3
o-cresol	95-48-7	Arnot Syracuse	5	6
Carbaryl	63-25-2	Arnot	5	14
4-chlorobenzoic acid		Aronson	5	
Quinoline	91-22-5	Arnot	4	14
Ethanol	64-17-5	Arnot	3	5
Ethylene glycol	107-21-1	Arnot	3	8
1,3,5- trimethylbenzene	108-67-8	Arnot	3	9
DNOP	117-84-0	Arnot	3	14
2,4-dimethylphenol	105-67-9	Arnot	2	4
Catechol	120-80-9	Arnot	2	4
Propanal	123-38-6	Arnot	2	4
2,4-dichlorophenol	120-83-2	Arnot	2	6
Acetophenone	98-86-2	Arnot	2	6
3-pentanone	96-22-0	Arnot	2	8
Pentyl acetate	628-63-7	Arnot	2	12
Diphenylamine	122-39-4	Arnot	2	15
2-hexanone	591-78-6	Arnot	1	5
Cyanazine	21725-46-2	Aronson		15

Table 4.2 Half-life 16-40 days

Chemical	Cas No	Source of data	No. of Data Points	Median Half-life (d)
Toluene	108-88-3	EMBK FW	10	28
		EMBK SW	19	79
		Syracuse (FW+SW)	37	
		Arnot	4	18
Di-(2-ethylhexyl)-phthalate	117-81-7	Arnot	9	21
		Aronson	19	74
		Aronson (soil)	23	67
Benzene	71-43-2	Arnot	8	40
		Syracuse	3	
Hexadecane	544-76-3	EMBK	8	
2,4,5-trichlorophenoxy acetic acid	93-76-5	Arnot	7	25
Dimethoate	60-51-5	Arnot	5	18
Cyanazine	21725-46-2	Arnot	5	38
Carbofuran	1563-66-2	Arnot	5	40
3,4-dichlorophenol	95-77-2	Arnot	4	18
Captan	133-06-2	Arnot	4	19
Malathion	121-75-5	Arnot	4	20
2-(2,4-dichlorophenoxy)acetic acid	94-75-7	Arnot	4	21
Mecoprop	7085-19-0	Arnot	4	25
Propoxur	114-26-1	Arnot	4	25
Dichloromethane	75-09-2	Arnot	4	30
1,3-dimethylbenzene	108-38-3	Arnot	3	19
		Syracuse		
1,4-dimethylbenzene	106-42-3	Arnot	3	19
		Syracuse		

1,2-dimethylbenzene	95-47-6	Arnot Syracuse	3	22
1,2,4-trimethylbenzene	95-63-6	Arnot	2	18
Chloroethane	75-00-3	Arnot	2	18
Methyl methacrylate	80-62-6	Arnot	2	18
4-chlorophenol	106-48-9	Arnot	2	20
Styrene	100-42-5	Arnot	2	21
EPTC	759-94-4	Arnot	2	30
2,4,6-trichlorophenol	88-06-2	Arnot	2	39
1,2,4-trimethylbenzene	95-63-6	Arnot	2	18

Table 4.3 Half-life 41-60 days

Chemical	Cas No	Source of data	No. of Data Points	Median Half-life (d)
Fluorene	86-73-7	EMBK Arnot Syracuse (Sediment)	9 2 11	46
Methyl parathion	298-00-0	EMBK	6	
Diazinon	333-41-5	Arnot	4	43
Diallate	2303-16-4	Arnot	4	46
1,2,4-trichlorobenzene	120-82-1	Arnot	4	61
Dibenzofuran	132-64-9	Arnot	3	52
Methyl parathion	298-00-0	Arnot	2	43
Acenapthene	83-32-9	Arnot	2	57
Isopropylbenzene	98-82-8	Arnot	1	57
Ethalfluralin	55283-68-6	Aronson		46
Chlorothalonil	1897-45-6	Aronson Arnot	5 4	55.8 49

Table 4.4 Half-life >61 days

Chemical	Cas No	Source of data	No. of Data Points	Median Half-life (d)
4-chloroaniline	106-47-8	EMBK (FW) EMBK (SW) Aronson (soil)	10 11	133 139
Atrazine	1912-24-9	Arnot	7	96
Trifluralin	1582-09-8	Arnot Aronson	7	106 180
Linuron	330-55-2	Arnot	6	111
Dieldrin	60-57-1	Arnot Aronson	6	792 1000
Chlordane	12789-03-6	Arnot Aronson	6	1072 54
Chloropyrifos	2921-88-2	Arnot Aronson	5	69 88
Dicamba	1918-00-9	Arnot	5	69
Simazine	122-34-9	Arnot	5	81
Aldicarb	116-06-3	Arnot	5	131
Aldrin	309-00-2	Arnot Aronson	5	161 120
TCCD	1746-01-6	Arnot	5	648
Hexachlorobenzene	118-74-1	Arnot	5	1245
Pyrene	0129-00-0	Aronson Aronson (soil) Syracuse	2 18 5	
Isopropalin	33820-53-0	Arnot	4	63
Phenanthrene	85-01-8	Arnot	4	67
4-chlorobiphenyl	2051-62-9	Aronson	4	67

