Follow-up study on the predictive capacity of the 3T3 Neutral Red Uptake cytotoxicity assay to correctly identify substances not classified for acute oral toxicity under the EU CLP system (LD_{50} > 2 000 mg/kg)

Final Study Report

Prepared by the European Centre for the Validation of Alternative Methods (ECVAM)

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LIST OF ACRONYMS AND ABBREVIATIONS

ATC	Acute toxic class method
BRD	Background review document
C	Classified
CLP	Classification, labelling and packaging
CSC	Chemicals Selection Committee
CV	Coefficient of variation
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EC	European Commission
ECBC	Edgewood Chemical Biological Centre
ECVAM	European Centre for the Validation of Alternative Methods
EINECS	European Inventory of existing commercial chemical substances
EPA	Environmental Protection Agency
ESR	Existing substances regulation
EU	European Union
FAL	FRAME Alternatives Laboratory
FDP	Fixed dose procedure
FN	False negative
FP	False positive
GD	Guidance document
GHS	Globally harmonised system of classification and labelling
HSL	Health and Safety Laboratory, UK
IC_{50}	Concentration producing 50% inhibition of the endpoint measured
IHCP	The Institute for Health and Consumer Protection
IIVS	The Institute for In Vitro Sciences
JRC	Joint Research Center
LD_{50}	Dose that produces lethality in 50% of test animals
MEIC	Multicentre Evaluation of *In Vitro* Cytotoxicity
MSDS	Material Safety Data Sheets
MW	Molecular weight
NA	Not applicable
NCD	New chemical database
NHK	Normal human keratinocytes
NICEATM	NTP Interagency Centre for the Evaluation of Alternative Toxicological Methods
NIEHS	US National Institute of Environmental Health Sciences
NIOSH	National Institute for Occupational Safety and Health
NPV	Negative predictive value
NRU	Neutral red uptake
NTP	National Toxicology Program
OECD	Organisation for Economical Cooperation and Development
ORATS	Online European Risk Assessment Tracking System
PC	predictive capacity
PPV	Positive predictive value
RAR	Risk assessment report
RC	Registry of Cytotoxicity

RF	Range finding
ROC	Receiver operating characteristic
RTECS	Registry of Toxic Effects for Chemical Substances
SD	Standard deviation
SDS	Sodium dodecyl sulphate
3T3	BALB/c mouse fibroblasts
TG	Test Guideline
TN	True negative
TP	True positive
UC	Unclassified
UDP	Up and down procedure
UN	United Nations
VC	Vehicle control
ZEBET	Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch (German Centre for Documentation and Evaluation of Alternatives to Animal Experiments)

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1.0 GENERAL INTRODUCTION

1.1 Current regulatory testing requirements and classification schemes for acute oral toxicity

Currently, for regulatory purposes, all the accepted methods for determining the acute oral toxicity are based on *in vivo* experiments that estimate the LD_{50} value (i.e. the single dose of a substance that can be expected to cause death in 50% of the animals in an experimental group). They include three approved refinement and reduction alternative methods (modifications of the classical LD_{50} test) described in the Organisation for Economical Cooperation and Development (OECD), Test Guidelines TG 420 (Fixed Dose Procedure, FDP), TG 423 (Acute Toxic Class Method, ATC) and TG 425 (Up and Down Procedure, UDP) (OECD, 2001a,b,c). All three methods are sequential tests where the outcome of the previous step/dose determines the next dose to be tested. Of these three methods, for FDP the number of animals required/test is 5–7, ATC uses on average 7 animals, and UDP about 6–9 animals. The main endpoint for FDP is evident toxicity while ATC and UDP use lethality as endpoint. FDP and ATC provide an estimated LD_{50} range, whereas UDP gives an LD_{50} point estimate together with confidence interval (Anon 2006, Creton et al., 2010).

Acute oral toxicity data are required for agrochemicals and biocides, and, depending on the country, they may also be required for industrial chemicals. Acute toxicity testing in animals of cosmetic ingredients and finished products is banned in the EU (Anon 2003). There are no requirements of acute oral toxicity testing of food additives, flavourings, food-contact materials, pharmaceuticals, or veterinary medical products (Seidle et al. 2010).

The major driver for conducting acute oral toxicity studies is for classification and labelling to allow substances to be categorised according to their potential hazards and the dose required to cause toxicity (Creton et al. 2010; Seidle et al. 2010). In order to increase consistency among diverse national and sectoral frameworks, the Globally Harmonised System of Classification and Labelling (GHS) was developed under the auspices of the United Nations (UN 2007). The implementation of GHS around the

world is resulting in some differences, due to the flexibility provided by the GHS modular design.

In the EU, the new regulation on classification, labelling and packaging (CLP) (Anon 2008) of substances and mixtures entered into force in 2009 to align previous EU legislation Dangerous Substances Directive (Directive 67/548/EEC, and the Dangerous Preparations Directive (Directive 1999/45/EC) to the GHS. According to this new regulation, the chemicals are allocated in one of 4 toxicity categories based on their acute oral toxicity properties according to the following cut-off criteria:

- category 1: $LD_{50} \leq 5$ mg/kg
- category 2: $5 < LD_{50} \leq 50$ mg/kg
- category 3: $50 < LD_{50} \leq 300$ mg/kg
- category 4: $300 < LD_{50} \leq 2\,000$ mg/kg.

Under this EU CLP classification scheme the limit dose is 2 000 mg/kg beyond which the chemicals do not require to have a hazard label for acute oral toxicity.

For the purpose of the present validation study and based on the EU CLP classification scheme, chemicals are assigned to two groups according to the 2 000 mg/kg cut-off limit and, therefore, a chemical will be categorised as 'classified' if the $LD_{50} \leq 2\,000$ mg/kg b.w. and as 'unclassified' if the $LD_{50} > 2\,000$ mg/kg b.w.

In the US, the Occupational Safety and Health Administration also accepts a limit dose of 2 000 mg/kg. However, other US Federal agencies require testing to a limit dose of 5 000 mg/kg to support a non-label designation (unclassified if $LD_{50} > 5\,000$ mg/kg). According to the GHS the chemicals are classified into 5 toxicity categories according to the following cut-off criteria:

- category 1: $LD_{50} \leq 5$ mg/kg
- category 2: $5 < LD_{50} \leq 50$ mg/kg
- category 3: $50 < LD_{50} \leq 300$ mg/kg
- category 4: $300 < LD_{50} \leq 2\,000$ mg/kg
- category 5: $2\,000 < LD_{50} \leq 5\,000$ mg/kg

1.2 Historical background and rationale for the use of *in vitro* cytotoxicity assays to predict acute oral toxicity

Several international programmes have explored in the past the possibility to use cell-based methods to predict acute oral toxicity. The first study in which this concept was investigated was the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) programme. The MEIC was initiated in 1983 to investigate the relevance of *in vitro* cytotoxicity test methods for human acute toxicity by correlative comparisons (Bondesson et al. 1989). The programme involved 96 laboratories worldwide, testing 50 reference chemicals using laboratory-specific *in vitro* cytotoxicity assays (Ekwall et al. 1998a, 1998b, 1999, 2000). The chemicals were selected to represent different chemical classes for which reference acute oral lethality data were available from humans (human lethal whole-blood concentration) and rodents (oral LD_{50} values). Rat and mouse oral LD_{50} data were collected from the Registry of Toxic Effects for Chemical Substances (RTECS) at the National Institute for Occupational Safety and Health (NIOSH).

In the MEIC programme, the *in vitro* tests correlated well with acute oral lethality data in humans (clinical and forensic human lethal blood concentrations), whereas rat and mouse oral LD_{50} correlations with human lethal dosage were only relatively good. These results indicated that *in vitro* basal cytotoxicity assays, compared to rodent acute oral toxicity tests, might be more accurate when estimating human acute oral lethality. The study further showed that the best correlations were found with human cell lines (Ekwall et al. 1998b). However, it was concluded that improvements in the prediction of human acute oral lethality were necessary before *in vitro* cytotoxicity assays could replace animal tests (Ekwall et al. 1998c).

In addition to these experimental studies, William Halle compiled the so-called Registry of Cytotoxicity (RC), which is a database of rodent acute oral LD_{50} values originally derived from RTECS, and published IC_{50} values from diverse *in vitro* cytotoxicity assays (Halle 1998, 2003). The printed version of RC (Halle 2003) compiled available data on over 350 different chemicals and has been a significant source of reference chemicals, containing a wide range of *in vivo* toxicity data from rodent acute oral studies, and allowing correlation of IC_{50} results with corresponding LD_{50} data for systematic evaluation of predictive capacity among *in vitro* methods.

More recently, RC has become a maintained database (unpublished, in electronic format) subject to continuous update and expansion, managed by The Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET, Germany). At present, the electronic version of RC comprises approximately 550 chemicals.

Among the many chemical classes represented in the RC, three classes of chemicals known to be problematic in *in vitro* cytotoxicity assays included neurotoxicants, insecticides, and chemicals requiring metabolic activation. The RC method, based on the comparison of the IC_{50} values and the LD_{50} values by using linear regression analysis, was able to predict the acute oral LD_{50} values for 252 out of 347 chemicals, and the intravenous LD_{50} for rats and/or mice for 117 out of 150 chemicals. The results were highly reproducible (Halle 2003).

In 1994 the concept of invoking *in vitro* data to determine starting doses for rodent acute oral toxicity tests, thereby reducing the number of animals used, was proposed at a workshop coordinated by the European Centre for the Validation of Alternative Methods (ECVAM) (Seibert et al. 1996). Essentially, it was proposed that the regression equation from correlation of RC database IC_{50} versus LD_{50} could be applied to estimate unknown LD_{50} values for a novel chemical from IC_{50} values measured as basal cytotoxicity *in vitro*, which would then be taken as a starting dose for the *in vivo* experiment (Spielmann et al. 1999).

In 2000, the US National Institute of Environmental Health Sciences (NIEHS), the National Toxicology Program (NTP), and the US Environmental Protection Agency (EPA) jointly sponsored an International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, to review the implementation of *in vitro* basal cytotoxicity assays in regulatory screening testing strategies (ICCVAM 2001). The workshop concluded that no *in vitro* cytotoxicity test method (or battery of assays) was available to replace the animal experiment. Moreover, it was concluded that none of the *in vitro* models reviewed had been adequately evaluated for reliability and relevance, leaving their applicability to generating information for acute oral toxicity testing open to further validation. However, there was agreement that *in vitro* basal cytotoxicity test methods would be useful for estimating the starting dose for rodent

acute oral toxicity studies (ICCVAM 2001, Botham 2004). In addition, the ICCVAM workshop recommended further development, optimisation, and validation of *in vitro* test methods with focus on target organ specificity and mechanistic factors such as absorption, distribution, metabolism, and excretion, which act to modulate lethality of xenobiotic response.

Subsequently, the adoption of OECD Test Guidelines (TG) 420, TG 423, TG 425 which are based on a stepwise procedure with the use of a minimum number of animals per step, resulted in a significant reduction of animal use. Also the OECD Guidance Document 24 on Acute Oral Toxicity Testing recommends the use of results from *in vitro* toxicity tests, amongst others, to assist in selecting the starting dose, particularly in cases where minimal prior information on the chemical is available (OECD, 2001d).

Following the 2000 ICCVAM workshop, the NTP Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) and ECVAM conducted a joint validation study of the Neutral Red Uptake (NRU) basal cytotoxicity assay performed in two standard cell systems: a human cell system (normal human keratinocytes, NHK), and a rodent cell system (BALB/3T3 cell line). The study involved 72 reference chemicals (12 representatives of each GHS toxicity category, including not classified) where MEIC and RC were major source databases. The study report is accompanied by a refined database re-compiling ~500 LD_{50} values obtained from ~200 study references (Anon 2006). The peer review panel of the NICEATM/ECVAM validation study concluded that the NRU basal cytotoxicity test method using the 3T3 mouse embryonic fibroblast cell line may be useful in a weight-of-evidence approach to determine the starting dose for acute oral *in vivo* toxicity protocols. The results of this study also showed that the overall accuracy of the 3T3 NRU test method for correctly predicting each of the GHS acute oral toxicity classification categories was low (around 30 %) (Anon 2006).

Although it is expected that the use of *in vitro* methods will reduce the number of animals required for each toxicity test, to date the cytotoxicity assays are recognised only as additional tests that can be used for estimating the initial doses for acute oral systemic toxicity tests *in vivo*.

Based on the results of the NICEATM/ECVAM validation study OECD has adopted a Guidance Document (GD No 129) that describes methods to determine the *in vitro* basal cytotoxicity of test substances using NRU assays and the use of the *in vitro* data to determine starting doses for *in vivo* acute oral systemic toxicity tests (OECD 2010).

1.3 Rationale for the use of the 3T3 NRU assay to identify compounds not classified for acute oral toxicity

The results of MEIC (Clemedson et al 1996), the Halle RC (Halle 2003), and the NICEATM/ECVAM international validation study (Anon 2006) have all shown a correlation of around 60 - 70% between *in vitro* IC_{50} cytotoxicity data and oral rat LD_{50} values. Furthermore, these studies indicated that with the *in vitro* cytotoxicity test methods, the precision of prediction of low systemic toxicity from cytotoxicity data is much better than the prediction of high systemic toxicity (Figure 1), suggesting that the 3T3 NRU test method could be useful to discriminate between chemicals with LD_{50} value $\leq$ 2 000 mg/kg b.w. (classified chemicals) and chemicals with LD_{50} > 2 000 mg/kg b.w. (unclassified), according to the new EU CLP classification system (Anon 2008).

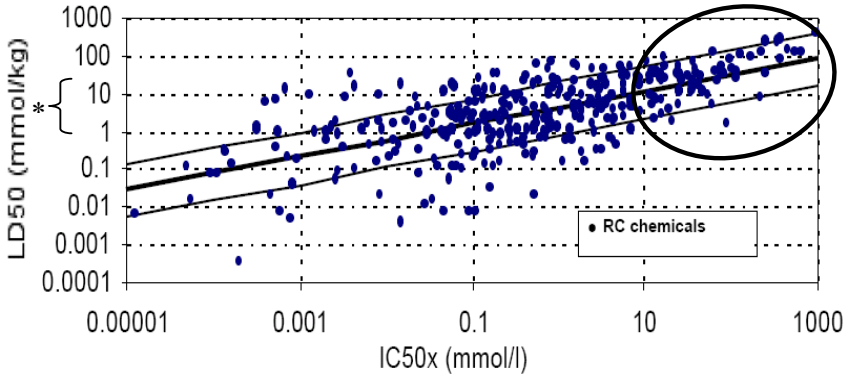

Figure 1. Registry of Cytotoxicity regression between cytotoxicity (IC_{50x}) and rodent acute oral log LD_{50} values of 347 chemicals (taken from Halle 2003). The circle indicates the part of the regression where the best prediction is obtained (low systemic toxicities).

* The left brace on the y-axis shows the range of *in vivo* LD_{50} mmol/kg values corresponding to a LD_{50} cut-off value of 2 000 mg/kg depending on the molecular weight of the chemical used (in the present study the range of molecular weights of interest for non-classified chemicals was from 60.06 to 1 228 Daltons, see Table 3).

An analysis performed on dossiers from New Chemical Database (NCD) maintained at the Institute for Health and Consumer Protection (JRC, Ispra) (http://ecb.jrc.ec.europa.eu) until 2008, showed that most of the industrial chemicals tested for regulatory purposes (~96.24%) in the EU fall into two categories of the GHS classification system, i.e. harmful (9.4% – 300 < LD_{50} ≤ 2 000 mg/kg) and not classified (86.6% – LD_{50} > 2 000 mg/kg) (Bulgheroni et al. 2009).

The follow-up study was motivated by two assumptions. First, it was assumed that the 3T3 NRU cytotoxicity assay may allow discrimination of a large fraction of the EU CLP unclassified compounds without giving false negative results. Secondly, it was assumed that the high prevalence of unclassified chemicals is the same in the whole population of chemicals registered (i.e. the proportion of chemicals with LD_{50} > 2 000 mg/kg b.w. is approximately 87%). Therefore, it was envisaged that the use of this test method in a tiered testing approach could significantly reduce *in vivo* testing for acute oral toxicity.

1.4 Aim of the study

The aim of this ECVAM validation study was to assess the predictive capacity (sensitivity, specificity, accuracy, positive predictive value and negative predictive value) of the 3T3 NRU cytotoxicity test to determine if a test chemical correctly falls into one of the two categories, unclassified (LD_{50} > 2 000 mg/kg), or classified (LD_{50} ≤ 2 000 mg/kg). The study used the test method protocol and the IC_{50}-LD_{50} regressions validated in the NICEATM/ECVAM validation study (Anon 2006).

In addition, two protocol modifications were assessed: one version of the 3T3/NRU protocol adapted to an automated platform and an abbreviated version of the validated protocol that was targeted at resolving acute oral toxicities around the 2 000 mg/kg cut-off value. The aim of this additional testing was to assess whether these variants of the validated protocol would generate similar data on the basis of the test chemicals selected and to assess, therefore, to which extent may be used for purposes of identifying negatives (LD_{50} > 2 000 mg/kg b.w.).

This method could be used as the first step of a tiered approach to identify the unclassified chemicals that would not need to be tested further in the *in vivo* acute oral toxicity tests.

These approaches could contribute to the reduction and refinement of the use of animals for acute oral toxicity testing.

2.0 ORGANISATION OF THE STUDY

2.1 Participating Laboratories

The objective of this validation study was to assess the ability of the 3T3 NRU test method to discriminate between classified (toxic/hazardous) ($LD_{50} \leq 2\ 000$ mg/kg) and unclassified ($LD_{50} > 2\ 000$ mg/kg) chemicals according to the current EU CLP system for acute oral toxicity.

In this case, according to the ECVAM's modular approach to validation (Hartung et al. 2004), only one laboratory was required to assess the predictive capacity and applicability domain (modules 5 and 6), since the test definition, within- and between-laboratory reproducibility and transferability (modules 1–4) have been already extensively assessed during the previous NICEATM/ECVAM validation study from 2002 to 2005, and the test has proved to be reliable (Anon 2006).

For that reason, only one laboratory was appointed to apply the original manual protocol validated in the NICEATM/ECVAM validation study to a new set of reference chemicals with good *in vivo* data on acute oral toxicity.

The Health and Safety Laboratory (HSL, UK) was awarded by ECVAM a contract (contract number CCR.IHCP.C433987.XO) to test coded chemicals using the previously validated 3T3 NRU test method protocol (Annex A). The study started in November 2007.

In addition, two other laboratories volunteered to use the same set of test chemicals in two variations of the 3T3 NRU test method protocol:

- The Institute for Health and Consumer Protection (IHCP) of the Joint Research Centre (JRC, Italy) used an automated version of the 3T3 NRU test method protocol adapted to its robotic testing platform (see Section 5.6 and Annex B).

- The Institute for In Vitro Sciences (IIVS, US) used a less costly abbreviated version of the 3T3 NRU test method protocol which only tested concentrations expected to be near the cut-off LD_{50} value of 2 000 mg/kg b.w. (see Section 5.6 and Annex C). The

IIVS laboratory participated in the previous NICEATM/ECVAM validation study and was experienced in the test method.

2.2. Management of the Study

Since the study was planned as a follow-up of the NICEATM/ECVAM validation study, and was initially intended to be performed in only one laboratory, the Management Team consisted merely of two staff members of ECVAM.

2.3. Chemical Selection Committee

A Chemicals Selection Committee (CSC) was appointed to identify test chemicals to be used in the study. The CSC consisted of Manfred Liebsch (ZEBET, Germany), Thomas Cole (IHCP), Pilar Prieto (ECVAM) and Agnieszka Kinsner-Ovaskainen (ECVAM). Fifty-six industrial chemicals were selected for testing (see Section 3).

The testing chemicals were purchased by ECVAM from Sigma-Aldrich and coded. The distribution of chemicals and respective material safety data sheets (MSDS) was done in January 2008 by Sigma-Aldrich-Germany for the two European laboratories and Sigma-Italy for the laboratory in the US.

Figure 2 illustrates the organisation of the study with respect to the test methods included and the participating laboratories, as well as the responsibilities for management of the project, selection, coding and supply of test chemicals and the data analysis. ECVAM staff members coordinated the study, and ICCVAM was involved in the study as a liaison.

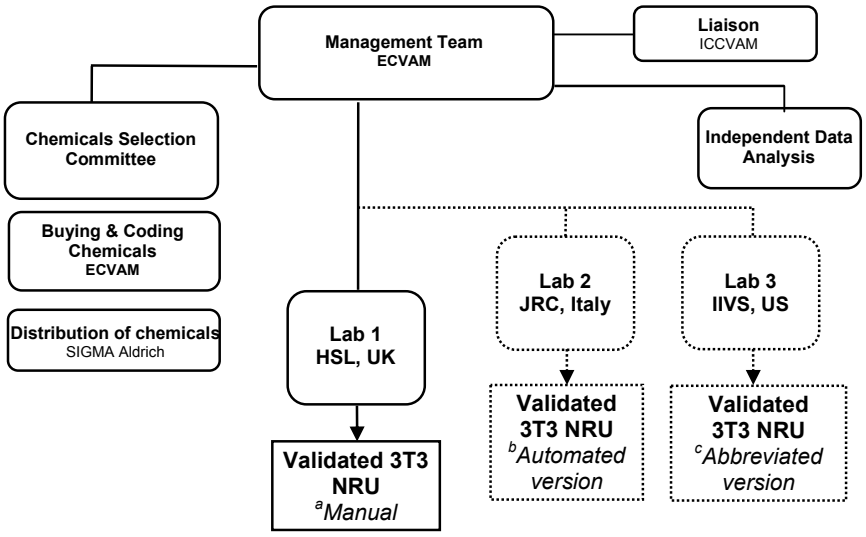

Figure 2. The organisation of the 3T3 NRU validation study

The core validation exercise concerned only Laboratory 1 (HSL, UK), which worked with the validated manual protocol.
Dashed line: Laboratories 2 (JRC, Italy) and 3 (IIVS, US) produced additional data on the basis of two protocol variants supporting a comparative analysis of protocol performance.
[a]Protocol available in Annex A.
[b]Automated version of validated protocol available in Annex B
[c]Abbreviated protocol only tested concentrations expected to be near the cut-off of LD_{50} = 2 000 mg/kg b.w.(Annex C).

2.4 Study timelines

At the end of October 2008, a meeting with the three laboratories was held at ECVAM during which the results from the solubility test performed in the three laboratories to select suitable solvents for the test chemicals, and to determine the solubility at the highest stock concentration to be used in the cytotoxicity assay were presented. Problems encountered were also discussed. The codes of the chemicals were not broken since the testing was not finalised in all laboratories. HSL presented the results obtained with the 9 substances selected to assess transferability of the validated 3T3 NRU test method protocol (Ethylene glycol, Sodium chloride, Boric acid, Sodium fluoride, Phenol, Potassium cyanide, Mercury chloride, Sodium arsenite, and Cycloheximide). Furthermore, in September 2009 a teleconference was held with the three laboratories and ICCVAM representatives to discuss the statistical analysis of the data.

Final reports from HSL, JRC and IIVS were received in October 2009, October 2008, and November 2008, respectively.

The decoding of test chemicals took place at the end of November 2009.

3.0 REFERENCE TEST CHEMICALS USED IN THE STUDY

3.1 Rationale and criteria used for selection of reference test chemicals

Test chemicals for which *in vivo* data were available for correlation with the *in vitro* measurements (IC_{50} values) were selected from three sources: 1) the ORATS (Online European Risk Assessment Tracking System) database, 2) the Registry of Cytotoxicity, 3) Annex I of Directive 67/548/EEC. Chemicals used in previous studies, in particular the NICEATM/ECVAM validation study and the EU FP6 integrated project ACuteTox (www.acutetox.eu, Clothier et al 2008), were excluded. Pre-defined selection criteria were applied, primarily to ensure the quality of the *in vivo* data and the practicability of testing the test chemicals *in vitro*. Pharmaceuticals and pesticides were excluded. Exceptions were two highly toxic compounds - brucine and aconitine (substances in toxicity category: GHS 1), which are natural alkaloids. Aconitine is a neurotoxin used in the past as a homicidal weapon and still with some limited application in herbal medicine. Brucine is used in industry, agriculture, and homeopathic medicine. Finally, a total set of 56 industrial chemicals, including cosmetic ingredients, were selected for independent coding and supply to the participating laboratories. The selected chemicals included a variety of molecular structures, balanced between two physical states (liquid and solid), as well as a wide range of physico-chemical properties. Moreover, the chemicals sample sizes were designed to distinguish chemicals with LD_{50} > 2 000 mg/kg b.w. (EU CLP classification system) (see 3.1.1).

3.1.1 General selection criteria

The primary goal of this validation study was to evaluate whether the validated 3T3 NRU test method was able to identify correctly the unclassified chemicals according to the EU CLP system for acute oral toxicity (i.e. LD_{50} > 2 000 mg/kg b.w.). Since the application of the test method was mainly intended for industrial chemicals and cosmetic ingredients, the main criterion was to include only industrial chemicals (excluding pharmaceutical and pesticides), with a statistically justified distribution:

1. ~50% compounds with LD_{50} > 2 000 mg/kg b.w.
2. ~50% compounds with $LD_{50} \leq$ 2 000 mg/kg b.w., classified in Annex I of Directive 67/548/EEC as very toxic, toxic or harmful

Moreover, it was sought to have a balance between liquid and solid chemicals.

3.1.2 Selection of classified chemicals ($LD_{50} \leq 2\ 000$ mg/kg b.w.)

Chemicals that were positive in acute oral dose tests, leading to an acute hazard label (very toxic, toxic, hazard, i.e. $LD_{50} \leq 2\ 000$ mg/kg) were selected from Annex I of Directive 67/548/EEC, which contains a list of harmonised classifications and labelling for chemicals or groups of chemicals, which are legally binding within the EU. The Annex I used for the selection of chemicals for the present study was valid until December 2010 after which it was replaced by Annex VI of the new EU CLP Regulation (Anon 2008).

For the first selection step the following criteria were applied:

1. All chemicals with the classification Xn (harmful), R22 (harmful if swallowed), T (toxic), R25 (toxic if swallowed), T+ (very toxic), R28 (very toxic if swallowed) were extracted from Annex I;
2. Only those compounds listed on EINECS (European Inventory of existing commercial chemical substances) were included (i.e. new chemicals, and others with no EC number were eliminated);
3. Only chemicals with data from rat oral acute toxicity experiments were included.
4. Chemicals with classification CMR (carcinogenic, mutagenic, reproductive toxicity), E (explosive), O (oxidising), F (flammable), and C (corrosive), were excluded;
5. In case of chemicals labeled Xn, the compounds classified as T/T+ by other routes (dermal, inhalation) and/or effect after prolonged exposure (R48) were excluded [e.g., T; R48/25 (Toxic: danger of serious damage to health by prolonged exposure if swallowed)];
6. In case of chemicals labelled T, the compounds classified T/T+ by other routes (dermal, inhalation) and/or T effect after prolonged exposure (R48) and/or cumulative effect (R33) were excluded.

The search in Annex I for chemicals with risk phrase R22 only (i.e. Xn, harmful by oral exposure) resulted in a total of 531 chemicals. Eliminating chemicals with no EC number (i.e., including only those with EINECS number) reduced the list to 296 chemicals. Further elimination of compounds with classifications CMRs, E, O, F, C,

and T/T+ by alternative dose route/chronic exposure, as well as entries with mixed CAS numbers led to a list of 157 chemicals**.**

The search in Annex I for chemicals with risk phrase R25 (i.e. T, toxic by oral exposure) excluding combination with other exposure routes resulted in a total of 339 chemicals. Eliminating chemicals with no EC number (i.e., including only those with EINECS number) reduced the list to 265 chemicals. Elimination of compounds with classifications CMR, E, O, F, C, R31, R32, T+ by alternative dose route, T by chronic exposure (R48, R33), as well as entries with mixed CAS numbers further reduced the list to 87 chemicals.

The search in Annex I for chemicals with risk phrase R28 (i.e., T+ toxic by oral exposure) excluding combination with other exposure routes resulted in 150 chemicals. Eliminating chemicals with no EC number (i.e., including only those with EINECS number) reduced the list to 119 chemicals. Elimination of compounds with classifications CMR, E, O, F, C, T+ with R33 (cumulative effects) and entries with mixed CAS numbers decreased the number of candidates to 76.

Summing up the first search resulted in a total of 320 chemicals:

- 157 chemicals classified as hazardous by acute oral exposure (Xn, R22 [200 mg/kg < LD_{50} ≤ 2 000 mg/kg];
- 87 chemicals classified as toxic by acute oral exposure (T, R25 [25mg/kg < LD50 ≤ 200 mg/kg];
- 76 chemicals classified as very toxic by acute oral exposure (T+, R28 [LD50 ≤ 25 mg/kg].

The list was further reduced by excluding:

- Pesticides and pharmaceuticals;
- Chemicals used in previous studies i.e. the NICEATM/ECVAM validation study and the EU ACuteTox project;
- Chemicals with inconsistent or missing LD_{50} data.

This second selection resulted in a list of a total of:

- 38 chemicals classified as hazardous (Xn, R22);

- 11 chemicals classified as toxic (T, R25);
- 7 chemicals classified as very toxic (T+, R28).

This list was further reduced by applying the following exclusion criteria:

- Chemicals not available in Sigma-Aldrich;
- Chemicals potentially difficult to handle in the laboratory or *in vitro* (based on indication from the physico-chemical properties);
- Insoluble metals;
- Chemicals with uncertain classification (LD_{50} value not compatible with official EU classification in Annex I).

The final list contains a total of 30 chemicals of which 19 were solids and 11 liquids. In addition, 8 chemicals are used as cosmetic ingredients, according to the European Commission (EC) database CosIng (http://ec.europa.eu/consumers/cosmetics/cosing/) (see Tables 1 and 2).

3.1.3 Selection of unclassified chemicals (LD_{50} > 2 000 mg/kg b.w.)

To select the chemicals with LD_{50} > 2 000 mg/kg b.w., two sources were consulted: 1) the ORATS database and 2) the RC.

The ORATS, (freely accessible at http://ecb.jrc.it/esis/index.php?PGM=ora) provides information on the progress of implementation of Council Regulation (EEC) 793/93 on existing substances, which has been revoked and replaced by the Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), introducing a comprehensive framework for the evaluation and control of "existing" chemicals. The system contains 4 lists of priority chemicals (a total of 141 chemicals, collected from 1994) which require immediate attention because of their potential effects to man or the environment, together with the Risk Assessment Reports (RAR). These dossiers contain (among many others information) LD_{50} values reviewed by experts in the field.

The RC is part of the ZEBET database and provides *in vitro* IC_{50} values as well as acute oral toxicity data (LD_{50}) for rats and mice for approximately 550 chemicals. The LD_{50} values come from the RTECS database at the NIOSH.

The selection criteria applied were the following:

1. Only chemicals with an LD_{50} > 2 000 mg/kg bw. were included;
2. Chemicals not available (based on the CAS number) from Sigma-Aldrich were excluded;
3. Only chemicals with rat oral LD_{50} values were included;
4. Chemicals with reported LD_{50} values > 2 000 mg/kg bw. but classified in Annex I as "harmful", "toxic" or "very toxic" if swallowed (risk phrase R22, R25, R28) were excluded;
5. Only industrial chemicals were included;
6. Flammable and highly flammable compounds were excluded;
7. Chemicals were excluded if their LD_{50} values showed large differences between RC and Annex I since they were considered confounding.

The final list contains 26 chemicals from which 13 were liquids and 13 solids. In addition, 19 chemicals are used as cosmetic ingredients, according to EC database CosIng (http://ec.europa.eu/consumers/cosmetics/cosing/) (see Tables 1 and 3).

Table 1. Summary of the steps and criteria for the selection of the test chemicals

Selection of classified chemicals (LD_{50} ≤ 2 000 mg/kg b.w.)	**Annex I Dir 67/548/EEC**			**Total Number**
	Xn	T	T+	
Starting pool Including only data from rat oral exposure experiments	531	339	150	**1020**
Only EINECS	296	265	119	680
First reduction: excluding chemicals with - CMR, E, O, F, C classification - T/T+ by alternative dose routes - T/T+ by chronic exposure (R48, R33) - Entries with mixed CAS	157	87	76	320
Second reduction: excluding pesticides and pharmaceuticals, chemicals used in previous studies, chemicals with inconsistent or missing LD_{50} values	38	11	7	56
Third reduction: excluding chemicals not available in SIGMA, potentially difficult to handle, insoluble metals, chemicals with uncertain classification	22	6	2	**30** (19 solids, 11 liquids) 8 in CosIng

Table 1. Summary of the steps and criteria for the selection of the test chemicals

Selection of unclassified chemicals (LD_{50} > 2000 mg/kg b.w.)	**ORATS and RC**	**Total Number**
Starting pool		**691**
Including: chemicals with rat oral LD_{50} > 2000 mg/kg values; industrial chemicals Excluding: chemicals not available in SIGMA, flammable and highly flammable chemicals; inconsistent classification when compared to RC and/or Annex I		**26** (13 liquids, 13 solids) 19 in CosIng

Xn = harmful; T = toxic; T+ = very toxic; R48 = prolonged exposure; R33 = cumulative effects; RC = Registry of Cytotoxicity; ORATS = Online European Risk Assessment Tracking System; CMR = carcinogenic, mutagenic, reproductive toxicity; E = explosive; O = oxidasing; F = flammable; C = corrosive; CosIng = the European Commission database with information on cosmetic substances and ingredients

3.2 Procurement, coding, and distribution of the reference test chemicals

The chemicals were coded by ECVAM (Annex D) and purchased from Sigma-Aldrich. The distribution of chemicals and respective MSDS was done by Sigma-Aldrich-Germany for the two European labs and Sigma-Italy for the laboratory in the US. Due to current law concerning the shipment of chemicals, Sigma-Aldrich was not allowed to send non-identified toxic substances. Therefore, each laboratory nominated a person, independent from the study, who received the chemicals together with a sealed list of the coded items and MSDS (each in a separate sealed envelope, with the code displayed on top).

