# *Improvement of the OECD 306 screening test: Workshop held at CEFAS laboratories, Lowestoft, UK 17-18 February 2015 and subsequent ring test*

Co-organised by ECETOC, Cefas and Cefic-LRI

Workshop Report No. 34

EUROPEAN CENTRE FOR ECOTOXICOLOGY AND TOXICOLOGY OF CHEMICALS

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Workshop Report No. 34

Brussels, September 2017

ISSN-2078-7219-34 (online)

# ECETOC Workshop Report No. 34


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## *Improvement of the OECD 306 screening test*

CONTENTS

# SUMMARY

Previous ECETOC Workshops (ECETOC 2007 and 2013), have highlighted concerns with biodegradation screening tests (BSTs) including the OECD 306 marine biodegradability test which unlike the OECD 301 tests is not defined as a ready biodegradability test (OECD, 1992). The Cefic LRI funded ECO11 project investigated and validated enhancements of both freshwater and marine BSTs, which included increasing inocula to environmentally-relevant concentrations and extending the test duration to beyond the persistence half-life threshold criteria (ECETOC, 2013; Martin et al, 2017a).

The ECO11 project identified the need to develop and validate science-based alternatives or enhanced screening studies, and a potential opportunity arose to apply some of the findings to potentially improve marine biodegradability OECD 306 assessments. Marine biodegradability tests are an intrinsic part of offshore chemical control schemes and developing an improved marine biodegradability OECD 306 test has been recognised as an important issue from both a regulatory and contract research organisation (CRO) perspective. Robust tests are recognised as essential to allow the effective identification and prioritisation of persistence chemicals in the marine environment at the screening level. This led to the organisation and conduct of a workshop to discuss improvements to the OECD 306 test.

A summary of the key workshop discussions is provided in this report. The outcome of the workshop ultimately led to the agreement to evaluate some potential improvements via the conduct of an OECD 306 ring test. The ring test is being managed by Newcastle University, as an extension of the Cefic LRI ECO11 project.

# 1. INTRODUCTION

Biodegradation is a significant but poorly understood process that can result in the loss or transformation of a chemical in the environment. The prediction of biodegradation is critical in determining persistence, eventual environmental concentrations, likely exposure and ultimately the risk of long-term adverse effects of chemical substances on biota, including humans. A series of international standardised biodegradation screening tests (BSTs) including ready biodegradation tests (RBTs; OECD 301 and 310), the marine biodegradation test (OECD 306) and inherent biodegradation tests (IBTs; OECD 302), in addition to simulation tests (OECD 303, 307, 308, 309 and 314) have been developed and approved by the Organisation for Economic Co-operation and Development (OECD) to measure the relative biodegradability of substances. BSTs are highly prescribed and conservative regulatory tests (OECD, 2006) that have historically formed the foundation for measuring the biodegradability of chemicals in regulatory frameworks for hazard and environmental risk assessments.

However, in recent years, regulatory emphasis (e.g. European guidelines on the registration, evaluation, authorisation and restriction of chemicals (REACH)) has shifted from identifying chemicals that are readily or rapidly biodegradable to identifying chemicals that are potentially persistent in the environment. REACH suggested a number of enhancements to improve the environmental relevance of BSTs (ECHA, 2016). The OSPAR Commission, which oversees the OPSAR Convention, a mechanism by which 15 governments and the EU cooperate to protect the marine environment of the North-East Atlantic, does not currently consider these enhancements for testing chemicals of the offshore industry. Marine biodegradability assessments are an intrinsic part of offshore chemical control schemes.

Previous ECETOC Workshops (ECETOC 2007 and 2013) have highlighted concerns with the BSTs including the OECD 306 (OECD, 1992) and the need to develop and validate science-based alternatives or enhanced screening studies. The Cefic LRI funded ECO11 project investigated and validated enhancements of both freshwater and marine BSTs (OECD 301 and 306), which included increasing inocula to environmentally-relevant concentrations and extending the test duration to beyond the persistence half-life threshold criteria (ECETOC, 2013; Martin et al, 2017a). Developing an improved OECD 306 test is recognised as essential to allow the effective identification and prioritisation of persistence chemicals in the marine environment at the screening level.

