Workshop on the findings and implications of the ring test for improving the OECD 306 marine biodegradation test

3-4 May 2018

Newcastle University,

Newcastle-upon-Tyne, United Kingdom

Newcastle University
UK | Malaysia | Singapore

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LRI
Long-Range Research Initiative

Aim of the workshop

A series of standardised biodegradation screening tests (BSTs; such as the OECD 301 series and OECD 306) have been developed to measure the relative biodegradability of chemicals. Recently, regulatory emphasis has shifted from measuring biodegradation towards prioritisation of chemicals based on persistence. In their current guise, BSTs are ineffective as screens for persistence [1,2,3]. They are prone to high levels of variation and produce a large number of fails, many of which can be considered false negatives, whereby a chemical fails a test not because of its recalcitrance, but rather because the test itself has failed [4,5,6]. Under certain circumstances, this could lead to unnecessary additional biodegradation, bioaccumulation and toxicity tests and therefore contravention of the 3 R's (replacement, reduction and refinement) principles in animal research [7,8.]

A Cefic-LRI sponsored and ECETOC organized workshop to discuss improvements to the marine OECD 306 biodegradation test was delivered in 2015 [9]. During this workshop, methodological improvements to BSTs were discussed, based on the above research, in addition to clarifying guidance on testing and interpretation of results obtained from marine BSTs. Methodologically: (i) increasing bacterial cell concentrations to better represent the bacterial diversity inherent in the sampled environments [10]; and (ii) increasing test durations to investigate extended lag phases observed in marine assessments, were recommended to be validated in a multi-institutional ring test.

From 2016-18, an international Cefic LRI funded ring test was coordinated by Newcastle University, comparing an improved marine BST (comprising seawater concentrated with tangential flow filtration), a respirometric marine BST (comprising non-concentrated seawater), and a standard OECD 306 closed bottle test across 13 laboratories in the UK, Norway, Germany, Italy, Canada, USA and Japan. Five test chemicals, representing a range of different biodegradation potentials, were used to validate the new method and included; a positive reference compound (sodium benzoate), a negative reference compound (pentachlorophenol) and three compounds with variable reported degradation (4-nitrophenol, triethanolamine and hydrolysed polyacrylamide).

This workshop will be the first opportunity to discuss the findings from this ring test including implications and recommendations for environmental risk assessment approaches with perspectives presented from CROs, industry, academia and regulatory bodies. These results stem from the earlier Cefic-LRI project ECO-11.

The workshop will consist of:

- Presentations on the biodegradation, microbial sequencing and environmental data from the ring test.
- Presentations from academics working on associated topics, a research-led CRO, perspectives from industry and regulatory bodies.
- Panel discussions presenting the perspectives from academia, industry and regulatory bodies on the recommendations from the ring test for improved persistence and biodegradation testing.
- Breakout sessions discussing workshop presentations, testing experiences and recommendations for an improved OECD 306 test.
- Theory in practice sessions demonstrating analysis conducted for the improved marine BST (tangential flow filtration for cell concentration methods, flow cytometry for cell counts and DNA sequencing for microbial profiling) and discussions on their relevance for future testing at CROs.

Participants are invited to contribute to a poster session on “Biodegradation & Persistence Assessment”. Please indicate interest in contributing to the poster session on the registration form.

The Cefic-LRi sponsored workshop will be delivered by the ring test organising committee coordinated by Newcastle University.

