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Biologische Testverfahren zur ökotoxikologischen Charakterisierung von Abfällen

ABSCHLUSSBERICHT

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15. **Zusätzliche Angaben**		
16. **Kurzfassung** Die ökotoxikologische Charakterisierung von Abfällen ist Bestandteil ihrer Bewertung als gefährlich oder nicht-gefährlich wie in der Europäischen Abfallliste beschrieben. Trotz der Umsetzung in nationales Recht in der Abfallverzeichnisverordnung 2001 fehlt bislang die methodische Ausgestaltung des aus dem Gefahrstoffrecht abgeleiteten Gefährlichkeitskriterium (H14 „ökotoxisch“). Ausgehend von den Empfehlungen der CEN Richtlinie 14735 (2005) wurde ein internationaler Ringtest durch BAM, FH Giessen-Friedberg und ECT GmbH organisiert, an dem insgesamt 67 Laboratorien aus 15 Ländern teilnahmen. Im Ringtest wurden stellvertretend drei Abfallarten getestet: eine mit Schwermetallen belasteten Asche aus einer Müllverbrennungsanlage (INC), ein hoch mit organischen Schadstoffen (PAK) belasteter Boden sowie ein Abfall aus konservierten Hölzern, der viel Kupfer und andere Schwermetalle enthielt. Die Proben durch die BAM vorbehandelt (u.a. Trocknung, Siebung und Homogenisierung) und verteilt. Parallel zur biologischen Testung erfolgte eine chemische Untersuchung der Eluate und Feststoffproben. Die im Ringversuch eingesetzte Basis-Testbatterie bestand aus drei aquatischen (Algen-, Daphnien und Microtoxtests) und drei terrestrischen (akuter Regenwurm- und Pflanzentest mit Hafer und Rübse). Zusätzlich wurden Daten aus zehn zusätzlichen - fünf aquatischen (inklusive einem Gentoxtest) und fünf terrestrischen – Tests gewonnen. Fast alle Tests wurden nach ISO-Richtlinien durchgeführt, wobei die jeweilige Toxizität mittels EC50-Werten angegeben wurde. Die Datenauswertung erfolgte nach ISO (2002) bzw. Environment Canada (2005) Empfehlungen. Insgesamt wurden neben einer hohen Zahl von Referenztestdaten 634 Datensätze aus der Basis-Batterie und 204 Datensätze aus den Zusatztests erstellt. Nur wenige Ergebnisse waren nicht akzeptabel (z.B. aufgrund fehlender Referenzdaten) und noch weniger Daten wurden als statistische oder biologische Ausreißer identifiziert. Zum Beispiel variierte die Akzeptanzrate bei den Tests der Basis-Batterie zwischen 74,1% (Algentest) und 92,6% (Daphnientest). Methodisch traten keine Probleme auf, auch wenn weitere Angaben zur Feuchteeinstellung in den terrestrischen Tests sowie Details der Referenztestung und –auswertung notwendig erscheinen. Unabhängig vom Testsystem verursachte SOI immer die geringsten und WOO die stärksten Wirkungen, während die EC50-Werte von INC eine mittlere Toxizität anzeigten. Unter den aquatischen Tests reagierten die Daphnien sowie eine Algenart am empfindlichsten, während in den Feststofftests die Pflanzen immer stärker als die Regenwürmer reagierten. Aufgrund der Ergebnisse aus der erweiterten Testbatterie konnten Vorschläge für eine Modifizierung der bestehende Basis-Testbatterie erarbeitet werden. So könnte z.B. der akute Regenwurmtest durch einen anderen Test mit Bodeninvertebraten mit höherer Sensitivität ersetzt werden. Die zusätzlichen Untersuchungen verbesserten den Kenntnisstand zur Abfalltestung beträchtlich, (z. B. der Einsatz von Kunsterde als Kontroll-substrat). Ein Vergleich von Ringtestergebnissen mit den (wenigen) Literaturangaben ergab eine gute Übereinstimmung. Die Ergebnisse des Ringtests zeigen, dass die Kombination einer biologischen Testbatterie und chemischer Analytik für die ökotoxikologische Charakterisierung von Abfällen notwendig ist. Ergänzt durch im Abschlussbericht vorgeschlagene Modifikationen ist die Basis-Testbatterie gut für die Gefährdungs- und Risikobeurteilung von Abfällen geeignet. Die weitere, wahrscheinlich multi-variate Auswertung der Ringtest-daten wird die Identifikation derjenigen Tests verbessern, die am besten für die ökotoxikologische Charakteri-sierung von Abfällen geeignet sind. Schließlich unterstützen die Ringtesterfahrungen auch die in der CEN-Richtlinie 14735 gemachten Vorschläge für die Durchführung ökotoxikologischer Tests mit Abfällen.		
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15. **Supplementary Notes**

16. **Abstract**
The ecotoxicological characterization of waste is part of their assessment as hazardous or non-hazardous according to the European Waste List. Despite its transfer into national law in the waste list ordnance 2001 no methodological recommendations have been provided to cover the hazard criterion (H14 "ecotoxicity") which was taken over from the legislation on dangerous substances. Based on the recommendations of CEN guideline 14735 (2005), an international ring test was organised by BAM, FH Giessen-Friedberg and ECT GmbH. In total, 67 laboratories from 15 countries participated in the ring test. It was performed with three representative waste types: an ash from an incineration plant mainly contaminated with heavy metals, a soil containing high concentrations of organic contaminants (PAHs) and a preserved wood waste contaminated with copper and other heavy metals. Samples were prepared by BAM (e.g. inter alia dried, sieved and homogenised) and distributed. Parallel to the biological testing the eluates and solid samples were chemically characterized. The basic test battery used in the ring test consisted of three aquatic (Algae test, *Daphnia* acute test and Microtox test) and three terrestrial (earthworm acute and plant test with two species (oat, rape)) tests. In addition, data were submitted for ten additional tests (five aquatic (including a genotoxicity test) and five terrestrial ones). Almost all tests were performed according to ISO guidelines, providing EC50 values as measurement of toxicity. Data evaluation was done following recent recommendations made by ISO (2002) and Environment Canada (2005).
Besides a high number of reference test data, 634 data sets were produced in the basic test battery and 204 data sets in the additional tests. Only few data sets were not acceptable (e.g. due to lack of reference data) and even less results were identified as statistical or biological outliers. For example, in the case of the basic test battery the acceptance rate varied between 74.1% (Algae test) and 92.6% (Daphnia test). Methodologically, no problems occurred but further guidance on moisture determination in the terrestrial tests as well as details concerning reference testing and data evaluation for several tests are needed. Independently which test system is considered, SOI always caused the lowest effects and WOO was most toxic, while the EC50 values of INC show an intermediate toxicity. Among the aquatic tests, daphnids and one algal species were the most sensitive ones, while plants were always more sensitive than earthworms in the solid waste samples.
Based on the test results from additional tests proposals for the modification of the existing basic test battery could be made. For example, the earthworm acute test could be replaced by another soil invertebrate test with higher sensitivity. Further work performed in parallel to the ring test improves waste testing considerably (e.g. the use of artificial soil as control substrate). A comparison of the ring test results with literature data published so far revealed a good agreement. The results of this ring test support confirm that a combination of a battery of biological tests and chemical residue analysis is needed for an ecotoxicological characterization of wastes. With small modifications proposed in this report the basic test battery is considered to be well suitable for the hazard and risk assessment of wastes. Further, probably multi-variate evaluation of the ring test results will improve the identification of those tests most qualified for the ecotoxicological characterization of wastes. Finally, the experiences made in the ring test support also the proposals made in CEN guideline 14735 (2005) concerning the performance of such tests.

17. **Key Words**
European Union Union, waste, laboratory, eluate tests, soil tests, invertebrates, micro-organisms, plants, ring tests

18. **Charge**	19.	20.

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A. Summary: Overview on the overall results of the ring test

A.1 Introduction

A.1.1 Legal background

The European Waste List (2001/118/EC (EC 2001)) is a harmonized list of about 850 different waste types, thus forming a consistent waste classification system across the EU. It is intended to be a catalogue of all wastes, grouped according to generic industry, process and waste type. So far, the list was amended three times and can be revised according to the Waste Framework Directive. It includes 850 waste six-digit-codes in 20 chapters, defining 405 waste types as hazardous waste material and 200 waste types in so called “mirror entries”. A mirror entry is defined as follows: Wastes with potential to be either hazardous or non-hazardous depending on their composition and the concentration of dangerous substances. Right now, 14 hazard criteria for the characterization of hazardous waste types were defined. The criteria H3 to H8, H10, H11 (flammable, irritant, harmful, toxic, carcinogenic, corrosive, teratogenic, mutagenic) are based on concentration of dangerous substances. The criterion H14 “ecotoxic” lacks an assessment and testing strategy. In addition, no specific threshold values have been defined so far. Details of the legal background are provided by Moser & Kessler (2008).

During an international workshop organised by UBA and JRC in Ispra (Italy) in September 2005 the participants agreed that biological test systems should be used for the ecotoxicological characterization of waste. A distinct need for a harmonised test battery was identified, to be developed and validated in the framework of CEN TC 292. In addition, a general agreement was reached that this test battery shall address the property of ecotoxicity of waste by using at this stage test organisms, as representatives for various ecosystems or compartments and various trophic levels

(CEN 2005). Clearly, for the validation of such a test battery it is necessary to perform an international ring test. In this contribution the main experiences and results of this ring test are summarised.

A.1.2 Aims of the ring test

The validation of test procedures is an essential part in the standardisation process of each eco-toxicological test. The framework of European standardisation (CEN) demands an internationally conducted ring test with typical test substrates which provides valid information on the practicability and the reproducibility of the results for the respectively employed test procedures. Therefore, the main aim of this ring test was the establishment of a test battery for the ecotoxicological characterisation of wastes (H14), using the EN 14735 standard “Characterization of waste – Preparation of waste samples for ecotoxicity tests” (CEN 2005) as the basis.

In this context, the following issues were addressed:

- the validation of the preparation of the test substrates according to this standard;
- the assessment of the suitability of the basic test battery (i.e. three aquatic and two terrestrial tests) in terms of practicability and sensitivity, including the question whether modifications of existing test methods are necessary or not;
- the evaluation of the uncertainty level of the results for the various tests;
- the identification of recommendations concerning a test battery for routine use.

Finally, open questions and needs for future research had to be selected.

A.1.3 Organisation of the ring test

In agreement with recommendations published by international standardisation organisations (e.g. OECD 2005a) or in the scientific literature (e.g. Römbke & Moser 2002) the ring test was organised in a way that a high number of participants was selected, who represented various countries and institutional backgrounds as well as a broad range of experience with the selected ecotoxicological tests. Main activities of the ring test were centralized:

- Umweltbundesamt (UBA) Dessau, Germany:

  General organisation and communication between participants and third parties as well as the organisation of a demonstration workshop (June 21 – 22, 2006; Berlin-Marienfelde, Germany) and the final meeting (June 29, 2007; Berlin, Germany) and the final report. In this role, the UBA was supported by a Scientific Advisory Board, consisting of four scientists including a representative of CEN.
- Federal Institute for Materials Research and Testing (BAM), Berlin, Germany:

  Preparation, characterization and distribution of the three test substrates (differing strongly in their chemical and physical properties).
- ECT Oekotoxikologie GmbH (ECT) Flörsheim, Germany:

  Scientific co-ordination of the ring test including the preparation of Standard Operation Procedures (SOPs) and forms (paper, EXCEL-file), the organisation of a training workshop (December 15, 2005; Berlin, Germany) and the compilation of the final report.
- University of applied sciences (FH) Giessen-Friedberg, Giessen, Germany:

  Statistical assessment of the ring test results according to ISO 5725-2 (2002).

The institutions involved in the organisation of the ring test are shown in Figure 1.

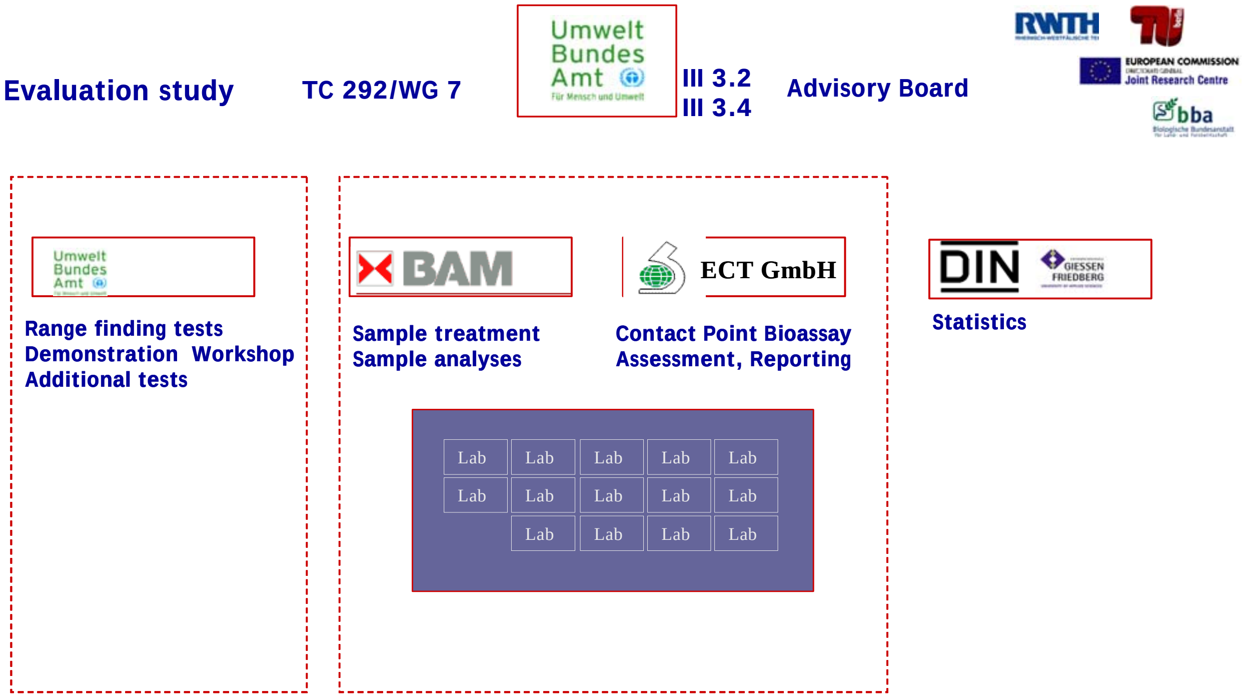

Fig. 1: Overview on the organisation of the EU ring test for the evaluation of wastes

A total of 67 laboratories took part in this ring test and 64 laboratories returned data to the organisers (according to their respective number of test systems). The participants were based in 15 countries: Austria (2), Belgium (5), Czech Republic (4), France (4), Germany (23), Ireland (1), Italy (5), The Netherlands (1), Norway (1), Portugal (4), Slovak Republic (1), Spain (4), Sweden (3), UK (1), USA (1). The participants represented 16 universities, 16 public research institutions, 28 contract laboratories and three companies (no data were delivered by the latter group).

A.2 Methods

A.2.1 Test substrates

The test materials were selected upon consultation with CEN's committee CEN TC 292/WG7. Their processing, characterisation and distribution was conducted by BAM (Berlin). Details of their selection, procession and characterization are given in Becker et al. (2008). The overall process is shown in Figure 2. The three materials covered a wide range of toxicity and different matrices of the main waste flows.

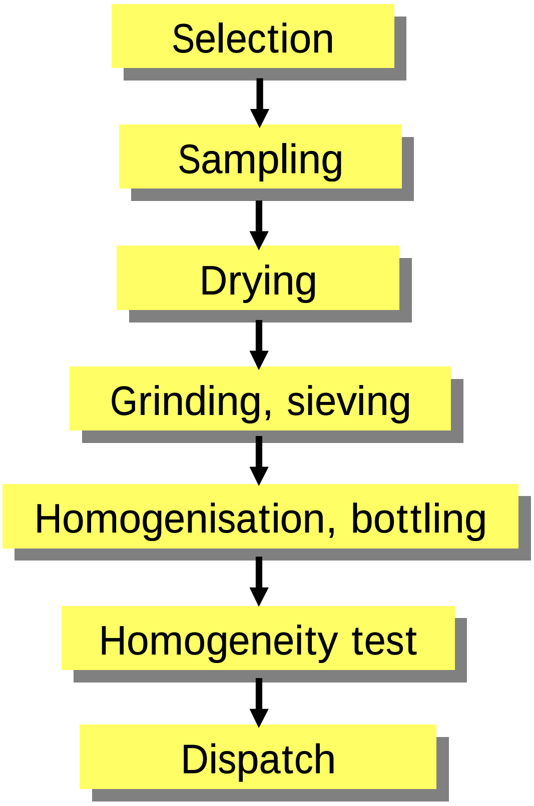

Fig. 2. Schematic processing of the three test substrates: from selection to dispatch

Municipal waste incineration ash (INC)

The starting material (719 kg) was obtained form a Dutch incineration plant for municipal waste („bottom ash“) and was processed at BAM (drying, sieving [< 4 mm], homogenisation). 318 kg were bottled and distributed among the participants according to their specific demand. Along with partly high concentrations of heavy metals (Cu 6,800 mg/kg; Zn 2,639 mg/kg; Pb 1,623 mg/kg) a high pH (about 10.5) was observed.

PAH contaminated soil (SOI)

The polluted sandy soil originates from a former gasworks site in Berlin (Germany), which was dried, sieved (< 4 mm) and homogenised at BAM. A total of about 680 kg of the starting material was used. Besides a high PAH content (sum of the 16 EPA-PAK: 840 mg/kg) only a minor amount of mineral oil hydrocarbons was detected (152 mg/kg).

Waste wood (WOO)

This substrate was a mixture of treated and untreated wood samples from a commercial timber processing plant, which were treated with copper-based wood preservatives according to the regulations of different European countries. The starting material was grounded with a cutting mill (< 4 mm). The obtained amount of 900 kg was homogenised and 617 kg were bottled in containers between 0.5 kg and 10 kg and dispatched to the participants. This substrate demanded a complicated homogenisation procedure due to its low bulk density and poor flowability. The copper content was high (2,110 mg/kg), while the pH was low (pH ≈ 4.8).

The preparation of the three test substrates followed the provisions laid down in CEN 14735 (2005). The main steps of the procedures are shown in Figure 3. As stated above, the main preparation of the tests substrates was performed by BAM, while the preparation of the eluates was conducted by the individual partners. In an own SOP the handling of the waste samples was described, starting from the moment the samples arrived in the laboratory (including details like sample labeling, storage conditions and so on). In addition, each laboratory was asked to determine basic parameters of the test substrates like pH, conductivity, TOC and the concentration of the main pollutants for the eluates as well as pH, organic matter content and water holding capacity for the solid samples. However, due to limited resources this was not always possible. All information gained was compiled in specific forms provided by the ring test organizers.

In order to perform tests following a dose-response design the eluates as well as the waste samples had to be diluted using an appropriate dilution material (e.g. OECD reconstituted water (OECD 2004) or OECD artificial soil (OECD 1984)). Both for the control and the dilution steps the same material had to be used. Details of the preparation of the dilutions as well as the design of the tests were laid down in the guidance papers for individual tests and were part of the information given during the demonstration workshop.

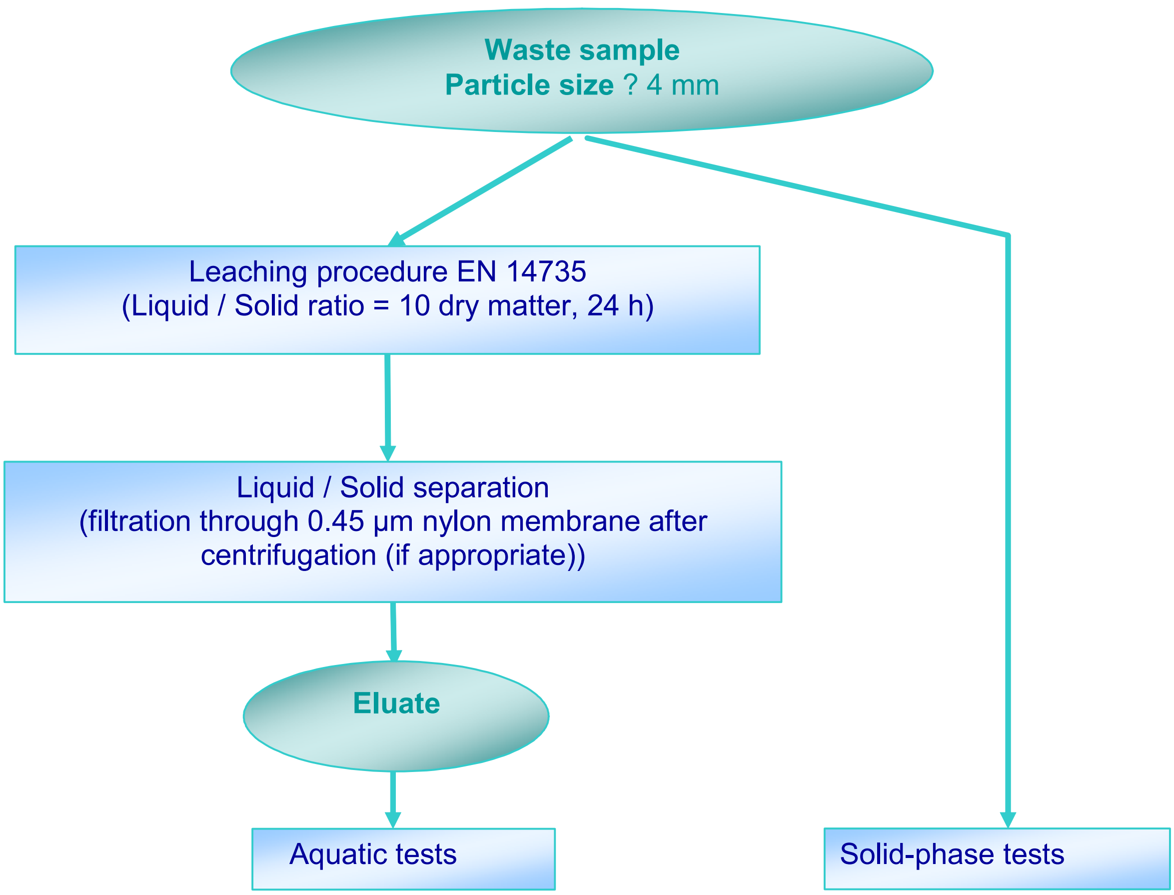

Fig. 3: Overview on the treatment of the three test substrates in the ring test

A.2.2 Basic test methods

The eco-toxicological characterisation of wastes is laid down in European standard CEN 14735 (2005), which describes the sample preparation and provides an informative collection of appropriate test procedures for the investigation of wastes. This collection of test procedures was condensed to a basic test battery, containing three aquatic and three terrestrial procedures, which are briefly characterised in the following (Table 1). Details of the test performance of these five tests are given by Weltens (2008), Pandard (2008), Pattard (2008a), Moser et al. (2008) and Förster et al. (2008).

Table 1: Brief overview on the five tests belonging to the basic test battery

Name	**Guideline**	**Species**
Eluate (aquatic) waste tests:		
Algae	ISO 8692 (2004a)	*Desmodesmus subspicatus, Pseudo-kirchneriella subcapitata*
Daphnia	ISO 6341 (1996)	*Daphnia magna*
Luminescent bacteria	ISO 11348-1/2 (2005)	*Vibrio fischeri* (3 sources)
Solid (terrestrial) waste tests:		
Earthworms (acute)	ISO 11268-1 (1997)	*Eisenia fetida, Eisenia andrei*
Plants	ISO 11268-2 (2004b)	*Avena sativa, Brassica napus*

Only tests which fulfilled the validity criteria given in the individual guidelines, which included a reference test (not in the case of plant tests since here no reference substance is formally required) and which were performed according to the basic Operation Procedures (SOPs) provided by the organisers were considered to be acceptable for assessment. In the case of the luminescent bacteria tests the sensitivity is mainly proven by performing limit tests; therefore, the reference tests following a dose-response design were not used for the acceptance check of these tests.

A total of 634 data sets were produced in the basic test battery. In detail, the results of 143 algae tests, 161 Daphnia tests, 154 luminescent bacteria tests, 52 earthworm tests and 124 plant tests were submitted by the participants.

A.2.3 Additional test methods

In addition, ten additional test procedures (five aquatic and five terrestrial ones) regarded as being potentially appropriate for the determination of the eco-toxicity were performed in the ring test. These test methods are briefly characterized in Table 2. Details of the test performance of the additional waste eluate tests are given by Moser (2008), Wadhia (2008) and Eisenträger (2008). The respective information provided for the additional solid waste tests is provided by Scheffczyk et al. (2008), Riepert (2008), Amorim et al. (2008), Natal da Luz et al. (2008) and Neumann-Hensel et al. (2008). Since the experience with these additional test methods in general is lower than for the methods used in the basic battery and since no SOPs were prepared for them, the acceptance of test data sets was handled on a case-by-case basis (i.e. in general no tests with a reference substance were required).

A total of 204 data sets were produced in the additional test battery. In detail, the results of 51 *Lemna* tests, 20 *Brachionus* tests, 12 *P. putida* bacteria tests, 10 *Ceriodaphnia* tests and 23 umu tests were submitted by the participants for waste eluates. The respective numbers for the solid waste tests are: 21 Collembola tests, 17 earthworm reproduction tests, 10 earthworm avoidance tests, 12 enchytraeid tests and 28 *Arthrobacter* tests.

