

***Workshop on Biodegradation
and Persistence
26-27 June 2007, Holmes Chapel***

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1. EXECUTIVE SUMMARY

This two-day workshop sponsored and organised by ECETOC and co-sponsored by the Environment Agency for England and Wales, took place at Cranage Hall, Holmes Chapel, UK on the 26-27 June 2007.

The aim of the workshop was to identify the key areas of research that were needed to further progress made in the assessment of biodegradation of chemicals, during the REACH (Registration, Evaluation and Authorisation of Chemicals) RIP (REACH Implementation Projects) Endpoint Working Group on Biodegradation. The principle areas covered were those that addressed enhanced screening tests and higher tier tests.

There were 38 attendees, with people from academia, industry and the regulatory community. Appendix 1 gives the list of attendees and Appendix 2 the meeting programme.

The primary outcome of the meeting was agreement on a number of research activities that, if funding was available (e.g. through the CEFIC Long Range Research Initiative) would help improve the current approaches to assessing the evaluation of the persistence of chemicals in the environment. These activities are briefly described in Table 1 and in Appendices 3-10.

Screening studies

The workshop agreed that current tests used to assess ready biodegradability ('ready tests') were not very good at differentiating persistent chemicals and, because they vary widely in their test conditions and contain many historical differences, they also generate very variable data. More information could be extracted from ready tests (e.g. shape of the degradation curve) and with the implementation of REACH there is an opportunity to consider systematic standardisation of the ready tests (e.g. substrate concentration, inoculum size etc).

There was a broad consensus that an enhanced tier of biodegradation screening studies are 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. Whilst extension of the test duration and conducting studies using higher test volumes posed little concern to regulatory members of the workshop, it was felt that some validation and standardisation was needed with respect to working with higher biomass levels and acclimated inocula. It was envisaged that such enhanced tests could contribute to a weight of evidence approach to decide if a chemical is persistent.

The group also decided that, in order to prevent confusion over the terms acclimation and adaptation, the terminology "deliberate pre-exposure of the inoculum to the test chemical" should

be used to describe adapted or acclimated inocula. Some participants recommended the use of highly adapted systems as a positive screen for persistence i.e. chemicals that could not degrade in such systems can be assumed to persist. The workshop concluded that adaptation should be taken into account in any assessment of the persistence of a chemical (pre-exposure should be at environmentally realistic concentrations).

It was discussed whether there were new analytical and labelling techniques becoming available that may overcome a) the technical limitations associated with conventional endpoints (O_2 , CO_2 and DOC) and b) the requirement for radiolabelled material to study fate, including biodegradation, at low substance concentrations.

The workshop also addressed the possibility of measuring half-lives from a ‘battery’ of tests, thus generating a distribution of half-lives. These could be compared to single values and assessed for their usefulness within the regulatory decision making process. This approach was potentially very powerful when part of a strategy that involved rapid screening tests and addressed microbial diversity.

Higher tiered studies

There was a general consensus that there are no soil, sediment or water biodegradation studies that accurately simulate biodegradation in the ‘natural’ environment and the phrase ‘simulation tests’ was misleading. There are higher tiered biodegradation studies (e.g. OECD 307, 308 and 309) that use environmental media that can describe degradation under conditions that have a greater environmental relevance than the ready biodegradation test. However, many of these studies do pose considerable problems associated with their interpretation. Problems identified included:

- Selection of the most appropriate compartment for testing;
- how to deal with bound residues;
- how to discriminate between degradation half-lives for persistency assessments and rates of dissipation from water or sediment;
- how to correct for temperature differences and is it really necessary.

Selecting the most appropriate inoculum is important in generating relevant half-life data but this is not as simple as deciding which compartments most of the chemical ends up in (for example >5% in any compartment signifies realistic presence under Canadian legislation).

It was proposed that the OECD 307 and 308 tests could be extended to 180 days when appropriate to match the P criteria. There was also an agreement that further discussions and more guidance on the interpretation of results from these studies was needed.

Recommendations for future research

The topics listed in Table 1 (see section 3.7) were developed into RfPs (see Appendices 3-10). Although not given a priority during the workshop, in the process of writing this report, the authors have sought an indication of the priority for the work from the workshop attendees. The following RfPs were considered to be of very high priority for PBT assessment and/or risk assessment, with a good chance for success:

- **Validation set of chemicals for biodegradation research**
This short and targeted project was seen as being essential to all the other projects, in that an agreed set of chemicals with a range of biodegradation behaviours would be extremely useful in helping to ensure that methods were “tuned” to their task and not overly protective or too powerful.
- **Addressing bound residues**
This project was also seen as short term, being a review of the issues and science, to be followed by a workshop that could address the issues and design the research necessary to investigate the issue of bound residues and put them into a proper context. This could also be an ECETOC Task Force, followed by a CEFIC-LRI funded workshop;
- **Microbial density and diversity and the ratio of chemical to microbial mass**
This was one of two, large projects that could be considered as fundamental to the discussions about how to test chemicals for their biodegradation in the environment and how to generate the values for half-lives needed for PBT and risk assessment. This project was seen as focussing on the methodologies for generating test systems and to address the impact of diversity/density and how these might be considered in screening tests and then for the development of low cost enhanced screening tests;
- **Measurement of biodegradation half-lives and identifying sources of variability**
This second project was seen as focussing more on the generation of half-lives that were relevant to assessment of chemicals in the environment and which supported a ‘probabilistic’ approach to the assessment, including the identification of environmental half-life bands.

2. BACKGROUND

The ability of certain chemical substances to persist in the environment is an issue of global concern that requires careful consideration in environmental risk assessment. This is especially true when this ability is coupled with bioaccumulative (B) and toxicological (T) properties, i.e. when it is classed as a PBT or vPvB substance. However, assessing environmental persistence of chemical substances is not straightforward. Persistence cannot be directly measured; it can only be inferred from the continued presence of a substance (or of metabolites, when they are persistent) in the environment or the lack of observed degradation data in the environment (ECETOC, 2003).

Degradation is an important process that can result in the loss or transformation of a chemical substance in the environment. Degradation of organic chemicals in the environment influences exposure and, hence, it is a key parameter for estimating the risk of long-term adverse effects on biota. Degradation rates, or half-lives, are determined in, or default rates assigned from, laboratory-based degradation tests. These tests can be simple screening tests (e.g. the OECD 301 and 310 ready biodegradability tests) or relatively complex higher tiered 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).

Information on the degradability of chemicals is an essential requirement for hazard assessment (e.g. for classification and labelling), environmental risk assessment (for chemical safety assessment) and persistency assessments (for PBT/vPvB assessment). At the STEP (Simulation Testing for Environmental Persistence) workshop held in 2004 a number of potential improvements to the biodegradation screening tests were identified. These included extending the test duration for poorly water soluble substances, conducting the study at lower test substance concentrations and using information on primary biodegradation. The STEP workshop concluded that “a number of enhancements for increasing the reliability of the ‘ready’ screening tests had been identified” and “that further guidance needed to be developed in this area as a matter of urgency”.

During the preparation of the technical guidance documents for REACH (Registration, Evaluation and Authorisation of Chemicals) the use of degradation data against the three regulatory endpoints identified above was carefully considered. In developing this guidance further modifications and enhancements were identified by the REACH Implementation Project (RIP 3.3) endpoint working group on degradation. These enhancements were aimed at increasing the environmental relevance and predictability of biodegradation and environmental persistence. Members of this endpoint working group felt that the enhancements identified by the STEP workshop and the proposed REACH guidance merited broader discussion by interested stakeholders from regulatory authorities, industry and academia.

In order to maintain the momentum of these valuable discussions, and to communicate the relevant outputs of the REACH Implementation Projects to a broader community of scientists, ECETOC and the Environment Agency for England and Wales agreed to co-organise a workshop to help improve the scientific basis for persistency and biodegradation assessments. In doing so it was necessary for the workshop to consider: a) the design, application domain and limitations of existing biodegradability tests; b) the technical challenges faced in the design of new biodegradability tests to assess the persistence of chemicals in the environment; and c) how biodegradability data should be interpreted and analysed, especially data from higher tier biodegradability tests.

Thirty-eight experts (Appendix 1), from thirteen countries, who had experience in biodegradation testing, microbiology, environmental fate, environmental chemistry or regulating persistent chemicals attended the workshop. Nine regulatory agencies were represented and the remaining participants were equally split between academia and industry. The workshop was held at Cranage Hall, Holmes Chapel, UK on the 26-27 June 2007. The workshop had a series of formal presentations highlighting issues at the screening and higher tiered testing levels. There were three syndicate and plenary sessions to focus on, and prioritise, the key issues (Appendix 2).

To address the aims of the workshop the participants were set the following objectives:

- Identify methodologies to improve assessment of biodegradation:
 - the gap between ready tests and higher tier soil, sediment and water tests;
 - the design and interpretation of higher tier tests;
- identify research to address knowledge gaps in the proposed methodologies;
- draft research plans including potential funding opportunities, collaborations and timelines to develop improve tests to address persistence.

3. WORKSHOP OVERVIEW

3.1 Screening studies

The aim of this session was to identify areas where the ready biodegradation tests (RBT) could be further improved to help better predictions of biodegradation be made. A series of presentations introducing the issues relating to screening studies was followed by a syndicate session.