[1] ECETOC, Persistence of chemicals in the environment. Workshop Report No. 90; 2003.;
[2] ECETOC, Workshop on biodegradation and persistence. Workshop Report No. 10. **2007**.;
[3] ECHA, Guidance on information requirements and chemical safety assessment. Chapter R.11: PBT assessment. Version 2.0. **2014**.;
[4] Martin TJ, Snape JR, Bartram A, Robson A, Acharya K, Davenport RJ. Environmentally relevant inoculum concentrations improve the reliability of persistent assessments in biodegradation screening tests. *Environmental Science and Technology* **2017**, 51 (5), 3065-3073.
[5] Kowalczyk A, Martin TJ, Price OR, Snape JR, van Egmond RA, Finnegan CJ, Schafer H, Davenport RJ, Bending GD. Refinement of biodegradation tests methodologies and the proposed utility of new microbial ecology techniques.*Ecotoxicology and Environmental Safety* **2015**, 111, 9-22.
[6] Goodhead, AK, Head, IM., Snape, JR, Davenport, RJ. Standard inocula preparations reduce the bacterial diversity and reliability of regulatory biodegradation tests. *Environmental Science and Pollution Research.* **2013**, 21 (16), 9511–9521.
[7] EC. Technical guidance document on risk assessment: Part II; in 797 support of Commission Directive 93/67/EEC on Risk Assessment for 798 new notified substances; Commission Regulation (EC) No 1488/94 799 on Risk Assessment for existing substances; Directive 98/8/EC of the 800 European Parliament and of the Council concerning the placing of 801 biocidal products on the market; EC: Brussels, **2003**.
[8] Martin TJ, Goodhead AK, Acharya K, Head IM, Snape JR, Davenport RJ. High Throughput Biodegradation-Screening Test to Prioritize and Evaluate Chemical Biodegradability. *Environmental Science and Technology* **2017**, 51(12), 7236-7244.
[9] ECETOC, Improvement of the OECD 306 screening test: Workshop held at CEFAS laboratories, Lowestoft, UK 17-18 February 2015 and subsequent ring test. Workshop Report No. 34. **2017**
[10] Martin TJ, Goodhead AK, Snape JR, Davenport RJ. Improving the ecological relevance of aquatic bacterial communities in biodegradability screening assessments. *Science of the Total Environment* **2018**, 627, 1552-1559

Programme day 1: Thursday, 3rd May 2018

8.30- 9.00	Arrival and registration at Research Beehive in the Old Library building, Newcastle University, Newcastle-upon-Tyne
9.00- 9.10	Welcome to Newcastle University. Health and safety induction Russell Davenport & Amelie Ott *Newcastle University*
9.10- 9.25	**The OECD 306 Ring Test - One step towards an improved understanding of persistence assessment of chemicals in the 21st century** Graham Whale *Shell Health*
9.25- 10.10	**Preliminary results of the ring test to improve the OECD 306** Russell Davenport *Newcastle University*
10.10- 10.40	Coffee and poster session
10.40- 10.55	**EOSCA: The Industry perspective** Ian Still *EOSCA*
10.55- 11.10	**Marine biodegradation testing at NIVA** Aina Charlotte Wennberg *Norwegian Institute for Water Research*
11.10- 11.25	**A regulators view on environmental persistence testing** Pippa Curtis-Jackson *Environment Agency UK*
11.25- 11.30	**Process and timelines for OECD test guideline development** Russell Davenport *Newcastle University*
11.30-11.40	Group photo
11.40- 11.50	Preparing questions for the panel discussion
11.50- 12.25	**Panel discussion** Panel: Russell Davenport, Amelie Ott, Tim Martin, Graham Whale, Ian Still, Aina Charlotte Wennberg and Pippa Curtis-Jackson; Facilitator: Jason Snape
12.25- 13.10	Lunch and poster session
13.10- 13.25	**Application of long term exposure for adaptation of microbial communities in persistency testing** Baptiste Poursat *University of Amsterdam*
13.25- 13.40	**Determining biodegradation kinetics of hydrophobic chemicals in mixtures at low concentrations** Philipp Mayer *Technical University of Denmark*
13.40- 15.10	**Breakout session 1: Results of the ring test** Group 1: Facilitator Graham Whale, Rapporteur Russell Davenport Group 2: Facilitator Jason Snape, Rapporteur Baptiste Poursat
15.10- 15.30	**Groups report back incl. questions**
15.30- 16.00	Coffee

16.00- 17.00	**Breakout session 2: Regulatory aspects** Group 1: Facilitator Graham Whale, Rapporteur Russell Davenport Group 2: Facilitator Jason Snape, Rapporteur Baptiste Poursat
17.00- 17.20	**Groups report back incl. questions**

Close of first day

19.30	Complimentary dinner at Blackfriars Restaurant

Programme day 2: Friday, 4th May 2018

9.00- 9.30	Report to Research Beehive in the Old Library building; luggage storage facilities can be provided if required
9.30- 11.30	**Theory in practice sessions** – Group 1: Tangential flow filtration for cell concentration – Group 2: Flow cytometry for cell counts – Group 3: DNA Sequencing for microbial profiling
11.30- 12.00	**Science required to improve biodegradation screening tests at CROs**