Table 2: Brief overview on the ten tests belonging to the additional test battery

Name	**Guideline**	**Species**
Eluate (aquatic) waste tests:		
Aquatic macrophyte	ISO 20079 (2004c)	*Lemna minor*
Rotifer	ISO/CD 20666 (2007)	*Brachionus calyciflorus*
Sludge bacteria	ISO 10712 (1995)	*Pseudomonas putida*
Water flea	AFNOR 90-376 (2000)	*Ceriodaphnia dubia*
Umu Genotoxicity	ISO 13829 (2000)	*Salmonella typhimurium*
Solid (terrestrial) waste tests:		
Collembola	ISO 11267 (1999)	*Folsomia candida*
Earthworm repro.	ISO 11268-2 (1998)	*Eisenia fetida, Eisenia andrei*
Enchytraeidae	ISO 16387 (2004d)	*Enchytraeus albidus, E. crypticus*
Earthworm avoidance	ISO 17512-1 (2006)	*Eisenia fetida, Eisenia andrei*
Arthrobacter contact	DIN 38412-48 (2002)	*Arthrobacter globuliformis*

A.2.4 Test data evaluation

The evaluation of results was done following ISO 5725-1 (1994) and ISO 5725-2 (2002), however, the logarithms of the EC_{50}-values were used. All EC_{50} calculations were performed using the statistical program ToxRat (2006); i.e. in those cases where test participants did not use probit analysis themselves the respective data sets were re-calculated in order to improve comparability of the results. Since the test procedures in question are tedious and elaborate compared with trace analytical investigations replicate determinations within a given laboratory and within short intervals of time were not possible. Thus, repeatability and reproducibility were evaluated using the results on the variability in different laboratories.

In addition, it was looked at which results were outside of the range geometric mean plus minus two-fold standard deviation (warning limit approach; Environment Canada 2005). Another aspect considered was the factor between minimum and maximum values of each respective test. Details of the evaluation process are given by Donnevert (2008). Results regarding reproducibility and repeatability of the data are given also by Pandard & Van der Wielen (2008).

A.3 Results

A.3.1 Results of reference tests

In parallel to the tests with the three waste materials most of the participating laboratories secured the sensitivity of the test species by performing tests with specific reference substances (Table 3). These tests were evaluated separately (aquatic reference tests: Pattard 2008b); terrestrial reference tests: Haller et al. 2008).

First of all, the data sets are large. For instance, in the case of the earthworm tests the number of results with the reference substance chloroacetamide is considerably higher (52) than the number of data provided in the original ring test when the range of expected results was defined (18; Edwards 1984). Secondly, only few participants (4 – 13%) did not perform reference tests when required. Even when such reference tests were not absolutely necessary as in the case of the luminescent bacteria and the plants, a considerable number of participants (47 and 19%, respectively) provided such data. Finally, only in very few cases results from these tests gained data which are outside of the required range (0 – 5%). Therefore, independently from the EU waste ring test the reference data will be used to improve the recommendations given in the existing guidelines (in particular for the algae and plant tests).

Table 3: Overview on the performance reference tests and the acceptance of their results (for details see Pattard (2008b) and Haller et al. (2008)). Data given in % of the overall number and in absolute numbers (in brackets)

Test system	**No. test data sets**	**No. Ref. tests**	**Results not in range**
Algae	**100%** (143)	**86%** (123)	**5%** (7)
Daphnia	**100%** (161)	**96%** (155)	**3%** (6)
Luminescent bacteria	**100%** (154)	**47%** (73)	-
Earthworms	**100%** (52)	**87%** (37)	**0%** (0)
Plants	**100%** (124)	**19%** (23)	-

A.3.2 Basic test methods

In a first step, the acceptance of the provided data was determined. Using the validity criteria given in the respective guidelines as well as the results of the reference tests as mentioned in the previous chapter those data were identified as useful for further assessment. In addition, in a few cases test results were not accepted because they were considered as outliers, either due to statistical or biological (EC_{50} < / > as the overall mean by a factor of ten). The outcome of this exercise for the five basic tests and separately for the three test substrates is given in Table 4. Note that the differences between the three sources of luminescent bacteria and the two plant species were too small to be considered here.

The rate of acceptance was very high and varied between 74.1 (Algae tests) and 92.6% (*Daphnia* tests). In a first attempt the acceptance rate of the algae tests was considerably lower (slightly less than 70%), because the required range of reference results was set too low in the current guidelines. Following a discussion with ISO representatives responsible for the standardisation of this test the range was adapted and the rate reached 74.1% which is only slightly less with those rates found in the other basic tests. All further evaluation is based on the data identified as accepted here.

Table 4: Number of accepted tests data sets in percent of the total number of tests performed, separately for the five basic tests and the three test substrates

Test system	**No. tests**	**INC**	**SOI**	**WOO**	**Sum**	**% Total**
Algae	143	35	35	36	106	**74.1**
Daphnia	161	47	51	51	149	**92.6**
Lumi. bacteria	154	45	42	53	140	**90.9**
Earthworms	52	13	14	14	41	**78.8**
Plants	124	37	35	35	107	**86.3**

A compilation of the individual results is provided in Annex I. In modification of ISO 5725-2 (2002) this annex contains not only the mean EC_{50} values and information about their reproducibility (i.e. the 95%-confidence intervals) but also the factor between the respective minima and maxima (after exclusion of outliers). Tests in which no EC_{50} values could be determined (most often with the substrate SOI) were not considered for the general assessment. Further details of the test results are given by Weltens (2008), Pandard (2008), Pattard (2008), Moser et al. (2008) and Förster et al. (2008).

Before comparing the results of the individual tests the most important methodological experiences from the five basis tests are briefly summarised. In fact, the performance of the five tests almost never did cause problems, although most of the participating laboratories were not familiar with the biological testing of waste material or eluates.

Concerning the algae tests the most striking outcome was the clear difference in sensitivity between the two species: *P. subcapitata* is by a factor of four (tests with INC) to five (tests with WOO) more sensitive than *D. subspicatus*. No modifications became necessary in the *Daphnia* test. When performing the tests with luminescent bacteria, comparable differences were found like in the algae tests: depending on the source (fresh / liquid versus freeze bacteria) different sensitivities were found. In the tests with the waste substrate WOO the sensitivity differed by a factor of about five, showing that the freeze bacteria are more sensitive. While the earthworm tests were running fine, participants asked for more guidance on how determining the moisture of the test substrate mixtures. In addition, there is strong concern about suitability of the currently used reference chloroacetamide, which is considered to be mutagenic. Finally, in the case of plants the situation is similar: the tests themselves are working but in a few cases it became difficult to find and maintain the optimal moisture level when setting up the tests. From the very beginning it was clear that the two plant species had to be handled separately due to their different taxonomy and physiology.

Concerning the reproducibility of the accepted test results the factor between the lowest and the highest EC_{50} values are compared for each test system (and species / strains) separately (Table 5). In addition, EC_{50} values outside of the warning limits were not taken into consideration. However, since always less than two data sets were outside of the warning limits, this exclusion did not influence the evaluation considerably.

In terms of toxicity, these results can be assessed in various ways:

Toxicity of the three waste substrates: Independently which test system is considered, SOI always caused the lowest effects and WOO was most toxic, while the EC_{50} values of INC were found somewhere between these extremes.

Sensitivity of the individual test systems in aquatic and terrestrial tests: The waste substrate INC caused the strongest effects on daphnids and the algae *P. subcapitata*, followed by the second algal species *D. subspicatus*, while the luminescent bacteria reacted much less. In contrast, SOI impacted only (weakly) the luminescent bacteria but showed no toxicity for algae and daphnids. The waste substrate WOO was highly toxic to all aquatic species; only the liquid/fresh luminescent bacteria reacted less strongly. In the case of the terrestrial tests plants were always more impacted by the three waste substrates than earthworms. However, with the exception of WOO which was strongly affecting *B. napus*, the difference between the terrestrial EC_{50} values was always lower than the factor of two. Again with the exception of WOO, where there was a clear difference, *B. napus* was equally sensitive as *A. sativa*.

Reproducibility of the test results: The difference between the lowest and highest EC_{50} values was in most cases small, which is not surprising for the waste substrate SOI where due to its low toxicity effect values can only slightly vary (actually, just by a factor of 2 – 3). The EC_{50} values for the highly toxic WOO differed in two cases by a factor less than five and in only one case by a factor clearly higher than 100. In the tests with the waste INC only in the algae tests factors higher than ten were observed. In general, in the terrestrial tests smaller differences were found.

Table 5: EC_{50} values (% waste) and their minimum - maximum factor based on all accepted data minus those outside of the warning limits (see the Annex I). N.a. = not applicable: no EC_{50} determinable due to low toxicity.

Test system: **Species / strain**	**EC_{50} values (% waste)**			**EC_{50}s: Minimum–maximum factor**		
	INC	**SOI**	**WOO**	**INC**	**SOI**	**WOO**
D. subspicatus	8.79	>100	1.34	15	n.a.	2
P. subcapitata	2.61	>100	0.22	20	n.a.	27
D. magna	2.79	>100	0.5	10	n.a.	33
Lumi-Bacteria: all	35.4	63.39	2.69	8	2	114
Lumi-Bacteria: freeze	30.83	65.77	0.73	8	2	20
E. fetida / E. andrei	44.98	>100	18.97	2	n.a.	5
A. sativa	29.44	56.75	10.96	4	2	10
B. napus	23.88	62.95	2.64	6	3	11

A.3.3 Additional test methods

Details of the test performance of the additional waste eluate tests are given by Moser (2008), Wadhia (2008) and Eisenträger (2008). The respective information provided for the additional solid waste tests is provided by Scheffczyk et al. (2008), Riepert (2008), Amorim et al. (2008), Natal da Luz et al. (2008) and Neumann-Hensel et al. (2008). Since the additional test methods were not the main focus of the ring test, only the most important results will be presented here (Table 6). In general, the number of data sets per test system were relatively low (aquatic: 10 – 51; terrestrial: 10 – 28); thus, with the exception of the *Lemna* test (51 data sets) any discussion of the reproducibility of the test results is premature. All results were accepted except those without raw data and those violating the respective validity criteria. No methodological problems were reported by the participants.

Table 6: Number of accepted (= total number minus those without raw data) tests and EC_{50} values (% waste) of the additional test systems (Note: in the case of the earthworm avoidance and the *Arthrobacter* tests NOEC values are listed)

Test system	**No. tests**	**INC**	**SOI**	**WOO**
Lemna minor	51	> 50	> 50	2.0
P. putida	12	23.1	>80	0.2
B. calicyflorus	20	5.0	>100	0.1
C. dubia	10	4.8	>90	0.1
Collembola	18	26.0	47.9	5.0
Enchytraeidae	12	31.8	>100	14.6
Earthworm Reproduction	14	16.1	>50	4.1
Earthworm Avoidance	10	10.25	75	2.6
Arthrobacter Contact test	28	22.77	12.4	0.6

As in the case of the basic tests, WOO caused the strongest effects in all test systems and SOI the least ones, with INC showing intermediate results. For all waste substrates, the tests with *C. dubia* and *B. calicyflorus,* followed by the test with P*. putida*, were the most sensitive ones. In comparison to the tests with the basic test battery, several non-basic tests could become alternatives in terms of sensitivity. The *Lemna* test was clearly the least sensitive method, but it could provide information of the plant-related toxicity in coloured or turbid waste eluates, where an application of algae test is not possible. Among the solid waste tests, the enchytraeid reproduction test was the least sensitive method, while the earthworm avoidance and in particular the *Arthrobacter* tests were the most sensitive ones. However, the results of both tests are difficult to assess so far since partly EC_{50} values are missing. Compared to the basic test battery almost all zoological tests were more sensitive than the earthworm acute test.

In total, 23 umu tests were performed in the ring test. With the exception (cytotoxicity?) of two WOO tests, no genotoxicity was found at the highest eluate concentration (67%).

A.3.4 Further work performed in the ring test

In addition to the tests with the basic and the additional test batteries the participants of the ring test performed additional work, partly referring to the same data but evaluating them in a different way. For example, Hofman et al. (2008) characterised and compared various batches of OECD artificial soil provided by participating laboratories. Postma & Van der Sloot (2008) studied the relationship between toxicants, availability and effects in the waste substrates. In addition, the results of the basic aquatic tests which those being available as miniaturised toxkits were compared by Persoone & Wadhia (2008).

A.3.5 Comparison with literature data

So far, the experiences with testing wastes in ecotoxicology are very limited (Kostka-Rick 2004). In addition, data are often published in “grey” reports (e.g. Deventer et al. 2004). Despite the fact that it was not the main aim to compare the results of this ring test with the few experiences published in the literature some first examples can be presented here. For instance, the LC_{50} value determined for INC in artificial soil (44.98%) in the acute earthworm tests is in good agreement with the range of LC_{50} values determined for twelve German incineration ashes (11.5 and 43.6%; Römbke &

Moser 2007). Comparable incineration ashes from France with very low heavy metal contents showed a lower toxicity in the earthworm acute test (LC_{50} values 40 – 76%), but no details are known about the conditions in these tests (Quilici et al. 2004). As a general tendency, soils contaminated with PAHs usually cause only low effects on earthworms. For example, after mixing a PAH-contaminated soil (sum of EPA-PAH: about 3000 mg/kg) with artificial soil an LC_{50} of 32.9% was determined (Potter et al. 1999). This result seems to be comparable with the results of the ring test where the substrate SOI contained about 840 mg/kg PAH.

Results from earthworm tests with copper-contaminated wood substrate are not known. On the other hand, the heavy metal copper is highly toxic for earthworms. Mainly reproduction but less mortality is the most sensitive endpoint (Rundgren & Van Gestel 1998). For example, in the field significant effects on earthworm populations were found starting at copper concentrations somewhere between 30 and 100 mg/kg soil (Belotti 1998). Assuming a copper concentration of 2000 mg/kg in WOO it seems clear that the effects observed in the ring test occurred in an order of magnitude comparable to the one determined in soil studies. Further comparisons with literature data have to be performed for the other basic tests as well.

A.4 Discussion

A.4.1 Organisation of the ring test

The results of this validation study show that the Standard CEN 14735 is basically suitable for the evaluation of the ecotoxicity of wastes under practical conditions. This statement is based on three facts:

- The high number of participating laboratories (64) and, subsequently, the high number of valid data sets (at lest ten per test);

- The low number of non-acceptable test results combined with an even lower number of statistical outliers or data outside of the range of the mean plus/minus 2 sd, ending up in about 1000 data sets (including reference tests).

These statements are subject to the provisions set by CEN TC 292.

Detailed results of the tests with the basic test battery and the additional tests will be provided in the elaborated final report of the EU-wide ring test, to be published as a book by the Umweltbundesamt (Dessau) in 2008.

A.4.2 Methodological consequences

According to the results of the ring test, practicability and sensitivity of the aquatic tests with Algae, daphnids and Bacteria could be proven. Among the terrestrial tests, the plant test can be recommended while the acute earthworm test – despite its practicability – should be replaced because of its low sensitivity. Also the reference substance chloroacetamide should be replaced by a chemical like boric acid which is not toxic to humans (Römbke & Ahtiainen 2007). In addition, details of the performance (in particular the validity criteria and the range of reference results) of the tests with Algae and luminescent Bacteria have to be clarified. Some, mainly aquatic, tests can probably be automatised and/or miniaturised without a decline of practicability and sensitivity.

Open questions refer to details of the preparation of the eluates (e.g. concerning the influence of the first separation step (centrifugation) or the establishment of new methods like the short-term column percolation test (ISO/DIS 21268-3). Additional data analyses are needed in order to assess the conditions of the leaching test (volume collected after filtration, type of filtration membrane and so on). Another area of further evaluation is the comparison between the chemical analyses performed on the eluates and the biological test results. Further research is needed in the area of non-basic tests, since with exception of the *Lemna* test, the number of data sets for these tests was too small to prepare a detailed evaluation. Their sensitivity is partly very high, meaning that for example the Collembola or earthworm reproduction test might replace the earthworm acute test. Special attention should be given to the Earthworm Avoidance test and, in particular, the *Arthrobacter* test, which may be included in the basic test battery due to their combination of practicability and sensitivity.

However, more experience with different kinds of waste materials is necessary for all ecotoxicological test systems discussed so far.

A.5 Summary and Recommendations

The outcome of this ring test supports the recommendations made in the literature (e.g. Wundram & Bahadir 1999; Pandard et al. 2006; Wilke et al. 2007) that a combination of the results of a battery of biological tests and chemical residue analysis is needed for an ecotoxicological characterization of wastes. With small modifications (exchange of the acute earthworm test with a more sensitive soil invertebrate test) the basic test battery as used in the ring test is considered to be a good starting point for the hazard and risk assessment of wastes. However, further (probably multi-variate) evaluation of the ring test data will give deeper insight in identifying the most suitable and practical battery of ecotoxicological tests for the characterization of wastes. Finally, the experiences made in the ring test support also the proposals made in CEN guideline 14735 (2005) concerning the performance of such tests.

A.6 References

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Annex I: Summary of the test results: Explanations see below

Sample	Test	N	St-O	N-Ak	EC_{50}	LL	UL	U-O	M-M
INC	AL-ges.	48	0	13	4.15	0.47	36.64	78	59
INC	AL-Ds	21	0	7	8.79	1.17	65.77	56	15
INC	AL-Ps	27	0	6	2.61	0.46	14.79	32	20
INC	DA	54	0	7	2.79	1.06	7.35	7	10
INC	LB-ges	49	1	4	35.40	10.99	114.02	10	8
INC	LB-freeze	25	1	4	30.83	11.19	84.92	8	8
INC	EW	18	0	5	44.98	30.83	65.61	2	2
INC	PL-As	22	1	3	29.44	8.18	105.93	13	4
INC	PL-Bn	21	1	3	23.88	5.50	103.75	19	6
SOI	LB-ges	48	0	6	63.39	41.11	97.72	2	2
SOI	LB-freeze	25	0	4	65.77	47.21	91.62	2	2
SOI	PL-Bn	20	0	3	62.95	30.83	128.53	4	3
WOO	AL-ges	47	1	11	0.5	0.05	4.84	97	138
WOO	AL-Ds	21	0	6	1.34	0.75	2.38	3	2
WOO	AL-Ps	26	1	5	0.22	0.03	1.64	55	27
WOO	DA	53	0	2	0.34	0.06	1,84	31	33
WOO	LB-ges	57	1	4	2.69	0.26	27.93	107	114
WOO	LB-freeze	24	0	4	0.73	0.16	3.38	21	20
WOO	LB-liquid	32	1	0	5.508	2.000	15.171	8	3
WOO	EW	17	0	3	18.97	8.79	40.93	5	5
WOO	PL-As	21	0	3	10.96	2.79	43.05	15	9
WOO	PL-Bn	19	0	2	2.64	0.67	10.42	16	11

Legend: **INC:** Municipal waste incineration ash; **SOI**: PAH contaminated soil; **WOO:** Waste wood (Cu contamination). **AL**: Algae; **Ds:** *D. subspicatus*; **Ps:** *P.subcapitata*; **DA:** Daphnids; **LB:** Luminescent bacteria; **ges:** In total; **Freeze:** freeze-dried; **Liquid**: Liquid culture; **EW:** Earthworms; **PL**: Plants; **As**: *A. sativa*; **Bn:** *B. napus*. **p:** No. of participating laboratories; **N:** No. of reported data. **St-O:** Statistical outliers; **N-Ak:** Number of test data not accepted. **EC_{50}:** Effect value in % dilution (medium or OECD / LUFA soil). **UL/LL**: Upper / lower limit of the 95% confidence interval of the respective EC_{50}-value. **U-O:** Factor between UL and OL; **M-M:** Factor between minimum and maximum values

B. Contributions at the Final Project Workshop (Berlin; June 29, 2007)

B.1 *Welcome address* - M. Angrick

German Federal Environmental Agency

Dear Ladies and Gentlemen,

on behalf of the German Federal Environment Agency I warmly welcome you to the final meeting of the European Ringtest on the Ecotoxicity of waste. I also bring you the best regards from Ms. Penning, the head of Division III: Environmentally Compatible Engineering - Processes and Products, who is unfortunately not able to attend our meeting today.

First of all, I would like to give you a riddle:

You take 2.1 tons of 3 different waste materials, 67 committed lab teams from 16 countries, a very well organized project team in 4 different cities, an enormous number of daphnids, earthworms, algae, kilograms of standard soil and not naming an uncountable volume of data collection files, report sheets and statistical reports.

What will you get?

The result is a very successful ring test on the use of bioassays in waste and waste eluates.

We from the German Federal Environment Agency are very glad that we have been able to support and organize this ring test, bringing together so many experts and helping to meet the so called H14-challenge in European waste legislation.

Before opening the floor for the scientific details, I would like to thank the German Federal Ministry of the Environment for the encouragement and the financial support of the project.

I guess you all know that such a large project can not come to a success without the close and successful cooperation within the project team. Therefore I would like to thank ECT, in particular Mr. Römbke and Mr. Moser for being the central point in all questions regarding the test systems and the data collection. Secondly I want to thank Mr. Becker from the German Institute for Material Research and Testing for preparing and distributing the waste samples. A very important part in the ring test was the statistical assessment of the data sets. I want to thank Ms Donnevert from the University of Applied Sciences in Gießen for accepting the challenge. I would like to thank the German Institute for Standardisation, who financially supported the statistical assessment. I also want to thank you for hosting our final meeting today in this room with its wonderful view on downtown Berlin.

Last but not least, I want to thank you all for participating in the ring test, for doing all these tests at your own expense and as I was told by Ms Moser for being a dedicated and enjoyable H14 community. In order to honour your acknowledge your participation in the ring test, we will provide certificates for every lab, subsequent to the project completion in September.

Ladies and Gentlemen, today we will have the possibility to see and discuss the first results of the ring test, mainly the results of the basic test battery and some additional test systems. As always we will have limited time for discussion, nevertheless use the opportunity to ask your questions and to share your opinion with the other experts. Maybe this meeting is a starting point for further discussions and future co-operations.

The conclusions of the ring test can be started today and will continue within the next months. From our point of view, the experience and results gained in this project are very valuable. Therefore the German Federal Environment Agency is able to finance the set-up of a web-based data base, in which all results and documents are available for additional scientific assessments, open also for other experts.

Dear Colleagues, I am sure you all are looking forward to the results of the different test systems and maybe the results of your lab.

I now wish us all interesting presentations and fruitful discussions….

B.2 Welcome address - A. Kopp

Federal Ministry for the Environment, Nature Conservation and Nuclear Safety, Germany

Dear Ladies and Gentlemen,

first of all I would like to thank the organisation for giving me the opportunity to welcome you in the name of the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety to this final meeting of the European Ring test on the ecotoxicity of waste.

Let me extend a special thanks to all the lecturers for presenting the scientific results and experiences from the ring test, I'm sure you all are looking forward to the presentations and the experiences we will gain from it.

Before we start with the background and the details of this interesting project, I would like to open the meeting with some explanations about the idea of funding an European ring test for waste, from the point of view of an Environment Ministry. I also want to highlight some major points, which should be discussed on your way to an ecotoxicological characterization of waste.

The German Environment Ministry is involved in the discussions regarding the implementation of the European Waste List, since it has been enforced in the year 2000. We are familiar with the difficulties caused by the classification of hazardous waste according to the European Waste List in Germany, but also in other European member states. The characterisation of waste and the distinction between waste as “hazardous” or “non-hazardous” is currently one of the most discussed questions in the context of the European Waste List. In most of the European Member States the legal situation is similar: Waste is classified by depending on their chemically analysed composition and the concentrations of dangerous substances. The use of biological test systems for assessing the ecotoxicity of waste and for the identification of hazardous waste types are often limited to a few specific question, if used at all.

From our point of view, the ecotoxic potential of such heterogenous materials like waste, often accompanied by unknown or complex compounds, requires the use of evaluated and commonly accepted test methods, for solid waste testing as well as for waste eluates. The question of hazardousness is not only important for the classification, the labelling or the transport of waste. It is also very important in the current development of innovative resource protection strategies, in which the environmental risk of waste plays an important role in waste management and waste legislation and where biological test systems can be included in the risk assessments of waste and in the development of environmentally sound reuse scenarios.

Against the background of this upcoming question and due to the fact that waste specific test strategies and validated test methods are needed, the German Environment Ministry decided to fund an European interlaboratory project to validate biological test systems for waste and waste eluate tests.

Together with the German Environment Agency, our partner in the organisational and scientific support of the ring test, we are happy that we have been able to bring so many European experts together. Based on the efforts of every participant and the project team we have been able to gain a lot of experience in the biological testing of waste. We are now able to define suitable test batteries and to adapt existing test methods to the specific requirements of waste tests. We now have built the scientific platform, where we can identify significant biological effects and discuss the implementation of toxicity criteria or threshold values.

Our next steps will be to introduce our experiences and results to the standardisation committees, national and on the European level, and to develop helpful instruments for the waste authorities in Germany and perhaps in the other Member States.

The German Environment ministry is very glad, that our German environmental research project plan has built a sustainable platform for this important project, for the summarizing of expert knowledge and for the development of recommendations for the waste legislation. I hope that the European ring test will also be the starting point for new partnerships and for future projects.

I am sure, you are all eagerly awaiting for the final results of the ring test, therefore I wish us all interesting presentations and fruitful discussions.

