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Alternative approaches to standard toxicity testing

TQP ID 9 - 257430120 - NILU

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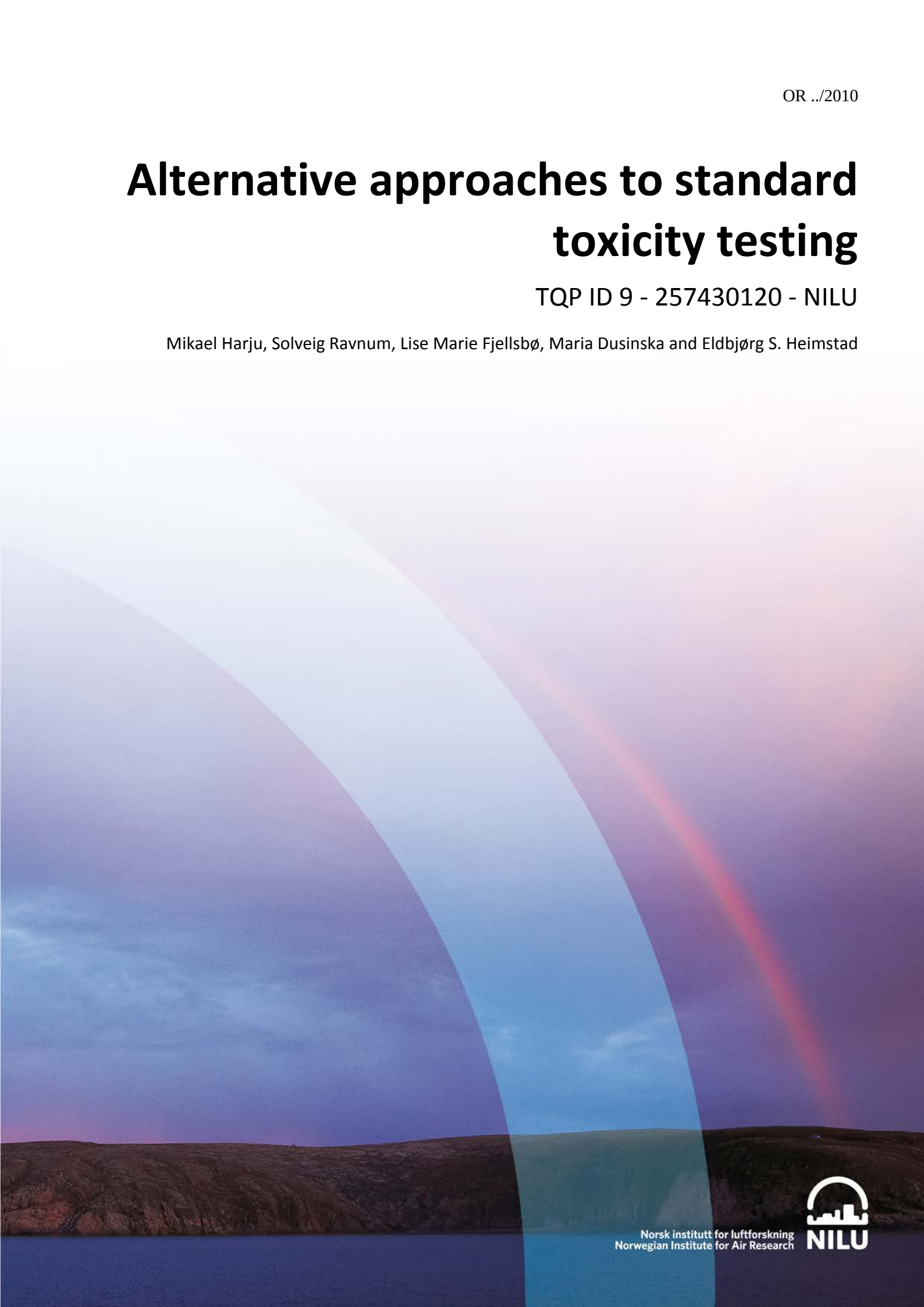

Norsk institutt for luftforskning
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Preface

The CO_2 Capture Mongstad (CCM) Project is in an early development phase of project development. The project is at the moment organized as a joint effort by Gassnova SF and Statoil, and is funded by the Norwegian government. All Frame Agreements and subsequent Call-Offs will be entered with Gassnova SF.

The purpose of the project is to plan and build a large scale CO_2 capture plant (the CCP). The facility will be situated next to the Mongstad Refinery on the Mongstad industrial site north of Bergen on the west coast of Norway.

An amine based CO2 capture plant may cause harmful emissions to the atmosphere. Amines and degradation products from reactions in the process and in the atmosphere are of particular concern, but there is limited knowledge about the behavior of these chemical compounds. Thus several studies will be initiated by the project to increase this knowledge.

The activity Alternative approaches to animal toxicity tests is one of several H&E TQP Amine activities that will be launched for the CCP development in the period up to project sanctioning. This report includes a review of Integrating Testing Strategies (ITS) including (Q)SAR and a data gap analysis on current knowledge level in relation to the information demands for the human risk assessment within REACH.

Contents

Summary

This report gives a recommendation for Integrated Testing Strategy (ITS), based on alternative approaches to avoid animal testing, to all Amine 9 compounds (nitramines, nitrosamines, amides and aldehydes) with specific focus on pre-selected list of N-nitrosamines and nitramine compounds provided by Company.

An extensive review of validated methods and available tests was performed. Following REACH and ECHA guidelines we performed analysis of available testing strategies for chemical substances with aim to propose Integrated Testing Strategy (ITS) for nitramines and nitrosamines and other compounds described in the scope of work.. Suggested ITS includes both validated OECD recommended methods as well as methods presently under validation by ECVAM, ICVAM or JaCVAM. Our approach to ITS take into consideration the 3Rs principle (reduction, replacement, refinement) with stress on alternative methods. We therefore recommend tier procedure using in vitro methods with consideration in vivo taking case by case approach.

Physical-chemical properties such as stability, water solubility and their characterization in testing media before and after the treatment, is integral part of ITS for nitramines and nitrosamines and other compounds described in the scope of the work.

First we propose *in vitro* cytotoxicity tests (OECD Guidance document No 129) as starting point for determination of IC50. Further we recommend performing *in vitro* phototoxicity assay (OECD TG 432, EU Test Method B.41). We recommend in vivo acute toxicity (OECD 425) test for determination of LD 50 to be performed for all compounds where data do not exist. Optionally OECD 436 for inhalation is suggested as this is the main route of exposure for these compounds.

For genotoxicity/mutagenicity first i*n vitro* prokaryotic, the OECD 471 Ames method has been suggested as starting point following by two additional mammalian mutagenicity tests, OECD 473 or 487 cytogenicity assay (chromosomal aberration or micronucleus), and i*n vitro* Comet assay or gene mutation assay OECD 476. All assays we recommend have to be performed with and without metabolic activation. When negative or equivocal results are obtained additional in vitro genotoxicity test with the same endpoints but on different cell system is recommended. Additionally *in vitro* transformation assay EU B21 is recommended to perform. If negative *in vitro* genotoxicity and carcinogenicity results are obtained case by case approach is suggested either with no further testing or with inclusion of in vivo carcinogenicity test. If positive results are obtained in genotoxicity, *in vivo* test with combined endpoints for chromosomal damage (OECD 471 or 487), gene mutation (OECD 473) and with *in vivo* comet assay (and possibly other such as toxicogenomics endpoints) is suggested without additional second *in vivo* experiment.

If positive results are obtained in i*n vitro carcinogenicity test* (B.21 the Cell Transformation assays) further i*n vivo carcinogenicity test* (the OECD 453 combined chronic toxicity/carcinogenicity study) might be suggested depending on production of compound and following case by case approach. For nitrosamines QSARs based on TD50 is also recommended. If substance is classified as a genotoxic or carcinogenic no reproductive toxicity tests are necessary.

If negative in mutagenicity/carcinogenicity studies, further reproductive toxicity following also tier strategy is proposed. The tier 1st includes initial testing with the Embryonic stem cell test (Invitox No 113) for in vitro embryotoxicity and the Micromass (Invitox 122) method for developmental toxicity testing, following by 2nd tier in vivo OECD 422 for determination of fetotoxicity. If the outcome shows that the substance is fetotoxic a 3rd tier is recommended with one of the following tests: OECD 415 or OECD 416.

As part of the ITS approach the feasibility for the use of QSAR to predict toxicity was evaluated. Available toxicity information and data were compiled with the use of several databases and literature searches for 23 nitrosamine and 14 nitramine compounds. Information and data gaps were evaluated in accordance to the information requirements in REACH Annex VII-X. A QSAR model was developed based on the present 12 project nitrosamine compound with tested TD_{50} endpoints and additional compounds with test data from literature. This model included at least two mode of actions for carcinogenicity and the final descriptors in the QSAR model did not indicate a mechanistic insight into the carcinogenic character of the substances, and is therefore considered to be a preliminary model. Due to Principle 5 (REACH Annex XI) and expert judgement, we recommend that this model is further refined or a new model is generated with larger/modified amount of compounds, selection of descriptors expected to be important for the mode of action, and/or for selected compounds with a common mode of action.

If the mode of action for carcinogenicity of nitramines can be expected to be similar to the mechanism of nitrosamines, it would be feasible to use both nitramines and nitrosamines when developing a QSAR model for TD_{50}. Nitramine compounds with available future test data of TD_{50} can be added to the nitrosamine compounds as long as they are part of the physical-chemical property domain.

(Q)SAR methods using an expert system such as Toxtree (implemented in the OECD (Q)SAR Toolbox) could be used to classify carcinogenic from non-carcinogenic of nitrosamine and possible nitramines with a fairly high accuracy. The methods used have a certain true positive rate such as 93.33% accuracy for chemicals with the structural alert alkyl and aryl N-nitroso group. These results could be used as supportive evidence to limit (or even reduce testing time) the testing scheme. If such a method would be admissible for legislative purposes we do not know at this time.

Abbreviations

AD	Applicability Domain. The physicochemical descriptor space spanned by a particular training set of chemicals. It offers the opportunity to assess whether a compound can be reliably predicted.
ADME	Adsorption Distribution Metabolism Effect
CAS	Unique numerical identifiers for chemical elements, compounds, polymers, biological sequences, mixtures and alloys
ECHA	European Chemicals Agency
ESAC	ECVAM Scientific Advisory Committee
DMEL	Derived-Minimal-Effect-Level
DNEL	Derived-No-Effect-Level
GLP	Good Laboratory Practice
IC_{50}	Inhibitory concentration 50%
ITS	Integrated Testing Strategy
LC_{50}	Lethal concentration 50%
LD_{50}	Median lethal dose (abbreviation for "Lethal Dose, 50%"), of a toxic substance or radiation is the dose required to kill half the members of a tested population after a specified test duration.
MLR	Multiple Linear Regression
NIOSH	National Institute for Occupational Safety and Health
NOAEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
OSHA	Occupational Safety and Health Administration
PCA	Principal Component Analysis
PBMK	Pharmacokinetic modeling
(Q)SAR	**(**Quantitative) Structure-Activity Relationship
3R	Reduce, refine and replace the use of animals for toxicity testing
REACH	Registration, Evaluation, Authorisation and Restriction of Chemical
SMILES	simplified molecular input line entry specification. SMILES is a specification for unambiguously describing the structure of chemical molecules using short ASCII strings
TD_{50}	The standardized measure of carcinogenic potency, TD_{50}, is the daily dose rate in mg/kg body weight/day to induce tumors in half of test animals that would have remained tumor-free at zero dose. Whenever there is more than one positive experiment in a species, the reported TD_{50} value is a Harmonic Mean calculated using the TD_{50} value from the most potent target site in each positive experiment.

Alternative approaches to standard toxicity testingAlternative approaches to standard toxicity testing

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1 Introduction

In December 2006, following several years of discussion and debate, the European Union (EU) finalized new legislation on the control of chemicals. This regulation is termed REACH for Registration, Evaluation, Authorisation and Restriction of CHemicals. It entered into force on 1st June 2007 to streamline and improve the former legislative framework on chemicals of the European Union (EU). REACH places greater responsibility on industry to manage the risks that chemicals may pose to the health and the environment. In principle REACH applies to all chemicals: not only chemicals used in industrial processes but also in our day-to-day life, for example in cleaning products, paints as well as in articles such as clothes, furniture and electrical appliances. REACH aims to ensure a high level of protection of human health and the environment, including the promotion of alternative methods for testing of substances. The regulation also aims to enhance the competitiveness of the European chemical industry and increase transparency with regard to information on chemicals. Importantly, the promotion of non-animal testing is also a listed objective.

The aim of REACH is to improve the protection of human health and the environment through the better and earlier identification of the intrinsic properties of chemical substances. Manufacturers and importers will be required to gather information on the properties of their chemical substances, which will allow their safe handling, and to register the information in a central database run by the European Chemicals Agency (ECHA) in Helsinki. The main tasks of ECHA are to manage the databases necessary to operate the system, co-ordinate the in-depth evaluation of suspicious chemicals and run a public database in which consumers and professionals can find hazard information.

One of the main reasons for developing and adopting the REACH Regulation was that a large number of substances have been manufactured and placed on the market in Europe for many years, sometimes in very high amounts, and yet there is insufficient information on the hazards that they pose to human health and the environment. There is a need to fill these information gaps to ensure that industry is able to assess hazards and risks of the substances, and to identify and implement the risk management measures to protect humans and the environment.

It has been known and accepted since the drafting of REACH that the need to fill the data gaps would result in an increased use of laboratory animals for the next 10 years. At the same time, in order to minimise the number of animal tests, the REACH Regulation provides a number of possibilities to adapt the testing requirements and use existing data and alternative assessment approaches instead.

Experience with for instance the OECD High Production Volume Chemicals Programme has clearly demonstrated that when substances of similar structure and toxicity profiles are assessed as a group (category) substantial savings in the number of tests can be achieved.

Potential registrants are required to obtain data on the hazards of their substances as specified in the Annexes VII-X of REACH. Annex VI of REACH provides a basic four steps procedure for fulfilling the information requirements. The procedure comprises the following steps: (i) Gather and share existing information; (ii) Consider information needs; (iii) Identify information gaps; and (iv)Generate new data/Propose testing strategy. Furthermore, as mentioned previously, testing on vertebrate animals should only be undertaken as a last resort.

The development of reliable alternative methods is in general a long and expensive process and the limited availability of acceptable alternative methods is placing increasing pressure on the efforts to provide alternatives to animal testing methods. Most toxicological endpoints require an integrated testing approach such as the combination of *in-vitro* and *in-silico methods* or the use of test batteries owing to the fact that many alternative methods are not suitable as purely stand-alone methods. New testing technologies such as methods based on toxicogenomics, metabolomics and proteomics as well as high-throughput technologies are promising tools that need to be evaluated for their suitability and validity as well. An integrated testing strategy (ITS) allows for a more holistic and effective approach to carry out risk assessment, which is central to the regulatory process.

Article 13(3) of the REACH Regulation requires that new tests shall be carried out in accordance with the test guidelines included in Commission Regulation No. 440/2008 or in accordance with other international test methods recognised by the European Commission or ECHA. In addition, in Annexes VII to X on standard information requirements, the use of various OECD test guidelines is required in cases where no EU test method exists (e.g. OECD TG 414, 421 and 422). Article 13(3) also specifies that information may be generated using other methods provided the conditions defined in Annex XI of the REACH Regulation are met. These include inter alia that the result is sufficient for the purposes of classification and labelling and/or risk assessment, and that adequate and reliable documentation of the applied method is provided (see Annex XI of the REACH Regulation for more information). Moreover, a specific requirement is introduced in Article 13(4) of the REACH Regulation for ecotoxicological and toxicological tests. Since June 1st 2008, new tests of this kind have to be carried out in compliance with the principles of Good Laboratory Practice (GLP) provided for in Directive 2004/10/EC, as no other international standard has so far been recognised as being equivalent. In case of physico-chemical testing it may be desirable but it is not mandatory to have tests performed according to GLP standard. The Guidance on information requirements and chemical safety assessment contains specific Integrated Testing Strategies for each endpoint (e.g. for aquatic toxicity, mutagenicity), which should be consulted before new tests are performed.

There is therefore a need to replace the standard test programme for chemicals with an intelligent test strategy. For this it is necessary to link information about the toxicity of substances with other information in order to develop a tailor-made test programme for each substance.

Among the different alternative methods that can be used in the hazard assessment of chemicals are the so-called non-testing methods, which comprise (Q)SAR models and other, less formalised, approaches based on the grouping of chemicals (read-across and chemical category formation). To address animal welfare concerns, the REACH legislation explicitly expresses the need to use non-testing methods to reduce the extent of experimental testing.

(Q)SAR models are models that relate molecular structure to a measured activity or property of that same structure. Typical activities are experimental determined toxicity endpoints or physicochemical properties. The process typical include calculation of various atomic and molecular properties which should be selected preferably on a mechanistic approach for the endpoint of interest. The relationship is most often described by mathematical regressions. The central principle of qualitative structure-activity relationships (SAR) and quantitative structure-activity relationships (QSAR) is that the activity of molecules is reflected in their structure. Hence, similar molecules have similar activities. The (Q)SAR approach therefore assumes that the structure of a molecule (e.g., its geometric, electronic properties etc.) contains the features responsible for its physical, chemical, and biological properties. Serafimova & co-workers have recently published a review of QSAR models, databases and software tools for predicting genotoxicity and carcinogenicity (Serafimova et al., 2010).

On the basis of the general rules in REACH Annex XI, the registrant may adapt the standard testing regime based on the use of existing data such as data on human health and historical human data as long as the quality of the data is assured based on the stipulated conditions/criteria. An approach with ‘weight of evidence’ might be applied, based on several independent sources of information, which individually might be regarded to be insufficient as basis for drawing any conclusions on the compounds toxicity or unwanted property. Qualitative or quantitative structure activity relationship models (Q)SARs may be used as an indication of a substances dangerous property or give important mechanistic understanding of the compound. *In vivo* animal testing may be supplemented by (Q)SAR if certain criteria have been met, these include the scientific validity of the model (an unambiguous algorithm), that the substance falls within the applicability domain of the (Q)SAR, quality measures of the algorithm which include the definition of endpoint (TD_{50}, LD_{50}, NOAEL etc), measures of goodness-of-fit, robustness and predictivity. Also, if possible, a mechanistic interpretation of the chemicals for which the models can generate reliable predictions.

Alternatively or in a combination with (Q)SAR, an approach using compound grouping and read-across is possible. Substances whose physicochemical and structural similarity having similar ecotoxicological or toxicological endpoint, generally also having a similar mechanistic action, may be considered as a group or category. Read-across of a substance is achieved by the interpolation

(prediction) from reference substances within this group or category to other substances in this group, thus avoiding the need to test all compounds for all endpoint required in REACH.

2 Objectives

The aim of this study was to identify and document methods for alternative approaches to standard toxicity testing using laboratory animals (in vivo tests) for a to nitramines, nitrosamines, amides and aldehydes with specific focus on pre-selected list of N-nitrosamines and nitramine compounds provided by Company.

There were two main objectives:

- Propose an Integrated Testing Strategy (ITS) based on alternative approaches to standard toxicity testing using laboratory animals (in vivo tests) to avoid unnecessary suffering for animals, and to reduce time and cost.

- Evaluate the feasibility for use of (Q)SARs and/or read-across for predicting toxicity for given substance groups

A list of the specific substances is given in Appendix A. The list consists of two groups; i.e. nitrosamines and nitramines. The work was carried out without physical experiments or programming.

Alternative in vitro methods to animal testing were evaluated and validated against existing human and in vivo/in vitro data taking into consideration threshold concept, exposure levels and extent of possible exposure, dose-response relationship, route of exposure (with focus on inhalation) and time scale, key toxicological and ecotoxicological information, Methods which gave clear dose response relationship with low background level of damage and were able to identify the key toxicological endpoints were of preference. As final outcome, concepts and approach to integrating testing strategies of compounds of concern such as nitrosamines and nitramine were aimed at. The validation status for all identified methods were aimed at, as well as documentation of references and potential method improvements, test facilities and suppliers are documented. Focus was on *in vitro* and *in silico* methods, and their validation against existing in vivo and human data was performed.

(Q)SAR model development as part of ITS was aimed at for at least one substance per group, dependent on the requirements of sufficient amount of data for existing endpoints, the fulfilment of OECD criteria for (Q)SAR models and that the structures were within the applicability domain (chemical category) of the model.

An extensive review including literature and database was performed to gather and compile relevant physicochemical, toxicological and ecotoxicological data of the substances followed by data gap analysis in accordance to the requirements specified in REACH Annex VII-X. Integrated testing strategy for the substances

was evaluated for the purpose of diminishing animal testing. The work was performed in accordance to what is stated in the standard information requirements for substances in REACH regulation (REACH 2006a, REACH 2006b) and the guidance on information requirements and chemical safety assessment of OECD (OECD, 2004). The validation status for all identified methods, references and potential methods improvements, test facilities and suppliers were documented.

In short this procedure for fulfilling the information requirements were followed. (i) Gather information; (ii) Consider information needs; (iii) Identify information gaps; and (iv) Propose testing strategy were testing on animals was aimed as a last resort.

Where data and necessary information is missing, additional experimental studies and more comprehensive QSAR models were suggested for future work.

3 Approach & evaluation of available methods

3.1 REACH Guidance on Integrative testing strategy (ITS)

The main objective of Integrated Testing Strategy (ITS) is to give guidance on a stepwise approach to hazard identification with respect to toxicity. A principle of the strategy is that the results of one study are evaluated before another study is initiated, since information from some toxicity studies can give valuable information to other endpoints. The strategy seeks to ensure that the data requirements are met in the most efficient and humane manner so that animal usage and costs are minimized. The ITS must provide advice on how the REACH Annexes VI to XI (Regulation (EC) No 1907/2006, Directive 67/548/EEC) information requirements for toxicity tests can be met. Careful consideration of existing toxicological data, exposure characteristics and current risk management procedures is recommended to ascertain whether the fundamental objectives of the ITS have already been met.

A four step process has been developed for clear decision making:

Stage 1) Gather existing information according to Annex VI
Stage 2) Consider information according to Annex VII to X
Stage 3) Identify gaps (and adequacy of all available data for classification and labelling and /or risk assessment, or to fulfill the criteria for waiving)
Stage 4) Generate new data / propose testing strategy

ECHA has produced a document containing guidance on REACH explaining the RECAH obligations and how to fulfil them: “Guidance on information requirements and chemical safety assessment”, Chapter R.7A: Endpoint specific guidance (REACH 2008). A summary of the guidance on the different integrated testing strategies of the document is given below.

Integrated testing strategy for physico-chemical properties; adsorption and desorption.
The majority of substances registered under REACH will require a full physico-chemical data set according to Annex VIII. For higher tonnage chemicals, some additional physico-chemical data may be required according to Annex IX. These data consist of information that are used to assess the physical hazards and help predict toxicological or environmental hazards, fate and behaviour.

For substances where tests needed to fulfil the information requirement, consideration should be given to the order in which the tests are conducted. For some endpoints more than one technique is described. Several tests are inter-related.

Endpoints that need to be addressed are:
Melting/freezing point, Boiling point, Relative density, Vapour pressure, Surface tension, Water solubility. Partition coefficient N-octanol/water, Flash point, Flammability, Explosive properties, Self-ignition temperature, Oxidising properties, Granulometry, Adsorpition/Desorption, Stability in organic solvent and degradation products, Dissociation constant, Viscosity.

In this ITS we consider only biological tests.

Integrated testing strategy for irritation/corrosion.
The guidance on strategy suggests a stepwise approach for developing adequate and scientific data for assessment, evaluation and classification of the corrosive and irritating properties of a substance. Guidance for integrated testing strategy is also provided by the rules of Directive 67/548/EEC; the specific rules for adaptation from standard information requirements in column 2 of Annexes VII-X, and by the general rules for adaptation from standard information requirements in Annex XI.

Risk assessment of the irritating potential of a substance is normally made in a qualitative way provided the substance has been classified as irritant or corrosive to the skin. Existing test guidelines do not contain dose response assessment, so that a quantitative analysis will often not be possible, hence hazard identification and appropriate classification is the most important factors of the recommended strategy.

ITS for skin corrosion/irritation
Part I: Retrieving existing information (existing physico-chemical properties, existing human data, existing animal data from *irritation/corrosivity studies*, existing data from general toxicity studies via the dermal route and from sensitization studies, existing (Q)SAR and read-accross, existing in vitro data).
Part II: Weight of evidence analysis and judgement (taking all the existing and relevant data into account, if there is sufficient information to make a decision of whether classification/labeling is necessary, and how to do it).
Part III: Generation of new information by testing new in vitro and in vivo tests (before new in vivo tests are performed, the use of in vitro tests should be fully exploited (Article 25 of REACH) by using the general rules of Annex XI which allow to adapt to the standard rules for testing in Annexes VIII to X).

ITS for eye irritation
Part I: Retrieving existing information (conclusion from the information strategy on *skin irritation/corrosion*, existing physico-chemical properties, existing human data, existing animal data from irritation studies, existing data on acute dermal toxicity, existing (Q)SAR and read-accross, existing in vitro data).
Part II: Weight of evidence analysis and judgement (taking all the existing and relevant data into account, if there is sufficient information to make a decision of whether classification/labeling is necessary, and how to do it).
Part III: Generation of new information by testing new in vitro and in vivo tests (before new in vivo tests are performed, the use of in vitro tests should be fully exploited (Article 25 of REACH) by using the general rules of Annex XI which allow to adapt to the standard rules for testing in Annexes VIII to X).

Integrated testing strategy for sensitisation.
The recommended strategy is a guidance on a stepwise approach for developing adequate and scientific data for assessment, evaluation and classification of the sensitising properties of a substance. The recommended testing strategy for this endpoint takes account of existing data on toxicity, exposure characteristics as well as specific rules for adaptation from standard information requirements (column 2 of Annexes VII-X) and some specific rules for adaptation from standard information requirements (Annex XI) of the Directive 67/548/EEC.

ITS for skin sensitisation
1. Gather and evaluate existing information (human, animal, in vitro, (Q)SAR, read-accross and chemical category data) on skin sensitisation according to Annex VI, step I.
2. Consider required information needs (Annex VII:8.3) and make an overall weight of evidence assessment.
3a) Perform the available in vitro tests
3b) Perform the appropriate in vivo test (LLNA, reduced LLNA or other).

For sensitization via skin exposure, OECD recommends a two tier testing. The first tier consists testing of chemical with TG 429 using Lymph node assay or Mouse ear swelling test. If no definite answer is obtained from the above then TG 406- Guinea pig maximization test is recommended.

ITS for respiratory sensitization
1. Gather data on whether the substance is a skin sensitizer/R43.
2. Gather data on whether the substance is a di-isocyanate.
3. Gather data on whether the substance has any structural alerts (acid anhydride, platinum salt etc).
4. Judge whether there are any other good reasons to suppose potential respiratory snsitisation hazard (human data, animal data, (Q)SAR, in vitro tests).

Integrated testing strategy for acute toxicity.
The guide on strategy is providing advice on how the REACH Annex VII and VIII (Directive 67/548/EEC) information requirements for acute toxicity can be met using the most humane methods, sufficient for hazard assessment, risk assessment of Derived–No-Effect-Level (DNEL) derivation, but also being

valuable for repeated dose toxicity studies when it comes to target organ toxicity and dose selection.
The standard information requirements for acute toxicity under the REACH Regulations (Directive 67/548/EEC) define that acute toxicity testing via the oral route is required if the yearly tonnage level is >1 t/y (Annex VII), but further that *acute oral toxicity testing is not necessary if the substance is corrosive to the skin or if a study on acute toxicity by the inhalation route is available* (column 2 in Annex VII). Further, acute toxicity via at least one other route of exposure but oral is necessary, if the yearly tonnage level is >10 t/y (Annex VIII-X). Depending on the nature of the substance and the likely route of human exposure, specific adaptations exist (column 2 Annex VIII and Annex VIII Section 8.3). But *acute oral toxicity testing is not necessary if the substance is corrosive to the skin* (Annex VII). If there is any reason for believing acute toxicity at non-corrosive levels, based on existing data, this should be addressed.

ITS for acute toxicity
Stage 1. Gathering of existing information by reviewing the existing data, according to Annex VI (human or animal data, physicochemical properties, (Q)SARs, in vitro test data). For non-corrosive substances, the results of eye irritation and skin sensitisation studies (Annex VII) may provide useful information of the potential for systemic toxicity. All human and test data should be considered. For example data from different in vitro studies (basal cytotoxicity and dermal penetration studies), systemic effects from other studies, route of human exposure, physico-chemical properties, dermal or respiratory toxicity of structurally related compounds can all be used to choose whether the route of exposure should be inhalation or dermal in the acute toxicity in vivo test.

Stage 2. Consideration of information needs according to the relevant Annex VII to X. A thorough evaluation of the collected information is carried out, the available data must be relevant and reliable to fulfill the REACH requirements. When acute toxicity via a second route is required, the choice of route (dermal or inhalation) depends on the nature of the substance and on the likely route of human exposure. If exposure is possible via inhalation, or if physico-chemical properties indicate that such exposure may occur, then testing via inhalation for acute toxicity should be conducted. Testing for acute dermal toxicity is unnecessary if the following effects are observed from other studies: systemic toxicity in skin/eye irritation and/or skin sensitization studies, death in an acute oral toxicity study with potential for dermal absorption, systemic toxicity in an acute oral toxicity study with potential for high dermal absorption, or just potential for high dermal exposure.

Stage 3. Identification of data gaps (and adequacy of all data for classification and labeling and/or risk assessment, or to fulfill the criteria for waiving). Identify what additional information is required in order to classify the substance in order to perform a risk assessment. If the substance can be excluded from acute toxicity testing if it does not appear necessary (Annex XI), for example: if a Weight of Evidence (WoE) shows that available data is sufficient for adequate hazard characterization and the exposure of the substance is controlled, or if the substance is not bio-available via a specific route and any possible local effects are characterized, or if testing via the inhalation route proves irrelevant due to

physico-chemical properties. If the available data is contradictory or insufficient, additional in vitro studies, (Q)SARs, read-across should be performed before conducting any in vivo study

Stage 4. Generating new data /propose new testing strategy. If data gaps need to be filled, new data must be generated (Annex VII and VIII). New tests on animals should only be performed as a last resort, and the standard OECD guidelines should normally be used. If the substance is not corrosive, and it has potential for acute toxicity, and it exhibits human exposure due to physico-chemical properties, and it is not possible to establish toxicity with in vitro tests, then acute toxicity testing on animals is suggested. The most likely route of exposure must be decided, if human exposure by inhalation is identified, then the recommended testing strategy by inhalation should be chosen (OECD GD 39: OECD TG's 433 and 436 or OECD TG 403 and EU B.2). Decision on dose selection might be chosen from available validated in vitro tests.

Integrated testing strategy for repeated dose toxicity

The objective in this strategy is to give guidance on a stepwise approach to hazard identification with regard to repeated dose toxicity. Information generated in this strategy should be suitable for classification and labeling according to the criteria in Annex VI in Directive 67/548/EEC. *Testing for repeated dose toxicity is not required for chemicals produced at tonnage levels less than 10 tonnes per annum (t/y).* The Annexes VII-X of the REACH regulation provide the standard information requirements in Column 1 and specify triggering and waiving possibilites for the specific endpoints in Column 2. Annex XI of the REACH regulation contains basic approaches /rules for adaptation of the standard testing regime in Annexes VII-IX.

ITS for repeated dose toxicity

Step 1: Collecting all existing information relevant for repeated dose toxicity (Annex VI).
Step 2: Consider required information needs.
Step 3: Evaluation of the available information.
Step 4: Decision on whether to propose further studies.

Utilisation of the different tests at each of the tonnage levels:

10 t/y or more (Annex VIII), 100 t/y or more (Annex IX) and 1000 t/y or more (Annex X).

Testing does not appear scientifically necessary:

The substance can be excluded from repeated dose toxicity testing if it does not appear necessary (Annex XI), for example: if a Weight of Evidence (WoE) shows that available data is sufficient for adequate hazard characterization and the exposure of the substance is controlled, or if the substance is not bio-available via a specific route and any possible local effects are characterized, or if testing via the inhalation route proves irrelevant due to physico-chemical properties, or if the substance belongs to a group or a category of substances that have a common functionality or breakdown products or sufficient information of the toxicological properties, testing of all individual category members may not be necessary.

Integrated testing strategy for reproductive toxicity

The recommended strategy is based on a Weight of Evidence approach to give sufficient information to support risk assessment, and to give adequate information to consider whether classification is warranted. The ITS permits informed decisions on reproductive toxicity potential in a step-by-step tiered manner, within the production tonnage related requirements framework of REACH Annexes VII to X. *Testing for reproductive toxicity is not required for chemicals produced at tonnage levels less than 10 tonnes per annum (t/y).*

ITS of reproductive toxicity

Available toxicological data, exposure characteristics, and current risk management procedures is necessary to ascertain whether the objectives of the ITS have already been met. If it is concluded that further testing is required, then a series of decision points are defined.

The ITS provides a three-stage process for clear decision making, relevant for all tonnage levels > 10 t/y.

Stage 1. A series of preliminary questions to consider before deciding whether any testing for reproductive toxicity potential is required (relevant for all tonnage levels > 10 t/y).