Chloroethene	75-01-4	Arnot	4	76
Diuron	330-54-1	Arnot	4	101
Triallate	2303-17-5	Arnot	4	106
Anthracene	120-12-7	Arnot	4	174
Methoxychlor	72-43-5	Arnot	4	191
Benzo(a)pyrene	50-32-8	Arnot Syracuse (sediment)	4 17	284
Benz(a)anthracene	56-55-3	Arnot Syracuse	4	301
Fluoranthene	206-44-0	Arnot	4	306
2,4,5-trichlorophenol	95-95-4	Arnot	4	366
Chrysene	218-01-9	Arnot Aronson Aronson (soil)	4 2 15	532
Cyclohexane	110-82-7	Arnot	3	79
3'3-dichlorobenzidine	91-94-1	Arnot	3	93
Hexachloroethane	67-72-1	Arnot	3	138
Bromacil	314-40-9	Arnot	3	240
2,3,4,6-tetrachlorophenol	58-90-2	Arnot	2	98
Bis(2-chloroethyl)ether	111-44-4	Arnot	2	104
2,4-dinitrotoluene	121-14-2	Arnot	2	104
1,2-dichloroethane	107-06-2	Arnot	2	140
Pentachlorophenol	87-86-5	Aronson Arnot	 7	94.6 146
Bromethalin	63333-35-7	Aronson		178
Pentachlorobenzene	608-93-5	Aronson		270
Hydra-methylnon	67485-29-4	Aronson		383
TCDD	1746-01-6	Aronson		562

Lindane (HCH)	58-89-9	Arnot Aronson	5	392 792
Heptachlor	76-44-8	Arnot Aronson	5	824 2000
DDT	50-29-3	Aronson		3800

FW - Freshwater

SW - Seawater

Appendix 5: Summary of measured data and results of RBT for intermediate list reference chemicals (listed in bin order)

Chemical	Initial Bin Assessment	Ref Std	Freshwater data			All aquatic data			Standardised test result
			Data points	Half-life range (d)	Median half-life (d)	Data points	Half-life range (d)	Median half-life (d)	
Aniline	1	Yes	36	0.4-150	5	49	0.4-150	5.7	Pass RBT
Anthroquinone	1	Yes	-	-	-	-	-	-	Pass RBT
m-cresol	1		13	0.7-12.2	1.4	20	0.7-28.8	1.75	Pass RBT
Di-n-butyl phthalate	1		18	0.9-48.5	5	29	0.6-48.5	2.8	Pass RBT
Naphthalene	1		18	0.9-150	11.5	95	0.9-150	11.3	Pass RBT
Nitrilotriacetic acid	1		28	0.5-7.5	1.9	33	0.5-22	2.3	Pass RBT
1-octanol	1	Yes							Pass RBT
Phenol	1		36	0.1-11.6	1.1	44	0.1-22	2	Pass RBT
Sodium benzoate	1	Yes	1	1.9	1.9	13	1-9	3	Pass RBT
Toluene	1		14	0.02-83.3	1		0.02-150	25	Pass RBT
2,4-dichlorophenol	1								Pass RBT
Benzene	1		6	1.8-36	15.4	8	1.8-72	15.4	Variable RBT
4-nitrophenol	1/2	Yes	18	1.3-77	2.5	36	1.3-150	12.5	Variable RBT

Diethylene glycol	2	Yes	-	-	-	-	-	-	Variable RBT Pass inherent
Di iso-octyl phthalate	1/2	Yes	0	-	-	-	-	-	Variable RBT Pass inherent
Hexadecane	1/2	Yes	6	1.4-29	24	29	1.4-150	26	Variable RBT
Nonylphenol	2		2	8.5-12.4	10.45	10	4-150	14.2	Fail RBT
Pentaerythritol	1/2		-	-	-	-	-	-	Variable RBT
4-fluorophenol	2								
2,4-dintrotoluene	2								Fail RBT Pass inherent
1,3,5-trimethylbenzene	2		-	-	-	-	-	-	Fail RBT (36% after 28 days, 56% after 60 days) (Acclimated Pass 72%)
4-chloroaniline	3		10	93-150	133	23	8-150	116	Variable RBT
Chlorothalonil	3/4								
Cyclododecane	3		-	-	-	-	-	-	<10% - Fail RBT but not considered P
Cyclododeca-1,5,9-triene	3								Fail RBT

Diethyl hexyl phthalate	3		29	5.3-150	53.3	30	4.5-150	52	
Di iso-tridecyl adipate	3	Yes	-	-	-	-	-	-	Fail RBT
Fluorene	3		0			9	11-150	36.7	Fail RBT
Methyl parathion	3		2	3.3-3.7	3.5 (Prim degradation)	4	3.6-150	3.9	Fail RBT
Pentachlorophenol	2/3	Yes	6	50-150	128				Fail RBT
m-terphenyl	3		2	15-51	-	-	-	-	Fail RBT
2,4-dibutylphenol	3								Fail RBT
2,2,4,6,6-pentamethylheptane	3/4		1	-	-	-	-	-	Fail RBT
1,2,4-trichlorobenzene	3		-	-	-	6	5.5-150	105	Fail RBT
2,4,5-trichlorophenol	3								
Trans-decalin	3/4		-	-	-	-	-	-	Fail (0%) (Acclimated Fail 15.7%)
Atrazine	3/4		-	-	-	-	-	-	Variable RBT
Benzo(a)anthracene	4								Fail RBT
Benzo(a)pyrene	4		7	85-150	150	25	0.2-150	150	Fail RBT
Chlorobenzilate (P) Chlorobenzilate (U)	4		8 6	6-150 75-150	30.8 150	15*	6.6-150*	29*	Fail RBT