B.3 Talk – H. Moser

Interlaboratory test for the ecotoxicological characterisation of waste and waste eluates

- Background and Structure -

Heidrun Moser

German Federal Environmental Agency
Section "Hazardous Waste Management"

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Background — European Waste List

The European Waste List 2000/532/EC

- is a harmonizes list of about 850 different waste types.
- replaces the 94/3/EC List of Waste and the 94/904/EC List of hazardous waste.
- forms a consistent waste classification system across the EU.
- is intended to be a catalogue of all wastes, grouped according to generic industry, process and waste type.
- gives the basic for all national and international waste reporting obligations, waste statistics and the transboundary transport of waste regulated by the Basel Convention.
- was amended three times so far and can be revised according to the Waste Framework Directive
- includes 850 waste six-digit-codes in 20 chapters, defining 405 waste types as hazardous waste material and 200 waste types in so called "mirror entries".
- defines 14 Hazard criteria for the characterization of hazardous waste types

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Background — Mirror Entries

Waste in mirror entries

- The majority of hazardous " mirror entries" refers to the content of "dangerous substances"
- Few hazardous "mirror entries" refer to specific hazardous properties or the presence of a specific hazardous component

For Example: MW Incineration ash:

Chapter	19:	Wastes from waste management facilities, off-site waste water treatment plants and the preparation of water intended for human consumption
Sub-Chapter	19 01:	wastes from incineration or pyrolysis of waste

19 01 11* bottom ash and slag containing dangerous substances

19 01 12 bottom ash and slag other than those mentioned in 19 01 11

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Background — Hazard Criteria

H 1	„Explosive" – may explode when under effect of flame or sensitive to shocks
H 2	„Oxidising" – exhibit highly exothermic reactions in contact with other substances
H 3 A	„Highly flammable" – Liquids with flash point <21°C, catch fire on contact with air, readily ignited, flammable gases, evolve highly flammable gas on contact with water
H 3 B	„Flammable" – Liquids having flashpoint between 21°C and 55°C
H 4	„Irritant" – Non corrosive substances which cause inflammation on contact with skin
H 5	„Harmful" – if inhaled, ingested or penetrate the skin may involve limited health risks
H 6	„Toxic" – may involve serious, acute or chronic health risks and even death
H 7	„Carcinogenic" – may induce cancer or increase its incidence
H 8	„Corrosive" – may destroy living tissue on contact
H 9	„Infectious" – substances containing viable micro-organisms or their toxins which known or believed to cause disease in man or other living organisms
H 10	„Toxic for reproduction" – affect the incidence of non-heritable adverse effects in the progeny and/or male or female reproductive functions or capacity
H 11	„Mutagenic" – may induce hereditary genetic defects or increase their incidence
H 12	Substances which release toxic gases in contact with water, air or an acid.
H 13	"Sensitizing"
H 14	„Ecotoxic" – may present risks for one or more sectors of the environment
H 15	Wastes capable by any means after disposal of yielding another substance which possess any of the characteristics listed by this annex.

How is ecotoxity of waste defined actually?

H14: Ecotoxic – may present risks for one or more sectors of the environment

67/548/EEC Directive on Dangerous Substances
1999/45/EC Directive on Dangerous Preparations

- **Compounds correlated to R-phrases:**

R- Phrases	Description
R50-53	Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
R50	Very toxic to aquatic organisms
R51-53	Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
R52-53	Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment
R52	Harmful to aquatic organisms
R53	May cause long-term adverse effects in the aquatic environment
R54	Toxic to flora
R55	Toxic to fauna
R56	Toxic to soil organisms
R57	Toxic to bees
R58	May cause long-term adverse effects in the environment
R59	Dangerous for the ozone layer

How is ecotoxity of waste defined actually?

H14: Ecotoxic – may present risks for one or more sectors of the environment

Concentration limits for H14

- Total concentration of = 0.25% of one or more substances classified as dangerous for the environment with R phrases R50-53
- Total concentration of = 2.5% of one or more substances classified as dangerous for the environment with R phrases R51-53
- Total concentration of = 25% of one or more substances classified as dangerous for the environment with R phrases R52-53
- Total concentration of = 0.1% of one or more substances classified as dangerous for the environment with R phrases R59

Background Testbattery for the Ring test

Workshop UBA and JRC, September 2005

EUROPEAN COMMISSION Joint Research Centre

Problems around Soil and Waste III
The H-14 Criterion and (Bio)analytical Approaches for Ecotoxicological Waste Characterization.

Download: http://ies.jrc.cec.eu.int/366.html

Some of the major conclusions:

- Clear agreement that **biological test systems** should be used for the ecotoxicological characterization of waste.
- A distinct need for a **harmonised test battery** was identified
- to be developed and validated in the framework of **CEN TC 292**
- A general agreement that this test battery shall address the property of ecotoxicity of waste by using at this stage **test organisms, as representatives for various ecosystems or compartments and various trophic levels.**

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Background Critical issues

Critical issues touched upon were:

- The determination of a **test strategy**, whether waste should be assessed in a basic characterization procedures or more often on a routine base.
- The **application** of the test strategy, for all potentially ecotoxic waste, for specific waste codes or only for waste in mirror entries?
- Can we develop **toxicity criteria** for each test system and **threshold values** based on bioassays to classify waste as hazardous?
- Needs the **fate of the waste** to be considered for classification?
- Apart from the technical and scientific questions some questions remain to be answered by the **National Legislation and Regulatory Authorities** in the Member States as they require **political consensus**

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Background Definition of test battery

EN 14735: Characterisation of waste – Preparation of waste samples for ecotoxicity tests

Terrestrial test methods	Aquatic test methods
Earthworm – Acute toxicity	*Daphnia magna* – Effects of mobility
Earthworm – Effects on reproduction	*Daphnia magna* – Effects on reproduction
Collembola – Effects on reproduction	*Ceriodaphnia dubia* – Reproduction test
Coleoptera – Reproduction test	*Brachionus calicyflorus* – Reproduction test
Enchytraeid – Reproduction test	*Vibrio fischeri* – Luminescent bacteria test
Soil Flora – Inhibition of root growth	*Pseudomonas putida* – Inhibition of growth
Soil Flora – Effects on emergence and growth	Freshwater algal growth inhibition test
Ammonium oxidation – Rapid test	*Lemna minor* – Growth inhibition test
Mineralisation and nitrification	Freshwater fish acute toxicity test
Juvenile land snails – Inhibition on growth	Marine copepads – Acute toxicity test
	Marine algal growth inhibition test
	Salmonella / Microsome test
	UMU test

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The Ring test project in numbers:

How many participants have provided data sets so far?

Registered participants in total:	67
Number of participants who provided data:	60
Participants who have announced data sets but did not deliver:	4
Number of participants who definitely cancelled participation:	3

The Ring test project in numbers:

Geographical background of the participants

Austria	2
Belgium	5
Czech Republic	4
France	4
Germany	23
Ireland	1
Italy	5
The Netherlands	1
Norway	1
Portugal	4
Slovak Republic	1
Spain	4
Sweden	3
United Kingdom	1
USA	1

In total 15 countries were represented.

The Ring test project in numbers:

Institutional background of the participants

Universities:	16
Public institutions	16
Contract laboratories:	28
Industry*:	0

* one chemical company agreed to participate but did not deliver

The Ring test project in numbers:

Standard test battery (5 tests): Number of delivered data sets

	Promised	Delivered	Percentage
Algae tests	129	141	109 %
Daphnia tests	150	161	107 %
Bacteria tests	138	151	109 %
Earthworm tests	69	52	75 %
Plant tests	150	124	83 %

The Ring test project in numbers:

Additional aquatic tests (5 tests): Number of delivered data sets

	Promised	Delivered	Percentage
Lemna tests	42	48	114 %
Brachionus tests	15	20	133 %
P. putida tests	15	12	80 %
Ceriodaphnia tests	15	10	67 %
umu tests	15	23	153 %

The Ring test project in numbers:

Additional terrestrial tests (5 tests): Number of delivered data sets

	Promised	Delivered	Percentage
Collembola tests	24	21	88 %
Earthworm Reproduction tests	27	14*	52 %
Earthworm Avoidance tests	24	6*	25 %
Enchytraeid tests	21	9*	43 %
Arthrobacter tests	21	22	105 %

* Several tests still ongoing

The Ring test project in numbers:

A great, big thanks to you all for…..

	Data sets
Basic aquatic test battery:	453
Basic terrestrial test battery:	176
Additional aquatic tests:	113
Additional terrestrial tests:	72
SUM	814

Not included in this sum:	Data sets
Reference testing:	116
Other additional tests:	≈50
Analytical work	
Characterization of test substrates	
Comparison of eluate qualities	
Monitoring of sample stability	
Investigation of artificial soils	
etc.	

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What's next under scientific aspects?

- Certificates for all participants in August 2007
- Final assessment of all data in September 2007 (additional terrestrial tests still ongoing, e.g.)
- Implementation of the data base and integration of the data until November 2007
- Publication of all raw data and statistical assessments in the ring test report (sent to all participants in December 2007)
- Different publications of the results
- Integration of the results and experiences in the evaluation study of EN 14735 (→ see presentation of Pascal Pandard)

Remark: The participants are allowed to publish their own data or comparisons of their ring test data with other results. Please remark the origin of the data or the ringtest background in your publications. Thanks

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What's next in waste legislation process?

- Development of a national recommendation of actions, together with colleagues from the Laender
- Discussion of the outcome of the ring test and the national recommenations with other European authorities
- Notification?

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B.4 Talk – Roland Becker

Sample preparation and analytical results

Workshop 29. June 2007, Berlin

Roland Becker
Ute Kalbe
Andreas Buchholz
Holger Scharf

Federal Institute for Materials Research and Testing (BAM)

BAM I Department of Analytical Chemistry; Reference Materials 1

Selection and preparation of the test materials

To produce 3 ring test materials covering the major waste types in Europe

-> Technical restrictions

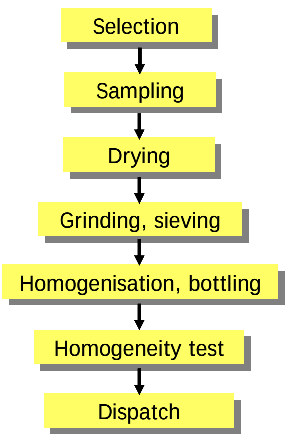
-> Increasing number of participants during the project

-> Problems with candidate materials

BAM I Department of Analytical Chemistry; Reference Materials 2

Preparation of Reference Materials

- Typical reference materials project:
  - Batch of identical units
- This project:
  - Incineration ash (INC)
    67 units between 100 g and 16.7 kg
  - Waste wood (WOO)
    67 units between 15 g and 31 kg
  - Gasworks soil (SOI)
    67 units between 0.5 kg and 31 kg



BAM I Department of Analytical Chemistry; Reference Materials 3

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INC

BAM

Incineration ash

- Requirement: Dry material, < 4 mm
- 719 kg “Bottom Ash“ received
- Wet, particles up to several cm
- Drying, sieving and bottling
- Final demand for 67 laboratories: 318 kg
- Main pollutant: Heavy metals and very high pH (~ 10.5)

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BAM

> 10 mm

> 8 mm

BAM I Department of Analytical Chemistry; Reference Materials 5

Umwelt Bundes Amt

Bottling (INC, SOI)

BAM

Sample divider

BAM I Department of Analytical Chemistry; Reference Materials 6

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Bottling of INC

BAM

Bottled unit (~ 13 kg, 10 L)	Tinplate container, PTFE foil	Spinning riffler (for smaller units)
		

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WOO

BAM

Waste wood

- Dry material, < 4 mm
- 900 kg starting material (mixture of treated and untreated wood, "As-free")
- Main toxic component: Cu-based wood preservative (concentration range as currently prescribed for various European countries)
- Homogenised and bottled in containers as for material 1 (INC)
- Different unit sizes according to the need of participants:

100 g – 30 kg (total amount bottled: 617 kg)

Cu concentration in the bottled material: (2.11 ± 0.06) g/kg

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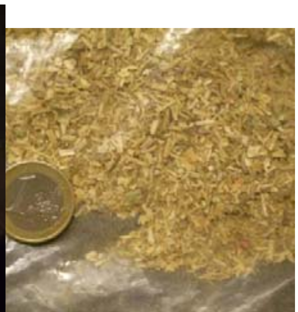
WOO

BAM

Waste wood, test sample



< 4 mm

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WOO, bottling

Umwelt Bundes Amt

BAM

SOI

Soil

- Origin: Former gasworks site (Berlin, Germany)
- Material was air-dried to constant weight
- Dry material was sieved, upper particle size limit 4 mm
- The homogenised material was bottled as INC
  (total bottled amount: ~ 680 kg)
- Predominant pollutant: Polycyclic aromatic hydrocarbons (PAH)

Total content of 16 EPA-PAH (HPLC-DAD):	(840 ± 125)	mg/kg
Total petrol hydrocarbons (GC):	(152 ± 30)	mg/kg
Water content:	(0.98 ± 0.014)	%

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Dispatch

- INC and WOO: End of October 2006
- SOI: End of November 2006
- Containers authorised for hazardous materials
- Materials packed in Teflon foil
- Seeds for the plant tests included

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Homogeneity study

BAM

To prove the similarity of the units of a batch

- Selection of a representative analyte

INC: Heavy metals, Cd, As, Co, Cr, Cu, Hg, Mn, Ni, Pb, V, Zn

WOO: Cu

SOI: Polycyclic aromatic hydrocarbons (16 EPA-PAH)

- Withdraw subsamples of selected units
- Specify the sample intake, conduct analyses
- 1-way ANOVA

→ Evaluation of "between-bottle" variability of the analyte content (ISO-Guide 35)

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INC, homogeneity

BAM

Metal content in the bottled material:

Metal	Method	Mean from homogeneity study (mg/kg)	Variability between bottled units (%)
Cd	ICP-OES	6.6	7.3
As	ICP-OES	7.4	6.9
Co	ICP-OES	19	7.2
Cr	ICP-OES	212	6.3
Cu	ICP-OES	6500	48
Hg	CV-AAS	37	17
Mn	ICP-OES	800	5.7
Ni	ICP-OES	211	11.2
Pb	ICP-OES	1623	18.7
V	ICP-OES	42	2.7
Zn	ICP-OES	2639	7.4

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WOO, Characterisation

BAM

pH (in water, 26.8 °C): (5.41 ± 0.04)
according to DIN ISO 10390

Elemental analysis: C: (46.44 ± 0.43) %
Sample size: 100 mg N: (0.277 ± 0.091) %
H: (6.535 ± 0.044) %

Total carbon: (37.4 ± 1.12) %
Total inorganic carbon: <0.2 %
Sample size: 80 mg

Water content (gravimetric, 105 °C): (8.77 ± 0.11) %
Sample size: 5 g

Water content (Karl-Fischer-Titration): (8.23 ± 0.10) %
Sample size: 50 mg

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WOO, homogeneity

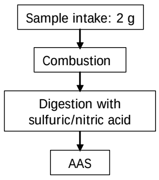
1 Sample from each of 9 bottled units was analysed for Cu in triplicate

=> „between bottle“ variability

4 Samples of 1 bottled unit was analysed for Cu in triplicate

=> „within bottle“ variability

Sample	Copper content (g/kg)			
No.	Subsample 1	Subsample 2	Subsample 3	Mean
1	2.11	2.24	2.24	2.20
2	2.10	2.15	2.17	2.14
3	2.09	2.17	2.30	2.19
4	2.16	2.00	2,23	2.13
5	1.91	2.07	2.11	2.03
6	2.08	2.01	2.04	2.05
7	2.13	2.11	2.00	2.08
8	2.01	2.13	2.11	2.08
9	2.05	2.10	2.09	2.08
Mean	-	-	-	**2.11**
SD	-	-	-	0.06 (2.6 %)
10a	2.01	1.99	2.09	2.03
10b	2.23	2.02	1.97	2.08
10c	2.13	2.21	2.26	2.20
10d	2.22	2.02	1.90	2.05
Mean	-	-	-	**2.09**
SD	-	-	-	0.07 (3.2 %)



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SOI, Characterisation

TPH: (152 ± 30) mg/kg
This content is close to the background value for TPH observed in many environmental samples including those regarded as not contaminated.

pH (in water, 26.2 °C): (8.36 ± 0.02)
according to DIN ISO 10390

Elemental analysis: C: (4.98 ± 2.47) %
Sample size: 100 mg N: (0.085 ± 0.028) %
H: (0.304 ± 0.028) %

Total carbon: (2.73 ± 0.99) %
Total inorganic carbon: (0.099 ± 0.004) %
Sample size: 80 mg

Water content (gravimetric, 105 °C): (0.980 ± 0.014) %
Sample size: 4.5 g

Water content (Karl-Fischer-Titration): (1.29 ± 0.68) %
Sample size: 50 mg

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SOI, homogeneity

- **100 g were withdrawn from 10 bottled units and analysed in quadruple for the 16 EPA-PAH (ASE/Methanol; HPLC/DAD)**
- **“Within-bottle“ and “between bottle“ variabilities were determined (ANOVA)**

	Mean (mg/kg)	Within bottle variability (%)	Between bottle variability (%)
Naphthalene	n.d.	-	-
Acenaphthylene	n.d.	-	-
Acenaphthene	7.2	30.6	26.6
Fluorene	4.2	23.6	21.0
Phenanthrene	69.1	19.0	19.5
Anthracene	23.4	18.5	19.4
Fluoranthene	182	14.7	14.6
Pyrene	146	14.1	14.3
Benz[*a*]anthracene	87.2	13.4	12.9
Chrysene	69.4	13.2	12.6
Benzo[*b*]fluoranthene	78.6	12.6	10.2
Benzo[*k*]fluoranthene	31.0	11.9	11.5
Benzo[*a*]pyrene	59.0	12.5	10.4
Dibenz[*a,h*]anthracene	9.4	12.3	8.92
Benzo[*ghi*]perylene	34.7	11.2	8.41
Indeno[1,2,3-*cd*]pyrene	35.2	10.4	8.83
Sum	836		

BAM I Department of Analytical Chemistry; Reference Materials 18

Eluates

Comparability of eluate preparation

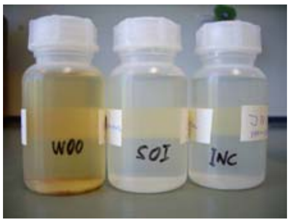
- Eluates to be prepared as prescribed in the following presentation
- To be analysed by the participants
- A selected number laboratories may sent eluates samples to BAM
- Quantification of selected metals in the eluates

  INC: Cu, Ni, Pb

  WOO, SOI: Cu

- ~ 100 mL in PE bottles
- pH adjusted to 1-2 with HNO_3

Eluates



Selected participants sent eluates to BAM

- ~ 100 mL in PE bottles
- pH adjusted to 1-2 with HNO_3

Eluates were received at BAM from:

Institute	City	Country
University Bremen	Bremen	Germany
Mälardalen University	Västerås	Sweden
ECT Oekotoxikologie GmbH	Flörsheim	Germany
Hydrotox GmbH	Freiburg	Germany
Ecotoxicological center Bratislava s.r.o. B	Ivanka pri Dunaji	Slovak Republic
RWTH Aachen	Aachen	Germany
Universität für Bodenkultur Wien, IFA-Tulln	Tulln	Austria

Eluates, measurements at BAM

No.	As (µg/L)	Cd (µg/L)	Cr (µg/L)	Cu (µg/L)	Hg (µg/L)	Mn (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
INC-02	< 1	0.4	24.0	271	< 0.1	26.4	4.2	76.2	169
INC-15	< 1	0.2	10.9	89.8	< 0.1	< 2	1.9	12.3	3.7
INC-22	< 1	< 0.1	26.4	75.4	< 0.1	< 2	< 1	11.8	< 2
INC-23	< 1	< 0.1	33.5	157	< 0.1	< 2	< 1	13.4	< 2
INC-32	< 1	0.2	17.0	136	< 0.1	40.8	5.5	1960	232
INC-59	< 1	< 0.1	19.6	169	< 0.1	1.4	< 1	19.2	7.3
INC-62	< 1	< 0.1	21.3	172	< 0.1	< 2	< 1	18.6	4.8

No.	As (µg/L)	Cd (µg/L)	Cr (µg/L)	Cu (µg/L)	Hg (µg/L)	Mn (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
WOO-02	74.3	1.2	246	30100	< 0.1	1840	10.6	13.8	453
WOO-15	81.4	1.6	282	32600	< 0.1	1990	10.7	9.5	418
WOO-22	74.7	0.9	262	26200	< 0.1	1820	14.9	7.7	431
WOO-23	82.2	1.0	269	30400	< 0.1	1930	13.4	18.7	426
WOO-32	61.7	0.9	222	20400	< 0.1	1350	8.7	27.4	504
WOO-59	82.6	1.0	294	30700	< 0.1	1810	9.5	8.3	407
WOO-62	79.1	1.1	272	30800	< 0.1	1910	9.9	6.4	422

No.	As (µg/L)	Cd (µg/L)	Cr (µg/L)	Cu (µg/L)	Hg (µg/L)	Mn (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
SOI-02	< 1	0.3	4.4	76.4	< 0.1	39.8	2.3	29.9	79.4
SOI-15	< 1	0.7	3.1	12.9	< 0.1	3.4	3.6	13.2	5.2
SOI-23	3.0	0.3	7.4	37.9	< 0.1	16.2	< 1	14.4	25.7
SOI-32	< 1	0.1	4.4	38.3	< 0.1	29.6	2.3	149	146
SOI-59	4.3	0.2	10.7	52.4	< 0.1	31.5	1.9	24.5	35.0
SOI-62	3.4	0.1	7.3	42.1	< 0.1	20.5	2.3	16.6	17.7

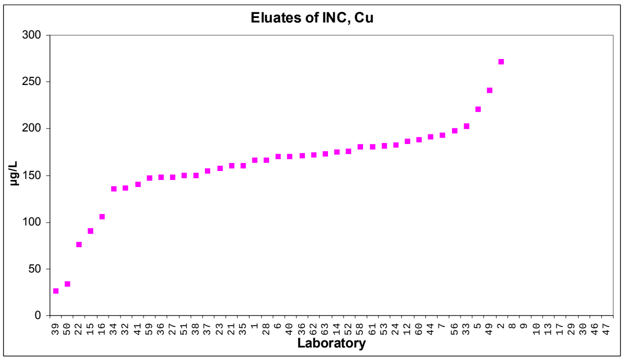
All eluates filtrated (Cellulose acetate, 0.2 µm)

Eluates, results

	INC				WOO				SOI			
Laboratory	pH	Dry matter (%)	Moisture (%)	Copper (µg/L)	pH	Dry matter (%)	Moisture (%)	Copper (mg/L)	pH	Dry matter (%)	Moisture (%)	Copper (µg/L)
1	n.d.	97,8	2,2	166	n.d.	90,9	9,2	29,4	n.d.	99,2	0,8	12,4
2	10,7	97,8	2,2	271	5,3	91,4	8,6	30,1	8,4	99,4	0,6	76,4
5	10,8	97,8	2,2	220	5,0	91,1	n.d.	28,5	8,1	99,0	n.d.	66,0
6	10,6	97,7	2,3	170	4,8	90,6	9,4	19,2	8,0	99,2	0,9	8,3
7	11,8	97,5	2,5	193	4,4	90,7	9,3	31,3	7,6	99,2	0,8	17,4
8	10,8	97,3	2,7	<50	5,4	90,9	9,1	32,2	8,4	99,2	0,8	<50
9	9,3	97,7	2,3	n.d.	4,9	92,6	7,4	n.d.	8,0	99,2	0,8	n.d.
10	9,7	97,9	2,1	n.d.	4,9	93,1	6,9	n.d.	7,2	99,2	0,8	n.d.
12	n.d.	98,2	1,8	186	n.d.	91,5	9,3	32,3	n.d.	99,5	0,5	32,3
13	10,6	97,7	2,4	n.d.	5,0	90,8	10,1	n.d.	7,8	99,2	0,8	n.d.
..	..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..	..
37	n.d.	97,2	2,8	154	n.d.	91,4	8,6	31,5	n.d.	99,3	0,7	<100
38	5,5	97,7	2,3	150								
39	n.d.	98,0	2,0	26	n.d.	91,0	9,0	23,2	n.d.	99,0	1,0	9,6
40	10,7	98,0	2,0	170	5,3	49,3	4,3	30,3	8,1	99,2	0,8	<70
41	10,5	97,5	2,5	140	5,3	89,9	9,1	30,0	7,3	99,0	0,7	12,6
44	10,9	97,7	2,3	191	5,5	99,2	0,8	31,6	7,7	91,0	9,0	47,0
46	9,7	97,7	2,3	n.d.	5,5	91,5	8,5	n.d.	7,9	99,1	0,9	n.d.
47	9,9	97,5	2,5	n.d.	5,3	90,0	10,1	n.d.	8,1	99,2	0,8	n.d.
49	10,6	98,1	2,9	240	5,4	91,6	8,4	81,6	8,7	99,3	0,7	32,2
50	n.d.	n.d.	n.d.	34	n.d.	n.d.	n.d.	27,6	n.d.	n.d.	n.d.	6,8
51	10,4	97,6	2,4	150	5,4	91,4	8,6	31,5	8,5	99,3	0,7	31,0
52	n.d.	n.d.	n.d.	175	n.d.	n.d.	n.d.	29,4	n.d.	n.d.	n.d.	27,0
53	10,7	98,9	1,2	181	5,4	92,8	7,8	31,2	7,9	99,8	0,2	40,7
56	n.d.	97,3	n.d.	197	n.d.	90,7	9,3	32,9	n.d.	98,9	1,1	20,6
58	10,7	97,8	2,2	180	5,2	90,5	9,5	30,0	8,4	99,0	1,0	<20
59	10,7	97,8	2,2	126 / 169	5,3	91,0	9,0	n.d. / 30,7	8,5	99,2	0,8	<10 / 52,4
60	n.d.	97,7	2,3	188	n.d.	99,2	0,8	35,1	n.d.	90,5	9,5	16,6
61	10,4	97,6	2,4	180	4,6	90,8	9,2	32,9	8,2	99,2	0,8	<50
62	n.d.	97,9	2,1	172	n.d.	91,1	8,9	30,8	n.d.	99,3	0,7	42,1
63	9,9	97,6	2,4	173	4,9	89,6	10,4	36,9	7,9	99,0	1,0	14,0
64	10,3	98,1	1,9	n.d.	5,2	94,9	5,1	n.d.	8,9	97,7	2,3	n.d.