Close of workshop

Ring test organising committee

- **Angelika Baumbusch**, Environment Agency, Norway: angelika.baumbusch@miljodir.no
- **Russell Davenport**, Newcastle University, UK: russell.davenport@newcastel.ac.uk
- **Bruno Hubesch**, CEFIC LRI Programme, Belgium: bhu@cefic.be
- **Tim Martin**, Newcastle University, UK: timothy.martin@ncl.ac.uk
- **Amelie Ott**, Newcastle University, UK: a.i.g.ott2@newcastle.ac.uk
- **Nik Robinson**, EOSCA, UK: secretary@eosca.eu
- **Bob Rowles**, Cefas, UK: bob.rowles@cefas.co.uk
- **Jason Snape**, AstraZeneca, UK: jason.snape@astrazeneca.com
- **Ian Still**, EOSCA, UK: ian.still@ecolab.com
- **Graham Whale**, Shell Health Risk Science Team, UK: graham.whale@shell.com
- **Lucy Wilmot**, ECETOC, Belgium: lucy.wilmot@ecetoc.org

Platform abstracts

The OECD 306 ring test - one step towards an improved understanding of persistence assessment of chemicals in the 21st century

Graham Whale

Shell Health, Manchester, UK
E-mail contact: graham.whale@shell.com

The environmental hazard and risk assessment of chemicals is based primarily on their Persistence (P), Bioaccumulation (B) and Toxicity (T), the so-called PBT properties. Persistence of chemicals in the environment is a cornerstone of chemical assessment because this determines exposure and is therefore a key parameter for estimating the risk of long-term adverse effects on biota. For example, marine biodegradability screening tests (BST's) are an intrinsic part of offshore chemical control schemes and the OSPAR Harmonised Offshore Chemical Notification Format (HOCNF) registration process. However, there is increasing concern that progress in the scientific understanding of the factors which influence chemical persistence as assessed in BSTs are not fully incorporated in existing regulations and environmental risk assessments.

This concern has been recognised and many of the short comings of existing biodegradation studies have been discussed in a series of multi stakeholder ECETOC workshops where the emphasis was on identifying research required to better understand the key issues and ideally propose methods to mitigate the limitations. In fact, the recent CEFIC LRI persistence related projects including the Eco 11 and the Eco 11.2 OECD 306 Ring Test owe their origins to these ECETOC Workshops. As these and other industry related persistence research projects (e.g. CONCAWE) have generated new data and insights these need to be communicated to regulators and the wider scientific community to ensure regulations are built on sound and credible science. As part of this process the results and key learnings from the OECD 306 Ring Test will be linked to other research initiatives with the aspiration being to initiate a paradigm shift in the way persistence assessment of chemicals is undertaken in the 21st Century.

Preliminary results of the ring test to improve the OECD 306

Amelie Ott[1], Timothy Martin[1], Graham Whale[2], Jason Snape[1,3], Robert Rowles[4], Bruno Hubesch[5] and Russell Davenport[1]

[1]School of Engineering, Newcastle University, Newcastle upon Tyne, UK
[2]Shell Health, Manchester, UK
[3]AstraZeneca Global Environment, Alderley Park, UK
[4]Centre for Environment, Fisheries and Aquaculture Science (Cefas), Lowestoft, UK
[5]European Chemical Industry Council (Cefic), Brussels, Belgium
E-mail contact: russell.davenport@ncl.ac.uk

A series of standardised biodegradation screening tests (BSTs; e.g. OECD 301, 306) have been developed to measure the relative biodegradability of chemicals. Recently, regulatory emphasis has shifted from measuring biodegradation towards prioritisations on chemical persistence. In their current guise, BSTs are ineffective as screens for persistence. They are prone to high levels of variation and produce a large number of fails, many of which can be considered false negatives, wherby a chemical fails a test not because of its recalcitrance, but rather because the test itself has failed.

An ECETOC funded workshop to discuss improvements to marine biodegradation testing was delivered in 2015. During this workshop, methodological improvements to BSTs were discussed, in addition to clarifying guidance on testing and interpretation of results obtained from marine BSTs. Methodologically: (i) increasing bacterial cell concentrations to better represent the bacterial diversity inherent in the sampled environments; and (ii) increasing test durations to investigate extended lag phases observed in marine assessments, were recommended to be validated in a multi-institutional ring test.