Jason Snape (AstraZeneca, UK) gave an introductory overview of enhanced screening tests and how they may be used to determine persistence with particular reference to REACH requirements. The difficulties in assessing persistence were described. A weight of evidence approach considering the factors that influence persistence was recommended. Ready tests are designed to screen out chemicals that will rapidly biodegrade in all environments. However, they are not truly standardised and have a high potential for conflicting or variable results. This may lead to difficulties in interpretation in terms of persistence. Ready tests can be modified without reducing the stringency of the test philosophy e.g. by testing at low concentrations and by enhancing the bioavailability of poorly soluble chemicals. Enhanced screening tests are more relevant for assessing persistence, especially if pre-adaptation of the inoculum is included.

Steve Robertson (Environment Agency, UK) described the work of the EU PBT WG. Their goal was to prioritise the chemicals that require further action, to advise on further work and to develop and standardise principles for assessing PBTs. Of the 117 substances reviewed to date, 26 have been confirmed as PBTs. The lessons learned from these assessments are that QSARs provide useful supporting information but cannot be used alone and that ready biodegradation tests remain useful at the screening level especially when mineralisation, although less than the passmark, was observed. The limitations of ready tests, e.g. testing poorly soluble substances can be overcome by enhancing the test design but the modifications need to achieve general acceptance. Such modifications are worth pursuing because simulation testing is difficult to conduct and interpret and resource intensive.

Although marine biodegradability is a key endpoint for persistency assessment under REACH, there is a methodology gap for cost effective and relevant screening tools. Aourel Mauffret (Andalusia Institute of Marine Science, Spain) described a study to develop a new test system that would decrease variability of results. The goal was to develop a reliable and efficient inoculum for marine biodegradability screening tests. Biofilms were established by colonising glass beads in an open flow through system of seawater. The colonised beads were used as inoculum in several test systems. Linear alkylbenzene sulphonate (LAS) was used as model compound and spiked at environmentally relevant concentrations. The extent of LAS mineralisation depended strongly on the volume of colonised beads. Acclimation enhanced the biodegradation rate. Low temperatures slowed down the mineralisation rate, however, the bacterial origin and their field conditions played a key role in their response to cold stress.

Gérald Thouand (University of Nantes, France) focussed on the inoculum as the main source of variability in biodegradation tests and showed that a clear understanding of the identity of an inoculum can help to perform better biodegradability tests. The stringency of the RBTs was demonstrated by showing that the level of bacteria present in such a test varied by one to two orders of magnitude. Therefore there was a 20-80% chance of a false negative i.e. a biodegradable compound being assigned as persistent (based on an OECD 301B type study).

The main parameter to take into account is the cell density which must be fixed above 10^{11} total bacteria/litre (final concentration) and when the cell density is adjusted to this level, the ratio S_0/X_0 (i.e. f/m) should be considered. This threshold value results in a higher probability of introducing competent organisms into the flask. A strategy on how to address environmental biodegradation and its variability was described by Dr Thouand. He proposed that when testing chemicals, a number of sources of inocula should be used, with proven high diversity, in a battery approach, thus describing a probability of biodegradation. When addressing selection of inocula: think about:

- the probability of biodegradation (referred to as ‘probability concept’ in the rest of the document);
- a pre-evaluation of the diversity;
- the use of standard arrays of micro-organisms.

Eugene Madsen (Cornell University, USA) described how Stable Isotope Probing (SIP) could be applied to biodegradation processes. SIP is a relatively new research technique that allows the members (taxonomic groups and their genes) of microbial communities actively involved in biodegradation to be identified. The basic principles of SIP were reviewed, as were the results of a case study involving naphthalene biodegradation in surface sediments. SIP may offer a unique way to diagnose and understand situations in which organic compounds considered ‘biodegradable’ have been found to persist. SIP could also help validate lab to field extrapolation i.e. prove the degraders in the lab and field are one and the same.

3.2 Syndicate Discussions

Following the presentations, participants worked in four syndicates to address a number of questions designed to identify how screening tests could be modified to provide better assessments of biodegradability.

The potential to change microbial densities and the impact of microbial diversity on biodegradation potential in the context of developing enhanced biodegradation screening studies was discussed by syndicate A.

The REACH Endpoint Working Group for degradation has identified a number of possible enhancements to RBTs used as an initial screen of persistence. These included extending the test duration, increasing the test volume, enhancing the biomass levels and allowing for acclimation. Whilst extension of the test duration and conducting studies using higher test volumes posed little concern to regulatory members of the syndicate, it was felt that some validation and standardisation was needed with respect to working with higher biomass levels and acclimated inocula.

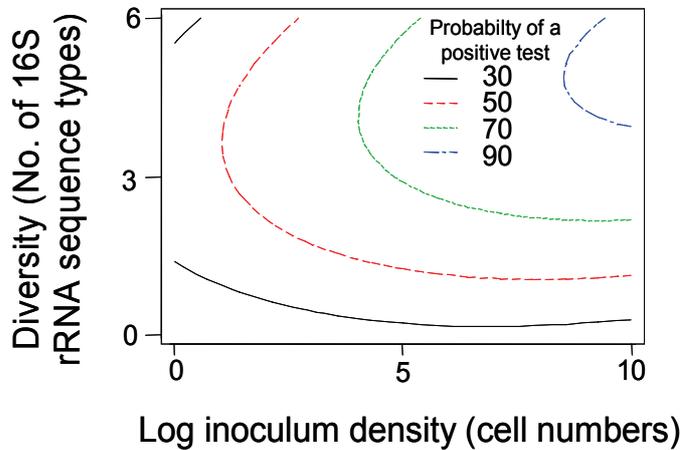
Investigations (Thouand *et al*, 1995; 1996) have already demonstrated the relationship between cell density and the probability of para-nitrophenol being degraded in river water. Mauffret *et al* (2007) have also demonstrated the importance of increasing microbial cell densities and its environmental relevance, by colonising glass beads, to assess the marine biodegradability of LAS. Finally, research by Ingerslev and Nyholm (2000) found that low test volumes increased (i) the lag phase prior to the onset of degradation of para-nitrophenol and 2,4-D and (ii) the chance of false negatives. Ingerslev and Nyholm (2000) attributed this to the absence of competent degraders at lower test volumes.

The syndicate concluded that research was necessary to explore the relationship between microbial density, microbial diversity, test volume and the probability for observing biodegradation (see Figure 1). It was agreed that these investigations should be conducted with a much wider defined reference set of chemicals that had varying degrees of environmental persistency and included some chemicals for which no degradation should be observed. There was a broad consensus that such an approach would improve the predictability of biodegradation at the screening level and offer a more scientific basis for prioritisation based on environmental persistence.

It was agreed that methods for biomass concentration, (e.g. centrifugation, filtration and colonisation of glass beads and other inert supports), were worthy of further investigation. The syndicate were particularly interested in comparing membrane filtration techniques with the colonisation techniques of Mauffret *et al* (2007) to see if the need for extensive colonisation periods could be avoided. In following such an approach it was agreed that some protocols were required to maintain the health of the inoculum e.g. protection from protozoan grazing.

However, it was recognised that increasing biomass levels may cause problems with the signal to noise ratio associated with the traditional semi-specific endpoints such as dissolved organic carbon (DOC) removal, CO₂ evolution and oxygen demand. Therefore either a strategy to reduce background carbon levels would be required or studies would need to be conducted with specific chemical analysis. It was also recognised that test systems that were purged with air (e.g. OECD 301B) would need to be used to prevent vessels going anoxic at higher biomass levels.

Figure 1: Establishing the relationship between cell density, inoculum diversity and the probability of observing biodegradation.



To validate whether increasing biomass density is an appropriate enhancement approach it was agreed that a matrix that examines the impact of density and volume should be used that incorporates some level of redundancy e.g. conducting a study with a volume of 10 litres with 30 mg/l biomass and comparing it to a study with a volume of 100 ml that contained 3000 mg/biomass.

Within the context of inoculum composition and inoculum variability a discussion was held with regard to the merits of the MITI I (OECD 301C) and MITI II (OECD 302C) studies. The philosophy of using fresh samples from no fewer than ten sites, mainly in areas where a variety of chemicals were used was broadly supported as it has the potential to maximise the microbial diversity and broaden the geographical relevance of the inoculum. However, the conditioning of the inocula with glucose and peptone (MITI I) or synthetic sewage (MITI II) for a minimum of 28 days counter-balanced these diversity gains. The syndicate felt that both MITI studies should be evaluated without the conditioning regime. There was also a broad consensus that with a biomass level of 100 mg/l and substance concentration of 30 mg/l that the MITI II could be seen as equivalent to an enhanced screening study as it lacked the degradative power associated with either the Zahn-Wellens (OECD 302B) or SCAS (OECD 302A) tests.

Syndicate B was asked to address the issue of adaptation. There was initial confusion over the terms acclimation and adaptation. Some considered acclimation the process whereby a sample from the field was allowed to acclimate to conditions in the laboratory, for example the temperature and light conditions. Others considered it the process, in which a sample was acclimated through exposure to the test material. Adaptation was seen by most as the microbial response to this exposure resulting in enhanced biodegradation capabilities. To avoid further

confusion, the group decided to use the terminology “deliberate pre-exposure of the inoculum to the test chemical” to clarify what was meant by adapted or acclimated inocula.