1.1) Has the substance already been classified for effects on fertility as Reproductive Toxicity Category 1 or 2 (R60) and development as Reproductive Toxicity Category 1 or 2 (R61)? If the answer is no, proceed to 1.2). If yes, it may or may not be relevant to proceed to Stage 2.

1.2) Is the substance classified as a genotoxic carcinogen (Carcinogen Category 1 and Mutagen Category 3 or Carcinogen Category 2 and Mutagen Category 3) or a germ cell mutagen (Mut. Cat. 1 or 2)? If the answer is no, proceed to 1.3).). If yes, it may or may not be relevant to proceed to Stage 2.

1.3) Does the substance exhibit low toxicological activity, negligible systemic absorption and no significant human exposures? If yes no further testing is required. If no, proceed to Stage 2.

Stage 2. Evaluation of the available toxicological database and consideration of reproductive toxicity alerts. Considering data for substances with a similar structure or causing toxicity with a similar mode of action. If sufficient data exist to conclude that the substance does not present a reproductive toxicity hazard or that further data are unlikely to change the classification, the no further testing is required. If sufficient data does not exist, go to Step 3.

Stage 3. Relevant reproductive and developmental toxicity tests triggered by tonnage level or alerts in Stage 1 and 2. Four internationally harmonized guideline studies are listed in the REACH Annexes that can be used at Stage 3 to provide the necessary information to support classification, risk assessment and identification of N(L)OAELs. The tests listed in the REACH annexes are: OECD 421/422, OECD 414/EU B.31, and OECD 416/EU B.35.

Integrated testing strategy for mutagenicity

The recommended testing strategy is a flexible stepwise approach for hazard identification on the mutagenicity of the substance, to obtain sufficient data to be able to carry out risk characterization, classification and labeling. Minimization of animal use and costs are taken into account. It is preferred that tests as described in OECD Guidelines or EU Directive 67/548/EEC are used when possible. For a proper coverage of the mutagenicity potential of a substance, information on gene mutations, structural chromosome aberrations and numerical chromosome aberrations is required. This may be obtained from available data from tests on the substance or by predictions using chemical grouping, read-across or (Q)SAR techniques. Physico-chemical properties, toxicokinetic and toxicodynamic parameters, other toxicity data, data on structurally similar chemicals must also be considered. In vitro tests are particularly useful for gaining information of the mutagenicity potential, and these tests are given a critical role in this testing strategy. Substances classified in category 1, 2 or 3 for mutagenicity and/or category 1 or 2 for carcinogenicity will usually not require additional testing in order to meet the requirements of the REACH Annexes VII-X.

If the *in vitro* genotoxicity/mutagenicity tests are positive (OECD TG 471, 480, 481, 473, 476, 487, 479, 482), then *in vivo* genotoxicity/mutagenicity testing has to be considered (OECD TG 474, 475, 477, 478, 483, 484, 485, 486). In case of positive results for *in vivo* tests in somatic cells, then germ cell mutagenicity is suspected. Today there is no established non-animal germ cell mutagenicity approach available to replace e.g. the mouse heritable translocation assay (OECD TG 485).

Standard information requirements at Annex VII:
A preliminary assessment of mutagenicity is required for substances at the Annex VII tonnage level of REACH. All available information must be included, at least data from a gene mutation test in bacteria. If the bacterial test is positive, it is possible that the substance is being genotoxic in mammalian cells, so further testing must be considered, for example the in vitro gene mutation study in mammalian cells (OECD 476).

Standard information requirements at Annex VIII:
Information on gene mutations, and structural and numerical chromosome aberrations is required for substances at the Annex VIII tonnage level of REACH. At least one further test in addition to the gene mutation test in bacteria is required; for example an in vitro mammalian cell test capable of detecting both structural and numerical chromosome aberrations, referred to as an in vitro cytogenicity study and an in vitro micronucleus study. There are two different tests that cover these requirements: The in vitro chromosome aberration test (OECD 473) and the in vitro micronucleus test (OECD TG 487), both are cytogenetic assays. These tests can also be used as alternatives to the first in vitro mammalian cell test (OECD 476) specified under Annex VII. The Annex VIII tonnage level requires the in vitro mammalian cell test (OECD 476) in the second part of the standard information when the results of the bacterial gene mutation test and the in vitro cytogenicity or in vitro micronucleus tests are all negative, in order to detect in vitro mutagens that give negative results in the other two tests. When adequate information from a reliable in vivo test capable of detecting gene mutations is available, no in vitro mammalian cell gene mutation test is required.

Provided all in vitro tests have given negative results, normally no in vivo tests will be required to fulfill the standard information requirements of Annex VIII.

Requirements for testing beyond the standard levels specified for Annex VII and VIII:
Concerns raised by positive results from in vitro tests can justify further testing. The chemistry of the substance, data on analogous substances, toxicokinetic and toxicodynamic data, and other toxicity data will also influence further testing.

1. Substances that are negative in the standard set of in vitro tests.
These substances are considered to be non-genotoxic. Only very few substances have shown genotoxicity in vivo, but not in vitro. Knowledge about the metabolic profile may indicate that further in vitro tests, or an in vivo test, may be needed (together with an alternative to rat liver S-9 mix, a reducing system, a metabolically active cell line like HepG1 or genetically engineered cells).

2. Substances that are positive in the standard set of in vitro tests.
REACH Annex VII defines substances for which only a bacterial gene mutation test is positive, should be studied further according to the requirements of Annex VIII. Regarding Annex VIII, when both the mammalian tests are negative, but the bacterial test is positive, it will be necessary to decide further testing on a case-by-case basis. In REACH Annex VIII, when positive results show in an in vitro mammalian cell mutagenicity test, then somatic cell in vivo testing is required. For example the rodent bone marrow or mouse peripheral blood micronucleus test (OECD TG 474), a rodent bone marrow clastogenicity study (OECD TG 475), a Comet assay (single cell gel electrophoresis) for detection of DNA breaks, a test for gene mutations in a transgenic rodent model, or a rat liver unscheduled DNA synthesis (UDS) test. In vivo genotoxicity test may be incorporated into a short-term repeated dose toxicity test (28 days).

3. Substances that give positive results in an in vivo test for genotoxic effects in somatic cells.
Substances that give positive results in in vivo tests for genotoxic effects in somatic cells, should always be considered for the potential to affect germ cells. In order to minimize animal use, the possibility to combine germ cell genotoxicity tests and reproductive toxicity tests may be considered. Also such substances can be studied further to establish whether they specifically act as aneugens and threshold for their genotoxic activity can be identified.

Integrated testing strategy for carcinogenicity.
The REACH guide to testing strategy describes how to decide whether or not a standard carciogenicity study or any further testing is required. REACH only specifies a carcinogenicity test for substances at the Annex X tonnage level (>1000 t/y). Below 1000 t/y the main concern is genotoxic chemicals. Carcinogenic process is a multi-step process, and chemically-induced cancer may be induced by many pathways or modes of action. Substances that are genotoxic carcinogens can be detected by in vitro or in vivo mutagenicity tests. Carcinogens that act by non-genotoxic modes of action are mode difficult to identify, because short-term tests are not available, and may therefore go unidentified. For

substances where there is no concern for mutagenic activity and no toxicological indicators of concern for carcinogenicity, no further consideration of carcinogenic potential is necessary.

The step-by step approach is a guidance to assessment of carcinogenicity (the hazard, the underlying mode of action, and the potency) for substances at each of the tonnage levels specified in Annex VII to X of REACH.

Step 1. Gather and assess all available data from toxicity tests and non-tests such as read-across/proper chemical category and suitable predictive models, and examine the Weight of Evidence.
Step 2. Consider if the standard information requirements are met,
Step 3. Confirm that information requirements of Annexes VII and VIII are fulfiled, make proposals to confirm with Annexes IX and X.

Substances at Annexes VII, VIII and IX
Any relevant test data that are already available such as repeated dose/reproductive/developmental toxicity studies and information from predictive techniques such as read-across, chemical grouping or (Q)SARs should be used to judge the hazard potential of this endpoint. The minimum information to be provided at these tonnage levels is similar to what is required for the mutagenicity endpoint. Positive results from in vitro mutagenicity provide an alert for possible carcinogenicity, and further testing for vitro and/or in vivo mutagenicity is required. This will not lead to classification of the substance as a carcinogen, but substances shown to be in vivo mutagens should be assumed to be potentially carcinogenic, and this should be taken into account in the risk assessment for this endpoint. There are ways to calculate cancer risks associated with human exposure, such as derivation of Derived-Minimal-Effect-Level (DMEL) and Derived –No-Effect-Level (DNEL).

Substances at Annex X
All substances at this tonnage level should be evaluated for carcinogenicity. All relevant data from toxicity studies and predictive techniques such as chemical grouping, read-across and (Q)SARs should be assessed. It may be suggested to supplement the data with in vitro or alternative shorter-term in vivo investigations, to avoid a carcinogenicity study. Substances classified as a category 1 or 2 mutagen does normally not require a carcinogenicity study. For substances classified as a category 3 mutagen it should first be considered whether a higher level of classification for mutagenicity can be tested first. Some non-genotoxic carcinogens will go unidentified and may be a risk to humans. There are ways to calculate cancer risks associated with human exposure, such as derivation of Derived-Minimal-Effect-Level (DMEL) and Derived –No-Effect-Level (DNEL). A carcinogenicity study may be justified if there are suspicions that the substance may be carcinogenic, and all other available information is not conclusive.

3.2 Toxicity data compilation

A list of the specific molecular structures of the compounds with corresponding cas.no was given by the Company. The molecular structures were used as first choice and cas.no. were checked for correctness. 2-dimensional structures were build and transformed to structural format SMILES and cas.no. were generated

and checked with the software ACD Chemsketch (freeware) or EPISUITE v4.0. The unique cas.no. were further used to search for data in the various databases, compiling toxicity and ecotoxicity data in various databases. Due to the high amount of data (compilation and data gap analysis) emphasis was made on toxicity data and inhalation end points.

Toxicity data for the substances of interest were collected in tables with the use of various available databases such as Toxnet, CPDB, and RTECS. Literature searches with ISIBase, PubMed and SciFinder were also performed in order to find information and data.

The mentioned databases are listed together with other useful databases and software in Appendix B.

Below is listed the most important databases for compiling toxicity data.

TOXNET: The TOXNET database of the US National Library of Medicine (NLM) (http://toxnet.nlm.nih.gov) is a cluster of different databases, collecting information on toxicology, hazardous chemicals, environmental health, and toxic releases. From the website, it is possible to search within and across the databases by several identifiers, such as chemical name, CAS number, molecular formula, classification code, locator code, and structure or substructure. Among the TOXNET databases, the Chemical Carcinogenesis Research Information System (CCRIS) and the GENE-TOX databases deal specifically with mutagenicity and carcinogenicity data. CCRIS contains over 9000 chemical records with animal carcinogenicity, mutagenicity, tumour promotion, and tumor inhibition test results provided by the National Cancer Institute (NCI). Test results have been reviewed by experts in carcinogenesis and mutagenesis. GENE-TOX was developed by the US EPA and contains genetic toxicology (mutagenicity) test data, resulting from expert peer review of the open scientific literature, on over 3000 chemicals.

CPDB: The Carcinogenic Potency Database (CPDB) (http://potency.berkeley.edu/cpdb.html) provides a unique resource of the results of 6540 chronic, long-term animal cancer tests on 1547 chemicals. The CPDB provides easy access to the bioassay literature, with qualitative and quantitative analyses of both positive and negative experiments that have been published over the past 50 years in the general literature through 2001 and by the National Cancer Institute/National Toxicology Program through 2004. The CPDB is downloadable in pdf, xls and txt formats, and is searchable by chemical name, CAS number, or author.

RTECS: The National Institute for Occupational Safety and Health (NIOSH) developed the Registry of Toxic Effects of Chemical Substances (RTECS) database, in this case accessed through the Canadian Centre for Occupational Health and Safety (CCOHS). Features of the RTECS Database is said to be a definitive toxicological database with supplemental information pertinent to both the chemical industry and the occupational safety and health community. This technical data is needed to assess workers' exposures to chemicals, particularly to lesser-known-and-used chemical substances. OSHA has designated RTECS as a primary source for toxicity data for Material Safety Data Sheets in its Hazard

Communications Rule. In recent years RTECS has grown to include more than 160,000 chemicals. The toxicological data are organized into six fields: primary irritation, mutagenic effects, reproductive effects, tumorigenic effects, acute toxicity and multiple dose toxicity. Each data line includes the citation to its bibliographic source. RTECS provides a host of reference data including, but not limited to: CAS Numbers, OSHA PELS, ACGIH TLVs, NIOSH RELS, Carcinogenic assessments, Beilstein Reference Numbers, and Bioassay results from the National Toxicology Program (NIOSH-National Institute for Occupational Safety and Health, http://www.cdc.gov/niosh/rtecs/RTECSfeatures.html, accessed online 3 September 2010).

3.3 Data gap analysis

The requirements stated in the REACH regulation (EC) No 1907/2006 Annexes VII-X were followed in detail to develop a gap analysis on existing toxicity data. Focus for the gap analysis were the information requirements for substances manufactured or imported in quantities of one tonne or more (Annex VII), 10 tonnes or more (Annex VIII), 100 tonnes or more (Annex IX) and 1000 tonnes or more (Annex X). The work followed what is stated in the respective Annexes; such as the specific rules for adaptation (column 2) and to provide any other relevant mutagenic or toxicological information that was available.

The information requirement with respect to mammalian toxicity in the REACH annexes VII through X, for all substances manufactured or imported in quantities from 1 ton to 1000 tons (Article 12(1)a-e) requires toxicological information on acute, short term (28-days), sub-chronic (90 days) and long-term ($\geq$ 12 months) repeated dose toxicity as well as reproductive toxicity. In the case the substance is determined to be a genotoxic carcinogen, a germ cell mutagen or the substance have a low toxicological activity with enough toxicokinetic data available to support the results, no reproductive toxicity studies need to be done (REACH, 2006a). Mutagenicity and genotoxicity is an important endpoint for all annexes and implies a considerable human health risk for the substance groups nitrosamines and nitramines. Based on the annexes, in case of positive results of in vitro mutagenicity in bacteria or in vitro cytogenicity (Chromosomal aberration or micronucleus study) or gene mutation in mammalian cells further mammalian in vivo genotoxicity/mutagenicity studies shall be performed. Further investigation might be necessary at 1000 tonnes and more depending on the quality and relevance of all the available data. Also a carcinogenicity study might be necessary if the substance has a widespread dispersive use or there is a risk for a long-term human exposure or that the substance already have been classified as a mutagen category 3.

3.4 Alternative methods versus in vivo studies

The review of existing knowledge was performed to gather available toxicological and ecotoxicological information. Existing data such as historical human data, acceptable and relevant data from tests not carried out according to the principles of Good Laboratory Practice (GLP), application of weight of evidence and use of (QSARs), in vitro methods and read-across. The approach followed the principles of the rules provided for in section 1 of Annex XI to REACH (REACH, 2006b). If

(Q)SAR methods were possible to conduct, OECD Principles for (Q)SAR validation was aimed at (OECD, 2004) and external validation was an objective. (Q)SAR reports were aimed at to be in accordance to the Guideline R.6 from ECHA (ECHA, 2008).

Existing information on in vitro alternative methods to measure relevant toxicological endpoints for detecting adverse health effects from exposure to nitrosamines, nitramines or in mixtures were compiled. Both *in vitro* and *in silico* methods were reviewed. Approach to provide the key toxicological and ecotoxicological information, preferable without animal testing and with emphasis on inhalation as uptake route was emphasized.

The promotion of alternative methods is increasingly favoured at the expense of conventional animal testing at European level and by European Commission but also in other industrialised regions such as USA and Japan. These initiatives are coordinated by OECD at the international level. As a consequence of the EU Directive 86/609/EEC, in 1991 the European Centre for the Validation of Alternative Methods (ECVAM) was founded which has become a unit of the Joint Research Centre of the EU Commission in Ispra, Italy, since 1992. Normally new alternative methods are validated at these facilities (ECVAM, JaCVAM, ICCVAM) before OECD validation and approval.

3.4.1 Sources of in vitro and in vivo toxicity methods

We searched for OECD recommended methods (both for *in vitro* and *in vivo* methods). Additionally, we searched for ECVAM, JaCVAM and ICCVAM validated methods waiting for approval or in validation process. Standard validated methods for mammalian (human related) toxicity studies as well as methods under validation process are described in a number of databases:

- OECD Guidelines for Testing of Chemicals (http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788)
- ECVAM - European Center for the Validation of Alternative Methods (http://ecvam.jrc.ec.europa.eu/)
- JaCVAM - Japanese Center for the Validation of Alternative Methods (http://jacvam.jp/en/index.html)
- ICCVAM - Interagency Coordinating Committee on the Validation of Alternative Methods at the National Toxicology Program (http://iccvam.niehs.nih.gov/)

In addition new methods are suggested as part of the alternative testing strategy:

- ISI Web of Science (http://apps.isiknowledge.com/)
- PubMed (http://www.ncbi.nlm.nih.gov/pubmed)

3.4.2 General considerations of in vitro tests

Currently available in vitro tests are accepted by regulatory bodies mostly for genotoxicity testing. It has been suggested that they can be used in future to determine the starting dose of *in vivo* studies, assist in evaluation of data from animal studies especially in identification of species differences, or to increase understanding of the toxicological mechanism of action of the substance. They cannot be used to replace testing in animals completely, although this may be possible in the future. *In vitro* data may be useful for predicting acute toxicity in humans and a range of tests have been investigated that permit calculation of an IC50 (inhibitory concentration 50%) value. It has been suggested that the results of *in vitro* cytotoxicity tests may be as predictive of acute oral toxicity in humans as rat or mouse data. However, this aspect needs to be further investigated (Kinsner-Ovaskainen et al 2009). ECVAM recently validated several in vitro methods and many of them are under validation (Zuang et al. 2009)

Main advantages of in vitro test methods:

- 3R (Reduce, refine and replace the use of animals for toxicity testing)
- Easy
- Cheap
- Less time consuming
- Good for studying mechanisms of toxicity at molecular and cellular level
- Potentially robust
- High-throughput is possible to test hundreds of chemicals per short time and it is possible to combine them with chip or robotic technology for performing or evaluation of the results
- Specific types of toxicity can be studied such as photogenotoxicity
- Human primary cells (e.g. lymphocytes) or stable cell lines can be used which might be closer to humans than animal systems
- Some assays can be adopted for fast in situ screening (in exposed environment or for biomonitoring)

Major disadvantages of in vitro models and test methods:

- Major disadvantage is that toxicokinetic studies cannot be perform by using in vitro system
- Most of the tests use a prediction model for correlating in vivo and in vitro data experimental data. For in vitro data these prediction models depend upon the concentration curves which are highly dependent upon factor like e.g. the cell density, serum concentration and quality of the media. A slight change in any of the factors may result in a shift in the concentration curves and incorrect use of in vitro data in combinations with in vivo experiments
- Correlation of experimental data (in vitro and in vivo) with human data is challenging even in cases where human primary cells or cell lines derived from humans are used
- There is lack of metabolic activation for some in vitro systems which can be partly overcome with using external metabolic systems (feeder cells or co-cultivation with potent metabolically active cells, S9 fraction, using metabolically active cells and cell lines, see below).

- Current in vitro methods for testing reproduction toxicity either do not include metabolic activation or introduction of metabolic systems is not yet included in the validation program. Presence of metabolic activation system is relevant for testing the toxicity of nitrosamines and nitramines.

3.4.3 Selection of methods based on endpoints

Toxicokinetics and metabolism

In vitro system is simpler, lack in vivo complexity, metabolic activation is in most cases missing and toxicokinetic cannot be studied. There are several methods which try to overcome this problem, though there are not satisfactory yet, and cannot fully replace animal testing (Coecke 2006):

1. Co-culture of indicator cells with drug metabolizing competent cells.
2. Addition of S9-mix.
3. A promising novel method is the development of genetically engineered cell lines capable of phase I and phase II metabolism preferably by enzymes of human origin (Pelkonen et al 2005).
4. Liver on a Chip. A revolution in cancer research. The idea reflects the fact that the liver is a key player both in many diseases and in the process of testing new drugs (Linda Griffith, MIT; http://spectrum.mit.edu/articles/features/liver-on-a-chip/).
5. The skin and penetration *in vitro* test, OECD TG 428.
6. The intestinal absorption model, Caco-2 cell monolayers (Le Ferrec et al 2001, Prieto et al 2004).
7. *In silico* models of drug absorption (Bergstrøm 2005).
8. "In combo" approach of ADME estimation, using *in silico* tools and *in vitro* screening (Waterbeemd 2005).
9. *In vitro* hepatic biotransformation enzyme induction [Hepa RG (inhouse); Hepa RG (commercial) and cryopreserved human hepatocytes] (3 test methods Ongoing validation study (ECVAM with ICCVAM and JaCVAM involvement) (Zuang et al., 2009)

Skin irritation and corrosion

Validated alternative methods accepted for regulatory use, and methods under development for determining skin corrosion exist that have replaced the animal tests:

1. In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test (TER), OECD TG 430
2. In vitro skin Corrotion: Human Skin Model Test, OECD TG 431
3. In Vitro Membrane Barrier Test Method for Skin Corrosion, OECD TG 435
4. EST-1000 method for skin corrosivity testing, EU Test Method B.40 bis, Compliant with OECD TG 431
5. Rat Transcutaneous Electrical Resistance (TER) skin corrosivity test, INVITTOX No 115 (ECVAM validated)

6. EPISKIN [R], finalised and scientifically accepted by ECVAM Scientific Advisory Committee (ESAC) in 2007, http://ecvam.jrc.it/index.htm.
7. EpiDerm[R], finalised and scientifically accepted by ECVAM Scientific advisory committee (ESAC) in 2007, http://ecvam.jrc.it/index.htm.
8. Skin Irritation Corrosion Rule Estimation Tool (SICRET), based on physicochemical properties or structural alerts (Walker et al 2005).
9. SkinEthic RHE assay, EU Test method B.46
10. Reconstructed Human Epidermis (RhE) Test Method, OECD Draft

Eye irritation and corrosion

Two *in vitro* tests are already validated by OECD, and others are still under validationg process or under development:

1. Bovine Corneal Opacity and Permeability (BCOP) test method, OECD TG 437
2. Isolated Chicken Eye (ICE) test method, OECD TG 438
3. Cytosensor Microphysiometer test, INVITTOX Protocol 102 – Validated by ESAC as to be used as part of tiered testing strategy
4. Fluorescein Leakage test, INVITTOX Protocol 71– Validated by ESAC as part of tiered testing strategy
5. Slug Mucosal Irritation (SMI) Assay, Under validation at ECVAM
6. Rule-based non-testing approach based on physicochemical properties and structural alerts (Gerner et al 2005).

Skin sensitization

For skin sensitisation, no OECD validated *in vitro* method exist. The alternative test methods for skin sensitization are:

1. OECD TG 429 Local Lymph Node Assay (LLNA) was modified in terms of reduction of animal use to a reduced version of LLNA (rLLNA) using only the equivalent of the high dose group from the full LLNA to be used as screening test to distinguish between sensitizers and non-sensitizers, was accepted by ESAC in 2007, http://ecvam.jrc-it/index.htm.
2. A method of measurement of cytokine expression in keratinocytes, in co-culture with dendritic cells or in reconstituted epidermis is under development (Casati et al 2005).
3. Sens-it-iv, a recent EC FP6 project (started in 2005), development of animal free test strategies for skin and lung sensitization (http://www.sens-it-iv.eu).
4. VITOSENS, sensitisation test on human dendritic cells, is operational and under external validation (Zuang et al., 2009)
5. (Q)SAR to predict skin sensitization (Robersts et al 2007 and Patlewicz et al 2007).

Inhalation sensitizing
To our knowledge, there are no validated methods for testing sensitization from exposure via inhalation. OECD has validated one method, TG 413, which measures subchronic inhalation toxicity and is a 90 day study which measures subchronic inhalation toxicity after repeated exposure via inhalation route for 28 days. It can be used for determing NOAEL and LOAEL but does not provide any indication of sensitization.

Acute toxicity
In vitro and *in vivo* tests methods for oral/chronic oral/inhalation toxicity were assessed in order to get an overview of adverse effects and an estimate of dosages for further toxicity assays (table 3.1).

Basal cytotoxicity with *in vitro* test methods may have the potential to predict quantitative aspects of acute toxicity (Botham 2004, ICCVAM/NICEATM 2001a, b, 2006, Stokes et al 2008; Zuang and Hartung 2005), e.g:

1. BALB/c 3T3 mouse fibroblast (3T3) The Neutral Red Uptake (NRU) test.
2. Normal human epidermal keratinocytes (NHK) NRU test.

These methods are now included in OECD No. 129 Guidance document (OECD, 2009a) on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests and may be used in a weight-of-evidence approach to determine the starting dose for current *in vivo* acute oral toxicity protocols, i.e., Fixed Dose Procedure (OECD guideline 420, Acute Toxic Class Method (OECD guideline 423), Up and Down Procedure (OECD guideline 425). Additionally, *in vitro* cytotoxicity test (3T3 Neutral Red Uptake) for identifying substances with acute oral LD50 > 2000 mg/kg b.w has been validated by ECVAM (Zuang et al, 2009). Several not yet OECD-validated *in vitro* tests such as clonogenic (colony forming ability) and proliferation assays are also promising cytotoxicity tests to be used in addition to the above methods, and as more independent methods in future.

ACuteTox is a recently finished EC FP6 integrated project developing alternative methods consisting of a database with in vitro and in vivo data on acute toxicity (http://www.acute-tox.org). The components have to be integrated into a validated in vitro test battery or testing scheme combined with a prediction model for data extrapolating aiming at hazard identification of acute toxicity to humans (Gennari 2004). Several acute toxicity in vitro methods were pre-validated within A-Cute-Tox such as the trans-epithelial resistance (TER) method and the enhanced paracellular permeability (PCP) methods.

For *in vivo* toxicity there exist improved and combined methods, where more data can be gathered with fewer animals tested. For example the previous OECD 401 method for oral toxicity (LD50) is now improved into the OECD 420, 423 and 425 methods, and the OECD 403 method for inhalation toxicity (LC50) is now being improved, and a new alternative to this is the OECD 436 method.
OECD 420 is mostly used for drug testing and determination of LD 50 of compounds with no or low toxicity. For environmental compounds and industrial chemicals OECD 425 or OECD 423 are recommended. However, OECD 425 gives more exact calculation of LD50.

Table 3.1 Validated methods for acute oral, chronic oral and inhalation toxicity

OECD validated - In vivo assays	
423	Acute Toxic Class (ATC) Method for acute oral toxicity testing (replacing 401)
420	Fixed Dose Procedure (FDP) for acute oral toxicity testing (replacing 401)
425	Up-and-Down Procedure for acute oral toxicity testing (replacing 401)
407	Repeated Dose 28-day Oral Toxicity Study in Rodents
408	Repeated Dose 90-Day Oral Toxicity Study in Rodents
409	Repeated Dose 90-Day Oral Toxicity Study in Non-Rodent
403	Acute Inhalation Toxicity
412	Subacute Inhalation Toxicity: 28-Day Study
413	Subchronic Inhalation Toxicity: 90-day Study
436	Acute Inhalation Toxicity - Acute Toxic Class Method
OECD drafts – In vitro assays	
OECD Draft Guidance 129 (2009)	Neutral Red Uptake (NRU) test with human cells (normal human epidermal keratinocytes (NHK)) (NHK NRU assay) and mouse BHK 3T3fibroblasts 3T3 NRU tests Using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests.
ECVAM validated – In vitro assays	
ECVAM follow up validation study completed (2009)	*In vitro* cytotoxicity test (3T3 Neutral Red Uptake) for identifying substances with acute oral LD50 > 2000 mg/kg b.w
Scientifically validated – In vitro assays	
	In vitro cytotoxicity tests (colony forming ability, relative growth, growth activity)

Subacute and (sub) chronic toxicity

1) **The test for subacute toxicity** with 28 days of exposure (OECD TG 407, 410 and 412).

Conventional *in vivo* tests for subacute or subchronic toxicity cannot be replaced at present or in the near future by in vitro or other alternative methods (Prieto et al 2006).

Phototoxicity

Please see table below for methods available for detecting phototoxicity.

Table 3.1b Validated methods for acute phototoxicity in vitro

OECD and EU validated - In vitro assays	
OECD TG 432 (2004) EU Test Method B.41	3T3 NRU Phototoxicity Test (photo-irritation); 3T3 NRU Phototoxicity Test: Application to UV Filter Chemicals
ECVAM validation process In vitro assays	
ECVAM feasibility study completed	Tiered testing strategy to predict phototoxicity (3T3 NRU PT and reconstructed human epidermis models)
JaCVAMsponsored	Produced reactive oxygen species (ROS) and photostability study
JaCVAM	Test method battery to predict phototoxicity (yeast growth inhibition Phototoxicity assay and red blood cell photohemolysis assay)

Neurotoxicity

No *in vitro* models for neurotoxicity testing have been validated or have been accepted for regulatory purposes. The development of *in vitro* neurotoxicity tests are present at the research level. A number of *in vitro* models for studying the blood-brain barrier (BBB) are currently available, but need to be refined (ECVAM's Workshop 49, Prieto et al 2004, Garberg 2005). In vitro methods for developmental neurotoxicity (DNT) testing into international hazard and risk strategies has been discussed by ECVAM (Coecke et al 2007) and by the TestSmart DNT programme (Lein et al 2007).

Genotoxicty and Mutagenicity

Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms. *Genotoxicity* is a broader term and refers to processes which alter the structure, information content or segregation of DNA and are not necessarily associated with mutagenicity.

Table 3.2 give lists of validated test methods (*in vitro* and *in vivo*) for testing mutagenicity.

Several *in vitro* tests for genotoxicity and mutagenicity testing are currently accepted at the OECD level **(**OECD TG 471, 480, 481, 473, 476, 487, 479, 482) and several of them are under validation either as separate tests (comet assay) or in combination of two genotoxicity tests (Comet assay in vitro and micronucleus test in vitro) in 3D skin models. Comet assay is a robust, quick, well established method, which is under validation by EVCAM/JaCVAM and used as valuable test by Pharmaceutical industry and other industries, and results are accepted by regulatory bodies. Final validation study is expected in autumn 2010, and submission of validation results and draft TG is expected early 2011 (https://www.oecd.org/dataoecd/22/61/41339719.pdf).

As already mentioned, the limitations are: insufficient metabolic capacity, comparison of i*n vitro/in vivo* doses, specific characteristics of commonly used rodent cell lines may not be representative for the in vivo situation, and finally target organ specific mode of action can give misleading *in vitro* results (Hengstler et al 2003).

Table 3.2 Validated methods for genotoxicity/mutagenicity

OECD validated - In vivo assays	
474	Mammalian Erythrocyte Micronucleus Test
475	Mammalian Bone Marrow Chromosome Aberration Test
477	Genetic Toxicology: Sex-Linked Recessive Lethal Test in Drosophila melanogaster
478	Genetic Toxicology: Rodent Dominant Lethal Test
483	Mammalian Spermatogonial Chromosome Aberration Test
484	Genetic Toxicology: Mouse Spot Test
485	Genetic toxicology, Mouse Heritable Translocation Assay
486	Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells in vivo
JaCVAM/ECVAM/ICVAM validation process – In vivo assays	
JaCVAM ECVAM/ICVAM	In vivo Comet assay (Single-Cell Gel Electrophoresis [SCGE] Technique)
OECD validated – In vitro assays	
471	Bacterial Reverse Mutation Test
473	In vitro Mammalian Chromosome Aberration Test
476	In vitro Mammalian Cell Gene Mutation Test
479	Genetic Toxicology: In vitro Sister Chromatid Exchange Assay in Mammalian Cells
480	Genetic Toxicology: Saccharomyces cerevisiae, Gene Mutation Assay
481	Genetic Toxicology: Saacharomyces cerevisiae, Miotic Recombination Assay
482	Genetic Toxicology: DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells in vitro
487	In Vitro Mammalian Cell Micronucleus Test
JaCVAM validation process – In vitro assays	
JaCVAM ECVAM/ICVAM	In vitro Comet assay (Single-Cell Gel Electrophoresis [SCGE] Technique)

Carcinogenicity

Presently, no single *in vitro* test or combination of tests is considered to be sufficient to replace *in vivo* tests. Carcinogenesis caused by chemicals are complex and multistep processes of long-term toxicity where yet much remains to be discovered on the mechanistic level (Hengstler et al 2003, Bolt et al 2004). Many substances causing cancer are non-genotoxic, meaning they cause cancer without damaging the DNA, and several *in vitro* assays have been established for their detection (Sakai et al 2002), but they are not finally approved by the OECD (OECD 2001, 2006d): the Syrian hamster embryo cell assay (SHE), the C3H10T1/2 assay and the BALB/c3T3 assay. Table 3.3 give lists of test methods (*in vitro* and *in vivo*) for testing carcinogenicity.