## Workshop information

The concerns above, from both regulators and contract research organisations (CROs), acted as a catalyst to hold a workshop to discuss improvements to the OECD 306. The Centre for Environment, Fisheries and Aquaculture Science (Cefas) hosted the two-day ECETOC/Cefic workshop on 18 – 19 February 2015 in Lowestoft, UK. It focused on highlighting the outcomes of the ECO11 research, discussing the deficiencies of biodegradation tests in marine water, considering reliable and pragmatic improvements to the OECD 306 method and offered a practical demonstration of inocula concentration with tangential flow filtration (TFF). Thirty-seven participants attended, representing academia, industry and regulatory bodies. A questionnaire was circulated to the participants before the workshop to gauge interest, capability and capacity in performing enhanced biodegradation tests. Questionnaire responses indicated a particular demand from CROs for an enhanced marine test and significant interest to participate in a ring test of an improved OECD 306.

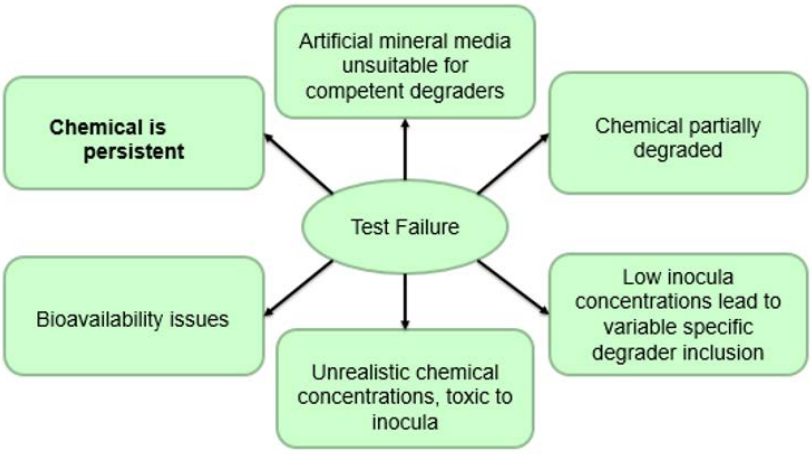
Presentations were given on the results from ECO11, experiences with enhanced biodegradability tests under REACH and problems with existing seawater testing methodology, including marine BODIS testing. Breakout sessions discussed concerns with existing marine biodegradability guidelines and the logistics for a potential ring test of an improved version of the OECD 306 test. The main discussion points can broadly be separated into two areas: Issues relating to the OECD 306 test protocols; and Issues relating to the interpretation of the data generated by the OECD 306. Where possible, the consensus view is reported here.

# 2. OECD 306 TEST FOR BIODEGRADABILITY IN SEAWATER: FAULTS AND IMPROVEMENTS

The OECD 306 test uses marine water both as the aqueous phase and the source of microorganisms. Nutrients are added to the seawater and the concentration of test chemicals is considerably higher than realistically expected in the marine environment. The method is not a test for ready biodegradability, partly because no additional inoculum is used other than the microorganisms already present in seawater. The test does not simulate the marine environment since nutrients are added and unrealistically high test substance concentrations are used. The new subsection "biodegradability in seawater" was therefore proposed for the OECD 306 test. The test can be run as a shake flask or closed bottle method for a period of 28 days with a possible extension up to 60 days for the shake flask method (OECD, 1992, 2006).

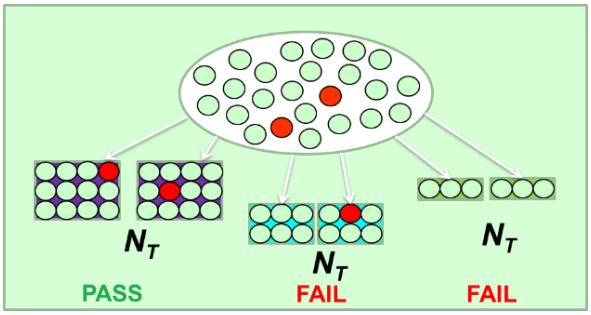
The OECD 306 test produces a large number of fails, many of which can be considered to be false negatives. A range of reasons why a fail may be encountered are shown in Figure 1, only one of which is that the chemical is persistent.