BAM I Department of Analytical Chemistry; Reference Materials 22

Eluates, INC

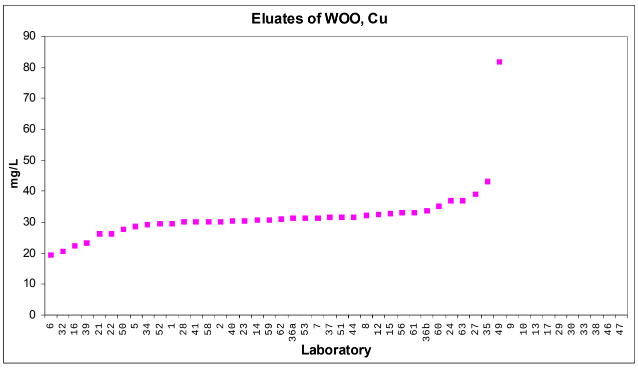


Mean: 161 µg/L

SD: 47 µg/L

BAM I Department of Analytical Chemistry; Reference Materials 23

Eluates, WOO



Mean: 32 mg/L

SD: 9.5 mg/L

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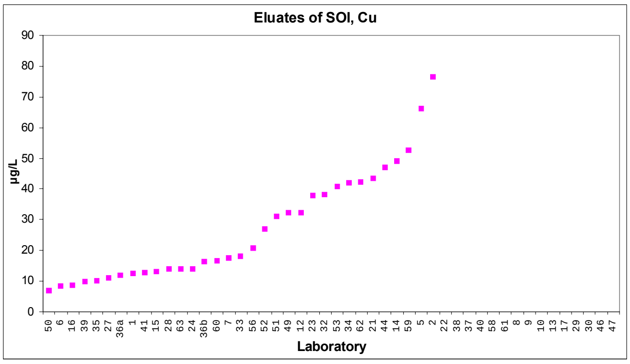
Eluates, SOI

BAM



Mean: 27 µg/L

SD: 18 µg/L

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Conclusions

BAM

- **The ring test materials display sufficient homogeneity**

- **Future interlaboratory comparisons:**

  - Consider technical restrictions for preparation and dispatch of the materials

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B.5 Talk – G. Donnevert

Berlin
June 29, 2007

European Ring Test –
Ecotoxicological Characterisation of Waste

Statistical assessment

Gerhild Donnevert

University of applied sciences (FH) Gießen-Friedberg, Germany

Content

Statistical evaluation following ISO 5725-2

ISO 5725-2 Accuracy (trueness and precision) of measurement methods and results

Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method

- ➢ Internationally accepted standard for the evaluation of interlaboratory tests for method validation in chemical analytics
- ➢ Performance data according to ISO 5725-2 are published in national and international standards for water analysis
- ➢ Suitable for the preliminary estimation of measurement uncertainty of an analytical method

Repeatability and reproducibility

Definitions according to ISO 5725-1

- Repeatability standard deviation s_r
  a measure of dispersion of the distribution under repeatability conditions, i.e. conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.
- Reproducibility standard deviation s_R
  a measure of dispersion of the distribution under reproducibility conditions, i.e. conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.

ISO 5725-2 - Requirements

- ✓ Every laboratory shall strictly follow the SOP!
- ✓ Homogenous test materials
- ✓ Quantifiable data only, test results < *x* or > *y* cannot be evaluated
- ✓ The analytical method yields results on a continuous scale (EC50)
- ✓ Approximately normally distributed data (log EC 50).
- ✓ For statistical evaluation a minimum number of 8 valid data sets is required.
- ? Uniform test results
  - ⇒ acceptance criteria
  - ⇒ recalculation

Acceptance of test results

All test results were evaluated in a tiered process:

1. Compliance with validity criteria given in the guideline?

Example: Mortality of earthworms < 10% in the control

Note: This check was always performed.

2. Test performance according to the SOP?

Example: Number of replicates correct? Test conditions measured?

Note: Clear non-compliance of the SOPs ➔ rejection of the test

3. Result of reference tests within the required range?

Example: *Daphnia* EC50 Potassium dichromate: 0.6 – 2.1 mg/L

Note: Strictly used for algae and Daphnia tests, partly used for bacteria and earthworm tests and not used for plants

Acceptance of test results

All three criteria were combined to determine whether a specific test was acceptable (i.e. useful for statistical and graphical assessment) or not.

Please note:

- This evaluation is still preliminary, since still data from reference tests are announced to be delivered.
- In "complex" situations (e.g. slightly missed ranges), a case-by-case decision was made
- For the Algae test, the required ranges (validity, reference testing) had to be extended.
- Finally, the number of data sets which were NOT ACCEPTED in this first run but gave reliable results are given separately.

Recalculation of test results

Goal: "uniform test results":

- Raw data from all participants

  Not always available

- Calculation of test results with the same algorithm

  Probit analysis using linear max. likelihood regression

- Calculation of the 95 % confidence limits

  Not always possible

In some cases big difference between reported and recalculated test result - for different reasons.

Adaption of the statistical evaluation

Problem: Mostly single test results

⇒ Repeatability could not be calculated according to ISO 5725-2 (no within-laboratory standard deviation)

Solution:

The confidence interval of a test result contains precision information from various treatments, i.e. some kind of repeatability information.

⇒ Standard deviation of laboratory test result is derived from upper and lower limit of the confidence interval

STD = (log UL - log LL)/4

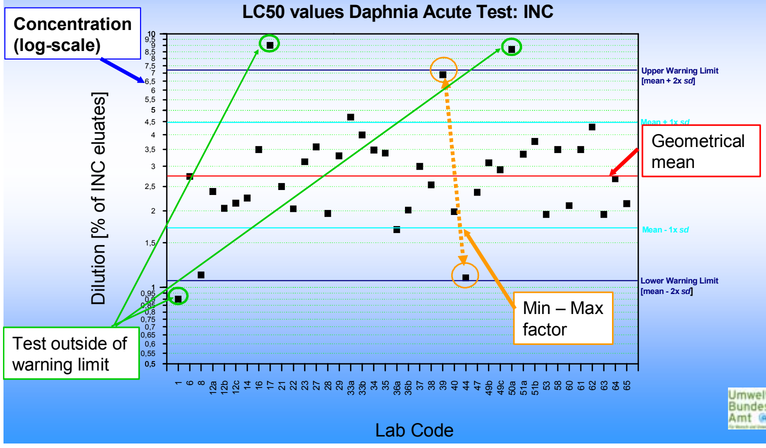
Presentation of test results

The precision of ring tests can be presented in several, very different ways. Here, two approaches were used:

- **Comparison of the factor between the lowest and the highest LC/EC50 values (e.g. Chapman 1995)**
  Basis: tests with chemicals spiked into standard media
  Outcome: a **factor of 4** is considered to be okay
  BUT: Complex substrate may lead to higher factors
- **Warning-Charts (Environment Canada 1999)**
  Originally developed for the interpretation of the results of reference tests in one laboratory

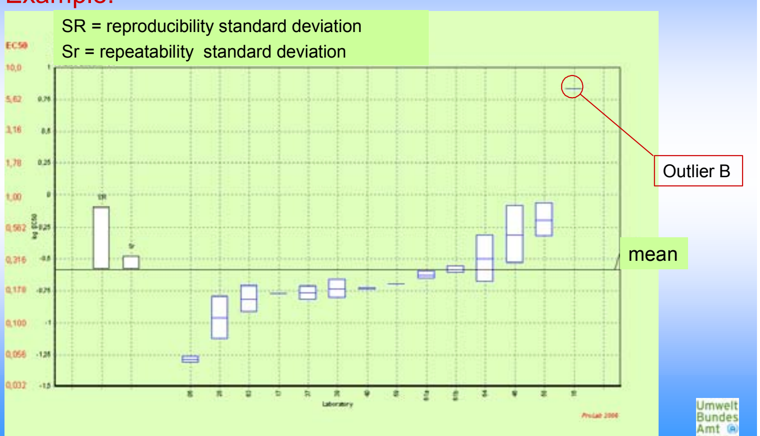
Presentation of test results

Example of Warning-Charts (Environment Canada 1999)



Presentation of results of statistical evaluation

Example:



Statistical outliers

- Assumption of normally distributed data implies the rejection of outliers. Statistical tests were performed to indicate two types of outliers:

  Type B: large systematic error

  Type C: high within-laboratory variance
- Laboratory test results may be considered to be an outlier. Decision is in the responsibility of the panel.

For the repeatability and reproducibility data presented in this meeting no outliers have been eliminated so far!

Open questions

- Was the acceptance procedure strict enough?
- Did we choose the right algorithm for recalculation?
- Is the confidence interval representative for repeatability standard deviation?
- Shall outliers be eliminated?

Acknowledgements

Many thanks to:

German Institute for Standardisation DIN
for funding the statistical assessment

Dr. Steffen Uhlig
for the intensive discussion of the statistical evaluation and the adaption of the software

B.6 Talk – J. Römbke

Berlin
June 29, 2007

European Ring Test –
Ecotoxicological Characterisation of Waste

Results of the basic test battery

Jörg Römbke & Gerhild Donnevert

ECT Oekotoxikologie GmbH, Flörsheim/Main, Germany
FH Gießen-Friedberg, Germany

Umwelt Bundes Amt

Content

1. Aim of this contribution
2. Short description of the basic test battery
3. Results (separately for each test):
   - Validation criteria and reference testing
   - Effects of the three waste samples
4. Discussion
   - Methodological consequences
   - Suitability of the five tests
5. Recommendations

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Aim of this contribution

The following questions will be answered:

- ▶ Which results were gained in the five tests?
- ▶ Are the five tests useful (i.e. practical, sensitive etc.) for the evaluation of waste samples?
- ▶ Are modifications of the existing tests for waste testing necessary? If yes, are comments in CEN 14735 sufficient?
- ▶ Which tests can be recommended for routine use?
- ▶ Are there any open questions?

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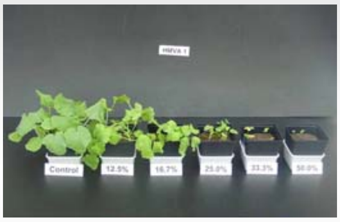
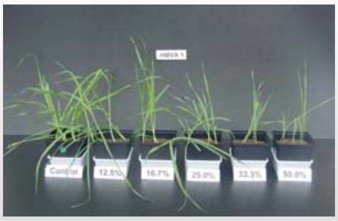
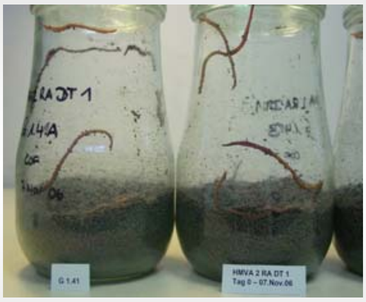
Overview: Basic test battery

Name	Guideline	Species
Algae	ISO 8692 (2004)	*D. subspicatus, P. subcapitata*
***Daphnia*:**	ISO 6341 (1996)	*D. magna*
Bacteria:	ISO 11348-1/2 (2005)	*V. fischeri* (3 sources)
Earthworms:	ISO 11268-1 (1993)	*E. fetida, E. andrei*
Plants:	ISO 11268-2 (1995)	*A. sativa, B. napus*

Results: Two examples of effects







Above: flight reaction of earthworms

Left: growth effects of two plant species

Tests with reference substances

Test system	Data set	No. Ref. tests	Not in range
Algae test	**100%** (141)	**69%** (97)	**18%** (26)
Daphnia tests	**100%** (161)	**96%** (155)	**2%** (4)
Bacteria test	**100%** (151)	**47%** (69)	- -
Plant tests	**100%** (124)	**15%** (18)	- -
Earthworm tests	**100%** (52)	**71%** (37)	**2%** (1)

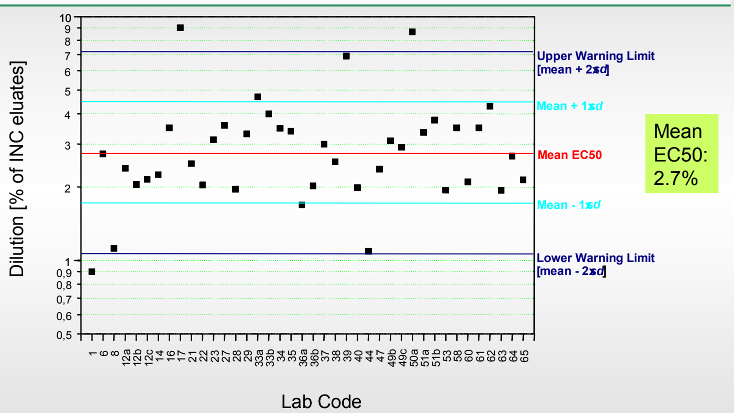
Validity of the tests

Test system	Data set	No. invalid	No. not SOP
Algae test	**100%** (141)	**27%** (20)	**0%** (0)
Daphnia tests	**100%** (161)	**2%** (1)	**5%** (3)
Bacteria test	**100%** (151)	**16%** (24)	**3%** (5)
Plant tests	**100%** (124)	**11%** (14)	**6%** (7)
Earthworm tests	**100%** (52)	**0%** (0)	8% (4)

Acceptance of the tests

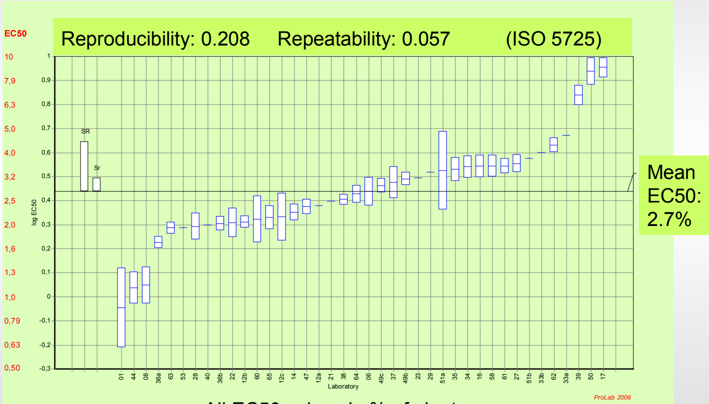
Test system	Data set	Accepted	"Acceptable"
Algae test	**100%** (141)	**62%** (88)	**91%** (129)
Daphnia tests	**100%** (161)	**93%** (150)	**96%** (154)
Bacteria test	**100%** (151)	**81%** (122)	**81%** (122)
Plant tests	**100%** (124)	**83%** (103)	**83%** (103)
Earthworm tests	**100%** (52)	**90%** (47)	**94%** (49)

Daphnia test: EC50 INC



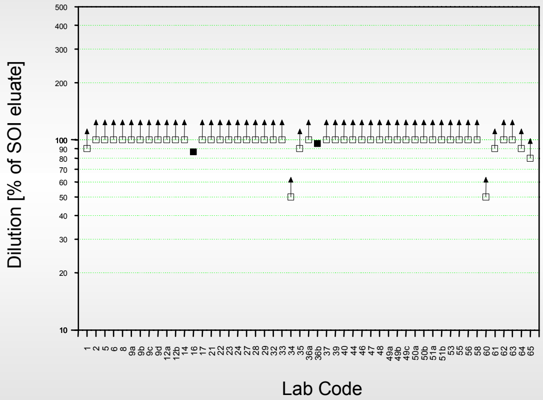
Min-max factor of all data about 10

Daphnia test: EC50 INC



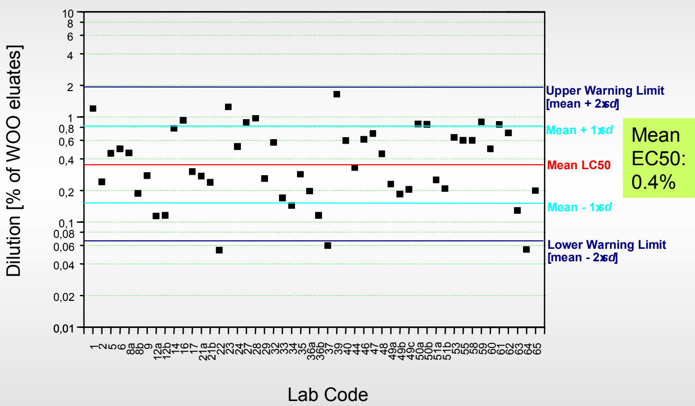
All EC50 values in % of eluate

Daphnia tests: EC50 SOI



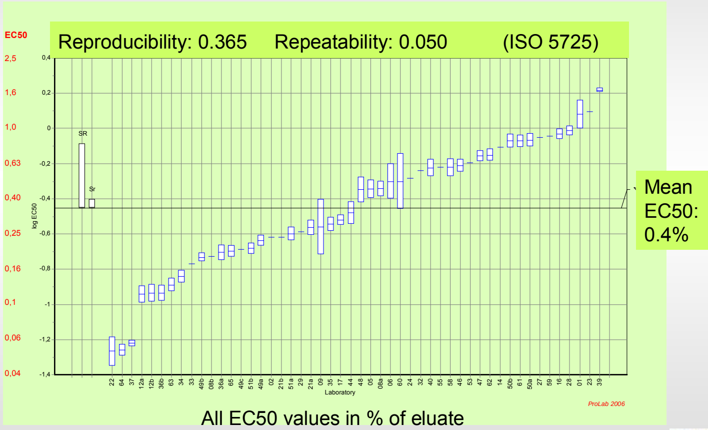
Out of 52 tests, only in two cases an EC50 could be determined.

Daphnia tests: EC50 WOO



High toxicity; min-max factor about 30

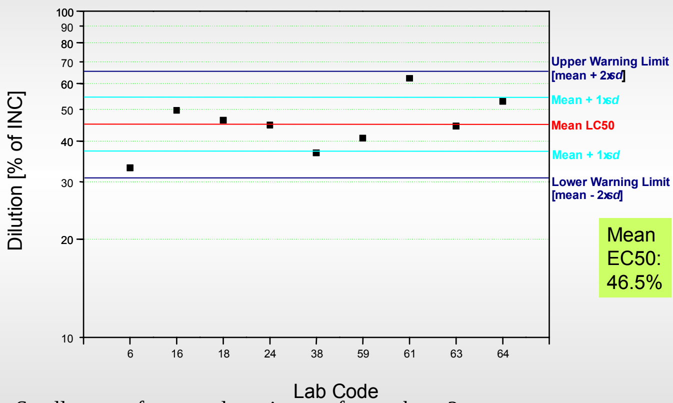
Daphnia tests: EC50 WOO



Daphnia: Summary of results

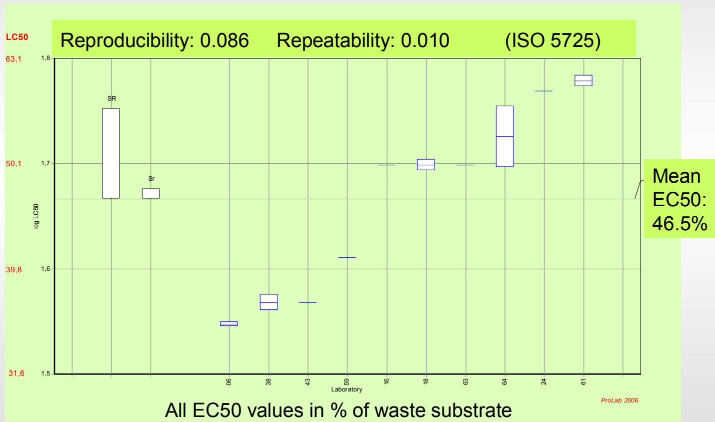
Participation:	45 labs 151 tests
Methodology:	Performance without problems
Acceptance status:	11 tests not accepted (7%)
Reference testing:	Required; 44 labs tested potassium dichromate
Sensitivity:	High; often the most sensitive test
	Almost no reaction in SOI tests
Reliability:	Only six tests outside the warning limits;
	min-max factor acceptable (10 – 30)
Recommendation:	Should be part of the final test battery

Earthworm tests: EC50 INC

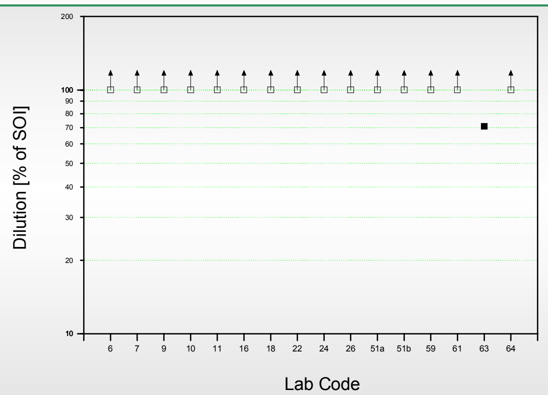


Small range of test results: min-max factor about 2.

Earthworm tests: EC50 INC

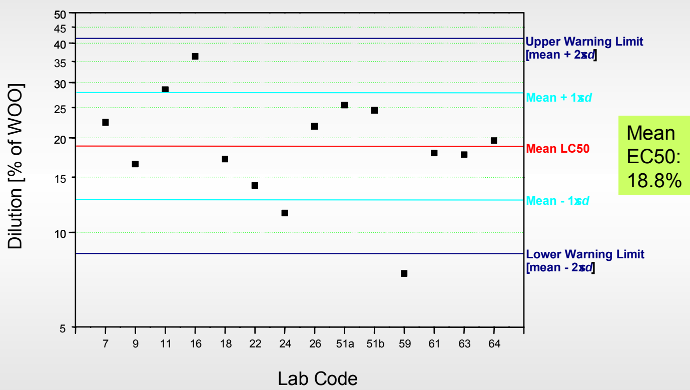


Earthworm tests: EC50 SOI



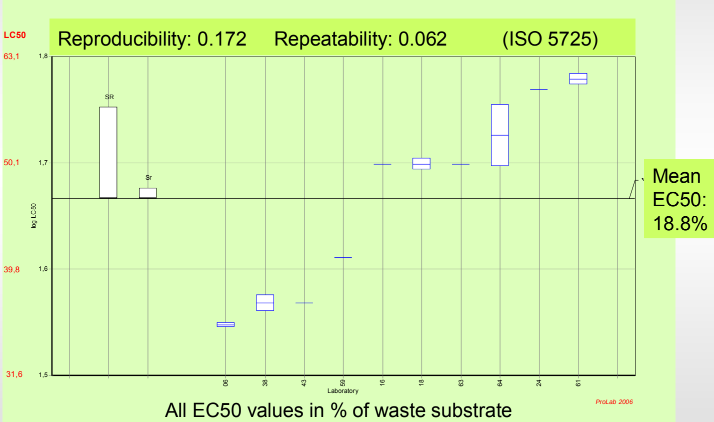
With one exception out of 16 tests: in 15 tests no effect of SOI at all.

Earthworm tests: EC50 WOO



Slightly wider range of toxicity: min-max factor of about 5.

Earthworm tests: EC50 WOO



Earthworm tests: Summary of results

Participation:	18 labs 52 tests
Methodology:	Performance without problems; guidance on moisture regulation necessary
Acceptance status:	5 tests not accepted (10%)
Reference testing:	Required; 11 labs with chloroacetamide data
Sensitivity:	Low; never the most sensitive test Almost no reaction in SOI tests
Reliability:	Only one test outside the warning limits; min-max factor very small (2 – 5)
Recommendation:	Worm test should be part of the final test battery – alternatives have to be checked

Reproducibility of aquatic tests

Minimum-maximum factor of EC values (all data)

Test system	INC	SOI	WOO
Algae test	55	?	135
D. subspicatus	15	?	4
P. subcapitata	55	?	135
Daphnia test	10	?	31
Bacteria test	8	2	114
(Freeze)	8	2	20
(Liquid/Fresh)	?	?	11

Independently from tests or substrates <2 data sets were outside the warning limits.

Reproducibility of soil tests

Test system	INC	SOI	WOO
	Min.-max. factor of LC/EC values (all data)		
Plant tests			
A. sativa	24	?	9
B. napus	6	3	11
Earthworm test	2	?	5

Independently from the tests or substrates <2 data sets were outside the warning limits

Sensitivity of aquatic tests

Test system	INC EC50	SOI EC50	WOO EC50
Algae test	5.4	> 50	0.6
D. subspicatus	8.3	> 50	1.2
P. subcapitata	3.8	> 50	**0.3**
Daphnia test	**2.7**	**> 100**	0.4
Bacteria test	**35.9**	**64.0**	2.5
(Freeze)	31.1	67.3	0.7
(Liquid/Fresh)	n.d.	n.d.	**6.1**

Sensitivity of soil tests

Test system	INC LC/EC50	SOI LC/EC50	WOO LC/EC50
Plant tests			
A. sativa	29.2	**> 100**	11.6
B. napus	**25.5**	**62.2**.	**2.8**
Earthworm test	**46.5**	**> 100**	**18.8**

Methodological test comparison A

Test system	Practicability (Costs, duration etc.)	Standardization (Intl. guideline?)
Algae test	High	Yes
Daphnia tests	High	Yes
Bacteria test	High	Yes
Plant tests	Low (2 species, 3 weeks)	Yes
Earthworm tests	Medium (2 weeks)	Yes

Methodological test comparison B

Test system	Validation criteria	Reference subst.
Algae test	Yes, but range(s) too narrow	
Daphnia tests	Okay	Okay: PDC
Bacteria test	Too many? Relevance?	Too many?
Plant tests	Okay	None so far. Boric acid?
Earthworm tests	Okay	Okay: not CA but boric acid?

Methodological test comparison C

Test system	Reproducibility (warning-limits / min-max factor)	Sensitivity
Algae test (two species)	Good	Medium
Daphnia tests	Good	High
Bacteria test (diff. strains)	Good	Low – high
Plant tests	Good	High
Earthworm tests	Good	Low

Open questions?

Test system	Work to be done
Algae test	Species sensitivity? Media?
Daphnia tests	None
Bacteria test	EC50 range of reference substances?
Plant tests	Species sensitivity?
Earthworm tests	Moisture regime?
ALL TESTS:	**Assessment of test battery**

Recommendations

Selection of test systems:

Aquatic tests: Algae, Daphnia, and bacteria tests

Terrestrial tests: Earthworm, and plant tests

However:

- ▶ Open issues have to be clarified
- ▶ Test modifications have to be included in standards
- ▶ More experience with different waste types is necessary
- ▶ Further research has to be performed, e.g.
  - search for an alternative to the earthworm acute test
  - simplification of existing methods (microplate?)
  - inclusion of genotox tests in the standard battery

Acknowledgements

We thank all participants of the ring test:

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And in particular those who did all the work!