Table 3.3 Validated methods for carcinogenicity

OECD validated – In vivo assays	
451	Carcinogenicity studies
452	Chronic Toxicity Studies
453	Combined Chronic Toxicity/Carcinogenicity Studies (alternative to 451 and 452)
ECVAM validation process- In vitro assays	
OECD draft review paper (No 31)* EU B.21	Cell Transformation Assays; Combination of different assays: 1.Syrian hamster embryo (SHE) assay 2.Balb/c assay 3.C3H/10T1/2 assay (Alternative to 451) http://www.oecd.org/dataoecd/59/29/36069919.pdf

*** Environment, Health and Safety Publications Series on Testing and Assessment No. 31.** Draft detailed review paper on cell transformation assays for detection of chemical carcinogens OECD document 2006, February 7

Reproductive and Developmental Toxicity

Three in vitro models have been formally validated by ECVAM and recommended as screening tests for developmental toxicity testing. These tests focus the endpoint embryotoxicity, and cover only certain aspects of developmental toxicity (Speilmann et al 2006). It has been recommended to improve the EST by supplementing it with a suitable in vitro metabolizing test (Pellizzer et al 2005, Coecke et al 2006).

1. **The embryonic stem cell test (EST)**
2. **The micromass test (MM)**
3. **The rat postimplantation whole embryo test (WEC)**

The ReProTech is an EC FP6 integrated research project for developing a predicting test strategy of reproductive toxicology and endocrine dirruption (Hareng et al 2005, Bremer et al 2005). **Combined test strategies** for improving the predictive value of in vitro reproductive toxicity testing are planned to be developed: 1) Combination of in vitro tests and (Q)SArs focusing on the blood-testis barrier and the blood-placental barrier (Hewitt et al 2007). 2) Combination of in silico approaches and physiologically based pharmacokinetic (PBPK) modeling (Verwei et al 2006).

Table 3.4 gives a list of validated test methods (*in vitro* and in *vivo* methods) for testing reproduction toxicity.

Table 3.4 Validated methods for reproduction toxicity

OECD validated - In vivo assays		**Main endpoint**
415	One generation	Reprod./Feto./Devel.
416	Two generation	Reprod./Feto./Devel.
414	Prenatal developmental	Devel.
426	Developmental neurotoxicity	Neurotoxicity
New TG Draft	Extended one generation (415+416+414+426)	Reprod./Devel./Neuro./Immuno.
407	Repeated dose 28 day oral	Endocrine disruption
421	Rep/dev toxicity	Reprod./Feto./Devel.

OECD validated - In vivo assays		**Main endpoint**
422	Combined 407+421 (422 is screening reproductive toxicity studies)	Reprod./Feto./Devel.
440	Uterotrophic Bioassay in Rodents: A short-term screening test for oestrogenic properties	Endocrine disruption
441	Hershberger Bioassay in Rats: A Short-term Screening Assay for (Anti)Androgenic Properties	Endocrine disruption
OECD validated – In vitro assays		
455	The Stably Transfected Human Estrogen Receptor-alpha Transcriptional Activation Assay; detection of potential estrogen receptor binding by measuring luciferase activity.	Hormone activity
New TG Draft	Steroidogenesis Assay; measuring of 17β-estradiol (E2) and testosterone (T).	Hormone activity
ECVAM validated – In vitro assays		
INVITTOX No 123 Whole embryo culture (WEC):	Embryo (malformation, retardation and death) after over a 48 hr exposure. Quantification of **developmental toxicity** by IC50 (50% inhibition of cell viability and growth), ICmax (malformations), ICnoec (total morphological score (TMS) as the sum of scores for all organs).	Developmental tox.
INVITTOX No 113 Embryonic stem cell test (EST):	Growth and differentiation of murine ESC D3, into spontaneously contracting cardiomyocytes (adjunct- OECD TG 414). Quantification of **embrytoxicity** by ID50 (50% inhibition of cardiac cell differentiation) and IC50D3 (50% viability of D3 cells) and IC503T3 (50% viability of 3T3 cells).	Embryotox.
INVITTOX No 122 Micromass assay (MM):	Detect inhibition and cell differentiation in rat micromass cultures of limb bud. Quantification of **developmental toxicity** by ID50 (50% inhibition of cell differentiation and number of foci) and IC50 (50% inhibition of cell viability and growth).	Developmental tox.

3.4.4 New approaches in testing: Pattern based systems (omics)

Toxicogenomics

Toxicogenomics is a novel technique combining toxicology with genomics, transcriptomics, proteomics or metabolomics. The aim of toxicogenomics is to elucidate the molecular mechanisms of a novel toxicological process and to derive the molecular expression patterns to be able to predict the toxicity or the genetic susceptibility of a compound. Toxicogenomics combines *in vitro* or *in vivo* assays with *in silico* techniques. Changes in gene expression (mRNA/protein profiling) upon exposure of a chemical *in vitro* or *in vivo* is performed and compared with previously gathered results by the use of appropriate databases, in order to recognize typical patterns of gene expression induced by specific chemicals (http://ctd.midibl.org/).

Toxicogenomics techniques have the potential to predict specific endpoints of toxicity after long-term carcinogenicity studies and long-term studies by using short-term *in vivo* exposure of laboratory animals, and will result in refinement and reduction of animal testing (Corvi et al 2006, OECD 2005b). The use of short-term animal tests in combination with whole genome transcriptional profiling (**transcriptomics**) to identify substance specific alterations in mRNA expression patterns have been reported (review: Hengstler 2006). Specific gene clusters are affected by the specific chemical compounds, and different subtypes of hepatotoxicity can be differentiated (Huang et al 2004, Waring et al 2001). Certain custom-made microarrays exist, where only a subset of the genes of a genome are present, for study of organ specific or endpoint specific gene expression: ERG chip with 200 estrogen-responsive genes (Hayashi et al 1991). In addition the RT-PCR ELISA kits is another alternative methods to whole genome profiling, combining a reverse transcription polymerase chain reaction with an enzyme-linked immune-absorbent assay. Toxicogenomics using **proteomics** techniques implies gathering of protein expression profiles from *in vitro* or *in vivo* assays and combining it with *in silico* techniques. Proteins expression profiles are analysed and quantified on 2D gels, and further sequenced and characterized my mass spectrometry (MS) analysis. The expression patterns of proteins are compared with appropriate databases to recognize typical patterns of protein expression induced by specific chemicals. Toxicogenomics combined with **metabolomics** implies analysis of specific patterns of enzyme expression upon exposure of a specific chemical, and further comparison with appropriate databases for recognition of affected metabolic pathways (http://www.genome.jp/kegg/).

Databases

There exist several databases for information and comparison of data on chemicals interacting with specific organic molecules or affecting specific pathways or causing specific diseases, thereby expanding the perspectives and the interpretations of the results.

The Comparative Toxicogenomics Database (CTD) elucidates molecular mechanisms by which environmental chemicals affect human disease (http://ctd.midibl.org/). The etiology of most chronic diseases involves interactions between environmental factors and genes that modulate important physiological processes. This assumption is supported by the many complex

diseases caused by reversible behaviours or avoidable exposures, and by the relatively rare number of diseases attributed to single gene mutations. Environmental factors are implicated in many common conditions such as asthma, cancer, diabetes, hypertension, immune deficiency disorders and Parkinson's disease; however, the molecular mechanisms underlying these correlations are not well understood. CTD includes manually curated data describing cross-species chemical–gene/protein interactions and chemical– and gene–disease relationships to illuminate molecular mechanisms underlying variable susceptibility and environmentally influenced diseases. These data will also provide insights into complex chemical–gene and protein interaction networks.

STITCH **(**http://STITCH.embl.de/) is a resource to explore known and predicted interactions of chemicals and proteins. Chemicals are linked to other chemicals and proteins by evidence derived from experiments, databases and the literature. STITCH contains interactions for over 74,000 small molecules and over 2.5 million proteins in 630 organisms.

The Pharmacogenomics Knowledge Base **(**www.PharmGKB.org) has the mission to collect, encode, and disseminate knowledge about the impact of human genetic variations on drug response. We curate primary genotype and phenotype data, annotate gene variants and gene-drug-disease relationships via literature review, and summarize important PGx genes and drug pathways.

The DrugBank database (http://www.drugbank.ca) is a unique bioinformatics and cheminformatics resource that combines detailed drug (i.e. chemical, pharmacological and pharmaceutical) data with comprehensive drug target (i.e. sequence, structure, and pathway) information.

The KEGG database (http://www.genome.jp/kegg/), Kyoto Encyclopedia of Genes and Genomes, was developed as a bioinformatics resource as part of the research projects of the Kanehisa Laboratories in the Bioinformatics Center of Kyoto University and the Human Genome Center of the University of Tokyo. Since 1995 they have been developing knowledge-based methods for uncovering higher-order systemic behaviors of the cell and the organism from genomic and molecular information, and associated bioinformatics technologies are being developed both for basic research and practical applications.

3.4.5 (Q)SAR and read across

Literature were searched for potential developed (Q)SAR models of the compounds of interest; i.e. nitrosamines and nitramines. In addition, a new software tools developed for the purpose of QSAR development was evaluated for the purpose of read across. OECD (Q)SAR Application Toolbox which contain tested and estimated toxicity data in addition to some QSAR models was evaluated for the purpose of data gap filling for toxicity endpoints. The development of the (Q)SAR Application Toolbox is made in order to increase the regulatory acceptance of (Q)SAR methods and to make (Q)SAR technology readily accessible, transparent, and less demanding in terms of infrastructure costs. The Toolbox is a software application intended to be used by governments, chemical industry and other stakeholders in filling gaps for (eco)toxicity data needed for assessing the hazards of chemicals. The Toolbox incorporates

information and tools from various sources into a logical workflow. Crucial to this workflow is grouping chemicals into chemical categories.

The seminal features of the Toolbox are:
1. Identification of relevant structural characteristics and potential mechanism or mode of action of a target chemical.
2. Identification of other chemicals that have the same structural characteristics and/or mechanism or mode of action.
3. Use of existing experimental data to fill the data gap(s).

The last step refers to read-across, trend analysis or QSAR model in order to fill the data gap.

The OECD Toolbox includes some databases with experimental data that can be used to support grouping and read-across: a) the ISSCAN database –1149 chemicals containing data for carcinogenicity and Ames mutagenicity; b) the EXCHEM database– 256 chemicals containing data for Ames mutagenicity, chromosomal aberrations and mouse micronucleus assay; c) the OASIS Genotox database – 2684 chemicals with data for Ames mutagenicity and chromosomal aberrations as well as data for metabolism. The Toolbox also includes the Danish EPA database containing predicted data of different genotoxicity and carcinogenicity endpoints for more than 166,000 chemicals.

The compiled data from the project for specific toxicity endpoints were imported as a database in the OECD Toolbox in case the implemented databases did not include these data. TD_{50} (mg/kg body wt/day) values were already included in the Toolbox as part of the ISSCAN database. LD_{50} values were not implemented as part of the toolbox and own database of 20 compounds with corresponding LD_{50} was imported and used for prediction of non-tested compounds.

In addition to this validated tool by OECD we have aimed at developing a QSAR model based on the available data from this project and other published data that can make a more trustworthy model with larger dataset. In house QSAR software ADMEWORKS ModelBuilder (version 3.0.60 Standard Edition 2006, Fujitsu Kyushu System Engineering Ltd) was used in order to calculate various molecular and atomic descriptive parameters. The same software was used to make regressions based upon Genetic Algorithm and Interactive Multiple Linear Regressions (Interactive MLR).

3.4.5.1 The OECD Principles for (Q)SAR Model Validation

To facilitate the consideration of a (Q)SAR model for regulatory purposes, it should be associated with the following information (OECD, 2007):

1) a defined endpoint
2) an unambiguous algorithm
3) a defined domain of applicability
4) appropriate measures of goodness-of-fit, robustness and predictivity
5) a mechanistic interpretation, if possible

According to Principle 1, a (Q)SAR should be associated with a "defined endpoint", where endpoint refers to any physicochemical, biological or environmental effect that can be measured and therefore modelled. The intent of this principle is to ensure transparency in the endpoint being predicted by a given model, since a given endpoint could be determined by different experimental protocols and under different experimental conditions. Ideally, (Q)SARs should be developed from homogeneous datasets in which the experimental data have been generated by a single protocol. However, this is rarely feasible in practice, and data produced by different protocols are often combined.

According to Principle 2, a (Q)SAR should be expressed in the form of an unambiguous algorithm. The intent of this principle is to ensure transparency in the description of the model algorithm.

According to Principle 3, a (Q)SAR should be associated with a "defined domain of applicability. The need to define an applicability domain expresses the fact that (Q)SARs are reductionist models which are inevitably associated with limitations in terms of the types of chemical structures, physicochemical properties and mechanisms of action for which the models can generate reliable predictions. This principle does not imply that a given model should only be associated with a single applicability domain. The boundaries of the domain can vary according to the method used to define it and the desired trade-off between the breadth of model applicability and the overall reliability of predictions.

According to Principle 4, a (Q)SAR should be associated with "appropriate measures of goodness-of–fit, robustness and predictivity." This principle expresses the need to provide two types of information: a) the internal performance of a model (as represented by goodness-of-fit and robustness), determined by using a training set; and b) the predictivity of a model, determined by using an appropriate test set. There is no absolute measure of predictivity that is suitable for all purposes, since predictivity can vary according to the statistical methods and parameters used in the assessment.

According to Principle 5, a (Q)SAR should be associated with a "mechanistic interpretation, wherever such an interpretation can be made. Clearly, it is not always possible to provide a mechanistic interpretation of a given (Q)SAR. The intent of this principle is therefore to ensure that there is an assessment of the mechanistic associations between the descriptors used in a model and the endpoint being predicted, and that any association is documented. Where a mechanistic interpretation is possible, it can also form part of the defined applicability domain (Principle 3).

GUIDANCE ON PRINCIPLE OF DEFINED ENDPOINTS

Importance of Quality of Measured Endpoint Data

Oral exposures to mammals are one of the defined endpoints in the OECD test guidelines but the measurement of the effects of the chemical on the animal is much more influenced by kinetic factors than intrinsic thermodynamic variations in chemical structure. As chemical structure varies, the exposure itself is no longer comparable from one chemical to another. The shifting exposure regime with different chemicals will mean that, for some chemicals, a response near the true

lethal potency of the chemical is measured whereas for another chemical only 10% of the true lethal potency is measured. In such cases, the endpoint is as much a measure of the toxicity of the chemical as it is an artifact of the way the chemical is tested (OECD, 2007).

GUIDANCE ON PRINCIPLE OF UNAMBIGUOUS ALGORITHMS

The algorithms used in (Q)SAR modelling should be described thoroughly so that the user will understand exactly how the estimated value was produced and can reproduce the calculations, if desired. Important regulatory endpoints are estimated for chemicals by selecting the proper (Q)SAR for the specific class of chemical (see application domain in Chapter 4), or a proper general (Q)SAR model(s) based upon a common toxic effect, computing the chemical-specific molecular descriptors required by the (Q)SAR model, and using those molecular descriptors in a mathematical algorithm to create an estimate of the endpoint for the chemical. The ability to reproducibly complete all three steps producing an estimate is an important part of the acceptance of (Q)SAR models. All three steps in producing estimated values may involve individual algorithms as is the case of mechanistic estimates of dose-response endpoints. For many binary endpoints where the (Q)SAR model is primarily a classification model, the algorithms may be an association with the presence or absence of important chemical substructures (OECD, 2007).

The following elements should be considered when assessing the algorithm:

1. The dataset of chemicals, end-point values and descriptor values.
2. A clear description of the derivation of the descriptors and how they were measured.
3. A clear description of the test and training sets and, if outliers were removed a clear justification for this.
4. The mathematical model(s) used to explore the descriptor and end-point relationship needs describing.
5. Statistical parameters describing how the model performs (see Chapter 5).
6. The parameters and their values which constitute the (Q)SAR.

Multiple Linear Regression (MLR)

When the endpoint needs to be modelled using more than one descriptor (selected by different approaches) then multiple linear regression (MLR) and /or multivariate techniques are applied. MLR is the most popular regression method, it produces a transparent and easily reproducible algorithm. As it can suffer of the use of correlated variables, this correlation must be carefully controlled. This is controlled in the software used in this project; i.e. ADMEWORKS ModelBuilder. The problem of possible overfitting (i.e. to many descriptors compared to compounds), common also to other modelling methods, must be also verified by statistical validations methods for predictivity. In ADMEWORSK ModelBuilder a recommended ratio of 6 of number of compounds to descriptors is given. The selection of descriptors in MLR can be performed a priori by the model developer on mechanistic basis or by evolutionary techniques such as Genetic Algorithms as well as methods like Principal Component Analysis (PCA) or Factor Analysis (FA) (OECD, 2007).

GUIDANCE ON PRINCIPLE OF A DEFINED DOMAIN OF APPLICABILITY OECD

Principle 3 states that a (Q)SAR should be associated with a defined domain of applicability and expresses the need to include supporting information with a (Q)SAR which will define the classes of chemicals with which the model performance will satisfy the regulatory requirements. There is no absolute boundary between reliable and unreliable predictions for a given model, but rather a tradeoff between the constraints of the applicability domain (AD) and the overall reliability of prediction for numerous chemicals. In general, the less constrained the AD, the more likely chemicals will be included for which the predictions will be less reliable. The more constrained the AD, the more chemicals will be encountered for which the end point cannot be predicted with the (Q)SAR. The balance within these tradeoffs depends on the requirements and can be determined by the user in the validation process within the specific regulatory context (OECD, 2007).

Recommendations for Deriving Applicability Domains

Ideally, the Applicability Domains (AD) should define the structural, physicochemical and response space of the model. This is because the best assurance that a chemical is predicted reliably is to have confirmation that the chemical is not an outlier in terms of its structural fragments (structural domain), its descriptor values (physicochemical domain) or its response values (response domain). When the AD is defined in more mechanistic terms, the (Q)SAR can predict reliably beyond the physicochemical and response space of the training set (OECD, 2007)

Hotelling's test

A common approach to distance analysis is to use the Hotelling's test and the associated leverage statistics. The leverage of a chemical provides a measure of the distance of the chemical from the centroid of its training set. Chemicals in the training set have leverage values between 0 and 1. A warning leverage (h*) is generally fixed at 3p/n, where n is the number of training chemicals, and p the number of descriptors plus one. A leverage value greater than the warning leverage is considered large (OECD, 2007)

Williams plot

To visualise the outliers in a model, i.e. outliers in both the descriptor space and the response space, a plot of standardised residuals (R) vs. leverages (or hat values, h), called the Williams graph is sometimes used, see Pavan et al. (2005) and Helguera et al. (2007). If chemicals in the training set have leverages greater than the warning leverage (0.07), these compound could be considered as outliers.

As with all statistical methods based on physicochemical descriptors, the leverage approach needs to be applied with care. While the observation that a chemical has a large leverage indicates that it is outside the descriptor coverage of the model, a chemical with small leverage can also be outside the AD for other reasons (e.g. a presence of a toxicophore that is not present in the training set). The inability to discriminate unequivocally between chemicals that are inside and outside the AD is common to all statistical methods based on physicochemical descriptors, and

this should be taken into account when applying the concept of the AD (OECD, 2007).

GUIDANCE ON THE PRINCIPLE OF MEASURES OF GOODNESS-OF- FIT, ROBUSTNESS AND PREDICTIVITY

For validation of QSAR model these strategies are suggested:

1. internal validation or cross-validation;
2. validation by dividing the data set into training and test compounds;
3. true external validation by application of model on external data and
4. data randomization or Y-scrambling

The need for information on the performance of (Q)SAR models is expressed by OECD Principle 4 which states that models should be associated with appropriate measures of goodness-of.fit and robustness (internal performance) and predictivity (external performance). The assessment of model performance is sometimes called statistical validation within the context of the assessment (OECD, 2007). Statistical validation techniques also provide a means of identifying .spurious. models based on chance correlations, i.e. situations in which an apparent relationship is established between the predictor and response variables, but which is not meaningful and not predictive.

Goodness-of fit, robustness and predictivity of MLR
Estimating the regression coefficients**.** Regression coefficients in MLR model can be estimated using the least squares procedure by minimizing the sum of the squared residuals. The aim of this procedure is to give the smallest possible sum of squared differences between the true dependent variable values and the values calculated by the regression model.

Assessing the relative importance of descriptors**.** If the variables are standardized to have mean of zero and standard deviation of one, then the regression coefficients in the model are called beta coefficients. The advantage of beta coefficients (as compared to regression coefficients that are not standardized) is that the magnitude of these beta coefficients allows the comparison of the relative contribution of each independent variable in the prediction of the dependent variable. Thus, independent variables with higher absolute value of their beta coefficients explain greater part from the variance of the dependent variable.

Assessing goodness-of-fit. To assess goodness-of-fit, the coefficient of multiple determination (R^2) is used. R^2 estimates the proportion of the variation of y that is explained by the regression. If there is no linear relationship between the dependent and the independent variables $R^2= 0$; if there is a perfect fit $R^2= 1$. R^2 value higher than 0.5 means that the explained variance by the model is higher than the unexplained one. The end-user(s) of a QSAR model should decide what value of R^2 is sufficient for the specific application of the model. One author has recommended that $R \geq 0.9$ for in vitro data and $R \geq 0.8$ for in vivo data can be regarded as good [Kubinyi, 1993].

Avoiding overfitting**.** The value of R^2 can generally be increased by adding additional predictor variables to the model, even if the added variable does not contribute to reduce the unexplained variance of the dependent variable. It follows that R^2 should be used with caution. This could be avoided by using another statistical parameter the so-called adjusted R2 (R2adj). R^2adj is interpreted similarly to the R^2 value except it takes into consideration the number of degrees of freedom. It is adjusted by dividing the residual sum of squares and total sum of squares by their respective degrees of freedom. The value of R^2adj decreases if an added variable to the equation does not reduce the unexplained variance.

From the calculated and observed dependent variable values the standard error of estimates could be obtained. The standard error of estimate measures the dispersion of the observed values about the regression line. The smaller the value of s means higher reliability of the prediction. However it is not recommended to have standard error of estimate smaller than the experimental error of the biological data, because it is an indication for an overfitted model (Wold et al., 1984).

The statistical significance of the regression model can be assessed by means of F-value. The F-value is the ratio between explained and unexplained variance for a given number of degrees of freedom. The higher the F-value the greater the probability is that the equation is significant. The regression equation is considered to be statistically significant if the observed F-value is greater than a tabulated value for the chosen level of significance (typically, the 95% level) and the corresponding degrees of freedom of F. The degrees of freedom of F-value are equal to p and n-p-1. Significance of the equation at the 95% level means that there is only a 5% probability that the dependence found is obtained due to chance correlations between the variables.

4 Results

4.1 Recommended strategy for testing

Physical-chemical characterisation of tested compounds including molecular weight, solubility in water and tested media, and stability should be preferable known before testing. During data compilation of physical-chemical properties (not shown in report) it was clear that many compounds lack experimental data. Based on the existing experimental and estimated physical-chemical data, most compounds should be water soluble (low LogKow values) and neutral at pH 7. Only a few compounds (with butyl groups) have a LogKow higher than 2.3. *In situ* characterisation (before and after the treatment) should be incorporated into ITS.

Potential health effects of Amine 9 compounds can be evaluated by toxicity studies using animals or cell cultures. Various endpoints are used to cover a variety of possible toxic responses, as described in the Tender Invitation:

- Acute toxicity
- Phototoxicity
- Mutagenicity/genotoxicity
- Carcinogenetic effects
- Reproduction toxicity and developmental toxicity
- Repeated dose toxicity (chronic toxicity)
- Subchronic toxicity
- Sensitisation
- Irritation/corrosion of skin and eyes

We regarded **acute toxicity, phototoxicity, mutagenicity**/**genotoxicity**, **carcinogenicity**, **reproduction toxicity** and **sensitisation** as the most important endpoints to consider, and therefore the focus in this report is on methods representing these endpoints. We recommend to start ITS with acute toxicity tests. We do not recommend repeated dose toxicity tests (chronic toxicity tests) and subchronic toxicity tests in our strategy, since the endpoints will be partly covered with the recommended reproduction toxicity test and the combined carcinogenicity/chronic toxicity test and other alternative *in vitro* and *in silico* tests.

The recommended integrated strategy for testing (ITS) described in this chapter provides advice on how the REACH Annexes VI to XI (Directive 67/548/EEC) information requirements for toxicity tests can be met. The strategy seeks to ensure that the data requirements are met in the most efficient and humane manner so that animal usage and costs are minimized. Together with the data gathered on the Amine 9 compounds collected in the compiled data spread sheet and in the gap analysis spread sheet (Appendix H and I) this testing strategy is guidance on a stepwise approach for developing adequate and scientific data for assessment, evaluation and classification of the toxic properties of the Amine 9 substances (e.g. nitramines, nitrosamines, as well as amides, aldehydes and amines) with specific focus on nitrosamines and nitramines.

4.1.1 Important points to consider for selecting appropriate methods

There are few important points to consider for the selection of appropriate methods for testing genotoxicity, carcinogenicity and reproductive toxicity concerning potential flue gas compounds like **nitrosamines** and **nitramines**:

- Nitrosamines can be converted to nitramines both *in vivo* and *in vitro* and vice versa (Fraser et al., 1980).
- The metabolites (through cytochromes P450) of nitrosamines (such as nitrosodialkylamines) are known to be responsible for the carcinogenicity through DNA-alkylation or DNA-oxidation (genotoxic carcinogen) (Ishikawa et al. 2007; Helguera et al., 2007; Lewis 1997).
- Nitramines can be reduced (deaminated) to *formaldehyde* and *ammonia* both *in vivo* and *in vitro*, and thus can act as tumor promoters. This process is reported to be responsible for the carcinogenicity of nitramines (as non-genotoxic carcinogen) (Frei et al., 1984)

This indicates that the standard *in vitro* tests are not sufficiently reassuring and that in order to ensure mutagenicity potential is adequately explored, there is a need for a metabolic activation system or appropriate metabolic system (metabolically active cells or feeder cells) for assessing the toxicity of nitramines and nitrosamines. For all in vitro experiments, to test compounds with and without metabolic activation system (either rat liver S-9 mix, a reducing system, a metabolically active cell line like HepG2 cells or genetically engineered cell lines) should be considered.

Generally, N-nitrosamines, such as *N*-nitrosodialkylamines, need to be metabolized in vivo by cytochromes P450 to achieve their biological activity (left hand side of figure 1), in contrast to *N*-nitrosamides, such as *N*-nitrosoureas, that do not need metabolic conversion (right hand side of figure 1). Activated metabolites of N-nitrosodialkylamines, α-hydroxynitrosamines, decompose spontaneously to alkanediazohydroxides, then to alkyldiazonium ions, which can react with biological nucleophiles such as nucleic acids and proteins (Preussmann and Stewart, 1984). The resulting DNA alkylation is an important factor in the carcinogenic or mutagenic activities of N-nitroso compounds.

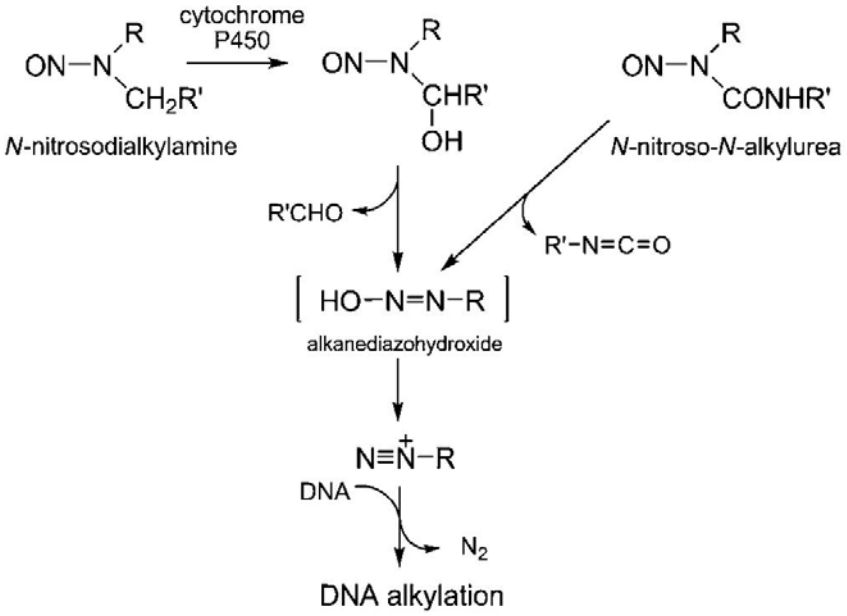


Figure 1: Activation of N-nitroso compounds. The character R or R' indicates any alkyl group (Ishikawa et al. 2007). Since nitramines can be converted (reduced) to nitrosamines it is likely to believe that the above mechanism may be relevant in addition to other potential carcinogenic action via reduction to formaldehyde and ammonia (W. Lijinsky, 1992).

These compounds are likely to be mutagenic and thus also potentially carcinogenic. They also can act potentially as tumor promoters (via formaldehyde) and thus can act as non genotoxic carcinogens. It is therefore important to choose methods and endpoints which may identify genotoxic endpoints on different levels (DNA breakage, base damage, point mutations, larger mutagenic and clastogenic changes as well as disruptions in cell divisions). In case of negative results in genotoxicity, possible non-genotoxic carcinogenicity should be tested. It is therefore important to address both initiation of carcinogenicity (genotoxic effects) as well as non-genotoxic carcinogenicity (promotions, proliferation, progression). Literature reviews have shown that these compounds are likely to release photooxidative products, and thus phototoxicity and photogenotoxicity should be also addressed (Michejda and Rydstrom 1984).

For reproductive toxicity, in the nirosamines group, the data from N-nitrosodimethylamine (62-75-9) indicated that it is a confirmed reprotoxic chemical with main effects being observed as fetotoxicity. This suggests the need for the methods which are mainly focussed upon measuring fetotoxic effects.

N-nitramines

As the N-nitrosamines needs to be activated metabolically to give cancerogenic intermediates, a similar metabolic activation have been reported for N-nitramines. A study established the mutagenicity of dimethylnitramine and nitromorpholine in a liquid-incubation system using the AMES test with Salmonella typhimurium strain TA 100 and TA 1530 in the presence of S9-liver fraction (containing

hepatic postmitochondrial supernatant obtained from 1254 aroclor-treated rats) (Khudolei et al. 1981). They registered that the metabolic activation of nitramines involved the conversion of nitramines to nitrosoamines due to the reduction of the nitro group with the following metabolic steps following that of nitrosoamines, with hydroxylation by the cytochrome P450 multifunction oxidases with subsequent heterolysis to the cancerogenic intermediate (Khudolei et al. 1981) while Malavelle et al. suggests that dimethylnitramine undergo hydroxylation in the presence of the rat liver (S9) to hydroxymethyl-methylnitramine which display a 100-fold increased activity in the TA 100-strain compared to the parental compound (Malaveille et al. 1983).

4.1.2 Testing strategy and recommendation of methods for acute toxicity, mutagenicity and carcinogenicity

4.1.2.1 Recommendation and rationale for choice of methods

We performed an extensive review of validated methods and other available tests and testing strategies following REACH and ECHA guidelines with stress on 3Rs - to exclude, reduce using of animals and minimise their suffering. We suggest using a tier approach focussing on in vitro and *in silico* methods where applicable. We also recommend case by case approach to exclude unnecessary *in vivo* experiments. We further propose *in vivo* experiments which combine two or three endpoints to reduce use of animals.

We recommend starting with the new *in vitro* methods, OECD draft 129 to estimate starting doses for acute oral systemic toxicity tests and/or *in vitro* cytotoxicity test (3T3 Neutral Red Uptake) for identifying substances with acute oral LD_{50} > 2000 mg/kg b.w. Optionally we recommend also additionally two non validated methods; clonogenic and proliferation assays. Though these have no officially OECD or ECVAM validated protocol, they are considered very promising, and give useful information especially for *in vitro* genotoxicity experiments. *In vitro* cytotoxicity tests can be used for determination of IC_{50}, which in future can be used to estimate LD_{50} and LC_{50} and thus can be useful for predicting acute toxicity. In combination with other endpoints they are also important for increasing our understanding of the toxicological mechanism of action. In addition, these tests are quick, inexpensive and by using cells from corresponding organs they can predict organ-specificity.

Due to the nature of the selected chemicals, in vitro phototoxicity test using OECD TG 432 3T3 NRU tests is recommended in this stage.

In vivo acute toxicity tests are necessary even if *in vitro* test data exist. In our approach we recommend OECD 425 method which gives most precise calculation of LD_{50} with confidential interval. For inhalation toxicity (LC_{50}), OECD 436 We recommend OECD 436 instead of improved 403 as this method is preferably used for environmental compounds and has advantage by using stepwise approach in testing. Concentrations obtained are used for setting dosages for further toxicity tests.

For genotoxicity/ mutagenicity, three different *in vitro* tests are usually initially chosen for 3 different endpoints, usually both prokaryotic and eukaryotic tests.

The OECD 471 Ames method (prokaryotic) is recommended in ITS as first genotoxicity test and has been used often for Amine 9 compounds with positive outcome (CAS No 62-75-9, 59-89-2, 1116-54-7 and 4164-28-7)., therefore we chose OECD 471 as starting point, followed by 2 eukaryote tests. The OECD 476 method is the only validated gene mutation method for mammalian cells, and it has been used to prove mutagenicity of three Amine 9 compounds (CAS No 62-75-9, 59-89-2). The OECD 473 assay and the i*n vitro* Comet Assay are both chosen in our strategy for DNA damage analysis, because the OECD 473 has been used with positive outcome for one Amine 9 compound (CAS No 59-89-2), and the in vitro Comet Assay is quick, inexpensive, easy to perform, well established and commonly used in many laboratories. The OECD 479 (SCE) and 482 (UDS) methods are either not specific, error prone and/or complicated to perform, so we do not recommend these methods. The OECD 480 and 481 methods of gene mutations in Saccharomyces, were not chosen to be of primary interest due to the possibility of species specific differences. The OECD 487 micronucleus method can be alternatively used for detecting mutagenicity and clastogenicity and also has been choosen in our strategy. If the outcome of the 3 selected tests is negative or equivocal, we suggest an additional *in vitro* genotoxicity test with the same endpoints but with other cell type.