***Figure 1: Possible reasons for a failing of an OECD 306 test (adapted from Martin, 2014)***




One of the most important reasons for potential test failure is that degradation of a chemical is dependent upon the inclusion of competent degraders within the inoculum. The likelihood of incorporating potential degraders in the test inoculum depends on the total number of bacterial cells in a test (Goodhead et al, 2008) (Fig. 2). As the number of individuals per sample ($N_T$) increases, for example by increasing inocula concentrations, the probability of specific degrader inclusion increases. By contrast, low inocula concentrations lead to the variable inclusion (and thus variable test outcome) or exclusion of specific degraders leading to a test fail. This has been termed the "biodegradation lottery". The aim is to deliver a test inocula which is more representative of the bacterial diversity expected in the sampled environment, which was addressed in the ECO11 project.

***Figure 2: Biodegradation lottery where specific degraders are represented by red circles and $N_T$ refers to the number of individuals in a sample (adapted from Martin, 2014)***




The OSPAR Commission (OSPARCOM) biodegradability in seawater ring test reported variable data for the reference substance (Elf Akvamiljo & IARE, 1996). It was suggested at the workshop that this might actually have been indicative of variability in the inclusion of competent degraders between the different marine inocula.

The concentration of the inoculum was originally considered in the OECD 306 guidelines; however, investigations in using ultra-filtered and centrifuged seawater, as well as marine sediments, were unsuccessful (OECD, 1992). The ECO11 project determined that membrane filtration (MF) and tangential flow filtration TFF are effective in increasing the total number of bacteria in aqueous samples, including seawaters, with minimal community composition bias, whilst also fulfilling a selection of scientific and practical criteria, comprised within the method selection framework. This framework assessed any differences between concentrated and non-concentrated inocula; as well as considering more practical concerns, such as throughput and cost. The TFF method, which offers a higher throughput (2.5-60 L/h) than MF, was demonstrated to the participants at the workshop[1]. The ECO11 project determined that increasing the total number of cells to environmentally relevant concentrations within a BST for persistence assessment resulted in no false positive or negative assignations of reference chemicals in freshwater tests, and generally a greater extent of biodegradation and slightly shortened lag phases for marine tests.

Participants at the workshop expressed concerns over the variation reported in OECD 306 tests, evident within and between testing facilities; with temporal and spatial variations in environmental inocula also appearing to impact the biodegradation test outcome. The concern that industry may lose confidence in testing facilities due to this variation was also raised. It was also suggested that it may lead to facilities seeking specific inocula that are more likely to degrade a test compound. This contravenes an OECD principle that a test should be reproducible, irrespective of the laboratory, country or date on which a test is conducted. There was a clear consensus that the OECD 306 needs to be more reliable. There was widespread support for a reliable validated test, which has regulatory acceptance. There was a discussion regarding the inclusion of different reference substances, with more 'difficult' compounds suggested to improve confidence in the improved protocol and the validity of degradation data obtained. One recommendation was to assess if a reference compound could be found where biodegradation after 28 days would be expected to be between 20% and 60%.

[1] https://youtu.be/EcT8848hDE4

The OECD 306 test guidelines classify a chemical as biodegradable in seawater if there is a 70% reduction in dissolved organic carbon (DOC), or the theoretical oxygen demand (ThOD) exceeds 60% during the 28-day test period. The DOC threshold is higher since cells incorporate some of the carbon into new cells (OECD, 1992). Participants at the workshop generally agreed that the threshold of 60% biodegradation in 28 days was established arbitrarily. The ECO11 project recommended extending test durations beyond the standard 28 days and potentially up to 120 days in some cases.

# 3. INTERPRETATION OF OECD 306 DATA

It was recognised that even if the OECD 306 is improved, there is still a requirement to establish better guidance on testing and interpreting the data obtained. This included: acknowledging that the OECD 306 is a screening test, and a fail in a screening test does not necessarily equate to environmental persistence; developing guidance on what can be done to improve assessments of likely persistence if a substance fails the OECD 306; identifying other acceptable methods for improving marine biodegradation testing; alignment on how to deal with extended lag phases from a regulatory perspective; and identifying more appropriate reference substances (ECETOC, 2013; Martin et al, 2017a).