It was agreed that most inocula are already pre-adapted to many chemicals currently in production if they are present in wastewater. The major benefit of pre-exposure would be for new to the world chemicals or those present at very low level in wastewater compared to the normal test concentration in a screening test. The challenge around any screening test for persistence is to get competent degraders in the inoculum and ensure that these organisms are relevant. Approaches to increase the competent degraders in the inoculum include adding a bigger inoculum, increasing test vessel size or increasing test material and inoculum levels together. Any such measures must ensure that the signal to noise (background in the blank) ratio remains sufficient to allow accurate quantification of the percent biodegraded. Such efforts have the potential to increase the level or the probability of having competent degrader by a single digit multiplier. In contrast, pre-exposure to the test chemical as a positive selective pressure has the potential to increase the level or probability of having competent degraders by orders of magnitude.

Despite the benefits of such a strengthened inoculum, there is a significant fear by the regulators of false positives, where a persistent chemical is misidentified as biodegradable. Hence, to be deemed acceptable these pre-exposure approaches must avoid the potential for false positives to occur.

Pre-exposure was postulated to act in several ways including:

- Inducing the synthesis of non-constitutive enzymes needed for the degradation procession;
- allowing the size of small population of competent degraders to reach a sufficient population size to ensure that they are represented in the initial inoculum or at high enough level in the test to effect biodegradation;
- facilitating the development of new phenotypes of competent degraders to appear as a result of positive selection of new genotypes resulting from mutation or other genetic changes.

The first two processes are seen as more predictable and less random than the third and possibly more broadly relevant in the normal environment. With this perspective, it was felt that any pre-exposure should facilitate the first two processes but not the third.

It was then agreed that the use of pre-exposed inocula may be acceptable if certain conditions are met including:

- The original inoculum is derived from the relevant habit where persistence is being assessed or a relevant upstream source (e.g. wastewater treatment plant for a freshwater stream);

- the pre-exposure occurs under environmental conditions relevant to those in this habitat and/or the screening test;
- the time frame of the pre-exposure is relevant and not excessive. For example, the time frame should not exceed normal chemical and cell residence times or the persistence criteria half-life in the relevant habitat. Examples include activated sludge age of treatment plants (ca 15d); flow time in rivers (3-10d), and the standard EU TGD criteria of 40d in the freshwater environment.

To make this proposition more acceptable, research to understand the variables associated with pre-exposure and adaptive responses in the field and lab would be useful including the role of test material concentration, time and growth conditions. Such research might simply consist of theoretical comparisons based upon Monod growth models. Importantly, there was a strong consensus that pre-exposure protocols need to be validated using reference chemicals, including the so called GRAPs (Generally Regarded as Persistent), to provide reassurance that the pre-exposure treatment has little risk of generating false positive results.

Despite their regulatory importance, it was stated that the only environmental situation accurately simulated by a ready test is the ready test. The value of the test has been its historic track record for identifying easily biodegradable chemicals not differentiating persistent ones.

The ready tests vary widely in their conditions and contain many historical vestigial differences, which make no ecological or analytical sense. Most have never been systematically standardised or optimised relative to signal to noise, test substance concentration, inoculum size etc. It may be time to address these differences and simplify the options.

The information available from a ready test including, for example shape of the curve, is not being fully utilised at present. UBA is considering a research project to better harvest this information. Other references include Nopens *et al* (2000) and Storhas *et al* (2001).

Based upon current convention and terminology, a ready-type test inoculated with a deliberately pre-exposed inoculum is no longer a ready test. These nuances can be a source of confusion for many.

Sequencing batch reactors (e.g. semi-continuous activated sludge, SCAS, units) are a very powerful method of applying selective pressure on the biomass. They can be an excellent system for pre-exposing inocula for screening tests. Activated sludge, sediment or soil can be used as inoculum. Removal of biomass at various intervals following addition of the test substance to the reactor, and using this as an inoculum for an RBT could be a way of measuring the ease/difficulty with which competent degraders can be established (i.e. binning). They have a proven history for this purpose and there is evidence in unpublished studies showing that not everything pre-exposed in a SCAS ultimately undergoes biodegradation in screening tests or the environment.

Syndicate C addressed f:m ratio (food to micro-organisms). Initial discussions focussed on why the f:m ratio was important in these screening tests. There were two main effects that were considered, firstly it was thought that the probability of biodegradation was enhanced if f:m is low (although at the same time it was also recognised that the substrate concentration needed to be above a critical concentration). Secondly the kinetics of degradation may alter as density of the micro-organisms (m) is reduced. Concern was also raised about the impact of changing the f:m ratio on the signal to noise ratio in the test system. While this was not thought to be a problem for tests where the specific methods are used for the endpoint, it is a problem when non-specific methods, e.g. CO₂, O₂ uptake, DOC are used. It was thought that more could be done to understand the source of the noise observed in screening tests, and if, as was suspected, background levels of organic carbon were one major source, attempts should be made to try and reduce these. Other influences on the rate of biodegradation included the bioavailability of the test substance, which will influence the f in the test system and the impact of selection pressure on micro-organisms.

Some suggestions as to how this might be overcome included measuring the probability of biodegradation, perhaps in a battery of tests (as discussed in the Thouand presentation). It was also thought that by including adaptation in the test the variability in the tests would also be reduced. Lowering the test concentration and/or increasing the microbial biomass levels were also seen as useful approaches that would be unlikely to lead to changes in the actual rates or kinetics, but could reduce the variability and increase the power of the tests. Finally understanding the diversity of inoculum sources and evaluating a range of inoculum sources were also seen as valuable approaches to developing knowledge in this area.

In the discussion, bioavailability of the substrate (test substance) was seen as very important. Many chemicals were failing RBTs, being limited by their solubility and the kinetics of getting into solution. It was suggested that the tests should address what was actually in solution and defining f based on this bioavailable fraction. Other suggestions included defining the available fraction of 'f' and extending the probability concept to low concentration cometabolism processes. The development of more sensitive non-specific methods was not considered to be a priority research topic in the context of these methods.

Methods to increase both diversity and density of microbial biomass of inoculum for use in screening studies were seen as being needed, as well as the development of methods to define the biodiversity of inocula.

Developing a list of reference substances to define the spectrum of persistence and non-persistence for validation of modified test methods was recommended to help address concern that too many modifications were being made and tests were becoming too powerful. Such an exercise could be very useful in supporting many of the research topics. It was also recognised that the kinetics of degradation altered with substrate concentration, characterisation of co-

metabolic processes and the extent to which these methods were representative of the environmental processes may need investigation.

Syndicate D looked at possible new techniques for measuring biodegradation endpoints in the modified ready test. Discussion started with a review of the technical difficulties of using current indicators of biodegradability – DOC, CO₂ and O₂. This enabled the group to focus on how these problems might be overcome with alternative techniques. The key problem is how to test at low concentrations (e.g. at µg/l concentrations for poorly water soluble substances or substances that would be toxic to micro-organisms at the concentrations used in current ready biodegradability tests).

The following ideas were discussed.

- Radiolabelled test chemicals
Use of ¹⁴C may often lead to sufficient sensitivity to detect CO₂ from low exposure concentrations. The disadvantages may include unavailability of a sample and insufficient specific activity of the test compound. There was also concern that if a radiolabelled sample was available then a higher level study would be preferred rather than an enhanced ready test.
- F:m ratio
Simply increasing the mass of both f and m, i.e. keeping the ratio the same, will potentially lead to higher analytical sensitivity simply because of increased chemical mass and its breakdown products.
- Flow cytometry
This is a developed technique often applied to determine the presence, number and some physiological details of viable microbes in water samples. As such, it has potential to compare microbial characteristics of microbial communities that have and have not been exposed to a chemical substrate in water based environments. It has high potential sensitivity. Substantial research would be needed to determine if it is possible to correlate some descriptor of the populations, e.g. growth to degradation in a quantitative way, e.g. a given growth rate may relate to a rate of biodegradation. Establishing such a relationship is not expected to be easily achieved in the near future.
- Use of non-¹⁴C isotopic samples, e.g. ¹³C
The disadvantage is lack of availability of labelled compounds but if available, they can be used for techniques such as stable isotope probing (SIP). This method is used to identify microbes that incorporate ¹³C into their genetic material and thereby are identified as being able to biodegrade the chemical. This technique may have future promise given its potential high sensitivity but requires substantial development of libraries of microbes for specific

chemicals. Since such development is not anticipated to occur in the near future, consideration in the context of improving current ready test methodology is unlikely.

- Genomics

As with SIP, genomic techniques have high potential sensitivity but lack the necessary understanding of responses to link to biodegradation. Because of this it is unlikely that genomic techniques would be suitable for an initial screening test. If simplifying physiological principles could be defined (for biodegradation of classes of chemicals or chemical functional groups), then assays for enzyme activity or genes encoding key enzymes could be developed. Before genomics-type assays can be useful in biodegradation studies, a substantial effort would need to be expended in correlating “omics”-based measurements to biodegradation. Biomarkers suffer from the same problems as genomics.

- Epifluorescence

Similar to flow cytometry.

- Calorimetry

Current technological advances in this technique have increased its sensitivity. Therefore, if breakdown pathways are assumed to be exothermic reactions, then increases in heat output above those from control inocula may indicate biodegradation of the substance. Further investigation into the sensitivity of this approach is needed relative to the use of CO₂ or O₂.