Table 2. Thirty chemicals selected for the 3T3 NRU validation study bearing an acute hazard label according to EU CLP classification scheme (classified chemicals)

EU CLP	Chemical name	CAS No	Melting point (ºC)	Vapour press. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/Use
1	Aconitine	302-27-2	204	1.38 E-20	717.2	1.37	645.74	≥ 95%	solid	0.13	310	T+; R28	Neurotoxin used in the past as a homicidal weapon and still with some limited application in herbal medicine
1	Brucine	357-57-3	178	3.13 E-10	633.7	1.41	394.46	98%	solid	0.98	3200 (15ºC)	T+; R28	Toxin (related to strychnine) used in industry, agriculture, and homeopathic medicine
2	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl) ammonium sulphate	25646-77-9	155	6.01 E-6	364.3	-	292.35	-	solid	0.98	-	T; R25	Industrial (photographic processing)
3	1-Phenyl-3-pyrazolidone	92-43-3	126	1.63 E-5	304.1	1.188	162.19	≥ 96.5%	solid	0.89	1.16 E+4	Xn; R22	Industrial (anti-oxidant, e.g., photochemicals)
3	Barium chloride	10361-37-2	963	3.39 E4	1560	3.856	208.23	99.999%	solid	-	-	T; R25	Industrial (reagent)
3	Copper sulphate	7758-98-7	200	7.3	-	3.603	159.61	≥ 99.99%	solid	-	-	Xn; R22	Industrial (reagent) Cosmetic (skin conditioning)**
3	Ethyl chloroacetate	105-39-5	-21.0	4.87	144.3	1.145	122.55	99%	liquid	0.94	1.94 E+4 (30ºC)	T; R23/24/25	Solvent, organic synthesis

Table 2. Thirty chemicals selected for the 3T3 NRU validation study bearing an acute hazard label according to EU CLP classification scheme (classified chemicals)

EU CLP	Chemical name	CAS No	Melting point (ºC)	Vapour press. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/Use
3	Malononitrile	109-77-3	32	0.125	218.5	1.049	66.06	> 99%	solid	-0.6	1.33 E+5	T; R23/24/25	Industrial (intermediate)
3	*P*-penzoquinone	106-51-4	115.7	0.1	174.0	1.256	108.09	98%	solid	0.2	1.11 E+4 (18ºC)	T; R23/25	Industrial (ingredient, reagent)
3	Resorcinol	108-46-3	111	1	280	1.272	110.11	98%	solid	0.8	7.17 E+5	Xn; R22	Industrial (reagent, intermediate) Cosmetic (hair dyeing, masking)**
3	Sodium salt of chloroacetic acid	3926-62-3	199 (decomp.)	0.259	189.0	1.393	116.48	98%	solid	0.22	-	T; R25	Industrial (reagent, intermediate)
4	1,2,4-Trichlorobenzene	120-82-1	17	0.46	213.5	1.454	181.45	> 99%	liquid	4.02	49	Xn; R22	Industrial (solvent: e.g., rubber, polystyrene manufacture)
4	1,2-Dichlorobenzene	95-50-1	-16.7	1.36	180	1.306	147.00	99%	liquid	3.43	156	Xn; R22	Industrial (solvent)
4	1-Naphthylamine	134-32-7	49.2	0.012 hPa/(30C) 0.004 hPa/(20C)	300.8	1.114	143.19	~ 98%	solid	2.25	1700 (20C)	Xn; R22	Industrial (intermediate, reagent for dyestuffs)
4	2,4,6-Tris(dimethylamino-methyl) phenol	90-72-2	-20	5.60 E-4	156 (decomp)	0.969	265.39	95%/ 70%	liquid	-0.66	>850 g/l (20ºC)	Xn; R22	Industrial (epoxy resin polymerisation accelerator)

Table 2. Thirty chemicals selected for the 3T3 NRU validation study bearing an acute hazard label according to EU CLP classification scheme (classified chemicals)

EU CLP	Chemical name	CAS No	Melting point (ºC)	Vapour press. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/Use
4	2,6-Diethylaniline	579-66-8	3.5	0.00383	235.5	0.906	149.23	98%	liquid	3.15	670	Xn; R22	Industrial (herbicide intermediate)
4	2-Chloro-4-nitroaniline	121-87-9	108	4.85 E-4	326.2	1.494	172.57	97%/ 99%	solid	2.12	933	Xn; R22	Industrial (intermediate for dyes, pigments)
4	2-Phenoxyethanol	122-99-6	14	0.007	245	1.107	138.16	-	liquid	1.16	2.67 E+4	Xn; R22	Industrial (e.g., additive in cosmetics) Cosmetic (preservative)**
4	Acetophenone	98-86-2	20	0.397	202	1.03	120.15	≥ 98%	liquid	1.58	6130	Xn; R22	Industrial (solvent, reagent, intermediate, ingredient in flavours & fragrances) Cosmetic (masking)**
4	Ammonium chloride	12125-02-9	340	1 (160.4C)	-	1.00	53.49	-	solid	-	-	Xn; R22	Industrial (various) Cosmetic (buffering, masking, viscosity controlling)**

Table 2. Thirty chemicals selected for the 3T3 NRU validation study bearing an acute hazard label according to EU CLP classification scheme (classified chemicals)

EU CLP	Chemical name	CAS No	Melting point (ºC)	Vapour press. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/Use
4	Benzaldehyde	100-52-7	-26.0	0.127	179	1.044	106.12	≥ 99.5%/ ≥ 98%	liquid	1.48	6570	Xn; R22	Industrial (reagent, intermediate, flavour) Cosmetic (denaturant, masking, solvent)**
4	Benzyl benzoate	120-51-4	21	2.24 E-4	323.5	1.118	212.24	≥ 99%	liquid	3.97	15.4	Xn; R22	Industrial (polymer plasticiser, solvent, food flavour) Cosmetic (antimicrobial, perfuming and solvent, restricted substance)**
4	Diallyl phthalate	131-17-9	-70	1.16 E-3	165-167	1.121	246.26	97%	liquid	3.23	182 (20ºC)	Xn; R22	Industrial (monomer, plasticiser, cross-linking agent)
4	Ethoxyquin	91-53-2	-	1.32 E-4	124	1.03	217.31	≥ 75%	liquid	3.87	17.5	Xn; R22	Industrial / Pesticide (anti-oxidant pet food preservative)
4	Maleic acid	110-16-7	130.5	5.19 E-6	160	1.59	116.07	99%	solid	-0.48	4.41 E+5	Xn; R22	Industrial (polyester monomer, reagent) Cosmetic (buffering)**

Table 2. Thirty chemicals selected for the 3T3 NRU validation study bearing an acute hazard label according to EU CLP classification scheme (classified chemicals)

EU CLP	Chemical name	CAS No	Melting point (ºC)	Vapour press. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/Use
4	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	101-72-4	74	7.11 E-5	161	1.040	226.35	-	solid	3.28	54.6	Xn; R22	Industrial (anti-oxidant: rubber, plastic, food)
4	Octyl 3,4,5-trihydroxybenzoate	1034-01-1	101	2.90 E-9	482.9	1.185	282.33	≥ 99%	solid	3.66	36	Xn; R22	Industrial (reagent, intermediate)
4	Phthalic anhydride	85-44-9	130.8	<0.01	295	1.530	148.12	≥ 99%	solid	1.6	6200	Xn; R22	Industrial (intermediate: production of plastics, resins)
4	Sodium cyanate	917-61-3	>300	813	-	-	65.01	96%	solid	-0.46	-	Xn; R22	Industrial /Pesticide (intermediate, reagent)
4	Tetramethylthiuram monosulphide	97-74-5	109.5	2.70 E-4	260.9	1.370	208.37	97%	solid	0.75	5.00 E+4	Xn; R22	Industrial (rubber vulcanisation or curing by polymer cross-linking)

* = Nomenclature is based on Annex I to Directive 67/548/EEC; ** = Cosmetic use from the European Commission database CosIng available at (http://ec.europa.eu/consumers/cosmetics/cosing/); MW = molecular weight

Table 3. Twenty-six chemicals selected for the 3T3 NRU validation study not requiring to bear an acute hazard label according to EU CLP classification scheme (unclassified chemicals)

Chemical name	CAS number	Melting point (ºC)	Vapour pres. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/ Use
1,2-Benzenedicarboxylic acid	68515-48-0	84.91	8.61 E-7	405.7	0.972 at 25°C	418.61	technical	liquid	9.37	0.20	not classified	Plasticiser for polyvinyl chloride (PVC) and vinyl chloride copolymers
2-(2-Butoxyethoxy)ethanol	112-34-5	-68	30 (130°C)	231	0.967 at 25°C	162.23	≥ 99%	liquid	0.56	1.00 E+6	not classified	Industrial use (textile, photographic) Cosmetic (restricted substance, masking, solvent in hair dye products, viscosity controlling)**, solvent
2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	79-94-7	178-181	1.76 E-11 (20°C)	417.9	2.057	543.87	97%	solid	7.2	1.00 E-3	not classified	Industrial (chemical, polymers), intermediate

Table 3. Twenty-six chemicals selected for the 3T3 NRU validation study not requiring to bear an acute hazard label according to EU CLP classification scheme (unclassified chemicals)

Chemical name	CAS number	Melting point (ºC)	Vapour pres. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/ Use
2-Butoxyethyl acetate	112-07-2	-63	0.29 (20°C)	192	0.942 at 25°C	160.21	99%	liquid	1.57	9000	not classified	Industrial, solvent Cosmetic (masking solvent)**
2-Ethylhexyl acrylate	103-11-7	-90	0.15 (20°C)	215-219	0.884 at 20°C	184.28	-	liquid	4.09	100	not classified	Industrial (textile, paper, pulp and board, intermediate)
4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	81-14-1	134-137	1.22 E-5	369	1.206	294.31	NA	solid	3.86	-	not classified	Cosmetic (restricted, masking, perfuming)**
Caprylic acid	124-07-2	15-17	1 (78°C)	237	0.91 at 25°C	144.21	≥ 99%	liquid	3.05	789 (30°C)	not classified	Industrial (intermediate), fertilizer, (non-agricultural) pesticide Cosmetic (cleansing, emulsifying, masking, perfuming, surfactant)**

Table 3. Twenty-six chemicals selected for the 3T3 NRU validation study not requiring to bear an acute hazard label according to EU CLP classification scheme (unclassified chemicals)

Chemical name	CAS number	Melting point (ºC)	Vapour pres. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/ Use
Diepoxide 126	2386-87-0	−37	1.8 E-5	363.4	1.17 at 25°C	252.31	-	liquid	1.78	-	not classified	Industrial (e.g. electrical-resin porcelains, coating curing agents)
Di-"isodecyl" phthalate	26761-40-0	-45.6	5.28 E-7	425.8	0.965 at 20°C	446.66	≥ 99.0%	liquid	10.36	0.28	not classified	Industrial (lubricant, softener, solvent, plasticizer)
Diisopropanolamine	110-97-4	42-45	2.65 E-3	249-250	1.004 at 25°C	133.19	≥ 98.0%	solid	-0.82	8.70 E+5	not classified	Industrial (chemical, paints, intermediate)
Dimethyldioctadecyl-ammonium chloride	107-64-2	-	-	-	-	586.5	≥ 97.0%	solid	9.42	-	not classified	Cosmetic (antistatic, hair conditioning)**
Edetic acid	60-00-4	250	4.98 E-13 (25°C)	614. 2	0.860 at 20	292.24	98.50%	solid	-3.86	1000l	not classified	Industrial (basic, agricultural, textile, electrical) Cosmetic (chelating)**

Table 3. Twenty-six chemicals selected for the 3T3 NRU validation study not requiring to bear an acute hazard label according to EU CLP classification scheme (unclassified chemicals)

Chemical name	CAS number	Melting point (ºC)	Vapour pres. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/ Use
Ethyl acetoacetate	141-97-9	−43	1 (28.5°C)	181	1.029 at 20°C	130.14	99%	solid	0.25	1.10 E+5 (17°C)	not classified	Industrial (intermediate, solvent), odour agent Cosmetic (perfuming)**
Glycerol triacetate	102-76-1	78	2.48 E-3	258-260	1.16 at 25°C	218.2	≥ 99%	liquid	0.25	5.80 E+4	not classified	Industrial (chemical, metal, paper, pulp and board, photographic, cigarette, absorbent, adhesive, solvent) Cosmetic (antimicrobial, film forming, masking, plasticiser, solvent)**, food additive

Table 3. Twenty-six chemicals selected for the 3T3 NRU validation study not requiring to bear an acute hazard label according to EU CLP classification scheme (unclassified chemicals)

Chemical name	CAS number	Melting point (ºC)	Vapour pres. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/ Use
Methenamine	100-97-0	280	0.004	252.7	1.428	140.19	≥ 99.5%	solid	-4.15	4.49 E+5 (12°C)	not classified	Industrial (basic, chemical, paper, pulb and board, adhesive) Cosmetic (preservative)**
Potassium sulfate	7778-80-5	-	3.35 E-5	-	2.66	174.26	≥ 99.0%	solid	-1.03	-	not classified	Industrial (agricultural, chemical, leather), food additive, fertilizer, Cosmetic (viscosity controlling)**
Sorbitan monolaurate	1338-39-2	176.35	8.23 E-13	516.1	1.032 at 25°C	346.46	-	liquid	4.47	-	not classified	Span® 20/solution, detergent Industrial (chemical, textile), food additive Cosmetic (emulsifying)**

Table 3. Twenty-six chemicals selected for the 3T3 NRU validation study not requiring to bear an acute hazard label according to EU CLP classification scheme (unclassified chemicals)

Chemical name	CAS number	Melting point (ºC)	Vapour pres. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/ Use
Triethanolamine	102-71-6	17.9-21	0.01 (20°C)	190-193 (5 mm Hg)	1.124 at 25°C	149.19	≥ 98%	liquid	7.76	1.00 E+6	not classified	Industrial (textile, photographic, construction, intermediate) Cosmetics (buffering, emulsifying, masking, surfactant, restricted substance)**
Triethylene glycol dimethacrylate	109-16-0	5.76	9.40 E-4	170-172 (5 mm Hg)	1.092 at 25°C	286.32	≥ 95.0%	liquid	1.88	3600	not classified	Cosmetic (nail conditioning)**
Tripotassium citrate	866-84-2	275	5.73 E-5	309.6	-	324.41	≥ 99%	solid	-0.28	6.06 E+5	not classified	Cosmetic (buffering, chelating)**
Tris(nonylphenyl) phosphite	26523-78-4	-	5.68 E-18	>360	0.99 at 25°C	689	-	liquid	21.56	-	not classified	Polymers industry, stabilizer Cosmetic (antioxidant)**

Table 3. Twenty-six chemicals selected for the 3T3 NRU validation study not requiring to bear an acute hazard label according to EU CLP classification scheme (unclassified chemicals)

Chemical name	CAS number	Melting point (ºC)	Vapour pres. mmHg (25ºC)	Boiling point (ºC)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25ºC)	Annex I (classification and risk phrases)*	Remarks/ Use
Trizinc bis(orthophosphate)	7779-90-0	-	1.41	158	-	386.11	99.999%	solid	-2.15	-	not classified	Industrial (agricultural, chemical, paper, pulp and board)
Tween 20	9005-64-5	-	2.16 E-22	695.8	1.095 at 25°C	1228	-	liquid	5.37	-	not classified	Viscous liquid, non-ionic detergent Cosmetic (emulsifying, surfactant)**
Urea	57-13-6	132-135	1.20 E-5	158.06	1.335 at 25°C	60.06	-	solid	-2.11	5.45 E+5	not classified	Industrial (agricultural, chemical, adhevise, intermediate), food additive, animal feedstuff additive, Cosmetic (antistatic, buffering, humectant, skin conditioning)**

Table 3. Twenty-six chemicals selected for the 3T3 NRU validation study not requiring to bear an acute hazard label according to EU CLP classification scheme (unclassified chemicals)

Chemical name	CAS number	Melting point (°C)	Vapour pres. mmHg (25°C)	Boiling point (°C)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25°C)	Annex I (classification and risk phrases)*	Remarks/ Use
Zinc distearate	557-05-1	128-130	8.58 E-6	359.4	-	632.33	10-12% as Zn	solid	14.44	-	not classified	Industrial (metal, paint, paper, pulp and board, intermediate, anti-adhesive) Cosmetic (cosmetic colorant, viscosity controlling, anticaking, restricted substance)**

Table 3. Twenty-six chemicals selected for the 3T3 NRU validation study not requiring to bear an acute hazard label according to EU CLP classification scheme (unclassified chemicals)

Chemical name	CAS number	Melting point (°C)	Vapour pres. mmHg (25°C)	Boiling point (°C)	Density (g/cm^3)	MW	Purity	Solid/ liquid	Log Kow	Water Sol. mg/l (25°C)	Annex I (classification and risk phrases)*	Remarks/ Use
Zinc oxide	1314-13-2	251.64	2.64 E-12	583.59	-	81.39	99.999%	solid	1.53	6431	not classified	Industrial (agricultural, chemical, electrical, paints, adhesive, intermediate), food additive, insulating Cosmetic (UV absorber, bulking, skin protecting, restricted substance)**

* = Nomenclature is based on Annex I to Directive 67/548/EEC; ** = Cosmetic use from the European Commission database CosIng available at (http://ec.europa.eu/consumers/cosmetics/cosing/); MW = molecular weight

4.0 COLLECTION OF *IN VIVO* REFERENCE DATA

A database containing rodent LD_{50} values was produced and used to derive rat acute oral LD_{50} reference values for the 56 reference test chemicals tested in the present validation study. The LD_{50} values were used to assess the predictive capacity of the 3T3 NRU test method in respect to its ability to correctly distinguish test chemicals having $LD_{50} \leq 2\,000$ mg/kg b.w. or $LD_{50} > 2\,000$ mg/kg b.w. In addition, to check the applicability of the approach in other parts of the world, where the GHS classification system is implemented (e.g. US Federal agencies), the capacity of the 3T3 NRU test method to distinguish test chemicals having $LD_{50} \leq 5\,000$ mg/kg b.w. or $LD_{50} > 5\,000$ mg/kg b.w, and the capacity to estimate the additional hazard classification categories for acute oral toxicity, specifically, the GHS category 5 ($2\,000 < LD_{50} \leq 5\,000$ mg/kg b.w.) was also addressed (see Section 13.0 and Annex E).

4.1 Strategy and criteria applied for the identification and selection of rodent acute oral LD_{50} reference data

Internet databases (e.g. ChemIDplus - http://chem.sis.nlm.nih.gov/chemidplus; HSDB, linked to the US National Library of Medicine Toxicology Data Network "Toxnet" - http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB), supported by original references, were used as main sources of LD_{50} data collection. Full printed copies of reference articles were obtained for review and archiving, including any further publications cited as sources of additional data not registered in the on-line databases. To minimise repetition, preference was given to primary citation sources, avoiding compilation of data occurring as a secondary reference.

There were several criteria the studies and data should meet in order to be acceptable as a reference value for the test chemical. Studies using the common unit of LD_{50} mg/kg were included and only data from rat or mice studies were collected, with no preference of the sex. The most appropriate administration route was considered to be oral; administration by gavage (stomach tube) was regarded as equivalent to oral administration.

4.2 Final rodent acute oral LD_{50} reference data used for the assessment of predictive capacity of the assay

In order to compare the *in vitro* 3T3 NRU data (IC_{50}) with the *in vivo* rat acute oral toxicity data (LD_{50}), arithmetic mean LD_{50} reference values for the 56 reference test chemicals were calculated using the databases and literature as explained in the section 4.1. For the present validation study, the values included in the calculations of the reference values were those rat acute oral LD_{50} values that had finite numbers. However, if no finite values were available from studies in rats, finite mouse acute oral LD_{50} values were used. Ranges, approximate values, and censored ('smaller/greater than') values were omitted, unless only LD_{50} values 'smaller/greater than' were available.

If a test chemical had multiple finite LD_{50} values available, the arithmetic mean of the LD_{50} values was calculated. When all LD_{50} values were censored, the lowest one was selected for further calculations.

Table 4 lists all the rat and mouse acute oral LD_{50} values (mg/kg) obtained from the databases and the literature. The table also shows whether the test chemical is, for the purpose of this validation study, considered classified ($LD_{50} \leq 2\,000$ mg/kg b.w.) or unclassified ($LD_{50} > 2\,000$ mg/kg b.w.) based on the obtained reference LD_{50} value, according to the current EU CLP classification scheme. In addition, the classification into the GHS toxicity categories is also included. The reference mean represents the reference value obtained using the above mentioned criteria. For three test chemicals (2,2',6,6'-Tetrabromo-4,4'-Isopropylidenediphenol, Triethylene glycol dimethacrylate, and Zinc oxide) only mouse data were used and for another three test chemicals (4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone, Tripotassium citrate, and Zinc distearate) censored ('higher than') values were included due to the unavailability of finite values. For 14 test chemicals only one rat value was available.

Of the total 56 test chemicals, the following distribution was obtained according to the EU CLP acute oral toxicity categories:

- 1 (2%) in category 1 ($LD_{50} \leq 5$ mg/kg)
- 2 (4%) in category 2 ($5 < LD_{50} \leq 50$ mg/kg)
- 5 (9%) in category 3 ($50 < LD_{50} \leq 300$ mg/kg)

- 16 (29%) in category 4 ($300 < LD_{50} \leq 2\,000$ mg/kg)
- 32 (57%) unclassified ($LD_{50} > 2\,000$ mg/kg).

For the purpose of this validation study, to discriminate between classified ($LD_{50} \leq 2\,000$ mg/kg b.w.) and unclassified ($LD_{50} > 2\,000$ mg/kg b.w.) test chemicals, 24 test chemicals belong to the group of classified and 32 to the group of unclassified, according to the EU CLP system.

According to the GHS acute oral toxicity categories, the distribution was the following:
- 1 (2%) in category 1 ($LD_{50} \leq 5$ mg/kg)
- 2 (4%) in category 2 ($5 < LD_{50} \leq 50$ mg/kg)
- 5 (9%) in category 3 ($50 < LD_{50} \leq 300$ mg/kg)
- 16 (29%) in category 4 ($300 < LD_{50} \leq 2\,000$ mg/kg)
- 11 (20%) in category 5 ($2\,000 < LD_{50} \leq 5\,000$ mg/kg)
- 21 (38%) unclassified ($LD_{50} > 5\,000$ mg/kg).

There were six test chemicals (1,2-Dichlorobenzene, 2,6-Diethylaniline, 2-Chloro-4-nitroaniline, 2-Phenoxyethanol; Octyl 3,4,5-trihydroxybenzoate and Phthalic anhydride) categorised as harmful (Xn, R22) according to Annex I (former EU scheme under Dangerous Substances Directive, see Table 2) that according to the calculated LD_{50} reference values were placed in the group of unclassified test chemicals (Table 4). Discrepancies between the assigned toxicity class and risk phrases and the LD_{50} values found in publicly available databases has been previously reported (Rudén and Hansson, 2003; Hoffmann et al., 2010).

The reference LD_{50} values reported in Table 4 are based on the arithmetic mean from several studies. The usual practice when several studies are available for the same chemical, is to assign classification based on a precautionary principle of applying the more severe result. Similarly, during risk assessment, toxicologists take into account the lowest LD_{50} value reported. The six test chemicals listed above would have been correctly assigned to EU CLP category 4 (as in Table 2), if the lowest LD_{50} value reported had been considered.

Table 4. Rodent acute oral reference LD_{50} values and test chemicals' classification into the acute oral toxicity categories using the reference oral LD_{50} value

Chemical number	Chemical	Mouse (mg/kg)	Rat (mg/kg)	Mouse mean (mg/kg)	Rat mean (mg/kg)	Reference mean* (mg/kg)	C/UC†	GHS toxicity category	EU CLP toxicity category
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	100-200	81 35		58	58	C	3	3
2.	1,2,4-Trichlorobenzene	766	756	766	756	756	C	4	4
3.	1,2-Benzenedicarboxylic acid		> 10 000 2 550		2 550	2 550	UC	5	UC
4.	1,2-Dichlorobenzene	4 450- 5 110	500 2 138 1 000 1 516 5 170		2 065	2 065	UC	5	UC
5.	1-Naphthylamine		300 779		540	540	C	4	4
6.	1-Phenyl-3-pyrazolidone	360 287	309 200 504 (204- 1 058)	324	255	255	C	3	3
7.	2-(2-Butoxyethoxy)ethanol	2 400 4 500	6 530 5 660 6 560 7 300 5 080 6 560 6 050	3 450	6 249	6 249	UC	UC	UC

Table 4. Rodent acute oral reference LD_{50} values and test chemicals' classification into the acute oral toxicity categories using the reference oral LD_{50} value

Chemical number	Chemical	Mouse (mg/kg)	Rat (mg/kg)	Mouse mean (mg/kg)	Rat mean (mg/kg)	Reference mean* (mg/kg)	C/UC†	GHS toxicity category	EU CLP toxicity category
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	10 000 3 200 4 400 4 500 5 000	> 2 000 > 5 000	5 420	> 2 000	5 420 (only mouse)	UC	UC	UC
9.	2,4,6-Tris(dimethylaminomethyl)phenol		1 200 2 400-2 600		1 200	1 200	C	4	4
10.	2,6-Diethylaniline		2 690 1 800		2 245	2 245	UC	5	UC
11.	2-Butoxyethyl acetate		2 400 7 030 3 000		4 143	4 143	UC	5	UC
12.	2-Chloro-4-nitroaniline	1 250	6 800-10 000 6 430	1 250	6 430	6 430	UC	UC	UC
13.	2-Ethylhexyl acrylate	4 400	5 720 5 600 6 700	4 400	6 007	6 007	UC	UC	UC
14.	2-Phenoxyethanol	16 500	1 260 1 400 1 900 13 700	16 500	4 565	4 565	UC	5	UC
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone		>10 000		> 10 000	> 10 000	UC	UC	UC

Table 4. Rodent acute oral reference LD_{50} values and test chemicals' classification into the acute oral toxicity categories using the reference oral LD_{50} value

Chemical number	Chemical	Mouse (mg/kg)	Rat (mg/kg)	Mouse mean (mg/kg)	Rat mean (mg/kg)	Reference mean* (mg/kg)	C/UC†	GHS toxicity category	EU CLP toxicity category
16.	Acetophenone	1 250 740	3 000 2 650 900 1 139 815	995	1 701	1 701	C	4	4
17.	Aconitine	1 1.8	5.97	1.4	6	6	C	2	2
18.	Ammonium chloride	1 300	1 650	1 300	1 650	1 650	C	4	4
19.	Barium chloride	430	300 150 118 500 400	430	294	294	C	3	3
20.	Benzaldehyde	28	1 300	28	1 300	1 300	C	4	4
21.	Benzyl benzoate	1 560	2 080 1 900	1 560	1 990	1 990	C	4	4
22.	Brucine	150	1	150	1	1	C	1	1
23.	Caprylic acid		1 283 > 2 000 10 080 > 13 000		5 682	5 682	UC	UC	UC
24.	Copper sulphate	87 369	300 794 610 960	228	666	666	C	4	4

Table 4. Rodent acute oral reference LD_{50} values and test chemicals' classification into the acute oral toxicity categories using the reference oral LD_{50} value

Chemical number	Chemical	Mouse (mg/kg)	Rat (mg/kg)	Mouse mean (mg/kg)	Rat mean (mg/kg)	Reference mean* (mg/kg)	C/UC†	GHS toxicity category	EU CLP toxicity category
25.	Diallyl phthalate	> 1 470 and < 2 150 > 1 000 and < 1 470	891 656 970 770 800-1 700		822	822	C	4	4
26.	Diepoxide 126		4 500		4 500	4 500	UC	5	UC
27.	Di-"isodecyl" phthalate		64 000 > 6 000		64 000	64 000	UC	UC	UC
28.	Diisopropanolamine	2 120	4 765 7 600	2 120	6 183	6 183	UC	UC	UC
29.	Dimethyldioctadecylammonium chloride		11 300 13 000		12 150	12 150	UC	UC	UC
30.	Edetic acid		4 500 > 2 000		4 500	4 500	UC	5	UC
31.	Ethoxyquin	1 584	800 2 420 1 000	1 584	1 407	1 407	C	4	4
32.	Ethyl acetoacetate		3 980		3 980	3 980	UC	5	UC
33.	Ethyl chloroacetate	350	50 180 235	350	155	155	C	3	3

Table 4. Rodent acute oral reference LD_{50} values and test chemicals' classification into the acute oral toxicity categories using the reference oral LD_{50} value

Chemical number	Chemical	Mouse (mg/kg)	Rat (mg/kg)	Mouse mean (mg/kg)	Rat mean (mg/kg)	Reference mean* (mg/kg)	C/UC†	GHS toxicity category	EU CLP toxicity category
34.	Glycerol triacetate	1 800 1 100 9 280 3 200- 6 400	6 400- 12 800 3 000	4 060	3 000	3 000	UC	5	UC
35.	Maleic acid	2 400	708	2 400	708	708	C	4	4
36.	Malononitrile	18.6 (13.7- 25.3)	14 25 60-(61)		19.5	19.5	C	2	2
37.	Methenamine	> 5 000 569 (511- 625)	9 200 > 5 000 > 20 000	> 5 000	9 200	9 200	UC	UC	UC
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	1 122 3 030 3 592	720 800 1 620	2 581	1 047	1 047	C	4	4
39.	Octyl 3,4,5-trihydroxybenzoate		2 710 1 960		2 335	2 335	UC	5	UC
40.	*p*-benzoquinone	25-50	130 5.6 100		79	79	C	3	3
41.	Phthalic anhydride	1 500 2 210 1 500	2 500- 5 000 1 500- 2 000 4 500	1 737	4 500	4 500	UC	5	UC

Table 4. Rodent acute oral reference LD_{50} values and test chemicals' classification into the acute oral toxicity categories using the reference oral LD_{50} value

Chemical number	Chemical	Mouse (mg/kg)	Rat (mg/kg)	Mouse mean (mg/kg)	Rat mean (mg/kg)	Reference mean* (mg/kg)	C/UC†	GHS toxicity category	EU CLP toxicity category
42.	Potassium sulfate		6 600		6 600	6 600	UC	UC	UC
43.	Resorcinol		370 980 301 533 489		535	535	C	4	4
44.	Sodium cyanate		1 500		1 500	1 500	C	4	4
45.	Sodium salt of chloroacetic acid	165 255	76 580	210	328	328	C	4	4
46.	Sorbitan monolaurate		33 600 > 39 800 41 250		37 425	37 425	UC	UC	UC
47.	Tetramethylthiuram monosulphide	1 360 1 200 818 1 150	400	1 132	400	400	C	4	4
48.	Triethanolamine	5 846	5 530	5 846	5 530	5 530	UC	UC	UC
49.	Triethylene glycol dimethacrylate	10 750	10 837 (10 557.36 ± 1 116.64)	10 750		10 750 (only mouse)	UC	UC	UC
50.	Tripotassium citrate		> 7 200		> 7 200	> 7 200	UC	UC	UC
51.	Tris(nonylphenyl) phosphite	> 10 000	10 000 19 500 > 10 000	> 10 000	14 750	14 750	UC	UC	UC

Table 4. Rodent acute oral reference LD_{50} values and test chemicals' classification into the acute oral toxicity categories using the reference oral LD_{50} value

Chemical number	Chemical	Mouse (mg/kg)	Rat (mg/kg)	Mouse mean (mg/kg)	Rat mean (mg/kg)	Reference mean* (mg/kg)	C/UC†	GHS toxicity category	EU CLP toxicity category
52.	Trizinc bis(orthophosphate)	30 000	> 5 000 30 000	30 000	30 000	30 000	UC	UC	UC
53.	Tween 20	> 33 000 > 33 000	> 33 000 40 370	> 33 000	40 370	40 370	UC	UC	UC
54.	Urea	11 500 13 000	14 300 15 000 8 471	12 250	12 590	12 590	UC	UC	UC
55.	Zinc distearate	> 10 000	> 5 000 > 10 000 > 5 000	> 10 000	> 5 000	> 5 000	UC	UC	UC
56.	Zinc oxide	7 950	> 15 000 > 5 000	7 950	> 5 000	7 950 (only mouse)	UC	UC	UC

* = the LD_{50} reference value was calculated according to the criteria in Section 4.2; † = C/UC = classified/unclassified, classified if the $LD_{50} \leq$ 2 000 mg/kg b.w., unclassified if the LD_{50} > 2 000 mg/kg b.w.

5.0 MODULE 1: TEST DEFINITION

5.1 Rationale for the proposed test method

A number of national and international studies and initiatives have shown the relationship between *in vitro* cytotoxicity and *in vivo* acute lethality (see Section 1.3) and the usefulness of the NRU cytotoxicity assays for estimating the *in vivo* starting dose for acute oral systemic toxicity tests. Therefore, NRU cytotoxicity assays could be used to reduce the number of animals required for the classification and labelling of chemicals for acute oral toxicity, as shown by the NICEATM/ECVAM validation study (Anon 2006).

The RC regression between cytotoxicity (IC_{50}) and rodent acute oral LD_{50} values has also indicated that the precision of the prediction from cytotoxicity data of low systemic toxicity is better than the prediction of high systemic toxicity, as previously described in Section 1.3 and presented in Figure 1. The results of the NICEATM/ECVAM validation study have shown that the overall accuracy of the 3T3 NRU cytotoxicity assay for correctly predicting each of the GSH acute oral toxicity classification categories was low (around 30%). With regard to the LD_{50} > 2 000 mg/kg limit dose, the results of the study also indicated that the 3T3 NRU test method had a high sensitivity of 98%. Of all the 22 chemicals included in the study with an LD_{50} > 2 000 mg/kg b.w., 18 chemicals were identified as false positives (18/22), but only one chemical was predicted as false negative (negative predictive value was 80%).

Taking into account the above results, and also the high prevalence (87% of 4 219) of unclassified substances (acute oral LD_{50} > 2 000 mg/kg) in the EU's New Chemical Database (Bulgheroni et al., 2009), it was assumed that the 3T3 NRU cytotoxicity assay may allow for "filtering" the substances unclassified according to the EU CLP classification system from the rest, leaving only the true and false positives to be further tested in animals.

However, since the chemical data set used for the purpose of the NICEATM/ECVAM validation study was selected in order to have a balanced representation among the different GHS toxicity categories (including the unclassified), the number of compounds with $LD_{50} \leq$ 2 000 mg/kg b.w. and LD_{50} > 2 000 mg/kg b.w. was

unbalanced (45 and 22, respectively), suggesting that it would be timely and warranted to confirm the capacity of the 3T3 NRU test method to identify the unclassified chemicals using a data set that includes a larger number of chemicals with LD_{50} > 2 000 mg/kg b.w.

5.2 Intended purpose/use of the test method

The purpose of this validation study was to assess the capacity of the 3T3 NRU cytotoxicity test method to give a simple "yes/no" answer in order to discriminate between classified (toxic/hazardous, $LD_{50} \leq$ 2 000 mg/kg) and unclassified (LD_{50} > 2 000 mg/kg) chemicals according to the EU CLP classification scheme for acute oral toxicity.

This method could be used as a first step in a tiered approach to identify unclassified chemicals.

By clearly identifying negatives (unclassified chemicals), i.e. no further testing needed, this approach would contribute to the reduction of the use of animals for acute oral toxicity testing because only the positives (classified chemicals) would need to be tested.

5.3 Human or environmental health endpoint addressed by the test method

The 3T3 NRU test method addresses the question whether a compound may be acutely toxic in humans after oral exposure.

5.4 Scientific basis – biological and mechanistic relevance and basis

Hazard classification for acute oral toxicity as currently performed for regulatory purposes, is either driven by estimating the LD_{50} using lethality as an endpoint (ATC, UDP methods) or is based upon the dose producing evident toxicity (FDP method) within a 24-hour period, following administration of the substance. The *in vitro* NRU cytotoxicity test method uses cell death as the endpoint. The elementary concept of basal cytotoxicity assays is that chemicals exert their toxic effects by causing injury to structures and functions universal to all cells, such as cell membranes. With the basal cytotoxicity assays it is possible to quantitatively estimate the number of viable cells

expressed as an IC_{50} value, e.g. the concentration that decreases cell viability by 50% in the cell culture.

There are several mechanisms that lead to cell death, such as disruption of cell membrane structure and/or function, of mitochondrial function, of protein turnover, and of energy production. Chemicals may induce these effects in several ways, such as by causing membrane lysis, denaturation (coagulation, precipitation) of proteins, saponification of lipids, and covalent interactions with macromolecules. The effect may also occur in distal organs via disruption of homeostatic signalling mechanisms (Gennari et al. 2004).

The NRU cytotoxicity assay was reported by Borenfreund and Puerner (1984) as a cell viability chemosensitivity assay for cells in monolayer based on the ability of viable cells to incorporate and bind neutral red (NR), a vital dye. They also were the first to publish a NRU protocol for 3T3 cells to objectively quantify cytotoxicity (Borenfreund and Puerner 1985). The cationic neutral red (3-amino-*m*-dimethylamino-2-methyl-phenazine hydrochloride) passively diffuses through the cell membranes and accumulates in lysosomes, binding by electrostatic hydrophobic bonds to anionic and/or phosphate groups. The retention of the dye inside the lysosomes is dependent on the ability of the cell to preserve the pH gradient via ATP production. As the dye is basically uncharged, it is able to enter the membranes. However, due to the proton gradient, the pH in lysosomes is lower than in the cell causing the dye to become charged and consequently retained in lysosomes. If the pH gradient is reduced or the integrity of cell membranes is compromised, such as due to exposure to toxic compounds, the dye uptake diminishes and/or it is not retained in the lysosomes. In this way, it is possible to distinguish viable, damaged or dead cells with the NRU assay.