4-chlorobiphenyl (P) 4-chlorobiphenyl (U)	4		8 5	0.2-5 62-150	3.8 62				Fail RBT
Chrysene	4								Fail RBT
Hexachlorobenzene	4								Fail RBT
Hexachlorohexane	4								Fail RBT
Musk xylene	4								Fail RBT

- includes soil data
- P- Primary biodegradation
- U- Ultimate biodegradation

Appendix 6: Summary data for intermediate list of reference chemicals[1]

Bin 1: Would normally pass a RBT and a modified RBT

Chemical Name	CAS No	Ready test result	ESR Report	Use in method development studies	Standard test reference chemical	Log K_{ow}	Water solubility (mg l^{-1})	VP (Pa)	HLC (Atm m^3 mol^{-1})	Commercial availability	14C Available
Aniline	62-53-3	Pass	Yes	Yes	OECD 301, 302, 306, 309, 310	1.08	2.00E+04	1.05E+02	4.7E-01	Yes	Yes
Sodium benzoate	532-32-1	Pass			OECD 301, 302, 306, 309, 310	-2.27	5.56E+05	4.89E-07	7.0E-11	Yes	Yes
1-octanol	111-87-5	Pass			OECD 310	2.80	5.40E+02	1.32E+01	2.1E+00	Yes	Yes
Anthraquinone	84-65-1	Pass				3.39	3.9E+00	5.1E-06	2.7E-04	Yes	
Phenol	108-95-2	Pass	Yes	Yes		1.51	2.62E+04	4.30E+01	1.5E-01	Yes	Yes
Di-n-butylphthalate	84-74-2	Pass	Yes			4.61	6.40E+00	3.00E-02	3.6E-00	Yes	Yes
m-cresol	108-39-4	Pass				2.06	8.89E+03	2.23E+00	2.7E-01	Yes	
2,4-dichlorophenol	120-83-2					2.80	4.50E+03	1.20E+01	2.3E-00	Yes	
Naphthalene	91-20-3		Yes	Yes		3.17	1.42E+02	1.13E+01	4.8E-05	Yes	Yes
Nitrilotriacetic acid	139-13-9					-3.81	7.34E+05	9.54E-07	2.5E-10	Yes	Yes
Toluene	108-88-3	Pass	Yes	Yes		2.5	5.73E+02	3.79E+03	5.0E+02	Yes	Yes

1: Final recommendations for the reference set – identified in RED

Bin 2: Would normally pass an enhanced screening biodegradability test and currently fail the other screening tests

Chemical Name	CAS No	Ready test result	ESR Report	Use in method development studies	Standard test reference chemical	Log K_{ow}	Water solubility (mg l^{-1})	VP (Pa)	HLC (Atm m^3 mol^{-1})	Commercially available	14C Available
Diethylene glycol	111-46-6	Mixed		Yes	OECD 302A	-1.5	1.00E+06	3.50E-01	3.8E-05	Yes	
4-chloroaniline	106-47-8			Yes		1.72	3.90E+03	3.60E+00	1.6E-01	Yes	Yes
1,3,5-trimethylbenzene	108-67-8	Fail				3.63	1.20E+02	2.68E+02	6.58E-03	Yes	
2,4-dinitrotoluene	121-14-2	Fail	Yes			2.18	4.50E+02	9.58E-02	3.97E-07	Yes	
4-fluorophenol	371-41-5	Mixed		Yes		1.71	1.25E+04	3.04E+01	2.7E-01	Yes	
Benzene	71-43-2	Mixed	Yes			2.0	2.00E+03	1.26E+04	4.5E+02	Yes	Yes
Hexadecane	544-76-3	Mixed		Yes	OECD 302D					Yes	Yes
Di-iso octyl phthalate	27554-26-3	Mixed			ISO 10634	8.4	2.40E-04	8.00E+00	1.3E+07	Yes	
1,3,5-trichlorobenzene	108-70-3	Fail				4.19	1.43E+01	6.11E+01	1.6E+02	Yes	
4-nitrophenol	100-02-7	Pass		Yes	OECD 302A	1.9	7.51E+03	9.86E-02	7.1E-04	Yes	Yes
4-nonylphenol	84852-15-3	Mixed	Yes			5.8	1.50E+00	1.26E-02	6.3E-00	Yes	
Octylphenol	140-66-9	Mixed				5.3	4.80E+00	6.91E-02	3.0E-00	Yes	
Pentaerythritol	115-77-5	Fail (32.7%)		Yes		-1.7	1.00E+05	1.92E-06	4.6E-10	Yes	

1: Final recommendations for the reference set – identified in RED

Bin 3: Should normally fail any screening test whether modified RBT or an enhanced screening test