- Specific chemical analysis

Developments in analytical chemistry are continually improving detection/quantification limits. Although specific to a chemical the approach should be considered for testing at low concentrations. Given specific analysis determines primary biodegradation, it is likely that only some of the intermediates would be identified, so the residual DOC in test media at the end of the test could be determined to indicate if significant (soluble) intermediates may be present.

It was concluded that the current test endpoints are generally fit for purpose. Overcoming the limitations must be done in a cost effective way otherwise a higher tier test may be more advantageous because they can be more informative.

None of the alternative endpoints appears to offer a general advantage although use of radiolabelled test substances and sensitive specific chemical analysis can be useful when available and especially if primary biodegradation needs to be measured.

The syndicate also considered if additional information might be obtained out of current test data. For example, a pass in the ready test, including the 10-day window indicates a default half-life of 15 days and if the 10-day window is not achieved the rate is assumed to be 50 days. Are there

sufficient data on ready biodegradation tests and environmental half-lives to assess the realism of these defaults or to assign other values?

3.3 Plenary discussions on screening tests

The following additional topics were raised during the syndicate feedback session.

Metabolites – It was discussed to what extent metabolite identification was important at this stage in the assessment of a chemical's persistence. It was recognised that this would depend on a number of factors including the level of degradation observed and the method of measurement. It was recommended that measuring DOC at the end of a RBT might yield information about the nature of the metabolites. Thus for example, if a poorly soluble substance degraded to a soluble metabolite, this would be observed by an increase in DOC, especially if the soluble metabolite did not further degrade. It was recognised that there were issues to be resolved regarding identification of metabolites.

Mobile Genetic Elements – One aspect of the talk by Eugene Madsen related to whether the DNA fragments that had been identified in his work as being the main degraders for naphthalene were likely to move from micro-organism to micro-organism and whether this would be seen by SIP techniques. Within the discussion it was not felt that these fragments were significantly mobile or whether SIP would be useful in such examinations.

Representative inocula – The question was raised as to whether activated sludge derived inocula were representative of micro-organisms in the environment. The discussion highlighted the historical origin of this source, as it led to the most consistent responses in ring tests during the early developments of the RBTs. Hence there might be potential for enhanced RBTs to use alternative sources.

The research topics that had been identified were discussed in plenary to check that the workshop attendees were in general agreement (see section 3.7).

3.4 Soil, sediment and water tests

The aim of this session was to identify areas where the higher tier tests could be improved to help better predictions of biodegradation be made. A series of presentations introducing the issues relating to soil, sediment and water tests was followed by a syndicate session.

Jason Snape introduced the session by giving a presentation on the Intelligent Testing Strategy (ITS) that had been developed by RIP EWG 9 (Degradation) with specific emphasis on the pros

and cons of the approach. Two main challenges were identified in the implementation of this ITS. These were 1) the identification of the compartment(s) of significant exposure and concern (i.e. which environmental compartments should be tested) and 2) how to evaluate degradation half-lives for water and sediment from higher tiered biodegradation tests and compare these to the P and vP criteria.

Persistence of substances is normally evaluated firstly by a simple standard screening test for ready biodegradability in water. It is only when a substance appears 'not readily' degradable that a more detailed evaluation of persistence in the marine environment is relevant. In the scope of PTB assessment this will also concern substances with a potency to adsorb strongly on sediment and therefore other routes and processes than biodegradation in the water layer should be evaluated.

These processes are volatilisation, photolysis, biodegradation of photolysis products, adsorption and sedimentation, anaerobic transformation and formation of bound residues. Standard tests are available to determine first order rate constants for each of these processes under laboratory conditions. However, a procedure for the extrapolation of the results to a single overall half-life in the marine environment is failing.

Han Blok presented a case study showing how rate constants for various processes can be properly converted and integrated. To this end processes that occur on the surface of interfaces (e.g. volatilisation, adsorption and anaerobic degradation) have to be converted by defining the marine system by its surface/volume ratio. Processes that depend on a specific fraction of the volume (e.g. the UV radiated fraction in the case of photolysis) have to be converted after defining the specific volume/total volume ratio.

After conversion of the observed rates in the test system to the rates in the real marine system, the disappearance by the various processes can be ranked to assess the route of elimination. Also independent processes that occur simultaneously and processes that proceed in succession need to be properly combined.

Jon Ericson (Pfizer, USA) presented data on a number of human pharmaceuticals that had been evaluated in the aerobic (9 substances) and anaerobic (3 substances) water-sediment transformation test (OECD 308). Several issues were highlighted that would be of interest to the wider chemical industry. The environmental relevance of, and the need for, the anaerobic part of the study was questioned. The data presented showed only slightly longer DT_{50} for anaerobic samples compared to aerobic, as one might expect, with no difference in their degradation profiles. The presentation also compared DT_{50} data determined from the complete study (six time points) with a DT_{50} estimated based on the day 100 data alone. There were no major differences observed. Issues associated with irreversibly bound material and whether or not it could be considered as non-bioavailable and/or depleted from the system were also identified for

discussion. In cases where sorption is the predominant dissipation mechanism (e.g. cationic compounds) the experience to date would suggest the OECD 308 study becomes an expensive partitioning study. Given the current challenges, the OECD 308 study would be even more difficult to conduct and interpret without radiolabel test material.

Gary Roberts (AstraZeneca, UK) highlighted a number of challenges associated with the interpretation of higher tiered biodegradation data. The major issue identified was that it was difficult to obtain distinct half-lives for degradation in the water and sediment compartments of the OECD 308 study. For the substances being investigated, rapid dissipation was observed from the water phase ($DT_{50} < 2$ days). The applied radioactivity was binding to the sediment and being incorporated into the bound non-extractable fraction. It was not possible to determine if this bound material was parent compound or a degradation product(s). Concern was expressed that there was no regulatory guidance on how to deal with and interpret bound residues. The presentation highlighted that the test outputs (DT_{50} s) and the P criteria (half-lives) requirements were not consistent. A DT_{50} includes degradation, sorption and volatilisation.

In the final lecture, Roger Prince (ExxonMobil Biomedical Sciences, Inc., USA) discussed the relevance of temperature in deriving the rate of biodegradation of a substance. Temperature obviously plays an important role in determining microbial growth – after all, that is why refrigerators are so useful. But even there we can see that different microbes behave differently – red meat can be stored far longer than seafood, because the difference in temperature between the initial environment and the refrigerated one is far greater for red meat. Van't Hoff and Arrhenius are justly famous for developing a theoretical model to explain the temperature dependence of simple chemical reactions, and many microbiologists have been tempted to use a similar approach to understanding microbial growth. But in reality this is fraught with difficulties, and natural microbial communities do not usually display very clear cut temperature dependences. The simplest explanation is that natural communities are limited by a range of factors, and temperature is not necessarily the most important. How this should be accounted for in considering the likely persistence of a chemical in the environment is worth serious discussion.

3.5 Syndicate discussions

The syndicates were asked to address the questions (shown in italics at the start of each section) with the specific aim again of identifying knowledge gaps and research needs. Consideration was given on how these would be incorporated into a protocol and how relevant the different parameters would be in such a test. For example would microbial diversity generate so much variability that other changes would not be significant?

Syndicate E

Standard protocols, OECD 307, 308 and 309 are available for generating information relating to a chemical's potential to biodegrade in the environment. Consider the following list of parameters and address whether the questions that have been raised in the presentations can be overcome by changing these parameters. First rate the topics in order of priority, then address whether they are relevant and if so is there research that could be done to understand them better or could simple changes to the protocols achieve the same purpose?

- *Duration;*
- *temperature;*
- *primary, ultimate degradation versus half-lives and DT_{50s};*
- *acclimation of the micro-organisms;*
- *bioavailability of the chemical;*
- *dosing mechanisms to sediment/soil or to water?*

A common theme during these discussions was the need to share experience across industries on how higher tiered tests are used and data interpreted.

In terms of test duration, there was a need to match length of study to the P criteria and as such OECD 307 and 308 studies could be extended to 180 days. However, this was not considered to be a research requirement since shared experiences suggest the OECD 307 and 308 tests should be viable even up to one year with biomass maintained through out the duration. It was also suggested the OECD 309 could be run for longer durations under a semi-continuous feed regimen.

In discussions on temperature it was felt that if a test was conducted at 20°C using the Arrhenius equation to extrapolate results was not appropriate. Ideally, tests should be conducted at the environmental temperature at which the sample was collected to maintain microbial community. If not ideal, tests should at least be conducted within the normal seasonal temperature variation for that region (conditions to which the micro-organisms are pre-adapted). It was suggested that there was a research need to investigate whether laboratory conditions are realistic and representative of environmental conditions and to assess whether results could be extrapolated to the other temperature ranges or whether data needed be generated at two temperatures.

There was a discussion around the need for separate water and sediment tests, due to the difficulties in estimating DT_{50s} and in interpreting primary/ultimate degradation versus dissipative mechanisms (sorption) observed in this combined OECD 308 test system. For all tests there was recognition that more guidance was required regarding the interpretation of the results.

Further research was required in respect to the acclimation of organisms (e.g. to assess whether processes such as sieving has a significant impact to integrity, whether it is important to establish equilibrium, what are appropriate endpoints and is there a real impact on the test outcome?).

Bioavailability was discussed and it was agreed that there is a need to define what is meant by 'bound' in the context of soil and sediments. There were suggestions that bound material may be unavailable and therefore depleted from the system. There is a potential research requirement to link extraction techniques (e.g. different solvents, pH) to mechanism of binding and whether that is sufficient in itself to define what constitutes being bioavailable.