The NRU test method is one of the most used cytotoxicity tests and it has been applied to human, rodent and fish cell lines (Anon 2006, Knauer et al 2007). It is valuable for both basic and applied research and it has gained a regulatory acceptance as the viability endpoint for an *in vitro* assay for the evaluation of the phototoxicity of chemicals (OECD 2004). The applications of the assay also include toxin

determinations in biological and environmental samples as well as in biotechnological products and toxicity ranking of chemicals (Repetto et al. 2008).

The NRU cytotoxicity test method is quantitative and extremely sensitive. Compared to other cytotoxicity tests, such as tetrazolium salts, enzyme leakage or protein content, it is more sensitive, cheaper, has lower interference, and uses stable reagents. Furthermore, it is a relatively quick assay to perform (Repetto et al 2008).

5.5 Known limitations and drawbacks of the test methods

Chemicals that are insoluble in culture media, DMSO, or ethanol at concentrations high enough to induce >50% cytotoxicity or that are unstable in water may not be correctly predicted with the NRU test method. It is known that some chemicals form precipitates when dosing solutions are prepared or when they are added to the culture medium. Furthermore, some chemicals may cause precipitation of the dye resulting in over-prediction of the toxicity of these chemicals. As some chemicals are volatile, their volatile effects on the adjacent cells may be prevented by using plate sealers and subsequently acceptable results may be obtained (e.g. left and right mean of the vehicle controls in a 96-well plate do not differ by more than 15% from the mean of all vehicle controls). However, some solvents may react chemically with the plastic in the sealers.

If the test chemicals readily bind serum proteins their toxicity may be under-predicted due to the serum content of the culture medium. Also for chemicals that exert toxic effects only after being metabolised, the assay may give too low cytotoxicity prediction since the 3T3 cells have only a very limited capacity to metabolise xenobiotics (INVITTOX 1991). Some chemicals may specifically affect lysosomes and thus affect NRU binding and retention in the cell. Coloured chemicals with absorbance in the optical density range of NR may interfere with the test if they are soluble in the NR solvent and remain inside the cell after washing. The toxicity of chemicals that exert their effects by mechanisms not active in 3T3 cells (e.g., neurotoxic or cardiotoxic chemicals) are most probably under-predicted by the NRU assay.

The absorption, distribution, metabolism, and excretion of a single cell layer are not equivalent to the biokinetics of a whole organ/organism. Furthermore, the applied concentration of the chemical (nominal concentration) to the cells may not reflect the true situation of an organ *in vivo* after chemical exposure.

5.6 Protocols of the test methods

The test method protocol used in HSL is extensively described in the protocols validated in the NICEATM/ECVAM study (Anon, 2006). The test method protocol used in JRC is the automated version of the validated 3T3 NRU test method protocol and in IIVS is an abbreviated version of the validated 3T3 NRU test method protocol.

In all three laboratories the test method protocol uses 3T3 cells grown in 96-well plates. The cells are treated with test chemicals for 48 hours. After the treatment, NR is added to the cells. After 3 hours incubation a desorbing fixative (ethanol/acetic acid/water) is added to the wells for 20-45 minutes to extract the NR. The absorption is measured in a spectrophotometer. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of NR, as compared to the untreated or vehicle-treated controls.

The test method protocol used in JRC was adapted to a robotic automated platform. The plates were thus not inverted and blotted to remove the routine culture medium from the cells before the application of the test chemical, as done in the manual protocol, but the medium was aspirated by the washing station.

The test method protocol used in IIVS is based upon modifications of the procedures described in the 3T3 NRU validated protocol. Since the IIVS protocol was designed to discriminate between highly toxic test chemicals and those of minimal toxicity (as defined by a predicted rodent oral LD_{50} > 2 000 mg/kg b.w.), the assay utilised *in vitro* concentrations expected to be predictive of this range. Accordingly, the range of dilutions tested was typically 20 000 µg/ml to 80 µg/ml (unless test chemical solubility limits the maximum concentrations). Since the dose range was pre-defined, no range finding (RF) experiments were warranted or performed and each concentration of the test chemical was tested in three replicate wells (instead of 6 replicates as done in the validated test protocol) in at least two valid definitive

cytotoxicity tests. Accordingly, two chemicals could be tested in each 96-well plate, rather than only one. Wherever possible, an IC_{50} value was interpolated from the plot of the % of vehicle control (VC) versus the concentration of the test article or positive control. In cases where the % of VC was less than 50% throughout the tested concentration range, then the IC_{50} value was presented as less than the lowest concentration tested. In cases where the % of VC was greater then 50% throughout the tested concentration range, then the NRU_{50} value was presented as greater than the highest concentration tested. Two acceptance criteria had to be met for a result to be acceptable in IIVS:

1) The NR bioassay was accepted if the positive control compound caused an IC_{50} that fell within 2 standard deviations of the historical mean.
2) For each test plate, the left and the right mean of the VCs should not have differed by more than 15 % from the mean of all VCs.

The differences between protocols are presented in Table 5 and full versions of the protocols are presented in Annexes A-C.

Table 5. Differences between the protocols used by the three laboratories

Difference in protocols	HSL	JRC	IIVS
Removal of medium from 96 well plates	Manual inversion	Aspiration by robot	Manual inversion
Range finding experiment	Performed	Performed	Not performed, concentration range was predefined
Number of replicate wells per chemical	6	6	3
Number of chemicals per plate	1	1	2
Number of valid definitive cytotoxicity experiments	3	3	2
Data analysis	Hill function	Hill function	Interpolation between 2 concentrations surrounding the putative IC_{50}
Number of test acceptance criteria	3 (see section 5.6.2.)	3 (see section 5.6.2.)	2 (see section 5.6.)

5.6.1 Range finding (RF) experiment

In HSL and JRC, RF experiments were first performed to determine the appropriate concentration range for the main cytotoxicity assay. Eight different concentrations were used per RF experiment. If no cytotoxicity was observed, another RF experiment was performed at higher concentrations and within solubility limits. If needed, a more stringent solubility protocol was utilised to maximise the solubility and increase the stock concentration. In case of a biphasic concentration-response curve (i.e. cytotoxicity increases, plateaus, and increases again along with increasing concentrations) for NR uptake produced by the range finder test, the concentration range of the definitive test included the lowest concentration with 50% reduction in viability. Control wells, i.e. 1) wells with VC, and 2) wells containing treatment medium without cells, were included in each plate for each chemical and each concentration as indicated in Figure 3 below. A separate plate of positive control (Sodium Dodecyl Sulphate – SDS) concentrations was set up for each set of test chemical plates.

VC	VC	C1	C2	C3	C4	C5	C6	C7	C8	VC	VC
VC	VC	C1	C2	C3	C4	C5	C6	C7	C8	VC	VC
VC	VC	C1	C2	C3	C4	C5	C6	C7	C8	VC	VC
VC	VC	C1	C2	C3	C4	C5	C6	C7	C8	VC	VC
VC	VC	C1	C2	C3	C4	C5	C6	C7	C8	VC	VC
VC	VC	C1	C2	C3	C4	C5	C6	C7	C8	VC	VC
VC	VC	C1	C2	C3	C4	C5	C6	C7	C8	VC	VC
VC	VC	C1	C2	C3	C4	C5	C6	C7	C8	VC	VC

Figure 3. The 96-well plate configuration of the 3T3 NRU test method protocol

VC = vehicle control; C1-C8 = test chemical at 8 concentrations; grey shading = wells containing no cells.

5.6.2 Main cytotoxicity experiment

The definitive cytotoxicity assays to determine the IC_{50} values were performed with the test chemical at eight different concentrations across each plate, tested in six replicate wells per concentration in HSL and JRC, similarly as RF experiments, and three replicate wells in IIVS. The main experiment was repeated three times on three different days in HSL and JRC and twice in IIVS. The midpoint concentration of the range was determined as the one closest to the IC_{50} value obtained from the RF experiment. However, if only a slight or no cytotoxicity was observed in RF

experiments in HSL and JRC, the maximum concentrations for the definitive test were 250 mg/ml and 100 mg/ml, respectively, for chemicals soluble in culture medium and 2.5 mg/ml for chemicals soluble in DMSO, or maximum soluble concentration if the chemicals were not soluble at those concentrations. In IIVS, the maximum final concentrations tested were 20 mg/ml for culture medium soluble chemicals and 2.5 mg/ml for ethanol and DMSO soluble chemicals. The results had to meet all the acceptance criteria (for HSL and JRC see below, for IIVS see 5.6); if not, the test was repeated. For each set of test chemical plates, a separate plate with positive control (SDS) concentrations was set up.

The IC_{50} values were determined for each test chemical from the concentration-response curve and the acceptance of the test was evaluated. The test could be accepted in HSL and JRC if the results met the following criteria:

1) The positive control IC_{50} must be within two and a half (2.5) standard deviations of the historical mean established by the laboratory, and must have met criteria 2 and 3, and must have had an r2 (coefficient of determination) value calculated for the Hill model fit (i.e., from PRISM® software) ≥ 0.85.
2) The left and right mean of the VCs did not differ by more than 15% from the mean of all VCs.
3) At least one calculated cytotoxicity value must have been ≥ 0.0 % and $\leq$ 50.0 % viability and at least one calculated cytotoxicity value > 50.0 % and < 100 % viability.

   Exception: If a test had only one point between 0 and 100 % and the smallest dilution factor (i.e., 1.21) was used and all other test acceptance criteria were met, then the test was considered acceptable.

6.0 ASSESSMENT OF TEST CHEMICAL SOLUBILITY

6.1 Protocols used to assess solubility

The solubility of each test chemical was assessed according to the test method protocol of NICEATM/ECVAM validation study. The protocol is in Annex F.

Minor differences in the solubility testing protocols between the three laboratories are presented below.

In HSL, if the test chemical did not generate cytotoxicity in RF experiments, higher concentrations were tested. In case of insolubility as the limiting factor, an extended protocol was applied where the test chemical solution was incubated at 37ºC for 3 hours with occasional stirring. This extended solubility testing increased the solubility of 17 test chemicals out of 18 (Table 6). Only the solubility of 1,2-Benzenedicarboxylic acid was not increased.

In JRC for five test chemicals the highest solubility was determined as 500 000 µg/ml in DMSO.

In IIVS the maximum soluble concentration determined in solubility test with DMSO or ethanol as a solvent was 500 000 µg/ml, resulting in the highest concentration of 2 500 µg/ml administered to the cells, except for 1-Phenyl-3-pyrazolidone and Diallyl phthalate for which the highest concentrations tested were 2 000 µg/ml and 400 µg/ml, respectively. The typical starting test chemical concentration was 20 000 µg/ml in cell culture medium.

6.2 Summary of solubility results and conclusions

All laboratories reported several problems during the assessment of solubility of the test chemicals, such as precipitation of some test chemicals in the treatment medium or volatility, which can be observed as a vaporous cross contamination and subsequent reduced cell viability in the adjacent control wells.

For each laboratory, the solvents used for the test chemicals and the highest solubility concentrations are shown in Table 6. The same solvent was not always selected by the

three laboratories. The three laboratories used DMSO for 12 test chemicals (21%) and cell culture medium for 19 test chemicals (34%). For 22 test chemicals (39%) different solvents were selected by the three laboratories.

All three laboratories found three test chemicals, Trizinc bis(orthophosphate), Zinc distearate, and Zinc oxide, to be insoluble in all of the solvents (Table 6). HSL and JRC did not perform cytotoxicity studies on the three insoluble test chemicals. However, IIVS performed the cytotoxicity assay with the three test chemicals as they formed homogenous suspension at 800 µg/ml in culture medium after sonication and heating.

The final concentrations of each test chemical used in the cytotoxicity tests are summarised in Table 7.

When performing the main experiments, HSL observed precipitates with 2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol, 2-Butoxyethyl acetate, 4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone, Diisopropanolamine, Dimethyldioctadecylammonium chloride, and Tween 20, mostly at the highest concentrations in at least one of the three main tests (Table 8). In JRC precipitates were noticed with Barium chloride and Edetic acid (Table 8). IIVS further reported that for 4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone, Aconitine, Barium chloride, and Tetramethylthiuram monosulphide complete solubility was not achieved after diluting them in any of the solvents. However, these test chemicals formed homogenous suspensions at 400 to 800 µg/ml in culture medium after sonication and heating and, therefore, the cytotoxicity assay was performed by IIVS with these test chemicals. When IIVS prepared the 2X dilutions in the culture medium for the 4 test chemicals (2-Ethylhexyl acrylate, Barium chloride, Trizinc bis(orthophosphate) and Zinc oxide) for which the 200X dilutions were prepared in DMSO or ethanol, precipitates or suspensions were observed. For 1, 2, 4-Trichlorobenzene and 1,2-Dichlorobenzene precipitations were observed in several of the concentrations tested in all definitive tests performed.

Volatile effects were found in the three laboratories with some test chemicals and, therefore, plate sealers were used (Table 9). All three laboratories used plate sealers

for 1-Naphthylamine, Acetophenone, Ammonium chloride, Ethyl acetoacetate, and Ethyl chloroacetate. Furthermore, both HSL and IIVS found volatility with (4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate, 2,6-Diethylaniline, 2-Phenoxyethanol, Methenamine, *P*-benzoquinone, and Sodium cyanate). Volatility was noticed both in HSL and JRC with 2-Butoxyethyl acetate, and in JRC and IIVS with Benzaldehyde, Ethoxyquin, and Malononitrile. Only JRC reported volatile effects with Phthalic anhydride and IIVS with 2-Ethylhexyl acrylate. IIVS observed volatile effects with Benzaldehyde, Ethyl acetoacetate, and Ethyl chloroacetate throughout the tests even when plate sealers were used and two valid IC_{50} values could not be obtained. For these three test chemicals, the VC values from the wells next to the highest concentration were taken out of the mean VC calculation.

HSL did not perform the cytotoxicity assays with Aconitine, Brucine and Malononitrile. During solubility testing, Malononitrile became impossible to remove from the original vial and was, therefore, not tested further. Aconitine and Brucine were indentified as being highly toxic compounds by a person at HSL not involved in the validation study. HSL's Health and Safety Unit decided that further work with these two chemicals would not be carried out since, with such potent toxins, the safety of workers in the event of an accidental spill could not be ensured and was not covered by the Risk Assessment. The identification of these two chemicals was possible as they were provided in double-containers where the name on the label of the inner bottle could be identified. As explained in Section 3.2. the blind coding of the tests chemicals was done at each laboratory by an appointed person that was not involved in the validation study. For these two compounds the code label provided was stuck only to the outside container while the inner container was not coded. This happened at HSL only for these two chemicals, while in the other two laboratories the blind coding was done properly.

JRC reported a change in appearance of Diisopropanolamine (required storage under N_2) as it gradually transformed from powder to a solid lump. However, cytotoxicity assay was performed with this test chemical.

Table 6. Test chemical solubility results in HSL, JRC, and IIVS

Chem. Nr.	Chemical	Solvent			Maximum soluble concentration determined in solubility test (µg/ml)		
		HSL	JRC	IIVS	HSL	JRC	IIVS
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	CDM	CDM	CDM	20 000	20 000	40 000
2.	1,2,4-Trichlorobenzene	CDM	DMSO	DMSO	370*	500 000	500 000
3.	1,2-Benzenedicarboxylic acid	ETOH	DMSO	ETOH	200 000*	50 000	500 000
4.	1,2-Dichlorobenzene	DMSO	DMSO	DMSO	500 000*	500 000	500 000
5.	1-Naphthylamine	DMSO	DMSO	DMSO	200 000	200 000	500 000
6.	1-Phenyl-3-pyrazolidone	CDM	CDM	DMSO	2 000	2 000	400 000
7.	2-(2-Butoxyethoxy)ethanol	CDM	CDM	CDM	20 000	20 000	40 000
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	DMSO	DMSO	DMSO	200 000	20 000	500 000
9.	2,4,6-Tris(dimethylaminomethyl)phenol	CDM	CDM	CDM	345*	20 000	40 000
10.	2,6-Diethylaniline	DMSO	DMSO	DMSO	500 000*	500 000	500 000
11.	2-Butoxyethyl acetate	CDM	CDM	DMSO	10 400*	10 000	500 000
12.	2-Chloro-4-nitroaniline	DMSO	DMSO	DMSO	200 000	200 000	500 000
13.	2-Ethylhexyl acrylate	ETOH	DMSO	DMSO	100 000*	500 000	500 000

Table 6. Test chemical solubility results in HSL, JRC, and IIVS

Chem. Nr.	Chemical	Solvent			Maximum soluble concentration determined in solubility test (µg/ml)		
		HSL	JRC	IIVS	HSL	JRC	IIVS
14.	2-Phenoxyethanol	CDM	CDM	DMSO	20 000	20 000	500 000
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	DMSO	DMSO	CDM	200 000	20 000	400
16.	Acetophenone	DMSO	CDM	DMSO	500 000*	5 000	500 000
17.	Aconitine	DMSO	DMSO	CDM	20 000	100 000	800
18.	Ammonium chloride	CDM	CDM	CDM	20 000	20 000	40 000
19.	Barium chloride	DMSO	CDM	CDM	20 000*	200	400
20.	Benzaldehyde	DMSO	CDM	DMSO	500 000*	5 000	500 000
21.	Benzyl benzoate	DMSO	DMSO	DMSO	500 000*	500 000	500 000
22.	Brucine	DMSO	DMSO	CDM	20 000	100 000	800
23.	Caprylic acid	CDM	CDM	DMSO	2 000	5 000	500 000
24.	Copper sulphate	CDM	CDM	CDM	200	200	600
25.	Diallyl phthalate	DMSO	DMSO	DMSO	200 000	200 000	500 000
26.	Diepoxide 126	DMSO	DMSO	DMSO	500 000*	20 000	500 000
27.	Di-"isodecyl" phthalate	CDM	CDM	ETOH	200 000*	5 000	500 000
28.	Diisopropanolamine	DMSO	CDM	CDM	250 000*	20 000	40 000
29.	Dimethyldioctadecylammonium chloride	ETOH	CDM	ETOH	200 000	2 000	500 000
30.	Edetic acid	CDM	CDM	CDM	2 000	200	4 000
31.	Ethoxyquin	DMSO	DMSO	DMSO	200 000	200 000	500 000
32.	Ethyl acetoacetate	CDM	CDM	CDM	20 000	20 000	40 000

Table 6. Test chemical solubility results in HSL, JRC, and IIVS

Chem. Nr.	Chemical	Solvent			Maximum soluble concentration determined in solubility test (µg/ml)		
		HSL	JRC	IIVS	HSL	JRC	IIVS
33.	Ethyl chloroacetate	DMSO	CDM	DMSO	500 000*	20 000	500 000
34.	Glycerol triacetate	CDM	CDM	CDM	20 000	20 000	40 000
35.	Maleic acid	CDM	CDM	CDM	20 000	20 000	40 000
36.	Malononitrile	CDM[a]	CDM	CDM	2 000	20 000	40 000
37.	Methenamine	CDM	CDM	CDM	20 000	20 000	40 000
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	DMSO	DMSO	DMSO	200 000	2 000	500 000
39.	Octyl 3,4,5-trihydroxybenzoate	DMSO	DMSO	DMSO	200 000	200 000	500 000
40.	*P*-benzoquinone	DMSO	CDM	CDM	200 000	2 000	4 000
41.	Phthalic anhydride	DMSO	DMSO	CDM	200 000	200 000	400
42.	Potassium sulfate	CDM	CDM	CDM	20 000	20 000	40 000
43.	Resorcinol	CDM	CDM	CDM	20 000	20 000	40 000
44.	Sodium cyanate	CDM	CDM	CDM	20 000	20 000	40 000
45.	Sodium salt of chloroacetic acid	CDM	CDM	CDM	20 000	20 000	40 000
46.	Sorbitan monolaurate	DMSO	ETOH	DMSO	2 000*	200 000	500 000
47.	Tetramethylthiuram monosulphide	DMSO	DMSO	CDM	2 000	200 000	400
48.	Triethanolamine	CDM	CDM	CDM	20 000	20 000	40 000
49.	Triethylene glycol dimethacrylate	DMSO	DMSO	DMSO	200 000	200 000	500 000
50.	Tripotassium citrate	CDM	CDM	CDM	20 000	20 000	40 000
51.	Tris(nonylphenyl) phosphite	CDM	ETOH	CDM	20 000*	2 000	4 000

Table 6. Test chemical solubility results in HSL, JRC, and IIVS

Chem. Nr.	Chemical	Solvent			Maximum soluble concentration determined in solubility test (µg/ml)		
		HSL	JRC	IIVS	HSL	JRC	IIVS
52.	Trizinc bis(orthophosphate)	Insoluble	Insoluble	CDM	Insoluble	Insoluble	800
53.	Tween 20	CDM	CDM	CDM	20 000	20 000	40 000
54.	Urea	CDM	CDM	CDM	100 000*	200 000	40 000
55.	Zinc distearate	Insoluble	Insoluble	CDM	Insoluble	Insoluble	800
56.	Zinc oxide	Insoluble	Insoluble	CDM	Insoluble	Insoluble	800

CDM = Chemical Dilution Medium
DMSO = Dimethyl sulfoxide
ETOH = Ethanol
[a] Malononitrile was not tested at HSL in the cytotoxicity assay because it quickly became impossible to remove from the vial.
* Chemicals for which an extended solubility protocol was applied (see Section 6.1) since insufficient toxicity was obtained in the RF experiment.

Table 7. Concentration ranges and highest concentration solution used in each laboratory for the test chemicals

Chem. Nr	Chemical	RF/DF	HSL 1x final concentration (µg/ml)	JRC 1x final concentration (µg/ml)	IIVS 1x final concentration (µg/ml)	IIVS Highest 2X Dosing Solution as Clear Solution (µg/ml)
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	RF	10 000-0.001	10 000-0.001	-	-
		DF	10-0.67 (tests 1-3)	50-0.1 (test 1) 25-0.3 (tests 2, 3)	2 500-10.0	5 000 (tests 2, 3)
2.	1,2,4-Trichlorobenzene	RF	370-0.00004	1 000-0.0001 (tests 1-4) 2 500-0.00025 (test 5)	-	-
		DF	370-24.94 (tests 1-3)	-	2 500-10.0	213 (tests 2, 4)
3.	1,2-Benzenedicarboxylic acid	RF	1 000-0.0001 (test 1) 500-0.0001 (test 2)	1 000-0.0001 (tests 1-4) 250-0.00003 (test 5)	-	-
		DF	-	-	2 500-10.0	97 (test 1) 1 033 (test 2)
4.	1,2-Dichlorobenzene	RF	2 500-0.0003	1 000-0.0001 (tests 1, 2) 2 500-0.00025 (test 3)	-	-
		DF	-	-	2 500-10.0	470 (test 3) 213 (test 4)
5.	1-Naphthylamine	RF	1 000-0.0001	1 000-0.0001	-	-
		DF	1 000-4.71 (tests 1-3)	100-0.2 (tests 1-3)	2 500-10.0	470 (tests 2, 3)

Table 7. Concentration ranges and highest concentration solution used in each laboratory for the test chemicals

Chem. Nr	Chemical	RF/DF	HSL 1x final concentration (µg/ml)	JRC 1x final concentration (µg/ml)	IIVS 1x final concentration (µg/ml)	IIVS Highest 2X Dosing Solution as Clear Solution (µg/ml)
6.	1-Phenyl-3-pyrazolidone	RF	1 000-0.0001	1 000-0.0001	-	-
		DF	1 000-67.42 (tests 1, 2)	100-6 (tests 1, 2) 100-0.8 (test 3)	2 000-8.02	1 818 (test 2) 4 000 (test 3)
7.	2-(2-Butoxyethoxy)ethanol	RF	10 000-0.001	10 000-0.001	-	-
		DF	10 000-674.18 (tests 1-3)	10 000-163 (tests 1-3)	20 000-80.2	40 000 (tests 1, 2)
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	RF	1 000-0.0001	100-0.00001	-	-
		DF	100-6.74 (tests 1-3)	100-6 (tests 1-3)	2 500-10.0	470 (test 1) 97 (test 2)
9.	2,4,6-Tris(dimethylaminomethyl) phenol	RF	100-0.00001 (test 1) 345-0.00003 (test 2)	10 000-0.001	-	-
		DF	-	10 000-16.4 (tests 1-3)	20 000-80.2	40 000 (tests 1, 2)
10.	2,6-Diethylaniline	RF	2 500-0.0003 (tests 1, 2)	1 000-0.0001 (tests 1, 2) 2 500-0.00025 (test 3)	-	-
		DF	-	-	2 500-10.0	470 (test 3) 2 500 (test 4)
11.	2-Butoxyethyl acetate	RF	1 000-0.0001 (test 1) 5 200-0.0005 (tests 2, 3)	5 000-0.0005	-	-
		DF	5 200-350.58 (tests 1-3)	5 000-11 (tests 1-3)	2 500-10.0	5 000 (tests 1, 2)

Table 7. Concentration ranges and highest concentration solution used in each laboratory for the test chemicals

Chem. Nr	Chemical	RF/DF	HSL 1x final concentration (µg/ml)	JRC 1x final concentration (µg/ml)	IIVS 1x final concentration (µg/ml)	IIVS Highest 2X Dosing Solution as Clear Solution (µg/ml)
12.	2-Chloro-4-nitroaniline	RF	1 000-0.001	1 000-0.0001 (tests 1, 2)	-	-
		DF	200-3.53 (tests 1-3)	100-2 (tests 1-3)	2 500-10.0	213 (tests 1, 2)
13.	2-Ethylhexyl acrylate	RF	100-0.00001 (test 1)	1 000-0.0001 (tests 1-3)	-	-
			500-0.0001 (0.00005) (test 2)	2 500-0.00025 (test 4)		
		DF	-	-	2 500-10.0 (test 3)	NA (test 3)
					2 500-19.5	313 (test 4)
14.	2-Phenoxyethanol	RF	10 000-0.001	10 000-0.001	-	-
		DF	5 000-88.31 (tests 1-3)	10 000-112 (tests 1-3)	2 500-10.0	5 000 (tests 3, 4)
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	RF	1 000-0.0001	100-0.00001	-	-
		DF	500-8.83 (tests 1-3)	100-0.2 (tests 1, 2)	400-6.53	444 (test 1)
				100-1 (test 3)		247 (test 2)
16.	Acetophenone	RF	2 500-0.0003	2 500-0.00025	-	-
		DF	2 500-11.77 (tests 1-3)	2 500-398 (tests 1-3)	2 500-10.0	2 273 (test 3)
						1 033 (test 4)
17.	Aconitine	RF	-	100-0.00001	-	-
		DF	-	500-4 (tests 1-3)	400-6.53	247 (test 6)
						137 (test 7)

Table 7. Concentration ranges and highest concentration solution used in each laboratory for the test chemicals

Chem. Nr	Chemical	RF/DF	HSL 1x final concentration (µg/ml)	JRC 1x final concentration (µg/ml)	IIVS 1x final concentration (µg/ml)	IIVS Highest 2X Dosing Solution as Clear Solution (µg/ml)
18.	Ammonium chloride	RF	10 000-0.001	10 000-0.001 (tests 1, 2)	-	-
		DF	10 000-3.18 (tests 1-3)	10 000-5 (test 1) 10 000-16 (tests 2, 3)	20 000-80.2	40 000 (tests 2, 3)
19.	Barium chloride	RF	10-0.000001 (test 1) 1 000-0.0001 (test 2)	100-0.00001 (tests 1, 2) 10 000-0.001 (test 3)	-	-
		DF	-	10 000-112 (tests 1-4)	400-6.53	137 (test 1) NA (test 2)
20.	Benzaldehyde	RF	1 000-0.0001 (test 1) 2 500-0.0003 (test 2)	2 500-0.00025	-	-
		DF	-	2 500-20 (tests 1-3)	2 500-10.0	2 273 (tests 3, 4)
21.	Benzyl benzoate	RF	2 500-0.0003	1 000-0.0001 (test 1) 2 500-0.00025 (test 2)	-	-
		DF	-	1 000-0.5 (tests 1, 2) 2 000-1.0 (tests 3, 4)	2 500-10.0	1 033 (test 1) 213 (test 2)
22.	Brucine	RF	-	500-0.00005	-	-
		DF	-	500-0.8 (tests 1-3)	400-6.53	444 (test 1) 800 (test 2)

Table 7. Concentration ranges and highest concentration solution used in each laboratory for the test chemicals

Chem. Nr	Chemical	RF/DF	HSL 1x final concentration (µg/ml)	JRC 1x final concentration (µg/ml)	IIVS 1x final concentration (µg/ml)	IIVS Highest 2X Dosing Solution as Clear Solution (µg/ml)
23.	Caprylic acid	RF	1 000-0.0001	1 000-0.0001	-	-
		DF	1 000-67.42 (test 1-3)	1 000-2.0 (tests 1, 2) 2 500-4.0 (test 3)	2 500-10.0	5 000 (test 1) 2 273 (test 2)
24.	Copper sulphate	RF	100-0.00001	100-0.00001 (tests 1, 2)	-	-
		DF	500-8.83 (tests 1-3)	100-0.8 (test 1) 100-16 (tests 2, 3)	400-6.53	800 (tests 1, 2)
25.	Diallyl phthalate	RF	1 000-0.0001	100-0.00001	-	-
		DF	500-8.83 (tests 1-3)	1 000-8 (tests 1-3)	400-6.53	213 (tests 7, 8)
26.	Diepoxide 126	RF	2 500-0.0003	100-0.00001	-	-
		DF	2 500-11.77 (tests 1-3)	100-9.0 (test 1) 100-21 (tests 2, 3)	2 500-10.0	1 033 (test 2) 2 273 (test 3)
27.	Di-"isodecyl" phthalate	RF	200 000-0.02	1 000-0.0001 (test 1) 2 500-0.00025 (tests 2, 3)	-	-
		DF	-	2 500-4.0 (tests 1, 2) 2 500-28 (tests 3-6)	2 500-10.0	213 (test 1) 2 273 (test 2)

Table 7. Concentration ranges and highest concentration solution used in each laboratory for the test chemicals

Chem. Nr	Chemical	RF/DF	HSL 1x final concentration (µg/ml)	JRC 1x final concentration (µg/ml)	IIVS 1x final concentration (µg/ml)	IIVS Highest 2X Dosing Solution as Clear Solution (µg/ml)
28.	Diisopropanolamine	RF	1 250-0.0001	10 000-0.001 (tests 1, 2)	-	-
		DF	1 250-84.27 (tests 1-3)	5 000-82 (tests 1, 2) 5 000-293 (test 3)	20 000-80.2	40 000 (tests 1, 2)
29.	Dimethyldioctadecyl-ammonium chloride	RF	1 000-0.0001	1 000-0.0001	-	-
		DF	100-0.03 (tests 1-3)	1 000-8.0 (tests 1, 2) 50-0.4 (test 3)	2 500-10.0	213 (tests 1, 2)
30.	Edetic acid	RF	1 000-0.0001	100-0.00001 (test 1) 1 000-0.0001 (test 2)	-	-
		DF	1 000-67.42 (tests 1-3)	1 000-11 (tests 1-3)	2 000-8.02	1 818 (tests 1, 2)
31.	Ethoxyquin	RF	1 000-0.0001	1 000-0.0001	-	-
		DF	100-1.77 (tests 1-3)	1 000-0.5 (tests 1-3)	2 500-10.0	97 (tests 2, 3)
32.	Ethyl acetoacetate	RF	10 000-0.001	10 000-0.001	-	-
		DF	10 000-176.63 (tests 1-3)	10 000-0.6 (tests 1, 2) 10 000-16 (test 3)	20 000-80.2	40 000 (tests 3, 4)
33.	Ethyl chloroacetate	RF	1 000-0.0001	10 000-0.001	-	-
		DF	1 000-0.32 (tests 1-3)	100-0.8 (test 1) 100-6.0 (tests 2, 3)	2 500-10.0	5 000 (tests 3, 4)

Table 7. Concentration ranges and highest concentration solution used in each laboratory for the test chemicals

Chem. Nr	Chemical	RF/DF	HSL 1x final concentration (µg/ml)	JRC 1x final concentration (µg/ml)	IIVS 1x final concentration (µg/ml)	IIVS Highest 2X Dosing Solution as Clear Solution (µg/ml)
34.	Glycerol triacetate	RF	10 000-0.001	10 000-0.001	-	-
		DF	10 000-674.18 (tests 1-3)	10 000-585 (tests 1-3)	20 000-80.2	40 000 (tests 1, 2)
35.	Maleic acid	RF	10 000-0.001	10 000-0.001 (tests 1, 2)	-	-
		DF	1 000-17.66 (test 1) 2 000-35.33 (tests 2, 3)	10 000-78 (tests 1-3)	20 000-80.2	40 000 (tests 1, 2)
36.	Malononitrile	RF	-	10 000-0.001 (tests 1-3)	-	-
		DF	-	1 000-2.0 (tests 1-3)	20 000-80.2	40 000 (tests 3, 5)
37.	Methenamine	RF	10 000-0.001	10 000-0.001 (tests 1, 2)	-	-
		DF	1 000-67.42 (tests 1-3)	1 000-8.0 (tests 1, 2) 1 000-59 (test 3)	20 000-80.2	40 000 (tests 3, 4)
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	RF	1 000-0.0001	10-0.000001	-	-
		DF	10-0.18 (tests 1-3)	10-0.2 (tests 1-3)	2 500-10.0	213 (test 1) 44.1 (test 2)
39.	Octyl 3,4,5-trihydroxybenzoate	RF	1 000-0.0001	1 000-0.0001 (tests 1, 2)	-	-
		DF	0.2-0.0009 (tests 1-3)	1-0.06 (tests 1-3)	2 500-10.0	44.1 (tests 1, 2)
40.	*P*-benzoquinone	RF	1 000-0.0001	1 000-0.0001	-	-
		DF	100-1.77 (tests 1-3)	25-0.1 (tests 1, 2) 25-0.3 (test 3)	2 000-8.02	4 000 (tests 3, 4)

Table 7. Concentration ranges and highest concentration solution used in each laboratory for the test chemicals

Chem. Nr	Chemical	RF/DF	HSL 1x final concentration (µg/ml)	JRC 1x final concentration (µg/ml)	IIVS 1x final concentration (µg/ml)	IIVS Highest 2X Dosing Solution as Clear Solution (µg/ml)
41.	Phthalic anhydride	RF	1 000-0.0001	1 000-0.0001 (tests 1, 2)	-	-
		DF	1 000-67.42 (test 1)	1 000-2.0 (test 1)	400-6.53	800 (test 1)
			1 000-4.71 (tests 2, 3)	1 000-16 (tests 2, 3)		247 (test 2)
42.	Potassium sulfate	RF	10 000-0.001	10 000-0.001 (tests 1, 2)	-	-
		DF	10 000-2 633.3 (tests 1-3)	10 000-585 (tests 1, 2)	20 000-80.2	40 000 (tests 1, 2)
				10 000-2 098 (test 3)		
43.	Resorcinol	RF	10 000-0.001	10 000-0.001	-	-
		DF	500-8.83 (tests 1-3)	10 000-5.0 (tests 1-3)	20 000-80.2	40 000 (tests 1, 2)
44.	Sodium cyanate	RF	10 000-0.001	10 000-0.001 (tests 1, 2)	-	-
		DF	1 000-67.42 (tests 1-3)	1 000-8.0 (tests 1, 2)	20 000-80.2	40 000 (tests 3, 4)
				1 000-58 (test 3)		
45.	Sodium salt of chloroacetic acid	RF	10 000-0.001	10 000-0.001 (tests 1, 2)	-	-
		DF	1 000-67.42 (tests 1-3)	1 000-8.0 (tests 1, 2)	20 000-80.2	40 000 (tests 1, 2)
				1 000-37 (test 3)		
46.	Sorbitan monolaurate	RF	1.0-0.0000001 (test 1)	1 000-0.0001	-	-
			10-0.000001 (test 2)			
		DF	-	1 000-0.1 (tests 1, 2)	2 500-10.0	97 (tests 1, 2)
				1 000-1.6 (test 3)		

Table 7. Concentration ranges and highest concentration solution used in each laboratory for the test chemicals

Chem. Nr	Chemical	RF/DF	HSL 1x final concentration (µg/ml)	JRC 1x final concentration (µg/ml)	IIVS 1x final concentration (µg/ml)	IIVS Highest 2X Dosing Solution as Clear Solution (µg/ml)
47.	Tetramethylthiuram monosulphide	RF	10-0.000001	1 000-0.0001 (tests 1, 2)	-	-
		DF	10-0.18 (test 1)	10-0.08 (tests 1-3)	400-6.53	444 (test 1)
			10-0.05 (tests 2, 3)			800 (test 2)
48.	Triethanolamine	RF	10 000-0.001	10 000-0.001	-	-
		DF	10 000-674.18 (tests 1-3)	10 000-949 (tests 1-3)	20 000-80.2	40 000 (tests 1, 2)
49.	Triethylene glycol dimethacrylate	RF	1 000-0.0001	1 000-0.0001	-	-
		DF	500-2.35 (tests 1-3)	1 000-0.5 (tests 1-3)	2 500-10.0	2 273 (test 3)
						1 033 (test 2)
50.	Tripotassium citrate	RF	10 000-0.001	10 000-0.001	-	-
		DF	10 000-674.18 (tests 1-3)	10 000-16 (test 1)	20 000-80.2	40 000 (tests 1, 2)
				10 000-112 (tests 2, 3)		
51.	Tris(nonylphenyl) phosphite	RF	200-0.00002 (tests 1, 2)	10-0.000001 (tests 1-3)	-	-
		DF	-	-	2 000-8.02	1 818 (test 1)
						171 (test 2)
52.	Trizinc bis(orthophosphate)	RF	-	-	-	-
		DF	-	-	400-6.53	247 (test 1)
						NA (test 2)

Table 7. Concentration ranges and highest concentration solution used in each laboratory for the test chemicals

Chem. Nr	Chemical	RF/DF	HSL 1x final concentration (µg/ml)	JRC 1x final concentration (µg/ml)	IIVS 1x final concentration (µg/ml)	IIVS Highest 2X Dosing Solution as Clear Solution (µg/ml)
53.	Tween 20	RF	10 000-0.001	10 000-0.001	-	-
		DF	1 000-67.42 (tests 1-3)	5 000-8.0 (test 3) 10 000-16 (tests 1, 2)	20 000-80.2	40 000 (Tests 1, 2)
54.	Urea	RF	10 000-0.001 (test 1) 250 000-0.025 (test 2)	100 000-0.01	-	-
		DF	250 000-1 177.3 (tests 1-3)	100 000-1 119 (test 1) 100 000-1 633 (tests 2, 3)	20 000-80.2	40 000 (Tests 1, 2)
55.	Zinc distearate	RF	-	-	-	-
		DF	-	-	400-6.53	137 (Test 1)
			-	-		247 (Test 2)
56.	Zinc oxide	RF	-	-	-	-
		DF	-	-	400-6.53	137 (Test 1)
			-	-		NA (test 2)

RF = range finding; DF = definitive test; NA = not applicable as precipitates were found in all of the 2X dosing dilutions
The values reported in the table correspond to valid tests that met the acceptance criteria.