The initial *in vitro* bacterial and two mammalian genotoxicity/mutagenicity tests are generally followed by *in vivo* genotoxicity/mutagenicity tests. The OECD 474 *in vivo* micronucleus test and the OECD 475 *in vivo* chromosome aberration test are both validated and commonly used methods. They have also both been used with positive result for Amine 9 compounds (CAS No 62-75-9 and 59-89-2). The OECD 477 method with gene mutations in Drosophila, was not chosen to be of primary interest due to the possibility of species specific differences. The OECD 478, 483, 484 and 485 methods have not been previously documented to be particularly useful for validation of Amine 9 compounds, and were therefore not chosen. The OECD 486 UDS *in vivo* method is old and rough assay, not specific for DNA damage or repair and outcome is uninformative, so we do not recommend this method. In vivo comet assay to detect DNA damage in different organs and tissues seems very promising method and is recommended in our strategy in combined in vivo assay. If 2 out of 3 tests are positive in combined endpoint in vivo experiment then the compound is considered genotoxic, and likely to be carcinogenic and further long term carcinogenicity testing is considered unnecessary. If the outcome is negative, then the compound might be a non-genotoxic carcinogen and is recommended to be tested on carcinogenicity *in vitro* before any long term animal testing for carcinogenicity is considered *(see scheme figure 5).* The Cell transformation tests (OECD Draft 31, EU B.21) is the only available tests at this time. If results are positive for *in vitro* carcinogenicity, further long term carcinogenicity testing might also later be initiated using case by case approach. The long term rodent carcinogenicity test bioassay (OECD 451) is today commonly combined with chronic toxicity test assay (OECD 452), in the revised OECD 453 assay (a 12-24 months study). The OECD 453 method is therefore recommended in our strategy as an alternative method which addresses both chronic toxicity and carcinogenicity in the same assay. Time, cost and animals are reduced when choosing this method.
The methods chosen address specific endpoints that are highly relevant for the type of damage we expect from these compounds. The step-by-step strategy is

relevant for avoiding unnecessary experiments on animals by estimating the dosages and the toxicity with simpler assays *in vitro* (e.g. OECD 471, 476, 487 and 473), and thereby reducing the costs and the number of animals in the *in vivo* tests (e.g. OECD 474 and OECD 475). Finally the long term toxicity tests (Fig 4.8) are recommended to be performed only after judging the outcome from the genotoxicity tests (Fig 4.6 and 4.7), which also contributes to reducing time, cost and animals.

Time scale of *in vitro* and *in vivo* tests: In vitro cytotoxicity, Ames test and the Comet assay are quick and last up to two weeks. Chromosomal aberrations and micronuclei and gene mutation assay are more time consuming, experiments last up to one month. Neoplastic (morphological) transformation *in vitro* last about 4-6 weeks. Length of *in vivo* experiments depends if acute, sub-acute or chronic exposure is chosen, and last from one to six months. Carcinogenicity *in vivo* is long term two years study.

A recommendation for a step-by-step procedure for safety evaluation of Amine 9 compounds are shown in Table 4.1, 4.2, 4.3 and 4.4 and scheme (page 69, figure 5).

4.1.2.2 Step-by-step testing strategy

A recommendation for a step-by-step procedure for safety evaluation of CO_2 capture flue gas compounds can be followed by a 3 step procedure as shown in Table 4.1, 4.2, 4.3 and 4.4 and scheme (figure 5).

Step 1 Strategy for acute toxicity:

Table 4.1a Strategy for acute toxicity and phototoxicity in vitro

Acute Single Dose Toxicity		**Rational for choice of method(s)**
OECD Guidance document No 129	This method includes Neutral Red Uptake (NRU) test with rodent cells (mouse 3T3 fibroblasts) (3T3 NRU assay) to estimate starting dose for the below test.)	Useful for cytotoxicity testing to estimate starting doses for acute oral systemic toxicity tests and for genotoxicity
OECD TG 432 (2004) EU Test Method B.41	3T3 NRU Phototoxicity Test (photo-irritation); 3T3 NRU Phototoxicity Test:	This assay detects potential phototoxic chemicals
ECVAM follow up validation study completed (2009)	*In vitro* cytotoxicity test (3T3 Neutral Red Uptake) for identifying substances with acute oral LD50 > 2000 mg/kg b.w	This assays estimates LD 50 for compounds with low toxicity

Alternatively several non-OECD validated assays (scientifically validated) on different cells such as human hepatocyte HepG2, the human intestinal Caco2 cell line, and the human alveolar epithelial cell line A549, assay can be an option if they are performed under GLP conditions. The most promising assays are proliferation (growth activity) assay, relative growth or clonogenic, (colony forming ability, plating efficiency).

Table 4.1b Strategy for acute toxicity

Acute Single Dose Toxicity		**Rational for choice of method(s)**
OECD 425	Up-and-Down Procedure for acute oral toxicity testing (replacing 401)	There are a limit test and a main test. The limit test can be used efficiently to identify chemicals that are likely to have low toxicity.LD 50 values are calculated and it is possible to compute confidence intervals. This test is more used for environmental chemicals and unknown chemicals.
OECD 436	Acute Inhalation Toxicity - Acute Toxic Class (ATC) Method (replacing 403)	*Short time exposure within 14 days, or multiple doses within 24 hours, for determination of median lethal dose (LD50) or lethal concentration (LC50) for each sex at 95% confidence interval. Adverse effects. Concentrations obtained are used for setting dosages for further toxicity tests.*

Step 2 Genetic toxicity in vitro:

Perform 3 in vitro genotoxicity tests, addressing 3 different endpoints in this order:

a) OECD 471
b) OECD 487 OR 473
c) OECD 476
d) The comet assay in vitro as additional test or option for b or c

Table 4.2 Strategy for genetic toxicity *in vitro*

Genetic Toxicity		**Rational for choice of method(s)**
OECD 471	Prokaryote assay, reverse gene mutation test: Ames test.	*Validated method and the most commonly used initial method for genotoxicity screening. The method has been used with positive result for many Amine 9 compounds: 62-75-9, 59-89-2, 1116-54-7, 4164-28-7.*
OECD 473	Eukaryote assay, DNA damage effects/Chromosomal damage: In vitro mammalian cytogenetic test (chromosome aberrations).	*Validated and commonly used method. Mammalian systems used. Results are easy to screen. This endpoint was proven at epidemiological level as biomarker of cancer. The method has been used with positive result for the Amine 9 compound: 59-89-2.*
OECD 487	In Vitro Mammalian Cell Micronucleus Test	*Validated and commonly used method. Mammalian genotoxicity test system This endpoint was proven at epidemiological level as biomarker of cancer*
Comet Assay (ECVAM/ JaCVAM)	Eukaryote assay, DNA damage /DNA adduct formation in vitro Comet assay, Single-cell Gel Electrophoresis (SCGE) Technique.	*Human-Mammalian systems used. Under validation. Easy to perform, clear dose response, medium high throughput. Used in our laboratory, commonly used for testing. Used with positive outcome for at least 3 Amine 9 compounds: 62-75-9, 55-18-5, 59-89-2).*
OECD 476	In vitro mammalian cell gene mutation assay, the thymidine kinase (TK) locus assay or the hypoxanthine phosphoribosyltransferase assay (HPRT) assay.	*Validated and commonly used method. Mammalian systems used. Results are easy to screen. The method has been used with positive result for the Amine 9 compounds: 62-75-9, 59-89-2.*

➔ ***If negative results,* or equivocal,** additional in vitro test on different cell model but with same endpoint is recommended. If negative outcome, *the substance is likely to be non-genotoxic,* and is suggested to be tested for *in vitro* carcinogenicity (EU B21).

➔ ***If positive results,* 2 out of 3 po**sitive=> genotoxicity established, and the compound is determined to be a non-threshold substance. *Further in vivo genotoxicity test with 2-3 combined genotoxi*c endpoints is recommended *(table 4.3) only when the Annex VIII tonnage levels are reached (10-100 t/y) (table 4.3):*

Table 4.3 Strategy for genetic toxicity *in vivo*

In vivo genotoxicity tests:		**Rational for choice of method(s)**
OECD 474	Mammalian erythrocyte micronucleus test.	*Validated and commonly used method. This endpoint was proven at epidemiological level as biomarker of cancer It has been used with positive result for the Amine 9 compounds: 62-75-9 and 59-89-2.*
OECD 475	Mammalian bone marrow chromosome aberration test.	*Validated and commonly used method. This endpoint was proven at epidemiological level as biomarker of cancer It has been used with positive result for the Amine 9 compounds: 62-75-9 and 59-89-2.*
Comet assay Under JaCVAM/ECVAM validation	DNA damages/DNA adduct formation in vivo, Comet Assay, Single-cell Gel Electrophoresis (SCGE),	*Each organ – lung, liver, etc. can be used for specific organ genotoxicity. Easy to perform; clear dose response, medium high throughput. Used in our laboratory, commonly used for testing.*

➔ ***If positive results,*** *then the substance is considered a* ***genotoxic rodent carcinogen****. We suggest no further carcinogenicity testing is necessary. This is a* ***non-threshold substance.***

➔ ***If negative results,*** *then the substance is likely to be* ***non-genotoxic****. Go to table 4.4 and test for non-genotoxic carcinogenicity by in vitro carcinogenicity testing. If the tonnage levels, then in vivo carcinogenicity testing might be considered after the in vitro carcinogenicity tests*

Step 3 Carcinogenicity:

If the outcome of genotoxicity *in vitro* is negative we recommend testing compounds for neoplastic (morphological) transformation *in vitro* (OECD Draft 31, EU B.21) as first screening. This testing should be considered as part of battery of in vitro tests, e.g. in initiated in early step, immediately after outcome of in vitro genotoxicity is known (see scheme, Figure 5). Following REACH recommendation, depending on tonnage, further long term carcinogenicity testing might be considered. For *in vivo* carcinogenicity we recommend long term rodent carcinogenicity combined with chronic toxicity bioassay (OECD 453).

Table 4.4 Strategy for carcinogenicity tests.

Chronic toxicity / Carcinogenicity		**Rational for choice of method(s).**
OECD draft review paper 31, (EU B.21, ECVAM)	Cell transformation assays, combination of different assays: 1. Syrian Hamster Embryo (SHE). 2. BALB/C assay. 3. C3H/10T1/2 assay	*Alternative in vitro test for the in vivo OECD 451 test. Gives indication of possible in vivo test outcome.*
OECD 453	Combined Chronic Toxicity/ Carcinogenicity studies. 12-24 months.	*Validated method. Alternative fused test of the two common OECD 451 and OECD 452 methods. The OECD 451 method has been used for all Amine9 compounds that currently have a TD50 value. Results: carcinogenic properties, tumour incidence in relation to dose (TD_{50}), latency period, tumour multiplicity, potential for metastasis.*

- ➔ **If positive results** for *in vitro* carcinogenicity, the substance is considered **a carcinogen** but may be suggested for further in vivo testing (using case by case approach).
- ➔ **If negative results,** substance is considered **non-genotoxic, non carcinogenic compound** and should be tested for reproductive toxicity

Test evaluation forms of the recommended methods are described in Appendix F. For Cell transformation assay, this test can be included already in a battery of in vitro tests together with in vitro mutagenicity

4.1.3 Testing strategy and recommendation of methods for testing reproduction toxicity

If the substance of interest is classified as a genotoxic or carcinogenic or a germ cell mutagen, then further testing for reprotoxicity is normally not recommended.

Table 3.4 shows a list of validated in vitro and in vivo methods for reproductive toxicity testing. OECD is currently validating two *in vitro* methods, which measure the hormonal activity as their endpoint. The ECVAM validated tests assess the embryo and developmental toxicity. Since OECD and ECVAM validated in vitro tests do not measure fetotoxic effects in particular and do not include metabolic activation, both parameters which are relevant for testing nitramines and nitrosamines, we propose only to use these methods as initial tests for reproductive toxicity. Then further in vivo test methods for reproduction toxicity are proposed to be carried out.

The available reprotoxicity data from the nitrosamine group suggests that these chemicals mainly have effects on fetotoxicity. Based on this data, we can assume that the rest of the nitrosamines in the group are fetotoxic as well. There are four relevant methods (OECD 415, 416, 421, 422) which measure the fetotoxicity in addition to reproductive and developmental effects (Table 3.4). We therefore focussed on the advantages and disadvantages of these four methods. The OECD test method 422 measures several aspects of fetotoxicity and uses fewer animals (10 animals of each sex for 3 treatment groups and control) when compared to combination of OECD 407 (5 animals of each sex for 3 treatment groups and control) and 421 (10 animals of each sex for 3 treatment groups and control).

In summary, we recommend reproductive toxicity testing for those compounds which are considered as non-genotoxic and non-carcinogenic. A three tier study should be performed for testing the reproduction toxicity (Table 4.5). 1st tier includes initial testing with the EST method for embryotoxicity testing and the MM method for developmental toxicity testing. Continue with *in vivo* combined test for repeated dose and reproductive toxicity including measurements of developmental/reproductive toxicity (OECD 422). If no data on LD_{50} for the chemical is available, a test for LD_{50} determination (OECD 425) should be conducted before starting in vivo experiment. If outcome of OECD 422 is negative, no further testing is recommended. If the outcome is positive that the substance is fetotoxic and a third tier should be performed, with one of the following tests: OECD 415 or OECD 416.

Test evaluation forms of the recommended methods are described in Appendix F.

Table 4.5 Recommended reproduction toxicity testing protocol

Protocol for reproduction toxicity testing		
1st tier	INVITTOX 113 (EST) and INVITTOX 122 (MM)	
2nd tier	OECD 422	LD_{50} when no data available – suggest OECD 425
3rd tier	One or both of OECD 415, 416 depending on outcome of 2nd tier	

4.1.4 Testing strategy and recommendation of methods for testing sensitization

We do not recommend including sensitization tests, as the chemicals to be included in this strategy (nitrosamines and nitramines) are mostly assumed to be mutagenic, genotoxic, carcinogenic or reprotoxic. In these cases, sensitisation tests will not be necessary.

The value of performing such tests also depends on the exposure level. This information is not available yet, though we assume from preliminary numbers that the level will be far below to be of any concern for sensitization parameters.

Additionally, there are no validated methods for measuring sensitization from inhalation, an endpoint relevant for this project. However, it is assumed that skin sensitizers also are respiratory sensitizers, and these methods could therefore be considered though it is possible that only a few compounds will be also inhalation sensitizers.

Our conclusion is that if the Company would like to expand this strategy to other flue gas compounds which might be less toxic. There are a number of new alternative methods (some validated, some still under validation) for irritation/corrosion/sensitization which we can take into consideration in future.

4.2 In vitro non-validated methods for testing cytotoxicity, mutagenicity/genotoxicity and carcinogenicity in ITS

In suggested testing strategies we recommend several not fully validated methods. Among them the *in vitro* cytotoxicity assays are suggested that have not been fully validated yet, but they are known to contribute with valuable data in acute toxicity studies. The assays are also quick, inexpensive and easy to perform. Therefore we suggest *in vitro* cytotoxicity assays as a strategic starting point. Additionally, the in vitro Comet assay, (SCGE) (a eukaryote assay to measure DNA damage effects/DNA adduct formation) is currently being validated by JaCVAM. We contributed to development of this method and have much experience with the technique in our laboratory. In addition it is a quick, inexpensive and easy method to perform, widely accepted by industry and local regulatory bodies as genotoxicity tests. Finally, the Cell Transformation assays are chosen since they are the only currently available alternative methods to *in vivo* carcinogenicity tests. We also suggest in vivo Comet assay in cells from different tissues (liver, lung) in combined in vivo genotoxicity assay. We did not include toxicogenomic endpoints yet in our strategy but they seem very promising both in vitro and in vivo in combination with other methods.

As several non fully validated assays has been proposed for ITS find it important to test appropriateness of this approach and verify (‘validate’) this ITS against set of negative (nongenotoxic), positive (genotoxic) as well as set of amine 9 compounds.

4.3 Toxicogenomics; Recommendation for Future Strategy for Testing

Toxicogenomics; gene expression analysis of in vitro cell exposures. Toxicogenomics assays on exposed cells can give insight into novel adverse effect perspectives and discover new endpoints and effects without using in vivo methods. .Combined with appropriate databases e.g. CTD, comparison with other compounds giving similar effects can increase the knowledge base and assist hazard characterization. These tests should be considered in future testing strategies.

The use of toxicogenomics can be particularly valuable for quick screening of potential health impacts of new chemicals. Well established *in vitro* cell exposure models (the human alveolar epithelial cell line A549, the human intestinal Caco2 cell line or the human hepatocyte HepG2 cell line) can be used together with toxicogenomics in order to screen for putative adverse effects in organs like the lung, the gastrointestinal system (GI), or the liver. Full genome human microarrays contain microscopic spots of each of the 23 000 genes in the human genome on a glass slide. By harvesting RNA from *in vitro* exposed cell model systems, labeling with specific fluorophores, hybridizing to the full genome human microarrays, and scanning the emitted signals from each gene spot that is corresponding to the amount of bound RNA, then affected genes can be detected at an early stage, and further adverse health effects can be foreseen. The affected genes can be either over-activated or under-activated compared to a control exposure system, and these genes can be analyzed in software (Ingenuity) and databases (KEGG, CTD) for common molecular or metabolic pathways or common molecular interactions. Short-term *in vivo* exposures can also be analyzed with the same approach, to help avoid or at least minimize long-term *in vivo* exposures. Altogether, the use of toxicogenomics can be very informative as a pre-screening analysis, by interpreting early stages of activated molecular pathways in simple cell models, which most probably are equal to the pre-stages of later adverse effects on whole organs. By using this technique one can quicker approach the hidden information of putative adverse effects of a chemical, to better design more endpoint specific tests, to minimize the use of long-term animal testing, and to reduce the cost and time spent.

4.4 Data compilation and gap analysis

Available toxicological data from the RTECS, TOXNET, CPDB and IRIS EPA were compiled. The TD_{50} values for rat were evaluated and checked for agreement with the available TD_{50} values in OECD (Q)SAR Toolbox in the implemented ISSCAN Update 3 database. In addition other databases were searched for additional data and verification, see, Appendix B, for more information.

Schematic structure drawings of 23 nitrosamine and 15 nitramine compounds were provided by the Company, were thoroughly examined. We were not able to find CAS number for one of the nitramines and it was therefore impossible to find any information in the databases of this compound.

The standard information requirements for substances manufactured or imported in quantities of 1 through 1000 tonnes, according to annex VII-X in Regulation (EC) No 1907/2006, was the basis for the identification of information gaps. A schematic overview of these requirements is given in Appendix C.

Summary of compiled toxicity data is given in Appendix H and Summary of the data gap analysis is given in Appendix I.

Below follows an overview of the data gap analysis in accordance to REACH Annex VII-X:

Annex VII, Section 8, toxicological information, Section 8.1-8.3 requires information on skin corrosion, skin and eye irritation and skin sensitation; no data was found in databases.

Annex VII, Section 8.4, mutagenicity, nitrosamines have in many cases been extensively tested in vivo and in vitro, and many have been classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC) (http://monographs.iarc.fr/ENG/Classification/index.php) and EU (Directive EC 1907/2006 and Annex I of Directive 67/548/EEC). *N*-nitrosodimethylamine (CAS 62-75-9), *N*-nitroso-*N*-propylpropanamine (CAS 621-64-7), *N*-nitrosodiethanolamine (CAS 1116-54-7) is for example classified as a carcinogen category 2 according to the EU classification scheme. Many of the other non classified nitrosamines have a high degree of evidence based on in vitro and in vivo testing that they pose a significant risk to be mutagenic to microorganisms and in vitro cell cultures of rat, hamster and human. They have also been shown to have carcinogenic potency (Appendix H), usually on species of rat but also in some cases mouse, hamster, rabbit and guinea pig. Only *N*-(1-methylethyl)-*N*-nitroso-2-propanamine (CAS 601-77-4) and 4,4-dimethyl-3-nitroso-2-(propan-2-yl)-1,3-oxazolidine (CAS 39884-58-7) lack any information on mutagenicity or carcinogenicity while *N*-nitrosomethyl-(2-hydroxyethyl)amine (CAS 26921-68-6), *N*-ethyl-*N*-nitroso-2-propamamine (CAS 16339-04-1) and *N*-nitroso-1,3-oxazolidine (CAS 39884-52-1) only have one to two reports on its carcinogenicity and one or two reports on in vitro gene mutation. In contrary to nitrosamines, the nitramines only have a few reported results for carcinogenicity and mutagenicity. *N*-methyl-*N*-nitro-methanamine (CAS 4164-28-7) and *N*-nitromethanamine (CAS 598-57-2) have a reported carcinogenic potency (TD_{50}) value and were found to be positive for carcinogenicity but with equivocal result for in vitro mutagenicity. Also *N*-ethyl-*N*-nitroethanamine (CAS 7119-92-8) has been tested on rat, according to RTECS criteria it was assigned to be an equivocal tumorigenic agent.

In comparing the requirements in Annex VII, Section 8.4.1: In vitro gene mutation study in bacteria and Annex VIII, Section 8.4.2: in vitro cytogenicity or micronucleus study and Section 8.4.3: in vitro gene mutation in mammalian, specific rules of adaptation states that the study does not usually need to be conducted if adequate data from an in vivo cytogenicity test or the substance are known to be carcinogenic category 1 or 2 or mutagenic category 1, 2 or 3 and reliable in vivo mammalian gene mutation tests are available. Historical in vitro and in vivo tests as well as the classification by IARC and EU as to be carcinogenic to humans most probably are valuable in evaluating the need for further testing, especially for the nitrosamine group. Especially the compounds *N*-(1-methylethyl)-*N*-nitroso-2-propanamine (CAS 601-77-4) and 4,4-dimethyl-3-nitroso-2-(propan-2-yl)-1,3-oxazolidine (CAS 39884-58-7) would need to be tested according to Annex VIII, Section 8.4.2 and 8.4.3 if not (Q)SAR or cross reading is a viable approach. Most of the nitramines would need to be tested extensively according to Annex VII through X depending on the production volume. Annex IX, Section 8.4, specific rule of adaptation states that if there is a positive result in any of the in vitro genotoxicity studies in annex VII or VIII and there are no results available from an in vivo study already, specific in vivo studies should be aimed for (see Annex IX, Section 8.4). This statement suggests

that at least *N*-(1-methylethyl)-*N*-nitroso-2-propanamine and 4,4-dimethyl-3-nitroso-2-(propan-2-yl)-1,3-oxazolidine and most of the nitramines would need to be tested in case they are produced at a volume of 100 tonnes or more.

Annex X, Section 8.9.1: Carcinogenic study. Carcinogenic study may be proposed by registrant or agency depending on the long-term exposure risk to humans and if the compound is classified as a mutagen category 3 or there is evidence from the repeated dose studies that the substance is able to induce hyperplasia and/or pre-neoplastic lesions. Not necessary to be performed if the compound already is classified as a mutagen category 1 or 2 such as *N*-nitrosodimethylamine, a comprehensive list is found in Appendix I - Deliverable D1.Summary of data gap analysis These criteria, according to Annex VI of Directive 67/548, substances may become classified as carcinogenic, mutagenic, reprotoxic in category 1, 2 or 3:

Category 1 is used when the substance is **known to be** carcinogenic, mutagenic or reprotoxic for human. There is sufficient evidence that the substance is toxic for man, normally based on epidemiological data.

Category 2 is used when the substances **should be regarded as if** they are carcinogenic, mutagenic reprotoxic in human. There is sufficient evidence of toxicity in more than one other species.

Category 3 is used when substances **cause concern** for man, as to possible carcinogenic, mutagenic or reprotoxic effects. Normally there is evidence of toxicity in one species, and results are variable.

Many of the nitrosamines and all of the nitramines would not fulfil this criterion. By including the human cancer risk classification by the IARC monographs, volumes 1-100, additional nitrosamines could be excluded from additional testing. IARC classifies many of the nitrosamines to be group 2A (probably carcinogenic to humans) or 2B (possibly carcinogenic to humans). Two nitrosamines are classified as 2A while seven are classified as 2B see Appendix I and Table 4.6, below.

Table 4.6 International Agency for Research on Cancer (IARC)[a] classification scheme, Agents Classified by the IARC Monographs, Volumes 1–100

IARC Classification	
Group 1	Carcinogenic to humans
Group 2A	Probably carcinogenic to humans
Group 2B	Possibly carcinogenic to humans
Group 3	Not classifiable as to its carcinogenicity to humans
Group 4	Probably not carcinogenic to humans

[a] http://monographs.iarc.fr/ENG/Classification/index.php

Annex VII, Section 8.5, Acute toxicity: Only three nitrosamines lack acute toxicity data (LD_{50}, oral, rat) while two nitrosamines were administered subcutaneously, one on rat and one on hamster. Eight of the nitrosamines have only one study done on acute toxicity. Only *N*-nitrosodimethylamine, 4-nitrosomorpholine (CAS 59-89-2) and 1-nitroso-piperidine (CAS 100-75-4) have results on acute toxicity through the inhalation route. According to Annex VIII, specific rules of adaptation states that in addition to the oral route at least one additional route of exposure, i.e. inhalation needs to be tested for acute toxicity which also is the main probable route of exposure for these chemicals. Only *N*-methyl-*N*-nitromethanamine (CAS 4146-28-7) of the nitramines has any acceptable acute toxicity data (oral, rat) while *N*-ethyl-*N*-nitroethanamine (CAS 7119-92-8) and *N*-nitromethanamine (CAS 598-57-2) was studied for acute toxicity on mouse using intraperitoneal administration.

Annex VIII, Section 8.6, repeated dose toxicity for 28-days (Section 8.6.1) and 90-days (Section 8.6.2) has only been achieved for five nitrosamines for the 28-day study and four nitrosamines for the 90-day study and none of the nitramines have been tested in any repeated dose toxicity study. In annex IX, (Section 8.6.3) long term repeated toxicity study (12 month) should be performed. For this endpoint only *N*-ethyl-*N*-nitroso-ethanamine have any available data (2 studies). There are still uncertainties on the possibility to identify a NOAEL in the 28-day or 90-day study and the requirement that both female and male rats should be tested.

Annex VIII, Section 8.7, screening for reproductive toxicity, specific rules of adaptation clearly states that this type of study does not need to be conducted if the substance is known to be a genotoxic carcinogen or the substance is known to be a germ cell mutagen and that appropriate risk management measures are implemented. Many of the nitrosamines are genotoxic carcinogens, see appendix H, according to in vitro and in vivo testing. Those nitrosamines that lack data on carcinogenic potency (TD_{50}), fairly accurate estimates using (Q)SAR or cross-reading should be possible to apply. Nitramines on the other hand only two of 15 compounds have been tested and need to be further tested, primarily for genotoxicity as no (Q)SAR or a read-across approach is possible.

Annex VIII, Section 8.8: Toxicokinetics. We have limited knowledge on the toxicokinetic behaviour for these substances but such data might be available for some of the reports on acute or repeated dose toxicity. The toxicokinetic behaviour of *N*-nitrosodimethylamine was reported in the priority substances list assessment report from CESAR (http://www.hc-sc.gc.ca/ewh-semt/alt_formats/hecs-sesc/pdf/pubs/contaminants/psl2-lsp2/nitrosodimethylamine/ndma-eng.pdf, Accessed online 3 September 2010).

4.5 Recommended *in silico* model(s)

Based on the available and compiled tested mammalian toxicity data, the conclusion is that model development is only possible for one group of the compounds; i.e. nitrosamines for the prediction of the carcinogenic potency TD_{50} and acute toxicity LD_{50}.

A read-across approach as first attempt may be a choice in order to estimate the carcinogenic potency TD_{50} and acute toxicity LD_{50} values for the group of nitrosamines. These nitrosamines follow the rules to be classified as a group of similar chemicals. First, they all have a common functional group, secondly, there is a great likelihood of common mechanism of activity, i.e. similar precursors that have a significant likelihood to result in similar breakdown products, thirdly, these chemicals have a constant pattern in potency/toxicity across this group of chemicals relating chemical properties to activity. A suitable program for the development of the read across is the open source application available on the OECD website. This software tool have been developed under the OECD Quantitative Structure-Activity Relationships [(Q)SARs] Project, named "OECD (Q)SAR application toolbox". In addition, a QSAR modelling approach was performed by combining 12 nitrosamines with tested endpoints in this study (see Appendix A), with the set of different nitroso compounds from the work by Helguera and co-workers (2007). These data is based on the lowest observed TD_{50} values for rat, which was determined to be positive for carcinogenicity in the available literature (CPDB database with therein literature sources). The QSAR model approach followed the principles of OECD for QSAR model validation.

4.5.1 Available QSAR models of nitroso-compounds

Relevant existing quantitative (QSAR) and models for the compounds under investigation (nitrosamines) and similar substances have been explored and compiled based on literature and databases (Joint Research Centre (JRC, former ECB), Danish (Q)SAR database, OECD (Q)SAR Application Toolbox and other sources). The most relevant publications for nitroso-compounds is based on the lowest observed carcinogenic potency TD_{50} in male rats for the administration by the gavage route (Helguera et al. 2007) and administration in the water (Helguera et al. 2008a,b). Source of the data used in these studies was collected from the CPDB database (see Appendix B) containing TD_{50} from available literature sources. Comparison between the lowest observed TD_{50} (gavage) used in these studies and the TD_{50} reported in the CPDB, which is the harmonic mean calculated using the TD_{50} value from the most potent target site in each positive experiment, suggests that care of the use of especially sparse data should be taken into account. The difference between the lowest observed TD_{50} for male rat for gavage administration compared to the reported harmonic mean for female/male rat administered through the gavage route and through oral administration (water), is in many cases similar, but there are some instances that the deviance is quite large.

Performance of the published models (Helguera et al. 2007, 2008a,b) was satisfactory with overall good predictivity. The models, partially follows the OECD principles for the validation of QSARs (OECD, 2007), as they lack an external validation or test set, still the models seem to be promising. The training sets was able to account for 81-84% of the variance in the experimental activity

and with high values of cross-validation (internal performance of a model) suggesting that the model is robust and valid within the limits of the applicability domain. The Williams plot, using leverage values, was used for assessing the applicability domain of this dataset. The modeling was based on 35, 39 and 56 nitroso-compounds which is, at the low end, a somewhat small data set. The QSAR models used the Topological Substructural Molecular Design (TOPS-MODE) approach, which uses the spectral moments of the bond matrix to calculate the concentration of structural or physicochemical properties in regions of different sizes in the molecule. This approach was also used in combination with Abraham solute descriptors. They were able to correlate the length of the alkyl chains for the determination of carcinogenic potential and to discriminate between isomeric structures and the recognition of structural alerts in well known potent nitrosamines. Results suggest that a QSAR approach is feasible to determine TD_{50} for the nitrosamines.
Other approaches that has been used for (Q)SAR modeling/classification for the prediction of carcinogenic properties is the use of a novel type of support vector machine. In this study a set of 148 N-nitroso compounds was used for the training of the model using descriptors solely from molecular structure selected with stepwise linear discriminant analysis which were used as input into the support vector machine (Luan et al. 2005). The obtained results confirmed the discriminative capacity of the calculated descriptors resulting in a total accuracy of 95.2%, better than that of stepwise linear analysis alone (total accuracy of 89.8%).

4.5.2 Grouping of substances and read-across

According to the ECHA practical guide 6 (ECHA, 2010), the proper selection of a category/group is of uttermost importance. The category should be defined as having:

- common functional groups
- common precursor or breakdown products
- Constant pattern in changing potency
- Common constituents or chemical class

The first step in the OECD (Q)SAR toolbox is to gather all information about the compound excluding endpoints; i.e. to profile the target chemical(s). The Toolbox contains eighteen profilers, which form the basis of identifying potential analogue(s). These grouping methods are divided into four types, Predefined, Mechanistic, Empiric, and Custom. The Predefined profilers include; database or inventory affiliation, Substance type, OECD and USEPA categorization. The Mechanistic profilers include; Benigni/Bossa rulebase, superfragment, ECOSAR classification, OASIS acute toxicity mode of action, DNA binding, Protein binding, Organic functional groups, Cramer rules, Vehaar scheme, BfR rulebase for eye and skin irritation and BioWin MITI fragments, Empiric profilers include; Lipinski rules, Chemical elements and Groups of elements (OECD, 2009b). In addition the toolbox contains some metabolism profilers.

The current version of OECD Toolbox implements two so-called profilers and categories connected with genotoxicity and carcinogenicity (OECD, 2009b). The

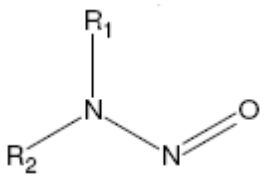
first one is the Benigni-Bossa rulebase (Benigni et al., 2008b) and the second is the OASIS DNA binding profiler developed by LMC Bourgas (Serafimova et al., 2007).