In terms of test design it was agreed that the dosing method used was dependant on test compound and that there was a need to validate different approaches and, where appropriate, run solvent controls. To aid interpretation an inert matrix should be used and volume effects taken into account. For practical reasons, the test substance used needs to be at a concentration that can be measured.

Syndicate F

Standard protocols, OECD 307, 308 and 309 are available for generating information relating to a chemical's potential to biodegrade in the environment. Consider the following list of parameters and address whether the questions that have been raised in the presentations can be overcome by changing these parameters. First rate the topics in order of priority, then address whether they are relevant and if so is there research that could be done to understand them better or could simple changes to the protocols achieve the same purpose?

- *Microbial density and diversity;*
- *microbial source – soil, sediment;*
- *microbial adaptation;*
- *substance concentration;*
- *cometabolism.*

The syndicate was of the opinion that it was the quantity of micro-organisms present in a test system not the density that determines the possibility of competent organisms being present (as demonstrated in the Mauffret presentation). The possibility of biodegradation could be increased by increasing the scale of the system without changing the microbial density but this immediately raises issues of practicality. The syndicate was of the opinion that more effort should be spent on understanding the role that microbial diversity plays in determining the probability of degradation in a test system. One approach could be to study whether the results in a series of replicate tests, for example, are different with inocula from different sources compared to those with inocula from the same source. The syndicate also felt it important to develop concepts and methods to

enable varying responses to be used in persistence assessment, perhaps similar to the use of species sensitivity distributions (SSDs) in toxicity assessment.

As far as adaptation is concerned, the group felt strongly that this should be taken into account in an assessment of the intrinsic persistence of chemicals. Exactly how this should be done is another matter, but this should certainly use pre-exposure at realistic levels.

Realistic (i.e. $< \mu\text{g/l}$) concentrations should preferably be used in test systems, as there are data showing that substrate concentrations have a significant effect on the type of kinetics of biodegradation. These data are, however, only available for a very limited set of chemicals and it is uncertain how generally applicable these data are. The syndicate strongly recommended that further research be carried out on this issue as it is conceivable that invalid extrapolations of biodegradation rates are made from the relatively high concentrations of chemicals in test systems to environmental levels. Biodegradation processes may differ in both kinetics and the reaction pathways at different substrate concentrations. This is another issue that should be investigated.

Other important issues relating to biodegradation at realistic concentrations versus those used in testing systems are the effects on the induction of genes required for biodegradation and the role of cometabolism. Cometabolism may play a more significant role at environmental levels than at higher concentrations and should therefore also be considered in persistency testing. Testing at realistic levels also raises a number of analytical issues and therefore more emphasis should be given to measuring primary degradation with the powerful analytical methods now available.

Syndicate G

Other than those described by OECD 307, 308 and 309, are there other methods that could be described that would generate the information necessary to enable an evaluation of a chemical's degradation. First rate the topics in order of priority. Do these methods simulate the compartments of interest? Are these methods available, short-term, long-term etc. Also consider:

- *Microbial density and diversity and source – soil, sediment, and microbial adaptation;*
- *substance concentration and its bioavailability;*
- *cometabolism;*
- *duration of test and temperature;*
- *primary, ultimate degradation versus half-lives and DT_{50s} .*

The important consideration was that these methods should simulate the compartments of interest and be as realistic as possible. It was agreed that the existing simulation tests have limited reality. For example the OECD 309 test, while capable of giving good data for some chemicals, is

considered a confounded study and it may be better to undertake assessment as two separate phases (a clean sediment and a pelagic phase). However, other draft protocols (OECD, 2007) are being developed to simulate releases to wastewater and it was suggested that these should be examined in more detail as these may stimulate further areas for research. There was discussion on the importance of cometabolism. This was considered an issue because there are a number of chemicals where the evidence indicates these can only be degraded in the presence of other carbon and energy sources. This is potentially very important when studying very low test substance concentrations and this is particularly relevant in the aquatic environment (especially sea water) because experience suggests that low test substance concentrations do not represent a reliable test given the additional sources of carbon that are present in the real world.

Discussions focused on the possibility of providing another energy source (secondary energy source) and on creating conditions to encourage a collaborative degradation. The analogy is that in toxicology there is growing concern about the presence and effects of multiple chemicals but in biodegradation we are still looking at these in isolation. Concern that many chemicals that are currently considered as persistent substances may reflect the fact that biodegradation of these chemicals will only occur in the presence of an additional carbon source (substrate). This leads to the question on how could this be demonstrated? It is known that mixed function oxygenases (MFOs) are not very specific but if MFOs are simulated this can help break down other substances. This led to discussions on whether it is possible to look at other mechanisms to bring micro-organisms and organics into a microbially challenged environment? A number of ideas were discussed including creating an inoculum by exposing micro-organisms to an organic cocktail which would provide an additional carbon source and may increase diversity and enhance possibility of cometabolism; operating as semi batch systems; seeding simulation tests, spiking tests with naturally occurring suspended solids? An RfP could be developed to look at these issues but prior to this it would be beneficial to prepare a white paper/review of how these could be resolved and in particular how to ensure the proposals addressed what may occur under realistic conditions in the environment.

When developing these proposals there is a need to ensure that regulators do not consider tests being developed as inappropriate (i.e. because it appears that tests will be using optimum conditions for degradation). Therefore design will still depend on use pattern to create release pattern and help simulate appropriate conditions to help ensure their acceptability by regulators.

It was agreed that these tests should not be too prescriptive but have to be designed to simulate a defined condition and there is more thought given to what is to be simulated before they are set up. Generic scenarios should simulate the conditions as laid down in the TGD otherwise case specific (i.e. tests for crop protection products different from those for household products).

In assessing primary versus ultimate degradation it was generally agreed that radiolabelled material is required to monitor disappearance of parent compound because it can be very difficult

to follow metabolites with cold chemistry particularly at low ($\mu\text{g/l}$) concentrations. Some considered that it may be easier with tritiated compounds. It was recognised that there was a requirement to reassess analytical methods which could be used to support biodegradation assessments (e.g. review of recent analytical method developments, labeling of compounds etc.). This is important since there is evidence (i.e. presence of a metabolite) that degradation only occurs at very low concentrations (e.g. some musk materials).

In terms of other tests – is there something between enhanced ready test and ‘simulation’ tests? One suggestion was to use a modified screening method by using more realistic concentrations but introducing waste waters (as a source of micro-organisms and organics) and using radiolabelled or appropriate analytical technique to look at disappearance of parent compound. This may help look at effects of cometabolism but there was some concern about its acceptability to regulators. On this point it was agreed that regulators want assessment of persistence under realistic conditions but there is a need to differentiate between PBT (intrinsic properties) and risk assessment (e.g. looking at fate in local sediments). There was consensus that the range of inherent tests currently available could be used to better understand persistence. This promoted discussion on the value on whether, for example, SCAS type tests should be used to establish how long it takes for a given microbial community takes to adapt to a xenobiotic or that even under optimum selection pressure the substance cannot be degraded (i.e. is persistent)? For such an approach it would be important to look at endpoints so not just carbon removal but loss of parent compound.

Syndicate H

*Consider the following issues and address research potential. Is the research feasible?
Is there any difference between marine or freshwater inocula sources, is the biochemistry different?
How do you deal with ranges of half-lives in addressing P criteria?
Can you normalise biodegradation half-lives between different tests e.g. through Organic Carbon or other parameters, particle size, microbial density etc?
Do soil degradation rates compare with sediment degradation rates?
Ditto marine (deep sea) versus near shore versus riverine?*

This syndicate primarily dealt with the question of how to get meaningful multi compartment half-life data from one or more biodegradation tests and how the inherent variability of these data could be addressed so that useful results are derived for chemical assessment. There are a number of potential factors which can influence half-lives and merit further research. These include whether there is a difference between marine and freshwater inocula sources for

addressing P criteria which could be linked to differences in biochemistry, salinity effects, differences in microbial densities and diversity etc.

Half-lives are needed to establish the P criteria for REACH, for environmental fate modeling (e.g. the TGD uses a nested multimedia model to calculate PECs - Simple Box). The persistence of a compound influences the mass distribution in the environment and hence helps identify the media which become important for risk assessment. Half-lives can also potentially be used to make inferences on metabolism for B criteria.

There was a suggestion that rather than giving actual numerical estimates of half-lives these should be assigned to a range with the size of the range varied according to the perceived accuracy of the determination (the half-life 'bin' concept). Examples are given in ECETOC (2003) and Beek *et al* (2001).

Research could be focussed on undertaking a range of battery tests using different inocula with a set of reference compounds to determine natural variability and use these to assess which parameters drive the variability between tests for the same media and different media. One issue would be to demonstrate if high biodiversity results in higher catabolic potential.

Another discussion point was whether it is possible to normalise biodegradation half-lives between different tests (e.g. through OC or other parameters, particle size, microbial density). It was agreed that there are several parameters that drive or that could be used to drive a normalisation process and that some are more important than others. However, the group believed that a review of the literature would be beneficial as a start to answering this question. It was generally agreed that bioavailability, as a function of K_{oc} could be used as a normalisation parameter, but the question of desorption remains. Therefore, more research is required to assess the rate at which 'bound' residues undergo desorption from solids. These rates will be affected by binding mechanisms which control desorption and hence bioavailability. One possible research approach would be to link extraction solvents with what is bioavailable and potential for toxicity to micro-organisms. It was felt that there was a lot of data on the mechanisms of binding and that the best approach would be to first prepare a white paper on the subject then hold a workshop to share the learning on extraction procedures.