Table 8. Test chemicals that formed precipitates in HSL, JRC, and IIVS

Chemical number	Chemical	HSL	JRC	IIVS
2.	1,2,4-Trichlorobenzene			x
4.	1,2-Dichlorobenzene			x
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	x		
11.	2-Butoxyethyl acetate	x		
13.	2-Ethylhexyl acrylate			x*
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	x		x
17.	Aconitine			x
19.	Barium chloride		x	x*
28.	Diisopropanolamine	x		
29.	Dimethyldioctadecylammonium chloride	x		
30.	Edetic acid		x	
47.	Tetramethylthiuram monosulphide			x
52.	Trizinc bis(orthophosphate)	x	x	x*
53.	Tween 20	x		
55.	Zinc distearate	x	x	x
56.	Zinc oxide	x	x	x*

* = IIVS observed precipitates in all of the 2x dosing dilutions (see also Table 7)

Table 9. Test chemicals that showed volatility in HSL, JRC, and IIVS

Chemical number	Chemical	HSL	JRC	IIVS
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	x		x
5.	1-Naphthylamine	x	x	x
10.	2,6-Diethylaniline	x		x
11.	2-Butoxyethyl acetate	x	x	
13.	2-Ethylhexyl acrylate			x
14.	2-Phenoxyethanol	x		x
16.	Acetophenone	x	x	x
18.	Ammonium chloride	x	x	x
20.	Benzaldehyde		x	x*
31.	Ethoxyquin		x	x
32.	Ethyl acetoacetate	x	x	x*
33.	Ethyl chloroacetate	x	x	x*
36.	Malononitrile		x	x
37.	Methenamine	x		x
40.	*P*-benzoquinone	x		x
41.	Phthalic anhydride		x	
44.	Sodium cyanate	x		x

x = chemicals were identified as volatile and plate sealers were used during the cytotoxicity assays; * = extreme volatility was observed even when plate sealers were used

6.3. Summary

All three laboratories used the same step-wise solubility protocol (see Annex F). For 39% of the test chemicals the laboratories used different solvents. The solubility of the test chemicals was not achieved for seven test chemicals in IIVS, but they were tested, whereas HSL and JRC found three chemicals to be insoluble in any of the solvents and no cytotoxicity assay was performed. In addition to the three insoluble chemicals, HSL did not perform the cytotoxicity assay with another three test chemicals due either to the high toxicity of the identified chemicals or to a change in physical properties. Annex G summarises the number of chemicals tested and not tested in each laboratory. For some of the test chemicals, all laboratories reported precipitate formation during the course of the cytotoxicity assay, and all three laboratories found volatile effects during RF experiments resulting in the use of plate sealers during the cytotoxicity tests for the test chemicals concerned. However, IIVS observed extreme volatility with three test chemicals despite using the plate sealers.

7.0 MODULE 2: WITHIN-LABORATORY REPRODUCIBILITY

7.1 Statistical approaches used for the assessment of within-laboratory reproducibility of experimental data

The within-laboratory reproducibility was assessed by two methods: 1) coefficients of variation (CV) analysis and 2) concordance between the toxicity predicted (either classified or unclassified) for each test chemical from the cytotoxicity assay. Both assessment methods were applied to the data obtained in HSL and JRC. As IIVS reported results only from two accepted tests per test chemical, the within-laboratory reproducibility was assessed only by the concordance between the predicted toxicity for each test chemical in each test.

CV% were calculated per chemical per laboratory by dividing the standard deviation (SD) by the arithmetic mean IC_{50} value and multiplying by 100 (Tables 10-12).

Table 13 shows the arithmetic mean of the two IC_{50} values obtained in IIVS from two accepted cytotoxicity assays.

Concordance between toxicity predictions was calculated per test chemical per laboratory, when at least two IC_{50} values were available. Each IC_{50} was considered individually. The agreement between the toxicity predictions (being either classified or unclassified) in each laboratory was assessed independently of the actual *in vivo* LD_{50} value. The regression models used to predict the toxicity (e.g. the LD_{50} values) from the NRU generated IC_{50} values are described in Section 7.2 below.

7.2 Predictions of LD_{50} values from the IC_{50} values

To obtain the predicted LD_{50} values from the IC_{50} values (μg/ml) as originally estimated in the laboratories, the validated regression models from the NICEATM/ECVAM validation study were applied according to the following formulas:

Validated millimole regression model:

$$\text{Log } LD_{50} \text{ (mmol/kg)} = 0.439 \log IC_{50} \text{ (mM)} + 0.621$$

Validated weight regression model:

$$\text{Log } LD_{50} \text{ (mg/kg)} = 0.372 \log IC_{50} \text{ (µg/mL)} + 2.024$$

7.3 Handling of censored data

In JRC and HSL whenever a cytotoxic effect was not observed (either in RF or main experiment) the characteristic value (IC_{50} value) was reported as higher than the maximum concentration tested. This happened for 8 test chemicals in JRC and for 11 test chemicals in HSL. In IIVS when the % of VC was less than 50% throughout the range of concentrations tested, then the IC_{50} value was presented as less than the lowest concentration tested (13 test chemicals). In cases where the % of VC was greater than 50% throughout the range of concentrations tested, then the IC_{50} value was presented as greater than the highest concentration tested (11 test chemicals). In statistical terminology these characteristic values are censored observations. An IC_{50} value expressed as higher than a certain concentration tested was considered as right censored value, while an IC_{50} value expressed as lower than a certain concentration tested was considered as left censored value.

The decision whether or not to include a censored IC_{50} value in the assessment of concordance between predicted toxicities was taken based on the estimated LD_{50} value. In other words, right censored values were included only if the corresponding predicted LD_{50} was > 2 000 mg/kg, and left censored values were included only if the predicted LD_{50} was ≤ 2 000 mg/kg after applying the two validated regressions (Tables 14-19).

When the millimole regression model was used, right censored IC_{50} values were excluded from 7 chemicals (1,2-Benzenedicarboxylic acid, 2,4,6-Tris(dimethylaminomethyl)phenol, 2-Ethylhexyl acrylate, Barium chloride, Benzaldehyde, Sorbitan monolaurate and Tris(nonylphenyl) phosphate) out of the 11 test chemicals with right censored observations in HSL (Table 14). Four chemicals (1,2-Dichlorobenzene, 2,6-Diethylaniline, Benzyl benzoate and Di-"isodecyl" phthalate) had accepted right censored IC_{50} values since the predicted LD_{50} values were > 2000 mg/kg. In JRC (Table 15) right censored IC_{50} values were excluded from 8 test chemicals (1,2-Benzenedicarboxylic acid, 1,2,4-Trichlorobenzene, 1,2-Dichlorobenzene, 2,6-Diethylaniline, 2-Ethylhexyl acrylate, Benzyl benzoate, Edetic

acid, Tris(nonylphenyl) phosphite). In IIVS (Table 16) right censored IC_{50} values were excluded from three out of the 11 test chemicals with right censored values (4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone, Barium chloride and Phthalic anhydride), and all left censored IC_{50} values were accepted since the predicted LD_{50} values were ≤ 2 000 mg/kg.

When the weight regression was used, all right censored IC_{50} values were excluded in HSL (Table 17) and in JRC (Table 18). In IIVS right censored IC_{50} values were excluded from 10 out of 11 test chemicals with right censored values, and all left censored IC_{50} values were accepted since the predicted LD_{50} values were ≤ 2 000 mg/kg (Table 19).

7.4 Results of the within-laboratory reproducibility assessment

HSL performed the main cytotoxicity assay for 39 out of 56 test chemicals. Three test chemicals were found to be insoluble during the solubility testing, one became impossible to remove from the vial, two were found to be highly toxic during the preparation of stock concentrations and could not be tested due to safety measures in the laboratory, and 11 test chemicals did not produce enough cytotoxicity in the RF experiments and, thus, for those chemicals no main cytotoxicity assay was performed. Table 10 shows the arithmetic mean IC_{50}, SD and CV% for 39 test chemicals for which at least three IC_{50} values were available.

JRC performed the main cytotoxicity assay for 47 out of 56 test chemicals. The laboratory found three chemicals to be insoluble in any of the solvents, and 6 of the test chemicals did not produce cytotoxicity in the RF experiments and no main cytotoxicity assay was performed. Table 11 shows the arithmetic mean IC_{50}, SD and CV% for 44 test chemicals for which at least three IC_{50} values were available.

IIVS performed the cytotoxicity assay for all 56 test chemicals. Due to the abbreviated version of the protocol the laboratory did not perform RF experiments, and only results from two accepted cytotoxicity tests were reported for each chemical. Table 13 shows the arithmetic means IC_{50}, for 37 test chemicals for which finite IC_{50} values were available. For Barium chloride, Dimethyldioctadecylammonium chloride, Ethoxyquin, Urea, and Sodium salt of chloroacetic acid, one of the two IC_{50} values

reported was censored and then, the average value shown in the table is based on the finite IC_{50} value.

The LD_{50} values predicted from the IC_{50} values obtained for each test per test chemical in each laboratory, using the millimole regression model are presented in Tables 14-16. The corresponding predicted LD_{50} values calculated using the weight regression model are shown in Tables 17-19. These tables also indicate the predicted LD_{50} censored values that were excluded since the criteria explained in Section 7.3 were not met.

7.4.1 Assessment of within-laboratory reproducibility using the CV% values

In HSL, the mean of all obtained CV% values was 28%, the median CV% was 20%, and CV% range was 4-107% (Table 10). There were six test chemicals in HSL with remarkably high CV%, i.e. > 60% (1-Naphthylamine, 2-Chloro-4-nitroaniline, 4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone, Dimethyldioctadecylammonium chloride, N-isopropyl-N'-phenyl-p-phenylenediamine, and Triethylene glycol dimethacrylate) of which Dimethyldioctadecylammonium chloride stands out with the clearly highest CV%, i.e. 107%.

In JRC, the mean of all CV% values was 19%, the CV% median was 17%, and CV% range was 2-50% (Table 11). Glycerol triacetate showed the highest CV%, i.e. 50%.

Table 12 summarises the CV results for HSL and JRC showing the mean, median and range of CVs values. The mean and median within-laboratory CV values were very similar for both JRC and HSL. Median CV values were always lower than the corresponding means, which indicated that large individual CV values skewed the CV distributions. Of the two laboratories, HSL had higher mean and median CV values.

Table 12 also shows CV results with respect to chemical form (solid, liquid) volatility, solubility, special storage conditions, and toxicity category (classified and unclassified) for HSL and JRC. There is not a particular chemical attribute that has a strong effect on variability of results obtained in HSL and JRC. Only precipitates appear to affect the within-laboratory variability assessed by CV.

Table 10. Within-laboratory reproducibility of the IC_{50} values in HSL

Chemical number	Chemical	Arithmetic mean IC_{50} (µg/ml)	SD IC_{50}	CV%
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate[a]	5.1	1.6	30.8
2.	1,2,4-Trichlorobenzene	141.2	42.3	30.0
3.	1,2-Benzenedicarboxylic acid	-[b,c]	-	-
4.	1,2-Dichlorobenzene	-[b,d]	-	-
5.	1-Naphthylamine[a]	30.5	20.7	67.9
6.	1-Phenyl-3-pyrazolidone	72.5	11.4	15.8
7.	2-(2-Butoxyethoxy)ethanol	2 017.7	529.7	26.3
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol[e]	47.8	2.8	5.8
9.	2,4,6-Tris(dimethylaminomethyl)phenol	-[b,c]	-	-
10.	2,6-Diethylaniline[a]	-[b,c]	-	-
11.	2-Butoxyethyl acetate[a,e]	1 134.3	80.3	7.1
12.	2-Chloro-4-nitroaniline	54.8	38.6	70.5
13.	2-Ethylhexyl acrylate	-[b,c]	-	-
14.	2-Phenoxyethanol[a]	416.3	22.1	5.3
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone[e]	94.7	58.7	62.0
16.	Acetophenone[a]	132.2	19.6	14.8
17.	Aconitine[f]	-	-	-
18.	Ammonium chloride[a]	380.5	75.1	19.7
19.	Barium chloride	-[b,c]	-	-
20.	Benzaldehyde	-[b,c]	-	-
21.	Benzyl benzoate	-[b,d]	-	-
22.	Brucine[f]	-	-	-
23.	Caprylic acid	418.4	97.7	23.4
24.	Copper sulphate	75.9	10.2	13.5
25.	Diallyl phthalate	112.7	37.7	33.5
26.	Diepoxide 126	127.4	24.5	19.3
27.	Di-"isodecyl" phthalate	-[b,d]	-	-
28.	Diisopropanolamine[e]	634.9	32.9	5.2
29.	Dimethyldioctadecylammonium chloride[e]	8.2	8.7	106.6
30.	Edetic acid	322.4	111.8	34.7
31.	Ethoxyquin	8.1	3.8	47.0
32.	Ethyl acetoacetate[a]	1 006.8	439.1	43.6
33.	Ethyl chloroacetate[a]	15.7	0.6	4.1
34.	Glycerol triacetate	4 284.2	551.4	12.9
35.	Maleic acid	362.8	128.9	35.5
36.	Malononitrile[f]	-	-	-
37.	Methenamine[a]	85.6	16.4	19.2
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	1.1	0.7	64.1
39.	Octyl 3,4,5-trihydroxybenzoate	0.1	0.0	31.9

Table 10. Within-laboratory reproducibility of the IC_{50} values in HSL

Chemical number	Chemical	Arithmetic mean IC_{50} (µg/ml)	SD IC_{50}	CV%
40.	*P*-benzoquinone[a]	2.5	0.6	24.0
41.	Phthalic anhydride	768.4	32.0	4.2
42.	Potassium sulfate	8 336.5	697.4	8.4
43.	Resorcinol	94.2	7.3	7.7
44.	Sodium cyanate[a]	269.1	16.7	6.2
45.	Sodium salt of chloroacetic acid	144.9	12.8	8.8
46.	Sorbitan monolaurate	-[b,c]	-	-
47.	Tetramethylthiuram monosulphide	0.2	0.1	43.0
48.	Triethanolamine	2 473.8	241.4	9.8
49.	Triethylene glycol dimethacrylate	223.2	177.3	79.5
50.	Tripotassium citrate	2 087.5	357.7	17.1
51.	Tris(nonylphenyl) phosphite	-[b,c]	-	-
52.	Trizinc bis(orthophosphate)[g]	-	-	-
53.	Tween 20[e]	367.2	120.0	32.7
54.	Urea	20 978.4	2 056.3	9.8
55.	Zinc distearate[g]	-	-	-
56.	Zinc oxide[g]	-	-	-

[a] volatile compound (plate sealer used)
[b] right censored IC_{50} value
[c] data available only from two tests
[d] data available only from one test
[e] precipitation found at the higher concentrations in at least one of the three tests
[f] not tested
[g] insoluble compound

Table 11. Within-laboratory reproducibility of the IC_{50} values in JRC

Chemical number	Chemical	Arithmetic mean IC_{50} (µg/ml)	SD IC_{50}	CV%
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	3.1	1.1	36.3
2.	1,2,4-Trichlorobenzene	-[b]	-	-
3.	1,2-Benzenedicarboxylic acid	-[b]	-	-
4.	1,2-Dichlorobenzene	-[b,c]	-	-
5.	1-Naphthylamine[a]	4.1	1.2	28.1
6.	1-Phenyl-3-pyrazolidone	18.4	3.4	18.5
7.	2-(2-Butoxyethoxy)ethanol	2 596.8	918.2	35.4
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	50.0	5.1	10.2
9.	2,4,6-Tris(dimethylaminomethyl)phenol	138.2	11.3	8.1
10.	2,6-Diethylaniline	-[b]	-	-
11.	2-Butoxyethyl acetate[a]	3 042.8	292.7	9.6
12.	2-Chloro-4-nitroaniline	35.3	5.0	14.1
13.	2-Ethylhexyl acrylate	-[b]	-	-
14.	2-Phenoxyethanol	911.4	37.0	4.1
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	71.0	22.1	31.1
16.	Acetophenone[a]	2 184.5	178.5	8.2
17.	Aconitine	316.8	124.1	39.2
18.	Ammonium chloride[a]	656.4	200.9	30.6
19.	Barium chloride[e]	-[b,c]	-	-
20.	Benzaldehyde[a]	1 047.0	51.6	4.9
21.	Benzyl benzoate	-[b,c]	-	-
22.	Brucine	59.6	4.6	7.7
23.	Caprylic acid	830.9	243.7	29.3
24.	Copper sulphate	70.8	4.0	5.7
25.	Diallyl phthalate	110.3	4.5	4.1
26.	Diepoxide 126	75.5	4.1	5.5
27.	Di-"isodecyl" phthalate	-[c]	-	-
28.	Diisopropanolamine[h]	804.3	140.1	17.4
29.	Dimethyldioctadecylammonium chloride	12.4	1.5	12.4
30.	Edetic acid[e]	315.3	97.4	30.9
31.	Ethoxyquin[a]	9.2	2.2	23.8
32.	Ethyl acetoacetate[a]	2 116.8	60.2	2.8
33.	Ethyl chloroacetate[a]	40.3	9.3	23.1
34.	Glycerol triacetate	4 361.2	2 184.2	50.1
35.	Maleic acid	967.0	95.5	9.9
36.	Malononitrile[a]	24.1	4.7	19.4
37.	Methenamine	236.7	56.2	23.8
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	1.1	0.4	32.5
39.	Octyl 3,4,5-trihydroxybenzoate	0.2	0.1	36.3
40.	*P*-benzoquinone	2.0	0.4	18.0

Table 11. Within-laboratory reproducibility of the IC_{50} values in JRC

Chemical number	Chemical	Arithmetic mean IC_{50} (µg/ml)	SD IC_{50}	CV%
41.	Phthalic anhydride[a]	755.4	99.6	13.2
42.	Potassium sulphate	7 013.9	535.5	7.6
43.	Resorcinol	127.3	11.5	9.0
44.	Sodium cyanate	334.0	60.4	18.1
45.	Sodium salt of chloroacetic acid	159.2	35.8	22.5
46.	Sorbitan monolaurate	114.9	18.9	16.5
47.	Tetramethylthiuram monosulphide	0.6	0.3	46.1
48.	Triethanolamine	2 483.6	291.2	11.7
49.	Triethylene glycol dimethacrylate	57.8	6.9	12.0
50.	Tripotassium citrate	1 753.8	144.5	8.2
51.	Tris(nonylphenyl) phosphite	-[b]	-	-
52.	Trizinc bis(orthophosphate)[g]	-	-	-
53.	Tween 20	224.9	91.7	40.8
54.	Urea	17 959.7	408.0	2.3
55.	Zinc distearate[g]	-	-	-
56.	Zinc oxide[g]	-	-	-

[a] volatile compound (plate sealer used)
[b] right censored IC_{50} value
[c] data available only from two tests
[e] precipitation
[g] insoluble compound
[h] the appearance of compound changed from powder form to a solid lump during testing

Table 12. Summary of CV% results for HSL and JRC

Toxicity class/ Attribute	HSL				JRC			
	n	Mean	Median	Range	n	Mean	Median	Range
All chemicals	39	28.3	19.7	4.1-106.6	44	19.1	17.0	2.3-50.1
Solids	25	30.5	21.9	4.2-106.6	28	19.7	18.0	2.3-46.0
Liquids	14	24.7	21.3	4.1-79.5	16	18.0	12.0	4.1-50.1
Volatile chemicals	11	22.1	19.2	4.1-67.9	10	16.4	16.3	2.8-30.6
Non-volatile chemical	28	30.7	24.8	4.2-106.6	34	19.1	17.0	2.3-50.1
Precipitates	6	36.6	19.9	5.2-106.6	1	-	-	30.9
No precipitates	33	26.7	19.7	4.1-79.5	43	18.7	16.4	2.3-50.1
Specific storage conditions	16	24.3	18.2	4.2-79.5	19	18.2	17.4	4.1-39.2
No specific storage conditions	23	31.0	30.0	4.1-106.6	25	19.8	16.4	2.3-50.1
No specific attributes	14	29.0	26.7	8.4-70.5	22	20.9	17.3	2.3-50.1
Classified ($LD_{50} \leq 2\ 000$ mg/kg)	17	27.4	24.0	4.1-67.9	21	19.7	18.5	4.1-46.1
Unclassified ($LD_{50} > 2\ 000$ mg/kg)	22	28.9	19.3	4.2-106.6	23	18.5	13.2	2.3-50.1

Table 13. Within-laboratory reproducibility of the IC_{50} values in IIVS[c]

Chemical number	Chemical	Arithmetic mean IC_{50}*
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate[a]	-[i]
2.	1,2,4-Trichlorobenzene[e]	184.5
3.	1,2-Benzenedicarboxylic acid[e]	-[b]
4.	1,2-Dichlorobenzene[e]	-[b]
5.	1-Naphthylamine[a,e]	-[i]
6.	1-Phenyl-3-pyrazolidone[e]	25.6
7.	2-(2-Butoxyethoxy)ethanol	2 670.0
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol[e]	32.1
9.	2,4,6-Tris(dimethylaminomethyl)phenol	264.0
10.	2,6-Diethylaniline[a,e]	1 143.5
11.	2-Butoxyethyl acetate	-[b]
12.	2-Chloro-4-nitroaniline[e]	27.2
13.	2-Ethylhexyl acrylate[a,e]	-[b]
14.	2-Phenoxyethanol[a]	439.5
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone[e,j]	-[b]
16.	Acetophenone[a,e]	265.0
17.	Aconitine[e,j]	-[b]
18.	Ammonium chloride[a]	334.0
19.	Barium chloride[e,j]	344.0[b,k]
20.	Benzaldehyde[a,e,l]	114.5
21.	Benzyl benzoate[e]	784.5
22.	Brucine	41.3
23.	Caprylic acid[e]	536.5
24.	Copper sulphate	-[i]
25.	Diallyl phthalate[e]	114.0
26.	Diepoxide 126[e]	128.5
27.	Di-"isodecyl" phthalate[e]	-[b]
28.	Diisopropanolamine	448.0
29.	Dimethyldioctadecylammonium chloride[e]	13.0[i,m]
30.	Edetic acid	203.5
31.	Ethoxyquin[a,e]	16.3[m]
32.	Ethyl acetoacetate[a,l]	627.5
33.	Ethyl chloroacetate[a,l]	29.4
34.	Glycerol triacetate	5 635.0
35.	Maleic acid	505.5
36.	Malononitrile[a]	-[i]
37.	Methenamine[a]	221.5
38.	*N*-isopropyl-*N*'-phenyl-p-phenylenediamine[e]	-[i]
39.	Octyl 3,4,5-trihydroxybenzoate[e]	-[i]
40.	*P*-benzoquinone[a]	-[i]
41.	Phthalic anhydride	-[b]

Table 13. Within-laboratory reproducibility of the IC_{50} values in IIVS[c]

Chemical number	Chemical	Arithmetic mean IC_{50}*
42.	Potassium sulphate	5 145.0
43.	Resorcinol	-[i]
44.	Sodium cyanate[a]	378.0
45.	Sodium salt of chloroacetic acid	87.2[i,m]
46.	Sorbitan monolaurate[e]	121.5
47.	Tetramethylthiuram monosulphide[e,j]	-[i]
48.	Triethanolamine	1 870.0
49.	Triethylene glycol dimethacrylate[e]	124.5
50.	Tripotassium citrate	1 935.0
51.	Tris(nonylphenyl) phosphate	-[b]
52.	Trizinc bis(orthophosphate)[e,j]	85.3
53.	Tween 20	248.5
54.	Urea	7 560.0[b,k]
55.	Zinc distearate[e,j]	216.5
56.	Zinc oxid[e,j]	-[i]

* as only two independent tests were performed, no SD was calculated as it would not be comparable with the other two laboratories with three independent tests available

[a] volatile compound (plate sealer used)

[b] right censored IC_{50} value

[c] data only from two accepted definitive tests

[e] precipitation

[i] left censored observation

[j] complete solubility not achieved in any of the solvents and chemicals produced homogeneous suspensions at 400-800 µg/ml in CDM after sonication and heating (see Table 6).

[k] IC_{50} value from only one of the two definite tests because the IC_{50} value from one test was right censored

[l] volatility was seen in all definite tests although plate sealer was used

[m] IC_{50} value from only one of the two definite tests, because the IC_{50} value from one of the test was left censored

7.4.2 Assessment of within-laboratory reproducibility using concordance of toxicity predictions

7.4.2.1 Millimole regression model

The concordances of toxicity predictions (i.e. classified or unclassified, based on the predicted LD_{50} values – see Section 7.2) obtained using the validated millimole regression model for each laboratory for each test and per test chemical are summarised in Tables 14-16.

In HSL the concordance between toxicity predictions was not assessed for 16 test chemicals due to the following reasons: for 6 chemicals all right censored IC_{50} values were excluded since the estimated LD_{50} were < 2 000 mg/kg b.w.; for one chemical only two independent tests were available and one of them generated an IC_{50} censored value that was excluded: three chemicals had only one test accepted; and 6 chemicals were not tested (see Section 6.2, Annex G). From the remaining 40 test chemicals only one (2-(2-Butoxyethoxy)ethanol) showed no concordance between the toxicity predicted between the three tests. All three results of 2-(2-Butoxyethoxy)ethanol differed by no more than 295 mg/kg from the 2 000 mg/kg LD_{50} threshold value.

In summary, the reproducibility in HSL assessed by concordance of toxicity predictions between different tests was 98% (39/40).

In JRC the concordance between toxicity predictions was not assessed for 9 test chemicals due to the following reasons: for 2 test chemicals all right censored IC_{50} values were excluded since the estimated LD_{50} were < 2 000 mg/kg b.w.; for 4 test chemicals only one right censored IC_{50} value was accepted since the estimated LD_{50} was > 2 000 mg/kg b.w.; and 3 test chemicals were not tested (see Section 6.2, Annex G). From the remaining 47 test chemicals three (2-(2-Butoxyethoxy)ethanol, Aconitine and Barium chloride) showed no concordance between the toxicity predicted between the tests.

In summary, the reproducibility in JRC assessed by concordance of toxicity predictions between different tests was 94% (44/47).

The within-laboratory reproducibility for IIVS was evaluated only by assessing the concordance between toxicity predictions obtained in the two accepted tests for each test chemical. The concordance between toxicity predictions was not assessed for 3 test chemicals due to the following reasons: for 2 test chemicals all right censored IC_{50} values were excluded since the estimated LD_{50} were < 2 000 mg/kg b.w. and for 1 chemical the right censored IC_{50} value was excluded leaving only one finite estimated LD_{50} value (see Section 6.2, Annex G). The rest of the 53 test chemicals showed concordance between the toxicity predicted between the two tests.

In summary, the reproducibility in IIVS assessed by concordance of toxicity predictions between different tests was 100% (53/53).

7.4.2.2 Weight regression model

The results of the concordance of prediction of toxicity when the weight regression model was used are summarised in Tables 17-19.

In HSL the concordance between toxicity predictions was not assessed for 17 test chemicals due to the following reasons: for 10 test chemicals all right censored IC_{50} values were excluded since the estimated LD_{50} were < 2 000 mg/kg b.w., for one test chemical only value was estimated and 6 test chemicals were not tested (Annex G). From the remaining 39 test chemicals, Triethanolamine showed no concordance between the toxicity predicted between the three tests. All three results differed by no more than 134 mg/kg from the 2 000 mg/kg LD_{50} threshold value.

In summary, the reproducibility in HSL assessed by concordance of toxicity predictions between different tests was 97% (38/39).

In JRC the concordance between toxicity predictions was not assessed for 10 test chemicals due to the following reasons: for 7 test chemicals all right censored IC_{50} values were excluded since the estimated LD_{50} were < 2 000 mg/kg b.w. and 3 test chemicals were not tested (Annex G). From the remaining 46 test chemicals three (2-(2-Butoxyethoxy)ethanol, 2-Butoxyethyl acetate and Glycerol triacetate) showed no concordance between the toxicity predicted between the three tests. For 2-

Butoxyethyl acetate the results of the three tests were within 134 mg/kg from the 2 000 mg/kg LD_{50} threshold value.

In summary, the reproducibility in JRC assessed by concordance of toxicity predictions between different tests was 93% (43/46).

In IIVS the concordance between toxicity predictions was not assessed for 10 test chemicals due to the following reasons: for 9 test chemicals all right censored IC_{50} values were excluded since the estimated LD_{50} were < 2 000 mg/kg b.w. and for 1 chemical the right censored IC_{50} value was excluded leaving only one finite estimated LD_{50} value (Annex G). From the remaining 46 test chemicals 2-(2-Butoxyethoxy)ethanol showed no concordance between the toxicity predicted between the two tests.

In summary, the reproducibility in IIVS assessed by concordance of toxicity predictions between different tests was 98% (45/46)

The comparison of results of concordance of predicted toxicities (either classified or unclassified) in each laboratory obtained either by using the millimole or the weight regression model are summarised in Table 20.