For all the nitrosamines compounds the following profiles were in common: Benigni/Bossa rulebase (Alkyl and aryl N-nitroso groups), Cramer rules (High (Class III)), Organic functional groups (which will have varying groups in addition to N-Nitroso for different N-nitroso compounds). In addition some compounds are profiled with binding to DNA and/or with Superfragment profiling, while others are not.

Benigni/Bossa is a rulebase for carcinogenicity and mutagenicity (which corresponds to the endpoint TD_{50}) and all nitrosamines were profiled in accordance to this rulebase. Benigni/Bossa was therefore chosen as main category in order to collect similar structures belonging to this category with tested TD_{50} values within the toolbox. This is the crucial step before filling data gap of the target molecule with unknown TD_{50}.

According to the Benigni/Bossa rulebase these nitrosamines are defined by the following structural alert (Benigni et al. 2008):

SA_21: alkyl and aryl N-nitroso groups



R1= Aliphatic or aromatic carbon
R2= Any atom/group

Read across approach was therefore performed using the category Benigni-Bossa rulebase (Alkyl- or Aryl N-nitroso compounds). Only the structural alerts are implemented in this profile. No final classifications on mutagenicity or carcinogenicity are provided as a result of using the category (OECD, 2009b). The selection was further optimized and narrowed (pruned) by using a Similarity sub-categorization, Threshold=80%; Dice (Atom pairs; Topologic torsions;Atom centered fragments;Path;Cycles). Database used for data filling of TD_{50} was ISSCAN Update 3 and own imported database of 12 compounds with tested TD_{50} values. All TD_{50} values of our 12 compounds were also found in agreement with the ISSCAN Update 3 database, and only one value was selected for each chemical.

Only three nitrosamine structures lacked LD_{50} values. Since acute toxicity is difficult to categorize to a common mode of action, structure similarity was chosen as the main and only categorization for finding similar structures with assumed same activity. Same default settings as above with the option Dice were used. No database with LD_{50} was found in the toolbox and our own data (20 structures with tested LD_{50} values) were imported into the database and used for filling data gap, see Appendix D. Within the time frame of the project, we have at present not evaluated these predicted values. Still, the read-across is a promising method for predicting both LD_{50} and TD_{50} values for new compounds as long as

there is a good structural correlation with a few compounds with known activity and similar active groups and the same mechanistic function such as those for N-nitrosoalkylamine. There is a correlation of the LD_{50} and TD_{50} reflecting a similar toxicokinetic behavior before the substance is metabolically activated to a potential carcinogen with a specific acute toxicity. Also it should be highly relevant to qualitatively predict positive/negative outcomes of carcinogenicity and mutagenicity of nitrosamines and possible also for nitramines, the latter group dependent on similar compounds in the database of the toolbox.

Please note that the read across method has the potential for further optimization and evaluation with the use OECD (Q)SAR toolbox. Some difficulties such as function and modules not working were observed. There is scheduled a new version release in October 2010 and probably new features, hopefully categories and (Q)SAR models.

There are genotoxicity as well as non-genotoxicity mechanisms of carcinogenicity. The best known genotoxicity mechanisms are those of DNA-binding. This means the DNA binding profiler provides a logical grouping method for qualitatively indentifying chemicals that are potential carcinogens and quantitatively indentifying chemicals that are potential mutagens (OECD, 2009b). For most of the N-nitrosamines in this project, no binding to DNA or proteins were profiled in the toolbox. Since in many cases biotransformation of a non-DNA-binding parent compound can lead to a DNA-binding metabolite, the metabolism profiler is often included either as a primary or secondary profiler for carcinogenesis and genotoxicity (OECD, 2009b). This mode of action is highly relevant for nitrosamine compounds. This procedure should be further explored for data gap filling of TD_{50} (read across or trend analysis) and evaluated as a tool for data gap filling of other endpoints such as classification of carcinogenicity and positive/negative outcomes of carcinogenicity and mutagenicity..

4.5.3 QSAR model development

The 12 nitrosamine compounds with tested endpoints of TD_{50} from rat were combined with the Helguera et al. (2007) study comprising in total 40 compounds. All TD_{50} data was transformed to the unit µmol/kg body wt/day (see Appendix E) and converted to the negative logarithm ($-\log_{10}(TD_{50})$) before model development.

These 40 compounds with TD_{50} values and 11 compounds lacking this endpoint were gathered for evaluating the applicability domain. 2-dimensional (2D) and 3-dimensional (3D) descriptors were calculated for all compounds and the evaluation was done by Principal Component Analysis (PCA) with the software SIMCA (v. 11.5, Umetrics Inc.).

The software ADMEWORKS ModelBuilder was used in order to calculate various 2D/3D structural descriptors, 372 descriptors in total. Standard procedures removing descriptors revealing 90 % correlation was followed prior to the use of Genetic Algorithm (Heimstad et al., 2009) to find the best descriptor set (with lowest number of descriptors) with highest correlation to $LogTD_{50}$. The regression of 33 training compounds was made with Interactive MLR in ADMEWORKS ModelBuilder with quality parameters for the regression such as

Adjusted R^2, Cross-validated (leave one out) R^2CV and prediction R^2 for test set of 7 compounds.

4.5.4 Applicability domain

Hotellings T2 can easily be visualized by using Principal Component Analysis (PCA). The PCA plot (Figure 2a) shows a scores scatter plot with the two first Principal Components t1 and t2. The PCA is a mathematical procedure that transforms a number of possibly correlated variables, in this case 372 physicochemical and 2D/3D variables/descriptors, into a smaller number of uncorrelated (orthogonal) variables called principal components, in this case t1 and t2. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. If a multivariate dataset is visualized where each variable is an axis in a high-dimensional data space, PCA supplies the user with a lower-dimensional picture, usually describing 50-90% of the variation in the multidimensional space. The PCA scatter plot (defined by the principal components t1 and t2) displays how the observations, in this case nitrosoamines, are situated with respect to each other in such a two-dimensional representation of the multidimesional space. These types of plots show the possible presence of outliers, groups, similarities and other pattern in the data. These two principal components describes 53% of the variation of the nitrosoamines of the physicochemical and 2D/3D descriptors used in this project (ADMEWorks ModelBuilder software) and defines the different properties of the obsevations (nitrosoamines). Simca-P draws the tolerance ellipse based on Hotelling‘s T2 (with a 95% confidence interval). Two marginal outliers was identified, which are outside of the Hotelling T2, *N*-Nitroso-*N*-methyl-*N*-tetradecylamine (CAS 75881-20-8) and *N*-Nitrosocimetidine (CAS 73785-40-7). The latter is the compound with lowest activity of all 40 compounds with tested TD_{50} values and the other has quite low activity too. Figure 2b is the loadings plot containing the correlation structure of the X-space (physicochemical and 2D/3D variables). Comparison of both plots gives information which variables have most or less importance for the grouping of nitrosamines in the physicochemical and 2D/3D domain. Variables located far away from the centre are the strongest properties, which have the strongest influence on the differentiation between compounds. As seen in Figure 2a, there is a grouping of the nitroso compounds with the highest carcinogenic potency (black boxes) which have a TD_{50} ($1 < -\text{Log } TD_{50} < 0$), “average” potency (red circles) ($0 < -\text{Log } TD_{50} < -1$) and “low” potency (blue diamonds) ($-1 < -\text{Log } TD_{50} < -5$) from the left side of Figure 2a to the right side, some mixing of the groups also occurs but there is a clear trend. The nitroso compounds with the strongest carcinogenic potency (black boxes, Fig 2a) are mostly located at the left side of the plot, by comparing this positioning with the loadings plot (Figure 2b), those variables or molecular properties that are positioned in the far left side of the plot have the strongest positive correlation on the carcinogenic potency while those molecular properties situated at the far right side of the plot have the strongest negative correlation with carcinogenic potency (see arrows in Figure 2a and b).

Based on the PCA the AD for the structural and physicochemical domain is clearly defined while the response space (Y-data) is more clearly defined by a histogram (Figure 3 a and b). By binning the nitroso compounds based on activity,

the normal distribution of the response (TD_{50}) data is visualized. Figure 3a shows the histogram of the TD_{50} for the lowest reported TD_{50} observed in literature (similar data as in Helguera et al. 2007) and Figure 3b, the harmonic mean of TD_{50} using the TD_{50} values from the most potent target site in each positive experiment. The distribution of the Y-data in the histogram should preferably be of a Gaussian distribution. In this case, the distribution is not optimal as the "low" carcinogenic potency data between ($-1 < -\text{Log } TD_{50} < -5$) is underrepresented. This underrepresentation of "low" carcinotoxicity data might give uncertainties in the validity of the QSAR model such as poor Q^2 (cross-validation), random correlations of physicochemical or structural properties with the Y-data and overprediction of the model (80% correlation coefficient when the reality is a 70 % correlation coefficient). One way to fix this problem is to find additional data with suitable structures which match the AD and mechanistic properties. The general suggestions is that the Y-data should span two or more orders of magnitude. For the nitroso compounds the bulk of the compounds is within 2 orders of magnitude.

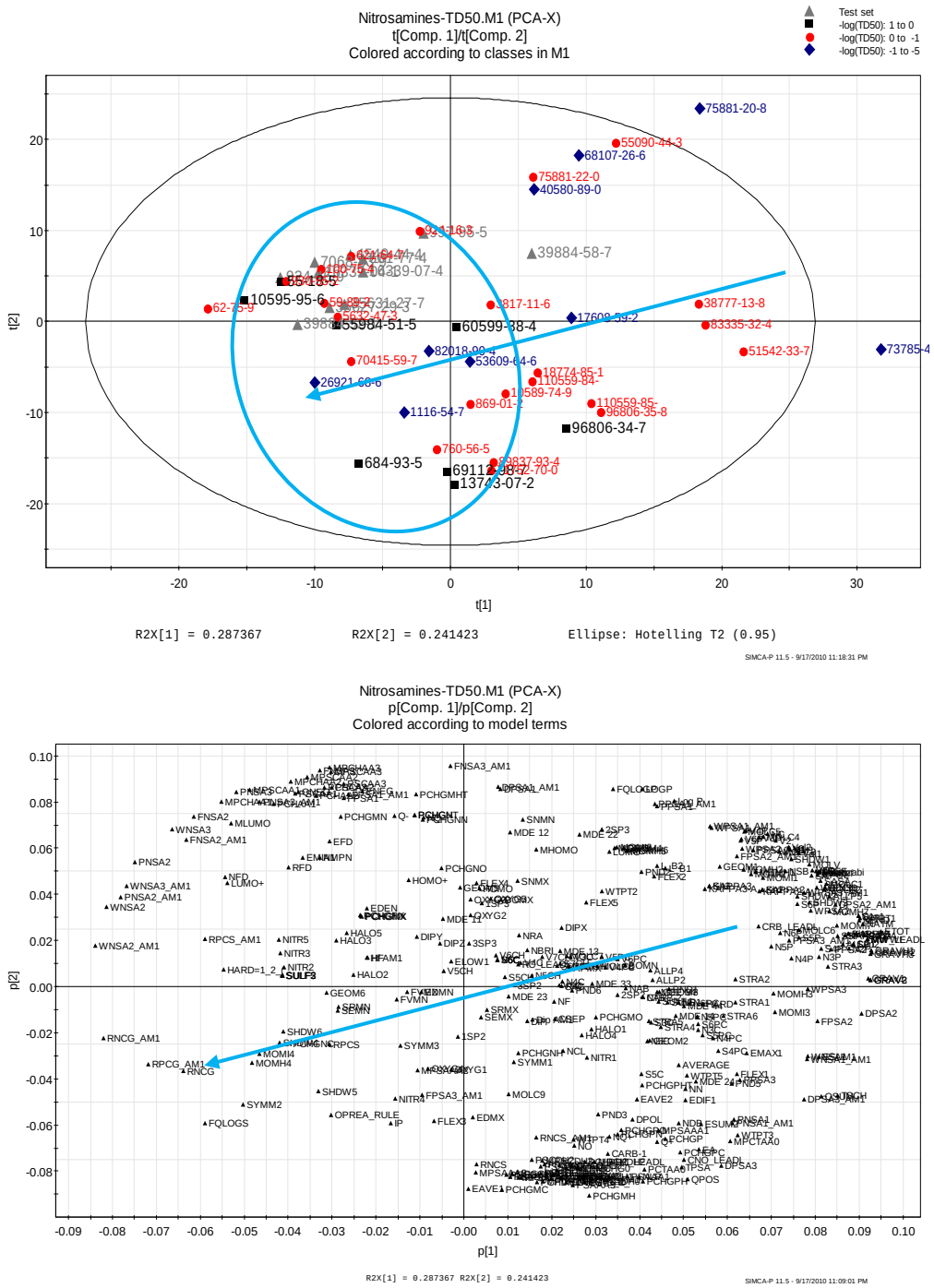


Figure 2: **Principal Component Analysis (PCA) plot: a) Scores scatter plot with the two first Principal Components, t[1] and t[2] describing 53% of the variation of the nitrosamines in the physicochemical and 2D/3D structural space. Two outliers identified, which are outside of the Hotelling T2 (95% confidence interval), b) Loadings plot containing the correlation structure of the X-space (physicochemical and 2D/3D variables). Comparison of both plots gives information which variables have most or less importance for the grouping of nitrosamines in the physicochemical/2D/3D domain.**

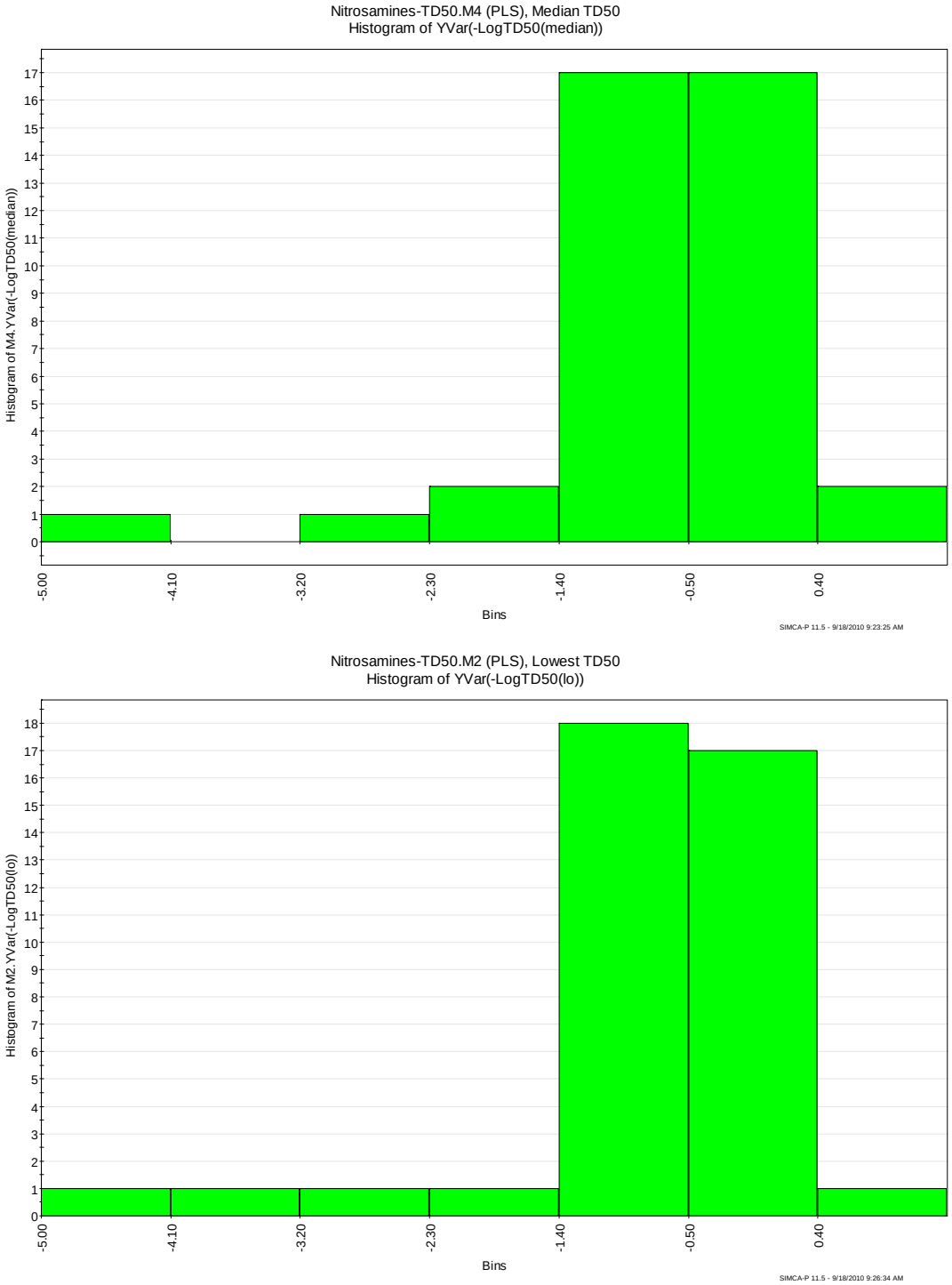


Figure 3: Histogram of the carcinogenic potency TD_{50} ($-Log_{10}(TD50)$): a) The lowest observed (reported in literature) TD_{50} of the nitrosamines, b) The harmonic mean using the TD_{50} values from the most potent target site in each positive experiment. The TD_{50} values are binned (x-axis) and the total numbers of binned nitrosamines are shown (Y-axis).

4.5.5 QSAR model based on 33 training and 7 test compounds

PCA analysis with Hotelling T2 revealed that the 40 compounds with the tested TD_{50} endpoints most probably were underrepresentated of "low" carcinotoxicity data, which might give uncertainties in the validity of the QSAR model. We have developed a QSAR model based on 33 training and 7 test compounds in accordance to the OECD validation requirements, but we recommend future model development for a larger group of nitrosamines and/or separated groups if various mode of action is a natural separation criteria. Due to possible outliers and the danger of not having a normally distributed data, further work needs to be performed to evaluate if the use of additional nitroso compound will strengthen the QSAR model. Some of the nitroso compounds have a slightly different mechanistic action such as between the groups nitrosoalkylamines and nitrosoureas (see figure 1) while compounds which are larger than the active site of the metabolically activating enzyme CYP 2E1 would indicate a different kinetic behaviour for a orally delivered compound where they may be transformed by the CYP 2A6 or is transformed/activated without the need metabolic activation. We have collected additional data of 80 other nitroso compounds which would need to be evaluated based on the AD, quality of data, and if they have an mechanistically similar function as the compounds under investigation. We suggests that the model below is a preliminary model that still needs to be refined further prior to regulatory use.

The test set of 7 compound constituted 20 % of the total set of 40 structures generated by a simple random splitting as part of an automatic procedure in the ADMEWORK ModelBuilder software.

Data pre-treatment included missing value test, zero test and correlation test (manually removes descriptors with correlation > 0.95) was performed prior to further QSAR development. The genetic algorithm was used to select the best parameter set for interactive Multiple Linear Regression (MLR) modelling. In all the models, as few descriptors as possible were aimed at to avoid overfitting; i.e. samples/parameters ratio equal or higher than 5.0 (Heimstad et al., 2009).

The figure below show the correlation between observed and predicted - $Log(TD_{50})$ values for the training (n=33) and the test set (n=7). Experimental and predicted values, for each chemical, are also given in Appendix E included predicted values for compounds lacking TD_{50} data.

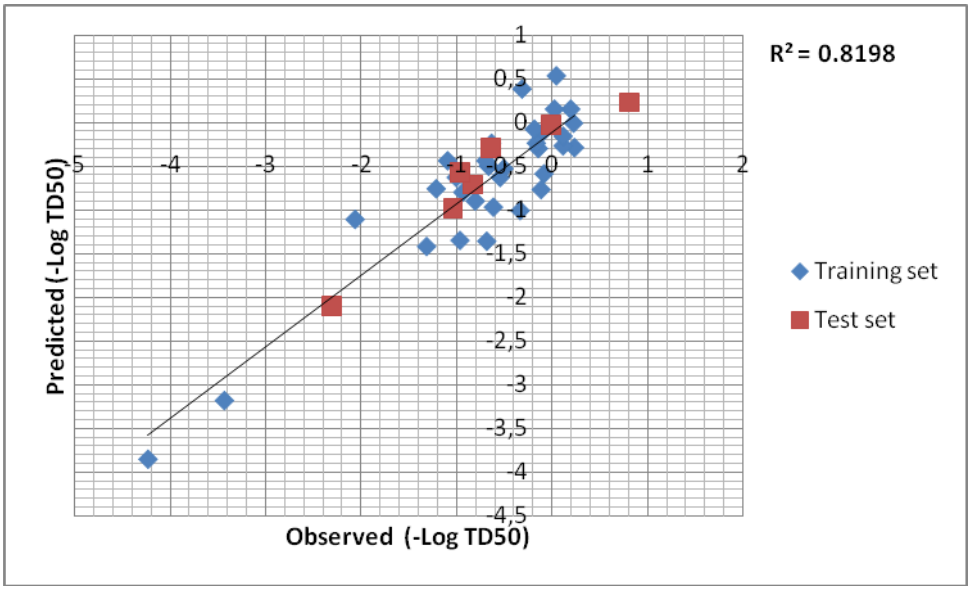


Figure 4: QSAR using Multiple Linear Regression. The training set which was used to train the QSAR is assigned blue diamonds while the test set (not used in model development) assigned red squares is used to verify the accuracy of the model.

The regression equation is given in the table below and the importance of descriptors to the regression is decreasing when going from left to right in the equation:

Training set: n=33
Test set: n=7 62-75-9, 924-16-3, 3817-11-6, 17608-59-2, 68107-26-6, 70415-59-7, 55984-51-5

Number of descriptors	Training set n=33 -Log(TD_{50})	R^2	Test set n=7 R^2	R2CV (Leave one out)
6	-0.685 +0.041**(OXYG2)** -0.244**(WTPT5)** +5.142**(FNSA1_AM1)** -3.339 **(GEOM3)** +0.910**(GEOM2)** +16.581**(HALO5)** Mean square error (MSE)= 2.09e-001 F-statistic = 19.70 p-value = 0.0000	0.819 Adj. 0.778	0.877 (MSE=0.128)	0.619

Explanation of the descriptors (and more detailed information is available in ADMEWORKS ModelBuilder documentation):

FNSA1-AM1:	Fractional negative charged partial surface area (MOPAC AM1)
OXYG2:	O:Total charge weighted atomic surface area of oxygen atoms
WTPT5:	Sum of atom IDs for nitrogen atoms
GEOM3:	Mass weighted Thickness
GEOM2:	Mass weighted Width

HALO5: X: Atomic charge weighted atomic surface area of halogen atoms/Total molecular surface area

GEOM2 and GEOM3 are 2D geometrical moment descriptors and WTPTF is a 2D weighted paths descriptor. Some 3D descriptors such as OXYG2 and HALO5 discriminate the various structures with the influence (and number) of oxygen and halogen atoms, respectively. FNSA1-AM1 is also a 3D descriptor based on semi-empirical quantum mechanics - MOPAC AM1.

Future studies with optimized QSAR model development with a larger set of structures, preferable comprising several low activity compounds, is recommended in order to explore if these descriptors are suitable for describing the carcinogenic nature of various nitrosamines. The 2D/3D descriptors in this equation point to general geometrical description of the nitrosamines, and may not necessarily explain the carcinogenic nature or the mode of action of the *N*-nitrosamines and/or their metabolites.

The Read Across and QSAR predicted endpoints of TD_{50} for untested compounds are given in Appendix D. There are relatively large discrepancies of the predicted endpoints for some of the compounds, reflecting differences between the read-across and the QSAR method, and the reason for this has not been evaluated at this stage.

5 Conclusion

Suggested Integrated testing strategy includes both validated OECD recommended methods as well as methods standardised and pre-validated by scientific consortia which are presently under validation by ECVAM, ICVAM or JaCVAM. Our approach takes into consideration 3Rs principle (reduction, replacement, refinement) with stress on alternative methods. We therefore recommend tier procedure using in vitro methods with consideration in vivo when necessary and in some cases taking case by case approach (see scheme, Figure 5).

Our ITS for all Amine 9 compounds but with specific stress on nitramines and nitrosamines include battery of assays with focus on use of alternative in vitro tests and combined endpoints *in vivo* tests (see scheme, figure 5). In this approach less *in vivo* tests are proposed using case by case approach and less animals will be used for testing. However, we strongly recommend verifying (validate) this approach experimentally with set of known mutagens and carcinogens and negative compounds as well as with set of Amine 9 compounds.

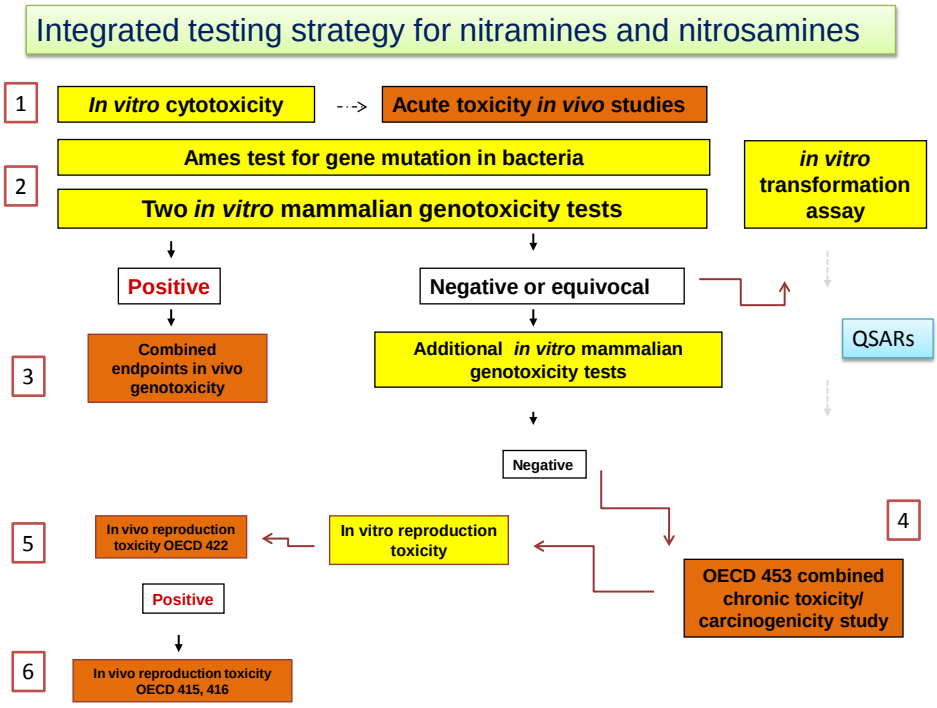


Figure 5: *Summary scheme of ITS for nitramines and nitrosamines*

Relevant test facilities and suppliers are given in Appendix G.

Conclusion of QSAR development

We recommend that Read Across and QSAR are viable methods for the prediction of carcinogenic potency and acute toxicity. We recommend that the currently developed Read Across method is further evaluated with different sub categorization (possible liver metabolism simulator) and input of specific variables such as the ionization potential, electrophilicity and hardness of the α-carbon of the molecules under study, as Read Across would benefit from finding a

linear correlation between structurally similar compounds with known TD_{50} or LD_{50} values. For QSAR modeling we suggest that a higher number of compounds are incorporated in future projects.

If there is proof enough that nitramines have a similar metabolic activation route as nitrosamines they might be possible to incorporate into a QSAR model based on nitrosamines, but as reports are contradictory further studies into the mechanism of nitramines for carcinogenic behavior is recommended since a common mechanism may be the most important factor for a scientific valid QSAR model (Veith, 2004). (Q)SAR methods using an expert system such as Toxtree (implemented in OECD QSAR Toolbox) could be used to classify carcinogenic from non-carcinogenic compounds with a fairly high accuracy. The methods used have a certain true positive rate such as 93.33% accuracy for chemicals with the structural alert "alkyl and aryl N-nitroso group". These results could be used as supportive evidence to limit (or even reduce testing time) the testing scheme. If such a method would be admissible for legislative purposes we do not know at this time.

In addition positive/negative outcomes of carcinogenicity/mutagenicity should be possible to predict with OECD QSAR toolbox through the use of Read-Across for nitrosamines and possible nitramines dependent on the available test data for similar compounds.

As stated in Principle 5 (REACH Annex XI), a (Q)SAR should be associated with a "mechanistic interpretation, wherever such an interpretation can be made. Clearly, it is not always possible to provide a mechanistic interpretation of a given (Q)SAR. The intent of this principle is therefore to ensure that there is an assessment of the mechanistic associations between the descriptors used in a model and the endpoint being predicted, and that any association is documented. The mechanistic basis of the model may be determined a priori (i.e. before modelling, by ensuring that the initial set of training structures and/or descriptors were selected to fit a pre-defined mechanism of action) or a posterior (i.e. after the modelling, by interpretation of the final set of training structures and/or descriptors).

The preliminary QSAR model developed for nitrosamines included at least two mode of actions for carcinogenicity and the final descriptors in the QSAR model do not indicate a mechanistic insight into the carcinogenic character of the substances. Most of the interaction of substances with bio-macromolecules are associated with size and shape structural parameters combined with electronic and/or molecular orbital energy parameters (Lewis et al, 1997; Heimstad et al., 2009).

Due to principle 5 and expert judgement, we recommend that this model is further refined or a new model is generated with larger/modified amount of compounds, selection of descriptors expected to be important for the mode of action, and/or for selected compounds with a specific common mode of action.

6 Recommendation for option

6.1 Recommendation for future ITS verification: The novelty of suggested ITS approach

The approach we used in suggested testing strategy for toxicity testing of nitramines and nitrosamines and other "Amine 9" compounds, focuses on the use of available alternative tests to animal testing. Our ITS includes both, fully validated OECD methods and several non-validated or partially validated but still not approved by OECD. This novel approach implements *in vitro* cytotoxicity tests as starting points for systemic toxicity. In first screening, *in vitro* comet assay as fast robust and easy method to perform is included into battery of *in vitro* assays. *In vitro* transformation assay is suggested early in the screening following *in vitro* assays for genotoxicity. Additionally we suggest to include *in vivo* comet assay to be performed on cells from several tissues (liver, lung, mammalian erythrocytes) in combination with micronucleus assay or chromosomal aberrations) and possibly other tests in one in vivo experiments. We did not implement toxicogenomics yet in our strategy but this approach seems to be very important and useful and should be further explored.

As these chemicals are likely to be metabolized a battery of assays with metabolic activation system (S 9 fractions) especially for genotoxicity is recommended. Many of these chemicals may also be phototoxic therefore we also suggest to include *in vitro* phototoxic assay but we strongly recommend to address also photogenotoxicity. The comet assay is considered in our strategy but it should be considered also for photogenotoxicity in near future as *in vitro* comet assay is fast, robust and has great potential to oxidized DNA lesions additionally to DNA breaks.

As this is novel approach with several non fully validated assays proposed, we strongly recommend to test its usefulness and appropriateness and to verify ('validate') several methods in this ITS against the set of positive and negative controls (known non-genotoxic, genotoxic compounds) as well as set of unknown "Amine 9" compounds.

Physical-chemical properties are integrated part of ITS but we did not have chance to fully explorate methods and suggest strategy for "Amine 9" compounds.

As in vivo tests are time consuming, our recommendations for near future work are:

- To explore testing strategy for physical-chemical properties, in situ characterisation of "Amine 9" compounds and their metabolites by exploring the existing approaches
- To further explore mechanisms of toxicity of all "Amine 9" compounds with focus also on other possible type of toxicity and their endpoints (immunotoxicity, neurotoxicity etc.) and suggest alternative testing strategies for them.
- To further explore toxicogenomics approach and its implementation to testing strategies

For experimental work we suggest:

- To test 6-9 “Amine 9” compounds for cytotoxicity (to estimate LD50, LC50) and phototoxicity using OECD validated NRU method and compare with Clonogenic and cell proliferation assays as well as to compare results with existing in vivo data. In case data are not available we propose further testing using OECD 420 for acute toxicity and compare with outcome of QSAR
- We also suggest in pilot study to perform 2 in vitro genotoxicity assays (OECD one cytogenetic assay either OECD 473 or 487 and point mutation OECD 479) with the comet assay using positive (preferably from Amine 9 group), negative genotoxic compounds with several unknown “Amine 9” compounds. We suggest set 3 genotoxic, 3 nongenotoxic and 6-9 “Ammine 9” unknown compounds.
- We suggest to test 6-9 “Amine 9” compounds for photogenotoxicity
- We suggest to test 6-9 “Amine 9” compounds for in vitro carcinogenicity with 1-3 positive (preferably from “Amine 9” group) and negative controls.

6.2 Recommendation for future QSAR work

It is concluded that the developed QSAR model for TD_{50} endpoints of nitrosamines is a preliminary model. We recommend further work to evaluate if a more mechanistic interpretable model is possible to make, either through the selection of specific descriptors or by choosing substances where more or less evidence is present for a common mode of action. At this stage, without proper evaluation of this preliminary model, we would not recommend to use this model. As for using this type of QSAR for regulatory purposes we would only suggest to use it as a weight of evidence in association with historical data and/or other relevant tests. In the case of nitrosamines the weight of evidence of structurally similar compounds might be enough in combination with a QSAR and Read-Across.