Chemical Name	CAS No	Ready test result	ESR Report	Use in method development studies	Standard test reference chemical	Log K_{ow}	Water solubility (mg l^{-1})	VP (Pa)	HLC (Atm m^3 mol^{-1})	Commercial availability	14C Available
Di-isotridecyl adipate	26401-35-4	Fail (38%)			OECD 302D	>10	<0.0001	6.36E-07	7.1E+04	Yes	
o-terphenyl	199-26-8	Fail				5.5	6.0E-01	1.0E-02	8.6E-01	Yes	
Cyclododeca-1,5,9-triene	4904-61-4	Fail				5.48	0.39	9.76E-00	7.32E-02		
Cyclododecane	294-62-2	Fail				6.12	0.11	3.1E-00	1.4E-00	Yes	
2,4-dibutylphenol	96-76-4	Fail				5.52	0.6	2.1E-03	6.11E-05	Yes	
Chlorothalonil	1897-45-6					3.7	26	7.60E-05	1.9E-02	Yes	
Diethylhexylphthalate	117-81-7		Yes			7.6	0.27	1.89E-05	9.2E+02	Yes	
Fluorene	86-73-7					4.02	1.69	8.00E-02	5.9E-00	Yes	
Methyl parathion	298-00-0			Yes		2.75	38	4.67E-04	1.4E-01	Yes	Yes
Pentachlorophenol	87-86-5	Fail		Yes		4.74	14	1.47E-02	1.2E-01	Yes	Yes
2,2,4,6,6-pentamethylheptane		Fail				5.9	1.6E-01	2.0E+02	2.2E+05		
1,2,4-trichlorobenzene	120-82-1					3.93	49	6.13E+01	2.2E+02	Yes	
2,4,5-trichlorophenol	95-95-4					3.45	1200	6.76E-01	1.2E-00	Yes	
Trans-decalin	493-02-7	Fail (0%) (Acclimated Fail 15.7)				4.20	6.5	1.05E+02	2.7E+03	Yes	

1: Final recommendations for the reference set – identified in RED

Bin 4: Should never pass a modified RBT or an enhanced screening test

Chemical Name	CAS No	Ready test result	ESR Report	Use in method development studies	Standard test reference chemical	Log K_{ow}	Water solubility (mg l^{-1})	VP (Pa)	HLC (Atm m^3 mol^{-1})	Commercial availability	14C Available
Musk xylene	81-15-2	Fail				4.45	0.8	8.5E-05	7.7E-09	Yes	
Hexachlorobenzene	118-74-1	Fail				5.86	0.19	2.40E-03	6.0E-01	Yes	Yes
Benzo(a)pyrene	50-32-8	Fail				6.11	0.0016	7.32E-07	3.2E-03	Yes	Yes
Hexachlorohexane	58-89-9	Fail		Yes		4.26	4	0.0344	4.8E-00	Yes	Yes
Atrazine	1912-24-9	Variable		Yes		2.8	35	0.0038	3.8E-03	Yes	Yes
Benzo(a)anthracene	56-55-3	Fail				5.52	0.029	3.62E-05	2.8E-01	Yes	
Chlorobenzilate	510-15-6					3.99	2.53	2.93E-04	2.0E-03	Yes	
4-chlorobiphenyl	2051-62-9					4.61	5.8	1.40E+00	3.7E-00	Yes	
Chrysene	218-01-9	Fail				5.81	0.003	8.31E-07	5.3E-01	Yes	

1: Final recommendations for the reference set – identified in RED

Appendix 7: Summary of data for chemicals considered but rejected as not suitable for inclusion in reference chemicals list

The following chemicals were initially considered as candidates for the final reference list but were excluded on the grounds that their biodegradability could not be assessed with sufficient confidence to place them in either bin 1, 2 or 3.

4-nitrophenol (4-NP): This chemical is classified in the United States as a priority pollutant. The CICAD mononitrophenols document (WHO, 2000) reports a large variability in the results from a number of RBTs but concludes that 4-NP is inherently biodegradable under aerobic conditions (depending on origin and density of inoculum and the applied test method). Results from different tests point to a possible bacteriotoxic effect of 4-nitrophenol at concentrations above 300 mg l^{-1} (Gerike and Fischer, 1979, 1981; Nyholm *et al*., 1984; Kayser *et al*., 1994).

It has been used as a model compound by several authors (e.g. Gerike and Fischer, 1979, 1981; Nyholm *et al*., 1996, 2003) and shows erratic behaviour in both standardized tests and during method development studies. Sometimes mineralisation is rapid, and on other occasions, mineralisation occurs only after a considerable lag phase. Torang and Nyholm (2005) considered 4-nitrophenol to be readily biodegradable after a lag phase. At 100 ug l^{-1} in natural surface waters they reported that the lag phase was reduced from 10 days to <1 day following an adaptation period of between one and five weeks. 4-nitrophenol is currently being used by Davenport *et al*., (2009) as a reference standard in their studies to investigate the importance of microbial density and diversity in inocula for use in ready tests. Initial results indicate a high probability of biodegradation (>70% parent compound degradation) with enhanced inocula concentrations and extended test duration (60 days) with low variability between inocula from 6 different locations for activated sludge and river water. Greater variation was observed when a 28-day test was used. 4-NP is recommended as a reference chemical for the modified SCAS test (OECD 302A) (OECD, 1981b). It is therefore considered highly likely to pass an enhanced test (using increased biomass). 4-NP has a water solubility of 12.4 g l^{-1}. Measured data (n=18) in non-standard tests suggest a median half-life of 2.5 days in the freshwater environment with a range from 1.3-77 days. 4-NP was rejected due to this variability.

Di-isooctylphthalate: Di-isooctylphthalate (di-2-ethylhexylphthalate) is recommended as a reference standard in ISO 10634 (ISO, 1995). Typical data (Roberts, personal communication, 2009) indicate a lag time of 3-8 days and mineralisation on day 28 of between 50 and 60%. Amendments such as pre-adsorbing on silica or the use of surfactant have a minor effect (sometimes positive and sometimes negative) on the extent of biodegradability. Nyholm (1990)

reported direct addition to the test media was as effective as sonication, emulsification or application on an inert adsorbent as means of dispersing the test chemical in the test solution. In all cases complete degradation (60-65% O_2 uptake) was reported. Di-isooctylphthalate is liquid at room temperature with a solubility of 0.29 ml l^{-1} and was initially considered as a candidate poorly water-soluble chemical for inclusion in bin 2.