This presentation will report the findings from an international ring test of an improved marine BST, whereby an improved marine BST comprising inocula concentrated by tangential flow filtration, a modified marine BST comprising seawater and a standard OECD 306 closed bottle test were compared across 13

laboratories in the UK, Norway, Germany, Italy, Canada, USA and Japan. Five test chemicals including a positive reference compound (sodium benzoate), a negative reference compound (pentachlorophenol) and three compounds with variable reported degradation (4-nitrophenol, triethanolamine and hydrolysed polyacrylamide), were used to provide a range of biodegradation potentials by which to validate the new method.

Biodegradation data for the five chemicals, in the three test systems used, across the 13 participating laboratories will be presented. The need for clearer guidance on biodegradation testing and interpretation will be discussed, with particular reference to test variability and extended lag phases frequently encountered in marine biodegradation assessments. The role which microbial communities play in chemical biodegradation and the extent to which microbial community analysis can explain inter- and intra-laboratory variation in biodegradation test outcome will also be discussed.

EOSCA: The industry perspective

Ian Still

European Oilfield Speciality Chemicals Association (EOSCA)
1 East Craibstone St, Aberdeen, AB11 6YQ, UK
Email contact: Ian.Still@ecolab.com

The regulation of chemicals approved for offshore use by the Oil & Gas industry in the North Sea has been carried out using the framework of the OSPAR Convention and the Contracting Parties (CPs) have implemented a Harmonised Mandatory Control Scheme (HMCS) for the approval of offshore chemicals since 2001.

The HMCS assessment process requires an evaluation of environmental fate for products at substance level. This includes biodegradation data for all organic substances in the product being evaluated. The accepted biodegradation test protocols have focused on marine biodegradation with the OECD 306 being mandatory with additional data for OECD 301 (freshwater) and BODIS test protocols being (generally) accepted.

The criteria of; persistent; inherent; and ready biodegradation; determined by < 20%; > 20 % < 60%; and > 60% biodegradation in 28 days are used in the HMCS regulatory assessment as part of the OSPAR pre-screening process.

This presentation will briefly discuss the industry's views regarding the implications of the results of the OECD 306 for the HMCS and the pros and cons of introducing a modified 306 test and/or extending the test duration. It also poses some questions for other stakeholders to discuss the regulatory viability of introducing a modified OECD 306 test.

Marine biodegradation testing at NIVA

Aina Charlotte Wennberg

Section for Ecotoxicology and Risk Assessment,
Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349 Oslo, Norway
Email contact: aina.charlotte.wennberg@niva.no

The Norwegian Institute for Water Research (NIVA) is Norway's foremost professional competence center for environmental and resource issues relating to the field of water. NIVA carries out research and monitoring, as well as innovation and development work. The Section for Ecotoxicology and Risk Assessment has both research projects on national and international level, as well as contract research assignments for industry. The ecotoxicology lab does ecotoxicology and biodegradation testing according to the OECD guidelines and ISO standards according to Good Laboratory Practice (GLP).

NIVA has a research station in the Oslo Fjord with fixed lines and pumps for access to both deep (60m) and surface seawater. The water is transported to the main lab in Oslo in 45 minutes where biodegradation testing is carried out in climate controlled rooms. The closed bottle test set up has been the preferred test set up, always with good results with the reference substance (aniline). Chemical analysis is done in house by our accredited analytical lab.

NIVA participated in the ringtest for improving the OECD 306 guideline, using both closed bottle test set-up and the oxitop system. Tangential flow filtration for concentration of seawater was performed at the marine research station with direct access to seawater and good infrastructure for filtration. However, this step adds one extra workday to the test, resulting that the workload for the biodegradation testing would increase by about 20%. Oxitop systems reduces the workload compared to the closed bottle system, but needs much higher chemical test concentration with the risk of inhibition of microorganisms from some chemicals. Continuous monitoring of biodegradation using systems such as the oxitops enables to adjust incubation time to the course of degradation; if plateau is reached, the test can end, if there was a long lag phase before biodegradation started, the test can be prolonged.