Table 14. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in HSL

Chemical number	Chemical	Predicted LD_{50} (mg/kg)			Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3		
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	233.65 (C)	177.48 (C)	204.30 (C)	Yes	205.14 (C)
2.	1,2,4-Trichlorobenzene	651.65 (C)	600.42 (C)	770.70 (C)	Yes	674.26 (C)
3.	1,2-Benzenedicarboxylic acid	> 2 563.6 (UC)	> 1 891.0 censored excluded	-	NA	> 2 563.6 (UC)
4.	1,2-Dichlorobenzene	> 2 130.9 (UC)	-	-	NA	> 2 130.9 (UC)
5.	1-Naphthylamine	390.17 (C)	261.73 (C)	225.59 (C)	Yes	292.50 (C)
6.	1-Phenyl-3-pyrazolidone	492.37 (C)	435.68 (C)	496.37 (C)	Yes	474.81 (C)
7.	2-(2-Butoxyethoxy)ethanol	2 295.9 (UC)	1 849.3 (C)	1 970.9 (C)	No	2 038.7 (UC)
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	758.51 (C)	787.42 (C)	797.03 (C)	Yes	780.99 (C)
9.	2,4,6-Tris(dimethylaminomethyl)phenol	> 722.44 censored excluded	> 1 244.2 censored excluded	-	NA	NA
10.	2,6-Diethylaniline	> 2 149.0 (UC)	> 2 149.0 (UC)	-	Yes	> 2 149.0 (UC)

Table 14. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in HSL

Chemical number	Chemical	Predicted LD_{50} (mg/kg)			Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3		
11.	2-Butoxyethyl acetate	1 574.8 (C)	1 533.9 (C)	1 631.5 (C)	Yes	1 580.1 (C)
12.	2-Chloro-4-nitroaniline	248.97 (C)	548.29 (C)	442.08 (C)	Yes	413.12 (C)
13.	2-Ethylhexyl acrylate	> 588.75 censored excluded	> 1 193.4 censored excluded	-	NA	NA
14.	2-Phenoxyethanol	949.82 (C)	948.86 (C)	911.26 (C)	Yes	936.65 (C)
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	942.81 (C)	675.39 (C)	556.25 (C)	Yes	724.82 (C)
16.	Acetophenone	487.66 (C)	556.14 (C)	523.94 (C)	Yes	522.58 (C)
17.	Aconitine	not tested	-	-	NA	NA
18.	Ammonium chloride	496.05 (C)	507.20 (C)	578.43 (C)	Yes	527.23 (C)
19.	Barium chloride	> 229.46 censored excluded	> 1 732.6 censored excluded	-	NA	NA
20.	Benzaldehyde	> 1 187.1 censored excluded	> 1 774.9 censored excluded	-	NA	NA
21.	Benzyl benzoate	> 2 618.5 (UC)	-	-	NA	> 2 618.5 (UC)

Table 14. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in HSL

Chemical number	Chemical	Predicted LD_{50} (mg/kg)			Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3		
22.	Brucine	not tested	-	-	NA	NA
23.	Caprylic acid	1 058.1 (C)	860.92 (C)	953.46 (C)	Yes	957.49 (C)
24.	Copper sulphate	512.22 (C)	458.78 (C)	470.52 (C)	Yes	480.51 (C)
25.	Diallyl phthalate	589.89 (C)	798.05 (C)	778.93 (C)	Yes	722.29 (C)
26.	Diepoxide 126	699.41 (C)	818.04 (C)	818.04 (C)	Yes	778.49 (C)
27.	Di-"isodecyl" phthalate	> 27 213 (UC)	-	-	NA	> 27 213 (UC)
28.	Diisopropanolamine	1 111.7 (C)	1 076.3 (C)	1 125.3 (C)	Yes	1 104.4 (C)
29.	Dimethyldioctadecylammonium chloride	294.27 (C)	531.89 (C)	189.86 (C)	Yes	338.67 (C)
30.	Edetic acid	1 217.7 (C)	1 099.5 (C)	1 471.1 (C)	Yes	1 262.8 (C)
31.	Ethoxyquin	152.96 (C)	233.17 (C)	243.48 (C)	Yes	209.87 (C)
32.	Ethyl acetoacetate	1 596.6 (C)	1 185.0 (C)	1 165.9 (C)	Yes	1 315.8 (C)
33.	Ethyl chloroacetate	209.67 (C)	209.67 (C)	203.20 (C)	Yes	207.52 (C)

Table 14. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in HSL

Chemical number	Chemical	Predicted LD_{50} (mg/kg)			Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3		
34.	Glycerol triacetate	3 476.6 (UC)	3 139.2 (UC)	3 476.6 (UC)	Yes	3 364.1 (UC)
35.	Maleic acid	635.41 (C)	855.31 (C)	879.86 (C)	Yes	790.19 (C)
36.	Malononitrile	not tested	-	-	NA	NA
37.	Methenamine	512.22 (C)	433.97 (C)	464.85 (C)	Yes	470.34 (C)
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	50.90 (C)	106.89 (C)	98.53 (C)	Yes	85.44 (C)
39.	Octyl 3,4,5-trihydroxybenzoate	38.71 (C)	38.02 (C)	29.15 (C)	Yes	35.29 (C)
40.	*P*-benzoquinone	95.24 (C)	82.66 (C)	78.16 (C)	Yes	85.36 (C)
41.	Phthalic anhydride	1 301.5 (C)	1 261.4 (C)	1 261.4 (C)	Yes	1 274.7 (C)
42.	Potassium sulfate	4 141.8 (UC)	3 886.3 (UC)	3 898.1 (UC)	Yes	3 975.4 (UC)
43.	Resorcinol	422.69 (C)	419.71 (C)	445.96 (C)	Yes	429.46 (C)
44.	Sodium cyanate	521.37 (C)	494.17 (C)	504.26 (C)	Yes	506.60 (C)
45.	Sodium salt of chloroacetic acid	521.19 (C)	525.95 (C)	558.84 (C)	Yes	535.32 (C)

Table 14. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in HSL

Chemical number	Chemical	Predicted LD_{50} (mg/kg)			Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3		
46.	Sorbitan monolaurate	> 111.11 censored excluded	> 305.31 censored excluded	-	NA	NA
47.	Tetramethylthiuram monosulphide	35.41 (C)	51.15 (C)	40.90 (C)	Yes	42.49 (C)
48.	Triethanolamine	2 051.1 (UC)	2 127.2 (UC)	2 232.9 (UC)	Yes	2 137.1 (UC)
49.	Triethylene glycol dimethacrylate	897.93 (C)	1 423.7 (C)	737.29 (C)	Yes	1 019.6 (C)
50.	Tripotassium citrate	3 188.4 (UC)	2 739.8 (UC)	2 967.6 (UC)	Yes	2 965.3 (UC)
51.	Tris(nonylphenyl) phosphite	> 1 672.6 censored excluded	> 1 672.6 censored excluded	-	NA	NA
52.	Trizinc bis(orthophosphate)	not tested	-	-	NA	NA
53.	Tween 20	2 845.6 (UC)	2 670.0 (UC)	3 469.1 (UC)	Yes	2 994.9 (UC)
54.	Urea	3 117.1 (UC)	3 389.9 (UC)	3 328.8 (UC)	Yes	3 278.6 (UC)
55.	Zinc distearate	not tested	-	-	NA	NA
56.	Zinc oxide	not tested	-	-	NA	NA

C = classified ($LD_{50} \leq$ 2 000 mg/kg b.w.); UC = unclassified (LD_{50} > 2 000 mg/kg b.w.); NA =not applicable

Table 15. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	191.92 (C)	141.01 (C)	158.34 (C)	-	Yes	163.76 (C)
2.	1,2,4-Trichlorobenzene	> 1 603.9 censored excluded	> 1 603.9 censored excluded	> 1 603.9 censored excluded	> 2 398.1 (UC)	NA	> 2 398.1 (UC)
3.	1,2-Benzenedicarboxylic acid	> 1 394.9 censored excluded	> 2 563.6 (UC)	> 2 563.6 (UC)	> 2 563.6 (UC)	Yes	> 2 563.6 (UC)
4.	1,2-Dichlorobenzene	> 1 425.2 censored excluded	> 1 425.2 censored excluded	-	-	NA	NA
5.	1-Naphthylamine	115.63 (C)	142.48 (C)	117.51 (C)	-	Yes	125.21 (C)
6.	1-Phenyl-3-pyrazolidone	277.24 (C)	235.57 (C)	267.25 (C)	-	Yes	260.02 (C)
7.	2-(2-Butoxyethoxy)ethanol	1 821.8 (C)	2 516.4 (UC)	2 450.0 (UC)	-	No	2 262.7 (UC)
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	760.60 (C)	795.98 (C)	831.93 (C)	-	Yes	796.17 (C)
9.	2,4,6-Tris(dimethylaminomethyl)phenol	810.77 (C)	865.88 (C)	820.36 (C)	-	Yes	832.34 (C)

Table 15. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
10.	2,6-Diethylaniline	> 1 437.3 censored excluded	> 1 437.3 censored excluded	> 2 149.0 (UC)	-	NA	> 2 149.0 (UC)
11.	2-Butoxyethyl acetate	2 501.5 (UC)	2 490.9 (UC)	2 315.1 (UC)	-	Yes	2 435.8 (UC)
12.	2-Chloro-4-nitroaniline	370.24 (C)	332.54 (C)	373.38 (C)	-	Yes	358.72 (C)
13.	2-Ethylhexyl acrylate	> 1 617.8 censored excluded	> 1 617.8 censored excluded	> 1 617.8 censored excluded	> 2 419.0 (UC)	NA	> 2 419.0 (UC)
14.	2-Phenoxyethanol	1 341.7 (C)	1 327.0 (C)	1 295.4 (C)	-	Yes	1 321.3 (C)
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	555.53 (C)	664.91 (C)	738.93 (C)	-	Yes	653.12 (C)
16.	Acetophenone	1 852.5 (C)	1 724.0 (C)	1 801.0 (C)	-	Yes	1 792.5 (C)
17.	Aconitine	2 156.0 (UC)	2 161.3 (UC)	1 515.6 (C)	-	No	1 944.23 (C)
18.	Ammonium chloride	628.38 (C)	605.85 (C)	766.80 (C)	-	Yes	667.01 (C)
19.	Barium chloride	2 117.4 (UC)	1 625.3 (C)	-	-	No	1 871.3 (C)
20.	Benzaldehyde	1 217.7 (C)	1 182.0 (C)	1 233.4 (C)	-	Yes	1 211.0 (C)

Table 15. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
21.	Benzyl benzoate	> 1 751.3 censored excluded	> 2 618.5 (UC)	-	-	NA	> 2 618.5 (UC)
22.	Brucine	690.90 (C)	728.11 (C)	737.01 (C)	-	Yes	718.67 (C)
23.	Caprylic acid	1 391.4 (C)	1 084.0 (C)	1 393.1 (C)	-	Yes	1 289.5 (C)
24.	Copper sulphate	469.16 (C)	453.94 (C)	477.09 (C)	-	Yes	466.73 (C)
25.	Diallyl phthalate	733.87 (C)	726.76 (C)	708.37 (C)	-	Yes	723.00 (C)
26.	Diepoxide 126	620.67 (C)	605.86 (C)	635.63 (C)	-	Yes	620.72 (C)
27.	Di-"isodecyl" phthalate	2 343.3 (UC)	3 567.0 (UC)	-	-	Yes	2 955.1 (UC)
28.	Diisopropanolamine	1 134.4 (C)	1 212.7 (C)	1 320.2 (C)	-	Yes	1 222.5 (C)
29.	Dimethyldioctadecylammonium chloride	470.36 (C)	458.30 (C)	422.36 (C)	-	Yes	450.34 (C)
30.	Edetic acid	1 106.6 (C)	1 436.4 (C)	1 215.4 (C)	> 762.57 censored excluded	Yes	1 252.8 (C)

Table 15. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
31.	Ethoxyquin	200.74 (C)	248.45 (C)	226.56 (C)	-	Yes	225.25 (C)
32.	Ethyl acetoacetate	1 875.3 (C)	1 843.5 (C)	1 830.6 (C)	-	Yes	1 849.8 (C)
33.	Ethyl chloroacetate	295.94 (C)	348.56 (C)	294.00 (C)	-	Yes	312.83 (C)
34.	Glycerol triacetate	3 466.0 (UC)	4 027.2 (UC)	2 453.1 (UC)	-	Yes	3 315.4 (UC)
35.	Maleic acid	1 261.9 (C)	1 258.8 (C)	1 166.4 (C)	-	Yes	1 229.0 (C)
36.	Malononitrile	179.02 (C)	160.58 (C)	191.06 (C)	-	Yes	176.89 (C)
37.	Methenamine	665.79 (C)	815.69 (C)	719.93 (C)	-	Yes	733.81 (C)
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	74.68 (C)	94.61 (C)	100.97 (C)	-	Yes	90.09 (C)
39.	Octyl 3,4,5-trihydroxybenzoate	45.10 (C)	54.41 (C)	39.88 (C)	-	Yes	46.46 (C)
40.	*P*-benzoquinone	77.79 (C)	72.19 (C)	84.50 (C)	-	Yes	78.16 (C)
41.	Phthalic anhydride	1 192.4 (C)	1 338.6 (C)	1 259.8 (C)	-	Yes	1 263.6 (C)

Table 15. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
42.	Potassium sulfate	3 590.8 (UC)	3 823.7 (UC)	3 641.7 (UC)	-	Yes	3 685.4 (UC)
43.	Resorcinol	475.25 (C)	483.14 (C)	511.83 (C)	-	Yes	490.08 (C)
44.	Sodium cyanate	504.28 (C)	590.82 (C)	571.80 (C)	-	Yes	555.64 (C)
45.	Sodium salt of chloroacetic acid	489.18 (C)	590.62 (C)	587.32 (C)	-	Yes	555.71 (C)
46.	Sorbitan monolaurate	959.07 (C)	876.09 (C)	833.70 (C)	-	Yes	889.62 (C)
47.	Tetramethylthiuram monosulphide	80.68 (C)	62.93 (C)	54.72 (C)	-	Yes	66.11 (C)
48.	Triethanolamine	2 011.3 (UC)	2 219.3 (UC)	2 189.2 (UC)	-	Yes	2 139.9 (UC)
49.	Triethylene glycol dimethacrylate	567.91 (C)	581.18 (C)	626.43 (C)	-	Yes	591.84 (C)
50.	Tripotassium citrate	2 640.6 (UC)	2 779.6 (UC)	2 836.0 (UC)	-	Yes	2 752.1 (UC)
51.	Tris(nonylphenyl) phosphite	> 449.00 censored excluded	> 449.00 censored excluded	> 449.00 censored excluded	-	NA	NA
52.	Trizinc bis(orthophosphate)	not tested	-	-	-	NA	NA
53.	Tween 20	2 092.1 (UC)	2 240.1 (UC)	2 880.4 (UC)	-	Yes	2 404.2 (UC)

Table 15. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
54.	Urea	3 089.9 (UC)	3 073.9 (UC)	3 030.7 (UC)	-	Yes	3 064.8 (UC)
55.	Zinc distearate	not tested	-	-	-	NA	NA
56.	Zinc oxide	not tested	-	-	-	NA	NA

C = classified ($LD_{50} \leq 2\,000$ mg/kg b.w.); UC = unclassified ($LD_{50} > 2\,000$ mg/kg b.w.); NA = not applicable

Table 16. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in IIVS

Chemical number	Chemical	Predicted LD_{50} (mg/kg)		Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2		
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	< 692.32 (C)	< 692.32 (C)	Yes	< 692.32 (C)
2.	1,2,4-Trichlorobenzene	826.71 (C)	693.28 (C)	Yes	759.99 (C)
3.	1,2-Benzenedicarboxylic acid	> 3 833.0 (UC)	> 3 833.0 (UC)	Yes	> 3 833.0 (UC)
4.	1,2-Dichlorobenzene	> 2 130.9 (UC)	> 2 130.9 (UC)	Yes	> 2 130.9 (UC)
5.	1-Naphthylamine	< 185.98 (C)	< 185.98 (C)	Yes	< 185.98 (C)
6.	1-Phenyl-3-pyrazolidone	244.48 (C)	346.97 (C)	Yes	295.73 (C)
7.	2-(2-Butoxyethoxy)ethanol	2 450.4 (UC)	2 175.3 (UC)	Yes	2 312.8 (UC)
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	664.99 (C)	647.05 (C)	Yes	656.02 (C)
9.	2,4,6-Tris(dimethylaminomethyl)phenol	1 131.7 (C)	1 080.2 (C)	Yes	1 105.9 (C)
10.	2,6-Diethylaniline	1 129.0 (C)	1 818.9 (C)	Yes	1 474.0 (C)
11.	2-Butoxyethyl acetate	> 2 236.3 (UC)	> 2 236.3 (UC)	Yes	> 2 236.3 (UC)

Table 16. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in IIVS

Chemical number	Chemical	Predicted LD_{50} (mg/kg)		Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2		
12.	2-Chloro-4-nitroaniline	356.57 (C)	277.48 (C)	Yes	317.03 (C)
13.	2-Ethylhexyl acrylate	> 2 419.0 (UC)	> 2 419.0 (UC)	Yes	> 2 419.0 (UC)
14.	2-Phenoxyethanol	1 033.8 (C)	876.82 (C)	Yes	955.32 (C)
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	> 1 407.0 censored excluded	> 1 407.0 censored excluded	NA	NA
16.	Acetophenone	731.24 (C)	688.84 (C)	Yes	710.04 (C)
17.	Aconitine	> 2 186.5 (UC)	> 2 186.5 (UC)	Yes	> 2 186.5 (UC)
18.	Ammonium chloride	499.44 (C)	499.44 (C)	Yes	499.44 (C)
19.	Barium chloride	> 1 158.8 censored excluded	1 084.6 (C)	NA	1 084.6* (C)
20.	Benzaldehyde	454.02 (C)	462.81 (C)	Yes	458.42 (C)
21.	Benzyl benzoate	1 577.3 (C)	1 571.2 (C)	Yes	1 574.3 (C)
22.	Brucine	563.37 (C)	656.15 (C)	Yes	609.76 (C)

Table 16. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in IIVS

Chemical number	Chemical	Predicted LD_{50} (mg/kg)		Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2		
23.	Caprylic acid	1 084.5 (C)	1 060.8 (C)	Yes	1 072.6 (C)
24.	Copper sulphate	< 163.93 (C)	< 163.93 (C)	Yes	< 163.93 (C)
25.	Diallyl phthalate	736.58 (C)	730.93 (C)	Yes	733.75 (C)
26.	Diepoxide 126	846.38 (C)	714.44 (C)	Yes	780.41 (C)
27.	Di-"isodecyl" phthalate	> 3 975.0 (UC)	> 3 975.0 (UC)	Yes	> 3 975.0 (UC)
28.	Diisopropanolamine	937.56 (C)	958.00 (C)	Yes	947.78 (C)
29.	Dimethyldioctadecylammonium chloride	460.28 (C)	< 410.20 (C)	Yes	460.28* (C)
30.	Edetic acid	1 062.8 (C)	1 020.1 (C)	Yes	1 041.4 (C)
31.	Ethoxyquin	< 235.02 (C)	291.24 (C)	Yes	291.24* (C)
32.	Ethyl acetoacetate	1 100.2 (C)	1 069.1 (C)	Yes	1 084.6 (C)
33.	Ethyl chloroacetate	271.57 (C)	275.25 (C)	Yes	273.41 (C)
34.	Glycerol triacetate	3 859.7 (UC)	3 738.3 (UC)	Yes	3 799.0 (UC)

Table 16. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in IIVS

Chemical number	Chemical	Predicted LD_{50} (mg/kg)		Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2		
35.	Maleic acid	933.60 (C)	916.73 (C)	Yes	925.16 (C)
36.	Malononitrile	< 300.55 (C)	< 300.55 (C)	Yes	< 300.55 (C)
37.	Methenamine	642.41 (C)	781.05 (C)	Yes	711.73 (C)
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	< 240.44 (C)	< 240.44 (C)	Yes	< 240.44 (C)
39.	Octyl 3,4,5-trihydroxybenzoate	< 272.19 (C)	< 272.19 (C)	Yes	< 272.19 (C)
40.	*P*-benzoquinone	< 144.17 (C)	< 144.17 (C)	Yes	< 144.17 (C)
41.	Phthalic anhydride	> 957.24 censored excluded	> 957.24 censored excluded	NA	NA
42.	Potassium sulfate	3 319.1 (UC)	3 113.0 (UC)	Yes	3 216.1 (UC)
43.	Resorcinol	< 400.31 (C)	< 400.31 (C)	Yes	< 400.31 (C)
44.	Sodium cyanate	576.54 (C)	599.77 (C)	Yes	588.16 (C)
45.	Sodium salt of chloroacetic acid	< 413.14 (C)	428.60 (C)	Yes	428.60* (C)

Table 16. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the millimole regression in IIVS

Chemical number	Chemical	Predicted LD_{50} (mg/kg)		Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2		
46.	Sorbitan monolaurate	898.83 (C)	928.56 (C)	Yes	913.69 (C)
47.	Tetramethylthiuram monosulphide	< 190.38 (C)	< 190.38 (C)	Yes	< 190.38 (C)
48.	Triethanolamine	1 846.4 (C)	1 935.3 (C)	Yes	1 890.8 (C)
49.	Triethylene glycol dimethacrylate	921.52 (C)	722.94 (C)	Yes	822.23 (C)
50.	Tripotassium citrate	2 771.6 (UC)	2 974.0 (UC)	Yes	2 872.8 (UC)
51.	Tris(nonylphenyl) phosphite	> 4 596.1 (UC)	> 4 596.1 (UC)	Yes	> 4 596.1 (UC)
52.	Trizinc bis(orthophosphate)	818.04 (C)	844.57 (C)	Yes	831.31 (C)
53.	Tween 20	2 505.8 (UC)	2 582.3 (UC)	Yes	2 544.1 (UC)
54.	Urea	> 2 273.1 (UC)	2 096.3 (UC)	Yes	2 096.3* (UC)
55.	Zinc distearate	1 468.8 (C)	1 809.5 (C)	Yes	1 639.2 (C)
56.	Zinc oxide	< 112.35 (C)	< 112.35 (C)	Yes	< 112.35 (C)

C = classified ($LD_{50} \leq 2\,000$ mg/kg b.w.); UC = unclassified ($LD_{50} > 2\,000$ mg/kg b.w.); NA = not applicable; * = the IC_{50} finite value was reported as predicted LD_{50}

Table 17. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in HSL

Chemical number	Chemical	Predicted LD_{50} (mg/kg)			Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3		
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	215.09 (C)	170.38 (C)	191.96 (C)	Yes	192.48 (C)
2.	1,2,4-Trichlorobenzene	643.50 (C)	600.37 (C)	741.82 (C)	Yes	661.90 (C)
3.	1,2-Benzenedicarboxylic acid	> 1 380.4 censored excluded	> 1 066.6 censored excluded	-	NA	NA
4.	1,2-Dichlorobenzene	> 1 941.0 censored excluded	-	-	NA	NA
5.	1-Naphthylamine	466.31 (C)	332.46 (C)	293.12 (C)	Yes	363.96 (C)
6.	1-Phenyl-3-pyrazolidone	535.26 (C)	482.57 (C)	538.94 (C)	Yes	518.92 (C)
7.	2-(2-Butoxyethoxy)ethanol	1 973.0 (C)	1 642.5 (C)	1 733.6 (C)	Yes	1 783.1 (C)
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	434.31 (C)	448.30 (C)	452.93 (C)	Yes	445.18 (C)
9.	2,4,6-Tris(dimethylaminomethyl)phenol	> 586.14 censored excluded	> 929.11 censored excluded	-	NA	NA

Table 17. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in HSL

Chemical number	Chemical	Predicted LD_{50} (mg/kg)			Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3		
10.	2,6-Diethylaniline	> 1 941.0 censored excluded	> 1 941.0 censored excluded	-	NA	NA
11.	2-Butoxyethyl acetate	1 442.0 (C)	1 410.3 (C)	1 485.9 (C)	Yes	1 446.1 (C)
12.	2-Chloro-4-nitroaniline	291.62 (C)	569.31 (C)	474.37 (C)	Yes	445.10 (C)
13.	2-Ethylhexyl acrylate	> 586.14 censored excluded	> 1 066.64 censored excluded	-	NA	NA
14.	2-Phenoxyethanol	1 008.0 (C)	1 007.2 (C)	973.25 (C)	Yes	996.15 (C)
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	699.25 (C)	527.07 (C)	447.14 (C)	Yes	557.82 (C)
16.	Acetophenone	612.31 (C)	684.43 (C)	650.70 (C)	Yes	649.15 (C)
17.	Aconitine	not tested	-	-	NA	NA
18.	Ammonium chloride	912.69 (C)	930.05 (C)	1 039.6 (C)	Yes	960.78 (C)
19.	Barium chloride	> 248.89 censored excluded	> 1 380.4 censored excluded	-	NA	NA

Table 17. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in HSL

Chemical number	Chemical	Predicted LD_{50} (mg/kg)			Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3		
20.	Benzaldehyde	> 1 380.4 censored excluded	> 1 941.0 censored excluded	-	NA	NA
21.	Benzyl benzoate	> 1 941.0 censored excluded	-	-	NA	NA
22.	Brucine	not tested	-	-	NA	NA
23.	Caprylic acid	1 082.3 (C)	908.79 (C)	990.91 (C)	Yes	994.01 (C)
24.	Copper sulphate	557.73 (C)	508.01 (C)	519.01 (C)	Yes	528.25 (C)
25.	Diallyl phthalate	511.51 (C)	660.81 (C)	647.37 (C)	Yes	606.56 (C)
26.	Diepoxide 126	584.13 (C)	667.07 (C)	667.07 (C)	Yes	639.43 (C)
27.	Di-"isodecyl" phthalate	> 9 907.9 (UC)	-	-	NA	> 9 907.9 (UC)
28.	Diisopropanolamine	1 172.1 (C)	1 140.4 (C)	1 184.2 (C)	Yes	1 165.5 (C)
29.	Dimethyldioctadecylammonium chloride	187.83 (C)	310.17 (C)	129.57 (C)	Yes	209.19 (C)
30.	Edetic acid	871.44 (C)	799.22 (C)	1 022.82 (C)	Yes	897.82 (C)

Table 17. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in HSL

Chemical number	Chemical	Predicted LD_{50} (mg/kg)			Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3		
31.	Ethoxyquin	172.96 (C)	247.23 (C)	256.46 (C)	Yes	225.55 (C)
32.	Ethyl acetoacetate	1 610.5 (C)	1 250.9 (C)	1 233.9 (C)	Yes	1 365.1 (C)
33.	Ethyl chloroacetate	296.66 (C)	296.66 (C)	288.89 (C)	Yes	294.07 (C)
34.	Glycerol triacetate	2 435.8 (UC)	2 233.9 (UC)	2 435.8 (UC)	Yes	2 368.5 (UC)
35.	Maleic acid	778.94 (C)	1 002.01 (C)	1 026.33 (C)	Yes	935.76 (C)
36.	Malononitrile	not tested	-	-	NA	NA
37.	Methenamine	593.21 (C)	515.47 (C)	546.38 (C)	Yes	551.69 (C)
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	66.78 (C)	125.21 (C)	116.87 (C)	Yes	102.96 (C)
39.	Octyl 3,4,5-trihydroxybenzoate	47.67 (C)	46.94 (C)	37.49 (C)	Yes	44.03 (C)
40.	*P*-benzoquinone	161.35 (C)	143.10 (C)	136.47 (C)	Yes	146.97 (C)
41.	Phthalic anhydride	1 273.6 (C)	1 240.2 (C)	1 240.2 (C)	Yes	1 251.4 (C)
42.	Potassium sulfate	3 144.1 (UC)	2 978.9 (UC)	2 986.6 (UC)	Yes	3 036.5 (UC)

Table 17. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in HSL

Chemical number	Chemical	Predicted LD_{50} (mg/kg)			Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3		
43.	Resorcinol	565.43 (C)	562.05 (C)	591.69 (C)	Yes	573.05 (C)
44.	Sodium cyanate	867.71 (C)	829.20 (C)	843.53 (C)	Yes	846.81 (C)
45.	Sodium salt of chloroacetic acid	657.43 (C)	662.52 (C)	697.45 (C)	Yes	672.47 (C)
46.	Sorbitan monolaurate	> 105.68 censored excluded	> 248.89 censored excluded	-	NA	NA
47.	Tetramethylthiuram monosulphide	51.07 (C)	69.74 (C)	57.71 (C)	Yes	59.50 (C)
48.	Triethanolamine	1 866.1 (C)	1 924.6 (C)	2 005.4 (UC)	No	1 932.0 (C)
49.	Triethylene glycol dimethacrylate	679.76 (C)	1 004.6 (C)	575.20 (C)	Yes	753.18 (C)
50.	Tripotassium citrate	1 926.2 (C)	1 694.0 (C)	1 812.6 (C)	Yes	1 810.9 (C)
51.	Tris(nonylphenyl) phosphite	> 758.55 censored excluded	> 758.55 censored excluded	-	NA	NA
52.	Trizinc bis(orthophosphate)	not tested	-	-	NA	NA
53.	Tween 20	904.13 (C)	856.64 (C)	1 069.4 (C)	Yes	943.39 (C)

Table 17. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in HSL

Chemical number	Chemical	Predicted LD_{50} (mg/kg)			Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3		
54.	Urea	4 100.3 (UC)	4 402.4 (UC)	4 335.0 (UC)	Yes	4 279.2 (UC)
55.	Zinc distearate	not tested	-	-	NA	NA
56.	Zinc oxide	not tested	-	-	NA	NA

C = classified ($LD_{50} \leq 2\,000$ mg/kg b.w.); UC = unclassified ($LD_{50} > 2\,000$ mg/kg b.w.); NA = not applicable

Table 18. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	182.06 (C)	140.20 (C)	154.68 (C)	-	Yes	158.98 (C)
2.	1,2,4-Trichlorobenzene	> 1 380.4 censored excluded	> 1 380.4 censored excluded	> 1 380.4 censored excluded	> 1 941.0 censored excluded	NA	NA
3.	1,2-Benzenedicarboxylic acid	> 824.20 censored excluded	> 1 380.4 censored excluded	> 1 380.4 censored excluded	> 1 380.4 censored excluded	NA	NA
4.	1,2-Dichlorobenzene	> 1 380.4 censored excluded	> 1 380.4 censored excluded	-	-	NA	NA
5.	1-Naphthylamine	166.38 (C)	198.59 (C)	168.67 (C)	-	Yes	177.88 (C)
6.	1-Phenyl-3-pyrazolidone	329.01 (C)	286.59 (C)	318.93 (C)	-	Yes	311.51 (C)
7.	2-(2-Butoxyethoxy)ethanol	1 621.8 (C)	2 132.5 (UC)	2 084.7 (UC)	-	No	1 946.3 (C)
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	435.32 (C)	452.42 (C)	469.68 (C)	-	Yes	452.48 (C)
9.	2,4,6-Tris(dimethylaminomethyl)phenol	646.33 (C)	683.37 (C)	652.80 (C)	-	Yes	660.83 (C)

Table 18. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
10.	2,6-Diethylaniline	> 1 380.4 censored excluded	> 1 380.4 censored excluded	> 1 941.0 censored excluded	-	NA	NA
11.	2-Butoxyethyl acetate	2 134.5 (UC)	2 126.7 (UC)	1 998.9 (C)	-	No	2 086.7 (UC)
12.	2-Chloro-4-nitroaniline	408.18 (C)	372.68 (C)	411.11 (C)	-	Yes	397.32 (C)
13.	2-Ethylhexyl acrylate	> 1 380.4 censored excluded	> 1 380.4 censored excluded	> 1 380.4 censored excluded	> 1 941.0 censored excluded	NA	NA
14.	2-Phenoxyethanol	1 350.8 (C)	1 338.2 (C)	1 311.2 (C)	-	Yes	1 333.4 (C)
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	446.66 (C)	520.13 (C)	568.80 (C)	-	Yes	511.87 (C)
16.	Acetophenone	1 897.4 (C)	1 785.3 (C)	1 852.5 (C)	-	Yes	1 845.1 (C)
17.	Aconitine	970.04 (C)	972.07 (C)	719.62 (C)	-	Yes	887.24 (C)
18.	Ammonium chloride	1 115.2 (C)	1 081.2 (C)	1 320.1 (C)	-	Yes	1 172.2 (C)
19.	Barium chloride	1 636.1 (C)	1 307.6 (C)	-	-	Yes	1 471.8 (C)

Table 18. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
20.	Benzaldehyde	1 410.5 (C)	1 375.4 (C)	1 425.9 (C)	-	Yes	1 403.9 (C)
21.	Benzyl benzoate	> 1 380.4 censored excluded	> 1 941.0 censored excluded	-	-	NA	NA
22.	Brucine	467.47 (C)	488.72 (C)	493.77 (C)	-	Yes	483.32 (C)
23.	Caprylic acid	1 365.0 (C)	1 104.7 (C)	1 366.4 (C)	-	Yes	1 278.7 (C)
24.	Copper sulphate	517.74 (C)	503.47 (C)	525.14 (C)	-	Yes	515.45 (C)
25.	Diallyl phthalate	615.50 (C)	610.44 (C)	597.32 (C)	-	Yes	607.75 (C)
26.	Diepoxide 126	527.92 (C)	517.21 (C)	538.67 (C)	-	Yes	527.94 (C)
27.	Di-"isodecyl" phthalate	1 240.4 (C)	1 770.8 (C)	-	-	Yes	1 505.6 (C)
28.	Diisopropanolamine	1 192.4 (C)	1 261.7 (C)	1 355.9 (C)	-	Yes	1 270.0 (C)
29.	Dimethyldioctadecylammonium chloride	279.49 (C)	273.40 (C)	255.12 (C)	-	Yes	269.34 (C)
30.	Edetic acid	803.57 (C)	1 002.4 (C)	870.02 (C)	> 586.14 censored excluded	Yes	891.99 (C)

Table 18. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
31.	Ethoxyquin	217.77 (C)	260.89 (C)	241.28 (C)	-	Yes	239.98 (C)
32.	Ethyl acetoacetate	1 845.7 (C)	1 819.2 (C)	1 808.4 (C)	-	Yes	1 824.4 (C)
33.	Ethyl chloroacetate	397.26 (C)	456.36 (C)	395.06 (C)	-	Yes	416.23 (C)
34.	Glycerol triacetate	2 429.5 (UC)	2 758.9 (UC)	1 812.6 (C)	-	No	2 333.7 (UC)
35.	Maleic acid	1 393.1 (C)	1 390.2 (C)	1 303.3 (C)	-	Yes	1 362.2 (C)
36.	Malononitrile	348.07 (C)	317.45 (C)	367.82 (C)	-	Yes	344.45 (C)
37.	Methenamine	740.82 (C)	879.92 (C)	791.56 (C)	-	Yes	804.10 (C)
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	92.40 (C)	112.92 (C)	119.31 (C)	-	Yes	108.21 (C)
39.	Octyl 3,4,5-trihydroxybenzoate	54.26 (C)	63.60 (C)	48.89 (C)	-	Yes	55.58 (C)
40.	*P*-benzoquinone	135.92 (C)	127.58 (C)	145.80 (C)	-	Yes	136.43 (C)
41.	Phthalic anhydride	1 182.5 (C)	1 304.3 (C)	1 238.9 (C)	-	Yes	1 241.9 (C)
42.	Potassium sulfate	2 785.8 (UC)	2 938.2 (UC)	2 819.2 (UC)	-	Yes	2 847.8 (UC)

Table 18. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
43.	Resorcinol	624.46 (C)	633.24 (C)	664.96 (C)	-	Yes	640.88 (C)
44.	Sodium cyanate	843.56 (C)	964.71 (C)	938.34 (C)	-	Yes	915.54 (C)
45.	Sodium salt of chloroacetic acid	623.05 (C)	730.93 (C)	727.46 (C)	-	Yes	693.81 (C)
46.	Sorbitan monolaurate	656.51 (C)	608.04 (C)	583.02 (C)	-	Yes	615.86 (C)
47.	Tetramethylthiuram monosulphide	102.61 (C)	83.14 (C)	73.84 (C)	-	Yes	86.53 (C)
48.	Triethanolamine	1 835.4 (C)	1 995.0 (C)	1 972.1 (C)	-	Yes	1 934.1 (C)
49.	Triethylene glycol dimethacrylate	461.07 (C)	470.18 (C)	501.02 (C)	-	Yes	477.42 (C)
50.	Tripotassium citrate	1 641.8 (C)	1 714.8 (C)	1 744.2 (C)	-	Yes	1 700.3 (C)
51.	Tris(nonylphenyl) phosphite	> 248.89 censored excluded	> 248.89 censored excluded	> 248.89 censored excluded	-	NA	NA
52.	Trizinc bis(orthophosphate)	not tested	-	-	-	NA	NA
53.	Tween 20	696.67 (C)	738.22 (C)	913.50 (C)	-	Yes	782.80 (C)
54.	Urea	4 069.9 (UC)	4 052.0 (UC)	4 003.7 (UC)	-	Yes	4 041.9 (UC)

Table 18. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in JRC

Chemical number	Chemical	Predicted LD_{50} (mg/kg)				Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2	Test 3	Test 4		
55.	Zinc distearate	not tested	-	-	-	NA	NA
56.	Zinc oxide	not tested	-	-	-	NA	NA

C = classified ($LD_{50} \leq 2\,000$ mg/kg b.w.); UC = unclassified ($LD_{50} > 2\,000$ mg/kg b.w.); NA = not applicable

Table 19. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in IIVS

Chem nr	Chemical	Predicted LD_{50} (mg/kg)		Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2		
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl) ammonium sulphate	< 539.95 (C)	< 539.95 (C)	Yes	< 539.95 (C)
2.	1,2,4-Trichlorobenzene	787.25 (C)	678.17 (C)	Yes	732.71 (C)
3.	1,2-Benzenedicarboxylic acid	> 1 941.0 censored excluded	> 1 941.0 censored excluded	NA	NA
4.	1,2-Dichlorobenzene	> 1 941.0 censored excluded	> 1 941.0 censored excluded	NA	NA
5.	1-Naphthylamine	< 248.89 (C)	< 248.89 (C)	Yes	< 248.89 (C)
6.	1-Phenyl-3-pyrazolidone	295.75 (C)	397.90 (C)	Yes	346.82 (C)
7.	2-(2-Butoxyethoxy)ethanol	2 085.0 (UC)	1 884.8 (C)	No	1 984.9 (C)
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	388.49 (C)	379.58 (C)	Yes	384.03 (C)
9.	2,4,6-Tris (dimethylaminomethyl) phenol	857.40 (C)	824.20 (C)	Yes	840.80 (C)
10.	2,6-Diethylaniline	1 125.0 (C)	1 685.3 (C)	Yes	1 405.2 (C)
11.	2-Butoxyethyl acetate	> 1 941.0 censored excluded	> 1 941.0 censored excluded	NA	NA
12.	2-Chloro-4-nitroaniline	395.37 (C)	319.68 (C)	Yes	357.53 (C)
13.	2-Ethylhexyl acrylate	> 1 941.0 censored excluded	> 1 941.0 censored excluded	NA	NA
14.	2-Phenoxyethanol	1 083.1 (C)	941.99 (C)	Yes	1 012.5 (C)
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	> 981.67 censored excluded	> 981.67 censored excluded	NA	NA
16.	Acetophenone	863.11 (C)	820.51 (C)	Yes	841.81 (C)