Pentaerythritol: Pentaerythritol often exhibits variable results in an RBT. The SIDS (1998) report states that pentaerythritol fails OECD 301C and degradation of 32.7% after 28 days in 301F test is reported by ExxonMobil. Painter and King (1985) reported on the behaviour in an EEC ring test to evaluate biodegradability in an enclosed respirometric method for ready biodegradability. Lag phases varied from 3-15 days and passed the test 9 times out of 11. Other data include that of Gerike and Fischer (1979) who reported 0% after 28 days and 97% after 12 days. Kaiser (1998) reported 13-97% in OECD 301E studies. van Ginkel and Stroo (1992) reported 64% CO_2 production after 28 days and 71% after 42 days and after a 5-20 day lag, degradation was 50-80% after 28 days (Roberts personal communication, 2009). The UK Standing Committee of Analysts (SCA, 2005) published a method for estimating the extent of ready, ultimate biodegradability of organic compounds under aerobic conditions. Using this method pentaerythritol exhibited 84% degradation (mean of 6 replicates) after 28 days. Recent results indicate that pentaerythritol passes a RBT more easily that it used to and thus could not be placed in bin 2 with any confidence, and was therefore rejected.

Pentachlorophenol: Ingerslev *et al*., (1998) studied the aquatic biodegradability of pentachlorophenol (PCP) in a battery of tests including the modified OECD shake flask screening test (MOST) and more environmentally realistic surface water die-away tests at low concentrations (1-74,000 µg l^{-1}). Degradation of PCP was dependent on the chemical concentration and length of the adaptation period which in turn was affected by the type and density of the inoculum. Three different degradation profiles were found. In the first, rapid degradation took place after variable acclimation periods, during which no degradation was observed (e.g. tests in surface water or with low biomass secondary effluent) In the MOST test inoculated with secondary effluent at the recommended stringent low biomass a lag phase of 28-31 days was recorded followed by removal of PCP within a few days at all concentrations. In the second type, rapid degradation took place after a period of slow linear removal of PCP (e.g. experiments using unadapted activated sludge as inoculum. In the third type, slow linear degradation which in some cases occurred after a period with no degradation. In some surface water tests no degradation occurred. Lapertot *et al*., (2006) reported PCP was relatively resistant to biodegradability in a ready test based on BOD analysis but concluded that this may have been as a result of substrate inhibition. Nyholm *et al*., (1996) reported a first order half-life of 2.6 d^{-1} for non adapted sludge and 1.4 d^{-1} for adapted sludge whilst Ingerslev and Nyholm (2000)

reported median lag phases of between 41 and 71 days and median half-lives of between 9 and 39 days in surface water systems. Nyholm *et al*., (2003) studied pentachlorophenol in an activated sludge system. It was not biodegraded in systems with a sludge age of <8 days, which was thought to indicate degradation by slow growing specific degraders. Measured data (n=6) in non-standard tests suggest a median half-life of 128 days with a range from 50-150 days.

Trans decalin: Trans decalin failed a modified 301F test with 2.7% biodegradation after 56 days. It also failed after 56 days with acclimated inoculum with 52.6% biodegradation (XOM data).

Atrazine: Atrazine (2-choro-4-ethylamineo-6-isopropylamino-1,3,5-triazine) biodegradability has been extensively reviewed, by for example, Erikson and Lee (1989) and Ghosh and Philip (2006). Most of the research has been carried out with pure bacterial isolates. It has also been used as a difficult to degrade reference chemical in biodegradability method development studies. Ingerslev and Nyholm (2000) developed a shake flask test for determination of biodegradation rates of ^{14}C labelled chemicals at low concentrations in surface waters in which atrazine was not degraded at all whereas Lapertot *et al*., (2006) reported complete degradation of atrazine in 21 days in a closed bottle RBT based on BOD analysis. Satsuma *et al*., (2002) reported on the biodegradation of atrazine in a water/sediment microcosm. Biodegradation led to a transient accumulation of cyanuric acid followed by gradual mineralisation. Recently Satsuma (2009) has isolated, from a naturally derived river ecosystem, the microbial community responsible for the complete biodegradation of atrazine.

Appendix 8: List of chemicals used in CEFIC LRi environmental projects (www.cefic-lri.org)