B.7 Talk – A. Eisenträger

Berlin
June 29, 2007

European Ring Test –
Ecotoxicological Characterisation of Waste

Results of the additional aquatic and genotoxic test systems

Adolf Eisenträger

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Umweltbundesamt, Dessau, Germany
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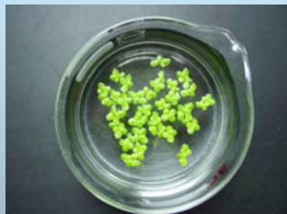
Aims of this contribution

- Short description of five additional aquatic tests
- Overview on the main results of these tests
- Comparison of the results with those gained in the basic test battery
- Discussion of the experiences
- Recommendations

Note that the performance of these tests was voluntary work.
Thus, the results were not completely evaluated statistically.

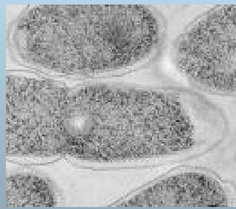
Characterization: *Lemna* test

Name: Duckweed growth inhibition
Guideline: ISO 20079 (2005)
Species: *Lemna minor*
Medium: Nutritive mineral medium (Steinberg)
Duration: 7 days
Parameter: Growth: frond number, frond area or dry weight or chlorophyll
Validity: Growth rate: 0.25 – 0.35/d; frond number growth rate ≥ 0.275/d
Reference: 3,5-Dichlorophenol (EC50: 1.8 – 3.6 mg/L)



Characterization: *P. putida* test

Name: *P. putida* growth inhibition test
Guideline: ISO 10712 (1995)
Species: *Pseudomonas putida*
Medium: Nutrient solution plus deionised water
Duration: 16 ± 1 hours
Parameter: Multiplication of cells
Validity: Multiplication factor ≥ 60 in the control
Reference: 3,5-Dichlorophenol; EC50: 10 – 30 mg/L

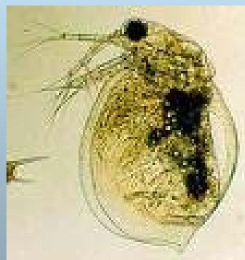


Characterization: *B. calicyflorus* test

Name: Chronic toxicity to *B. calicyflorus*
Guideline: ISO 20666 (2007)
Species: *Brachionus calicyflorus*
Medium: Synthetic recon-stituted water
Duration: 48 hours
Parameter: Population growth
Validity: Control reproduction
Reference: Sodium pentachloro-phenate: EC50 548 ± 232 µg/L

Characterization: *Ceriodaphnia* test

Name: Chronic toxicity test with *C. dubia*
Guideline: ISO 20665 (2007)
Species: *Ceriodaphnia dubia*
Medium: ELENDT M4
Duration: 7 days
Parameter: Mortality of adults, population growth
Validity: Control performance
Reference: Sodium pentachloro-phenate: EC50 170 - 330 µg/L



Characterization: umu test

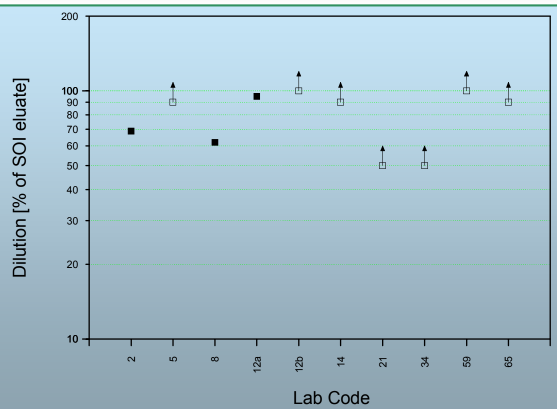
Name: Genotoxicity of water and wastewater
Guideline: ISO 13829 (2000)
Species: *Salmonella typhimurium*
Medium: TGA synthetic medium
Duration: 4 hours
Parameter: Induction of umuC gene
Validity: Induction rate > 2 in the control
Reference: 2-aminoanthracene
4-Nitro-quinoline-N-oxide

Results: Participation

Test	No. of labs	No. of tests	Unacceptable*
***Lemna minor*:**	14	48	10
***P. putida*:**	4	12	0
***B. calicyflorus*:**	7	21	0
***C. dubia*:**	4	10	0
Umu genotox:	6	21	3

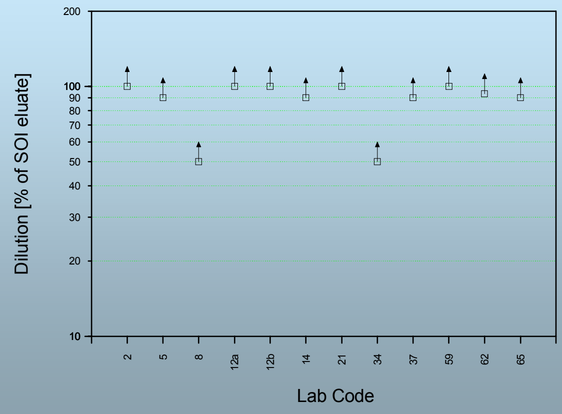
* only tests without raw data or being non-valid

Lemna: EC50 INC



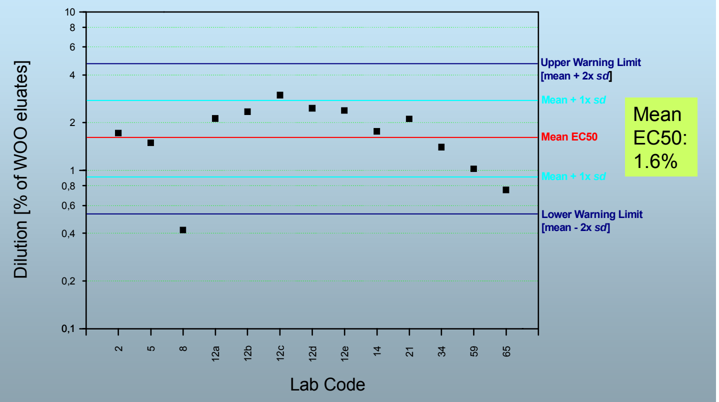
Indecisive results, but in any case only small effects of INC on *Lemna*

Lemna: EC50 SOI



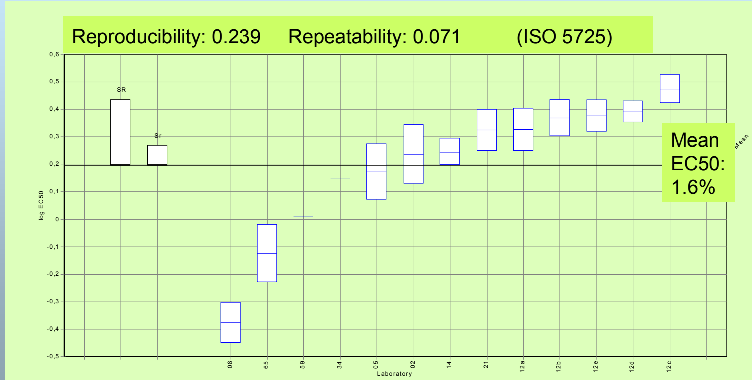
No effects at all of SOI on *Lemna*

Lemna: EC50 WOO



Only one test outside the warning limits; min-max factor of about 10

Lemna: EC50 WOO



All EC50 values in % of eluate

P. putida: EC50 INC + SOI + WOO

Lab Code	INC	SOI	WOO
1	6.8	>50.0	0.17
16	46.1	>80.0	0.19
23	(>80.0)	>80.0	0.12
37	16.4	>80.0	0.14
Mean	23.1	>80.0	0.15
Factor min - max	6.8	?	1.6

All EC50 values in % of eluate

B. calyciflorus: EC50 INC + SOI + WOO

Lab Code	INC	SOI	WOO
36	4.6	>90.0	0.06
47	5.1	>100	0.14
61	5.8	>90.0	0.11
62	6.0	>100	0.07
63	4.2	>90	0.13
64	4.4	>100	0.17
Mean	5.0	>100	0.11
Factor min - max	1.4	?	2.8

All EC50 values in % of eluate

Two further tests not shown here

Ceriodaphnia: EC50 INC + SOI + WOO

Lab Code	INC	SOI	WOO
38	4.6	-	-
61	2.4	>90.0	0.05
63	6.0	>90.0	0.10
64	6.1	>90.0	0.09
Mean	4.8	>90.0	0.08
Factor min -max	2.5	?	2.0

All EC50 values in % of eluate

umu: Results INC + SOI + WOO

Highest dilution (%) with an Induction Rate (IR) < 1.5

Example: IR without metabolic activation

Lab Code	INC	SOI	WOO
1	67	67	33
12	67	67	67
16	67	67	67
21	67	67	67
23	67	67	67
33	67	67	33

With exception (cytotox?) of two tests with WOO, no genotoxicity at the highest eluate concentration of 67%

Sensitivity of the additional tests

Test system	INC LC/EC50	SOI LC/EC50	WOO LC/EC50
Algae/plant tests			
D. subspicatus	12.5	> 50	1.2
P. subcapitata	5.8	> 50	0.7
L. minor	> 60	> 50	1.6
Animal tests			
D. magna	**2.7**	**> 100**	**0.4**
C. dubia	2.5	> 90	2.0
B. calicyflorus	5.0	> 100	0.1
Bacteria test			
V. fischeri (Freeze)	**31.1**	**67.3**	0.7
P. putida	6.8	> 50	1.6

Recommendations

Test performance

- No problems concerning the performance of these tests were reported
- With exception of the *Lemna* test, the number of data sets was too small to prepare a detailed evaluation

Test assessment

- Sensitivity partly very high, but no test was consistently more sensitive than others (incl. basic battery)
- More research and experience needed….
- Special case waste genotoxicity: Inclusion of umu-test or other genotox tests in basic battery?

B.8 Talk – Th. Moser

Berlin
June 29, 2007

European Ring Test –
Ecotoxicological Characterisation of Waste

Results of the additional terrestrial tests

Thomas Moser

ECT Oekotoxikologie GmbH, Flörsheim/Main, Germany

Aims of this contribution

- Short description of five additional terrestrial tests
- Overview on the main results of these tests
- Comparison of the results with those gained in the basic test battery
- Discussion of the experiences (so far possible)
- Recommendations

Note that the performance of these tests was voluntary work.
Thus, the results were not completely evaluated statistically.

Overview: Additional terrestrial tests

- ***Arthrobacter* solid contact test**
- **Collembola reproduction test**
- **Enchytraeid reproduction test**
- **Earthworm reproduction test**
- **Earthworm avoidance test**

Characterization: *Arthrobacter Test*

Name: *Arthrobacter* solid contact test
Guideline: DIN 38412 L48 (2002)
Species: *Arthrobacter globiformis*
Medium: Artificial Soil or field soils,e.g. LUFA
Duration: < one day
Parameter: Dehydrogenase activity
Validity: Increase of fluorescence
Reference: Zincnitrate tetrahydrate
Experience: Mainly from sediment tests, but usage in soil increasing

Characterization: *Collembola Test*

Name: Collembola reproduction test
Guideline: ISO 11268-1 (1999)
Species: *Folsomia candida*
Medium: OECD artificial soil
Duration: 28 days
Parameter: Mortality, reproduction
Validity: Control mortality and reproduction, CV reproduction
Reference: Phenmedipharm, dimethoate
Experience: Huge data set available

Characterization: *Enchytraeid Test*

Name: Enchytraeid reproduction test
Guideline: ISO 16387 (2002)
Species: *Enchytraeus albidus, E. crypticus*
Medium: OECD artificial soil
Duration: 28 days
Parameter: Mortality, reproduction
Validity: Control mortality, reproduction, CV reproduction
Reference: Carbendazim
Experience: Huge data set available

Characterization: *Earthworm chronic test*

Name:	Earthworm reproduction test
Guideline:	ISO 11268-2 (1998)
Species:	*Eisenia fetida, E. andrei*
Medium:	OECD artificial soil
Duration:	56 days
Parameter:	Biomass, reproduction
Validity:	Control mortality,repro-duction,CV reproduction
Reference:	Carbendazim
Experience:	Huge data set available

Characterization: *Earthworm avoidance test*

Name:	Earthworm avoidance test
Guideline:	ISO 17512 (2004)
Species:	*Eisenia fetida, E. andrei*
Medium:	OECD artificial soil
Duration:	48 h
Parameter:	Avoidance
Validity:	Control mortality
Reference:	Boric acid
Experience:	Good data base, quick screening method

Results: Participation

Test	**No. of labs**	**No. of tests**	**unacceptable***
***Arthrobacter*:**	6	22	3
Collembolans:	7	21	3
Enchytraeids:	3	9	-
Earthworm Reproduction:	5	14	-
Earthworm Avoidance:	2	6	-

*Only tests being non-valid or without raw data.

Results: *Arthrobacter* EC_{50} values

Lab Code	INC	SOI	WOO
2	43.4	3.2	1.3
12a	<21.9	<5.2	<0.8
12b	<20.1	<8.2	n.d.
29	17.3	27.8	<1.6
31	<12.5	<12.5	<25.0
33	7.6	15.2	0.53
Range	7.6 – 43.4	3.2 – 27.8	0.53 - <25.0

All EC50 values in % of solid material

Results: Collembola EC_{50} values

Lab Code	INC	SOI	WOO
11	16.60	75.09	5.20
15	10.06	29.04	4.69
16	20.22	73.40	10.66
18	34.27	72.36	1.20
22	9.10	16.60	3.30
58	65.80	20.70	4.85
Mean ± *sd*	26.0 ± 21.5	47.9 ± 28.5	5.0 ± 3.2
Factor min -max	7.2	4.5	8.9

All EC50 values in % of solid material

Results: Enchytraeid EC_{50} values

Lab Code	INC	SOI	WOO
16	12.23	<25	8.40
22	39.90	>100.0	8.10
66	34.2	>100.0	19.63
Mean ± *sd*	28.8 ± 14.6	n.a.	12.0 ± 6.6

All EC50 values in % of solid material

Results: Earthworm Repro EC_{50} values

Lab Code	INC	SOI	WOO
11	<12.50	<50.0	5.20
22	17.20	>50.0	>8.3
48	7.95	56.0	>6.3
51a	13.7	>100.0	3.4
51b	25.6	>100.0	4.9
Mean ± *sd*	16.1 ± 7.4	n.a.	4.5 ± 1.0

All EC50 values in % of solid material

Results: Earthworm Avoid NOEC values

Lab Code	INC	SOI	WOO
11	6.25	<25	<3.75
22	12.5	50.0	3.13

All NOEC values in % of solid material

Results: Summary I

	Mean EC_{50} values		
Test system	**INC**	**SOI**	**WOO**
Arthrobacter	7.6 – 43.4	3.2 – 27.8	0.53 - <25.0
Collembolans	26.0	**48.9**	5.0
Enchytraeids	28.8	n.d.	12.0
Earthworm Reproduction	**16.1**	n.d.	**4.5**
Earthworm Avoidance	6.25 – 12.5 NOEC	<25 – 50 NOEC	3.13 - <3.75 NOEC

All EC_{50} values in % of solid material

Results: Summary II

	Mean EC_{50} values		
Test system	INC	SOI	WOO
Collembolans	26.0	**48.9**	5.0
Earthworm Reproduction	**16.1**	n.d.	**4.5**
Plants Avena/Brassica	29.2 / 25.5	> 100 / 62.2	11.8 / 2.8
Earthworm Acute	46.5 (LC_{50})	> 100 (LC_{50})	18.8 (LC_{50})

All EC_{50} values in % of solid material

Recommendations

Test performance

- No problems concerning the performance of these Tests were reported
- The number of data sets was too small to prepare a detailed evaluation

Test assessment

- Sensitivity partly very high, the collembola or earthworm reproduction test might replace the earthworm acute test
- Special cases *Arthrobacter* and Avoidance-test: Inclusion in basic battery?

B.9 Talk – C. van der Wielen

European Ringtest –
Ecotoxicological characterisation of wastes

Results regarding reproducibility and repeatability

Claire Van der Wielen, Yves Marneffe, Philippe Maetz

Berlin, 29/06/2007, European Ringtest – Ecotoxicological characterisation of wastes

Plan

- Introduction
- Data and treatment
- Algae results
- *Daphnia magna* results
- Improvements
- Comments
- Future

Berlin, 29/06/2007, European Ringtest – Ecotoxicological characterisation of wastes

Introduction

- Conditions of repetability

Independent results of essais or analysis are obtained

- ✓Same method
- ✓Same test material
- ✓Same laboratory
- ✓Same operator
- ✓Same equipment
- ✓In a small time interval

Berlin, 29/06/2007, European Ringtest – Ecotoxicological characterisation of wastes

Introduction

- Conditions of reproductibility

Independent results of essais or analysis are obtained

✓Same method

✓Same test material

✓Different laboratories

✓Different operators

✓Different equipment

✓Time interval defined

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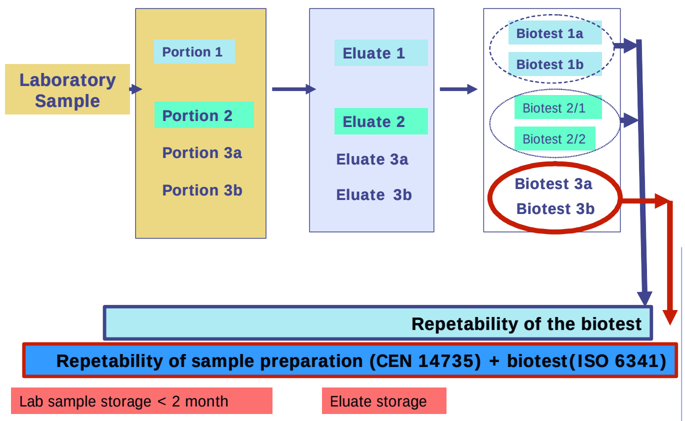
Treatment : R and r Conditions



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Data and treatment

◆Data: repeated tests from one lab

◆Data: accepted by UBA-ECT

◆Data: INC, WOO (almost no data for SOI)

◆Data: repeated results for test on eluates

➢ Reduced number, non homogenous data sets

➢ Treatment: evaluation of range

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Algae - INC

Participant [Code No.]	n	Mean	s	CV	Range	Range %	Conditions of R and r		
NEW							Eluate	Time	Syst.
1a,b	2	10,8	2,5	23	3,6	33	S	in //	V,μ
12a,b	2	3,9	2,5	64	3,5	91	S	in //	μ
12c,d	2	2,5	0,6	24	0,9	35	S	in //	V
12a,b,c,d	4	3,2	1,7	53	3,6	113	D		V,μ
49a,b,c	3	26,3	7,5	29	13,0	50	S	1 w	V
61a,b	2	6,8	0,6	9	0,9	13	S	in //	V,μ

		S,-,S	S,-,D	-,-,D
	Conditions	S,-,S	S,-,D	-,-,D
	n sets	3	2	3
Range %	Min	35	13	13
	Max	91	33	113

Algae - WOO

Participant [Code No.]	n	Mean	s	CV %	Range	Range %	Conditions R and r		
							Eluate	Time	Syst.
1a,b	2	1,2	0,07	6	0,10	9	S	in //	V, μ
12c,d	2	1,0	0,08	8	0,12	12	S	in //	μ
12b,c,d	3	1,2	0,29	25	0,56	87	D	1 m	V,μ
49a,b	2	1,4	0,02	1	0,02	2	S	1 w	V
51a,b	2	1,2	0,23	20	0,33	28	D	1 m	V
61a,b	2	0,2	0,02	7	0,03	10	S	in //	V,μ

		S,-,S	S,-,D	D,-,D
	Conditions	S,-,S	S,-,D	D,-,D
	n sets	2	4	3
Range %	Min	2	2	9
	Max	12	28	87

Algae : comments

- Range increases with integrating different test conditions
- Different biotest systems (vessel/microplate) do not seem to be the major factor of influence in the intralaboratory reproducibility
- Variability of INC data> WOO

Daphnia magna - INC

Participant [Code No.] NEW	n	LC50 Mean	s	CV	Range	Range %	Conditions of R and r E	B
33	2	4,34	0,49	11,40	0,70	16,13	S	in //
51	2	3,56	0,29	8,25	0,42	11,66	S	2 weeks
12	3	2,20	0,17	7,95	0,34	15,48	D	2 weeks
36	2	1,85	0,23	12,68	0,33	17,94	D	1 month

	n Labs	2	2	4
Range %	Min	12	15	12
	Max	16	18	18

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Daphnia magna - WOO

Participant [Code No.] NEW	n	Mean	s	CV	Range	Range %	Conditions of R and r E	B
50	2	0,85	0,01	0,74	0,01	1,05	S	in //
51	2	0,23	0,03	13,53	0,04	19,13	S	2 week
49	3	0,21	0,02	10,91	0,05	21,77	S	1 month
12	2	0,12	0,00	1,23	0,00	1,74	D	2 week
36	2	0,16	0,06	36,60	0,08	51,76	D	1 month
8	2	0,32	0,19	58,60	0,27	82,87	?	?
21	2	0,26	0,02	9,10	0,03	12,87	Eluate prep. differs	

	n Labs	3	2	7
Range %	Min	1	2	1
	Max	22	52	83

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Daphnia magna : comments

- INC: The same repeatability/range is observed starting from the same or different eluates (overall repeatability)
- Biotest repeatability (//, within one or two weeks) raise the question of storage
- Higher dispersion of the results for WOO>INC

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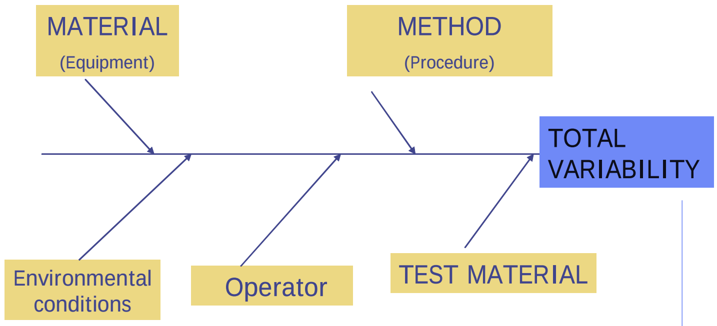
Improvement: Tool

- Causal effect diagram (Ishikawa):



Brainstorming (intralab, interlab)

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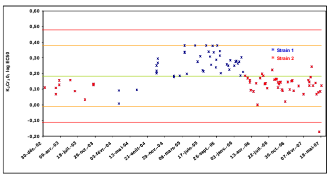
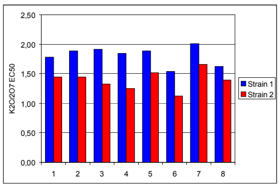
Improvement: by practice





- Shift in *Daphnia magna* control chart
- New strain, new operators,...: some results above the limit
- Test different hypothesis
- Conclusion: difference in sensitivity of the new strain
  - Identify the cause
  - Gain knowledge

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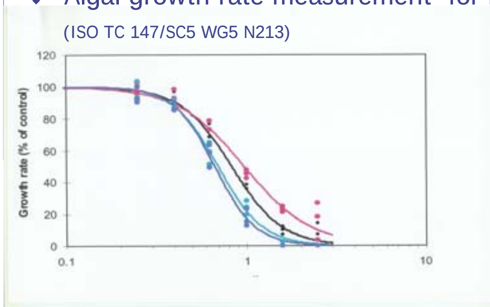
Improvement: Other endpoint?