If the mode of action for carcinogenicity of nitramines can be expected to be similar to the mechanism of nitrosamines, it would be feasible to include nitramines together with nitrosamines when developing a QSAR model for TD_{50}. The few nitramine compounds with experimental data (i.e. TD_{50}) can be added to the nitrosamine compounds as long as they are part of the physical-chemical property domain (AD) and that there is a similar mechanistic interpretation of the toxicokinetics of these compounds as for the nitrosamines.

Model development for LD_{50} for nitrosamines (and nitramines) should also be possible, although the mechanistic understanding is not clear in this case. We can probably assume a similar metabolic activation process and there exists a correlation between TD_{50} and LD_{50} data which may strengthen the development of the model.

(Q)SAR methods using an expert system such as Toxtree (implemented in the OECD (Q)SAR Toolbox) could be used to classify carcinogenic from non-carcinogenic compounds with a fairly high accuracy. The methods used have a certain true positive rate such as 93.33% accuracy for chemicals with the structural alert alkyl and aryl N-nitroso group. These results could be used as supportive evidence to limit (or even reduce testing time) the testing scheme. If such a method would be admissible for legislative purposes we do not know at this time.

Traditionally, the use of QSAR models for regulatory purposes has been conservative and at present the data generated by QSAR models is most often used to supplement experimental test data within weight-of-evidence approaches, including chemical categories and endpoint-specific integrated testing strategies (ITS). In the future it is however expected that (Q)SARs will be used increasingly for the direct replacement of test data, as relevant and reliable models become more available (Worth, 2009, Veith, 2004). It is further stated in Annex XI,1.3, “The Agency in collaboration with the Commission, Member States and interested parties shall develop and provide guidance in assessing which (Q)SARs will meet these conditions and provide examples.”

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Appendix A

List of substances

Nitrosamines			
CAS registry	**NAME (IUPAC)**	**SMILES**	**Code**
62-75-9	N-nitrosodimethylamine (NDMA)	CN(N=O)C	1
55-18-5	N-ethyl-N-nitroso-ethanamine (NDEA)	CCN(CC)N=O	1
10595-95-6	N-methyl-n-nitrosoethanamine	CN(CC)N=O	1
924-46-9	N-methyl-n-nitroso-1-propanamine	CCCN(N=O)C	3
621-64-7	N-nitroso-n-propyl-propanamine	O=NN(CCC)CCC	3
601-77-4	N-(1-methylethyl)-N-nitroso-2-propanamine	CC(C)N(N=O)C(C)C	2
924-16-3	N-butyl-n-nitroso-1-butanamine	O=NN(CCCC)CCCC	3
997-95-5	Bisisobutyl-N-nitrosamine	CC(C)CN(N=O)CC(C)C	3
16339-04-1	N-ethyl-n-nitroso2-propamamine	CCN(N=O)C(C)C	3
7068-83-9	N-methyl-n-nitroso1-butanamine	CCCCN(N=O)C	3
4549-44-4	N-ethyl-n-nitroso-1-butanamine	CCCCN(N=O)CC	3
5632-47-3	1-nitrosopiperazine	O=NN1CCNCC1	1
16339-07-4	1-methyl-4-nitroso-piperazine	O=NN1CCN(C)CC1	2
59-89-2	4-nitrosomorpholine	O=NN1CCOCC1	1
930-55-2	1-nitroso-pyrrolidine	O=NN(CCC1)C1	2
100-75-4	1-nitroso-piperidine	O=NN(CCCC1)C1	2
1116-54-7	N-Nitrosodiethanolamine (NDELA)	O=NN(CCO)CCO	1
26921-68-6	N-Nitrosomethyl-(2-hydroxyethyl)amine	N(CCO)(N=O)C	2
3817-11-6	4-(butylnitrosamino)-1-butanol	CCCCN(CCCCO)N=O	3
39884-52-1	N-Nitroso-1,3-oxazolidine	O1CN(CC1)N=O	2
35627-29-3	3-nitroso-1,3-oxazinane	N1(N=O)COCCC1	2
35631-27-7	5-methyl-3-nitroso-1,3-oxazolidine	CC1CN(N=O)CO1	2
39884-58-7	4,4-dimethyl-3-nitroso-2-(propan-2-yl)-1,3-oxazolidine	O=NN1C(OCC1(C)C)C(C)C	2
Nitramines			
CAS registry	**Name (IUPAC)**	**SMILES**	**Code**
4164-28-7	N-methyl-N-nitro- Methanamine	CN(C)N(=O)=O	1
7119-92-8	N-ethyl-N-nitro-ethanamine	O=N(=O)N(CC)CC	2
4164-29-8	Di-n-propylnitramine	O=N(=O)N(CCC)CCC	3
108249-27-0	N,N-bis(hydroxymethyl)nitramide	[O-][N+](=O)N(CO)CO	3
13084-48-5	2,2'-(nitroimino)diethanol	O=[N+]([O-])N(CCO)CCO	1
4164-32-3	N-Nitromorpholine	N(=O)(=O)N1CCOCC1	1
42499-41-2	1-nitropiperazine	[O-][N+](=O)N1CCNCC1	1
598-57-2	N-nitromethanamine	[O-][N+](=O)NC	1
19091-98-6	N-nitroethanamine	[O-][N+](=O)NCC	2
627-07-6	N-nitropropan-1-amine	[O-][N+](=O)NCCC	3
74386-82-6	2-(nitroamino)ethanol	[O-][N+](=O)NCCO	1
?	2-methyl-2-(nitroamino)propan-1-ol	[O-][N+](=O)NC(C)(C)CO	1
51883-27-3	N-nitroformamide	[O-][N+](=O)NC=O	2
32818-80-7	Metylnitroamino-methanol	[O-][N+](=O)N(C)CO	2
42499-46-7	2-(methylnitroamino)-ethanol	[O-][N+](=O)N(C)CCO	1

The compounds have been selected by company based on an evaluation of what nitrosamines and nitramines could be formed from the amines MEA, AMP, MDEA and piperazine.
Coding is based on the likely transformation products.
Codes:
1 – likely, 2 – possible, 3 – structurally similar to compounds that are likely or possible

Appendix B

List of useful databases and (Q)SAR related tools and software

Databases

Toxnet - Databases on toxicology, hazardous chemicals, environmental health, and toxic releases
http://toxnet.nlm.nih.gov/

The Carcinogenic Potency Database (CPDB)
http://potency.berkeley.edu/cpdb.html

The Global Portal to Information on Chemical Substances (eChemPortal)
http://webnet3.oecd.org/eChemPortal/Home.aspx
several databases in one

DSSTox Distributed Structure-Searchable Toxicity (DSSTox) Database Network
http://www.epa.gov/ncct/dsstox/index.html

OECD (Q)SAR Application Toolbox
(Tested and estimated endpoints included in the software tool)
http://www.oecd.org/document/54/0,3343,en_2649_34379_42923638_1_1_1_1,00.html

OSHA
http://www.osha.gov/
http://www.osha.gov/dts/chemicalsampling/toc/toc_chemsamp.html

Haz-Map (Information on Hazardous Chemicals and Occupational Diseases)
http://hazmap.nlm.nih.gov/index.html

ESIS (European chemical Substances Information)
http://ecb.jrc.ec.europa.eu/esis/

Danish (Q)SAR Database
http://130.226.165.14/index.html

Online chemical database with modelling environment
http://ochem.eu/

Chemspider
http://www.chemspider.com/RecordView.aspx?rid=280efd6b-dc30-4e5d-bc64-404ff3ab4f20

NHL ChemIdPlus Advanced (interface to Marvin) Physical & Toxicity data
http://chem.sis.nlm.nih.gov/chemidplus/

Look for chemicals
http://www.lookchem.com

SRC Syracuse university, PHYSPROP
http://www.syrres.com/what-we-do/product.aspx?id=133

http://www.syrres.com/what-we-do/databaseforms.aspx?id=386

(Q)SAR related software

The OECD (Q)SAR Application Toolbox (freeware)
http://www.oecd.org/env/existingchemicals/qsar
http://www.oecd.org/document/54/0,3343,en_2649_34379_42923638_1_1_1_1,00.html

Toxtree, European Chemicals Bureau (ECB) (freeware)
http://ecb.jrc.it/qsar/qsar-tools/index.php?c=TOXtrEE

DART ((Decision Analysis by Ranking Techniques) (freeware)
http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=DART

Web based software
http://esc.syrres.com/scripts/CASTScgi.exe
http://esc.syrres.com/scripts/CASTScgi.exe?CASNUM=1116-54-7

A Computer System to Evaluate the Carcinogenic Potential of Chemicals
http://www.epa.gov/oppt/sf/pubs/oncologic.htm

Java web application that allows the access to all the toxicity predictive models developed within the CAESAR Project.
http://www.caesar-project.eu/software/

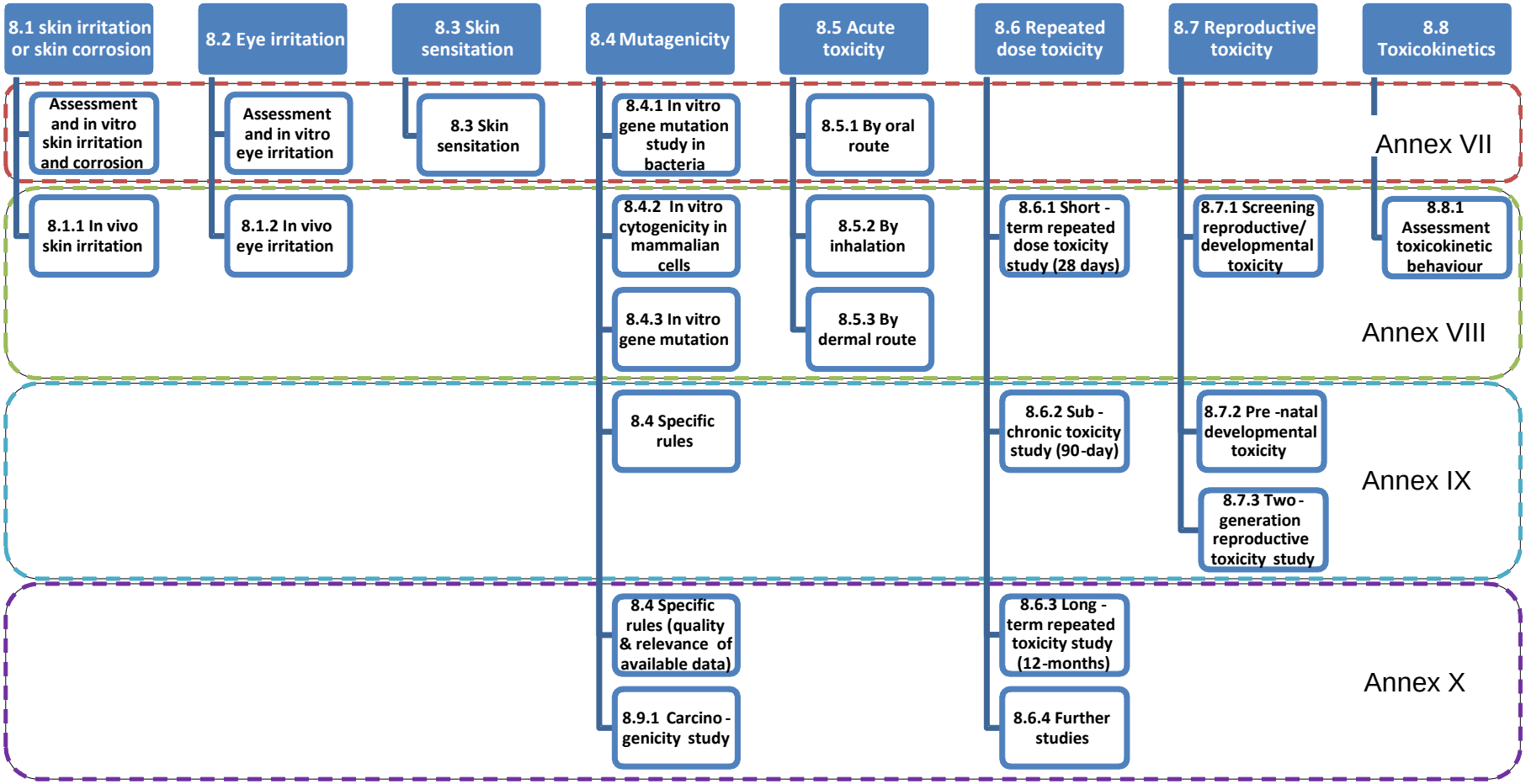
Google search: software read across ranking (Q)SAR
Overview:
http://www.scarlet-project.eu/results_SWs.html

Estimation Program Interface (EPI) Suite, EPI Suite v. 4.0 (Environmental fate)
http://www.epa.gov/oppt/exposure/pubs/episuite.htm

Marvin sketch web application (Physchem properties)
http://www.chemaxon.com/marvin/sketch/index.php

Appendix C

Standard toxicity information requirements for substances manufactured or imported in quantities of 1 through 1000 tonnes, according to annex VII-X in Regulation (EC) No 1907/2006

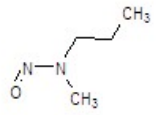
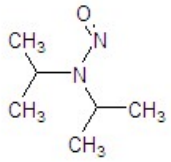
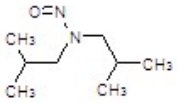
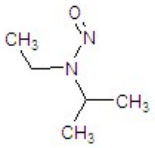
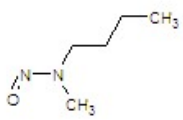

8.1 skin irritation or skin corrosion
8.2 Eye irritation
8.3 Skin sensitation
8.4 Mutagenicity
8.5 Acute toxicity
8.6 Repeated dose toxicity
8.7 Reproductive toxicity
8.8 Toxicokinetics
Assessment and in vitro skin irritation and corrosion
Assessment and in vitro eye irritation
8.3 Skin sensitation
8.4.1 In vitro gene mutation study in bacteria
8.5.1 By oral route
Annex VII
8.1.1 In vivo skin irritation
8.1.2 In vivo eye irritation
8.4.2 In vitro cytogenicity in mammalian cells
8.5.2 By inhalation
8.6.1 Short - term repeated dose toxicity study (28 days)
8.7.1 Screening reproductive/ developmental toxicity
8.8.1 Assessment toxicokinetic behaviour
8.4.3 In vitro gene mutation
8.5.3 By dermal route
Annex VIII
8.4 Specific rules
8.6.2 Sub - chronic toxicity study (90-day)
8.7.2 Pre -natal developmental toxicity
Annex IX
8.7.3 Two - generation reproductive toxicity study
8.4 Specific rules (quality & relevance of available data)
8.6.3 Long - term repeated toxicity study (12-months)
Annex X
8.9.1 Carcino - genicity study
8.6.4 Further studies

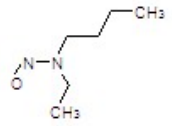
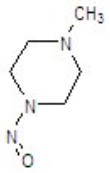
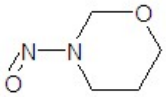
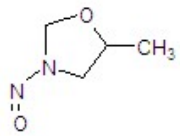
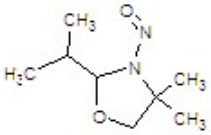
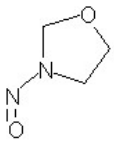
Appendix D

Read-across prediction of TD_{50} and LD_{50} endpoints compared to QSAR prediction of TD_{50}

Predicted values of TD_{50} (mg/kg body wt/day)

READ ACROSS prediction of TD_{50} with the use of OECD (Q)SAR Toolbox software
Database: ISSCAN Update 3
Category: Alkyl and aryl N-nitroso groups (Benigni/Bossa rulebase) + Subcategorized: Structure
Threshold=80%; Dice(Atom pairs;Topologic torsions;Atom centered fragments;Path;Cycles)

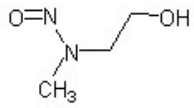
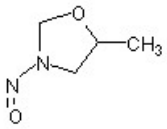
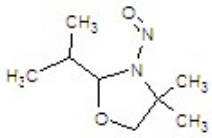
Cas.no.	2D structure	READ ACROSS TD_{50} mg/kg body wt/day	QSAR equation TD_{50} mg/kg body wt/day
924-46-9		1.14	0.18
601-77-4		2.96	11.42
997-95-5		0.204	5.25
16339-04-1		0.675	4.24
7068-83-9		2.29	0.57

4549-44-4		1.26	2.95
16339-07-4		0.640	5.82
35627-29-3		3.02	0.36
35631-27-7		2.33	0.70
39884-58-7		0.221	3.54
39884-52-1		0.46	0.21

READ-ACROSS prediction of LD_{50}

Databases: Own database of 20 structures with experimental values imported

Category: Similarity: Threshold=50%; Dice(Atom pairs;Topologic torsions;Atom centered fragments;Path;Cycles)

Cas.no.	2D structure	Predicted LD_{50} mg/kg
26921-68-6		1550
35631-27-7		444
39884-58-7		1930

Appendix E
QSAR model development and prediction of TD_{50} for non-tested compounds

-Log**(TD_{50})=**
-0.685 +0.041**(OXYG2)** -0.244**(WTPT5)** +5.142**(FNSA1_AM1)** -3.339 **(GEOM3)**
+0.910**(GEOM2)**+16.581**(HALO5)**

Project substances are marked in bold

		(µmol/kg body wt/day)	Experimental	Predicted
No	Cas.no	Experimental TD_{50}	-LogTD_{50}	-LogTD_{50}
	Training set			
2	**10595-95-6**	**0.57**	**0.24**	**-0.29**
3	13743-07-2	0.58	0.23	-0.01
4	60599-38-4	0.63	0.2	0.15
5	**55-18-5**	**0.76**	**0.12**	**-0.27**
6	96806-34-7	0.77	0.12	-0.16
7	684-93-5	0.9	0.05	0.53
8	69112-98-7	0.93	0.03	0.15
10	**59-89-2**	**1.21**	**-0.08**	**-0.59**
11	869-01-2	1.29	-0.11	-0.13
12	110559-84-7	1.3	-0.11	-0.77
13	83335-32-4	1.36	-0.14	-0.30
14	**621-64-7**	**1.43**	**-0.16**	**-0.24**
15	38777-13-8	1.53	-0.18	-0.08
16	760-56-5	2.06	-0.31	0.38
17	55090-44-3	2.13	-0.33	-1.01
18	89837-93-4	3.14	-0.5	-0.53
19	10589-74-9	3.43	-0.54	-0.63
20	96806-35-8	4.11	-0.61	-0.97
21	18774-85-1	4.3	-0.63	-0.24
23	71752-70-0	4.54	-0.66	-0.50
24	110559-85-8	4.58	-0.66	-0.50
25	51542-33-7	4.78	-0.68	-1.36
26	**930-55-2**	**4.88**	**-0.69**	**-0.44**
27	75881-22-0	6.29	-0.8	-0.90
29	**100-75-4**	**8.44**	**-0.93**	**-0.80**
30	**5632-47-3**	**9.21**	**-0.96**	**-1.35**
32	40580-89-0	10.13	-1	-0.63
34	**26921-68-6**	**12.39**	**-1.09**	**-0.44**
35	82018-90-4	16.14	-1.21	-0.76
36	**1116-54-7**	**20.65**	**-1.31**	**-1.42**
37	75881-20-8	114.62	-2.06	-1.11
39	53609-64-6	2694.4	-3.43	-3.18
40	73785-40-7	17132.17	-4.23	-3.85

	Test set			
1	55984-51-5	0.15	0.81	0.23
9	**62-75-9**	**1.03**	**-0.01**	**-0.03**
22	**924-16-3**	**4.37**	**-0.64**	**-0.29**
28	**3817-11-6**	**6.71**	**-0.83**	**-0.71**
38	17608-59-2	205.94	-2.31	-2.10
33	68107-26-6	11.06	-1.04	-0.98
31	70415-59-7	9.23	-0.96	-0.57
	Prediction set			
41	**39884-52-1**	**ND**	**ND**	**-0.32**
42	**924-46-9**	**ND**	**ND**	**-0.25**
43	**601-77-4**	**ND**	**ND**	**-1.94**
44	**997-95-5**	**ND**	**ND**	**-1.52**
45	**16339-04-1**	**ND**	**ND**	**-1.56**
46	**7068-83-9**	**ND**	**ND**	**-0.69**
47	**4549-44-4**	**ND**	**ND**	**-1.36**
48	**16339-07-4**	**ND**	**ND**	**-1.65**
49	**35627-29-3**	**ND**	**ND**	**-0.49**
50	**35631-27-7**	**ND**	**ND**	**-0.78**
51	**39884-58-7**	**ND**	**ND**	**-1.31**

Appendix F
Method description forms

TEST EVALUTION FORMS FOR ACUTE TOXICITY

TEST METHOD EVALUATION – OECD Guidance Document 129

ENDPOINT: Acute toxicity
TEST METHOD NAME: Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests
ENPOINT PARAMETER: Estimation of in vivo starting doses for acute toxicity
REFERENCE: ENV/JM/MONO(2010)20
VALIDATION STATUS: Unclassified
IN VIVO / IN VITRO TEST METHOD: In vitro
EXPOSURE ROUTE(S):
ANIMAL / CELL CULTURE INFORMATION:
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION:
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: The NRU assay is performed in a dose-response format to determine the concentration that reduces NRU by 50% compared to the controls (i.e. the IC_{50}). The IC_{50} value is used in a linear regression equation to estimate the oral LD_{50} value (dose that produces lethality in 50% of the animals tested), which is then used to determine a starting dose for acute oral systemic toxicity testing, using rats for the UDP, the ATC method, or FDP. The use of the NRU test method in a weight-of-evidence approach to determine starting doses for these acute oral systemic toxicity tests might reduce the number of animals required for the tests, and for relatively toxic substances, might reduce the number of animals that die or require humane euthanasia due to severe toxicity. For estimating starting doses, *in vitro* data should be considered along with all other data and information such as quantitative structure-activity relationship (QSAR) predictions, the LD_{50} of related substances, and other existing data to estimate a dose that is likely to be close to the actual LD_{50} value.
BRIEF TEST DESCRIPTION: The initial cytotoxicity test is performed to determine the starting doses for the main test.

The
NRU assays test eight concentrations of the test substance or the positive control by diluting the stock test substance solution in log dilutions to cover a large concentration range.
The main test of the cytotoxicity assays is performed to determine the IC_{50} value. The concentration closest to the range finder test IC_{50} value serves as the midpoint of the concentrations tested in the main test. Compared to the range finder test, the main test uses a smaller dilution factor for the concentrations tested.

SPECIFIC PRECAUTIONS:

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:

This form has been edited by:

Name	Organization	Date
Evy Sivesind	NILU	October-2010

TEST METHOD EVALUATION – ECVAM validation study: Method for discriminating toxic from non-toxic substances

ENDPOINT: Acute oral systemic toxicity / cytotoxicity
TEST METHOD NAME: The follow-up validation study of the BALB/3T3 neutral red uptake assay. Method for discriminating toxic from non-toxic substances
ENPOINT PARAMETER:
REFERENCE: ECVAM's ongoing activities in the area of acute oral toxicity
VALIDATION STATUS: ECVAM follow up validation study completed (2009) (BRD in preparation)
IN VIVO / IN VITRO TEST METHOD: In vitro
EXPOSURE ROUTE(S):
ANIMAL / CELL CULTURE INFORMATION:
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION:
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: Compounds which have an LD50 > 2000 mg/kg (i.e. in the EU are not classified as acutely toxic via the oral route) and will be identified using information from 28-days repeated dose toxicity studies.
BRIEF TEST DESCRIPTION: The evaluation was based on data retrieved from dossiers from the New Chemicals Database. Substances for which both the NOAEL values obtained from 28-days repeated dose studies in rats and the oral LD50 values were available in the submission files (1791 in total), were selected and included in the analysis. The substances were then grouped according to the GHS categories and from the obtained distribution the NOAELP 200 mg/kg was identified as the best threshold that could discriminate ''nontoxic" substances (LD50 > 2000 mg/kg b.w.) from the rest. This threshold allowed to correctly categorize 63% of the ''non-toxic" compounds. Less than 1% of compounds were misclassified as ''non-toxic" and all of them were falling in the harmful category (category 4). None of the toxic or very toxic substances were misclassified as ''non-toxic". The Negative Predictive Value (NPV) and the Positive Predictive Value (PPV) of this approach were 97% and 26.5%, respectively (for details of the analysis see Bulgheroni et al., 2009).
SPECIFIC PRECAUTIONS:

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:

This form has been edited by:

Name	Organization	Date
Evy Sivesind	NILU	October-2010

TEST METHOD EVALUATION-OECD 425

ENDPOINT: Acute oral toxicity
TEST METHOD NAME: Up and Down Procedure (UDP)
ENPOINT PARAMETER: Signs of toxicity and Mortality
REFERENCE: OECD 425
VALIDATION STATUS: YES OECD
IN VIVO / IN VITRO TEST METHOD: In Vivo
EXPOSURE ROUTE(S): Oral (Single dose by gavage). If single dose is not possible: smaller fractions within 24 hours.
ANIMAL / CELL CULTURE INFORMATION: Female Rats (8 and 12 weeks old)
NO. ANIMAL GENERATIONS TESTED: NA
TEST DURATION: 48 hrs to 14 days
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.): Weighing balance for weight measurement, Microscope for gross pathology
TEST PRINCIPLES: Acute oral toxicity refers to adverse effects occurring following oral administration of a single dose of a substance, or multiple doses given within 24 hours.
BRIEF TEST DESCRIPTION: The test is divided into two parts: a) Limit Test: It is a sequential test with 5 animals; performed when the test material is likely to be non toxic. Dose one animal at the test dose (2000/5000 mg/Kg). Animal Dies: Conduct the main test to determine the LD50. Animal Survives: Dose four additional animals sequentially so that a total of five animals are tested. (O=survival, X=death). If 3 animals X, limit test – terminated; main test - performed. The LD50 ≥ 2000/5000 mg/kg if ≥ 3 animal survival (OOOOO; OOOXO; OOOOX; OOOXX; OXOXO; OXOOO; OOXXO; OOXOO; OXXOO The LD50 is ≤ 2000/5000 mg/kg if ≥ 3 animals die (OXOXX; OOXXX; OXXOX; OXXX) b) Main Test: For each run, animals are dosed in a single ordered dose progression, one at a time, at 48 hrs. interval. The first animal is dosed a step below the level of the best estimate of LD50. Upon survival/death: Dose for the next animal is increased/decreased by a progression factor of 3.2 times the original dose. The dose progression factor is the antilog of 1/(the estimated slope of the dose-response curve) (for e.g. a progression of 3.2 corresponds to a slope of 2). If no information on the slope of the substance to be tested, a dose progression factor of 3.2 is used which gives a dose sequence of 1.75, 5.5, 17.5, 55, 175, 550, 2000 (or 1.75, 5.5, 17.5, 55, 175, 550, 1750, 5000 for

specific regulatory needs). If no estimate of the substance's lethality is available, dosing should be initiated at 175 mg/kg.
The main test is stopped when one of the stopping criteria is met.

a) 3 consecutive animals survive at the upper bound;
b) 5 reversals occur in any 6 consecutive animals tested;
c) at least 4 animals have followed the first reversal and the specified likelihood-ratios exceed the critical value.

Results of the main test procedure serve as the starting point for a computational procedure for calculation of LD50 and a confidence interval.

Interval between treatment groups: Usually at 48 hr interval or determined by the onset, duration, and severity of toxic signs.

Observation: At least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days.

SPECIFIC PRECAUTIONS: Maximum dose volume for administration: 1mL/100g of body weight or 2 mL/100g body weight for aqueous solutions.
Fasting before dose administration: Rats-overnight; Mice- 3-4 hrs
Fasting after dose administration: Rats- 3-4 hrs; Mice- 1-2 hrs

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION: Mortality or moribund animals; nature, severity and duration of toxic effects (such as changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma).

SUITABILITY EXPOSURE OF AIR SAMPLES: No

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMP[ ] WATER SA[ ]S:

COMMENTS:

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria Dusinska	NILU	August-2010

TEST METHOD EVALUATION – OECD 432

ENDPOINT: Acute toxicity
TEST METHOD NAME: 3T3 NRU phototoxicity test
ENPOINT PARAMETER: Identify the phototoxic potential of a test substance induced by the excited chemical after exposure to light
REFERENCE: OECD TG 432
VALIDATION STATUS: OECD validated
IN VIVO / IN VITRO TEST METHOD: In vitro
EXPOSURE ROUTE(S):
ANIMAL / CELL CULTURE INFORMATION:
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION:
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: The 3T3 NRU phototoxicity test is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of simulated solar light. Cytotoxicity in this test is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red when measured 24 hours after treatment with the test chemical and irradiation NR is a weak cationic dye that readily penetrates cell membranes by non-diffusion, accumulating intracellularly in lysosomes. Alterations of the cell surface of the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged or dead cells, which is the basis of this test.
BRIEF TEST DESCRIPTION: Balb/c 3T3 cells are maintained in culture for 24 h for formation of monolayers. Two 96-well plates are pre-incubated with eight different concentrations of the test substance for 1 h. Thereafter one of the two plates is exposed to the highest non-cytotoxic irradiation dose whereas the other plate is kept in the dark. Cytotoxicity in this test is expressed as a concentration-dependent reduction of the uptake of the Vital dye Neutral Red (NR) when measured 24 hours after treatment with the test chemical and irradiation. NR penetrates cell membranes by non-diffusion, accumulating in lysosomes. Alterations of the cell surface of the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes result in a decreased uptake

and binding of NR. It is thus possible to distinguish between viable, damaged or dead cells. To predict the phototoxic potential, the concentration responses obtained in the presence and in the absence of irradiation are compared, usually at the IC50 level, i.e., the concentration reducing cell viability to 50 % compared to the untreated controls.

SPECIFIC PRECAUTIONS: It is important that UV sensitivity of the cells is checked regularly and it is also important that cell culture conditions assure a cell cycle time within the normal historical range of the cells or cell line used.

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:

This form has been edited by:

Name	Organization	Date
Evy Sivesind	NILU	October-2010

TEST METHOD EVALUATION – OECD 436

ENDPOINT: Acute toxicity
TEST METHOD NAME: Acute Inhalation Toxicity – Acute Toxic Class Method
ENPOINT PARAMETER: hazard assessment for short-term exposure to a test article by inhalation
REFERENCE: OECD TG 436
VALIDATION STATUS: OECD validated
IN VIVO / IN VITRO TEST METHOD: In vivo
EXPOSURE ROUTE(S): Inhalation
ANIMAL / CELL CULTURE INFORMATION:
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION:
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: The test is based on a stepwise procedure; sufficient information is obtained on the acute inhalation toxicity of the test article during an exposure period of 4 hours to enable its classification. Other durations of exposure may apply to serve specific regulatory purposes. At any of the defined concentration steps, 3 animals of each sex are tested. Depending on the mortality and/or the moribund status of the animals, 2 steps may be sufficient to allow judgment on the acute toxicity of the test article. If evidence is provided that one sex is more susceptible than the other, then the test may be continued with the more susceptible sex only. The outcome of the previous step will determine the following step such that: a) No further testing is needed, b) Testing of three animals per sex, or c) Testing with 6 animals of the more susceptible sex only *i.e.* the lower boundary estimates of the toxic class should be based on 6 animals per test concentration group, regardless of sex.
BRIEF TEST DESCRIPTION: The test method is based on a stepwise procedure, each step using 3 animals of each sex (the preferred species is rat). Animals are exposed in inhalation chambers to a pre-defined concentration for 4 hours. Absence or presence of compound-related mortality of the animals at one step will determine the next step. The starting concentration is selected from one of four fixed levels corresponding to GHS categories 1-4 for gases, vapors or aerosols. Animals are observed daily for clinical signs of toxicity for a total of at least 14 days. Animals' body weights should be determined at least weekly. All the animals

should be subjected to gross necropsy.

SPECIFIC PRECAUTIONS: Animals in severe pain or distress should be humanely killed.

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:

This form has been edited by:

Name	Organization	Date
Evy Sivesind	NILU	October-2010

TEST EVALUTION FORMS FOR MUTAGENICITY /GENOTOXIXITY TESTING

TEST METHOD EVALUATION – OECD 471

ENDPOINT: Mutagenicity/Genotoxicity
TEST METHOD NAME: Bacterial Reverse Mutation Test
ENPOINT PARAMETER: The number of revertant colonies on both negative and positive control plates is the principal endpoint (prokaryote assay, reverse gene mutation by Ames test. Histidine forward and reverse gene mutation
REFERENCE: OECD TG 471
VALIDATION STATUS: OECD validated
IN VIVO / IN VITRO TEST METHOD: In vitro
EXPOSURE ROUTE(S): An appropriate minimal agar (e.g. containing Vogel-Bonner minimal medium E and glucose) and an overlay agar containing histidine and biotin or tryptophan, to allow for a few cell divisions, is used.
ANIMAL / CELL CULTURE INFORMATION: Salmonella typhimurium: TA98, TA100, TA1535, TA1537, or TA1538. Host-mediated assay
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION: All plates in a given assay should be incubated at 37°C for 48-72 hours. After the incubation period, the number of revertant colonies per plate is counted.
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. The suspensions are mixed with an overlay agar and plated immediately onto minimal medium. The treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium. For both techniques, after two or three days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.
BRIEF TEST DESCRIPTION: Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. In the preincubation method, the

treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium. For both techniques, after two or three days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

SPECIFIC PRECAUTIONS: It is recognized that certain classes of mutagens are not always detected using standard procedures such as the plate incorporation method or preincubation method. These should be regarded as "special cases" and it is strongly recommended that alternative procedures should be used for their detection. The following "special cases" could be identified (together with examples of procedures that could be used for their detection): azo-dyes and diazo compounds , gases and volatile chemicals, and glycosides. A deviation from the standard procedure needs to be scientifically justified.