It was agreed that the 'estimated' rate constant from a ready (or other biodegradation) test could not be extrapolated to other media, although many regulatory agencies often perform this extrapolation. This is because there are differences in cell densities, bioavailability and sorption. There is existing literature on the subject of intermedia half-life extrapolation based on key parameters or perceived differences in the catabolic potential of different media and the recommendation was that this could be used to help develop a strategy for this work and identify further research needs.

There was also discussion on whether the OECD 301 test could be developed to allow rate constants to be estimated. It was felt that a critical review paper addressing this question is needed particularly for deriving freshwater half-lives (potential link to f:m ratio work).

3.6 Plenary Discussions on Higher Tier Tests

The output from the four syndicates that had considered test conditions in the OECD 307, 308 and 309 tests (including temperature, duration, acclimation, bioavailability etc.; preparation and selection of the microbial inoculum in the same tests; whether the OECD tests simulate their respective environmental compartments; what the influence of microbial density and diversity, cometabolism, etc. is and how variation in rates of degradation in different compartments should be dealt with) were discussed in plenary.

An initial discussion of these topics led to the identification of a number of headline ideas. These were then discussed to identify those areas that were considered appropriate for research funding. The issues/topic areas raised in this session were combined with those from the screening studies discussions and are detailed in section 3.7.

3.7 Research proposals

The findings from the syndicate sessions on screening tests and higher tiered tests were discussed further in plenary and the research topics worth consideration for funding are listed in Table 1.

Table 1: Research topics and proposals for how to address

Research topic	Description	Action
Validation – test chemicals and test methods	Draw up a list of chemicals, with clearly agreed properties and an agreed persistency category. This list would cover chemicals that biodegraded rapidly as well as those that were very slow to biodegrade. The purpose of this reference set would be to establish a set of chemicals that further research could address, and help allay fears that methods that were too aggressive were not being developed. The workshop attendees rated this as a very important exercise, that together with ring tests of any protocols eventually developed, would further the regulatory acceptance of the methods.	A short targeted activity is required that involves regulators and industry. See Appendix 3.
Bioavailability and bound residues	Definition is needed to clarify ‘bound chemicals’, when bound chemicals can be bioavailable and when they are practically unavailable. The problem starts with their identification, proceeding to the impact on the interpretation of data when bound residues are identified within a study. This would link the use of extraction solvents with what fraction is bioavailable and, therefore, has potential for toxicity to micro-organisms and higher organisms. The research should be based initially on a review of the literature to investigate what bound residues can be degraded and if so at what rate; Is it the same for all types of compounds (organic, inorganic, neutral, ionic, etc)? What are the mechanisms of binding and what types of analytical methods are needed to identify such mechanism? What are the appropriate extraction methods to fractionate available residues while maintaining compound integrity? What is the effect of desorption on bioavailability? Are there parameters that could be used for normalisation?	The proposed research project would take the form of a literature study, followed by a workshop to consider the results of the review and then to make recommendations on the type of further research that is needed. See Appendix 4.
Temperature	Investigate whether rates of biodegradation determined at laboratory temperatures can be predictive of rates under a wider range of environmental conditions. For example, if conducting a test at 20 °C, is extrapolation to other temperatures using the Arrhenius equation appropriate or does the testing need to be done at different temperatures? Is it important to conduct the tests at the environmental temperatures where the inoculum was collected to maintain the microbial community? Is it possible to extrapolate from one test temperature to a range of temperatures in the environment, or is it necessary to generate data at two or more temperatures?	A literature review is needed to investigate whether laboratory conditions are representative of environmental conditions. The review, and (possible published paper), would recommend how to account for the influence of temperature when assessing the environmental relevance of laboratory derived data. See Appendix 5.

Table 1: Research topics and proposals for how to address (cont'd)

Research topic	Description	Action
Understanding the impact of low substrate concentration and cometabolism on biodegradation test data	The relationship between biological processes occurring in high concentration biodegradation tests and those at low concentrations including cometabolism needs to be understood. There are data to suggest that it is not practical to extrapolate from laboratory studies done at high concentrations to environmental conditions. If this is the case, it probably needs to be understood why and what should or could be done to overcome these concerns.	Review literature conduct laboratory studies at <10 µg/l and compare to studies conducted at higher concentrations. See Appendix 6.
Addressing the f.m, microbial biodiversity and density	Develop methods to increase both diversity and density of microbial biomass of inoculum for use in screening studies so that the likelihood of false negatives is reduced. This will require an understanding of the impact of the biomass and its density on the data generated. Address density versus volume and pre-concentration methods such as colonisation of glass beads. The aim would be to develop a strategy that can be used to assess chemicals, in fresh and marine waters, without having to conduct the full suite of approaches developed in this research. It was also considered whether there was a need to conduct research on new devices to simplify the test procedure (screening test with easy to use and disposable platform). In this case, a platform would be proposed to allow O ₂ , CO ₂ and cell biomass measurement for each flask to build a balance of the biodegradation. The platform would use disposable flasks with a sensor embedded into the bottom.	Research proposal to be developed. See Appendix 7. A reference set of chemicals with a wider range of half-lives would be required to validate the testing. See Appendix 3.
Investigating pre-exposure	Pre-exposure of an inoculum to the test chemical may result in more environmentally realistic responses in biodegradation tests. Systematically evaluate various sources of inocula, various test material concentrations, times for pre-exposure in simple batch, intermittently fed batch and continuously fed chemostat-type exposure systems on the biodegradation of representative non-persistent and known persistent chemicals in screening tests (e.g. OECD 301). Different pre-exposure protocols for inocula to test the biodegradation of problematic chemicals in screening biodegradation tests are needed with the goal of defining guidance on the proper use of pre-exposed inocula for persistence assessments.	Initially a workshop to review approaches and agree the research needed. Implementation of the research would be aimed at achieving agreement of the “approved” processes. See Appendix 8.

Table 1: Research topics and proposals for how to address (cont'd)

Research topic	Description	Action
Measuring half-lives and understanding the principle causes of variability	<p>Develop a battery of tests to determine natural variability within and between environmental compartments. If such data become available can they be used to support a probability approach (analogous to the use of SSDs for deriving PNECs) to assessing half-life/persistence?</p> <p>Differences between inocula should be investigated to determine if higher biodiversity means greater catabolic potential. Research on factors influencing half-lives such as the f:m ratio may help. Consider the regulatory issue of how to select a single value from the distribution of values for regulatory decisions. Literature on inter-media half-life extrapolation based on key parameters is available. A review of this would be a starting point.</p>	Review of existing literature followed by research programme. See Appendix 9.
New analytical tools for biodegradation assessments	<p>Provide information on the various techniques currently available and their applicability to both screening level and 'simulation' studies. Although there is a growing body of knowledge on appropriate techniques to support single substances such as pesticides and pharmaceutical products, there needs to be clearer guidance on analytical techniques for other molecules and complex substances in particular.</p> <p>In addition to assessing the latest capabilities of ¹⁴C or tritiated hydrogen techniques (and other types of radiolabelling), the work would include a review of potential new alternative methods, e.g. use of microfibrils to concentrate substances in soil and sediment samples, Fourier Transform Infra Red and NMR, improved LCMS/GGMS advanced GCxGC. This would lead to recommendations on whether these could be practical and feasible to apply to biodegradation assessments of chemicals at low concentrations in a range of media.</p>	Prepare critical review of novel alternative techniques that may be able to overcome the technical limitations of conventional endpoints (O ₂ , CO ₂ , DOC). Include a review of advances in radiotracer and analytical chemistry techniques. See Appendix 10.

CONCLUSIONS AND RECOMMENDATIONS

The workshop reached the following conclusions:

Screening studies

Despite their regulatory importance it was stated that the only environment simulated by a ready test is the ready test. The value of the ready test has been its historical track record for identifying easily biodegradable chemicals. It is increasingly recognised that current ready tests are not very good at differentiating persistent chemicals. As these ready tests vary widely in their test conditions and contain many historical differences the workshop agreed that with REACH being implemented it was an appropriate time to address these differences and simplify the options.

There was a broad consensus that an enhanced tier of biodegradation screening studies are required to aid in the prioritisation of PBT and vP/vB assessments. Enhancements discussed included extending the test duration, increasing the test volume, enhancing the biomass and allowing for acclimation. Whilst extension of the test duration and conducting studies using higher test volumes posed little concern to regulatory members of the workshop, it was felt that some validation and standardisation was needed with respect to working with increased biomass density and diversity and acclimated inocula. It was envisaged that such enhanced tests could contribute to a weight of evidence approach to decide if a chemical is persistent.

The group also decided that, in order to prevent confusion over the terms acclimation and adaptation, the terminology “deliberate pre-exposure of the inoculum to the test chemical” should be used to describe adapted or acclimated inocula. Some participants recommended the use of highly adapted systems as a positive screen for persistence i.e. chemicals that could not degrade in such systems can be assumed to persist.

Three issues were also identified that were equally applicable to both screening and higher tiered studies, these were:

- Analytical techniques;
- use of half-lives;
- cometabolism.