- Algal growth rate measurement for K2Cr207

(ISO TC 147/SC5 WG5 N213)



P. subcapitata

- Cell number
- In vivo fluorescence
- DCMU-enhanced fluorescence

Parameter	EC50	95% CI	EC10	95%CI
Cell numbers	0,83	0,77-0,88	0,41	0,34-0,47
Total cell vol.	0,99	0,93-1,07	0,38	0,33-0,45
In vivo fluoresc.	0,69	0,66-0,71	0,37	0,34-0,40
Fluoresc. with DCMU	0,66	0,64-0,89	0,38	0,35-0,41

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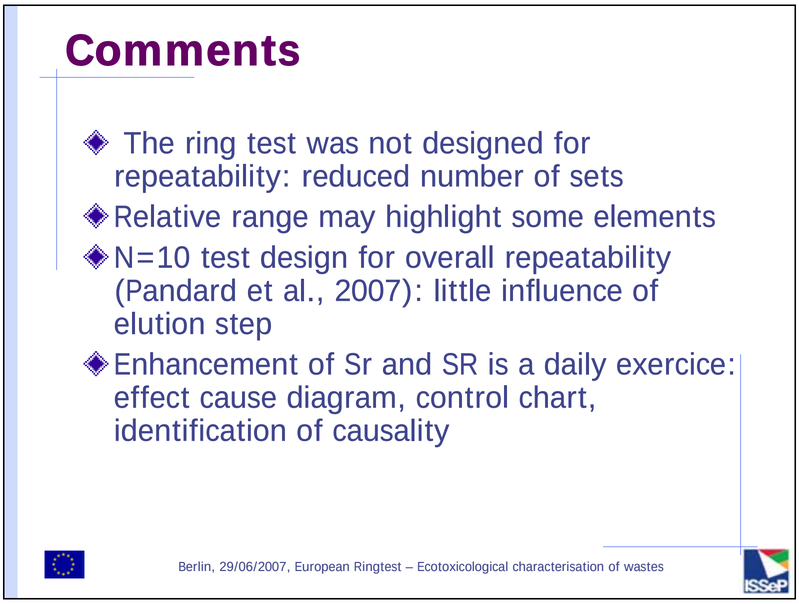
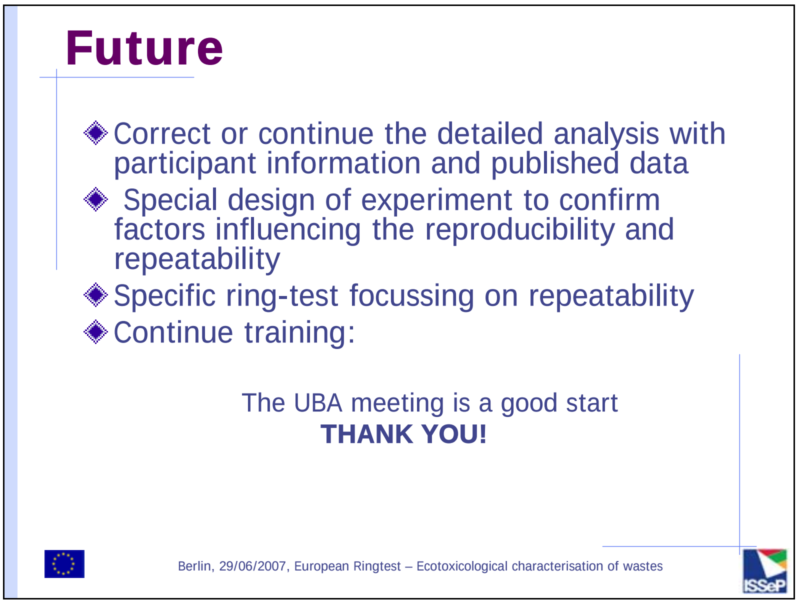
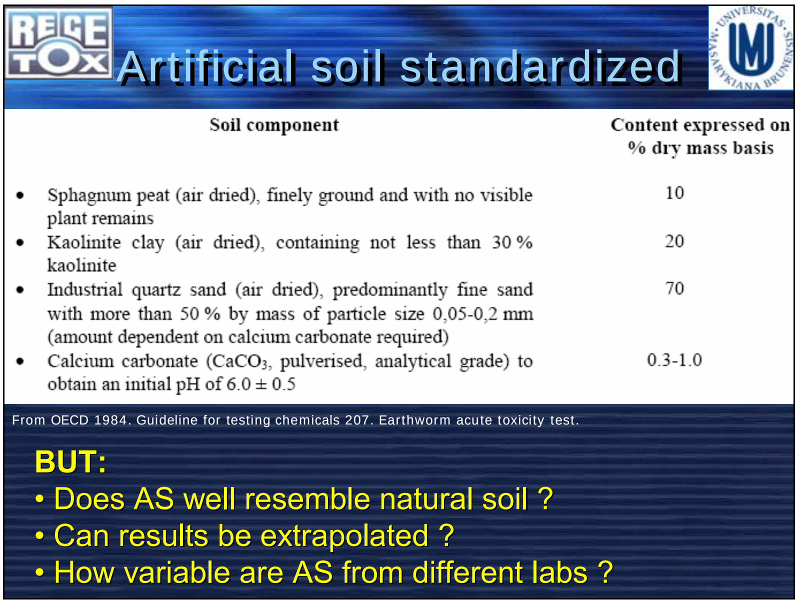
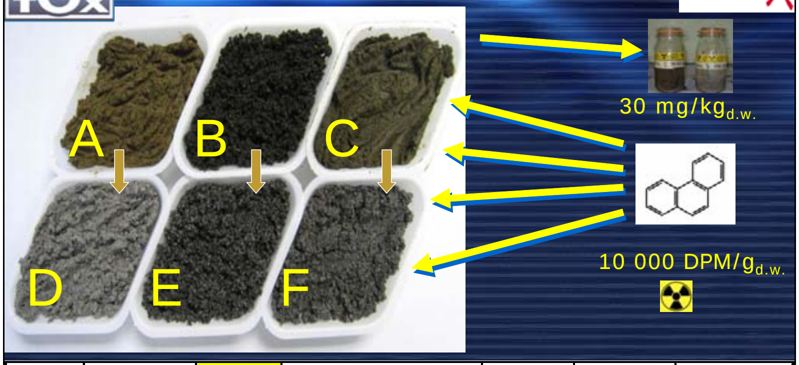

Comments
◆ The ring test was not designed for repeatability: reduced number of sets
◆ Relative range may highlight some elements
◆ N=10 test design for overall repeatability (Pandard et al., 2007): little influence of elution step
◆ Enhancement of Sr and SR is a daily exercice: effect cause diagram, control chart, identification of causality
Berlin, 29/06/2007, European Ringtest – Ecotoxicological characterisation of wastes


Future
◆ Correct or continue the detailed analysis with participant information and published data
◆ Special design of experiment to confirm factors influencing the reproducibility and repeatability
◆ Specific ring-test focussing on repeatability
◆ Continue training:
The UBA meeting is a good start
THANK YOU!
Berlin, 29/06/2007, European Ringtest – Ecotoxicological characterisation of wastes

B.10 Talk – J. Hofman



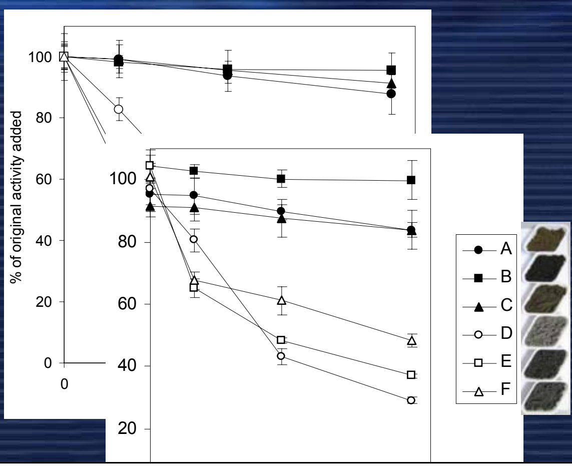
Hofman, Rhodes, Semple (2007)



Soil	Origin	C_{org} (%)	Particle size analysis		pH (H_2O)	WHC (%)	Dry matter (%)
			Clay	Sand			
A	arable	1.88	14.7	53.6	6.8	56.6	77.4
B	forest	9.02	13.3	41.3	5.9	56.7	53.4
C	grassland	7.62	14.1	52.8	7.3	58.3	63.0
D	artificial	1.88	20.0	76.1	7.3	56.1	80.7
E	artificial	9.02	20.0	61.3	7.2	56.4	65.5
F	artificial	7.62	20.0	64.2	7.3	54.9	71.7

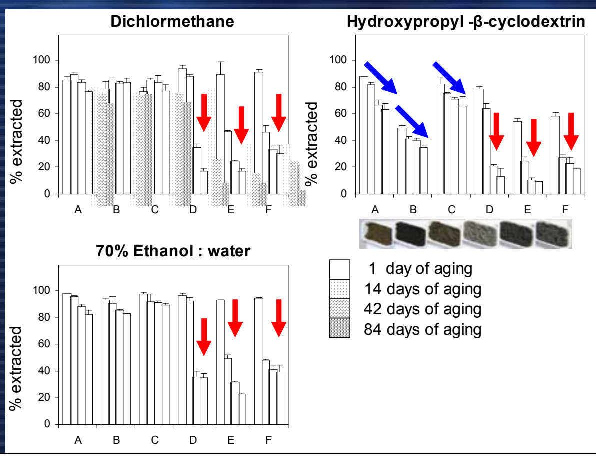
Hofman, Rhodes, Semple (2007)

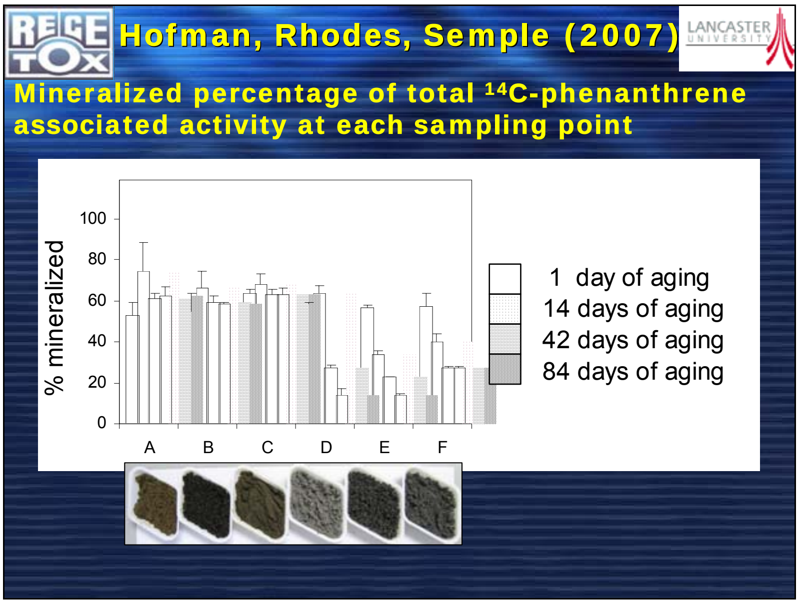
Total content of the phenanthrene in the soils

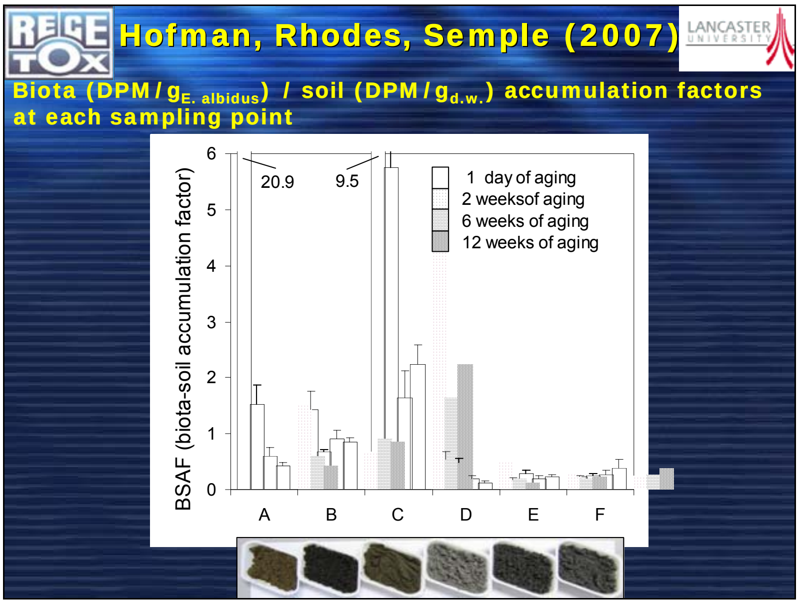


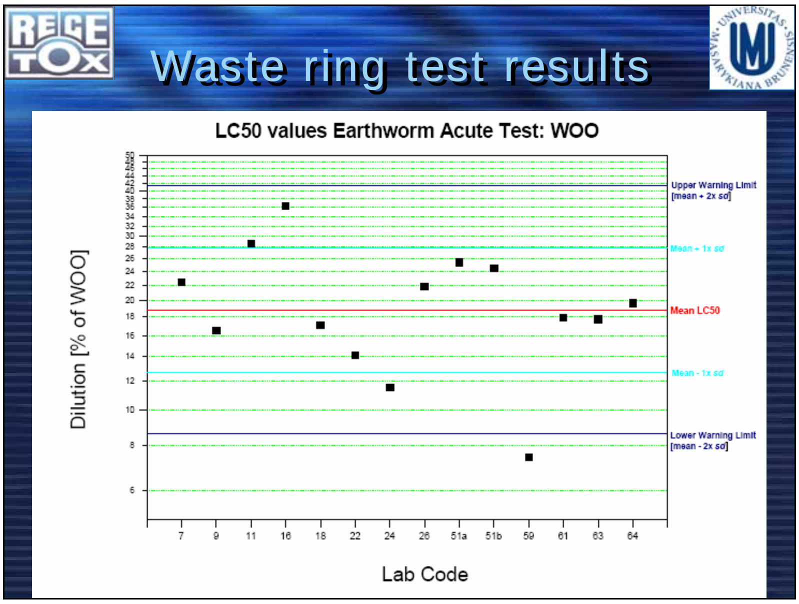
Hofman, Rhodes, Semple (2007)

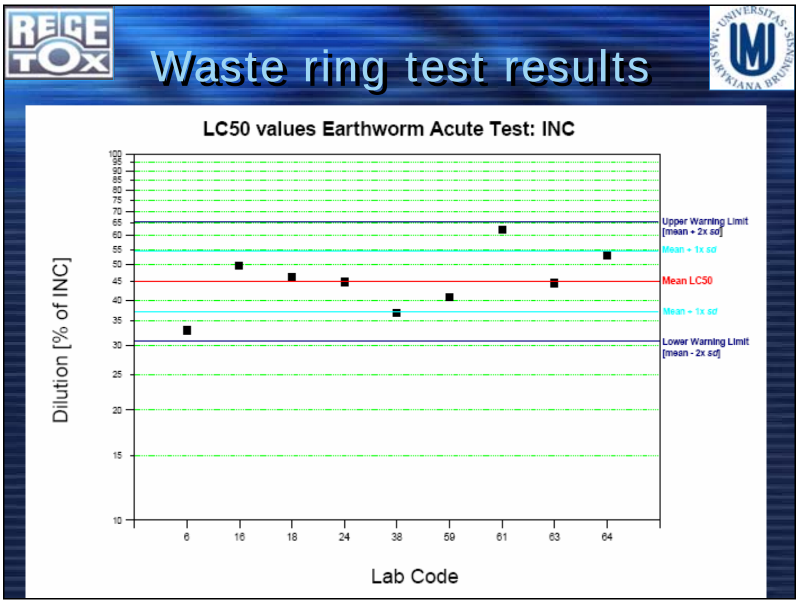
Extracted percentage of total ^{14}C-phenanthrene associated activity at each sampling point



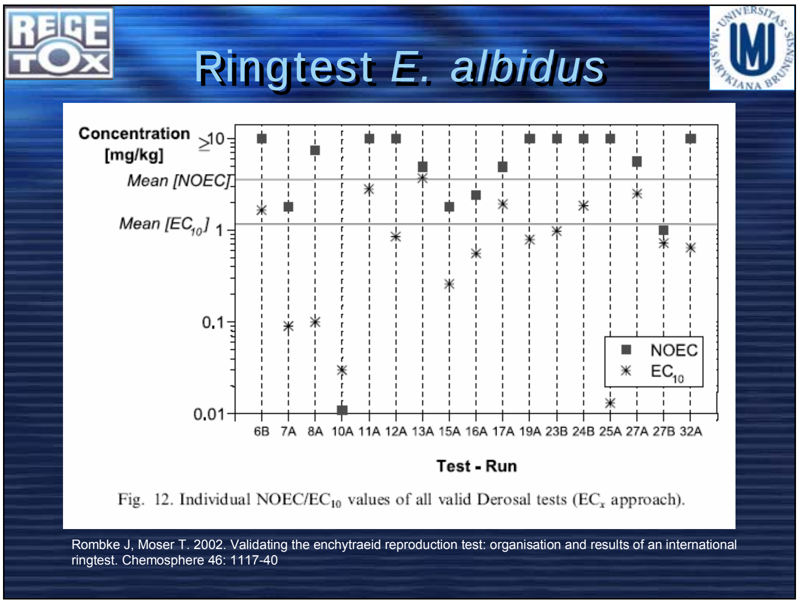

Hofman, Rhodes, Semple (2007)
LANCASTER UNIVERSITY
Mineralized percentage of total 14C-phenanthrene associated activity at each sampling point
% mineralized
100
80
60
40
20
0
A B C D E F
1 day of aging
14 days of aging
42 days of aging
84 days of aging


Hofman, Rhodes, Semple (2007)
LANCASTER UNIVERSITY
Biota (DPM/gE. albidus) / soil (DPM/gd.w.) accumulation factors at each sampling point
BSAF (biota-soil accumulation factor)
6
5
4
3
2
1
0
20.9
9.5
A B C D E F
1 day of aging
2 weeksof aging
6 weeks of aging
12 weeks of aging

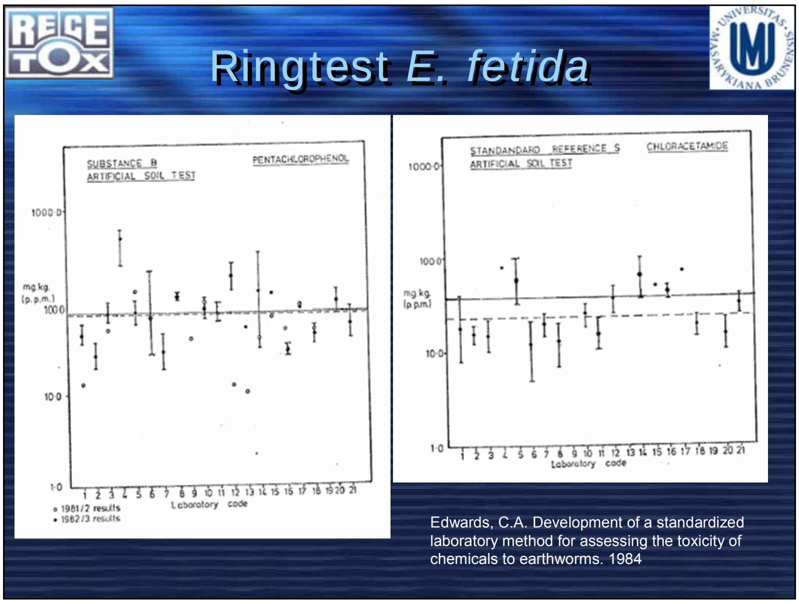

Waste ring test results
LC50 values Earthworm Acute Test: WOO
Dilution [% of WOO]
Upper Warning Limit [mean + 2x sd]
Mean + 1x sd
Mean LC50
Mean - 1x sd
Lower Warning Limit [mean - 2x sd]
7 9 11 16 18 22 24 26 51a 51b 59 61 63 64
Lab Code



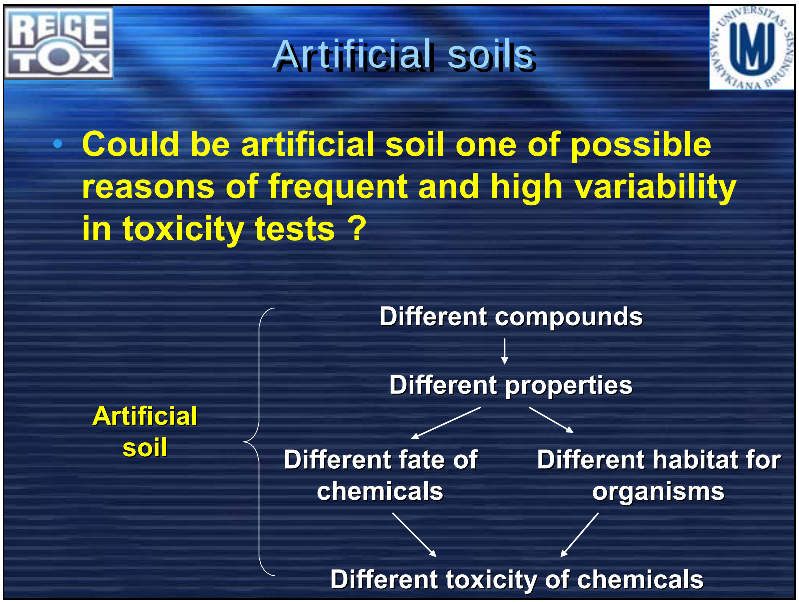
Waste ring test results
LC50 values Earthworm Acute Test: INC
Dilution [% of INC]
Lab Code
Upper Warning Limit [mean + 2x sd]
Mean + 1x sd
Mean LC50
Mean - 1x sd
Lower Warning Limit [mean - 2x sd]
6
16
18
24
38
59
61
63
64

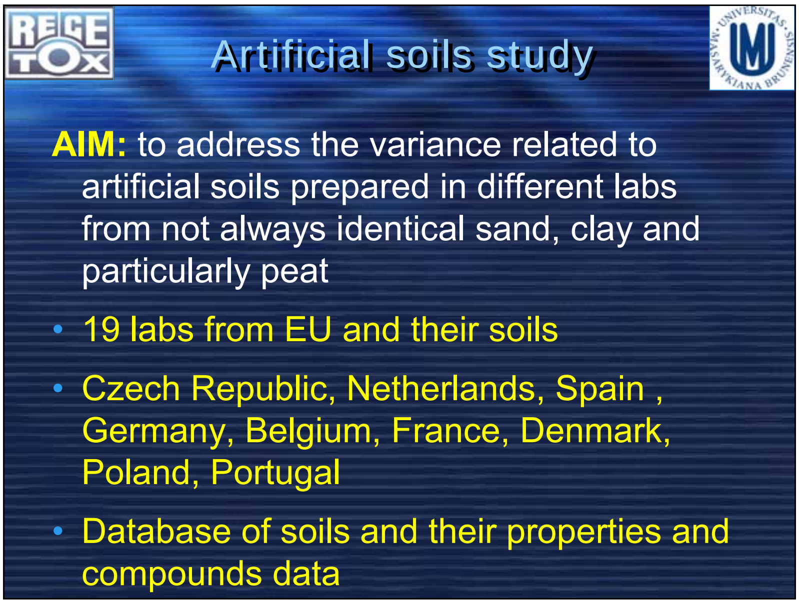


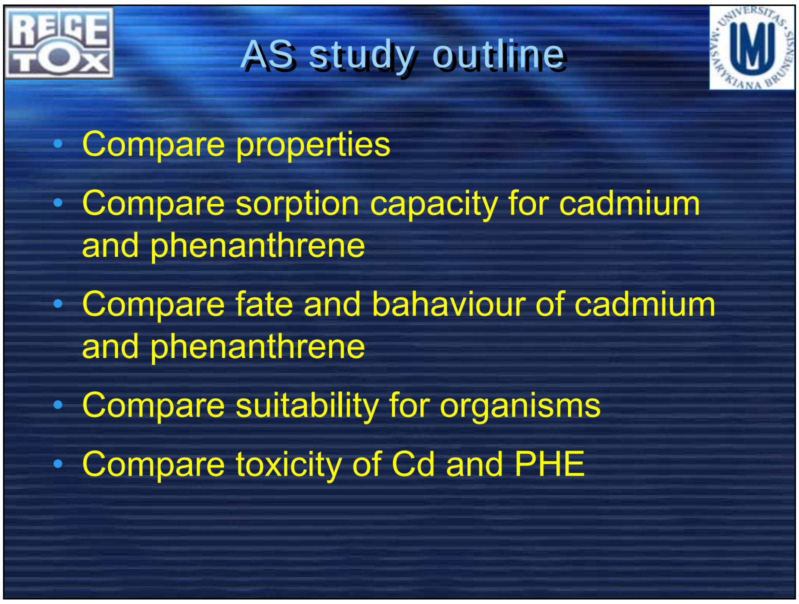
Ringtest E. albidus
Concentration [mg/kg]
Mean [NOEC]
Mean [EC10]
NOEC
EC10
6B 7A 8A 10A 11A 12A 13A 15A 16A 17A 19A 23B 24B 25A 27A 27B 32A
Test - Run
Fig. 12. Individual NOEC/EC10 values of all valid Derosal tests (ECx approach).
Rombke J, Moser T. 2002. Validating the enchytraeid reproduction test: organisation and results of an international ringtest. Chemosphere 46: 1117-40



Ringtest E. fetida
Edwards, C.A. Development of a standardized laboratory method for assessing the toxicity of chemicals to earthworms. 1984

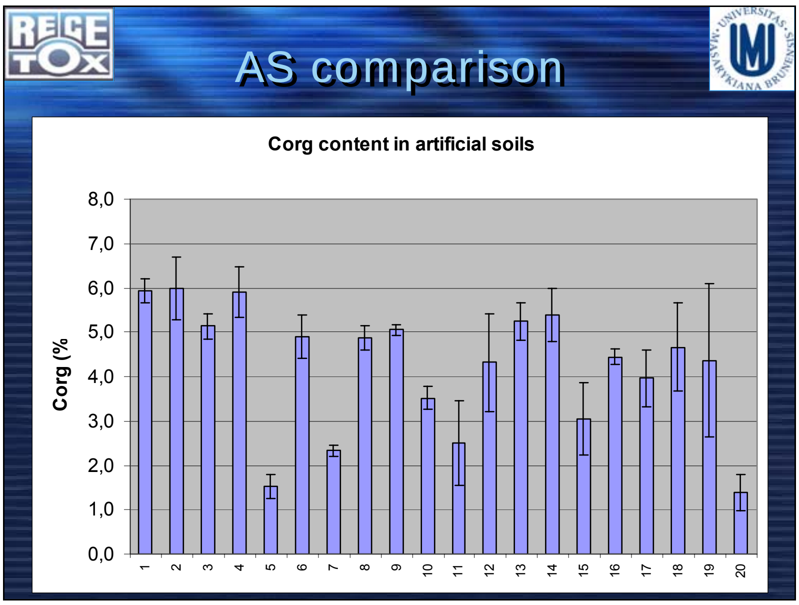

Artificial soils
Could be artificial soil one of possible reasons of frequent and high variability in toxicity tests ?
Artificial soil
Different compounds
Different properties
Different fate of chemicals
Different habitat for organisms
Different toxicity of chemicals

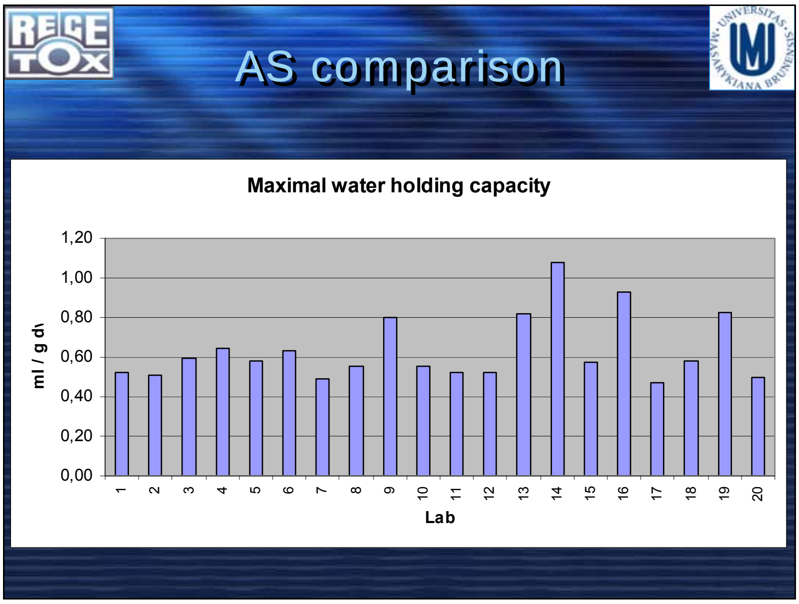

Artificial soils study
AIM: to address the variance related to artificial soils prepared in different labs from not always identical sand, clay and particularly peat
19 labs from EU and their soils
Czech Republic, Netherlands, Spain , Germany, Belgium, France, Denmark, Poland, Portugal
Database of soils and their properties and compounds data