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:
Compound tested:
Nitrosodimethylamine, CAS 62-75-9
N-Nitrosomorpholine, CAS 59-89-2
Dimethylnitramine, CAS 4164-28-7
Nitrosodiethanolamine, CAS 1116-54-7
Result: Positive

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria Dusinska	NILU	August-2010

TEST METHOD EVALUATION – OECD 473

ENDPOINT: Mutagenicity/Genotoxicity
TEST METHOD NAME: In vitro Mammalian Chromosome Aberration Test
ENPOINT PARAMETER: The percentage of cells with structural chromosome aberration(s) is the principal endpoint. Concurrent measures of cytotoxicity for all treated and negative control cultures in the main aberration experiment(s) are also endpoints in this project. Individual culture data is provided.
REFERENCE: OECD TG 473
VALIDATION STATUS: OECD validated
IN VIVO / IN VITRO TEST METHOD: In vitro
EXPOSURE ROUTE(S): Cell cultures are exposed to the test substance (liquid or solid) both with and without metabolic activation.
ANIMAL / CELL CULTURE INFORMATION:
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION: The cells are exposed to the test substance for 3-6 hours, and sampled at a time equivalent to about 1.5 normal cell cycle length after the beginning of treatment. If negative results, both with and without activation, an additional experiment without activation is done with continuous treatment until sampling at a time equivalent to about 1.5 normal cell cycle lengths.
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: Cell cultures are exposed to the test substance both with and without metabolic activation. At predetermined intervals after exposure of cell cultures to the test substance, they are treated with a metaphase-arresting substance (e.g. Colcemid® or colchicine), harvested, stained and metaphase cells are analysed microscopically for the presence of chromosome aberrations.
BRIEF TEST DESCRIPTION: The in vitro chromosome aberration test may employ cultures of established cell lines, cell strains or primary cell cultures. Cell cultures are exposed to the test substance (liquid or solid) both with and without metabolic activation during about 1.5 normal cell cycle lengths. At least three analysable concentrations of the test substance are used. At each concentration duplicate cultures is normally used. At predetermined intervals after exposure of cell cultures to the test substance, the cells are treated with a metaphase-arresting substance, harvested, stained. Metaphase cells are analysed microscopically for the presence of chromosome aberrations.
SPECIFIC PRECAUTIONS: Care should be taken to avoid conditions which would lead to positive results which do not reflect intrinsic mutagenicity and may arise from changes

in pH, osmolality or high levels of cytotoxicity.

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:
Compounds tested:
N-Nitrosomorpholine CAS 59-89-2
Results: Positive (except 3) VH10 human cells = negative result).

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria Dusinska	NILU	August-2010

TEST METHOD EVALUATION – OECD 487

ENDPOINT: Genotoxicity
TEST METHOD NAME: In Vitro Mammalian Cell Micronucleus Test
ENPOINT PARAMETER: Genotoxicity test for the detection of micronuclei (MN) in the cytoplasm of interphase cells
REFERENCE: OECD TG 487
VALIDATION STATUS: OECD validated
IN VIVO / IN VITRO TEST METHOD: In vitro
EXPOSURE ROUTE(S):
ANIMAL / CELL CULTURE INFORMATION: Various rodent cell lines (CHO, V79, CHL/IU, and L5178Y) and human lymphocytes The use of the human TK6 lymphoblastoid cell line (35), HepG2 cells (36) (37) and primary Syrian Hamster Embryo cells (38) has been described, although they have not been used in validation studies.
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION:
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: This is a genotoxicity test for the detection of micronuclei in the cytoplasm of interphase cells. Micronuclei may originate from acentric chromosome fragments (i.e. lacking a centromere), or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. The assay detects the activity of clastogenic and aneugenic test substances in cells that have undergone cell division during or after exposure to the test substance.
BRIEF TEST DESCRIPTION: Cell cultures of human or mammalian origin are exposed to the test substance both with and without an exogenous source of metabolic activation unless cells with an adequate metabolizing capability are used. Concurrent solvent/vehicle and positive controls are included in all tests.

During or after exposure to the test substance, the cells are grown for a period sufficient to allow chromosome or spindle damage to lead to the formation of micronuclei in interphase cells. For induction of aneuploidy, the test substance should ordinarily be present during mitosis. Harvested and stained interphase cells are analysed for the presence of micronuclei. Ideally, micronuclei should only be scored in those cells that have completed mitosis during exposure to the test substance or during the post-exposure period, if one is used. In cultures that have been treated with a cytokinesis blocker, this is achieved by scoring only binucleate cells. In the absence of a cytokinesis blocker, it is important to demonstrate that the cells analysed are likely to have undergone cell division during or after exposure to the test substance.

SPECIFIC PRECAUTIONS: It is important to demonstrate that cell proliferation has occurred in both the control and treated cultures, and the extent of test substance-induced cytotoxicity or cytostasis should be assessed in the cultures (or in parallel cultures) that are scored for micronuclei.

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:

This form has been edited by:

Name	Organization	Date
Evy Sivesind	NILU	October 2010

TEST METHOD EVALUATION- OECD 474

ENDPOINT: Mutagenicity/Genotoxicity
TEST METHOD NAME: Mammalian Erythrocyte Micronucleus Test
ENPOINT PARAMETER: The frequency of micronucleated immature (polychromatic) erythrocytes is the principal endpoint. The frequency of micronucleated immature (polychromatic) erythrocytes is the principal endpoint. The number of mature (normochromatic) erythrocytes in the peripheral blood that contain micronuclei among a given number of mature erythrocytes can also be used as the endpoint of the assay when animals are treated continuously for 4 weeks or more.
REFERENCE: OECD TG 474
VALIDATION STATUS: OECD validated
IN VIVO / IN VITRO TEST METHOD: In vivo
EXPOSURE ROUTE(S): Usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes may be acceptable when justified.
ANIMAL / CELL CULTURE INFORMATION: Bone marrow of rodents or mice is recommended, but other appropriate mammals may also be used
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION: With one single treatment of test substance: Samples of bone marrow are taken between 24 and 48 hours after treatment. Samples of peripheral blood are taken between 36 and 72 hours. If 2 or more daily treatments, samples should be collected once between 18 and 24 hours following the final treatment for the bone marrow and once between 36 and 48 hours following the final treatment for the peripheral blood.
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: Animals are exposed to the test substance by an appropriate route. If bone marrow is used, the animals are sacrificed at appropriate times after treatment, the bone marrow extracted, and preparations made and stained. When peripheral blood is used, the blood is collected at appropriate times after treatment and smear preparations are made and stained. For studies with peripheral blood, as little time as possible should elapse between the last exposure and cell harvest. Preparations are analyzed for the presence of micronuclei.
BRIEF TEST DESCRIPTION: The mammalian in vivo micronucleus test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic

apparatus of erythroblasts, by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents (mice or rats).
The purpose of the micronucleus test is to identify substances (liquid or solid) that cause cytogenetic damage which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage. Animals are exposed to the test substance by an appropriate route (usually by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection). Bone marrow and/or blood cells are collected, prepared and stained. Preparations are analyzed for the presence of micronuclei. Each treated and control group must include at least 5 analysable animals per sex. Administration of the treatments consists of a single dose of test substance or two daily doses (or more). The limit dose is 2000 mg/kg/body weight/day for treatment up to 14 days, and 1000 mg/kg/body weight/day for treatment longer than 14 days.

SPECIFIC PRECAUTIONS: If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test. Weight variation of animals should be minimal and not exceed ± 20% of the mean weight of each sex.

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:
Compound tested:
Nitrosaminedimethylamine, CAS 62-75-9 and N-Nitrosomorpholine, CAS 59-89-2.
Result: No conclusion, Positive/Positive, Positive, no conclusion

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria Dusinska	NILU	August-2010

TEST METHOD EVALUATION – OECD 475

ENDPOINT: Mutagenicity/Genotoxicity
TEST METHOD NAME: Mammalian Bone Marrow Chromosome Aberration Test
ENPOINT PARAMETER: The mitotic index should be determined as a measure of cytotoxicity in at least 1000 cells per animal for all treated animals (including positive controls) and untreated negative control animals. The number of cells scored, the number of aberrations per cell and the percentage of cells with structural chromosome aberration(s) should be evaluated. Different types of structural chromosome aberrations should be listed with their numbers and frequencies for treated and control groups.
REFERENCE: OECD TG 475
VALIDATION STATUS: OECD validated
IN VIVO / IN VITRO TEST METHOD: In vivo
EXPOSURE ROUTE(S): Usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes may be accepted when justified.
ANIMAL / CELL CULTURE INFORMATION: Bone marrow cells of rodents are used.
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION: Samples should be taken at two separate times on one day. The samples are taken between 12-18 hours after treatment. For optimum time of chromosome aberration detection, a later sample collection 24 hr after the first sample time is recommended. If dose regimens of more than one day are used, one sampling time at 1.5 normal cell cycle lengths after the final treatment should be used.
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES:
BRIEF TEST DESCRIPTION: Animals are exposed to the test substance (liquid or solid) by an appropriate route. The animals are sacrificed at appropriate times after treatment. Prior to sacrifice, animals are treated with a metaphase-arresting agent. Chromosome preparations are then made from the bone marrow cells and stained, and metaphase cells are analysed for chromosome aberrations. Each treated and control group must include at least 5 analysable animals per sex. The limit dose is 2000 mg/kg/body weight/day for treatment up to 14 days, and 1000 mg/kg/body weight/day for treatment longer than 14 days.

SPECIFIC PRECAUTIONS: If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test. Weight variation of animals should be minimal and not exceed ± 20% of the mean weight of each sex.

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria Dusinska	NILU	August-2010

TEST METHOD EVALUATION – OECD 476

ENDPOINT: Mutagenicity/Genotoxicity
TEST METHOD NAME: In vitro Mammalian Cell Gene Mutation Test
ENPOINT PARAMETER: Gene mutations, HPRT assay. Data should include cytotoxicity and viability determination, colony counts and mutant frequencies for the treated and control cultures. Survival (relative cloning efficiencies) or relative total growth should be given. Individual culture data should be provided. Negative results need to be confirmed.
REFERENCE: OECD TG 476
VALIDATION STATUS: OECD validated
IN VIVO / IN VITRO TEST METHOD: In vitro
EXPOSURE ROUTE(S): Cells in suspension or monolayer culture are exposed to the test substance, both with and without metabolic activation, for a suitable period of time and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection.
ANIMAL / CELL CULTURE INFORMATION: mammalian
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION: Each locus has a defined minimum time requirement to allow near optimal phenotypic expression of newly induced mutants (HPRT and XPRT require at least 6-8 days, and TK at least 2 days).
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: The objective is to detect possible mammalian mutagens and carcinogens induced by chemical substances.
BRIEF TEST DESCRIPTION: Mammalian cells in suspension or monolayer culture are exposed to, at least four analysable concentrations of the test substance, both with and without metabolic activation, for a suitable period of time. They are subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. It is recommended to utilise at least 106cells. Cytotoxicity is usually determined by measuring the relative cloning efficiency (survival) or relative total growth of the cultures after the treatment period. The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each selected locus and cell type, to allow near-optimal phenotypic expression of induced mutations. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells,

and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted.

SPECIFIC PRECAUTIONS: Care should be taken to avoid conditions which would lead to
results not reflecting intrinsic mutagenicity. Positive results which do not reflect intrinsic mutagenicity may arise from changes in pH, osmolality or high levels of cytotoxicity.

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:
Compound tested: Dimethylnitrosamine, CAS: 62-75-9, Results: positive

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria Dusinska	NILU	August-2010

TEST METHOD EVALUATION – Draft guideline Comet Assay

ENDPOINT: Genetic toxicity/mutagenicity

TEST METHOD NAME:
In vitro Comet Assay, Single-cell Gel Electrophoresis (SCGE) Technique.

ENPOINT PARAMETER:
DNA damage at the level of individual cells, measured by increased (%) tail intensity.

REFERENCES:

http://cometassay.com/index.htm

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12) Erkekoğlu P, Baydar T. Effect of allyl isothiocyanate (AITC) in both nitrite- and nitrosamine-induced cell death, production of reactive oxygen species, and DNA damage by the single-cell gel electrophoresis (SCGE): does it have any protective effect on HepG2 cells? Int J Toxicol. 2010 May-Jun;29(3):305-12.

VALIDATION STATUS: Under validation by JaCVAM

IN VIVO / IN VITRO TEST METHOD: In vitro

EXPOSURE ROUTE(S): In vitro cell exposure

ANIMAL / CELL CULTURE INFORMATION: Whole blood, lymphocytes, bone marrow, solid organs e.g. liver, primary or stable cell lines both monolayer cell cultures or suspension cell cultures.

NO. ANIMAL GENERATIONS TESTED:

TEST DURATION: Test itself with short term treatment last one day including analysis. Together with cell cultivation and depending on duration of treatment 3-5 days

INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.): The Comet assay does not require any special apparatus or chemicals. It can usually be performed on materials found in most laboratories. **Comet Assay Slides:** Regular unpolished glass microscope slides precoated with agarose work well for routine Comet assay use. **Electrophoresis Tank**: Horizontal electrophoresis tank with passive re-circulation. **Miscellaneous**: 1. Aluminium slide trays (Shandon Lipshaw) 2. Plastic slide trays (Shandon Lipshaw) 3. Slide Marker: Securline®.

Power pack,

Fluoresecence microscope

TEST PRINCIPLES: The Comet Assay or single cell gel electrophoresis (SCGE) assay is a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells (Singh et al., 1988). It combines the simplicity of biochemical techniques for detecting DNA single strand breaks, incomplete excision repair sites, alkali-labile sites, and cross-linking, with the

single cell approach typical of cytogenetic assays.
With inclusion of leasion specific enzyme specific DNA lesions such as oxidized purines and pyrimidine, DNA alkylation and bulk DNA lesions can be detected (Collins et al., 1996)

BRIEF TEST DESCRIPTION:

PROCEDURE:

The Comet Assay is based on the ability of negatively charged loops free DNA strands to be drawn through an agarose gel in response to an electric field. The extent of DNA migration depends directly on the DNA damage present in the cells. In this assay, a suspension of cells is mixed with low melting point agarose and spread onto a microscope glass slide. Following lysis of cells with detergent at high salt concentration, DNA unwinding and electrophoresis is carried out at a specific pH. Unwinding of the DNA and electrophoresis at neutral pH (7-8) predominantly facilitates the detection of cross links; unwinding and electrophoresis at pH 12.1-12.4 facilitates the detection of single and double strand breaks, incomplete excision repair sites, cross links; while unwinding and electrophoresis at a pH greater than 12.6 expresses alkali labile sites (ALS) in addition to all types of lesions listed above (Miyamae et al., 1997). When subjected to an electric field, the DNA migrates out of the cell, in the direction of the anode, appearing like a 'comet'. The size and shape of the comet and the distribution of DNA within the comet correlate with the extent of DNA damage (Fairbairn et al., 1995). Principles of image analysis are described by B Vilhar.

SPECIFIC PRECAUTIONS:

NOTE: Modification of method for detection of specific DNA lesions: specific DNA lesions consisting of strand breaks after treatment with alkali either alone or in combination with certain enzymes (e.g. endonucleases which detect specific lesions) increases DNA migration, (Collins et al., 1996). DNA-DNA and DNA-protein cross-links result in retarded DNA migration compared to those in concurrent controls (Tice et al., 2000)

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:

Compounds tested:
Nitrosodimethylamine, CAS 67-75-9
Nitrosodiethylamine, CAS 55-18-5
Nitrosomorpholine, CAS 59-89-2

Results: Positive for all three (approx. 300% increased tail intensity)

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria	NILU	August-2010

Dusinska			

TEST EVALUATION FORMS FOR CARCINOGENICITY TESTING

TEST METHOD EVALUATION – Draft guideline EU B.21

ENDPOINT: Cardinogenicity/Mutagenicity

TEST METHOD NAME: In vitro mammalian - cell transformation assay

ENPOINT PARAMETER: The Syrian Hamster Embryo *in vitro* cell transformation assay
Dose range finding study.
The BALB/c 3T3 cell transformation assay
This project aims to determine the carcinogenic potential of genotoxic and non-genotoxic chemicals. Clone A31-1-1 is derived from BALB/c 3T3 cell line originated from BALB/c mouse embryo. cultures.
The C3H/10T1/2 assay

REFERENCE: B.21- ENV/JM/MONO(2007)18

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12: Slamenová D, Dusinská M, Gábelová A, Bohusová T, Oravec C. An evaluation of three pesticides: piritione, supercypermethrin and metolachlor in transformation bioassays of BHK21 and hamster embryo cells. Cell Biol Toxicol. 1992 Oct-Dec;8(4):217-31. PubMed PMID: 1493583.

13: Slamenová D, Dusinská M, Gábelová A, Bohusová T, Ruppová K. Decemtione (Imidan)-induced single-strand breaks to human DNA, mutations at the hgprt locus of V79 cells, and morphological transformations of embryo cells. Environ Mol Mutagen. 1992;20(1):73-8. PubMed PMID: 1639085.

VALIDATION STATUS: Under validation at ECVAM

IN VIVO / IN VITRO TEST METHOD: In vitro

EXPOSURE ROUTE(S): in vitro cell exposure

ANIMAL / CELL CULTURE INFORMATION: different rodents (hamsters, mice).
Found results from: Fischer rat embryo (RLV/1706) cells and Mouse (BALB/c-3T3) cells

NO. ANIMAL GENERATIONS TESTED:

TEST DURATION: The Syrian Hamster Embryo *in vitro* cell transformation assay
The cultures will be incubated for a period of 7 days to allow colony development.
The BALB/c 3T3 cell transformation assay
The medium is replaced with fresh medium and changed twice a week during 3½ weeks and once a week during the following 2 weeks.
The C3H/10T1/2 assay
Medium with promoter is replaced at least once per week for the duration of the assay incubation,

which is from 6 to 8 weeks in for the two-stage assay.

INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):

TEST PRINCIPLES: The Syrian Hamster Embryo *in vitro* cell transformation assay consists in seeding target SHE cells at clonal density onto a feeder layer of X-irradiated SHE cells. Twenty four hours after seeding feeder cells, the target cells are seeded onto the feeder layer at a density appropriate to obtain 25-45 colonies per plate (60 mm diameter) and are treated 24 hours later.

The BALB/c 3T3 cell transformation assay
It is used as a sensitive and stable cell line for the focus formation assay. Genotoxic chemicals produce foci in the cultures subjected to a standard protocol or emphasized by post-treatment with tumor promoter TPA: non-genotoxic chemicals are evaluated for promoting activity of carcinogenesis in a two-stage method where cultures are first treated with a known carcinogen and then with a test chemical.

The C3H/10T1/2 assay
Low-passage frozen stock cultures should be obtained from a reliable source and expanded by
one or two passages, then cryopreserved in liquid nitrogen until required for assays. Passages 8 to 15 have been generally available and are recommended for their low-backgrounds of spontaneous transformants. The American Type Culture Collection currently supplies frozen cultures of clone 8 at passage 10. Subsequent culture passages should be performed prior to cells reaching confluence, which minimizes the appearance of transformed variants in the stock cultures. Seeding 2.5×10^4 cells per 100 mm dish or 75 cm^2 flask would start typical stock cultures, with passage at 7 days incubation.

BRIEF TEST DESCRIPTION: The objective is to provide an overview of the three main CTAs; (the Syrian hamster embryo cell (SHE), the BALB/c 3T3 and the C3H10T1/2 assays) and to correlate them with *in vivo* rodent assays and assess their performances in predicting chemical carcinogenicity.

SPECIFIC PRECAUTIONS:

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES:

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:
Compounds tested: Aniline CAS 62-53-3, Results**:** positive and negative

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria	NILU	August-2010

Dusinska		

TEST METHOD EVALUATION- OECD 453

ENDPOINT: Chronic toxicity / Carcinogenicity
TEST METHOD NAME: Combined Chronic Toxicity / Carcinogenicity Studies
ENPOINT PARAMETER: General: Suvival data, body weight / changes, food consumption, toxicokinetic data, opthalmoscopy, haematology, clinical chemistry. Clinical findings: signs of toxicity, incidence of any abnormality, nature, severity and duration of clinical obersvation. Necropsy data: Terminal body weight, organ weights and their ratios, necropsy findings – incidence and severity of abnormalities. Histopathology: Non neoplastic histopathological findings, neoplastic histopathological findings, correlation between gross and microscopic findings, detailed description of all treatment-related histopathological findings including severity gradings, report of any peer review of slides.
REFERENCE: OECD TG 453
VALIDATION STATUS: OECD validated
IN VIVO / IN VITRO TEST METHOD: In vivo
EXPOSURE ROUTE(S): Focus on oral route. Dermal or inhalation need careful modifications
ANIMAL / CELL CULTURE INFORMATION: Primarily rodent species, by use of other species, appropriate modifications must be taken (OECD Guidance Document No 116)
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION: Chronic phase: Normally 12 months, can be shorter (e.g.6 or 9) and longer (e.g. 18 or 24). Deviations from 12 months must be justified. Carcinogenicity phase: Normally 24 months
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: The objective of a combined chronic toxicity/carcinogenicity study is to identify carcinogenic and the majority of chronic effects, and to determine dose-response relationships following prolonged and repeated exposure.
BRIEF TEST DESCRIPTION: The rat is typically used for this study. For rodents, each dose group and concurrent control group intended for the carcinogenicity phase of the study should contain at least 50 animals of each sex, while for the chronic toxicity phase of the study should contain at least 10 animals of each sex. At least three dose levels should be used, in addition to the concurrent control group for both the chronic toxicity phase and the carcinogenicity phase of the study. For chronic phase, the test substance is administered daily in graduated doses to several groups of test animals, one dose level per

group. For carcinogenicity, phase, the test substance is administered daily to several groups of test animals for a major portion of their life span.

The observations permit the detection of neoplastic effects and a determination of carcinogenic potential as well as the general toxicity.

The three main routes of administration are oral, dermal, and inhalation. The Test Guideline focuses on the oral route of administration.

SPECIFIC PRECAUTIONS: Both sexes should be used. A sufficient number of animals should be used to have a throughout biological and statistical evaluation (e.g. for rodent – 50 animals of each sex)

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:

SUITABILITY EXPOSURE OF AIR SAMPLES: Requires modifications

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐

COMMENTS:

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria Dusinska	NILU	August-2010

TEST EVALUTION FORMS FOR REPRODUCTIVE TOXICITY

TEST METHOD EVALUATION-OECD 422

ENDPOINT: Reproduction/Developmental Toxicity
TEST METHOD NAME: Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test
ENPOINT PARAMETER: Toxic effects in P generation: No. of animals at the start, during and end of the test, the time of death, No. of fertile animals and pregnant females, time of onset, duration, and severity of toxic effects such as No. of implantations, post-implantation loss, counting of corpora lutea, duration of gestation, sensory and motor assessments, haematological test, clinical biochemistry, signs of difficult or prolonged parturition and all signs of toxicity including mortality. Gross necropsy and histopathology, microscopic findings of the male genital tract. Body weight changes and organ weight data, Food and water consumption, pertinent behavioural changes Toxic effects in F1 generation: No. and sex, stillbirths, live births, runts, post natal growth, body weight changes and presence of grossly visible abnormalities.
REFERENCE: OECD 422
VALIDATION STATUS: Yes OECD
IN VIVO / IN VITRO TEST METHOD: In Vivo
EXPOSURE ROUTE(S): Oral (gavage, diet or drinking water); Other routes require modifications
ANIMAL / CELL CULTURE INFORMATION: Rat; Other species require modifications
NO. ANIMAL GENERATIONS TESTED: F1
TEST DURATION: 54 days
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.): Microscope, Weighing machine
TEST PRINCIPLES: The study will provide evaluations of reproduction/developmental toxicity associated with administration of repeated doses. In particular, since emphasis is placed on both general toxicity and reproduction/developmental toxicity endpoints, the results of the study will allow for the discrimination between reproduction/developmental effects occurring in the absence of general toxicity and those which are only expressed at levels that are also toxic to parent animals. It could provide an indication of the need to conduct further investigations and could provide guidance in the design of subsequent studies.
BRIEF TEST DESCRIPTION: The test substance is administered in graduated doses to at least 3 test groups and a control group of males (at least 4 weeks) and females (approximately 54 days). Dosing is

done based on information from any existing toxicity and toxicokinetic data in 10 animals/ group/ sex. The highest dose level- inducing toxic effects but not death or severe suffering. Thereafter, a descending sequence of dose levels dosage related response and no-observed-adverse effects (NOAEL) at the lowest dose level. Two to four fold intervals between dosages is used. If in the limit test, no observable toxic effects are observed at 1000mg/Kg/b.w/day , then a full study using several dose levels is not necessary.
Mating is done at full sexual maturity (Sprague Dawley rats 10 weeks, Wistar rats -12 weeks) at 1:1 ratio. No. of pregnant females: 8/ group.
Termination of study Males: 28 days; Females: Day 3 post-partum, or 24-26 days for (non copulated females)
Observation: General clinical observation (Daily); Measurement of food/water consumption during pre-mating, pregnancy and lactation (Weekly); Weight of the P males and females (Day 1 of dosing and weekly, at termination), Weight of the pups: (Day 0 or 1, 4), Weight of pregnant females: (Day 0 or 7, 14 and 20 and within 24 hours of parturition (day 0 or 1 post-partum) and day 4 post-partum. duration of gestation, Number and sex of live pups, still births, live births, runts, Haematology, clinical biochemistry, Functional observations (at the end of the study), Pathology: Gross necroscopy and histopathology (at the end of the study). Effect of the substance on fertility, pregnancy, maternal and suckling behaviour, and growth and development of the F1 offspring.

SPECIFIC PRECAUTIONS:
Maximum dose volume for administration: 1mL/100g of body weight or 2 mL/100g body weight for aqueous solutions.
Diet: constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight
Gavage: given at similar times each day, and adjusted at least weekly to maintain a constant dose level in terms of animal body weight

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION: The dose of the test substance related to presence / absence, incidence / severity of abnormalities, including gross lesions, identified target organs, infertility, clinical abnormalities, affected reproductive and litter performance, body weight changes, effects on mortality, histopathology of the testis and epididymus, fertility data and any other toxic effects.

SUITABILITY EXPOSURE OF AIR SAMPLES: Require modifications

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMP☐ WATER SA☐S:

COMMENTS: Offers only limited means of detecting post-natal manifestations of prenatal exposure, or effects that may be induced during post-natal exposure.

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria Dusinska	NILU	August-2010

TEST METHOD EVALUATION-OECD 415

ENDPOINT: Reproduction Toxicity
TEST METHOD NAME: One-Generation Reproduction Toxicity Study
ENPOINT PARAMETER: Number and sex of pups, stillbirths, live births, Weight, Measurement of food consumption, Pathology: Gross necropsy and Histopathology (ovaries, uterus, cervix, vagina, testes, epididymides, seminal vesicles, prostate, coagulating gland, pituitary gland and target organs), Pertinent behavioural changes, signs of difficult or prolonged parturition and all signs of toxicity, including mortality.
REFERENCE: OECD 415
VALIDATION STATUS: Yes OECD
IN VIVO / IN VITRO TEST METHOD: In Vivo
EXPOSURE ROUTE(S): Diet or drinking water (recommended); Other routes (also acceptable)
ANIMAL / CELL CULTURE INFORMATION: Mice / Rat (5-9 weeks)
NO. ANIMAL GENERATIONS TESTED: F1
TEST DURATION: Males: 56 days; Rats 70 days. Females: 2 weeks prior to mating, continue 3-weeks mating period, pregnancy and up to the weaning of the F1 offspring.
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.): Microscope, Weighing machine
TEST PRINCIPLES: The test substance is administered in graduated doses to several groups of males for at least 1 complete spermatogenic cycle and females for at least two complete oestrous cycles. Endpoints of reproductive toxicity are measured in F1 and P generation animals.
BRIEF TEST DESCRIPTION: Animals are dosed according to either limit test (low toxicity in repeated-dose studies) or at 3 treatment groups (high/ intermediate/ low) and a control group. The animals are then mated according to 1:1 or 1:2 ratio. Each test and control group: 20 pregnant females at or near term. Animals which fail to mate: Evaluated to determine the cause of the apparent infertility. Litter without standardised litter: Normal rearing of progency until weaning. Litter with standardisation: on day 4, the size of each litter is adjusted to 4 males and 4 females Observations: once daily. Measurement of food consumption: Weekly- During pre-mating and mating periods; Daily-Pregnancy; Same day as the litters- After parturition and during lactation. Weight of the animals: P males and females (Day 1 of dosing and weekly) Weight of the pups: (Day 1, 4 and 7 and weekly, until termination of the study)

Animals are then examined for gross necropsy and histopathology and all signs of toxicity, including mortality are reported.

SPECIFIC PRECAUTIONS:

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION: Evaluated in terms of observed effects, necropsy and microscopic findings. The dose of the test substance related to presence / absence, incidence / severity of abnormalities, including fertility, clinical abnormalities, body weight changes, effects on mortality and any other toxic effects. Provides estimation of a no-effect level and adverse effects on reproduction, parturition, lactation and postnatal growth.

SUITABILITY EXPOSURE OF AIR SAMPLES: Require modifications

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMP[ ] WATER SA[ ]S:

COMMENTS: Extrapolation of the results of the study to man is valid to a limited degree, although it can provide useful information on no-effect levels and permissible human exposure.
It is not designed to determine specific cause and effects in all cases and will require modifications to study substances administered by the inhalation route.

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria Dusinska	NILU	August-2010

TEST METHOD EVALUATION-OECD 416

ENDPOINT: Reproduction Toxicity
TEST METHOD NAME: Two-Generation Reproduction Toxicity Study
ENPOINT PARAMETER: P and F1 generation: No. of fertile and pregnant animals, No. of animals showing signs of toxicity, description of signs of toxicity: time of onset, duration, and severity of any toxic effects. Integrity and performance of the male and female reproductive systems, including gonadal function, sperm count and motililty, the oestrus cycle, mating behaviour, conception, gestation, parturition, lactation, and weaning. F1 and F2 Pups: Number and sex of pups, stillbirths, live births, body and organ weight, growth and development, measurement of food and water consumption. Body and organ weight: P and F1 generation: uterus, ovaries, testes, epididymides (total and cauda), prostate, seminal vesicles with coagulating glands and their fluids (as one unit); brain, liver, kidneys, spleen, pituitary, thyroid and adrenal glands and known target organs; F1 and F2 pups: Brain, spleen and thymus Histopathological examination and necropsy findings with special attention to the organs of the reproductive system.(ovaries, uterus, cervix, vagina, testes, epididymides, seminal vesicles, prostate, coagulating gland, pituitary gland and target organs).
REFERENCE: OECD 416
VALIDATION STATUS: Yes OECD
IN VIVO / IN VITRO TEST METHOD: In Vivo
EXPOSURE ROUTE(S): oral (by diet, drinking water or gavage) unless another route of administration (e.g. dermal or inhalation) are more appropriate
ANIMAL / CELL CULTURE INFORMATION: Rat, Parental males and females (5-9 weeks old), F1 males and females (weaning)
NO. ANIMAL GENERATIONS TESTED: F1 and F2
TEST DURATION: Parental animals: During growth and for at least 1 complete spermatogenic cycle in males (56 days-mice and 70 days-rat) and females during growth and for several complete oestrous cycles. Dosing is continued during mating, resulting pregnancies, weaning of their Fl offspring and to the Fl offspring during their growth into adulthood, mating and production of an F2 generation, until the F2 generation is weaned.
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.): Microscope, Weighing machine
TEST PRINCIPLES: The test substance is administered in graduated doses to several groups of males during growth and for at least 1 complete spermatogenic cycle (56 days-mice and 70 days-rat) and females during growth and for several complete oestrous cycles. Administration to the parental (P) animals is done during mating, resulting pregnancies, weaning of their Fl offspring and to the Fl offspring during their growth into adulthood, mating and production of an F2 generation, until the F2 generation is weaned. Clinical observations and pathological examinations: Performed on all animals for signs of toxicity with special emphasis on the integrity and performance of the male and female reproductive systems and on the growth and development of the F1 and F2 offspring.

BRIEF TEST DESCRIPTION: Animals are dosed for at least 10 weeks before the mating period, according to either limit test (1000 mg/kg b.w./ day) or at 3 dose levels (descending sequence) and a concurrent control with the highest dose level chosen with the aim to induce toxicity but not death or severe suffering. Dose: 2-4 fold intervals/ 7-days-a-week basis. The animals are then mated according to 1:1 ratio. Dosing is continued during the 2 week mating period, continue throughout pregnancy and up to the weaning of the F1 offspring and continue until termination.

Test and control group: Sufficient number of animals to yield preferably not less than 20 pregnant females at or near parturition.
Control group: Untreated group or a vehicle-control group with highest volume of vehicle being used.
Observation: Pairs without progeny are evaluated to determine the apparent cause of the infertility, Assess the integrity and performance of the male and female reproductive systems and in addition study the growth and development of the F1 and F2 generation.
Results: Estimation of a no-effect level and an understanding of adverse effects on reproduction, parturition, lactation, postnatal development including growth and sexual development. Provide information on the effects of repeated exposure to a substance during all phases of the reproductive cycle. Can be used in assessing the need for further testing of a chemical and to provide information on permissible human exposure.