It was highlighted that 60% degradation within an OECD 306 does not imply that the remaining 40% is not biodegradable, but only that it did not degrade within the confines of the OECD 306. Although 60% biodegradation in 28 days is used as a pass threshold, a 20% cut off is also used to differentiate between inherent and, in effect, non-biodegradable substances, by some regulators. Guidance on interpreting different datasets, for the same chemical, which fall either side of this cut-off, was requested.

The importance of aligning guidance on interpreting extended lag phases from a regulatory perspective was highlighted during the discussion sessions. Marine tests have been shown to exhibit significant lag phases: this was recognised in the OSPARCOM ring test and ECO11, as well as in the literature (e.g. Thouand et al, 1996; Torang and Nyholm, 2005). Based on REACH recommendations (ECHA, 2016), ECO11 investigated extending test durations beyond 28 days, but there is still considerable uncertainty over how this data could be used in the current offshore chemical regulatory context (Martin et al, 2017a). For example, if 19% degradation is observed in 28 days but 100% degradation is observed in 120 days, would regulators be willing to accept the extended test outcome. Case by case expert judgement may be required to address this issue.

Freshwater biodegradation data is accepted for predicting biodegradability in marine systems, if marine data is not available (CHARM, 2005). The value in using freshwater biodegradation data was highlighted, however it was agreed that the current application of a safety factor of 0.7 has no scientific justification. The safety factor requires 86% degradation in freshwater, to be accepted as a pass in marine water. This extent of degradation is not always achieved with positive reference compounds and makes no allowance for loss mechanisms, such as carbon being incorporated into new cells rather than evolved as carbon dioxide (ECETOC, 2013).

Physical chemical characteristics of test chemicals, such as solubility, can impact on test outcome. Additional guidance on how to assess and account for these factors is still required. The need for additional guidance in recognising and interpreting test design limitations for more complex substances was identified (e.g. UVCBs: Unknown or Variable composition, Complex reaction products or of Biological material).

The focus of the next steps were to organise a ring test of an improved protocol. However, this was viewed as a first step in a process to improve the marine biodegradation assessments, which should also include establishing clearer guidance. One of the key achievements of the workshop was that it encouraged open and honest debate between regulators, academia and the chemical industry.

# 4. RING TEST TO IMPROVE OECD 306

Participants agreed that suggested revisions to the OECD 306 screening test have to be evaluated with a ring test. There was considerable debate on the terminology of proposing an 'enhanced' or 'improved' ring test. An enhancement implies a significant change to the test protocol and has to be formulated under a new designation, whereas an improvement is seen as a refinement of the existing protocol, which may facilitate its acceptance. The aim of the ring test would be: i) to investigate the principle that using environmentally-realistic inocula concentrations, by increasing the total number of cells within the test system, results in a more reliable assessment of environmental persistence; and ii) to extend test durations to account for long lag phases reported in marine biodegradation test systems.

Further discussion identified the principles regarding methods and organisation of a ring test.

It was agreed to use natural instead of artificial seawater to best represent the marine environment.

The influence of temperature on the outcome of marine tests was discussed. Whilst this may be a factor of interest, it was agreed that the initial focus should be on improving the reliability of the existing methods and investigations into issues such as temperature could be the focus of future research.

Interest was expressed in analysing a wide range of inocula sources with high throughput screening tests to generate probability data for biodegradation, as discussed by Thouand et al, 2011, Goodhead et al, 2014 and Martin et al, 2017b. It was agreed that this represents an area for future evaluation and research, but will not be covered by the ring test.

The ring test has to assess if an improved inoculum minimises the number of false negative and delivers a more robust screening method with more consistent replication within and between laboratories. Subsequently, it is proposed to test substances which do not pass the current OECD 306 test but are shown to be biodegradable in higher tier, more extensive, tests. Here, chemicals with degradation rates currently observed between 20-60% are of particular interest. Twenty percent biodegradation represents a threshold under which OSPAR assumes persistency of the substance and no further tests are performed (OSPAR, 2012). The value is considered equivalent to half-life values derived from simulation tests submitted under REACH (EC 1907/2006) of 60 days in marine water (OSPAR, 2012). It is important that any new method for assessing environmental persistence, be neither overly protective nor overly powerful: the inclusion of positive and negative reference substances is therefore recommended. It was suggested that chemicals could be chosen to represent different persistency categories or bins, defined previously by Comber and Holt, 2010. Suggestions of substances from companies routinely requiring marine biodegradation data, particularly the oil industry, are welcomed. However, any compounds used would ideally have sufficient previous biodegradation data to confidently place them in a persistency bin.