Chemical	Project	Log K_{ow}
Ethanol	Eco 8 Fish alternatives project	-0.14
2,2,2-trichloroethanol	Eco 8 Fish alternatives project	1.21
4-fluorophenol	Eco 11 Biomass and diversity project	1.71
Diethylphthalate	Eco 8 Fish alternatives project	2.65
di-n-butylorthophosphate	Eco 8 Fish alternatives project	4.61
4-decylaniline	Eco 8 Fish alternatives project	6.04
Naphthalene	Eco 2a Persistence project, Eco 8 Fish alternatives project	3.30
1,2-dichlorobenzene	Eco 8 Fish alternatives project	3.28
Dichloromethane	Eco 8 Fish alternatives project	1.34
Tetrachloroethylene	Eco 8 Fish alternatives project	2.97
1,2,4-trichlorobenzene	Eco 8 Fish alternatives project	3.93
Aniline	Eco 8 Fish alternatives project	1.08
4-chlorophenol	Eco 8 Fish alternatives project	2.16
2,4,6-trichlorophenol	Eco 8 Fish alternatives project	3.45
3,4-dichloroaniline	Eco 8 Fish alternatives project Eco 1c Soil and sediment toxicity project,	2.37
Allyl alcohol	Eco 8 Fish alternatives project	0.21
Ethanal	Eco 8 Fish alternatives project	-0.17
Acrolein	Eco 8 Fish alternatives project	0.19
2-methyl-1,4-naphthoquinone	Eco 8 Fish alternatives project	2.21
2,3-dimethyl-1,3-butadiene	Eco 8 Fish alternatives project	3.13
2,2-methylenebis(4-chlorophenol)	Eco 8 Fish alternatives project	4.34
4-fluoroaniline	Eco 8 Fish alternatives project	1.2
2,2-methylene-3,4,6-trichlorophenol	Eco 8 Fish alternatives project	6.92
Hexachlorobenzene	Eco 3b Estuary modelling Eco 14a In vivo bioconcentration project	5.73
n-decane	Eco 1a Biomagnification project	6.3

n-tridecane	Eco 1a Biomagnification project	6.73
Chlorinated n-tridecane	Eco 1a Biomagnification project	6.8-7.0
Malathion	Eco 8 Fish alternatives project	2.29
Disulfotan	Eco 8 Fish alternatives project	3.86
4-nonylphenol	Eco 14b In vivo fish bioconcentration project	5.8
n-dodecanol	Eco 14b In vivo fish bioconcentration project	5.1
diisopropylnaphthalene	Eco 14b In vivo fish bioconcentration project	6.1
Hexadecylamine	Eco 14b In vivo fish bioconcentration project	6.7
n-tridecene	Eco 14a In vivo fish bioconcentration project	6.6
Endosulfan	Eco 14a In vivo fish bioconcentration project	2.23-3.62
Chlorpyrifos	Eco 14a In vivo fish bioconcentration project Eco 14b In vivo fish bioconcentration project	4.9
Pentachlorobenzene	Eco 14a In vivo fish bioconcentration project	5.2
4-t-octylphenol	Eco 14a In vivo fish bioconcentration project	5.3
Rotenone	Eco 8 Fish alternatives project	4.31
2,4-dintrophenol	Eco 8 Fish alternatives project	1.73
Pentachlorophenol	Eco 8 Fish alternatives project Eco 1c Soil and sediment toxicity project Eco 2a Persistence project Eco 15 Trophic magnification factors project	4.74, 5.02, 5.12
Permethrin	Eco 8 Fish alternatives project	7.43
Lindane	Eco 8 Fish alternatives project	3.72
Phenobarbital	Eco 8 Fish alternatives project	1.33
Hexamethylenetetramine	Eco 8 Fish alternatives project	
2-aminoethanol	Eco 8 Fish alternatives project	-1.61
DDT	Eco 1c Soil and sediment toxicity project Eco 2a Persistence project Eco 14b In vivo bioconcentration project Eco 14 Bioconcentration project	6.79, 6.2
Benzo(a)pyrene	Eco 1c Soil and sediment toxicity project, Eco 1c Biomagnification project Eco 2a Persistence project Eco 3b Estuary food web modelling	6.11, 6.06
Pyrene	Eco 3b Estuary food web modelling	6.58
Chrysene	Eco 1a Biomagnification project	5.86
2,4-dichlorophenol	Eco 1c Soil and sediment toxicity project Eco 2a Persistence project	2.8
Trinitrotoluene	Eco 1c Soil and sediment toxicity project	1.99

Phenol	Eco 2a Persistence project	1.46
4-hydroxybenzoate	Eco 11 Biomass and diversity project	
PCB 194	Eco 3a POPs modeling project	
PCB 180	Eco 3a POPs modeling project	
PCB 153	Eco 1a Biomagnification project Eco 3a POPs modeling project Eco 3b Estuary food web modelling project	
PCB 118	Eco 3b Estuary food web modelling project	
PCB 101	Eco 3b Estuary food web modelling project	
PCB 77	Eco 3a POPs modelling project	
PCB 52	Eco 3a POPs modelling project	
PCB 53	Eco 14b In vivo bioconcentration project	
PCB 28	Eco 3a POPs modelling project	
PCB 8	Eco 3a POPs modelling project	
Atrazine	Eco 3a POPs modelling project	2.61
Isoproturon	EEM 2 Terrestrial modelling project	
Eco 8 Extended List		
Salicylanilide	Eco 8 Fish alternatives project	
2-hydroxyethylether	Eco 8 Fish alternatives project	
2,3,4,5-tetrachlorophenol	Eco 8 Fish alternatives project	4.09
Triethylene glycol	Eco 8 Fish alternatives project	-1.75
4-nitrophenol	Eco 11 Biomass and diversity project Eco 8 Fish alternatives project	1.91
Pyridine	Eco 8 Fish alternatives project	
n-cresol	Eco 8 Fish alternatives project	2.06
2-chlorophenol	Eco 8 Fish alternatives project	
2,4-dimethylphenol	Eco 8 Fish alternatives project	
N-methylaniline	Eco 8 Fish alternatives project	

Appendix 9: Testing the Biodegradability of Poorly Water-Soluble Substances - Appendix 7.9-3 Guidance on information requirements and chemical safety assessment Chapter R.7b: Endpoint specific guidance

This appendix discusses the technical issues associated with conducting biodegradability assays with poorly water-soluble substances and the data-reporting requirements that would improve confidence in the data generated for such substances. The OECD (1995) and ISO Guidance 10634 (1995) for testing poorly water-soluble substances form the basis of discussion. Whilst the focus of this document will be towards methods for assessing the ready biodegradability of poorly water-soluble substances (OECD 301 series and the OECD 310 test) the issues equally apply to other biodegradability assays.