Table 19. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in IIVS

Chem nr	Chemical	Predicted LD_{50} (mg/kg)		Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2		
17.	Aconitine	> 981.67 censored excluded	> 981.67 censored excluded	NA	NA
18.	Ammonium chloride	917.98 (C)	917.98 (C)	Yes	917.98 (C)
19.	Barium chloride	> 981.67 censored excluded	928.11 (C)	NA	928.11 (C)
20.	Benzaldehyde	611.38 (C)	621.39 (C)	Yes	616.38 (C)
21.	Benzyl benzoate	1 263.3 (C)	1 259.1 (C)	Yes	1 261.2 (C)
22.	Brucine	393.24 (C)	447.47 (C)	Yes	420.35 (C)
23.	Caprylic acid	1 105.1 (C)	1 084.6 (C)	Yes	1 094.9 (C)
24.	Copper sulphate	< 212.40 (C)	< 212.40 (C)	Yes	< 212.40 (C)
25.	Diallyl phthalate	617.42 (C)	613.40 (C)	Yes	615.41 (C)
26.	Diepoxide 126	686.60 (C)	594.75 (C)	Yes	640.68 (C)
27.	Di-"isodecyl" phthalate	> 1 941.0 censored excluded	> 1 941.0 censored excluded	NA	NA
28.	Diisopropanolamine	1 014.5 (C)	1 033.2 (C)	Yes	1 023.9 (C)
29.	Dimethyldioctadecylammonium chloride	274.40 (C)	< 248.89 (C)	Yes	274.40* (C)
30.	Edetic acid	776.53 (C)	750.00 (C)	Yes	763.26 (C)
31.	Ethoxyquin	< 248.89 (C)	298.49 (C)	Yes	298.49* (C)
32.	Ethyl acetoacetate	1 174.6 (C)	1 146.4 (C)	Yes	1 160.5 (C)
33.	Ethyl chloroacetate	369.36 (C)	373.60 (C)	Yes	371.48 (C)
34.	Glycerol triacetate	2 661.4 (UC)	2 590.3 (UC)	Yes	2 625.8 (UC)
35.	Maleic acid	1 079.2 (C)	1 062.7 (C)	Yes	1 070.9 (C)

Table 19. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in IIVS

Chem nr	Chemical	Predicted LD_{50} (mg/kg)		Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2		
36.	Malononitrile	< 539.95 (C)	< 539.95 (C)	Yes	< 539.95 (C)
37.	Methenamine	718.71 (C)	848.14 (C)	Yes	783.42 (C)
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	< 248.89 (C)	< 248.89 (C)	Yes	< 248.89 (C)
39.	Octyl 3,4,5-trihydroxybenzoate	< 248.89 (C)	< 248.89 (C)	Yes	< 248.89 (C)
40.	*P*-benzoquinone	< 229.27 (C)	< 229.27 (C)	Yes	< 229.27 (C)
41.	Phthalic anhydride	> 981.67 censored excluded	> 981.67 censored excluded	NA	NA
42.	Potassium sulfate	2 606.2 (UC)	2 468.4 (UC)	Yes	2 537.3 (UC)
43.	Resorcinol	< 539.95 (C)	< 539.95 (C)	Yes	< 539.95 (C)
44.	Sodium cyanate	944.91 (C)	977.09 (C)	Yes	961.00 (C)
45.	Sodium salt of chloroacetic acid	< 539.95 (C)	557.02 (C)	Yes	557.02* (C)
46.	Sorbitan monolaurate	621.39 (C)	638.76 (C)	Yes	630.08 (C)
47.	Tetramethylthiuram monosulphide	< 212.40 (C)	< 212.40 (C)	Yes	< 212.40 (C)
48.	Triethanolamine	1 707.1 (C)	1 776.4 (C)	Yes	1 741.7 (C)
49.	Triethylene glycol dimethacrylate	694.86 (C)	565.70 (C)	Yes	630.28 (C)
50.	Tripotassium citrate	1 710.6 (C)	1 815.9 (C)	Yes	1 763.3 (C)
51.	Tris(nonylphenyl) phosphite	> 1 786.4 censored excluded	> 1 786.4 censored excluded	NA	NA
52.	Trizinc bis(orthophosphate)	544.92 (C)	559.86 (C)	Yes	552.39 (C)
53.	Tween 20	811.78 (C)	832.71 (C)	Yes	822.25 (C)
54.	Urea	> 3 137.6 (UC)	2 929.6 (UC)	Yes	2 929.6* (UC)

Table 19. Within-laboratory reproducibility of the toxicity (classified/unclassified) predicted by the IC_{50} value using the weight regression in IIVS

Chem nr	Chemical	Predicted LD_{50} (mg/kg)		Concordance between tests	Mean predicted LD_{50} (mg/kg) from tests
		Test 1	Test 2		
55.	Zinc distearate	707.75 (C)	844.62 (C)	Yes	776.19 (C)
56.	Zinc oxide	< 212.40 (C)	< 212.40 (C)	Yes	< 212.40 (C)

C = classified ($LD_{50} \leq 2\ 000$ mg/kg b.w.); UC = unclassified ($LD_{50} > 2\ 000$ mg/kg b.w.); NA = not applicable; * = the IC_{50} finite value was reported as mean predicted LD_{50}

Table 20. Comparison of millimole and weight regressions by laboratory for their performance in predicting classified and unclassified test chemicals (LD_{50} > 2 000 mg/kg b.w.) and the number of chemicals excluded in each analysis

	Number of tested chemicals excluded from analysis		Number of chemicals with concordant predictions between tests (% concordance)		Chemicals with discordant predictions between tests	
	Millimole regression	**Weight regression**	**Millimole regression**	**Weight regression**	**Millimole regression**	**Weight regression**
HSL	10	11	39/40 (98%)	38/39 (97%)	2-(2-Butoxyethoxy)ethanol	Triethanolamine
JRC	6	7	44/47 (94%)	43/46 (93%)	2-(2-Butoxyethoxy)ethanol Aconitine Barium chloride	2-(2-Butoxyethoxy)ethanol 2-Butoxyethyl acetate Glycerol triacetate
IIVS	3	10	53 (100%)	45/46 (98%)	-	2-(2-Butoxyethoxy)ethanol

7.5. Summary

Overall, the analysis of CVs for HSL and JRC shows that there are no big differences in the obtained CV values between the two laboratories (Table 12). When the values were assessed in both laboratories according to different chemical characteristics (e.g. volatility or solubility), the CV% values were high mainly in case of those test chemicals that formed precipitates during the 3T3 NRU cytotoxicity assay. Physical form, volatility and specific storage conditions did not have a remarkable effect, as shown by the mean, median and ranges of CV values in each laboratory for chemicals that had some of these attributes compared with others that do not. When the chemicals were grouped by toxicity category (classified/unclassified) the mean CV values were similar (for classified chemicals 27.4% in HSL and 19.7% in JRC and for unclassified chemicals 28.9% in HSL and 18.5% in JRC) to the overall mean CV values (28.3% in HSL and 19.1% in JRC). Table 12 also shows that JRC consistently obtained lower mean CV values and smaller differences between mean and median values compared to HSL. This is in line with the expectation that the automated version of the 3T3 NRU protocol is less variable. When compared to the values obtained in the previous NICEATM/ECVAM validation study (Anon 2006, p. 7-36), the CV values were similar, except for the volatile chemicals, where both HSL and JRC had remarkably lower mean CV values (22.1% and 16.4%, respectively) compared to the NICEATM/ECVAM validation study (31%). Therefore, the present validation study supports the former within-laboratory results of the NICEATM/ECVAM validation study.

The concordance of toxicity predictions (classified or unclassified according to the LD_{50} cut-off limit of 2 000 mg/kg b.w.) was high for each laboratory with both regressions: 98% for HSL, 94% for JRC, and 100% for IIVS with the millimole regression and 97% for HSL, 93% for JRC, and 98% for IIVS with the weight regression. The test chemicals that showed discordant predictions of toxicities among tests were different depending on the regression used. In HSL discordant predictions of toxicities were obtained with only one chemical with each of the regression models: 2-(2-Butoxyethoxy)ethanol when the millimole regression was used and Triethanolamine when the weight regression was used. In JRC three discordant predictions were obtained with each of the regression models, where one test

chemical, (2-(2-Butoxyethoxy)ethanol), was common in both cases. For IIVS, discordant toxicity predictions among tests were found with one chemical [(2-(2-Butoxyethoxy)ethanol] only with the weight regression.

Overall, the within-laboratory reproducibility obtained in each laboratory by calculating the concordance of predictions of toxicity (classified or unclassified) among tests for each chemical, did not differ significantly when the millimole or weight regression model was used. The main difference found was that the weight regression resulted in an increase in the number of test chemicals excluded from the analysis due to right censored IC_{50} values, which could not be accepted because the estimated LD_{50} value was < 2 000 mg/kg b.w. This was particularly remarkable in IIVS due to the abbreviated protocol used in this laboratory (see Annex C).

In addition, in both HSL and JRC the chemicals that had discordant predictions changed depending on the regression model used.

All the test chemicals with discordant predictions harbour properties that may have influenced the outcome of the results. The special storage conditions required for 2-(2-Butoxyethoxy)ethanol (to be stored under nitrogen), Triethanolamine (hygroscopic, to be kept under argon, sensitive to air), and Aconitine (to be kept under argon, sensitive to CO_2) could explain the discordant predictions for those chemicals. Barium chloride is hygroscopic and both JRC and IIVS have reported precipitates. JRC and HSL reported volatility for 2-Butoxyethyl acetate and HSL reported also precipitates. In addition, this chemical requires minimal exposure to air. Glycerol triacetate is a viscous test chemical and problems could have been encountered during preparation of solutions.

8.0 MODULE 3: TRANSFERABILITY

The transferability of the 3T3 NRU test method was assessed only in HSL and JRC who had no previous experience with the test method. In this validation study, IIVS has used an abbreviated version of the validated 3T3 NRU test method protocol and, in addition, IIVS had been one of the three lead laboratories that participated in the NICEATM/ECVAM validation study and, thus, was familiar with the test method.

8.1. Transfer of the validated 3T3 NRU test method protocol to the HSL laboratory

To establish the transferability of the 3T3 NRU test method protocol at HSL, the main cytotoxicity test was carried out following the original validated protocol with a selection of nine chemicals from those listed in the BRD (Section 3 in Anon 2006) of the NICEATM/ECVAM validation study. The selected chemicals (Ethylene glycol, Sodium chloride, Boric acid, Sodium fluoride, Phenol, Potassium cyanide, Mercury chloride, Sodium arsenite and Cycloheximide) represented the different acute oral toxicity categories as well as the unclassified chemicals according to both the GHS and EU CLP classification schemes. The IC_{50} values for these chemicals from the results of the NICEATM/ECVAM validation study (Anon 2006) span five orders of magnitude (Table 21).

The concentrations tested in the 3T3 NRU test method were chosen to span a log scale, centred on the published IC_{50} value, using a dilution factor of 1.47. In the first cytotoxicity experiment performed, Ethylene glycol, Sodium arsenite and Cycloheximide induced a decrease in cell viability greater than 88% at all the concentrations tested. When the experiments were repeated with fresh stocks of these chemicals which were kindly donated by ECVAM, improved concentration-response curves were obtained, with at least two data values of < 50% viability and at least two data values of > 50% viability. For these 3 chemicals IC_{50} values from only one definitive test were reported, while for the other 6 chemicals two definitive tests were performed.

The obtained IC_{50} values were compared with the published values from the BRD of the NICEATM/ECVAM validation study, (summarised in Table 21). The values varied from those published by 0.4 to 2.0-fold.

Table 21. Comparison of 3T3 NRU published IC_{50} geometric means reported from the NICEATM/ECVAM validation study with the IC_{50} geometric means obtained by HSL with 9 selected chemicals.

Chemical	Obtained IC_{50} (µg/ml) geometric mean	IC_{50} (µg/ml) geometric mean Published**	Animal LD_{50} (mg/kg b.w.)***	GHS hazard classification	EU CLP hazard classification
Ethylene glycol	40 600*	24 317	8 567	UC	UC
Sodium chloride	9 110	4 730	2 998	5	UC
Boric acid	3 495	1 850	2 660	5	UC
Phenol	93.1	66.3	414	4	4
Sodium fluoride	93.9	78	180	3	3
Potassium cyanide	81.8	34.6	10	2	2
Sodium arsenite	0.325*	0.759	41	2	2
Mercury chloride	6.13	4.12	1	1	1
Cycloheximide	0.144*	0.187	2	1	1

* = only one IC_{50} value was available; ** = values taken from BRD of the NICEATM/ECVAM validation study (Section 5, Table 5-8); *** = LD_{50} data are from BRD of the NICEATM/ECVAM validation study (Section 3, Table 3-2)

SDS was included in all tests performed throughout the validation study as a positive control that would induce cytotoxicity with an IC_{50} of 41.7 µg/ml (cited by the BRD of NICEATM/ECVAM validation study, as the geometric mean of IC_{50} values from the three testing laboratories). Since HSL initially lacked historical data on SDS, these data were generated during the course of the validation study. A positive control plate was set up in each test of the cytotoxicity assay and the data collated, and the average IC_{50} values and standard deviations were calculated.

A range of acceptable IC_{50} values for SDS were derived from the values obtained encompassing 2.5 times the standard deviations:

Hill function value: 38.8 ± 8.60 µg/ml; range: 17.3 – 60.3 µg/ml

This HSL-generated value was very similar to the value from the NICEATM/ECVAM validation study (41.7 µg/ml) and was used as part of the acceptance criteria for subsequent experiments performed with the validation set of test chemicals (see acceptance criteria in Section 5.6.2).

8.2. Transfer of the validated 3T3 NRU test method protocol to the JRC laboratory

The IHCP robotic testing platform of JRC performed the 3T3 NRU test method following the NICEATM/ECVAM validated study to evaluate the transferability of the 3T3 NRU manual protocol to the automated platform during the period from January to April 2007 (Norlén et al. 2007). Of the total chemicals tested in the study, 12 are listed in the BRD of the NICEATM/ECVAM validation study. The results of the 12 chemicals are summarised in the Table 22.

Table 22. Comparison of IC_{50} values obtained by the JRC robotic testing platform using the automated 3T3 NRU test method protocol with those reported by the NICEATM/ECVAM validation study for 12 selected chemicals

Chemical	IC_{50} (µg/ml) obtained (SD)	IC_{50} (µg/ml) geometric mean Published*	Animal LD_{50} (mg/kg b.w.)**	GHS hazard classification	EU CLP hazard classification
2-Propanol (sealed)	5 215,1 (1083.1)	3 618	5 843	UC	UC
Acetaminophen	30.9 (2.1)	47.7	2 404	5	UC
Acetylsalicylic acid	1 518.3 (737.8)	676.4	1 000	4	4
Atropine sulphate	240.2 (101.4)	76.0	639	4	4
Carbamazepine	103.5 (12.4)	103.2	1 957	4	4
Valproic acid	1 666.4 (353.6)	916	1 695 (mouse)	4	4
Cadmium Cl	0.9 (0.4)	0.5	88	3	3
Caffeine	347.8 (107.3)	153	192	3	3
Verapamil HCl	39.9 (-)[a]	34.9	108	3	3
Colchicine	$2.4 \cdot 10^{-2}$ ($4.9 \cdot 10^{-3}$)	$3.4 \cdot 10^{-2}$	6 (mouse)	2	2
Cycloheximide	0.17 ($8.8 \cdot 10^{-2}$)	0.2	2	1	1
Mercury Cl	3.6 ($7.7 \cdot 10^{-2}$)	4.1	1	1	1

* = values taken from BRD of the NICEATM/ECVAM validation study (Section 5, Table 5-8); ** = LD_{50} data are from BRD of the NICEATM/ECVAM validation study (Section 3, Table 3-2); a = only one IC_{50} value available

The positive control test chemical was SDS. Since this was the first study with this assay undertaken using the JRC robotic testing platform, there were no "historical" data on which to define the interval of acceptable IC_{50} values for SDS (see acceptance criteria in Section 5.6.1). Therefore, the acceptance interval for the IC_{50} of SDS for the study was calculated retrospectively using the results from all valid SDS plates of the study carried out from January to April 2007 (Norlén et al. 2007), where the other

three acceptance criteria were met. The resulting acceptance interval for the IC_{50} of SDS was 39.9 - 59.7 µg/ml.

Test acceptance criteria for all other test plates were criteria 1 and 2. If SDS was not accepted in a test, then the corresponding test was not accepted and, therefore, all the plates were rejected.

An overview of all results for the accepted SDS-plates is shown in Figure 4. These results are an adaptation of Figure 7-5 on page 7-45 in the NICEATM/ECVAM BRD. The figure shows the variation of SDS IC_{50} values for different laboratories (and study phases) in the NICEATM/ECVAM validation study and the results from accepted SDS test plates in the robotic platform (second last column in Figure 4). The last column in the right hand side of the graph shows the results from SDS test plates obtained by performing the manual protocol in JRC.

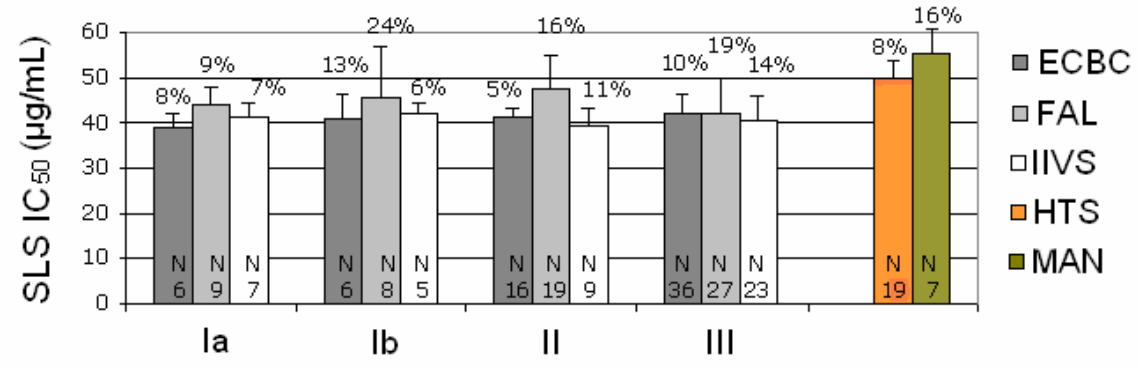

Figure 4. SDS IC_{50} for each laboratory, study phase, robotic platform tests and tests from ECBC (Edgewood Chemical Biological Centre, USA), FAL (FRAME Alternatives Laboratory, UK) and IIVS (Institute for In Vitro Sciences, USA).

The bars show mean IC_{50} values with standard deviations and the percent values above indicate intra-laboratory coefficients of variation (%). N = number of values HTS = high throughput screening; MAN = manual; SLS = sodium lauryl sulphate

The SDS test plates performed in the robotic platform have an IC_{50} mean of 50.0 µg/ml and standard deviation of 4.0 µg/ml. In general, both the standard deviation and the coefficient of variance for the SDS-plates performed in the robotic platform are

lower than for the other laboratories (and their different study phases). The IC_{50} mean of the plates performed in the robotic platform is higher than the IC_{50} means of the other laboratories that performed the cytotoxicity assay manually.

The results generated in this transferability phase of the 3T3 NRU test method protocol demonstrate the high precision achievable with the automated platform. The IC_{50} values for the chemicals tested compare very favourably with the data reported for the NICEATM/ECVAM validation study.

The Z-prime parameter was calculated to indicate the overall quality of the automated cytotoxicity assay. This parameter is used in the pharmaceutical sector and refers to the relationship between the test plate's signal dynamic range (in this case to the range of the optical density signals covering the viability responses from highest concentration to lowest concentration of test chemical) and the uncertainty associated with the signal measurements. For the Z-prime, the ideal value is 1 and with values between 0.5 and 1 the assay is considered to be "very good". In the present study, the Z-prime was above 0.5 for practically all the tests, therefore, indicating the high quality of the results generated by automated 3T3 NRU test method protocol (Norlén et al. 2007).

9.0 MODULE 4: BETWEEN LABORATORY REPRODUCIBILITY

As explained in Section 2 and according to the aim of this validation study only one laboratory, HSL, was appointed to evaluate the predictive capacity of the validated 3T3 NRU test method to discriminate between classified and unclassified chemicals (using a LD_{50} cut-off limit of 2 000 mg/kg b.w.) according to the current EU CLP classification system for acute oral toxicity. JRC and IIVS participated in the validation study with two variants of the validated protocol. Under these circumstances, a classical assessment of the variability between laboratories that used different protocols would not be applicable. However, we wanted to evaluate whether the automation of the validated test method protocol in JRC and/or the abbreviated protocol used by IIVS led to significant differences in terms of assigning the test chemicals to one of the two established toxicity categories (classified and unclassified).

Therefore, in this section, we present an evaluation of the variability between the three test method protocols by analysing the concordance between toxicity predictions obtained for each test chemical in the three laboratories using the three 3T3 NRU test method protocols. The concordance of toxicity predictions was calculated for each chemical using the validated regression models from the NICEATM/ECVAM validation study as previously described in Section 7.2, using as input the IC_{50} values obtained in each laboratory. Censored IC_{50} values were handled in the same manner as described in Section 7.3 (within-laboratory reproducibility).

Tables 23 and 24 summarise the concordances between laboratories of the predicted toxicities (classified and unclassified) based on the estimated LD_{50} values using the validated millimole and weight regression models, respectively. Trizinc bis(orthophosphate), Zinc distearate, and Zinc oxide were not tested in two laboratories (HSL and JRC). Furthermore, HSL did not test Aconitine, Brucine, and Malononitrile (see Section 6.2). Therefore, these 6 test chemicals were excluded from both regression analysis (Annex G).

9.1 Millimole regression model

From the remaining 50 test chemicals, and when the millimole regression was used, the assessment of concordance of toxicity predictions between the three laboratories was not possible for another nine chemicals. For these right censored IC_{50} values were excluded in at least one of the laboratories since the predicted LD_{50} after using the regression models was less than 2 000 mg/kg b.w. (as explained in Section 7.3). Annex G shows a summary of the number of chemicals excluded from the analysis in each laboratory. Of the remaining 41 test chemicals, 36 (88%) showed a concordance in toxicity predictions between all three laboratories, whereas for five chemicals (12%) the predictions were discordant between the laboratories (Table 23). Notable is that all the five chemicals had some special characteristics that may have led to the divergent results observed.

- For 1,2,4-Tricholorobenzene JRC reported only one acceptable measurement. In addition, this chemical reacted with the plastic test tubes during solubilisation and therefore all dilutions were prepared in glass tubes. Furthermore, IIVS observed variability for all four definitive tests with this test chemical as precipitates were observed in several of the concentrations tested.
- Triethanolamine required to be kept under argon, thus, if not stored correctly, its properties may have changed in the course of the study, which subsequently may have affected the study results.
- 2,6-Diethylaniline needed to be handled and stored under nitrogen. It was found to be volatile by both HSL and IIVS. Furthermore, JRC obtained two right censored IC_{50} values of which one was excluded since the estimated LD_{50} was smaller than 2 000 mg/kg b.w..
- 2-Butoxyethyl acetate also required special handling conditions in order to minimize exposure to air. It exhibited volatile effects in HSL and JRC. Precipitates were observed at HSL. IIVS reported only censored IC_{50} values for this test chemical.
- For Benzyl benzoate both HSL and JRC obtained only right censored IC_{50} values. Moreover, HSL applied the extended solubility protocol (see 6.1) but Benzyl benzoate still failed to be sufficiently cytotoxic on the 3T3 cells.

Between HSL and JRC the comparison of concordance of predicted toxicities could not be performed for 13 test chemicals (23%, 13/56) of which 7 chemicals were excluded from analysis due to censored IC_{50} values that did not meet the acceptance criteria (Section 7.3), and 6 chemicals were not tested in at least one laboratory. From the remaining 43 test chemicals, concordance of predictions between the two laboratories was observed for 41 test chemicals (95%). For two test chemicals (1,2,4-Trichlorobenzene and 2-Butoxyethyl acetate) there was no concordance between the predicted toxicities (5%).

Between IIVS and JRC the comparison of concordance of predicted toxicities could not be performed for 7 test chemicals, of which 4 were excluded from analysis due to censored IC_{50} values that did not meet the acceptance criteria mentioned in Section 7.3 in one of the laboratories, and 3 test chemicals were not tested in JRC. From the remaining 49 test chemicals the predicted toxicity was concordant for 44 test chemicals (90%) and discordant for 5 test chemicals (1,2,4-Tricholorobenzene, 2,6-Diethylaniline, Aconitine, Benzyl benzoate and Triethanolamine) (10%).

Between IIVS and HSL the comparison of concordance of predicted toxicities could not be performed for 14 test chemicals (6 were not tested in HSL and 8 were excluded due to censored IC_{50} values that did not meet the criteria set in Section 7.3). From the remaining 42 test chemicals, the predicted toxicity was concordant for 38 test chemicals (90.5%) and discordant for 4 test chemicals (2,6-Diethylaniline, 2-Butoxyethyl acetate, Benzyl benzoate and Triethanolamine) (9.5%).

Table 23. Comparison of toxicity predicted (classified/unclassified) in each laboratory by the IC_{50} value using the millimole regression

Chem nr	Chemical	HSL	JRC	IIVS*	Concordance between labs	*In vivo* LD_{50} (mg/kg)	Correct prediction HSL	Correct prediction JRC	Correct prediction IIVS
1.	(4-Ammonio-*m*-tolyl)ethyl (2-hydroxyethyl)ammonium sulphate	205.14 ± 28.09 (C)	163.76 ± 25.89 (C)	< 692.32 (C)	yes	58 (C)	yes	yes	yes
2.	1,2,4-Trichlorobenzene	674.26±87.36 (C)	> 2 398.05** (UC)	759.99 (C)	no	756 (C)	yes	no	yes
3.	1,2-Benzenedicarboxylic acid	> 2 563.6** (UC)	> 2 563.6 (UC)	> 3 833.0 (UC)	yes	2 550 (UC)	yes	yes	yes
4.	1,2-Dichlorobenzene	> 2 130.9** (UC)	censored excluded	> 2 130.9 (UC)	NA	2 065 (UC)	yes	censored excluded	yes
5.	1-Naphthylamine	292.50 ± 86.50 (C)	125.21 ± 14.99 (C)	< 185.98 (C)	yes	540 (C)	yes	yes	yes
6.	1-Phenyl-3-pyrazolidone	474.81 ± 33.94 (C)	260.02 ± 21.76 (C)	295.73 (C)	yes	255 (C)	yes	yes	yes
7.	2-(2-Butoxyethoxy)ethanol	2 038.7 ± 230.89 (UC)	2 262.7 ± 383.34 (UC)	2 312.8 (UC)	yes	6 249 (UC)	yes	yes	yes
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	780.99 ± 20.05 (C)	796.17 ± 35.66 (C)	656.02 (C)	yes	5 420 (UC)	no	no	no
9.	2,4,6-Tris (dimethylaminomethyl) phenol	censored excluded	832.34 ± 29.45 (C)	1 105.9 (C)	NA	1 200 (C)	censored excluded	yes	yes
10.	2,6-Diethylaniline	> 2 149.0* (UC)	> 2 149.0** (UC)	1 474.0 (C)	no	2 245 (UC)	yes	yes	no
11.	2-Butoxyethyl acetate	1 580.1 ± 48.99 (C)	2 435.8 ± 104.69 (UC)	> 2 236.3 (UC)	no	4 143 (UC)	no	yes	yes

Table 23. Comparison of toxicity predicted (classified/unclassified) in each laboratory by the IC_{50} value using the millimole regression

Chem nr	Chemical	HSL	JRC	IIVS*	Concordance between labs	*In vivo* LD_{50} (mg/kg)	Correct prediction HSL	Correct prediction JRC	Correct prediction IIVS
12.	2-Chloro-4-nitroaniline	413.12 ± 151.75 (C)	358.72 ± 22.73 (C)	317.03 (C)	yes	6 430 (UC)	no	no	no
13.	2-Ethylhexyl acrylate	censored excluded	> 2 419.0** (UC)	> 2 419.0 (UC)	NA	6 007 (UC)	censored excluded	yes	yes
14.	2-Phenoxyethanol	936.65 ± 21.99 (C)	1 321.3 ± 23.65 (C)	955.32 (C)	yes	4 565 (UC)	no	no	no
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	724.82 ± 197.97 (C)	653.12 ± 92.27 (C)	censored excluded	NA	> 10 000 (UC)	no	no	censored excluded
16.	Acetophenone	522.58 ± 34.26 (C)	1 792.5 ± 64.65 (C)	710.04 (C)	yes	1 701 (C)	yes	yes	yes
17.	Aconitine	not tested	1 944.3 ± 371.23 (C)	> 2 186.5 (UC)	NA	6 (C)	not tested	yes	no
18.	Ammonium chloride	527.23 ± 44.69 (C)	667.01 ± 87.15 (C)	499.44 (C)	yes	1 650 (C)	yes	yes	yes
19.	Barium chloride	censored excluded	1 871.3* (C)	1 084.6** (C)	NA	294 (C)	censored excluded	yes	yes
20.	Benzaldehyde	censored excluded	1 211.0 ± 26.34 (C)	458.42 (C)	NA	1 300 (C)	censored excluded	yes	yes
21.	Benzyl benzoate	> 2 618.5** (UC)	> 2 618.5** (UC)	1 574.3 (C)	no	1 990 (C)	no	no	yes
22.	Brucine	not tested	718.67 ± 24.46 (C)	609.76 (C)	NA	1 (C)	not tested	yes	yes
23.	Caprylic acid	957.49 ± 98.64 (C)	1 289.5 ± 177.96 (C)	1 072.6 (C)	yes	5 682 (UC)	no	no	no

Table 23. Comparison of toxicity predicted (classified/unclassified) in each laboratory by the IC_{50} value using the millimole regression

Chem nr	Chemical	HSL	JRC	IIVS*	Concordance between labs	*In vivo* LD_{50} (mg/kg)	Correct prediction HSL	Correct prediction JRC	Correct prediction IIVS
24.	Copper sulphate	480.51 ± 28.08 (C)	466.73 ± 11.77 (C)	< 163.93 (C)	yes	666 (C)	yes	yes	yes
25.	Diallyl phthalate	722.29 ± 115.06 (C)	723.00 ± 13.16 (C)	733.75 (C)	yes	822 (C)	yes	yes	yes
26.	Diepoxide 126	778.49 ± 68.49 (C)	620.72 ± 14.89 (C)	780.41 (C)	yes	4 500 (UC)	no	no	no
27.	Di-"isodecyl" phthalate	> 27 213** (UC)	2 955.1* (UC)	> 3 975. (UC)	yes	64 000 (UC)	yes	yes	yes
28.	Diisopropanolamine	1 104.4 ± 25.27 (C)	1 222.5 ± 93.27 (C)	947.78 (C)	yes	6 183 (UC)	no	no	no
29.	Dimethyldioctadecyl-ammonium chloride	338.67 ± 175.28 (C)	450.34 ± 24.97 (C)	460.28# (C)	yes	12 150 (UC)	no	no	no
30.	Edetic acid	1 262.8 ± 189.83 (C)	1 252.8 ± 168.09 (C)	1 041.4 (C)	yes	4 500 (UC)	no	no	no
31.	Ethoxyquin	209.87 ± 49.56 (C)	225.25 ± 23.88 (C)	291.24# (C)	yes	1 407 (C)	yes	yes	yes
32.	Ethyl acetoacetate	1 315.8 ± 243.36 (C)	1 849.8 ± 23.03 (C)	1 084.6 (C)	yes	3 980 (UC)	no	no	no
33.	Ethyl chloroacetate	207.52 ± 3.73 (C)	312.83 ± 30.95 (C)	273.41 (C)	yes	155 (C)	yes	yes	yes
34.	Glycerol triacetate	3 364.1 ± 194.81 (UC)	3 315.4 ± 797.76 (UC)	3 799.0 (UC)	yes	3 000 (UC)	yes	yes	yes
35.	Maleic acid	790.19 ± 134.61 (C)	1 229.0 ± 54.26 (C)	925.16 (C)	yes	708 (C)	yes	yes	yes

Table 23. Comparison of toxicity predicted (classified/unclassified) in each laboratory by the IC_{50} value using the millimole regression

Chem nr	Chemical	HSL	JRC	IIVS*	Concordance between labs	*In vivo* LD_{50} (mg/kg)	Correct prediction HSL	Correct prediction JRC	Correct prediction IIVS
36.	Malononitrile	not tested	176.89 ± 15.35 (C)	< 300.55 (C)	NA	19.5 (C)	not tested	yes	yes
37.	Methenamine	470.34 ± 39.41 (C)	733.81 ± 75.91 (C)	711.73 (C)	yes	9 200 (UC)	no	no	no
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	85.44 ± 30.20 (C)	90.09 ± 13.72 (C)	< 240.44 (C)	yes	1 047 (C)	yes	yes	yes
39.	Octyl 3,4,5-trihydroxybenzoate	35.29 ± 5.33 (C)	46.46 ± 7.36 (C)	< 272.19 (C)	yes	2 335 (UC)	no	no	no
40.	*P*-benzoquinone	85.36 ± 8.85 (C)	78.16 ± 6.17 (C)	< 144.17 (C)	yes	79 (C)	yes	yes	yes
41.	Phthalic anhydride	1 274.7 ± 23.18 (C)	1 263.6 ± 73.18 (C)	censored excluded	NA	4 500 (UC)	no	no	censored excluded
42.	Potassium sulfate	3 975.4 ± 144.25 (UC)	3 685.4 ± 122.43 (UC)	3 216.1 (UC)	yes	6 600 (UC)	yes	yes	yes
43.	Resorcinol	429.46 ± 14.37 (C)	490.08 ± 19.25 (C)	< 400.31 (C)	yes	535 (C)	yes	yes	yes
44.	Sodium cyanate	506.60 ± 13.75 (C)	555.64 ± 45.48 (C)	588.16 (C)	yes	1 500 (C)	yes	yes	yes
45.	Sodium salt of chloroacetic acid	535.32 ± 20.50 (C)	555.71 ± 57.64 (C)	428.60# (C)	yes	328 (C)	yes	yes	yes
46.	Sorbitan monolaurate	censored excluded	889.62 ± 63.77 (C)	913.69 (C)	NA	37 425 (UC)	censored excluded	no	no
47.	Tetramethylthiuram monosulphide	42.49 ± 7.99 (C)	66.11 ± 13.27 (C)	< 190.38 (C)	yes	400 (C)	yes	yes	yes

Table 23. Comparison of toxicity predicted (classified/unclassified) in each laboratory by the IC_{50} value using the millimole regression

Chem nr	Chemical	HSL	JRC	IIVS*	Concordance between labs	*In vivo* LD_{50} (mg/kg)	Correct prediction HSL	Correct prediction JRC	Correct prediction IIVS
48.	Triethanolamine	2 137.1 ± 91.29 (UC)	2 139.9 ± 112.44 (UC)	1 890.8 (C)	no	5 530 (UC)	yes	yes	no
49.	Triethylene glycol dimethacrylate	1 019.6 ± 359.04 (C)	591.84 ± 30.68 (C)	822.23 (C)	yes	10 750 (UC)	no	no	no
50.	Tripotassium citrate	2 965.3 ± 224.30 (UC)	2 752.1 ± 100.57 (UC)	2 872.8 (UC)	yes	> 7 200 (UC)	yes	yes	yes
51.	Tris(nonylphenyl) phosphite	censored excluded	censored excluded	> 4 596.1 (UC)	NA	14 750 (UC)	censored excluded	censored excluded	yes
52.	Trizinc bis(orthophosphate)	not tested	not tested	831.31 (C)	NA	30 000 (UC)	not tested	not tested	no
53.	Tween 20	2 994.9 ± 419.94 (UC)	2 404.2 ± 418.99 (UC)	2 544.1 (UC)	yes	40 370 (UC)	yes	yes	yes
54.	Urea	3 278.6 ± 143.16 (UC)	3 064.8 ± 30.65 (UC)	2 096.3# (UC)	yes	12 590 (UC)	yes	yes	yes
55.	Zinc distearate	not tested	not tested	1 639.2 (C)	NA	> 5 000 (UC)	not tested	not tested	no
56.	Zinc oxide	not tested	not tested	< 112.35 (C)	NA	7 950 (UC)	not tested	not tested	no

* = only two independent tests were performed and therefore, SD was not calculated; ** = IC_{50} value available only from one test; # = only the IC_{50} finite value was used to estimate the LD_{50}

9.2 Weight regression model

From the 50 test chemicals tested in all three laboratories and when the weight regression model was used to estimate LD_{50} values from the IC_{50} values obtained in each laboratory, the concordance of toxicity predictions could not be evaluated for 15 test chemicals. Annex G shows a summary of the number of chemicals excluded from the analysis in each laboratory. For these chemicals, IC_{50} right censored values were excluded in at least one of the three laboratories since the estimated LD_{50} was smaller than 2 000 mg/kg b.w. (see criteria in Section 7.3). The predicted toxicities were concordant for the remaining 35 test chemicals tested by all three laboratories (100%).