NIVA is also working for improving the reproducibility and understanding the microbial dynamics during the OECD 306 test in our own research project. The effect of aging seawater before test set-up on both the course of biodegradation and on the microbial community is studied using flow cytometry in microscopy and will be presented in this talk.

A regulators view on environmental persistence testing

Pippa Curtis-Jackson

Environment Agency,
Red Kite House, Howbery Park, Wallingford, Oxfordshire OX10 8BD, UK
Email contact: pippa.curtisjackson@environment-agency.gov.uk

The Registration, Evaluation, Authorisation and Restriction of Chemicals Regulation (REACH; EC No.1907/2006) is an instrument of the European Union designed to protect human health and the environment as well as the free movement of substances individually, in mixtures and in articles. Registration is the responsibility of the manufacturer/importer of primarily industrial chemicals to the EU and is prioritised by tonnage band of the substance (tonnage/year/registrant; t/y/r).

Registration entails the provision of information about the properties of a substance, to enable appropriate risk management. The type of degradation data required increases in complexity the greater the quantity of substance supplied. Test substances registered in the lower tonnage bands (≤ 1 and 10 – 100 t/y/r) require only screening studies i.e. Ready Biodegradability (OECD 301 A to F) and/or Biodegradability in Seawater (OECD 306) and/or CO2 Headspace (OECD 310) and those registered in the higher tonnage bands (100 – 1000 and > 1000 t/y/r) require screening and simulation studies, i.e. Aerobic/anaerobic Transformation in Soil (OECD 307), Aerobic/anaerobic Transformation in Aquatic Sediment Systems (OECD 308) or Aerobic Mineralisation in Surface Water – Simulation Biodegradation Test (OECD 309).

This presentation aims to give a short introduction to environmental persistence testing under the REACH Regulation, including interpretation of data, significant endpoints and the importance of validity criteria. Moreover, the use of weight-of-evidence approaches and evaluation of all available information will be discussed.

Application of long term exposure for adaptation of microbial communities in persistency testing (CEFIC-LRI Eco29)

Baptiste A.J. Poursat[1,2], Martin Braster[2], Rick Helmus[1], Rob J.M. van Spanning[2], Pim de Voogt[1,3] and John R. Parsons[1]

[1]Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Science Park 904,
1098 XH Amsterdam, The Netherlands.
[2]Department of Molecular Cell Biology, Vrije Universiteit, de Boelelaan 1108, 1081 HZ Amsterdam, The Netherlands.
[3]KWR Watercycle Research Institute, Nieuwegein, The Netherlands.
Email contact: B.A.J.Poursat@uva.nl

Regulatory determination of the persistency of organic chemicals is mostly done using OECD ready biodegradability tests (RBTs). RBTs, however, suffer from several problems that lead to a high variability of the results and, hence, to difficulties in their interpretation. The origin and history of the inocula is one of the major causes of that variability. Nowadays, it is evident that results of RBTs change over time as microbial populations apparently adapt to metabolise previously persistent

chemicals. Several studies also show an improvement of the biodegradation rates even after a short period of pre-exposure to the tested chemical. As such, there is a need to assess the influence of this process on RBTs. We, therefore, use chemostat systems to expose activated sludge microbial communities to different chemicals for a long term and under defined conditions. Four model chemicals are used in this project; Carbamazepine, N-methylpiperazine, Metformin and 4-chloroaniline. The three first of these products can be considered as emerging pollutants and are environmentally persistent. The biodegradation capacity of pre-exposed inoculum is assessed in biodegradation testing, following the OECD 310 guideline, and changes in community structure are followed by Illumina amplicon sequencing in time. Removal of tested chemicals and their transformation products are determined by GC-FID and LC-MS/MS. The results of this experiment show enhanced biodegradation capacity of the inoculum after pre-exposure to N-methylpiperazine. These will result in a better understanding of the relationship between microbial adaptation and biodegradation performance. Moreover, microbial communities exposed to metformin were able to degrade its known persistent transformation product, guanylurea, which is persistent in fresh water. Ultimately they will also allow more realistic predictions of their biodegradation in the environment compared to those obtained using standard testing protocols.