OECD Evaluation of the Biodegradability of Poorly Soluble Substances

OECD requires that when assessing biodegradability of poorly soluble compounds OECD the following aspects should receive special attention (OECD, 1992: Annex III):

- While homogeneous liquids will seldom present sampling problems, it is recommended that solid materials be homogenised by appropriate means to avoid errors due to non-homogeneity. Special care must be taken when representative samples of a few milligrams are required from mixtures of chemicals or substances with large amounts of impurities.
- Various forms of agitation during the test may be used. Care should be taken to use only sufficient agitation to keep the chemical dispersed, and to avoid overheating, excessive foaming and excessive shear forces.
- An emulsifier which gives a stable dispersion of the chemical may be used. It should not be toxic to bacteria and must not be biodegradable or cause foaming under the test conditions.
- The same criteria apply to solvents as to the emulsifiers.
- It is not recommended that solid carriers be used for solid test substances but they may be suitable for oily substances.
- When auxiliary substances such as emulsifiers, solvents and carriers are used, a blank run containing the auxiliary substance should be performed.
- Any of the four respirometric tests (301 B, 301 C, 301 D, 301 F) can be used to study the biodegradability of poorly soluble compounds.

Whilst OECD raise a series of valid issues that require careful considerations in testing the biodegradability of poorly soluble substances they do not constitute explicit guidance. The only critical guidance provided is the applicability of a restricted range of the 301 test series (point 7) and the requirement of additional control vessels where emulsifiers, solvents and carriers are used (point 6). Tests conducted with draft OECD 310 test (Headpsace test) are also suitable for assessing the biodegradability of poorly soluble substances.

Whilst advocating the use of emulsifiers, solvents and carriers, none are specifically identified and no guidance is provided regarding the acceptable level of each that can be introduced into the

test system. Consequently, numerous approaches of introducing the test substance can be applied and this will make it difficult to identify a set of core acceptable or workable solutions.

ISO Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in aqueous medium

In 1995 the International Standards Organization (ISO) concluded that the development of a single method for evaluating the biodegradability of poorly water-soluble organic substances might not be realized in the immediate future. Consequently, ISO proposed a series of methods where the final selection was based on a judgment of the physico-chemical properties of the test substance (ISO, 1995).

The ISO standard (1995) addressed four techniques for preparing poorly water-soluble substances and introducing them into the test apparatus. It must be noted than for water-soluble test substances compounds are usually introduced into the test medium via a concentrated stock solution. The methods proposed by ISO for poorly soluble substances were 1) direct addition, 2) ultrasonic dispersion, 3) adsorption on an inert support, and 4) creating a dispersion or emulsion. All of these techniques proposed by ISO are suitable for including within the OECD 301 and 310 test guidelines. ISO does not provide any advice about the use of suitable poorly soluble reference standards. Each of the ISO methods will be described below with a brief commentary or assessment.

Direct addition

ISO proposed introducing the test compound by either 1) weighing the substance directly into the test vessel, 2) weighing the test compound on to an inert support (typically a glass cover slip or piece of foil) and introducing this into the test vessel, or 3) preparing a solution of the test substance in a volatile solvent are removing the solvent prior to testing. Direct addition is applicable for a variety of substances e.g. crystalline solids and non-viscous liquids. These are introduced using either high precision micro-pipettes or direct weighing. In the case of direct weighing some replicate-to-replicate variability can be expected for crystalline compounds as they are usually being introduced at the very low mg weight range. Whilst direct pipetting using viscous liquids can be problematic, the use of a cover slip or foil can over come this. However care should be taken to ensure that the cover slip remains face up, if this becomes inverted then the microbiota will not be able to access the test substance. It must be noted that control flasks will be needed where carrier solvents have been used to ensure that all the solvent has been eliminated. In this case the same volume of the solvent needs to be introduced into the test system as in the test flask, but without the test substance. Even low levels of respiration associated with the solvent will need to be accounted for when interpreting data from the test flasks. Whilst controls should be used for cover slips etc. it is unlikely that any background respiration will be observed. Direct addition, particularly via direct weighing (or pipetting) or using a support, should act as a 'bench mark' and be applied in the assessment of all poorly water-soluble substances i.e. they should be used in parallel to any of the other guidance methods recommended by ISO. Direct addition is likely to give the most conservative estimate of biodegradation.

Ultrasonic dispersion

ISO (1995) recommend that a dispersion of the compound can be prepared using an ultrasonic probe prior to introducing it into the test vessel. Specific guidance are provided with respect to the

frequency of the ultrasonication required to make a 20 times concentrated stock solution, however total carbon analysis is required to confirm the concentration achieved. It must be noted that this approach is not suitable for substances that undergo thermal decomposition and that a stable emulsion is rarely formed. Consequently, this may not be the most appropriate approach recommended within the ISO guidance. This is particular true when stable emulsions cannot be formed and large numbers of sacrificial test flasks are being prepared as the possibility exists for introducing reduced concentrations to each flask with time i.e. a concentration gradient. If this technique is to be applied to tests using sacrificial analysis (e.g. OECD 310) the test flaks need to be sacrificed randomly for analysis at each time point.