The comparison of predicted toxicity between HSL and JRC could not be done for 17 test chemicals (31%, 17/56) due to excluded censored IC_{50} values and/or not tested chemicals in at least one of the two laboratories. For 37 test chemicals (95%, 37/39) the predicted toxicities were concordant while for 2 test chemicals (2-Butoxyethyl acetate and Di-"isodecyl" phthalate) the predicted toxicities were discordant (5%, 2/39).

The comparison of concordance of predicted toxicities between JRC and IIVS could not be made for 15 test chemicals (3 were not tested in JRC and 12 were excluded due to IC_{50} censored values that did not meet the criteria set in Section 7.3). For the remaining 41 test chemicals, the predicted toxicity was concordant for all chemicals.

The comparison of predicted toxicities between HSL and IIVS could not be performed for 20 test chemicals, 6 of which were not tested in HSL and 14 were excluded due to right IC_{50} censored values that did not meet the established criteria (Section 7.3) in at least one of the two laboratories. For the remaining 36 test chemicals the predicted toxicity was concordant between the two laboratories (100%).

Table 24. Comparison of toxicity predicted (classified/unclassified) in each laboratory by the IC_{50} value using the weight regression

Chem nr	Chemical	HSL	JRC	IIVS*	Concordance between labs	*In vivo* LD_{50} (mg/kg)	Correct prediction HSL	Correct prediction JRC	Correct prediction IIVS
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	192.48 ± 22.36 (C)	158.98 ± 21.26 (C)	< 539.95 (C)	yes	58 (C)	yes	yes	yes
2.	1,2,4-Trichlorobenzene	661.90 ± 72.50 (C)	censored excluded	732.71 (C)	NA	756 (C)	yes	censored excluded	yes
3.	1,2-Benzenedicarboxylic acid	censored excluded	censored excluded	censored excluded	NA	2 550 (UC)	censored excluded	censored excluded	censored excluded
4.	1,2-Dichlorobenzene	censored excluded	censored excluded	censored excluded	NA	2 065 (UC)	censored excluded	censored excluded	censored excluded
5.	1-Naphthylamine	363.96 ± 90.79 (C)	177.88 ± 17.97 (C)	< 248.89 (C)	yes	540 (C)	yes	yes	yes
6.	1-Phenyl-3-pyrazolidone	518.92 ± 31.54 (C)	311.51 ± 22.16 (C)	346.82 (C)	yes	255 (C)	yes	yes	yes
7.	2-(2-Butoxyethoxy)ethanol	1 783.1 ± 170.68 (C)	1 946.3 ± 282.05 (C)	1 984.9 (C)	yes	6 249 (UC)	no	no	no
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	445.18 ± 9.69 (C)	452.48 ± 17.18 (C)	384.03 (C)	yes	5 420 (only mouse) (UC)	no	no	no
9.	2,4,6-Tris(dimethylaminomethyl) phenol	censored excluded	660.83 ± 19.79 (C)	840.80 (C)	NA	1 200 (C)	censored excluded	yes	yes
10.	2,6-Diethylaniline	censored excluded	censored excluded	1 405.2 (C)	NA	2 245 (UC)	censored excluded	censored excluded	no
11.	2-Butoxyethyl acetate	1 446.1 ± 37.98 (C)	2 086.7 ± 76.15 (UC)	censored excluded	NA	4 143 (UC)	no	yes	censored excluded

Table 24. Comparison of toxicity predicted (classified/unclassified) in each laboratory by the IC_{50} value using the weight regression

Chem nr	Chemical	HSL	JRC	IIVS*	Concordance between labs	*In vivo* LD_{50} (mg/kg)	Correct prediction HSL	Correct prediction JRC	Correct prediction IIVS
12.	2-Chloro-4-nitroaniline	445.10 ± 141.14 (C)	397.32 ± 21.40 (C)	357.53 (C)	yes	6 430 (UC)	no	no	no
13.	2-Ethylhexyl acrylate	censored excluded	censored excluded	censored excluded	NA	6 007 (UC)	censored excluded	censored excluded	censored excluded
14.	2-Phenoxyethanol	996.15 ± 19.84 (C)	1 333.4 ± 20.23 (C)	1 012.5 (C)	yes	4 565 (UC)	no	no	no
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	557.82 ± 128.83 (C)	511.87 ± 61.49 (C)	censored excluded	NA	> 10 000 (UC)	no	no	censored excluded
16.	Acetophenone	649.15 ± 36.09 (C)	1 845.1 ± 56.43 (C)	841.81 (C)	yes	1 701 (C)	yes	yes	yes
17.	Aconitine	not tested	887.24 ± 145.17 (C)	censored excluded	NA	6 (C)	not tested	yes	censored excluded
18.	Ammonium chloride	960.78 ± 68.81 (C)	1 172.2 ± 129.25 (C)	917.98 (C)	yes	1 650 (C)	yes	yes	yes
19.	Barium chloride	censored excluded	1 471.8* (C)	928.11** (C)	NA	294 (C)	censored excluded	yes	yes
20.	Benzaldehyde	censored excluded	1 403.9 ± 25.89 (C)	616.38 (C)	NA	1 300 (C)	censored excluded	yes	yes
21.	Benzyl benzoate	censored excluded	censored excluded	1 261.2 (C)	NA	1 990 (C)	censored excluded	censored excluded	yes
22.	Brucine	not tested	483.32 ± 13.96 (C)	420.35 (C)	NA	1 (C)	not tested	yes	yes
23.	Caprylic acid	994.01 ± 86.80 (C)	1 278.7 ± 150.67 (C)	1 094.9 (C)	yes	5 682 (UC)	no	no	no
24.	Copper sulphate	528.25 ± 26.11 (C)	515.45 ± 11.02 (C)	< 212.40 (C)	yes	666 (C)	yes	yes	yes

Table 24. Comparison of toxicity predicted (classified/unclassified) in each laboratory by the IC_{50} value using the weight regression

Chem nr	Chemical	HSL	JRC	IIVS*	Concordance between labs	*In vivo* LD_{50} (mg/kg)	Correct prediction HSL	Correct prediction JRC	Correct prediction IIVS
25.	Diallyl phthalate	606.56 ± 82.60 (C)	607.75 ± 9.38 (C)	615.41 (C)	yes	822 (C)	yes	yes	yes
26.	Diepoxide 126	639.43 ± 47.88 (C)	527.94 ± 10.73 (C)	640.68 (C)	yes	4 500 (UC)	no	no	no
27.	Di-"isodecyl" phthalate	> 9 907.9** (UC)	1 505.6* (C)	censored excluded	NA	64 000 (UC)	yes	no	censored excluded
28.	Diisopropanolamine	1 165.5 ± 22.62 (C)	1 270.0 ± 82.07 (C)	1 023.9 (C)	yes	6 183 (UC)	no	no	no
29.	Dimethyldioctadecyl-ammonium chloride	209.19 ± 92.18 (C)	269.34 ± 12.68 (C)	274.40# (C)	yes	12 150 (UC)	no	no	no
30.	Edetic acid	897.82 ± 114.11 (C)	891.99 ± 101.21 (C)	763.26 (C)	yes	4 500 (UC)	no	no	no
31.	Ethoxyquin	225.55 ± 45.78 (C)	239.98 ± 21.59 (C)	298.49# (C)	yes	1 407 (C)	yes	yes	yes
32.	Ethyl acetoacetate	1 365.1 ± 212.70 (C)	1 824.4 ± 19.24 (C)	1 160.5 (C)	yes	3 980 (UC)	no	no	no
33.	Ethyl chloroacetate	294.07 ± 4.49 (C)	416.23 ± 34.77 (C)	371.48 (C)	yes	155 (C)	yes	yes	yes
34.	Glycerol triacetate	2 368.5 ± 116.55 (UC)	2 333.7 ± 480.36 (UC)	2 625.8 (UC)	yes	3 000 (UC)	yes	yes	yes
35.	Maleic acid	935.76 ± 136.35 (C)	1 362.2 ± 51.07 (C)	1 070.9 (C)	yes	708 (C)	yes	yes	yes
36.	Malononitrile	not tested	344.45 ± 25.38 (C)	< 539.95 (C)	NA	19.5 (C)	not tested	yes	yes
37.	Methenamine	551.69 ± 39.14 (C)	804.10 ± 70.39 (C)	783.42 (C)	yes	9 200 (UC)	no	no	no

Table 24. Comparison of toxicity predicted (classified/unclassified) in each laboratory by the IC_{50} value using the weight regression

Chem nr	Chemical	HSL	JRC	IIVS*	Concordance between labs	*In vivo* LD_{50} (mg/kg)	Correct prediction HSL	Correct prediction JRC	Correct prediction IIVS
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	102.96 ± 31.61 (C)	108.21 ± 14.06 (C)	< 248.89 (C)	yes	1 047 (C)	yes	yes	yes
39.	Octyl 3,4,5-trihydroxybenzoate	44.03 ± 5.68 (C)	55.58 ± 7.45 (C)	< 248.89 (C)	yes	2 335 (UC)	no	no	no
40.	*P*-benzoquinone	146.97 ± 12.88 (C)	136.43 ± 9.12 (C)	< 229.27 (C)	yes	79 (C)	yes	yes	yes
41.	Phthalic anhydride	1 251.4 ± 19.27 (C)	1 241.9 ± 60.94 (C)	censored excluded	NA	4 500 (UC)	no	no	censored excluded
42.	Potassium sulfate	3 036.5 ± 93.23 (UC)	2 847.8 ± 80.08 (UC)	2 537.3 (UC)	yes	6 600 (UC)	yes	yes	yes
43.	Resorcinol	573.05 ± 16.23 (C)	640.88 ± 21.30 (C)	< 539.95 (C)	yes	535 (C)	yes	yes	yes
44.	Sodium cyanate	846.81 ± 19.46 (C)	915.54 ± 63.71 (C)	961.00 (C)	yes	1 500 (C)	yes	yes	yes
45.	Sodium salt of chloroacetic acid chloroacetate	672.47 ± 21.79 (C)	693.81 ± 61.31 (C)	557.02# (C)	yes	328 (C)	yes	yes	yes
46.	Sorbitan monolaurate	censored excluded	615.86 ± 37.36 (C)	630.08 (C)	NA	37 425 (UC)	censored excluded	no	no
47.	Tetramethylthiuram monosulphide	59.50 ± 9.46 (C)	86.53 ± 14.68 (C)	< 212.40 (C)	yes	400 (C)	yes	yes	yes
48.	Triethanolamine	1 932.0 ± 69.91 (C)	1 934.1 ± 86.32 (C)	1 741.7 (C)	yes	5 530 (UC)	no	no	no
49.	Triethylene glycol dimethacrylate	753.18 ± 223.91 (C)	477.42 ± 20.94 (C)	630.28 (C)	yes	10 750 (only mouse) (UC)	no	no	no

Table 24. Comparison of toxicity predicted (classified/unclassified) in each laboratory by the IC_{50} value using the weight regression

Chem nr	Chemical	HSL	JRC	IIVS*	Concordance between labs	*In vivo* LD_{50} (mg/kg)	Correct prediction HSL	Correct prediction JRC	Correct prediction IIVS
50.	Tripotassium citrate	1 810.9 ± 116.13 (C)	1 700.3 ± 52.72 (C)	1 763.3 (C)	yes	> 7 200 (UC)	no	no	no
51.	Tris(nonylphenyl) phosphite	censored excluded	censored excluded	censored excluded	NA	14 750 (UC)	censored excluded	censored excluded	censored excluded
52.	Trizinc bis(orthophosphate)	not tested	not tested	552.39 (C)	NA	30 000 (UC)	not tested	not tested	no
53.	Tween 20	943.39 ± 111.69 (C)	782.80 ± 115.08 (C)	822.25 (C)	yes	40 370 (UC)	no	no	no
54.	Urea	4 279.2 ± 158.60 (UC)	4 041.9 ± 34.26 (UC)	2 929.6# (UC)	yes	12 590 (UC)	yes	yes	yes
55.	Zinc distearate	not tested	not tested	776.19 (C)	NA	> 5 000 (UC)	not tested	not tested	no
56.	Zinc oxide	not tested	not tested	< 212.40 (C)	NA	7 950 (only mouse) (UC)	not tested	not tested	no

* = only two independent tests were performed and therefore, SD was not calculated; ** = IC_{50} value available only from one test; # = only the IC_{50} finite value was used to estimate the LD_{50}

9.3. Comparison of the millimole and weight regression results

When the weight regression model was used to predict the toxicities from the IC_{50} values obtained in each laboratory, the number of chemicals excluded (right IC_{50} censored values that resulted in an estimated LD_{50} value smaller than 2 000 mg/kg b.w. or were not tested) was higher than when the millimole regression was used (21 and 15 test chemicals excluded, respectively). Table 25 shows that with the millimole regression the number of test chemicals excluded due to right IC_{50} censored values that could not be accepted in HSL, JRC and IIVS was 6, 2, and 2, respectively. With the weight regression the number of test chemicals excluded due to right IC_{50} censored values that could not be accepted in HSL, JRC and IIVS was 10, 7 and 9, respectively.

Table 25. Test chemicals excluded in each laboratory when the millimole and weight regressions were used to predict the toxicity (classified or unclassified) of the test chemicals from the obtained IC_{50} values.

Chem Nr.	Chemical	Millimole regression			Weight regression		
		HSL	JRC	IIVS	HSL	JRC	IIVS
2.	1,2,4-Trichlorobenzene					X	
3.	1,2-Benzenedicarboxylic acid				X	X	X
4.	1,2-Dichlorobenzene		X		X	X	X
9.	2,4,6-Tris(dimethylaminomethyl)phenol	X			X		
10.	2,6-Diethylaniline				X	X	
11.	2-Butoxyethyl acetate						X
13.	2-Ethylhexyl acrylate	X			X	X	X
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone			X			X
17.	Aconitine						X
19.	Barium chloride	X			X		
20.	Benzaldehyde	X			X		
21.	Benzyl benzoate				X	X	
27.	Di-"isodecyl" phthalate						X
41.	Phthalic anhydride			X			X
46.	Sorbitan monolaurate	X			X		
51.	Tris(nonylphenyl) phosphite	X	X		X	X	X

When the millimole regression was used 5 chemicals showed discordant toxicity predictions between the three laboratories (1,2,4-Trichlorobenzene, Triethanolamine, 2,6-Diethylaniline, 2-Butoxyethyl acetate, and Benzyl benzoate), while with the

weight regression the toxicity predicted was concordant for all the chemicals for which comparisons were possible. It is noteworthy that 4 out of the 5 test chemicals that showed discordant prediction of toxicities with the millimole regression, were excluded from the weight regression due to right censored IC_{50} values resulting in estimated LD_{50} values smaller than 2 000 mg/kg b.w. In addition, for Triethanolamine the predicted toxicities between the three laboratories were concordant with the weight regression.

9.4. Summary

The variability between protocols (between-laboratory reproducibility) was assessed by analysing the concordance of toxicity predictions between the three laboratories using the mean LD_{50} values estimated from the IC_{50} values using both millimole and weight regressions.

With the millimole regression, there was 88% (36/41) concordance of toxicity predictions between the three laboratories and five test chemicals showed discordant predictions. With the weight regression, 100% (35/35) concordance of toxicity predictions between the three laboratories was obtained. For Triethanolamine the prediction of toxicity among the three laboratories changed to concordant with the weight regression analysis, while the other 4 test chemicals that resulted in discordant prediction of toxicities with the millimole regression, were excluded from the analysis using the weight regression, because the censored IC_{50} values did not meet the inclusion criteria described in Section 7.3. All the test chemicals with discordant predictions had some solubility or volatility problems, and/or required some special storage conditions. These may well have affected the accuracy of results and subsequent predictions.

The concordance of toxicity predictions were slightly higher between HSL and JRC (95%, 41/43) than between HSL and IIVS (90.5%, 38/42) or JRC and IIVS (90%, 44/49) with the millimole regression. With the weight regression, the concordance between HSL and JRC was also 95% (37/39), and 100% between HSL and IIVS and between JRC and IIVS. Between HSL and JRC the predicted toxicities were discordant for two chemicals: 2-Butoxyethyl acetate was discordant with both regression analyses; 1,2,4-Trichlorobenzene was discordant with the millimole

regression and was excluded in the weight regression analysis due to censored IC_{50} values, and Di-"isodecyl" phthalate was discordant only with the weight regression analysis.

Between HSL and IIVS and between JRC and IIVS, 4 and 5 test chemicals were discordant with the millimole regression, respectively. With the weight regression analysis only the predicted toxicity of Triethanolamine changed to concordant, in all three laboratories, while the other test chemicals were excluded due to IC_{50} censored values that did not meet the criteria established in Section 7.3.

Therefore, although the 3T3 NRU test method protocols used by HSL, JRC and IIVS were slightly different, they resulted in highly concordant predictions of toxicity (classified and unclassified) between the three laboratories.

10.0 MODULE 5: PREDICTIVE CAPACITY (PC)

10.1 Description of the statistical approaches used to assess the predictive capacity

For the purpose of this validation study the ability of the 3T3 NRU test method to distinguish classified from unclassified test chemicals, according to the current EU CLP classification scheme (limit dose 2 000 mg/kg b.w.), was assessed separately for each of the three test method protocols evaluated.

Since the validation study was conceived based on the previous findings from Halle's RC and NICEATM/ECVAM validation study, the aim was to determine how good the *in vitro* 3T3 NRU test method was in making the correct predictions of unclassified (correctly predicting an LD_{50} > 2 000 mg/kg b.w.) or classified ($LD_{50} \leq 2$ 000 mg/kg b.w.) using the regression models from the NICEATM/ECVAM validation study (millimole and the weight regression models).

In addition, a receiver operating characteristic curve (ROC) was used in order to determine the optimum IC_{50} for distinguishing between classified and unclassified test chemicals. For this purpose the LD_{50}-IC_{50} data pairs from the expanded RC were used (~550 chemicals). The optimum IC_{50} selected was then tested with the data obtained from the 3T3 NRU test method in each laboratory.

With both approaches, the parameters used to assess the predictivity of the assay were sensitivity, specificity, accuracy, positive predictive value and negative predictive value.

The sensitivity of a test is the ability of the test to correctly classify positive chemicals (for the purpose of this validation study, these are chemicals with $LD_{50} \leq 2$ 000 mg/kg b.w.). The specificity is the ability of the test to correctly classify negative chemicals (for the purpose of this validation study these are chemicals with LD_{50} > 2 000 mg/kg b.w.) .
Sensitivity is calculated using the following formula:

$$\text{Sensitivity} = [TP/(TP+FN)]*100$$

Specificity is calculated using the following formula:

$$\text{Specificity} = [TN/(FP+TN)]*100$$

Where:

TP – true positive

TN – true negative

FN – false negative

FP- false positive

The Positive Predictive Value (PPV) is defined as the probability that the chemical is actually positive when we get a positive test result. PPV is calculated using the following formula:

$$PPV = [TP/(TP+FP)]*100$$

The Negative Predictive Value (NPV) is defined as the probability that the chemical is actually negative when we get a negative test result. NPV is calculated using the following formula:

$$NPV = [TN/(FN+TN)]*100$$

Finally, accuracy is the proportion of true results (both true positives and true negatives) in the whole set of test chemicals tested using the method. Accuracy is calculated using the following formula:

$$\text{Accuracy} = [(TP+TN)/(TP+FP+FN+TN)]*100$$

The NPV and PPV of a test method are heavily dependent on the prevalence. Therefore, in view of the purpose of this validation study, the proportion of unclassified chemicals identified by the 3T3 NRU test method was calculated taking into consideration the prevalence of classified ($LD_{50} \leq 2\ 000$ mg/kg b.w.) and unclassified ($LD_{50} > 2\ 000$ mg/kg b.w.) chemicals. According to the analysis performed by Bulgheroni et al (2009) on the data from the EU NCD, 87% of the 4

219 industrial chemicals notified in EU until March 2008 fall into the unclassified category (according to EU CLP scheme chemicals with rat oral LD_{50} > 2 000 mg/kg b.w.) and 13 % belong to the EU CLP categories 1-4 (chemicals with rat oral LD_{50} ≤ 2 000 mg/kg b.w.). The following formula is used:

% of unclassified chemicals = (1-0.13)*Specificity + 0.13*(1-Sensitivity)

10.2 Regression analysis

The estimated LD_{50} values derived from the *in vitro* IC_{50} values obtained in each laboratory using the validated regression models were compared with published *in vivo* LD_{50} values obtained from the databases and literature (as described in Section 4 and Table 4).

10.2.1 Millimole regression analysis

One of the two regression analyses used to evaluate the predictive capacity of the 3T3 NRU test method was the millimole regression. For this analysis, the estimated LD_{50} values (mg/kg) were calculated from the IC_{50} millimolar values (see Section 7.2). Table 23 in Section 9 shows the estimated LD_{50} values as well as the categorisation of the test chemicals into classified or unclassified according to the 2 000 mg/kg cut-off limit.

<u>HSL</u>

With the millimole regression, in HSL the classified (LD_{50} ≤ 2 000 mg/kg)/unclassified (LD_{50} > 2 000 mg/kg) prediction could be obtained for 44 test chemicals. As for the rest of the chemicals, either the IC_{50} values were censored and had to be excluded from the analysis (according to the criteria in Section 7.3), or the chemical was not tested (Table 23, Section 9, Annex G). For 28 test chemicals (64%) the toxicity was correctly predicted; 15 test chemicals were over-predicted and one test chemical (Benzyl benzoate) was under-predicted. Seven out of the 11 true negatives (unclassified test chemicals) are used as cosmetic ingredients (see Tables 2 and 3).

Table 26. Prediction of acute oral toxicity (classified/unclassified) by the millimole regression in HSL

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified ($LD_{50} \leq 2\,000$ mg/kg)	Unclassified ($LD_{50} > 2\,000$ mg/kg)	Total
3T3-predicted toxicity (mg/kg)	Classified ($LD_{50} \leq 2\,000$ mg/kg)	17	15	32
	Unclassified ($LD_{50} > 2\,000$ mg/kg)	1	11	12
	Total	18	26	44

* = reference oral LD_{50} value in mg/kg from Table 4

Sensitivity: 17/18*100 = 94.4%
Specificity: 11/26*100 = 42.3%
PPV: 17/32*100 = 53.1%
NPV: 11/12*100 = 91.7%
Accuracy: (17+11)/44*100 = 63.6%
FP = 15
FN = 1

Taking into account the sensitivity and specificity of the 3T3 NRU test method at HSL and the prevalence of positive (13%) and negative (87%) chemicals in NCD, the proportion of unclassified chemicals identified by the test method was 38%.

JRC

With the millimole regression, results from five test chemicals could not be included in the analysis either because they were right censored IC_{50} values that had to be excluded since the estimated LD_{50} was smaller than 2 000 mg/kg b.w. or because the chemical was not tested (Annex G). From the remaining 51 test chemicals, 34 (67%) were correctly predicted as classified ($LD_{50} \leq 2\,000$ mg/kg) or unclassified ($LD_{50} > 2\,000$ mg/kg) (Table 23 Section 9). Fifteen chemicals were over-predicted and 2 chemicals were under-predicted (1,2,4-Trichlorobenzene and Benzyl benzoate). Eight out of the 12 true negatives (unclassified test chemicals) are used as cosmetic ingredients (see Tables 2 and 3).

Table 27. Prediction of acute oral toxicity (classified/unclassified) by the millimole regression in JRC

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified ($LD_{50} \leq 2\,000$ mg/kg)	Unclassified ($LD_{50} > 2\,000$ mg/kg)	Total
3T3-predicted toxicity (mg/kg)	Classified ($LD_{50} \leq 2\,000$ mg/kg)	22	15	37
	Unclassified ($LD_{50} > 2\,000$ mg/kg)	2	12	14
	Total	24	27	51

* = reference oral LD_{50} value in mg/kg from Table 4

Sensitivity: 22/24*100 = 91.7%
Specificity: 12/27*100 = 44.4%
PPV: 22/37*100 = 59.5%
NPV: 12/14*100 = 85.7%
Accuracy: (22+12)/51*100 = 66.7%
FP: 15
FN: 2

Taking into account the sensitivity and specificity of the 3T3 NRU test method at JRC and the prevalence of positive (13%) and negative (87%) chemicals in NCD, the proportion of unclassified chemicals identified by the test method was 40%

IIVS

With the millimole regression, two test chemicals were excluded from analysis since the IC_{50} values were right censored and the estimated LD_{50} values were smaller than 2 000 mg/kg b.w. (Annex G). From the remaining 54 test chemicals the toxicity of 35 test chemicals was correctly predicted (65%). Eighteen test chemicals were over-predicted and one chemical (Aconitine) was under-predicted. Eight out of the 12 true negatives (unclassified test chemicals) are used as cosmetic ingredients (see Tables 2 and 3).

Table 28. Prediction of acute oral toxicity (classified/unclassified) by the millimole regression in IIVS

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified ($LD_{50} \leq 2\,000$ mg/kg)	Unclassified ($LD_{50} > 2\,000$ mg/kg)	Total
3T3-predicted toxicity (mg/kg)	Classified ($LD_{50} \leq 2\,000$ mg/kg)	23	18	41
	Unclassified ($LD_{50} > 2\,000$ mg/kg)	1	12	13
	Total	24	30	54

* = reference oral LD_{50} value in mg/kg from Table 4

Sensitivity: 23/24*100 = 95.8%
Specificity: 12/30*100 = 40.0%
PPV: 23/41*100 = 56.1%
NPV: 12/13*100 = 92.3%
Accuracy: (23+12)/54*100 = 64.8%
FP: 18
FN: 1

Taking into account the sensitivity and specificity of the 3T3 NRU test method at IIVS and the prevalence of positive (13%) and negative (87%) chemicals in NCD, the proportion of unclassified chemicals identified by the test method was 35%.

Table 29 summarises the over and under-predictions in each laboratory when the millimole regression model was used.

Table 29. Over and under-prediction of toxicity in each laboratory using millimole regression

Chemical number	Chemical	Over-predicted			Under-predicted		
		HSL	JRC	IIVS	HSL	JRC	IIVS
2.	1,2,4-Trichlorobenzene					X	
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	X	X	X			
10.	2,6-Diethylaniline			X			
11.	2-Butoxyethyl acetate[a]	X					
12.	2-Chloro-4-nitroaniline	X	X	X			
14.	2-Phenoxyethanol[a]	X	X	X			
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone[a]	X	X				
17.	Aconitine						X
21.	Benzyl benzoate[a]				X	X	
23.	Caprylic acid[a]	X	X	X			
26.	Diepoxide 126	X	X	X			
28.	Diisopropanolamine	X	X	X			
29.	Dimethyldioctadecylammonium chloride[a]	X	X	X			
30.	Edetic acid[a]	X	X	X			
32.	Ethyl acetoacetate[a]	X	X	X			
37.	Methenamine[a]	X	X	X			
39.	Octyl 3,4,5-trihydroxybenzoate	X	X	X			
41.	Phthalic anhydride	X	X				
46.	Sorbitan monolaurate[a]		X	X			
48.	Triethanolamine[a]			X			
49.	Triethylene glycol dimethacrylate[a]	X	X	X			
52.	Trizinc bis(orthophosphate)			X			
55.	Zinc distearate[a]			X			
56.	Zinc oxide[a]			X			

[a] cosmetic use according to the EC database CosIng (see Tables 2 and 3)

When the test chemicals were classified according to the LD_{50} cut-off value of 2 000 mg/kg b.w., the toxicity of 24 test chemicals (8 unclassified and 16 classified), was correctly predicted in the three laboratories and over-predicted for 12 test chemicals (Table 23). Three chemicals were under-predicted: 1,2,4-Trichlorobenzene in JRC, Aconitine in IIVS, and Benzyl benzoate in HSL and JRC (Table 29).

Aconitine (principal alkaloid of *Aconitum napelus L.*) is the only test chemical under-predicted in IIVS. This is a highly toxic cardiotoxin and neurotoxin used in the past as

a homicidal weapon and still with some limited application in herbal medicine. The official EU CLP acute oral toxicity category is 1 ($LD_{50} \leq 5$ mg/kg). The cardiotoxicity and neurotoxicity of aconitine is due to its action on the voltage-sensitive sodium channels of the cell membranes of excitable tissues, including the myocardium, nerves and muscles (Chan, 2009). This chemical is also listed in the EC database of cosmetic ingredients (CosIng), however, as part of Annex II that contains substances which must not form part of the composition of cosmetic products. As indicated in Section 5.5, chemicals that exert their effects by mechanisms not active in 3T3 cells, are most likely to be under-predicted. Furthermore, Aconitine was found to be insoluble in all solvents in IIVS and it was tested at the lowest concentration (400 μg/ml). Although measures were taken to minimize precipitates, they could not be avoided. This test chemical was not tested in HSL (see section 6.2) and it was correctly predicted as classified in JRC with an estimated LD_{50} of 1 944.3 ± 371.23 mg/kg b.w., very close to the cut-off limit of 2 000 mg/kg b.w.

Benzyl benzoate was under-predicted in HSL and JRC. The official EU CLP acute oral toxicity category is 4 ($300 < LD_{50} \leq 2\ 000$ mg/kg). It has been reported that oral ingestion of large doses of Benzyl benzoate resulted in progressive lack of coordination, CNS excitation, seizures, and death (McEvoy 1993). In industry it is used as a plasticizer and a solvent, and it is also used as an antiparasitic pesticide (insecticide) (Heukelbach & Feldmeier 2006). Furtheremore, Benzyl benzoate is also a cosmetic ingredient but with some restrictions (Table 2).

In JRC, only one acceptable measurement of 1,2,4-Trichlorobenzene was obtained. The official EU CLP acute oral toxicity category is 4 ($300 < LD_{50} \leq 2\ 000$ mg/kg). It is used in industry as a solvent for making chemicals, and exposure to high levels may affect the liver, lungs, kidney and central nervous system (den Besten et al. 1991).

Furthermore, during the solubility test JRC reported that 1,2,4-Trichlorobenzene and Benzyl benzoate reacted with the plastic of the tubes during solubilisation.

10.2.2 Weight regression analysis

The second regression analysis used to assess the predictive capacity of the 3T3 NRU test method was the weight regression. For this analysis, the estimated LD_{50} values (mg/kg) were calculated from the IC_{50} values (µg/ml) using the formula of the validated weight regression model (see Section 7.2). Table 24 in Section 9 shows the estimated LD_{50} values as well as the categorisation of test chemicals into classified or unclassified according to the LD_{50} 2 000 mg/kg cut-off limit.

HSL

With the weight regression, sixteen test chemicals were excluded from the analysis since either they had right censored IC_{50} values based on the criteria explained in Section 7.3 or they were not tested in HSL (Table 24, Section 9, Annex G). From the remaining 40 test chemicals, the analysis showed correct prediction of toxicity for 21 test chemicals (53%). Nineteen test chemicals were over-predicted and none was under-predicted. Benzyl benzoate, which was under-predicted in HSL with the millimole regression, was not included in the weight regression analysis since the right censored IC_{50} values resulted in LD_{50} values that were smaller than 2 000 mg/kg b.w. and were excluded. Three out of the 4 true negatives (unclassified test chemicals) are used as cosmetic ingredients (see Tables 2 and 3).

Table 30. Prediction of acute oral toxicity (classified/unclassified) by the weight regression in HSL

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified ($LD_{50} \leq 2\,000$ mg/kg)	Unclassified ($LD_{50} > 2\,000$ mg/kg)	Total
3T3-predicted toxicity (mg/kg)	Classified ($LD_{50} \leq 2\,000$ mg/kg)	17	19	36
	Unclassified ($LD_{50} > 2\,000$ mg/kg)	0	4	4
	Total	17	23	40

* = reference oral LD_{50} value in mg/kg from Table 4

Sensitivity: 17/17*100 = 100%
Specificity: 4/23*100 = 17.4%
PPV: 17/36*100 = 47.2%
NPV: 4/4*100 = 100%
Accuracy: (17+4)/40*100 = 52.5%
FP: 19
FN: 0

Taking into account the sensitivity and specificity of the 3T3 NRU test method at HSL and the prevalence of positive (13%) and negative (87%) chemicals in NCD, the proportion of unclassified chemicals identified by the test method was 15%.

JRC

With the weight regression, ten test chemicals were excluded from the analysis since they had right censored IC_{50} values based on the criteria explained in Section 7.3 or chemicals that were not tested in JRC (Table 24, Section 9, Annex G). From the remaining 46 chemicals, the analysis showed correct prediction of toxicity for 26 test chemicals (57%). Twenty test chemicals were over-predicted and none was under-predicted. Benzyl benzoate and 1,2,4-Trichlorobenzene, which were under-predicted in JRC with the millimole regression, were not included in the weight regression analysis due to right censored IC_{50} values resulting in LD_{50} values smaller than 2 000 mg/kg b.w. The 4 true negatives (unclassified test chemicals) are used as cosmetic ingredients (see Tables 2 and 3).

Table 31. Prediction of acute oral toxicity (classified/unclassified) by the weight regression in JRC

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified ($LD_{50} \leq 2\,000$ mg/kg)	Unclassified ($LD_{50} > 2\,000$ mg/kg)	Total
3T3-predicted toxicity (mg/kg)	Classified ($LD_{50} \leq 2\,000$ mg/kg)	22	20	42
	Unclassified ($LD_{50} > 2\,000$ mg/kg)	0	4	4
	Total	22	24	46

* = reference oral LD_{50} value in mg/kg from Table 4

Sensitivity: 22/22*100 = 100%
Specificity: 4/24*100 = 16.7%
PPV: 22/42*100 = 52.4%
NPV: 4/4*100 = 100%
Accuracy: (22+4)/46*100 = 56.5%
FP: 20
FN: 0

Taking into account the sensitivity and specificity of the 3T3 NRU test method at JRC and the prevalence of positive (13%) and negative (87%) chemicals in NCD, the proportion of unclassified chemicals identified by the test method was 15%.

IIVS

With the weight regression, nine test chemicals were excluded from the analysis due to exclusion of right censored IC_{50} values since the estimated LD_{50} value was smaller than 2 000 mg/kg b.w. (Table 24, Section 9, Annex G). For the remaining 47 test chemicals, the results of the analysis showed correct prediction of toxicity for 26 test chemicals (55%). Twenty one test chemicals were over-predicted and none was under-predicted. Aconitine, which was under-predicted in JRC with the millimole regression, was not included in the weight regression analysis since it had right censored IC_{50} values resulting in LD_{50} values smaller than 2 000 mg/kg b.w. The 3 true negatives (unclassified test chemicals) are used as cosmetic ingredients (see Tables 2 and 3).