Microbial community analysis can be helpful in identifying sources of variation in the outcome of biodegradation tests, both within and between laboratories. It is not recommended as a standard practice in a revised biodegradation test due to the expense, however it is proposed as part of the ring test. This will ultimately provide evidence linking microbial community composition and diversity with biodegradation test outcome, allowing further insight into the biodegradation data.

It was suggested that at least six CROs (preferably, but not necessarily exclusively, operating under GLP conditions) would be the minimum required to participate in the ring test to encourage regulator acceptance. The CROs will be instructed to undertake the test at their own cost. However, academia will assist in concentrating the inoculum and setting up the experiment, and will conduct the data analysis including microbial profiling and final reporting.

# 5. UPDATE ON RING TEST PROJECT (AUGUST 2017)

The workshop organising committee accepted a proposed phased approach to a ring test project, prepared by Newcastle University. The OECD 306 Ring Test is an extension of the ECO 11 Cefic LRI Project and has been approved and funded by Cefic. The 3 phases of the test project are as follows:

- Phase I Development of the test protocol in which the ring test arrangements and guidelines will be finalised;
- Phase II Initiation and execution of the ring test;
- Phase III Data analysis including microbial profiling and preparation of the report.

The project was initiated in 2016 and the ring test coordination is managed by Newcastle University, UK, and monitored by a steering committee made up of members of the original organising committee. Amelie Ott was employed at Newcastle University to drive the consultation and agreement on the ring test format and oversee the initiation of the tests (Phases I and II). CROs from Europe (UK, Norway, Germany and Italy), North America (USA and Canada) and Japan are participating in the ring test with a target date to complete reporting by Q2 2018.

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# APPENDIX A: WORKSHOP PROGRAMME

| Programme Day 1: Tuesday 17 February 2015 | | |
|---|---|---|
| **11.00 – 13.00** | **Arrival and registration at CEFAS Laboratories, Lowestoft**<br>Register on-site at main reception area. Refreshments to be served. | |
| **13.00 – 13.15** | **Welcome to CEFAS. Health and safety induction** | **Bob Rowles**<br>CEFAS |
| **13.15 – 13.40** | **Biodegradability and marine biodegradability testing: the rationale behind the introduction of modified and enhanced biodegradability tests under REACH** | **Jason Snape**<br>AstraZeneca<br>**Malyka Galay-Burgos**<br>ECETOC |
| **13.40 – 14.00** | **Existing practices and problems in seawater testing methodology** | **Graham Whale**<br>Shell |
| **14.00 - 14.20** | **Problems with the Marine BODIS test** | **Linda Gioia**<br>CEFAS |
| **14.20 – 14.50** | **Coffee and open discussion regarding delegates' experiences and perspectives with seawater testing methodologies** | |
| **14.50 – 15.15** | **Putting the "bio" back into biodegradation testing** | **Russell Davenport**<br>Newcastle University |
| **15.15 – 15.45** | **Methods of providing inocula for enhanced biodegradation screening tests** | **Timothy Martin**<br>Newcastle University |
| **15.45 – 17.15** | **Coffee and breakout discussion** (based on questionnaire, but also to consider other questions – split into 2 or 3 groups depending on numbers) | |
| **17.15 – 17.45** | **Groups report back** | |