Adsorption onto an inert support

ISO (1995) recommend the use of silica gel, glass filter or any other non-biodegradable inert supports that do not release organic carbon into the test media. Supporting evidence is required to demonstrate that the support is inert and carbon free and the amount of support used should be minimal. Silica-based gels that are used for chromatography represent an inert support that has been used successfully. The test compound is usually introduced into the inert support at the required concentration via a carrier solvent (e.g. acetone or dichloromethane). Rotary evaporation and oven drying are then used to remove the solvent. A parallel procedure is required using the inert support and carrier solvent without the test substance for use in the control test flasks. Inert supports can also be used with insoluble solids. Prior to testing the carbon level of the inert support containing the test chemical or the specific chemical contained in the inert support needs to be quantitatively determined and compared to nominal. The required amount of the inert support can then be directly weighed into the test vessel. Any biodegradation of the solvent should be taken into account through the use of parallel control vessels. This procedure is applicable for compounds that will not be lost during the rotary evaporation and oven drying procedures. It does enable the amount of material to be directly weighed into the test flask to be increased thus increasing accuracy between replicate test flasks.

Dispersion with an emulsifying agent

ISO (1995) recommend using emulsifying agents to enhance the available of the poorly soluble test substance that are non-biodegradable and non-toxic under the conditions of the biodegradation test. Synperonic PE/P94, Synperonic PE/P103 or Tween 85 have been identified as commercial substances that could be used as emulsifying agents. Carrier solvents that are also non-toxic and non-biodegradable are also required to form these emulsions. ISO recommends that three emulsions be prepared prior to selecting the most homogeneous emulsion for use in the biodegradation test. Very clear guidance is also provided that states that the degradation observed in the control vessel (solvent and emulsifier with no test compound) must not exceed 10% of the degradation observed in the test flasks for the test to be consider valid. Supporting evidence should be provided to demonstrate that neither the solvent or the emulsifying agents are toxic to microbes or are biodegradable.

Minimum Test and Data Requirements for Poorly Water-Soluble Substances

The following information should be reported:

- Information on the chemical's water solubility, vapour pressure and adsorption characteristics are essential.
- The solubility of the chemical in other solvents should be stated (especially those being used to disperse the chemical in emulsifications and on to inert supports).
- The chemical structure or formula should be identified in order to calculate theoretical values and/or check measured values of parameters, e.g. ThOD, ThCO2, DOC, TOC, and COD. Information on the purity or the relative proportions of major components of the test material is required in order to interpret the results obtained, especially when the result lies close to the pass level.
- Information on the toxicity of the test substance, or any emulsifiers or carrier solvents, to bacteria may be very useful for selecting appropriate test concentrations and preparation strategies.
- Any pre-treatment of the compound before the test.
- The method of test substance introduction should be described in detail with supporting evidence especially regarding the use of solvents, emulsifiers and inert supports.
- Nominal versus measured carbon concentrations where inert supports and emulsions are used to generate concentrated stock preparations of the test substance prior to use. This should include the degree of recovery.
- Duration of any pre-treatment.
- Rate of degradation observed in the control flasks (treatment minus test substance).
- Suitable positive reference poorly soluble data (see below).

Conclusions and recommendations on biodegradability testing of poorly water-soluble chemicals

There is no single method for assessing the biodegradability of poorly water-soluble substances. The state of the science has not changed since ISO published its guidance in 1995. A combination of approaches should be used and these should at the very minimum be compared to biodegradation observed by direct addition. Direct addition will usually provide the most conservative estimate of biodegradation.

Normal positive reference substances such as sodium acetate, sodium benzoate, aniline or glucose offer little support in the assessment of poorly soluble substances other than demonstrate that the inoculum is active. In order to 'bench mark' methods to assess poorly soluble substances common poorly soluble reference substances should be used. Two examples are provided in the Annexes of the ISO guidance. These are biodegradation curves for diisooctylphthalate (where adsorption on inert support and dispersion with an emulsifying agent enhances degradation compared to direct addition) and anthraquinone (where adsorption on inert support and dispersion with an emulsifying agent enhances degradation compared to direct addition). In both cases the use of ultrasonication did not provide any significant benefit.

Greater confidence in the methods for increasing the availability of poorly soluble substances will be gained by using either diisooctylphthalate or anthraquinone as a positive control. The reference control should be introduced to the test system by direct addition and the choice of preparation. Therefore for any given biodegradation assessment there will need to be the following series of flasks:

- Blank Control (inoculum and media with no test compound);
- Positive reference for biodegradation (sodium acetate, sodium benzoate, aniline or glucose);
- Poorly soluble positive control (either diisooctylphthalate or anthraquinone introduced by direct addition);
- Test substance (introduced by direct addition for conservative assessment); Direct addition control;
- Test substance with choice of introduction (e.g. adsorption on an inert support);
- Poorly soluble positive control using the same choice of introduction as the test substance;
- Choice of introduction control (e.g. inert support and solvent without the test substance).

The above set of flasks appears onerous but they do not constitute a great deal of extra effort or expense. The long-term value of providing the additional information will be one of greater confidence in assessing poorly-soluble material against agreed bench mark standards.