Determining biodegradation kinetics of hydrophobic chemicals in mixtures at low concentrations

Heidi Birch, Rikke Hammershøj and Philipp Mayer

DTU Environment, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark
E-mail contact: philm@env.dtu.dk

A partitioning-based experimental platform was developed[1,2] and applied to determine primary biodegradation kinetics of 53 hydrocarbons at ng/L to µg/L concentrations covering C8−C20, 11 structural classes, and several orders of magnitude in hydrophobicity and volatility[2,3]: (1) Passive dosing from a loaded silicone donor was used to set the concentration of each hydrocarbon in aqueous mixture stock solutions; (2) these solutions were combined with environmental water samples in gastight auto sampler vials for 1−100 days incubation, and (3) automated solid phase microextraction (SPME) coupled to GC-MS was applied directly on these test systems for measuring primary biodegradation relative to abiotic controls. First order biodegradation kinetics were obtained for 40 hydrocarbons in activated sludge filtrate, 18 in seawater, and 21 in lake water. Water phase half-lives in seawater and lake water were poorly related to hydrophobicity and volatility but were, with a few exceptions, within a factor of 10 or shorter than BioHCwin predictions. The most persistent hydrocarbons, 1,1,4,4,6-pentamethyldecalin, perhydropyrene, 1,2,3,6,7,8-hexahydropyrene, and 2,2,4,4,6,8,8-heptamethylnonane, showed limited or inconsistent degradation in all three environmental media. This biodegradation approach can cover a large chemical space at low substrate concentrations, which makes it highly suited for optimizing predictive models for environmental biodegradation in aqueous media.

[1]Birch H, Andersen HR, Comber M & P Mayer. 2017. Biodegradation testing of chemicals with high Henry's constants – Separating mass and effective concentration reveals higher rate constants. Chemosphere 174: 716-21.
[2]Birch H, Hammersholt RH, Comber M & P Mayer. 2017. Biodegradation of hydrocarbon mixtures in surface waters at environmentally relevant levels - Effect of inoculum origin on kinetics and sequence of degradation. Chemosphere 184: 400-7.
[3]Birch H, Hammershøj R & P Mayer. 2018. Deter-mining Biodegradation Kinetics of Hydrocarbons at Low Concentrations: Covering 5 and 9 Orders of Magnitude of Kow and Kaw. Environmental Science & Technology 52, 2143−2151.

Questions breakout session 1 & 2

Breakout session 1: Results of the Ring Test (13.40- 15.10)

1. Are you comfortable with the proof of principle shown in the ring test?
2. What are the (dis)advantages of each test method examined ($OECD306_{CB}$, $mBST_{MR}$, $imBST_{MR}$)? How do they compare to each other?
3. What are the pros and cons to running a longer test up to 120 days?
4. What are the pros and cons to using the TFF system in an improved test?
5. How can an improved test account for variations in seawater cell concentrations and nominal concentration factors?
6. How can an improved test account for varying degradation potentials (source/ season) in seawater?
7. Are further reference compounds (next to a positive control) required to describe the seawater quality? If yes, which?
8. How do (different) manometric test systems affect the test? How can this be accounted for in an improved OECD 306?
9. Should it be common practice to routinely characterise the seawater in more depth for the tests (e.g. cell counts, microbial profiling)?

Breakout session 2: Regulatory Aspects (16.00- 17.00)