On the first of these issues there were discussions on whether there were new analytical and labelling techniques becoming available that may overcome a) the technical limitations associated with conventional endpoints (O₂, CO₂ and DOC) and b) the requirement for radiolabelled material to study fate, including biodegradation, at low substance concentrations. (Although in certain instances this may be overcome, simply by increasing the mass of both f and m, i.e.

keeping the ratio the same, could potentially lead to improved analytical sensitivity because of increased breakdown products).

On the second issue a large number of system specific and microbiological factors are involved in the biodegradation of a given chemical in the various environmental compartments. Consequently the use of one half-life or rate constant as the all encompassing number to characterise the behaviour of a chemical is fundamentally flawed. There was a suggestion that rather than giving actual numerical estimates of half-lives these should be assigned to a range with the size of the range varied according to the perceived accuracy of the determination (i.e. the half-life bin concept). It was considered that by measuring half-lives from a ‘battery’ of tests, half-life distributions could be generated. These could be compared to single values and assessed for their usefulness within the regulatory decision making process. The process that could be used to test this approach is shown in Figure 2.

Figure 2 Strategy for testing biodegradability variability

Pre-requisites for project development

1. Test set of chemicals with generally agreed rates of biodegradation
 2. Screening array for diversity assessment of inocula sources
 3. Inocula preparation for biodegradation platform
 4. Development of biodegradation platform
-

Testing of hypothesis

1. Test chemicals using the biodegradation platform
 2. Develop process for assigning to ECETOC ‘biodegradation bins’
 3. Use the same process to test ‘unknown’ chemicals
 4. Validate for PBT and risk assessment purposes
-

The third issue identified related to the fact that a number of chemicals can only be degraded in the presence of other carbon and energy sources (i.e. are cometabolised) and may not be linked to microbial growth. The issue of cometabolism is particularly important when studies are performed at very low (ng/l) substance concentrations and is particularly relevant in seawater environments where additional sources of carbon and energy are present in the real world.

Higher tiered studies

There was a general consensus that there are no biodegradation studies that accurately simulate biodegradation in the ‘natural’ environment. There are higher tiered biodegradation studies (e.g. OECD 307, 308 and 309) that use environmental media that can describe degradation under

conditions that have a greater environmental relevance than the ready biodegradation test. However, many of these studies do pose considerable problems associated with their interpretation. Problems identified included:

- Selection of the most appropriate compartment for testing;
- how to deal with bound residues;
- how to discriminate between degradation half-lives for persistency assessments and rates of dissipation from water or sediment;
- how to correct for temperature differences and is it really necessary.

Selecting the most appropriate inoculum is important in generating relevant half-life data but this is not as simple as deciding which compartments most of the chemical ends up in (for example >5% in any compartment signifies realistic presence under Canadian legislation). The ITS currently being developed under REACH seems to favour marine systems if a chemical has potential for slow degradation because its exposure is considered inevitable. Further discussion of this issues and its scientific rationale would have been useful.

Biodegradation data were presented at the workshop for 12 chemicals across three presentations using the OECD water sediment transformation test (OECD 308). These data indicated that most of the applied radioactivity (^{14}C) was becoming increasingly associated with non-extractable material present in the sediment (bound residue) as the study progressed. Attempts with various solvents had failed to result in this material being released from the sediment and as such it is not possible to distinguish parent compound from possible metabolites. Consequently, there needs to be some guidance on how to interpret these data as the ^{14}C material may no longer be bioavailable for degradation or toxicity.

Recommendations for future research

The topics in Table 1 were developed into RfPs (see Appendices 3-10). Although not given a priority during the workshop, in the process of writing this report, the authors have sought an indication of the priority for the work. The following RfPs were considered to be of very high priority for PBT assessment and/or risk assessment, with a good chance for success:

- Validation set of chemicals for biodegradation research
This short and targeted project was seen as being essential to all the other projects, in that an agreed set of chemicals with a range of biodegradation behaviours would be extremely useful in helping to ensure that methods were ‘tuned’ to their task and not overly protective or too powerful.

- Addressing bound residues

This project was also seen as short term, being a review of the issues and science, to be followed by a workshop that could address the issues and design the research necessary to investigate the issue of bound residues and put them into a proper context. This could also be an ECETOC Task Force, followed by a CEFIC-LRI funded workshop.

- Microbial density and diversity and the ratio of chemical to microbial mass

This was one of two large projects that could be considered as fundamental to the discussions about how to test chemicals for their biodegradation in the environment and how to generate the values for half-lives needed for PBT and risk assessment. This project was seen as focussing on the methodologies for generating test systems and to address the impact of diversity/density and how these might be considered in screening tests and then for the development of low cost enhanced screening tests.

- Measurement of biodegradation half-lives and identifying sources of variability

This second project was seen as focussing more on the generation of half-lives that were relevant to assessment of chemicals in the environment and which supported a 'probabilistic' approach to the assessment, including the identification of environmental half-life bands.

ABBREVIATIONS

DOC	Dissolved Organic Carbon
DT ₅₀	Disappearance time (50%)
EA	Environment Agency
EU TGD	European Union Technical Guidance Document
f:m	Ratio of the food concentration (f) to microbial concentration (m)
GC-MS	Gas Chromatography-Mass Spectrometry
GRAP	Generally Regarded As Persistent
ITS	Intelligent Testing Strategy
LC-MS	Liquid Chromatography-Mass Spectrometry
MFO	Mixed Function Oxygenase
NMR	Nuclear Magnetic Resonance
OECD:	Organisation for Economic Cooperation and Development
P	Persistent
PBT	Persistent, Bioaccumulative, Toxic
PEC	Predicted Environmental Concentration
PNEC	Predicted No Effect Concentration
OC	Organic Carbon
RBT	Ready Biodegradation Test
REACH	Registration, Evaluation and Authorisation of Chemicals
RfP	Request for Proposal
RIP EWG	Reach Implementation Project Endpoint Working Group
QSAR	Quantitative Structure Activity Relationship
S0:X0	Substrate concentration (time 0): biomass concentration (time 0)
SCAS	Semi-Continuous Activated Sludge
SIP	Stable Isotope Probing
SSD	Species Sensitivity Distribution
UBA	Umweltbundesamt (German Federal Environment Agency)
UV	Ultraviolet
vP	very Persistent
vPvB	very Persistent, very Bioaccumulative

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APPENDIX 2: WORKSHOP PROGRAMME

Tuesday 26 June 2007

09.00-09.10 **Welcome and Introduction** Chair

Session 1: METHODOLOGY AND INTERPRETATION – SCREENING STUDIES

09.10-09.40 **RIP 3.3 Degradation – Screening Studies** Jason Snape
AstraZeneca

09.40-10.00 **Use of degradation data in the PBT WG** Steve Robertson, Environment Agency
Henrik Tyle, Danish EPA

10.00-10.20 **An innovative tool for marine biodegradability screening** Aourell Mauffret
Instituto de Ciencias Marinas de Andalucia

10.20-10.40 **Impact of microbe densities and new strategies to implement biodegradability tests** Gerald Thouand
Nantes University

10.40-11.00 **Combining Stable Isotope Probing (SIP) with biodegradation – What might be the practical benefits for industry?** Eugene Madsen
Cornell University

11.10-13.00 **Syndicate Session 1**
Address further proposals for enhanced biodegradation tests

13.00-14.00 Lunch

14.00-15.00 **Report back – 15 minutes/syndicate**

15.00-16.00 **Plenary discussion**

16.00-16.15 Coffee Break

Tuesday 26 June 2007 (cont'd)**Session 2: METHODOLOGY AND INTERPRETATION
HIGHER TIER – SOIL, SEDIMENT AND WATER TESTS**

16.15-16.30	RIP 3.3 Degradation – Higher tier + ITS – Where we are – What are the pros/cons?	Jason Snape AstraZeneca
16.30-16.50	Evaluation of persistence in the marine environment by combining standard tests for various processes	Han Blok J. Block Consult
16.50-17.05	Evaluation of OECD 308 for assessing biodegradation of pharmaceuticals	Jon Ericson Pfizer
17.05-17.20	Dissipation versus degradation: Challenges in interpreting higher tiered biodegradation test data	Gary Roberts AstraZeneca
17.20-17.40	Optimum temperatures and correction of biodegradation kinetics	Roger Prince Exxonmobil
19.00-21.00	Workshop dinner	

Wednesday 27 June 2007

08.30-09.45	Syndicate session 2 Proposals for higher tier soil, sediment and water testing	
09.45-10.00	Coffee Break	
10.00-11.00	Feedback	
11.00-11.30	Discussion of research priorities and allocation to syndicates	
11.30-13.00	Syndicate session 3 Drafting of outline RfPs – Identify key players and other research parameters, Budget, time, etc., also lead contact	
13.00-14.00	Feedback – for communications	
14.00-15.00	Lunch	
		Close of Workshop

APPENDIX 3: RfP 1 - VALIDATION CHEMICAL SET

Aim of the research

To establish a list of chemicals, with an agreed set of properties and characterised degradation behaviour. The purpose of this reference set would be to establish a set of chemicals that further research could address, and help allay fears that methods that were too aggressive were not being developed.