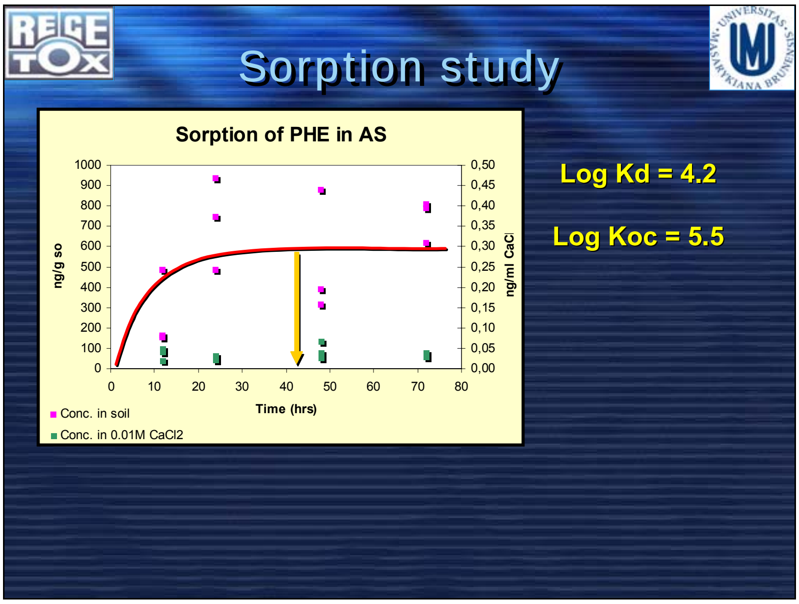

AS study outline
Compare properties
Compare sorption capacity for cadmium and phenanthrene
Compare fate and bahaviour of cadmium and phenanthrene
Compare suitability for organisms
Compare toxicity of Cd and PHE

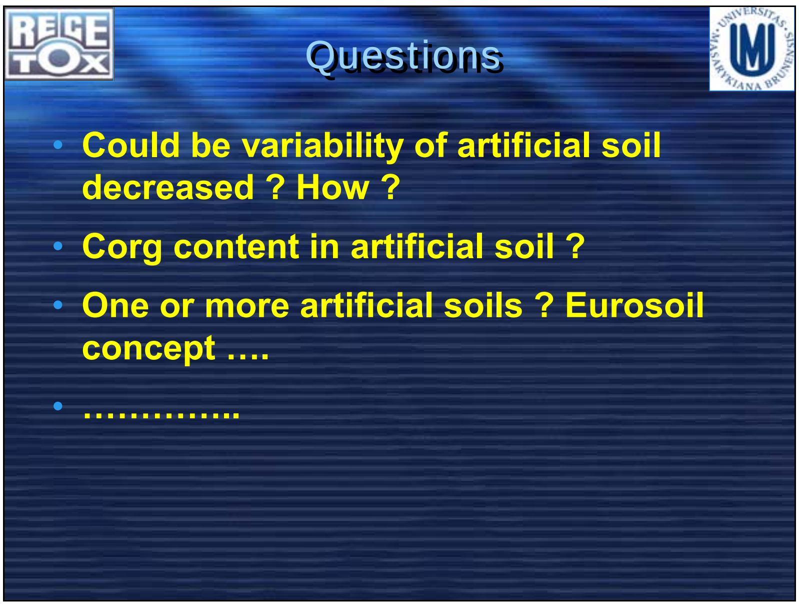

REGETOX
How does AS look like ?
UNIVERSITAS MASARYKIANA BRUNENSIS


REGETOX
How does AS look like ?
UNIVERSITAS MASARYKIANA BRUNENSIS

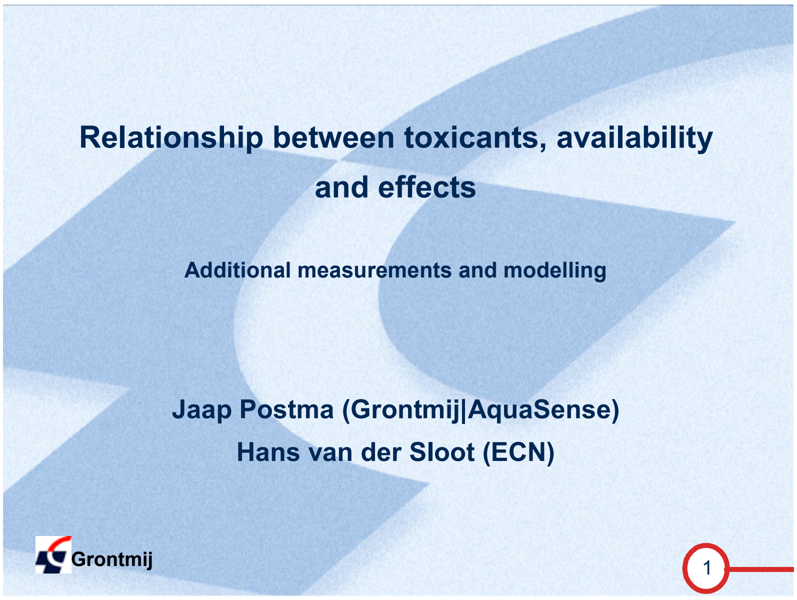

REGETOX
AS comparison
UNIVERSITAS MASARYKIANA BRUNENSIS
Corg content in artificial soils
Corg (%
8,0
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

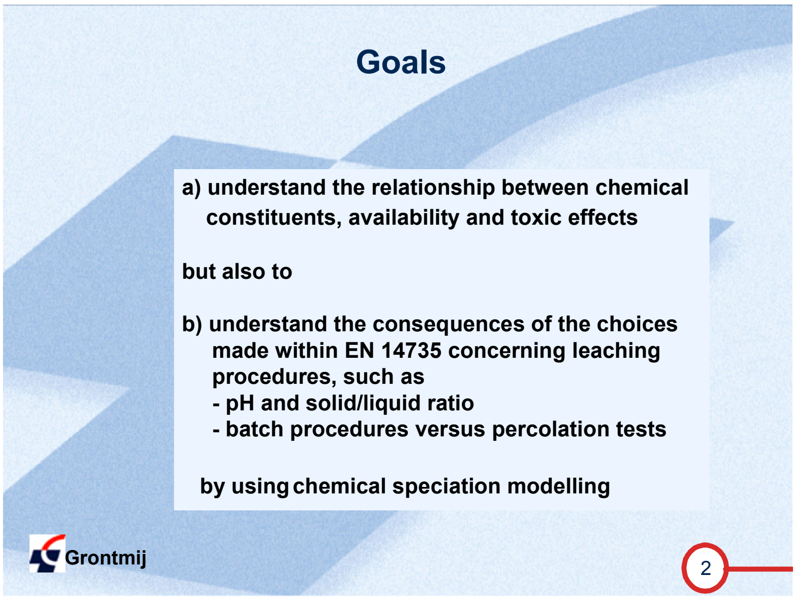

AS comparison
Maximal water holding capacity
ml / g dw
1,20
1,00
0,80
0,60
0,40
0,20
0,00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Lab

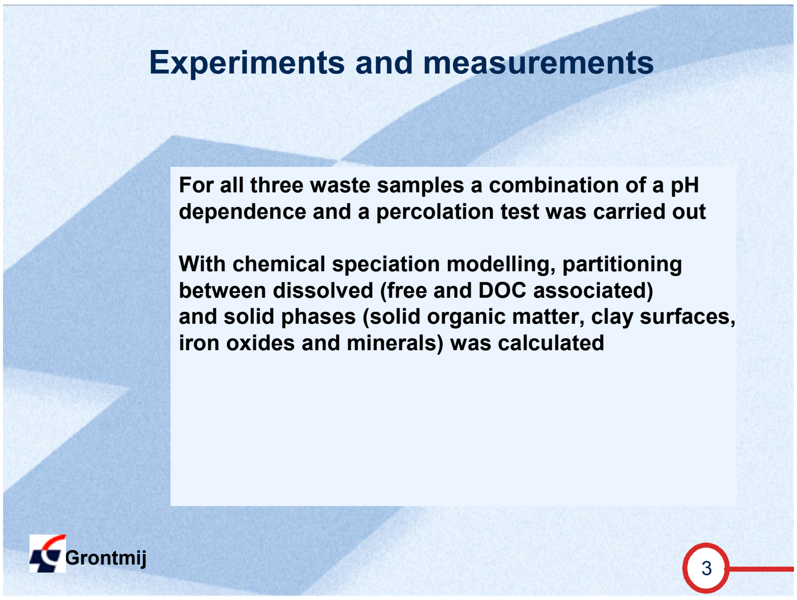

Sorption study
Sorption of PHE in AS
ng/g so
ng/ml CaCl
Time (hrs)
Conc. in soil
Conc. in 0.01M CaCl2
Log Kd = 4.2
Log Koc = 5.5


Questions
Could be variability of artificial soil decreased ? How ?
Corg content in artificial soil ?
One or more artificial soils ? Eurosoil concept ….
…………..

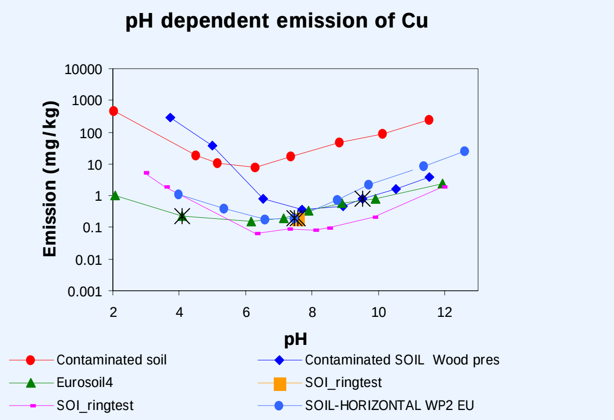
B.11 Talk – J. Postma







Data examples (1)



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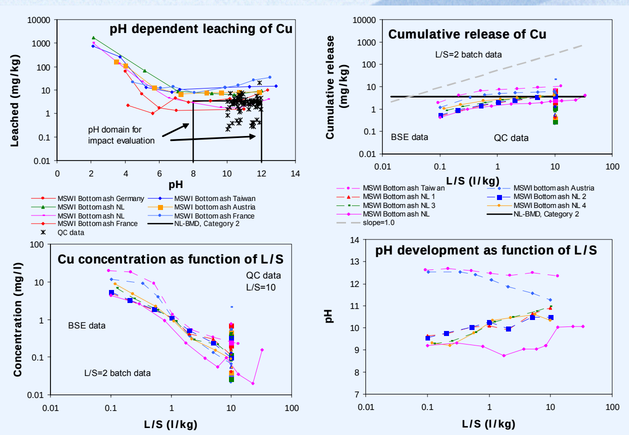
Data examples (2)



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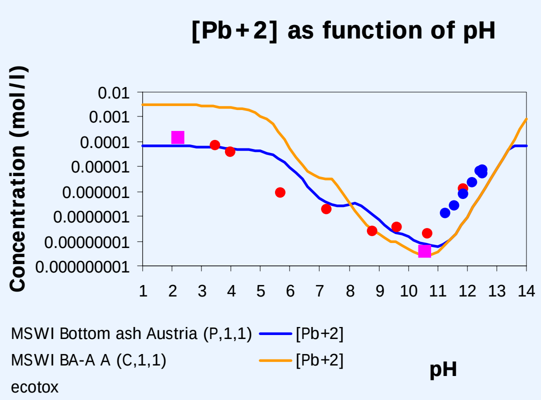
5

Data examples (3)



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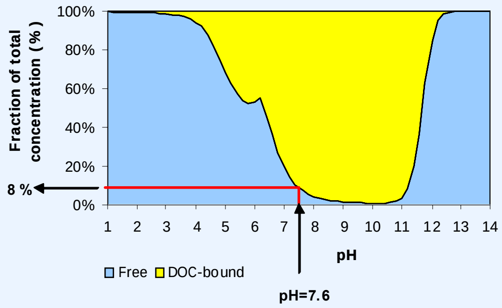
Data examples (4)



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7

TU in undiluted eluates, Wood

Besides some other such as chromium, especially copper was increased (± 22 mg/l !)

	EC_{50} copper (mg/l)	TU-values for copper
Microtox	0,13	170
D. magna	0,024	919
P. subcapitata	0,15	147

So, copper is clearly a main toxicant

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1) *D. magna*, copper and wood

i) Actual EC_{50}-value = 0,2 vol%
ii) Copper conc. has dropped to 0,044 mg/l
iii) Still, 2 TU at the EC_{50}-value

However,

a) not all copper is bioavailable
b) Interactions with DOC exist

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1) *D. magna*, copper and wood

	Test solutions (vol%)		
	100	0.50	0.20
Total Cu (mg/l)	22.1		
Free Cu	0.5		
Total Cu, test solution		0.110	0.044
"Expected" free-Cu		0.002	0.001
"Modelled" free-Cu		0.015	0.006
(%)	2.1	13.6	13.6

Conclusion: Toxicity is likely be caused by copper, but TU = ±0.5 and interactions with DOC

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2) Microtox, copper and wood

i) Actual EC_{50}-value = 0,4 vol%
ii) Copper conc. has dropped to 0,088 mg/l
iii) = 0.7 TU at the EC_{50}-value

However,

a) again, not all copper is bioavailable
b) Interactions with salts exist

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2) Microtox, copper and wood

	Test solutions (vol%)		
	100	0.52	0.26
Total Cu (mg/l)	22.1		
Free Cu	0.5		
Total Cu, test solution		0.115	0.057
"Expected" free-Cu		0.002	0.001
"Modelled" free-Cu		0.052	0.024
(%)	2.1	45.2	42.0

Conclusion: Toxicity is likely be caused by copper, but TU = ±0.4 and interactions with salts

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3) Algae, copper and incinerator ash

	Test solutions (vol%)		
	100	20	10
Total Cu (mg/l)	0.6		
Free Cu	0.019		
Total Cu, test solution		0.126	0.063
"Expected" free-Cu		0.004	0.002
"Modelled" free-Cu		0.010	0.005
(%)	3.1	8.0	7.3

Conclusion:
-Cu is not enough to cause toxicity (TU< 0.1)
-What is causing the interaction with copper availability?

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3) Algae, major ions and incinerator ash

	Undiluted eluate	Control medium algae	At 10 vol% (± EC_{50}-algae)
Concentration (mg/l)			
Ca	320	4,9	36,4
Mg	0,27	3,2	2,9
Na	168	25,3	39,6
K	40	0,46	4,4
Ratios			
Ca/Mg	1185	1,5	12,6
Na/K	4.2	55,4	9,0

Conclusion:
-Might Ca/Mg ratio cause toxicity?

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Overall conclusions

1) **chemical analyses, speciation modeling and TU-calculations were very useful in finding causative relations between toxicants and effects in organisms**

2) **bioavailability changes over the dilution range due to several interactions (DOC, Ca levels and salt) caused (at least in these cases) an increase in observed toxicity**

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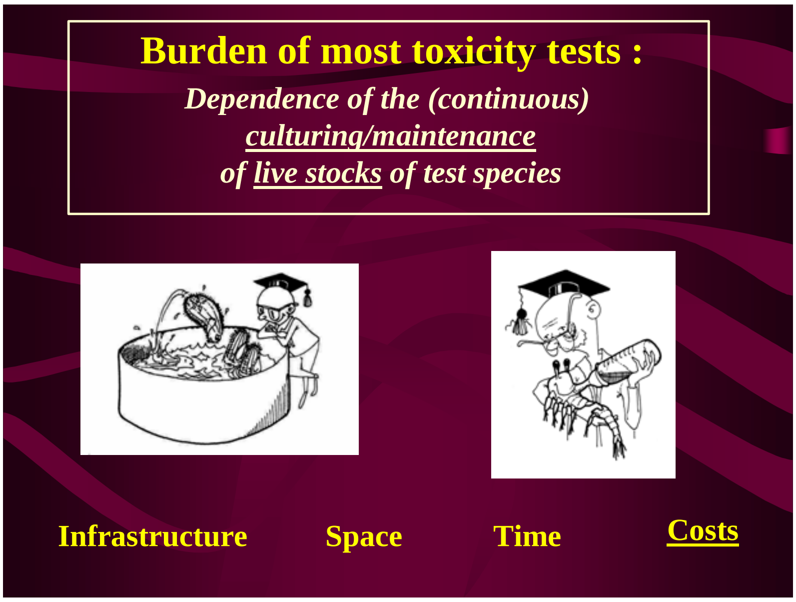
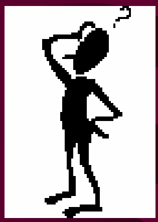
B.12 Talk – G. Persoone



MICROBIOTESTS

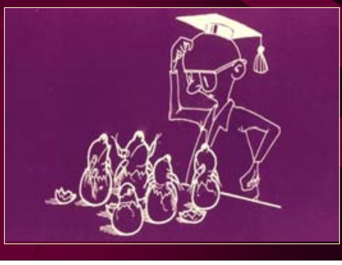
as alternatives to “conventional” toxicity tests



Research on microbiotests at the Laboratory for Biological Research in Aquatic Pollution (Ghent University – Belgium)

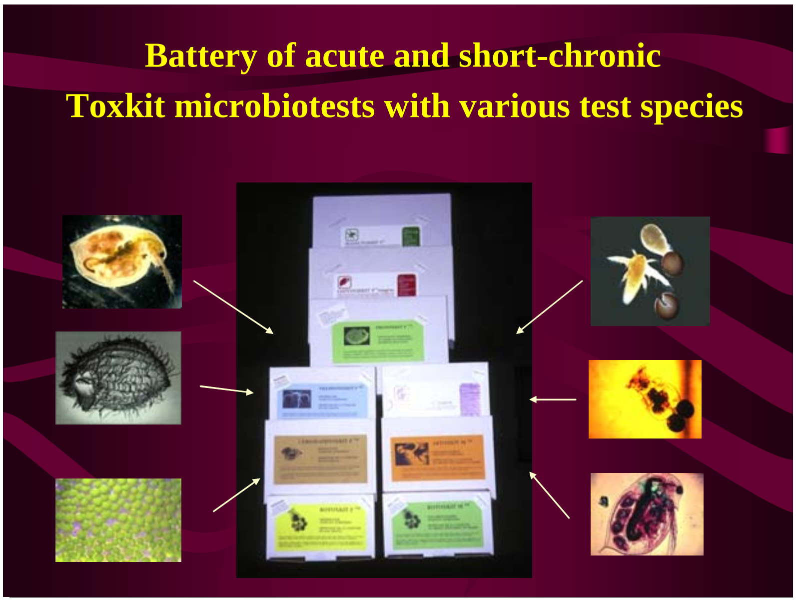
Basic approach :

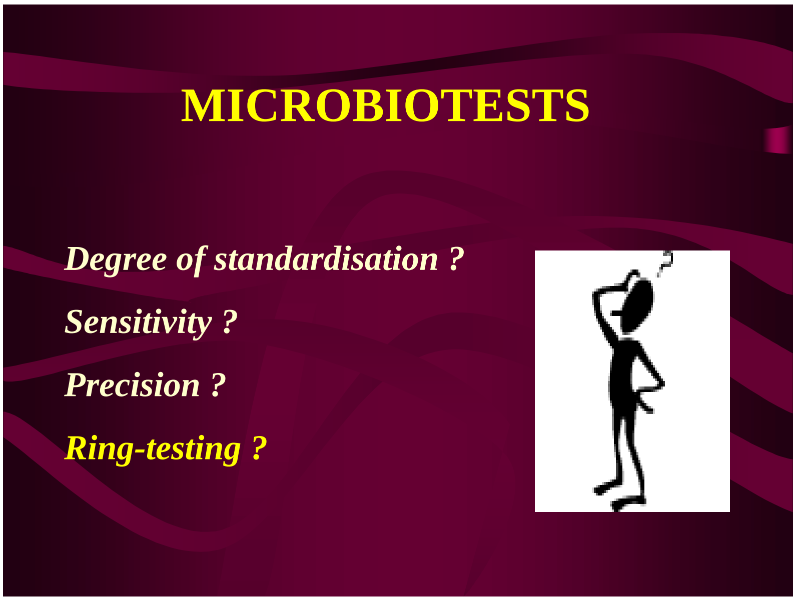
Use of dormant or immobilized stages of selected aquatic organisms from which the test biota can be obtained “on demand”

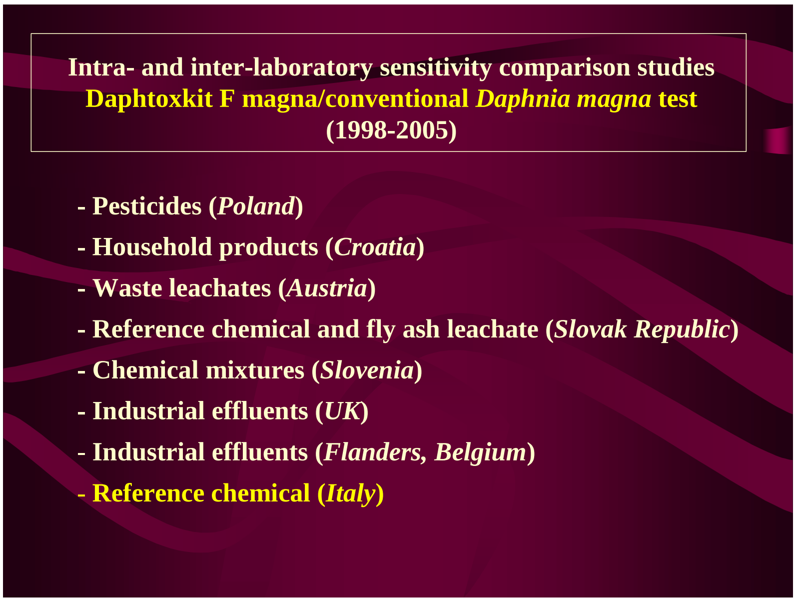


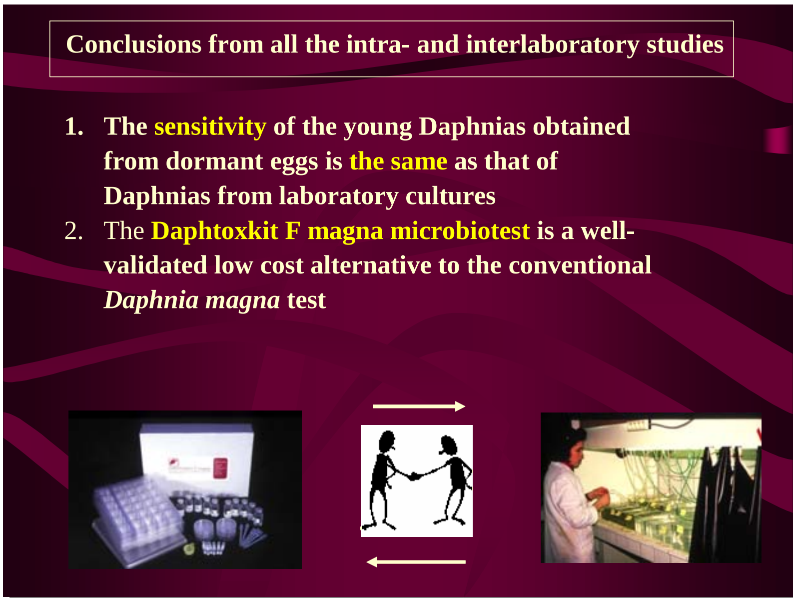
TOXKIT microbiotests

- Independent of culturing/maintenance of live stocks of test species
- User-friendly
- Miniaturised
- Low cost

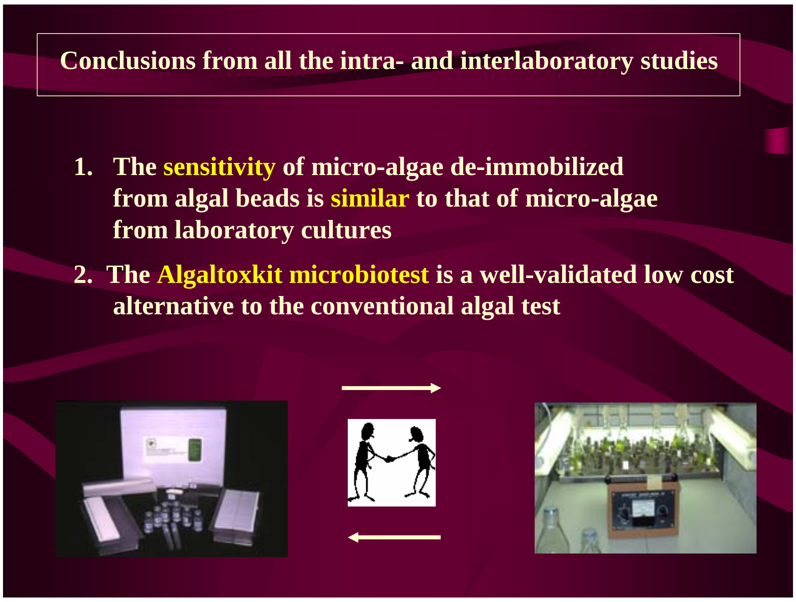

Battery of acute and short-chronic
Toxkit microbiotests with various test species


MICROBIOTESTS
Degree of standardisation ?
Sensitivity ?
Precision ?
Ring-testing ?