*Close of first day*

## Programme day 2: Wednesday 18 February 2015

| | |
|---|---|
| **09.30 – 10.00** | Report to CEFAS main reception<br>Luggage storage facilities can be provided if required |
| **10.00 – 11.00** | Morning discussion. Opportunity to ask questions on presentations or follow up on discussions from day 1 |
| **11.00 – 13.00** | **Group 1 tangential flow filtration practical demonstration to be followed by fluorescence microscopy session**<br>**Timothy Martin and Russell Davenport**<br><br>**Group 2 - Mini syndicate session**<br>**Facilitator Graham Whale, Rapporteur Bob Rowles**<br>**Questions for mini syndicate session:**<br>What are the major concerns regarding the use of OECD 306 and BODIS tests (Ideally from a regulatory, academic and industry perspective)?<br>Can the application of 'correction factors' to freshwater biodegradation tests be justified on scientific grounds?<br>Should amendments/enhancements to ready biodegradability tests currently permitted for freshwater assessments be equally applicable to marine tests?<br>How would you improve the OECD 306 test design and assessment of persistence of chemicals in marine waters?<br>Is a ring test of an enhanced OECD 306 essential? |
| **13.00 – 13.30** | **Lunch** |
| **13.30 – 15.30** | **Group 2 tangential flow filtration practical demonstration, to be followed by fluorescence microscopy session**<br><br>**Group 1 - Mini syndicate session**<br>**Facilitator Jason Snape, Rapporteur tbc**<br>**Questions for mini syndicate session:**<br>What are the major concerns regarding the use of OECD 306 and BODIS tests (Ideally from a regulatory, academic and industry perspective)?<br>Can the application of 'correction factors' to freshwater biodegradation tests be justified on scientific grounds?<br>Should amendments/enhancements to ready biodegradability tests currently permitted for freshwater assessments be equally applicable to marine tests?<br>How would you improve the OECD 306 test design and assessment of persistence of chemicals in marine waters?<br>Is a ring test of an enhanced OECD 306 essential? |

| | |
|---|---|
| **15.30 – 17.00** | **Report back from syndicates**<br>**Confirmation of need for a revised OECD 306 test including:-**<br>Recommendations for revisions to the OECD test<br>Suggested compounds for ring test<br>Ring test design principles (inoculum source(s), inoculum characterisation, test compound concentration, temperatures, test duration etc.)<br>Expressions of interest in participating in ring test (assuming access to equipment)<br>**Agreement on next steps/future meetings** |

*Close of Workshop*

# APPENDIX B: WORKSHOP PARTICIPANTS

| First name | Name | Affiliation |
|---|---|---|
| Inger-Lisa | Andersen | Baker Hughes, Norway |
| Angelika | Baumbusch | Norwegian Environment Agency, Norway |
| Petra | Bäverbäck | Schlumberger, Div: M-I SWACO, Norway |
| Michiel | Claessens | DuPont, Belgium |
| Alex | Clark | EOSCA, UK |
| Alex | Criddle | Lubricants UK Ltd, UK |
| Margot | Cronin* | Marine Institute, Ireland |
| Russell | Davenport | Newcastle University, UK |
| Geneviève | Deviller | Solvay, France |
| Ray | Drake | Chemex - Cambridge, UK |
| Tone Karin | Frost | Statoil Research Centre, Norway |
| Malyka | Galay Burgos | ECETOC, Belgium |
| Rhona | Garrioch | Opus Ltd, UK |
| Linda | Gioia | Cefas, UK |
| Randi | Hagemann | Statoil Research Centre, Norway |
| Bruno | Hubesch | Cefic, Belgium |
| Laurence | Libelo* | US EPA, USA |
| David | Liddy | MacDermid Offshore Solutions, UK |
| Susannah | Linington | BP Castrol Offshore, UK |
| Susan | Londesborough* | Finnish Safety and Chemical Agency, Finland |
| Heiko | Maischak | Dr. U. Noack-Laboratorien, Germany |
| Christopher | Malin | Nalco Champion, UK |
| Timothy | Martin | Newcastle University, UK |
| Chris | Mead | Harlan Laboratories, UK |
| Mattia | Ossola | Lamberti, Italy |

| | | |
|---|---|---|
| Nik | Robinson | EOSCA, UK |
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| Bob | Rowles | Cefas, UK |
| Mark | Shields | DECC, UK |
| Jason | Snape | AstraZeneca, UK |
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| Reidunn | Stokke | Norwegian Environment Agency, Norway |
| Kayashima | Takakazu | CERI, Japan |
| Kirit | Wadhia | Opus Ltd, UK |
| Aina Charlotte | Wennberg | NIVA, Norway |
| Graham | Whale | Shell, UK |

* Attended by webinar

# APPENDIX C: Authors (Workshop Organising Committee)

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Russell Davenport
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* Amelie Ott, though not on the Organising Committee for the workshop, made significant contributions to this workshop report.
** Left ECETOC July 2016

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D-2017-3001-251

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