Research deliverables

- Starting with existing data sources, e.g. ECETOC Persistence report, identify a set of chemicals with agreed properties and biodegradation behaviour. This list would cover chemicals that biodegraded rapidly as well as those that were very slow to biodegrade.
- Establish the properties for these chemicals, including physico-chemical as well as fate and partitioning properties.
- Test and agree the data with regulators and industry.
- Write up the results and publish the final outcome.

Cost and timing

€ 75k over 1 year.

APPENDIX 4: RfP 2 - BIOAVAILABILITY AND BOUND RESIDUES

Aim of the research

Clarify what is meant by ‘bound chemicals’, when bound chemicals can be bioavailable and when they are practically unavailable.

Research deliverables

The problem starts with the identification of bound residues, proceeding to the impact on the interpretation of data when bound residues are identified within a study. This would link the use of extraction solvents with what fraction is bioavailable and, therefore, has potential for toxicity to micro-organisms and higher organisms.

- Review the literature to investigate what bound residues can be degraded and if so at what rate? Is it the same for all types of compounds (organic, inorganic, neutral, ionic, etc)? What are the mechanisms of binding and what types of analytical methods are needed to identify such mechanism? What are the appropriate extraction methods to fractionate available residues while maintaining compound integrity? What is the effect of desorption on bioavailability? Are there parameters that could be used for normalisation?
- Draft a report highlighting these issues.
- Organise a workshop to discuss the report and to agree what conclusions can be drawn, how the data can be used in PBT and risk assessments and whether there is other research that is needed.
- Publish the report.

Cost and timing

€ 100k over 1 year.

APPENDIX 5: RfP 3 - UNDERSTANDING THE EFFECTS OF TEMPERATURE ON BIODEGRADATION

Aim of the research

Investigate whether rates of biodegradation determined at laboratory temperatures can be predictive of rates under a wider range of environmental conditions.

Research deliverables

- Review the available literature that addresses the impact of temperature on biodegradation. Address whether it is important to conduct the tests at the environmental temperatures where the inoculum was collected to maintain the microbial community and whether it is possible to extrapolate from one test temperature to a range of temperatures in the environment, or whether it is necessary to generate data at two or more temperatures?
- Make recommendations as to how this could be accounted for when addressing the environmental relevance of laboratory derived data.
- Publish the results, including the literature review.

Cost and timing

€ 50k over 1 year.

APPENDIX 6: RfP 4 - UNDERSTANDING THE IMPACT OF LOW SUBSTRATE CONCENTRATIONS ON BIODEGRADATION

Aim of the research

To understand microbiological, physiological and genetic controls of metabolism at very low concentrations. There is a need to better understand the relationship between biological processes occurring in high concentration biodegradation tests and those at low concentrations including cometabolism. It is important to be able to relate laboratory studies to environmental conditions and if there are changes depending upon concentration, how these may be taken into account.

Research deliverables

- Conduct a literature review and assess data. Use these data to design studies in this research.
- Undertake laboratory studies that measure the kinetics of biodegradation at low concentrations (<10 µg/l) and compare with studies run at 100-1000 µg/l.
- Identify the factors that are different between these concentrations.
- Propose how laboratory studies run at greater than, or equal to 100 µg/l may be used to predict environmental biodegradation at sub-µg/l concentrations.
- Publish the results.

Cost and timing

€ 250k over a 3-year period.

APPENDIX 7: RfP 5 - F:M AND MICROBIAL DENSITY, DIVERSITY

Aim of the research

- Develop methods to increase both diversity and density of microbial biomass of inoculum for use in screening studies.
- Understand the impact of the biomass and its density on the data generated. Address density v volume; include comparison of beads v other pre-concentration methods.
- Develop an approach that can be used to assess other chemicals, in fresh and marine waters, without having to conduct the full suite of approaches developed in this research.

Research deliverables

- Investigate the probability of biodegradation of a range of chemicals (validation set), addressing:
 - a range of inoculum sources;
 - test substance concentration;
 - variable microbial biomass levels.NB: The tests must not be conducted at concentrations of the test substance that are toxic to micro-organisms, even when biomass is changed.
- Signal to noise ratio:
 - identify/suggest sources of noise in such studies.
 - develop approaches that reduce the background levels of organic carbon (blanks).
 - are there alternative endpoints that address biodegradation?
- Develop a strategy/testing approach for how to address other chemicals and what conditions should be used in such tests.

Cost and timing

€ 400k over a 3-year period.

APPENDIX 8: RfP 6 - THE ROLE OF PRE-EXPOSURE CONDITIONS ON INOCULA SUCCESS IN SCREENING BIODEGRADATION TESTS AND THE RELEVANCE TO THE FIELD

Aim of the research

To better understand the effect of different pre-exposure scenarios on the response of microbial communities to ensure that any adaptation by the microbial community to the presence of the chemical has relevance to the field. This research will thus assess different scenarios for pre-exposure of microbial inocula to a test chemical, and the relevance of the resulting response to the field. Such research could involve the evaluation of different pre-exposure protocols on the biodegradation of problematic from a testing perspective, non-persistent chemicals along with definitively persistent chemicals in screening biodegradation tests. The ultimate goal would be to provide guidance on the proper use of pre-exposed inocula for persistence assessments.

Research deliverables

- Review all published information on this topic.
- Define the critical conditions (e.g. inoculum source, chemical and organism residence times, other substrates, chemical concentration, etc.) limiting microbial adaptation responses in different compartments in which persistence is important to establish acceptable and relevant conditions for pre-exposure.
- Systematically evaluate these variables during pre-exposure on the resulting biodegradation of representative non-persistent and known persistent chemicals in screening type tests (e.g. OECD 301).
- Relate the results to known chemical behaviour in the environment and develop a guidance document on acceptable conditions for pre-exposure.

Cost and timing

€ 300k over a 3-year period.

APPENDIX 9: RfP 7 - MEASURING HALF-LIVES AND ASSESSING VARIABILITY

Aim of the research

Biodegradation of chemicals in the environment is subject to an array of variables that mean that there is not one single degradation value for a chemical. This same variability can also be observed in laboratory test systems and it is important that the variability within and among media and the probably causes of this variability is understood.

Research deliverables

- A literature review of existing sources of biodegradation variability.
- A set of reference chemicals should be used (see Appendix 3).
- Determining which are the important processes and parameters driving this variability.
- Extrapolation of half-lives across media (normalisation potentials?).
- Ensure that bound residues are not addressed.
- Battery of inocula from different media to determine variability across media.
- Off-shore marine, coastal estuarine, (high solid loading), riverine freshwater, sediment (oligotrophic and eutrophic), soils.
- Consider aerobic test systems (note anaerobic systems may also need to be addressed).

Cost and timing

€ 500k over a 4-year period.

APPENDIX 10: RfP 8 - ANALYTICAL METHODS TO SUPPORT BIO-DEGRADATION ASSESSMENTS

Aim of the research

To help provide information on the various techniques currently available and their applicability to Tier 1 studies and ‘simulation’ studies. Although there is a growing body of knowledge on appropriate techniques to support single substances such as pesticides and pharmaceutical products there needs to be some clearer guidance regarding analytical techniques for other molecules and complex substances in particular.

Research deliverables

Literature review

- Identify the pros and cons of various existing methods (cold and hot) which have been commonly used for higher tiered tests and whether some of these could be used to provide additional information to support Tier 1 studies (For example, analysis of media from ready biodegradation tests where some analytical data could help to establish fate of the substance).
- Identify potential new alternative methods (e.g. use of microfibres to concentrate substances in soil and sediment samples) and demonstrate whether these will be practical and feasible to apply to biodegradation assessments in a range of media. Other alternatives include different types of probes (Fourrier Transform Infra Red and NMR), improved LCMS/GGMS and advanced GCxGC.
- Radiolabelling is recognised as an important method but there are still a number of issues which may affect the results. Research should focus at looking at the influence of labelling position and even atom which is labelled (^{14}C or ^3H). Experience in the pharmaceutical industry suggests that the position of label should be based on collaboration between biological (toxicologists) and analysts.
- Are there other types of ‘tags’ or even radiolabelling techniques?

Cost and timing

€ 75k over 1 year.

APPENDIX 11: ORGANISING COMMITTEE

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ECETOC WORKSHOP REPORTS

No.	Title
No. 1	Workshop on Availability, Interpretation and Use of Environmental Monitoring Data. 20-21 March 2003, Brussels
No. 2	Strategy Report on Challenges, Opportunities and Research Needs Arising from the Definition, Assessment and Management of Ecological Quality Status as Required by the EU Water Framework Directive Based on the Workshop EQS and WFD versus PNEC and REACH - Are They Doing the Job ? 27-28 November 2003, Budapest
No. 3	Workshop on Use of Human Data in Risk Assessment. 23-24 February 2004, Cardiff
No. 4	Influence of Maternal Toxicity in Studies on Developmental Toxicity. 2 March 2004, Berlin
No. 5	Workshop on Alternative Testing Approaches in Environmental Risk Assessment. 7-9 July 2004, Cr�cy-la-Chapelle
No. 6	Workshop on Chemical Pollution, Respiratory Allergy and Asthma. 16-17 June 2005, Leuven
No. 7	Workshop on Testing Strategies to Establish the Safety of Nanomaterials. 7-8 November 2005, Barcelona
No. 8	Workshop on Societal Aspects of Nanotechnology, 9 November 2005, Barcelona
No. 9	Workshop on the Refinement of Mutagenicity / Genotoxicity Testing, 23-24 April 2007, Malta