Intra- and inter-laboratory sensitivity comparison studies
Daphtoxkit F magna/conventional Daphnia magna test
(1998-2005)
- Pesticides (Poland)
- Household products (Croatia)
- Waste leachates (Austria)
- Reference chemical and fly ash leachate (Slovak Republic)
- Chemical mixtures (Slovenia)
- Industrial effluents (UK)
- Industrial effluents (Flanders, Belgium)
- Reference chemical (Italy)


Conclusions from all the intra- and interlaboratory studies
1. The sensitivity of the young Daphnias obtained from dormant eggs is the same as that of Daphnias from laboratory cultures
2. The Daphtoxkit F magna microbiotest is a well-validated low cost alternative to the conventional Daphnia magna test


Intra- and interlaboratory sensitivity comparison studies
Algaltoxkit F/conventional algal assay
(1998-2007)
- Waste leachates (Austria)
- Sediment pore waters (Flanders, Belgium)
- Reference chemical (Wallonia, Belgium)
- Reference chemical and fly ash leachate (Slovak Republic)
- Waste water treatment plant effluents (Denmark)
- Industrial effluents (UK)
- Industrial effluents (Flanders, Belgium)
- International Algaltoxkit Intercalibration Exercise


Conclusions from all the intra- and interlaboratory studies
1. The sensitivity of micro-algae de-immobilized from algal beads is similar to that of micro-algae from laboratory cultures
2. The Algaltoxkit microbiotest is a well-validated low cost alternative to the conventional algal test

EU Ecotox Waste Ringtest 2006-2007

Tests on waste eluates : Bacterial luminescence inhibition test
Algal growth inhibition test
Daphnia acute test

Tests on solid wastes : Earthworms acute test
Plant test

Additional or alternative test methodologies also welcome

During the course of the waste ringtest, it eventually appeared that several laboratories in different countries were performing tests according to the **Toxkit microbiotest** technology

In analogy to the recent international intercalibration exercises with the Daphtoxkit and the Algaltoxkit, an initiative was taken to collect Toxkit results from the waste ringtest in order to :

a) evaluate the interlaboratory precision of the Toxkit tests for wastes
b) make a first analysis of the correspondence of Toxkit results with the results of a few laboratories which had performed "conventional" tests with the same test species

Toxkit data were kindly provided by 13 participating laboratories

Name of laboratory	*Abbreviation*	*Number of different Toxkit tests performed*
MicroBioTests Inc. (Belgium)	MBT	5
Institut Provincial d'Hygiene et de Bactériologie (Belgium)	IPHB	2
EPAS (Belgium)	EPAS	2
AlControl Laboratories (United Kingdom)	ALC	4
Agenzia per la Protezione dell'Ambiente Tuscany (Italy)	ARPAT	1
Agenzia per la Protezione dell'Ambiente Grosseto (Italy)	ARPAG	1
Instituto do Ambiente (Portugal)	IDA	2
Mälardalen University (Sweden)	MALU	3
Technische Universität Braunschweig (Germany)	TUB	2
Insavalor-Polden (France)	POLD	1
INERIS (France)	INER	1
IRH Environnement (France)	IRH	1
Laboratoire Santé Environ. Hygiene de Lyon (France)	LSEH	1

The following Toxkit microbiotests have been performed on the 3 wastes in the framework of the EU ringtest :

Algaltoxkit F

Daphtoxkit F

Thamnotoxkit F

Chronic Rotoxkit F

Phytotoxkit

For a very first comparison of Toxkit results with results from conventional tests, data were available from tests performed by the organizing Institute UBA in Germany.

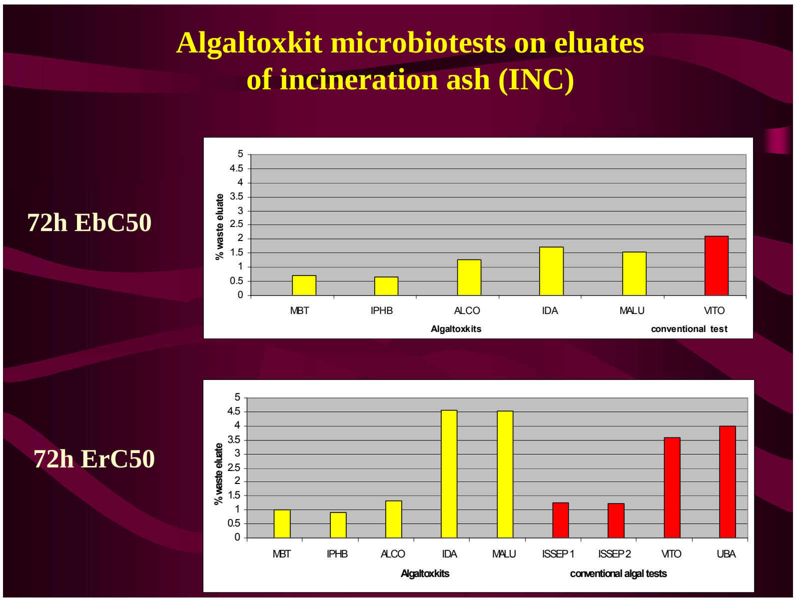
Results were also kindly provided by 2 Belgian Institutes :

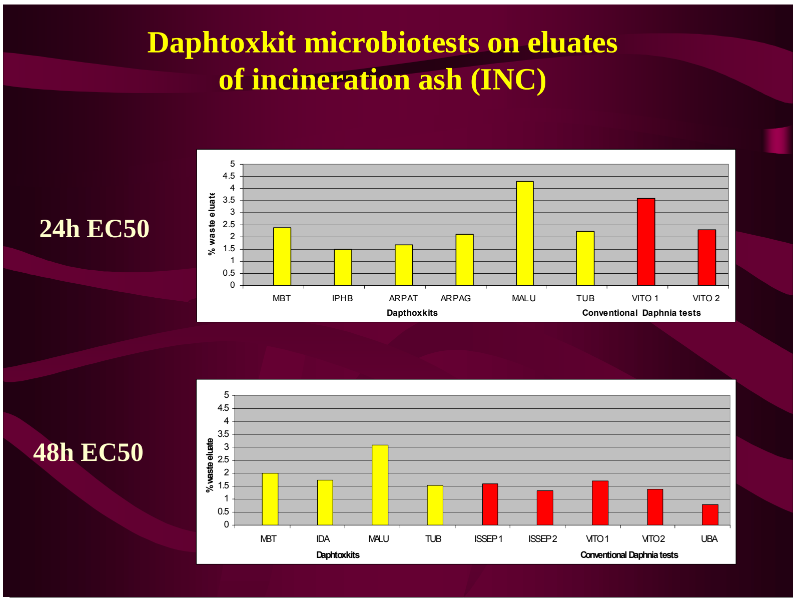
The VITO in Flanders

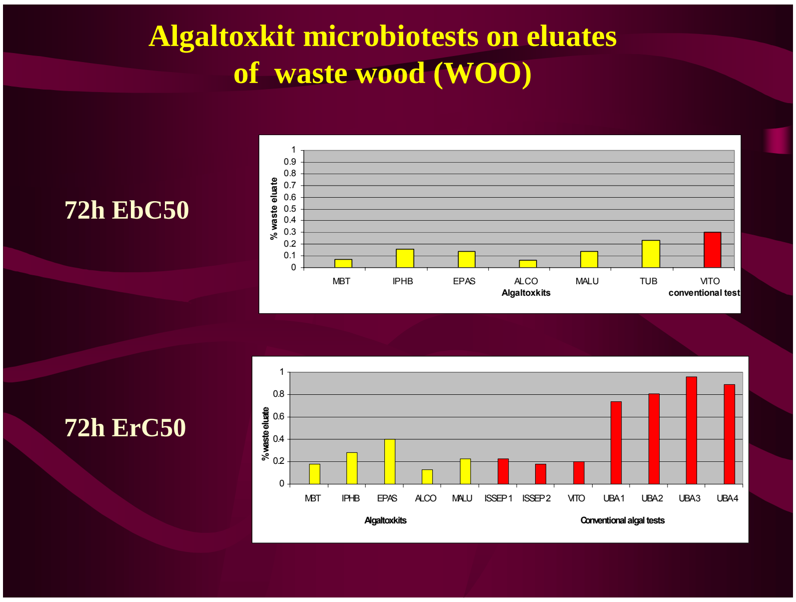
The ISSEP in Wallonia

First comments on the Toxkit results :

- **EC50's could not be calculated for all the tests because they were outside of the selected dilution range and the assays had not been repeated with the appropriate dilution range. These tests have therefore not been taken into consideration in this presentation.**
- **The gaswork soil (SOI) was found to be "not toxic" in all the Toxkit tests.**
- **For the Algaltoxkit tests, most laboratories provided 72h EbC50's. The corresponding ErC50's have been calculated for this presentation.**
- **For the Daphtoxkit tests not all the laboratories have provided both 24h and 48h EC50 values**

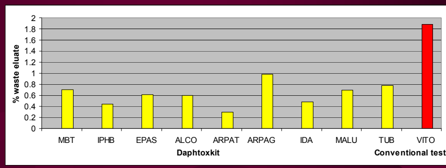

Algaltoxkit microbiotests on eluates
of incineration ash (INC)
72h EbC50
% waste eluate
MBT
IPHB
ALCO
IDA
MALU
VITO
Algaltoxkits
conventional test
72h ErC50
% waste eluate
MBT
IPHB
ALCO
IDA
MALU
ISSEP 1
ISSEP 2
VITO
UBA
Algaltoxkits
conventional algal tests


Daphtoxkit microbiotests on eluates
of incineration ash (INC)
24h EC50
% waste eluate
MBT
IPHB
ARPAT
ARPAG
MALU
TUB
VITO 1
VITO 2
Dapthoxkits
Conventional Daphnia tests
48h EC50
% waste eluate
MBT
IDA
MALU
TUB
ISSEP 1
ISSEP 2
VITO 1
VITO 2
UBA
Daphtoxkits
Conventional Daphnia tests

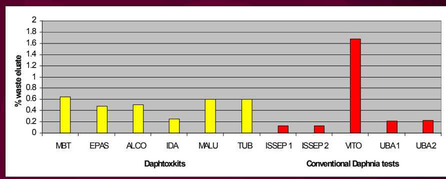

Algaltoxkit microbiotests on eluates
of waste wood (WOO)
72h EbC50
% waste eluate
MBT
IPHB
EPAS
ALCO
MALU
TUB
VITO
Algaltoxkits
conventional test
72h ErC50
% waste eluate
MBT
IPHB
EPAS
ALCO
MALU
ISSEP 1
ISSEP 2
VITO
UBA 1
UBA 2
UBA 3
UBA 4
Algaltoxkits
Conventional algal tests

Daphtoxkit microbiotests on eluates of waste wood (WOO)

24h EC50

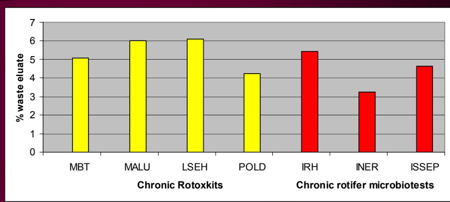


48h EC50



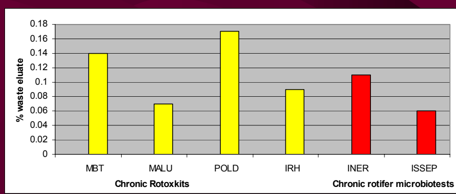
Chronic Rotoxkit microbiotests

48h EC50



INC waste

48h EC50



WOO waste

General conclusions

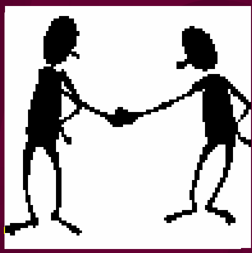
1. **Although the data set for conventional tests is very limited, the results of the Daphtoxkit and Algaltoxkit assays on waste eluates confirm the findings of previous ringtests, namely that :**

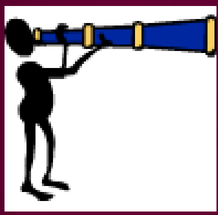
   the sensitivity of these microbiotests is identical to that of the conventional tests with the same test species.

2. **The EC50's of the Daphtoxkit and Algaltoxkit microbiotests are for most test results situated in a rather narrow range which is very similar to that of the conventional tests.**

3. **The chronic rotifer microbiotests are slightly less sensitive to the INC waste eluate, but slightly more sensitive to the WOO waste eluate, than the acute Daphnia tests**

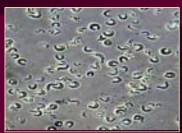
Overall, the results of the Toxkit assays in the present ringtest clearly reveal the value of microbiotests as reliable and cost-effective tools in a test battery, for the determination of the hazard of solid wastes.





Last minute first comparison of "all" the results of the conventional algae and Daphnia tests with the Algaltoxkit and Daphtoxkit microbiotests

72h ErC50's of *Pseudokirchneriella subcapitata* tests



	INC Convent.tests	INC Algaltoxkit	WOO Convent.tests	WOO Algaltoxkit
	n = 15	*n = 5*	*n = 12*	*n = 5*
Mean	6.9 %	2.5%	0.27 %	0.24 %
St.dev.	9.7	1.9	0.16	0.10
CV%	141 %	77 %	58 %	42 %

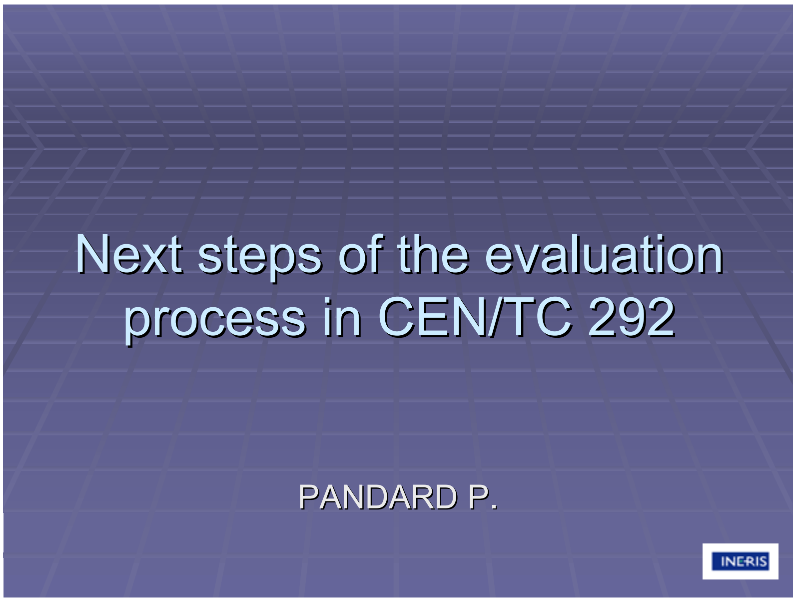
24h EC50's of *Daphnia magna* tests

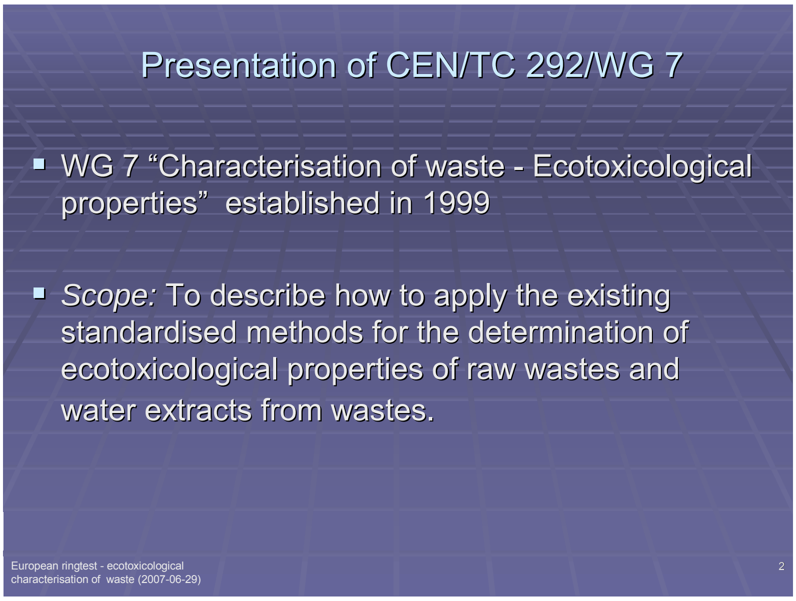
	INC		WOO	
	Convent.tests	Daphtoxkit	Convent.tests	Daphtoxkit
	n = 37	*n = 6*	*n = 47*	*n = 9*
Mean	2.85 %	2.57 %	0.51 %	0.62 %
St.dev.	1.10	1.00	0.36	0.20
CV%	39 %	39 %	69 %	32 %

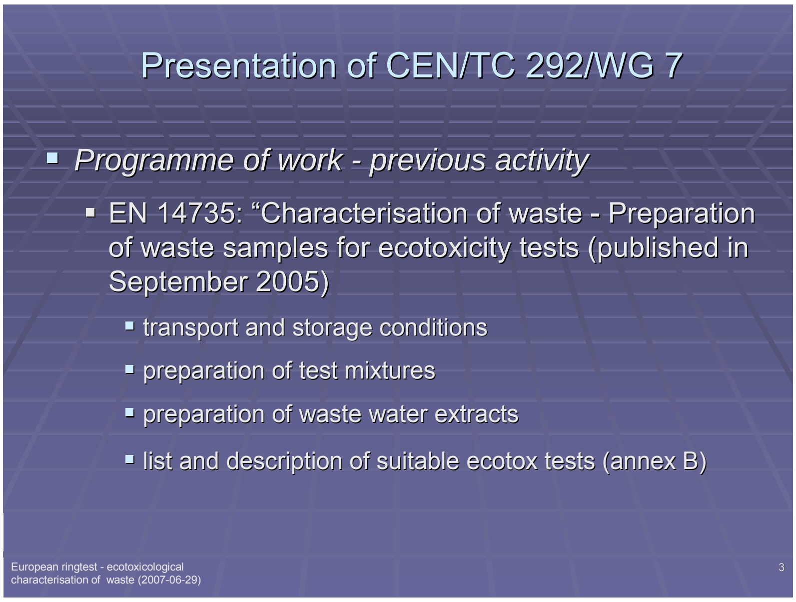
Time out

Thank you for your attention !

B.13 Talk – P. Pandard







Presentation of CEN/TC 292/WG 7

- *Programme of work - current activity*
  - WI 292050: “Characterisation of waste - Guidance on the use of ecotoxicity tests applied to waste” (preliminary work item)
    - guidance for the selection and use of ecotoxicity tests in order to identify the potential hazardous properties of wastes or to assess the risk related to a specific exposure scenario (basic ecotoxicological characterisation, re-assessment of treated waste, management of landfill sites, use of sludge in agriculture, use of mineral waste in road construction)
    - description of test limitations (confounding factors…)
    - possible modification of test design

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Use of ringtest results in the work programme of CEN/TC 292/WG 7

- Validation of EN 14735 “Characterisation of waste - Preparation of waste samples for ecotoxicity tests”
  - Suitability of test mixture and water extract preparation
  - Definition of the uncertainty of the results: reproducibility / repeatability according to ISO 5725 after removal of outliers (labs which didn’t not strictly follow the protocol of EN 14735)
    - leaching procedure (amount of waste / volume of eluate)
    - separation step (type of filter, porosity)

  => addition of a new clause or normative annex regarding uncertainty in the end result when revising the standard

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Use of ringtest results in the work programme of CEN/TC 292/WG 7

Repeatability studies - contribution of French labs

INC

	D. magna EC 50 48 h (%)			*P. subcapitata* ErC 50 72 h (%)			*V. fischeri* EC 50 30 min (%)		
	IPL/IRH (61)	INERIS (63)	POLDEN (64)	IPL/IRH (61)	INERIS (63)	POLDEN (64)	IPL/IRH (61)	INERIS (63)	POLDEN (64)
Number of tests	5	10	5	5	10	5	5	10	5
Min value	1.74	1.30	1.98	-	1.04	-	22.30	24.92	28.90
Max value	2.68	2.04	2.53	-	2.19	-	24.40	27.93	37.71
Mean value	2.37	1.62	2.32	-	1.37	-	23.44	26.75	34.35
Standard deviation (SD)	0.40	0.29	0.23	-	0.36	-	0.90	0.97	3.63
Coefficient of variation (%)	16.8	17.7	9.8	-	26.3	-	3.8	3.6	10.6
Mean value ± 2SD	1.57 – 3.17	1.04 – 2.20	1.86 - 2.78	-	0.65 – 2.09	-	21.65 – 25.23	24.81 – 28.69	27.09 – 41.61

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Use of ringtest results in the work programme of CEN/TC 292/WG 7

Repeatability studies - contribution of French labs

WOO

	D. magna EC 50 48 h (%)			*P. subcapitata* ErC 50 72 h (%)			*V. fischeri* EC 50 30 min (%)		
	IPL/IRH (61)	INERIS (63)	POLDEN (64)	IPL/IRH (61)	INERIS (63)	POLDEN (64)	IPL/IRH (61)	INERIS (63)	POLDEN (64)
Number of tests	5	10	5	5	9	5	5	10	5
Min value	0.23	0.10	0.042	-	0.14	-	0.48	0.43	0.64
Max value	0.35	0.14	0.059	-	0.20	-	0.64	0.63	1.02
Mean value	0.29	0.11	0.050	-	0.16	-	0.54	0.53	0.87
Standard deviation (SD)	0.05	0.01	0.0069	-	0.02	-	0.07	0.08	0.16
Coefficient of variation (%)	17.5	10.9	13.8	-	11.3	-	12.4	15.0	18.3
Mean value ± 2SD	0.19 - 0.39	0.09 - 0.13	0.036 - 0.064	-	0.12 - 0.20	-	0.40 - 0.68	0.37 - 0.69	0.55 - 1.19

Use of ringtest results in the work programme of CEN/TC 292/WG 7

- Validation of EN 14735 "Characterisation of waste - Preparation of waste samples for ecotoxicity tests"
  - improvement of annex B (list of suitable ecotox tests):
    - removal / addition of tests (*e.g.* Arthrobacter contact test?)
    - update of test limitations

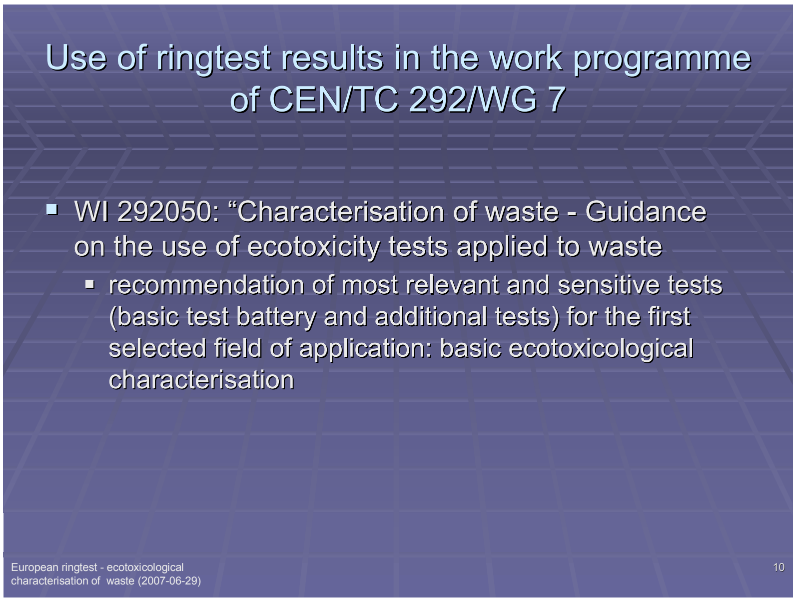
=> No proposal of testing strategy or threshold values

Use of ringtest results in the work programme of CEN/TC 292/WG 7

Annex B: example of table

***Daphnia magna* – Inhibition of mobility**

1.	**Title of the test**	Water quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea)
2.	**Harmonisation**	International
3.	**References**	EN ISO 6341
4.	**Principle**	Determination of the effect of chemicals, water samples and wastewater on mobility of young daphnids.
5.	**Test type**	Acute, static/semi-static
6.	**Test organism**	Daphnids
	Breeding stock	*Daphnia magna* Straus
	Age of test organism	< 24 h
	Feeding	None
7.	**Dilution medium**	Freshwater or synthetic reconstituted medium
	Volume	10 ml
8.	**Test conditions**	
	Test chamber size	20 ml
	Temperature	20 °C ± 2 °C
	pH	7,8 ± 0,2
	Light intensity/quality	Darkness
	Photoperiod	
9.	**Number of container, number of replicates**	5 daphnids per vessel, four replicates
10.	**Test duration**	24 h/48 h
11.	**Control**	Freshwater or synthetic reconstituted medium
12.	**Validity criteria**	Control mortality ≤ 10 % ; 0_2 concentration ≥ 2 mg/l ; sensitivity to $K_2Cr_2O_7$
13.	**Reference substance**	$K_2Cr_2O_7$: 0,6 mg/l ≤ EC 50 24h ≤ 2,1 mg/l
14.	**Statistics**	Regression, probits
15.	**Test parameter(s)**	Immobilisation
16.	**Endpoints**	EC 50
17. Application to wastes and water extracts from wastes: limitations and comments — Freshwater organisms may be affected by test mixtures that contain high amount of salts. That may occur when testing low dilutions of water extracts (e.g. dilutions factors 1/1, 1/2, 1/4). Survival may be affected by low oxygen content.		


Use of ringtest results in the work programme of CEN/TC 292/WG 7
WI 292050: "Characterisation of waste - Guidance on the use of ecotoxicity tests applied to waste
recommendation of most relevant and sensitive tests (basic test battery and additional tests) for the first selected field of application: basic ecotoxicological characterisation
European ringtest - ecotoxicological characterisation of waste (2007-06-29)
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