Table 32. Prediction of acute oral toxicity (classified/unclassified) by the weight regression in IIVS

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified ($LD_{50} \leq 2\,000$ mg/kg)	Unclassified ($LD_{50} > 2\,000$ mg/kg)	Total
3T3-predicted toxicity (mg/kg)	Classified ($LD_{50} \leq 2\,000$ mg/kg)	23	21	44
	Unclassified ($LD_{50} > 2\,000$ mg/kg)	0	3	3
	Total	23	24	47

* = reference oral LD_{50} value in mg/kg from Table 4

Sensitivity: 23/23*100 = 100%
Specificity: 3/24*100 = 12.5%
PPV: 23/44*100 = 52.3%
NPV: 3/3*100 = 100%
Accuracy: (23+3)/47*100 = 55.3%
FP: 21
FN: 0

Taking into account the sensitivity and specificity of the 3T3 NRU test method at IIVS and the prevalence of positive (13%) and negative (87%) chemicals in NCD, the proportion of unclassified chemicals identified by the test method was 11%.

Table 33 summarises the over-predictions in each laboratory with the weight regression. Table 34 shows the performance of the 3T3 NRU test method in each

laboratory with both regression analyses. Test chemicals used as cosmetic ingredients, are also highlighted in the table.

Table 33. Summary of over-predicted toxicities (false positive chemicals) in each laboratory using the weight regression model

Chemical number	Chemical	HSL	JRC	IIVS
7.	2-(2-Butoxyethoxy)ethanol[a]	X	X	X
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	X	X	X
10.	2,6-Diethylaniline			X
11.	2-Butoxyethyl acetate[a]	X		
12.	2-Chloro-4-nitroaniline	X	X	X
14.	2-Phenoxyethanol[a]	X	X	X
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone[a]	X	X	
23.	Caprylic acid[a]	X	X	X
26.	Diepoxide 126	X	X	X
27.	Di-"isodecyl" phthalate		X	
28.	Diisopropanolamine	X	X	X
29.	Dimethyldioctadecylammonium chloride[a]	X	X	X
30.	Edetic acid[a]	X	X	X
32.	Ethyl acetoacetate[a]	X	X	X
37.	Methenamine[a]	X	X	X
39.	Octyl 3,4,5-trihydroxybenzoate	X	X	X
41.	Phthalic anhydride	X	X	
46.	Sorbitan monolaurate[a]		X	X
48.	Triethanolamine[a]	X	X	X
49.	Triethylene glycol dimethacrylate[a]	X	X	X
50.	Tripotassium citrate[a]	X	X	X
52.	Trizinc bis(orthophosphate)			X
53.	Tween 20[a]	X	X	X
55.	Zinc distearate[a]			X
56.	Zinc oxide[a]			X

[a] use as cosmetic ingredient according to the EC database CosIng (see Table 2)

The test chemicals that were under-predicted (false negative results) in each laboratory with the millimole regression model were excluded from the weight regression analysis due to censored IC_{50} values that resulted in estimated LD_{50} values smaller than 2 000 mg/kg b.w. (see section 7.3).

Table 34. Comparison of millimole and weight regressions for their performance in predicting the acute oral toxicity categories (classified/unclassified) of the 56 test chemicals

	HSL		JRC		IIVS	
	Millimole regression n = 44	Weight regression n = 40	Millimole regression n = 51	Weight regression n = 46	Millimole regression n = 54	Weight regression n = 47
Sensitivity (%)	94.4	100	91.7	100	95.8	100
Specificity (%)	42.3	17.4	44.4	16.7	40	12.5
PPV (%)	53.1	47.2	59.5	52.4	56.1	52.3
NPV (%)	91.7	100	85.7	100	92.3	100
Accuracy (%)	63.6	52.5	66.7	56.5	64.8	55.3
FP	15	19	15	20	18	21
FN	1	0	2	0	1	0

Of the 56 test chemicals, 27 are used as cosmetic ingredients (48%, see Tables 2 and 3). The performance of the 3T3 NRU test method in predicting the two acute oral toxicity categories (classified and unclassified) of the 27 cosmetic ingredients in each laboratory with both regression analyses are summarised in Table 35.

Table 35. Comparison of millimole and weight regressions for their performance in predicting the acute oral toxicity categories (classified/unclassified) of the 27 test chemicals used as cosmetic ingredients

	HSL		JRC		IIVS	
	Millimole regression n = 22	Weight regression n = 21	Millimole regression n = 24	Weight regression n = 23	Millimole regression n = 26	Weight regression n = 24
Sensitivity (%)	83.3	100.0	85.7	100.0	100.0	100.0
Specificity (%)	43.8	18.8	47.1	23.5	42.1	17.7
PPV (%)	35.7	27.8	40.0	31.6	38.9	33.3
NPV (%)	87.5	100.0	88.9	100.00	100.0	100.0
Accuracy (%)	54.6	38.1	58.3	43.5	57.7	41.7
FP	9	13	9	13	11	14
FN	1	0	1	0	0	0

10.3 Receiver Operating Characteristic (ROC) Curve analysis

The second approach used to evaluate the predictive capacity of the 3T3 NRU test method to identify classified ($LD_{50} \leq 2\,000$ mg/kg b.w.) and unclassified ($LD_{50} > 2\,000$ mg/kg b.w.) test chemicals according to the EU CLP classification scheme, was based on applying to the data set the optimum IC_{50} value for distinguishing between classified and unclassified test chemicals, using the LD_{50} of 2 000 mg/kg b.w. as cut-off value. The optimal IC_{50} value was identified using the receiver operating characteristic (ROC) analysis on a training data set different from the validation data set.

A ROC Curve is a method of describing the accuracy of an assay separately from the decision thresholds. A ROC Curve is a plot of the true positive rate (Sensitivity) (Y axis) versus its false positive rate (1-Specificity) (X axis) for different cut-off points. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. It is known that as sensitivity increases, the specificity decreases, and the ROC curve shows precisely the magnitudes of these variations.

The RC data set (approx. 550 chemicals) was used as the training set to generate a prediction model to be used with the data set generated by each laboratory in the present validation study. A ROC curve was generated using the IC_{50} values and the classifications derived from experimental published LD_{50} values (Figure 5).

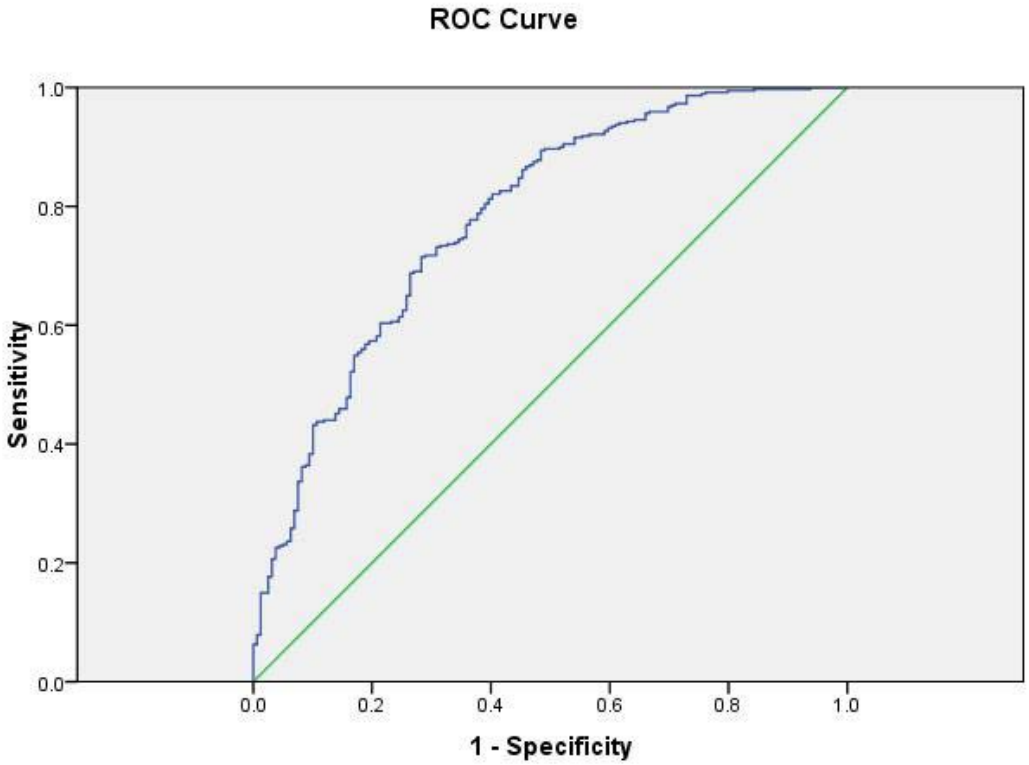

Figure 5. ROC Plot of the 3T3 NRU test method to discriminate between classified (LD_{50} ≤ 2 000 mg/kg b.w.) and unclassified (LD_{50} > 2 000 mg/kg b.w.) test chemicals based on the RC data set.

For each ROC curve, a list of cut-off values is generated (Table 36). There is not a precise rule to determine the best cut-off among the proposed list and the choice must be made according to the needs of the study and other practical considerations (e.g. a higher sensitivity is preferable if the aim is to correctly predict positive chemicals, even if there is a loss of specificity). Three different thresholds were chosen. The first one maximises specificity; the second gives the best balance between sensitivity and specificity, and the third maximises sensitivity.

Table 36. Criterion values and Coordinates of the ROC Curve

Positive if Less Than or Equal To	Sensitivity	1 - Specificity	Specificity
67.345686	.552	.176	.824
67.944742	.554	.176	.824
68.408295	.554	.182	.818
69.022893	.557	.182	.818
69.274559	.560	.182	.818
69.551204	.560	.189	.811
70.131893	.563	.189	.811
70.641682	.565	.189	.811
71.173328	.568	.189	.811
71.641200	.568	.195	.805
72.435719	.571	.195	.805
[a]73.297899	**.573**	**.195**	**.805**
73.889174	.573	.201	.799
75.090018	.573	.208	.792
76.736766	.576	.208	.792
77.769055	.579	.208	.792
77.992371	.582	.208	.792
78.326914	.582	.214	.786
78.890003	.584	.214	.786
80.469730	.587	.214	.786
82.044568	.590	.214	.786
83.296610	.592	.214	.786
85.906226	.595	.214	.786
88.445875	.598	.214	.786
89.597094	.601	.214	.786
90.698121	.603	.214	.786
91.627426	.603	.220	.780
91.988131	.603	.226	.774
93.021062	.603	.233	.767
95.041648	.606	.233	.767
96.564602	.606	.239	.761
97.117718	.606	.245	.755
98.186606	.609	.245	.755
98.990644	.611	.245	.755
99.219746	.614	.245	.755
99.410859	.614	.252	.748
99.750756	.617	.252	.748
100.268711	.620	.252	.748
100.622401	.622	.252	.748
100.801818	.625	.252	.748
101.111231	.625	.258	.742
103.573031	.628	.258	.742
106.384915	.630	.258	.742
107.435457	.633	.258	.742

Table 36. Criterion values and Coordinates of the ROC Curve

Positive if Less Than or Equal To	Sensitivity	1 - Specificity	Specificity
108.008879	.636	.258	.742
109.018341	.639	.258	.742
110.832022	.641	.258	.742
111.910630	.644	.258	.742
113.289964	.647	.258	.742
115.328611	.649	.258	.742
116.345391	.649	.264	.736
116.909193	.652	.264	.736
118.356568	.655	.264	.736
119.568800	.658	.264	.736
120.614893	.660	.264	.736
122.070331	.663	.264	.736
124.489707	.666	.264	.736
127.224080	.668	.264	.736
128.117715	.671	.264	.736
128.453640	.674	.264	.736
131.104834	.677	.264	.736
136.684293	.679	.264	.736
141.181662	.682	.264	.736
144.533635	.685	.264	.736
146.588332	.688	.264	.736
147.061477	.688	.270	.730
148.155517	.690	.270	.730
148.978988	.690	.277	.723
150.156620	.690	.283	.717
158.683509	.693	.283	.717
167.491701	.696	.283	.717
169.981090	.698	.283	.717
173.845656	.701	.283	.717
178.223092	.704	.283	.717
180.816506	.707	.283	.717
182.695181	.709	.283	.717
184.582195	.712	.283	.717
[b]186.363066	**.715**	**.283**	**.717**
188.021450	.715	.289	.711
190.293261	.717	.289	.711
193.352849	.717	.296	.704
195.261577	.717	.302	.698
196.966910	.717	.308	.692
199.562938	.720	.308	.692
203.462623	.723	.308	.692
206.644170	.726	.308	.692
208.500268	.728	.308	.692
210.288959	.731	.308	.692

Table 36. Criterion values and Coordinates of the ROC Curve

Positive if Less Than or Equal To	Sensitivity	1 - Specificity	Specificity
211.201704	.731	.314	.686
213.034410	.734	.314	.686
215.320020	.734	.321	.679
216.473059	.734	.327	.673
218.119310	.736	.327	.673
223.493456	.736	.333	.667
227.912054	.736	.340	.660
235.188756	.739	.340	.660
243.552842	.739	.346	.654
247.540458	.742	.346	.654
252.825733	.745	.346	.654
257.160928	.745	.352	.648
263.592600	.747	.352	.648
270.127478	.747	.358	.642
277.603899	.750	.358	.642
284.243150	.753	.358	.642
285.980103	.755	.358	.642
288.665175	.758	.358	.642
290.776433	.761	.358	.642
293.113464	.764	.358	.642
296.093679	.766	.358	.642
297.161871	.769	.358	.642
304.996499	.769	.365	.635
313.188970	.772	.365	.635
316.142588	.774	.365	.635
322.578254	.777	.365	.635
327.798113	.777	.371	.629
329.636132	.777	.377	.623
332.832773	.780	.377	.623
336.977883	.783	.377	.623
340.979435	.785	.377	.623
343.596754	.788	.377	.623
345.884229	.788	.384	.616
352.844348	.791	.384	.616
357.950639	.793	.384	.616
359.320420	.796	.384	.616
372.964042	.796	.390	.610
391.913697	.799	.390	.610
398.731140	.802	.390	.610
400.973338	.804	.390	.610
408.406123	.804	.396	.604
415.331188	.807	.396	.604
417.844940	.810	.396	.604
420.326537	.813	.396	.604

Table 36. Criterion values and Coordinates of the ROC Curve

Positive if Less Than or Equal To	Sensitivity	1 - Specificity	Specificity
426.233025	.813	.403	.597
434.441088	.815	.403	.597
445.471160	.818	.403	.597
457.375856	.821	.403	.597
463.856968	.821	.409	.591
466.626176	.821	.415	.585
475.067164	.823	.415	.585
483.698990	.826	.415	.585
[c]485.625682	**.826**	**.421**	**.579**
488.112729	.826	.428	.572
493.191475	.826	.434	.566
504.857536	.829	.434	.566
525.491029	.832	.434	.566
540.401262	.834	.434	.566
545.167869	.834	.440	.560
552.853458	.834	.447	.553
559.101397	.837	.447	.553
582.795651	.840	.447	.553
615.217473	.842	.447	.553
626.671774	.845	.447	.553
639.750187	.848	.447	.553

Test result variable(s): IC_{50} (µg/ml). Numbers in bold indicated the three selected thresholds: a = maximises specificity, b = best balance between sensitivity and specificity, c = maximises sensitivity

10.3.1 Analysis of the predictive capacity obtained using the ROC cut-off value

Predictive capacity (i.e. sensitivity, specificity, PPV, NPV and accuracy) was calculated for each laboratory applying the previously described thresholds to the set of 56 test chemicals tested in the present validation study.

Figure 5 shows the obtained ROC curve and Table 36 the coordinates of the ROC curve when applied to the LD_{50} cut-off value of 2 000 mg/kg b.w..

According to the ROC analysis, the IC_{50} cut-off value of 485.6 µg/ml (classified if the IC_{50} is smaller or equal to 485.6 µg/ml and unclassified if the IC_{50} is higher than 485.6 µg/ml) was chosen for the prediction of toxicity due to its high sensitivity.

Using this IC_{50} cut-off value, the predictive capacity of the 3T3 NRU test method protocol used in each laboratory was calculated.

HSL

Six test chemicals were not tested in HSL and three were excluded from the analysis (see Annex G) since they had right censored IC_{50} values smaller than the cut-off value of 485.6 (e.g. IC_{50} > 10 mg/ml). From the remaining 47 test chemicals the toxicity was under-predicted for 3 test chemicals (Barium chloride, Benzaldehyde, Benzyl benzoate) and over-predicted for 12 test chemicals, when an IC_{50} cut-off value of 485.6 μg/ml was used. Eight out of the 15 true negative (unclassified test chemicals) are used as cosmetic ingredients (see Tables 2 and 3).

Table 37. Prediction of acute oral toxicity (classified/unclassified) by applying an IC_{50} cut-off value of 485.6 μg/ml in HSL

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified	Unclassified	Total
Toxicity according to the IC_{50} 485.6 μg/ml cut-off value	Classified	17	12	29
	Unclassified	3	15	18
	Total	20	27	47

* = reference oral LD_{50} value in mg/kg from Table 4

Sensitivity: 17/20*100 = 85%
Specificity: 15/27*100 = 55.6%
PPV: 17/29*100 = 58.6%
NPV: 15/18*100 = 83.3%
Accuracy: (17+15)/47*100 = 68.1%
FP:12
FN: 3

Taking into account the sensitivity and specificity of the 3T3 NRU test method at HSL and the prevalence of positive (13%) and negative (87%) chemicals in NCD, the proportion of unclassified chemicals identified by the test method was 68%.

JRC

Three test chemicals were not tested in this laboratory and one was excluded from the analysis since the IC_{50} value was right censored and smaller than the threshold 485.6 μg/ml (Annex G). From the remaining 52 test chemicals the toxicity was under-predicted for 7 test chemicals (1,2,4-Trichlorobenzene, Acetophenone, Ammonium

chloride, Barium chloride, Benzaldehyde, Benzyl benzoate, and Maleic acid) and over-predicted for 11 test chemicals, when an IC_{50} cut-off value of 485.6 µg/ml was used. Ten out of the 17 true negatives (unclassified test chemicals) are used as cosmetic ingredients (see Tables 2 and 3).

Table 38. Prediction of acute oral toxicity (classified/unclassified) by applying an IC_{50} cut-off value of 485.6 µg/ml in JRC

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified	Unclassified	Total
Toxicity according to the IC_{50} 485.6 µg/ml cut-off value	Classified	17	11	28
	Unclassified	7	17	24
	Total	24	28	52

* = reference oral LD_{50} value in mg/kg from Table 4

Sensitivity: 17/24*100 = 70.8%
Specificity: 17/28*100 = 60.7%
PPV: 17/28*100 = 60.7%
NPV: 17/24*100 = 70.8%
Accuracy: (17+17)/52*100 = 65.4%
FP: 11
FN: 7

Taking into account the sensitivity and specificity of the 3T3 NRU test method at JRC and the prevalence of positive (13%) and negative (87%) chemicals in NCD, the proportion of unclassified chemicals identified by the test method was 57%.

IIVS

Three test chemicals were excluded from the analysis since the IC_{50} values were right censored and smaller than the threshold of 485.6 µg/ml (Annex G). From the remaining 53 test chemicals the toxicity was under-predicted for 2 test chemicals (Benzyl benzoate and Maleic acid) and over-predicted for 15 test chemicals, when an IC_{50} cut-off value of 485.6 µg/ml was used. Nine out of the 15 true negatives (unclassified test chemicals) are used as cosmetic ingredients (see Tables 2 and 3).

Table 39. Prediction of acute oral toxicity (classified/unclassified) by applying an IC_{50} cut-off value of 485.6 µg/ml in IIVS

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified	Unclassified	Total
Toxicity according to the IC_{50} 485.6 µg/ml cut-off value	Classified	21	15	36
	Unclassified	2	15	17
	Total	23	30	53

* = reference oral LD_{50} value in mg/kg from Table 4

Sensitivity: 21/23*100 = 91.3%
Specificity: 15/30*100 = 50%
PPV: 21/36*100 = 58.3%
NPV: 15/17*100 = 88.2%
Accuracy: (21+15)/53*100 = 67.9%
FP: 15
FN: 2

Taking into account the sensitivity and specificity of the 3T3 NRU test method at IIVS and the prevalence of positive (13%) and negative (87%) chemicals in NCD, the proportion of unclassified chemicals identified by the test method was 45%.

The test chemicals identified as false negatives (under-predicted toxicity) and false positives (over-predicted toxicity) in each laboratory are shown in Table 40.

Table 40. Over and under-prediction of toxicity generated in each laboratory by applying an IC_{50} value of 485.6 µg/ml to discriminate between classified and unclassified chemicals

Chem. Nr.	Chemical	Over-predicted			Under-predicted		
		HSL	JRC	IIVS	HSL	JRC	IIVS
2.	1,2,4-Trichlorobenzene					X	
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	X	X	X			
12.	2-Chloro-4-nitroaniline	X	X	X			
14.	2-Phenoxyethanol[a]	X		X			
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'- dinitroacetophenone[a]	X	X				
16.	Acetophenone[a]					X	
18.	Ammonium chloride[a]					X	
19.	Barium chloride				X	X	
20.	Benzaldehyde[a]				X	X	
21.	Benzyl benzoate[a]				X	X	X
23.	Caprylic acid[a]	X					

Chem. Nr.	Chemical	Over-predicted			Under-predicted		
		HSL	JRC	IIVS	HSL	JRC	IIVS
26.	Diepoxide 126	X	X	X			
28.	Diisopropanolamine			X			
29.	Dimethyldioctadecylammoium chloride[a]	X	X	X			
30.	Edetic acid[a]	X	X	X			
35.	Maleic acid[a]					X	X
37.	Methenamine[a]	X	X	X			
39.	Octyl 3,4,5-trihydroxybenzoate	X	X	X			
46.	Sorbitan monolaurate[a]		X	X			
49.	Triethylene glycol dimethacrylate[a]	X	X	X			
52.	Trizinc bis(orthophosphate)			X			
53.	Tween 20[a]	X	X	X			
55.	Zinc distearate[a]			X			
56.	Zinc oxide[a]			X			

[a] use as cosmetic ingredient according to the EC CosIng database (see Tables 2 and 3)

When the cut-off value 485.6 µg/ml was applied to the subset of 27 test chemicals used as cosmetics ingredients, the sensitivity was 71% in HSL, 29% in JRC, and 71% in IIVS, whereas the specificity was 50%, 59%, and 53%, in HSL, JRC and IIVS, respectively. The ROC analysis gave an overall accuracy for this type of chemicals of 50-58%. NPV was 80% in HSL, 67% in JRC, and 83% in IIVS. Most of the under-predictions observed in the analysis with all 56 test chemicals, are those with uses as cosmetic ingredients (Benzaldehyde, Benzyl benzoate Acetophenone, Ammonium chloride, and Maleic acid) as shown in Table 40.

Of the seven test chemicals with false negative prediction, Benzyl benzoate was common between the three laboratories, Barium chloride and Benzaldehyde were common in JRC and HSL, and Maleic acid in JRC and IIVS. Benzyl benzoate and 1,2,4-Trichlorobenzene were reported by JRC to react with plastic tubes during solubilisation and, therefore, glass tubes were used when preparing the dilutions. However, possible interaction with the plates during testing cannot be excluded, which may have affected the IC_{50} value. Acetophenone and Ammonium chloride were found to be volatile in all three laboratories and plate sealers were used. Barium chloride is a hygroscopic chemical that required specific storage conditions; it formed

precipitates in JRC and it was insoluble in all solvents in IIVS, where precipitates could not be avoided although measures (heating and sonication) were taken to avoid their formation. Benzaldehyde is sensitive to air, light and moisture and, therefore, required storage under nitrogen. JRC and IIVS reported volatility and in IIVS volatile effects were found even when plate sealers were used. Only Maleic acid had no specific characteristics that may have had an influence on the results. In IIVS, where two tests chemicals were tested in each 96-well plate, Maleic acid was tested in the same plate as (4-Ammonio-m-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate and the volatility of this test chemical may have affected the toxicity result of one of the first accepted tests for Maleic acid. However, the possible effects were considered insignificant since Maleic acid showed minimal cytotoxicity up to the highest concentration tested. All seven under-predicted chemicals fall under the EU CLP toxicity category 4 ($300 < LD_{50} \leq 2\ 000$ mg/kg), except Barium chloride that falls under toxicity category 3 ($50 < LD_{50} \leq 300$ mg/kg).

10.4 Summary

To evaluate the predictive capacity of the 3T3 NRU cytotoxicity assay to correctly categorise chemicals into either classified ($LD_{50} \leq 2\ 000$ mg/kg b.w.) or unclassified ($LD_{50} > 2\ 000$ mg/kg b.w.) according to the EU CLP classification scheme, two analyses were applied: 1) regression (both millimole and weight) models from the NICEATM/ECVAM validation study, and 2) ROC analysis.

With millimole regression analysis, the overall accuracy was 64% - 67%. The sensitivity, i.e. the ability to correctly categorise the classified chemicals ($LD_{50} \leq 2\ 000$ mg/kg b.w.), was 92% - 96%. The specificity, i.e. the ability to correctly categorise the unclassified chemicals ($LD_{50} > 2\ 000$ mg/kg b.w.), was 40% - 44%. The number of false negatives was very low (Benzyl benzoate in HSL and JRC, 1,2,4-Trichlorobenzene in JRC and Aconitine in IIVS), resulting in high negative predictive values in all three laboratories (i.e. 86% - 92%, see Table 34).

With the weight regression analysis, the overall accuracy was slightly lower in all three laboratories (53% - 57%), as shown in Table 34. The sensitivity was 100% for all the three laboratories. The specificity was low: 17% in HSL and JRC and 13% in IIVS. There were no false negatives with the weight regression, but it has to be noted

that the test chemicals that were under-predicted (false negative results) with the millimole regression (see above) were excluded from the weight regression analysis since they had right censored IC_{50} values that resulted in estimated LD_{50} values smaller than 2 000 mg/kg b.w. (see criteria in Section 7.3).

For the subset of 27 test chemicals used as cosmetic ingredients (48% of all those tested) the sensitivity, specificity, PPV and NPV of the 3T3 NRU test method was similar to the ones found for the whole set of test chemicals as shown in Tables 34 and 35.

Although the number of compounds with $LD_{50} \leq 2\,000$ mg/kg b.w. and $LD_{50} > 2\,000$ mg/kg b.w. in the NICEATM/ECVAM validation study was unbalanced (45 and 22, respectively), the results of that study with respect to the 2 000 mg/kg cut-off value showed sensitivities of 98% and 100% with the millimole and weight regression analyses, respectively. Only one chemical out of the 45 with an $LD_{50} \leq 2\,000$ mg/kg, was under-predicted with the millimole regression (negative predictive value was 80%) and none of the 45 classified chemicals was under-predicted with the weight regression analysis.

Therefore, the outcome of the current study further supports the results of the NICEATM/ECVAM validation study.

The predictive capacity of the 3T3 NRU test method to identify unclassified chemicals ($LD_{50} > 2\,000$ mg/kg b.w.) was also assessed with ROC analysis. An IC_{50} value of 485.6 µg/ml was identified as the best threshold since it gave high sensitivity with the training set (RC data). Applying this cut-off value to the data set obtained in this validation study, the sensitivity was 85% in HSL, 71% in JRC and 91% in IIVS. The specificity was 56% in HSL, 61% in JRC and 50% in IIVS. However, the number of under-predictions (false negatives) in each laboratory was increased compared to the ones obtained with the millimole regression analysis: 3 test chemicals in HSL (Barium Chloride, Benzaldehyde, and Benzylbenzoate), 7 test chemicals in JRC (1,2,4-Trichlorobenzene, Acetophenone, Ammonium chloride, Barium chloride, Benzaldehyde, Benzyl benzoate, and Maleic acid) and 2 test chemicals in IIVS

(Benzyl benzoate and Maleic acid). Benzyl benzoate and 1,2,4-Trichlorobenzene were common under both analyses.

Overall, the use of the regression analysis, and in particular the millimole regression, resulted in better performance of the 3T3 NRU test method to identify unclassified substances compared to the use of a selected IC_{50} cut-off value (ROC curve analysis), as shown by the lower rate of false negatives in all three laboratories with the regression analysis.

Furthermore, the high sensitivity (92% - 96%) and high negative predictive value (86% - 92%) obtained in this study indicate that negatives identified by the 3T3 NRU test method (40% - 44%) will most likely be correctly categorised as unclassified (LD_{50} > 2 000 mg/kg b.w.).

Assuming that the high prevalence (87% of 4 219) of unclassified substances (LD_{50} > 2 000 mg/kg b.w.) in the EU NCD is representative of all industrial chemicals, then the 3T3 NRU test method could allow reduction of animal toxicity testing by 35% - 40% when used as a first step in a testing strategy that limits animal testing to only those chemicals identified as classified ($LD_{50} \leq$ 2 000 mg/kg b.w.).

The data obtained with the three 3T3 NRU test method protocols resulted in comparable prediction of toxicities which indicate the suitability of the three test method protocols to identify unclassified chemicals according to the EU CLP classification scheme (limit dose of 2 000 mg/kg b.w.).

11.0 APPLICABILITY DOMAIN

The aim of the current validation study was to assess the ability of the 3T3 NRU test method to predict the toxicity category, i.e. classified ($LD_{50} \leq 2\ 000$ mg/kg b.w.) or unclassified ($LD_{50} > 2\ 000$ mg/kg b.w.) of 56 test chemicals. The test method is based on the ability of the cells to accumulate the neutral red dye that is reduced if the cells are damaged or dead (see Section 5.4).

The data presented in this report support the use of the 3T3 NRU test method to identify the unclassified chemicals according to the EU CLP classification scheme ($LD_{50} > 2\ 000$ mg/kg b.w.). The test method works for both industrial chemicals and cosmetic ingredients.

It has been previously shown that the 3T3 NRU test method is not suitable for chemicals that are toxic after being metabolised, as 3T3 cells have only a limited metabolic capacity (Anon, 2006). Furthermore, chemicals that exert their toxic effect by mechanisms not present in 3T3 cells, such as neurotoxic and cardiotoxic chemicals, may be under-predicted. Chemicals that are not soluble in culture medium, DMSO, or ethanol, are not compatible with the NRU test method.

In the present validation study, two regression models and a ROC analysis were performed to estimate the predictive capacity of the 3T3 NRU test method to identify unclassified chemicals ($LD_{50} > 2\ 000$ mg/kg b.w.). From these analyses, the millimole regression gave the best results overall in identifying the unclassified chemicals. Taking the outcome of the three laboratories together, only three chemicals were under-predicted (see Section 10.2.1.), of which Aconitine and 1, 2, 4-Trichlorobenzene had solubility problems (see Table 8). The evaluation of CV%-values showed that precipitating chemicals had clearly increased mean CV%-values compared to soluble chemicals (section 7.4.1 and Table 12). Solubility problems were also observed with many of the test chemicals for which the toxicity was over-predicted (Table 29). All these observations support the earlier indications that the 3T3 NRU test method is most suitable for soluble chemicals. Other chemical attributes (volatility, physical form, and specific storage conditions) had no remarkable effect.

Other studies (e.g. FP6 EU integrated project ACuteTox) have shown that chemicals with log Kow higher than 5 may have e.g. solubility problems and, therefore, can result in false predictions *in vitro*. In the present study, there were eight test chemicals with log Kow values > 5 (1,2-Benzenedicarboxylic acid, 2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol, Di-"isodecyl" phthalate, Dimethyldioctadecylammonium chloride, Triethanolamine, Tris(nonylphenyl) phosphate, Tween 20, and Zinc distearate) and all of them were categorised as unclassified based on the *in vivo* oral LD_{50} values (see Table 3). From these eight test chemicals, two (2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol and Dimethyldioctadecylammonium chloride) were over-predicted in all three laboratories and two (Triethanolamine and and Zinc distearate) in one laboratory (IIVS). Solubility problems were encountered with three (2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol, Dimethyldioctadecylammonium chloride, and Zinc distearate) out of the four over-predicted chemicals.

In addition, Aconitine is a well known neurotoxic and cardiotoxic alkaloid that exerts its toxic effect through interaction with voltage-sensitive sodium channels of the cell membranes of excitable tissues, including the myocardium, nerves, and muscles (Chang 2009). It has also been reported that exposure to high levels of 1, 2, 4-Trichlorobenzene may affect the liver, lungs, kidney and central nervous system (den Besten et al. 1991).

Therefore, the results obtained in this validation study support the limitations of the 3T3 NRU test method previously reported (Anon, 2006).

12.0 OVERALL CONCLUSIONS FROM THE STUDY

The overall results of this validation study, its high sensitivity (92% - 96%) and high negative predictive value (86% - 92%) with regard to the identification of negative chemicals (unclassified with LD_{50} > 2 000 mg/kg b.w.) with the millimole regression, show that the 3T3 NRU test method can be regarded as a valuable test method to screen-out the negative test chemicals (unclassified) when the method is used as a first step in a tiered approach for acute oral toxicity testing.

The study has shown that the test chemicals categorised as unclassified *in vivo* (LD_{50} > 2 000 mg/kg b.w.) are most likely categorised as unclassified also by the 3T3 NRU *in vitro* test method. Furthermore, assuming that the high prevalence (87%) of unclassified chemicals in the NCD is representative of all industrial chemicals, then the 3T3 NRU test method - which is able to correctly identify 40% - 44% of all true negatives - could allow reduction of animal toxicity testing by up to 40%. This assumes a tiered approach that limits animal acute oral toxicity testing to only those test chemicals identified as classified ($LD_{50} \leq$ 2 000 mg/kg b.w.) by the 3T3 NRU cytotoxicity assay.

The data obtained in this validation study also show that the three different 3T3 NRU test method protocols resulted in similar prediction of toxicities and, therefore, they can be used for the identification of unclassified chemicals according to the EU CLP classification scheme. The results have shown that the 3T3 NRU test method protocol is suitable for automation, and that the abbreviated version of the 3T3 NRU test method protocol targeted at resolving toxicities around the 2 000 mg/kg cut-off can also be used.

13.0 ADDITIONAL INFORMATION

The GHS was developed to promote increased consistency among diverse national and sectoral frameworks (UN 2007). The implementation of the GHS classification system around the world is resulting in some differences. In the EU, according to the new CLP regulation, chemicals are allocated in one of 4 acute oral toxicity categories and unclassified (LD_{50} > 2 000 mg/kg). In the US, some agencies require testing to a limit dose of 5 000 mg/kg to support a non-label designation (unclassified if LD_{50} > 5 000 mg/kg) and the chemicals are classified into 5 acute oral toxicity categories (see Section 1.1).

ICCVAM, in the framework of the ICATM (International Cooperation on Alternative Testing Methods) proposed to analyse the study data also with the LD_{50} threshold of of 5 000 mg/kg.

Therefore, in addition to the main goal of the validation study, we present in Annex E the outcome of the analysis performed to determine the predictive capacity of the 3T3 NRU test method protocols for estimating the additional hazard classification categories that are used by U.S. Federal agencies for acute oral toxicity, specifically, the GHS category 5 (2 000 $\leq$ LD_{50} $\leq$ 5 000 mg/kg) and GHS unclassified (LD_{50} > 5 000 mg/kg).

The evaluation of concordance, over-prediction, and under-prediction of these GHS toxicity categories was done using both NICEATM/ECVAM regressions models (i.e., weight and molar units). Moreover, the results of applying the optimum IC_{50} cut-off value, established from a training set (RC data set) for distinguishing between classified and unclassified test chemicals using the LD_{50} of 5 000 mg/kg as cut-off value is also presented in Annex E.

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