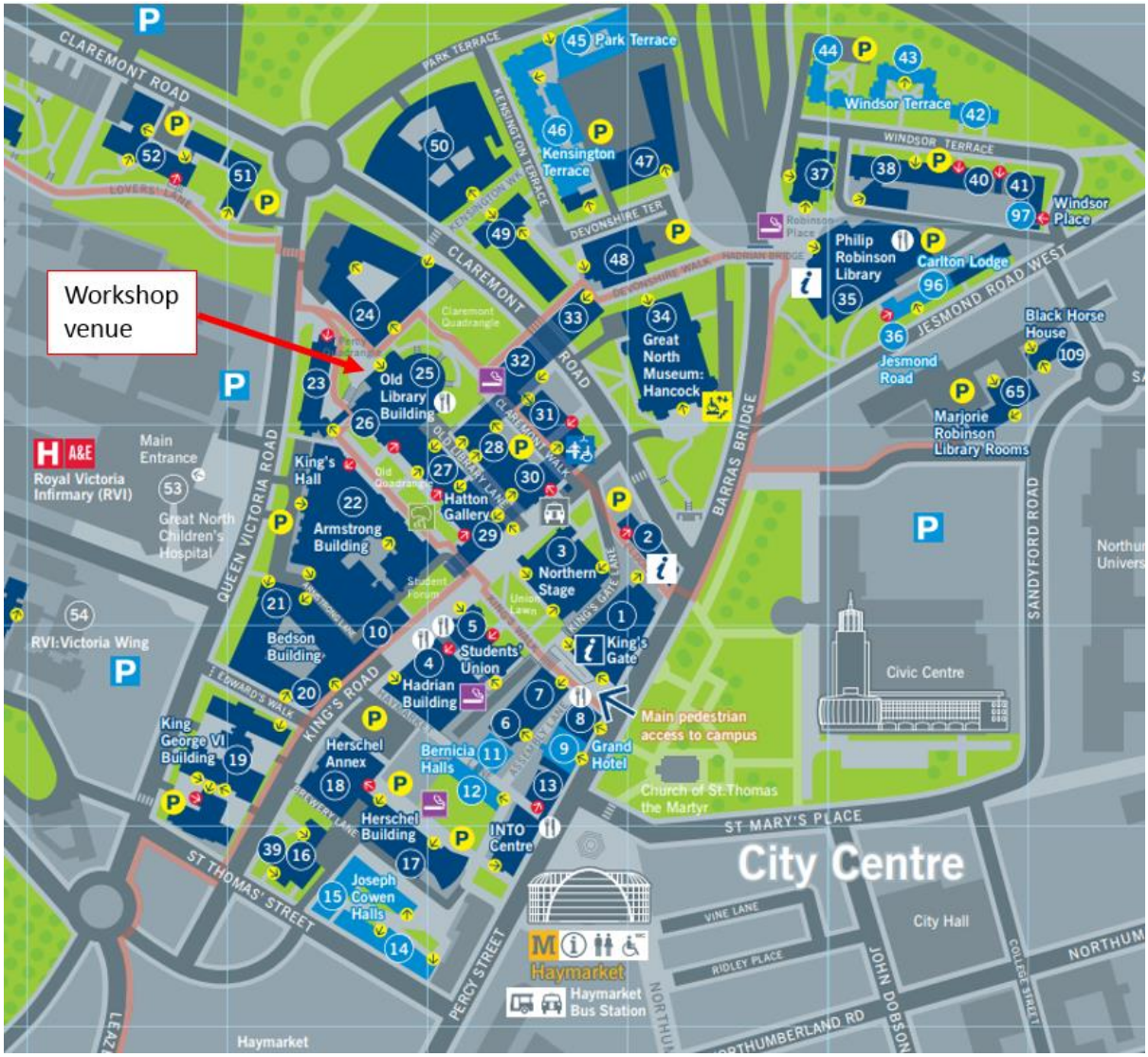
1. Which criteria should (not) be used to classify non persistent/ biodegradable chemicals in seawater?

   % degradation in x days? X days half-live? Lag phases?
2. What do we want to assess with the $imBST_{MR}$, ready biodegradability or persistency of a substance?
3. Can the the $imBST_{MR}$ be accepted as an enhanced ready test according to REACH guidance 7b?
4. How can the findings from the ring test be translated for water insoluble substances?
5. Is the existing OECD 306 and/or improved OECD 306 “fit for purpose” i.e. would it be better to use freshwater assessments?
6. What are the barriers (technical, logistic, regulatory or others) of running any of the marine biodegradation tests?

Venue

The workshop will take place in the “Research Beehive”, first floor of the Old Library Building at Newcastle University.

Address: Old Library Building, Claremont Rd, Newcastle-upon-Tyne, NE1 7RG



For the dinner on day 1, please join us on the 3rd May at 7.30 pm at the Blackfriars restaurant (Friars St, Newcastle upon Tyne NE1 4XN).

Registration and enquiries

Please register here https://forms.ncl.ac.uk/view.php?id=1327239.

If you are not able to attend the workshop, but are interested in hearing the talks electronically, please state your interest in the registration form.

Newcastle University, School of Engineering, Cassie Building,
Newcastle-upon-Tyne NE1 7RU, UK.
Amelie Ott, e-mail: **a.i.g.ott2@newcastle.ac.uk**