SPECIFIC PRECAUTIONS: Mating of siblings should be avoided, F1 offspring should not be mated
until they have attained full sexual maturity, stability of the test substance in the vehicle should be determined, Constant volume not exceeding 1 ml/100g b.w. should be used at all dose levels, constant dietary concentration in terms of the b.w. should be used, Gavage studies: Dose should be given at similar times each day, and adjusted to maintain a constant dose level in terms of animal b.w. with respect to placental distribution and last trimester of pregnancy.

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION: Evaluated in terms of observed effects, necropsy and microscopic findings. The dose of the test substance related to presence / absence, incidence / severity of abnormalities, including fertility, clinical abnormalities, body weight changes, effects on mortality and any other toxic effects. Provides estimation of a no-effect level and adverse effects on reproduction, parturition, lactation and postnatal growth.

SUITABILITY EXPOSURE OF AIR SAMPLES: -

GENERAL JUDGEMENT OF TEST:

SCORE OF TEST AIR SAMP☐ WATER SA☐S:

COMMENTS: Extrapolation of the results of the study to man is valid to a limited degree, although it can provide useful information on no-effect levels and permissible human exposure.
Males of the P generation need not be included in the evaluation: If data on spermatogenesis are available (e.g. 90 day study).

This form has been edited by:

Name	Organization	Date
Solveig Ravnum, Maria Dusinska	NILU	August-2010

TEST METHOD EVALUATION –ECVAM validated assays INVITTOX No 113

ENDPOINT: Classification and labelling, ranking of toxic potency
TEST METHOD NAME: Embryonic Stem Cell Test (EST)
ENPOINT PARAMETER: CELL DIFFERENTIATION: Inhibition of ES cell differentiation into cardiac myoblasts, measured by light microscopy CELL VIABILITY: Inhibition of 3T3 and ES cell viability determined by the MTT assay CELL PROLIFERATION: Inhibition of 3T3 and ES cell proliferation
REFERENCE: INVITTOX No 113
VALIDATION STATUS: ECVAM validated
IN VIVO / IN VITRO TEST METHOD: in vitro
EXPOSURE ROUTE(S):
ANIMAL / CELL CULTURE INFORMATION:
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION: 5 days
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: Two permanent mouse cell lines are used, ES cells (D3), to represent embryonic tissue, and fibroblasts (3T3 cells), to represent adult tissue. The test has been developed only after it was found that ES cells can be maintained in the undifferentiated stage in the presence of the cytokine leukemia inhibiting factor (LIF). When released from the undifferentiated stage, ES cells will form embryo bodies (EBs) and differentiate under appropriate conditions into the major embryonic tissues.
BRIEF TEST DESCRIPTION: Differentiation of ES cells. The mouse ES cell line D3 is cultured permanently in the presence of LIF, a differentiation inhibition factor. In the absence of LIF, ES cells start to differentiate spontaneously. Several concentrations of the test chemical are added to a stem cell suspension. Drops of ES cell suspension in supplemented DMEM (Dulbecco's Modified Eagle's Medium) are placed on the lids of 10 cm petri dishes ("hanging drop" culture according to Wobus al., 1991). After cultivation for 3 days the aggregates are transferred into bacterial (non tissue culture treated) petri dishes. 2 days later EBs are placed into 24-well plates (tissue culture treated) where further development of EBs proceeds into different embryonic tissues (Spielmann et al., 1995; Heuer et al., 1994a and b).

Differentiation into contracting myocardial cells is determined by light microscopy after another 5 days of culture.
SPECIFIC PRECAUTIONS:
CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:
SUITABILITY EXPOSURE OF AIR SAMPLES:
GENERAL JUDGEMENT OF TEST:
SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐
COMMENTS:
This form has been edited by:

Name	Organization	Date
Evy Sivesind	NILU	September 2010

TEST METHOD EVALUATION – ECVAM validated assays INVITTOX No 123

ENDPOINT: Classification and labelling, ranking of toxic potency
TEST METHOD NAME: Embryotoxicity Testing in Post-Implantation Embryo Culture
ENPOINT PARAMETER: Embryo morphology, functionalities, growth and cell viability
REFERENCE: INVITTOX No 123
VALIDATION STATUS: ECVAM validated
IN VIVO / IN VITRO TEST METHOD: In vitro
EXPOSURE ROUTE(S):
ANIMAL / CELL CULTURE INFORMATION:
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION:
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: Rat embryos with 1 to 5 somites are used in this method. In general, these embryos are relatively sensitive for xenobiotics as compared to older embryos. During 48 hours of culture, major aspects of organogenesis occur, including e.g. heart development, closure of the neural tube, development of ear and eye, brachial bars and limb buds. Interference during this period may lead to general retardation of growth and development or to specific malformations in one or several organ anlagen.
BRIEF TEST DESCRIPTION: Rat embryos are cultured on day 9.5 of gestation. To each embryo culture vessel 2 ml of rat serum is added. Compounds are dissolved and diluted in a proper solvent (e.g. Hanks' balanced salt solution), HBSS, DMSO or ethanol) and standard volumes of compound solutions are added to the culture serum in the culture vessel before addition of the embryo. The same volume of pure solvent is added to control cultures, a volume that is shown in prior experiments not to interfere with embryogenesis in culture. Embryos from each dam are distributed as evenly as possible over the control and various concentrations tested. Furthermore, the embryos are distributed so that the average initial somite number is nearly the same for each concentration. Compound concentrations are always tested in the presence of a similar number of concurrent controls. After 48 hours culture, each embryo is transferred to a petri dish containing HBSS (37^{o}C) and the embryos are scored in the same order in which they were put into culture.
SPECIFIC PRECAUTIONS:

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:
SUITABILITY EXPOSURE OF AIR SAMPLES:
GENERAL JUDGEMENT OF TEST:
SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐
COMMENTS:

This form has been edited by:

Name	Organization	Date
Evy Sivesind	NILU	September 2010

TEST METHOD EVALUATION – ECVAM validated assays INVITTOX No 122

ENDPOINT: Classification and labelling, ranking of toxic potency
TEST METHOD NAME: The Micromass Test – Method of Brown
ENPOINT PARAMETER: Cell differentiation: Alcian blue staining (cartilage-specific proteoglycan stain)
REFERENCE: INVITTOX No 122
VALIDATION STATUS: ECVAM validated
IN VIVO / IN VITRO TEST METHOD: In vitro
EXPOSURE ROUTE(S):
ANIMAL / CELL CULTURE INFORMATION:
NO. ANIMAL GENERATIONS TESTED:
TEST DURATION:
INSTRUMENT REQUIREMENTS (EXPOSURE, MEASUREMENTS ETC.):
TEST PRINCIPLES: The method is based on detecting the ability of a particular chemical to inhibit the formation of foci. Thus, positive chemicals will reduce the number of foci, or the number of cells within foci. The primary culture of limb bud cells of mammalian origin reproduces cartilage histogenesis, a fundamental step in the morphogenesis of the skeleton. Various functions, including cell proliferation, cell differentiation, cell to cell communication and cell to extracellular matrix interactions are implicated in this developmental process. Interference with these basic cell developmental functions may provide primordial teratogenic endpoints, and so this simple cell culture system appears to be a good model with which to study the teratogenic potential of chemical compounds.
BRIEF TEST DESCRIPTION: Embryos were obtained from Wistar rats on day 14 of gestation and the limb buds were isolated. Single cell suspension was prepared by trypsin action. The subsequent step, spotting of cells in 96 well plates, is the most critical: place with care the spot in the centre of the well and make the volume and number of cells within the spot as consistent as possible. The 96 well plates were placed in the incubator, then added medium with or without test chemical and left for 5 days. The total number of viable cells (i.e. IC50: 50% inhibition of cell viability and growth) and of differentiated cells (i.e. ID50: 50% inhibition of cells differentiation) and number of foci were determined.
SPECIFIC PRECAUTIONS:

CRITERIA FOR HAZARD EVALUATION CLASSIFICATION:
SUITABILITY EXPOSURE OF AIR SAMPLES:
GENERAL JUDGEMENT OF TEST:
SCORE OF TEST AIR SAMPLES: ☐ WATER SAMPLES: ☐
COMMENTS:

This form has been edited by:

Name	Organization	Date
Evy Sivesind	NILU	September 2010

Appendix G

Overview of laboratories in Europe

Searched in AltTox (www.alttox.org) and using Google

Name	Country	System	Short description	Services	Contact	Comment
APREDICA	USA/ UK	In vitro/ in vivo	Apredica provides preclinical contract testing services for the evaluation and optimization of the ADME, Toxicity, and Pharmacokinetic properties of drug candidates early in the drug-discovery process. We also provide *in vitro* testing services for cosmetics, agrochemicals, and other products for REACH compliance.		http://www.apredica.com/?gclid=CK32qqfqrqMCFdc43god6hph1Q	Low priority
Advanced Cell systems	Germany	In vitro	A wholly owned subsidiary of the German CellSystems® - specialises in research, development and production of sophisticated 3-dimensional cell culture models	• Non-destructive assays for cell viability • Measuring of proinflammatory mediators • Protein Profiling (ELISA & multiparameter analysis) • Characterisation of cell surface proteins and intracellular proteins • Proliferation studies on primary cells and cell lines • Histology & Immunohistochemistry • Lipid Profiling • Photo(cyto)toxicity testing • Molecular and biology analysis	http://www.advancedcellsystems.com/index.html	Low priority
An-eX Analytical Services, Ltd.	UK	In vitro	Dermal research and development including *in vitro* human skin permeation studies performed according to the OECD 428 and other appropriate guidelines	in vitro human skin permeation studies	http://www.an-ex.co.uk/resources/anex.html	
Austrian Research Centers (ARC) GmbH	Austria	in vivo / in vitro	CRO offering in vitro and in vivo toxicology services			Link not working, hard to find proper website

Name	Country	System	Short description	Services	Contact	Comment
Bionas GmbH	Germany		Bionas® analyzing system used with variety of cell types for testing toxic effects and other cell biology applications	•Developmental toxicity: The analysis of chemicals which cause functional and morphological effects on a developing organism. •Immunotoxicity: Investigation of immune modulating effects of compounds on cells of the immune system. •General toxicity: Effects of toxic compounds on cell lines and primary cells of any origin. •Cardiotoxicity: Cardiomyocytes are used for pharmacological and toxicological assays with a focus on long-term measurements. •Hepatotoxicity: Primary human and rat hepatocytes as well as hepatocytic cell lines can be investigated to predict liver toxicity of test compounds. •Environmental toxicity: Investigation of environmental toxins and contaminants like solvents and heavy metals, which cause problems by disrupting the functional properties in the living organism. •Nanotoxicity: The Bionas® analyzing system offers an alternative approach to animal testing to assess the toxicological risks of soluble chemicals and insoluble micro and nano particles.	http://www.bionas.de/en/Services/LaboratoryService.html	Promising
BSL BioService Scientific Laboratories GmBH	Germany	In vitro / in vivo	contract laboratory for in vitro and in vivo toxicology	•Cytotoxicity, Phototoxicity in vitro •Single dose toxicity •Repeated dose toxicity •Developmental and Reproductive Toxicity (DART) •Sensitization •Local tolerance studies (single or repeated applications) •Genetic Toxicology •Immunotoxicity •In vitro dermal absorption & toxicology	http://www.bioservice.com/services/toxicology.aspx	Promising
Centre for Advanced Research & Development on Alternative Methods (CARDAM)	Belgium	in vitro	in vitro contract testing, R&D, and related activities and consulting	In vitro contract research testing services in a GLP environment Regulatory required/approved methods Pre-clinical testing	http://www.cardam.eu/Cardam/Services+and+activities/	These we already know

Name	Country	System	Short description	Services	Contact	Comment
Covance	UK	In vitro / in vivo	Covance has worked with a wide range of industries, providing a full range of in vitro, in vivo and exploratory screening genetic toxicology assays, including multi-endpoint assays, designed to meet established scientific standards and evolving regulatory mandates.	Investigation of the origin of micronuclei (clastogenic or aneugenic) Transgenic models Comet assays DNA binding studies 28 day cytogenetic studies Photomutagenicity Cytogentic monitoring In vitro toxicology Cytotoxicity Phototoxicity	http://www.covance.com/products/nonclinical/toxicology/genetic-molecular-toxicology/index.php	Promising
Cultex® Laboratories	Germany	In vitro	The CULTEX® system is the platform for the in-vitro toxicological analysis of airborne substances, such as gases, volatile compounds and complex gas mixtures under realistic (in- and outdoor) atmospheric conditions.	•Development of new equipment configured to customer requirements •Concept and design using state-of-the-art CAD systems (computer aided design) and FEM simulation technologies (finite element method) in cooperation with our engineering office in Switzerland •Manufacture of all specified components in collaboration with our manufacturing partners in Germany (Askea Feinmechanik GmbH) and Japan (Yotsubishi Corporation/Sibata Scientific Technology Ltd.). Components are manufactured and assembled from stainless steel, boron silicate glass or engineering plastics •The original CULTEX® glass modules are manufactured exclusively by the Yotsubishi Corporation/Sibata Scientific Technology Ltd. in Tokyo (Japan) •Concurrent development of control software	http://www.cultex-laboratories.com/#	Promising
EDI GmbH	Germany	ex vivo / in vitro	ex vivo Instant Leukocyte Culture System; customized cell culture systems and immunoassays; in vitro immunotoxicology and immunopharmacology; co-cultures of immune cells with other tissues	pharmacological and toxicological expert reports assessments Immuno-pharmacology Immuno-toxicology Cell biology	http://www.edigmbh.de/home_e.html	Potential useful

Name	Country	System	Short description	Services	Contact	Comment
Eggcentris	Belgium	in vitro	in vitro tests to screen compounds for reproductive toxicity (effects on fertility and embryonic development)	Broadly applicable, multiparametric assays We screen compounds for their chemical and physical influence on fertility and embryonic development: 1. Toxicity testing: screening and ranking new chemical compounds to determine their effects on reproduction and fertility 2. Bio-activity determination: understanding the exact mechanism behind the biological end points of new drug compounds 3. Target validation: the essential intermediate step between screening and in vivo testing of drug compounds in their developmental phase # Project research Thanks to our intensive research and development activities, we can assist our customers with all their questions related to reproductive functions. We develop new testing concepts, fully adapted to the specific needs of our customers and the compounds they have under study. Quality control fertility management Fertility centers and their suppliers need to have their products tested for embryo toxicity. They can rely on EggCentris as a quality control lab.	http://www.eggcentris.com/index.php?ID=6993	
Epithelix Sàrl	Switzerland	in vitro	provides in vitro assays to pharmaceutical, biotech, cosmetic, and chemical industries using their own human cellular models	Aerosol exposure, toxicity of inhaled products, exposure of inhaled products, airway remodelling. Acute, long term and chronic in vitro studies and more	http://www.epithelix.com/content/view/7/7/lang,en/	Very useful
Eurofins-Biolab	Italy	In vitro / in vivo	specialised laboratory for in vitro and in vivo toxicology and ecotoxicology		http://www.biolab.it/htmbank/uk/COMPANY/company.htm	not so much about environmental exp

Name	Country	System	Short description	Services	Contact	Comment
FRAME Alternatives Laboratory	UK	In vitro	*in vitro* methods research and validation studies	* International alternative method validation studies. * Development of cytotoxicity assays as replacements for acute toxicity tests such as the kenacid blue test. * Development of replacements for the Draize eye irritancy test. The FRAME neutral red release assay is now available as a kit - Predisafe - marketed by Biopredic (France). The fluorescein leakage assay is used to model the barrier function of the eye. * Development of a 3D human skin model for irritancy testing. The model is also being used to investigate the way in which parasites penetrate human skin. * Development of models to investigate the effects of chemicals on the respiratory tract. * Development of an assay to predict phototoxicity. * Development of methods for embryotoxicity screening, using embryonic stem cell lines.	http://www.frame.org.uk/index.php	Mostly research, not sure how much they can do of services. But promising

Name	Country	System	Short description	Services	Contact	Comment
GenPharmTox BioTech AG	Germany	In vitro / in vivo	offers *in vitro* and *in vivo* testing of drugs and chem	**In vitro Services:** 1. Phototoxicity Test 2. Cytotoxicity / Hepatotoxicity Test 2.1. Screening Cytotoxicity / Hepatotoxicity Test 2.2. Standard Cytotoxicity / Hepatotoxicity Test 2.3. Advanced Cytotoxicity Test (V79 Cell BatteryTM) 3. AMES Test 4. HPRT Test 5. Mouse Lymphoma Assay 6. Comet Assay 6. Micronucleus Test BatteryTM, OECD Draft Guideline by J.M. Parry) 7. Chromosome Aberration Test **In vivo Services:** 1. General consideration 1.1. Animal Welfare 1.2. Homogeneity and Stability 1.3. Co-operations 2. Acute to Chronic Toxicity 3. Genotoxicity 4. Carcinogenicity 5. Reproduction Toxicity 6. Aquatic Toxicity 7. Biological Degradation / Bioaccumulation 8. Avian Toxicity	http://www.genpharmtox.de/services/Toxicology/toxycology.html	Promising, but maybe only for pharma products
GlycoMar	UK	in vitro	screening assay services using primary human cells and cell lines; contract research services; glycobiology and enzyme products	Cytotox, endotoxin	http://www.glycomar.com/screening_services.htm	drug mostly

Name	Country	System	Short description	Services	Contact	Comment
NOTOX	Netherlands	in vitro	in vitro GLP-compliant testing services include skin irritation, skin corrosion, eye irritation, and more	# Toxicology & ADME # General Toxicology # Genetic Toxicology # In vitro toxicology # Developmental & Reproduction Toxicology # Carcinogenicity # ADME & Kinetics # Safety Pharmacology # Special Toxicity Studies # Environmental Toxicology # Formulation development # Juvenile Toxicity studies # Lead Optimization # Single rat PK	http://www.notox.nl/vitro_toxicology.php	Very useful
Pharmacelsus	Germany	In vitro / in vivo	CRO offering *in vitro* and *in vivo* toxicology services	In vitro cytotoxicity extrapolation for estimation of in vivo LD50, Regulatory Genotoxicity Test # TOX-ESCAPE # Live-Cell Toxicology Screening Platform # Toxicology and Preclinical Development Unit # Flow Cytometry # Reactive oxygen species (ROS) # Apoptosis	http://www.pharmacelsus.de/content.php?type=10	Potential useful, with some alternative tests
Simcyp Limited		in silico	Simcyp ADME Simulator, workshops, and consultancy for ADME data interpretation and extrapolation	Simcyp Population-based ADME Simulator – Simcyp develops a user-friendly platform that integrates demographic, physiological, genomic and in vitro biochemical data to allow you to predict drug absorption and disposition in representative virtual patient populations. Extrapolation from in vitro to in vivo	http://www.simcyp.com/ProductServices/Simulator/	Mostly pharma, I think, but potential useful
Straticell	Belgium	in vitro	CRO for in vitro regulatory safety testing (e.g. REACH) and in vitro efficacy testing; also manufactures reconstituted skin models	Most of our relevant assays - see website	http://www.straticell.com	Very useful

Name	Country	System	Short description	Services	Contact	Comment
VitroCell Systems	Germany	in vitro	contract research using human cells; VitroCell chamber for in vitro studies of gases or complex mixtures	We offer customers from industry and research institutes new culture and exposure systems for in vitro studies of gases or complex mixtures. The VITROCELL® modules offer new possibilities for the characterization of the effects of airborne substances	http://www.vitrocell.com	potentially useful, especially for exposure part. Not much info if they perform tox tests themselves, or only the exposure system
XCellR8	UK	in vitro	contract research for development and optimization of cell-based methods and in vitro toxicology testing	In vitro toxicology testing, including validated and approved methods for raw materials or final formulations	http://www.x-cellr8.com/contractresearch.aspx	Potential useful
Molcode	Estonia	in silico	Molcode is a data holder for computationally predicted toxicological endpoints. All the predictions are based on Molcode developed and ECHA/JRC validated QSAR models and are in accordance with OECD criteria for QSARs.	C.1 / 3.3 Acute toxicity for fish (Danio rerio) C.1 / 3.3 Acute toxicity for fish (Fathead minnow) C.1 / 3.3 Acute toxicity for fish (Rainbow trout) C.2 / 3.2 Acute Toxicity for Daphnia C.11 / 3.6 Activated sludge respiration inhibition C.13 / 2.4a Bioconcentration factor: flow - through fish test C.17 Honeybees - acute contact toxicity test C.19 / 2.6 Organic carbon-sorption partition coefficient Koc B.1 tris / 4.2 Acute Oral Toxicity - Acute toxic Class Method B.1 tris / 4.2 Acute Oral Toxicity- in vitro (cytotoxicity) B.5 / 4.9 Acute toxicity: eye irritation/corrosion B.13/14 / 4.10 Mutagenicity: Reverse mutation test using bacteria B.42 /4.4 Skin sensitisation B.32 / 4.12 Carcinogenicity test (Female rat) 4.18a Estrogen receptor binding affinity 5.3 Permeability – Caco-2 monolayer 5.3 Permeability – PAMPA 5.4 Blood-brain barrier partition 5.9 Human Serum Albumin Binding Toxicity to Tetrahymena pyriformis	http://www.molcode.com/reach-service	Seems useful for QSAR

Name	Country	System	Short description	Services	Contact	Comment
SBW	Finland	in vitro / in vivo / in silico	Information on potential adverse effects and toxicity of your lead molecules is valuable already at the discovery stage of your project, and this data are highly crucial in selecting potential drug candidates for further development. We support you in the lead optimization and drug candidate selection with toxicology and safety pharmacology services. Our comprehensive research approach yields more information from a single study. Studies are done either fully under GLP standards or non-GLP with internal SOPs. Study reconstruction is ensured through adequate documentation of study conduct, and archiving of data.	* In vitro cytotoxicity, cell viability and apoptosis models * In vitro genotoxicity models * In vivo toxicity models * In vivo safety models * Cardiovascular safety models * Central nervous system safety models * Gastro-intestinal safety models * Respiratory system safety models * Regulatory guidance and consulting	http://www.sbw.fi/pre-clin	Potential useful
CometBiotech	Norway	in vitro	in vitro contract testing using human (mammalian model), human and environmental monitoring, R&D, and related activities and consulting	• In vitro cytotoxicity, cell viability (clonogenic assay, proliferation assay), • Phototoxicity and photogenotoxicity, • In vitro genotoxicity – comet assay, cytogenicity (micronucleus assay, chromosomal aberration) • Mammalian HPRT mutation assay • In vitro transformation assay (SHE) • In silico methods	NILU/Oslo University spin off company	Just established, GLP under preparation
LAB Research Inc (formerly Scantox)	Denmark / Hungary	in vivo / in vitro		Biological Control & Potency Testing General Toxicology Cytotoxicity & Genotoxicity Reproduction / Fertility Carcinogenicity Immunotoxicity Specializations Juvenile Toxicology Biocompatibility of Medical Devices Minipig Toxicology Pathology Services Pharmacology Services Dermal Studies	http://www.labresearch.com/home.htm	
contact laboratory.com (http://www.contractlaboratory.com/labclass/toxicology.cfm) can help finding appropriate laboratories for our needs						

Appendix H

Deliverable D.1 – Summary of Compiled Toxicity Data

Nitrosamines							
			ESTIMATES of CANCEROGENIC RISK to human[h] *http://www.epa.gov/IRIS/*				
CAS registry	name	IUPAC	Oral slope factor(s): per mg/kg/day	Inhalational unit risk(s): per ug/m3	Drinking water unit risk(s): per ug/L	Inhalation concentration at specified risk level E-06 (ug/m3)	Drinking water concentration at specified risk level E-06 (ug/L)
62-75-9	Nitrosodimethylamine (NDMA)	N-nitrosodimethylamine	5,10E+01	1,40E-02	1,40E-03	7,00E-05	7,00E-04
55-18-5	Nitrosodiethylamine (NDEA)	N-ethyl-N-nitroso-ethanamine	1,50E+02	4,30E-02	4,30E-03	2,00E-05	2,00E-04
10595-95-6	Nitrosomethylethylamine	n-methyl-n-nitrosoethanamine	2,20E+01	ND	6,30E-04	ND	2,00E-03
924-46-9	nitrosomethylpropylamine	n-methyl-n-nitroso-1-propanamine	ND	ND	ND	ND	ND
621-64-7	Nitrosodipropylamine	n-nitroso-n-propyl-propanamine	7,00E+00	ND	2,00E-04	ND	5,00E-03
601-77-4	diisopropylnitrosamine	N-(1-methylethyl)-N-nitroso-2-propanamine	ND	ND	ND	ND	ND
924-16-3	nitrosodibutylamine	n-butyl-n-nitroso-1-butanamine	ND	ND	ND	ND	ND
997-95-5	diisobutylnitrosamine	Bisisobutyl-N-nitrosamine	ND	ND	ND	ND	ND
16339-04-1	1-methyl-N-nitroso-diethylamine	n-ethyl-n-nitroso2-propamamine	ND	ND	ND	ND	ND
7068-83-9	Butylmethylnitrosamine	N-methyl-n-nitroso1-butanamine	ND	ND	ND	ND	ND
4549-44-4	Buthylethylnitrosamine	n-ethyl-n-nitroso-1-butanamine	ND	ND	ND	ND	ND
5632-47-3	nitrosopiperazine	1-nitrosopiperazine	ND	ND	ND	ND	ND
16339-07-4	1-methylnitrosopiperazine	1-methyl-4-nitroso-piperazine	ND	ND	ND	ND	ND
59-89-2	N-Nitrosomorpholine	4-nitrosomorpholine	ND	ND	ND	ND	ND
930-55-2	Nitrosopyrrolidine	1-nitroso-pyrrolidine	2,10E+00	6,10E-04	6,10E-05	2,00E-03	2,00E-02
100-75-4	Nitrosopiperidine	1-nitroso-piperidine	ND	ND	ND	ND	ND
1116-54-7	Nitrosodiethanolamine (NDELA)	N-Nitrosodiethanolamine	2,80E+00	ND	8,00E-05	ND	1,00E-02
26921-68-6	Nitrosomethylethanolamine	N-Nitrosomethyl-(2-hydroxyethyl)amine	ND	ND	ND	ND	ND
3817-11-6	butylbutanolnitrosamine	4-(butylnitrosamino)-1-butanol	ND	ND	ND	ND	ND
39884-52-1	N-nitroso-1,3-oxazolidine	N-Nitroso-1,3-oxazolidine (ChemId)	ND	ND	ND	ND	ND
35627-29-3	N-nitrosotetrahydro-1,3-oxazine	3-nitroso-1,3-oxazinane	ND	ND	ND	ND	ND
35631-27-7	N-nitroso-5-methyl-1,3-oxazolidine	5-methyl-3-nitroso-1,3-oxazolidine	ND	ND	ND	ND	ND
39884-58-7	N-nitroso-2-isopropyl-4,4-dimethyl-1,3-oxazolidine	4,4-dimethyl-3-nitroso-2-(propan-2-yl)-1,3-oxazolidine	ND	ND	ND	ND	ND

Nitramines							
CAS registry	**name**	**IUPAC**					
4164-28-7	Dimethylnitramine	N-methyl-N-nitro- Methanamine	ND	ND	ND	ND	ND
7119-92-8	Diethylnitramine	N-ethyl-N-nitro-ethanamine	ND	ND	ND	ND	ND
4164-29-8	Dipropylnitramine	Di-n-propylnitramine	ND	ND	ND	ND	ND
108249-27-0	Dimethanolnitramine (N,N-dimethylolnitramine)	N,N-bis(hydroxymethyl)nitramide	ND	ND	ND	ND	ND
13084-48-5	Diethanolnitramine	2,2'-(nitroimino)diethanol	ND	ND	ND	ND	ND
4164-32-3	Morpholine nitramine?, 4-Nitromorpholine	N-Nitromorpholine	ND	ND	ND	ND	ND
42499-41-2	Piperazine nitramine?, 1-nitropiperazine	1-nitropiperazine	ND	ND	ND	ND	ND
598-57-2 and 11328	Methylnitramine	N-nitromethanamine	ND	ND	ND	ND	ND
19091-98-6	Ethylnitramine, N-nitroethylamine	N-nitroethanamine	ND	ND	ND	ND	ND
627-07-6	Propylnitramine	N-nitropropan-1-amine	ND	ND	ND	ND	ND
74386-82-6	Ethanolnitramine	2-(nitroamino)ethanol	ND	ND	ND	ND	ND
?	Dimethylethanolnitramine	2-methyl-2-(nitroamino)propan-1-ol	ND	ND	ND	ND	ND
51883-27-3	N-nitroformamide	N-nitroformamide	ND	ND	ND	ND	ND
32818-80-7	[methyl(nitro)amino]methanol	Metylnitroamino-methanol	ND	ND	ND	ND	ND
42499-46-7	2-[methyl(nitro)amino]ethanol	2-(methylnitroamino)-ethanol	ND	ND	ND	ND	ND

Nitrosamines

				RTECS database[e]			
CLASSIFICATION OF CANCEROGENICITY[i]	***CLASSIFICATION IN EU[j]***			**Toxicity**			
by IARC, EPA and NTP	**Symbol**	**Risk**	**Safety**	**LD50 oral rat (mg/kg)**	**Classification Criteria for LD50[b]**	**LC50 inhalation rat**	**Human**
2A, regarded as carcinogenic to humans	T+, N	45-25-26-48/25-51/53	53-45-61	37/26[d]	EU class T+, very toxic[c]	Rat LC50 78 ppm (4h	20mg/kg/2.5Y (Woman LDLo oral)
2A, regarded as carcinogenic to humans	ND	ND	ND	220/280[d]	Harmful	ND	ND
2B, regarded as carcinogenic to humans	ND	ND	ND	90	Toxic	ND	ND
ND	ND	ND	ND	106 (sub[a])	-	ND	ND
2B, regarded as carcinogenic to humans	T, N	45-22-51/53	53-45-61	480	EU class T, Toxic	ND	ND
ND	ND	ND	ND	850	Harmful	ND	ND
2B, regarded as carcinogenic to humans	ND	ND	ND	1200	Harmful	ND	ND
ND	ND	ND	ND	5600 (sub[a], hamster)	-	ND	ND
ND	ND	ND	ND	1100	Harmful	ND	ND
ND	ND	ND	ND	130	Toxic	ND	ND
ND	ND	ND	ND	380	Harmful	ND	ND
ND	ND	ND	ND	2260	-	ND	ND
ND	ND	ND	ND	100	Toxic	ND	ND
2B, regarded as carcinogenic to humans	ND	ND	ND	282	Harmful	1000 mg/kg/10M (m	ND
2B, regarded as carcinogenic to humans	ND	ND	ND	900	Harmful	ND	ND
2B, regarded as carcinogenic to humans	ND	ND	ND	200	Toxic	>500 mg/m3/10M (r	ND
2B, regarded as carcinogenic to humans	T	45	45-53	7500	EU class T, Toxic	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	1800	Harmful	ND	ND
ND	ND	ND	ND	1500	Harmful	ND	ND
ND	ND	ND	ND	600	Harmful	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND

Nitramines

				RTECS database[e]			
CLASSIFICATION OF CANCEROGENICITY[i]:	***CLASSIFICATION IN EU[j]***			**Toxicity**			
by IARC, EPA and NTP	**Symbol**	**Risk**	**Safety**	**LD50 oral rat (mg/kg)**	**Classification Criteria for LD50[b]**	**LC50 inhalation rat**	**Human**
ND	ND	ND	ND	1095	Harmful	ND	ND
ND	ND	ND	ND	730 (IP, mouse)	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	500 (IP, mouse)	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND
ND	ND	ND	ND	ND	-	ND	ND

Nitrosamines

The Carcinogenic Potency Database (CPDB)[f] (accessed 30th, June 2010)	RTECS[e] and Toxnet[g] databases (accessed 12th, August 2010)			
	Carcinogenicity	**Mutagenicity**	**Reproductive data[e]**	
TD50 oral rat (mg/kg/day)	**Result**	**Result**	**TDLo (rat, oral, mg/kg body weight)**	**Effect**
0.0959[m,v]	Positive	Positive (mostly)	20/30/35/30/35/5(IP)/20(IV)/10(IP)/1.94 (oral, mouse)/0.5 (intraplacental)	Fetotoxicity/fetal death/abortion etc/classified as a reproductive effector (RTECS criteria)
0.0265[m,v]	Positive	Pos/neg/no concl.	200/158/51/180/180(IP)/70(IP)/140(IV)/100 (IP,mouse)/150(oral,hamster)/53.4(sub,hamster)/0.5 (intraplacental)	Fetotoxicity/fetal death/classified as a reproductive effector (RTECS criteria)
0,0503	Positive	Pos/neg/no concl.	ND	ND
ND (might be possible to determine)	Positive	Positive(8)	ND	classified as a reproductive effector (RTECS criteria)
0,186	Positive	No concl./ pos/pos	ND	classified as a reproductive effector (RTECS criteria)
ND	ND	Positive (1)	ND	ND
0,691	Positive	Pos/neg/no concl.	1200/1200/1000(IP)/30(sub)	Fetotoxicity/fetal death/classified as a reproductive effector (RTECS criteria)
ND	Uncertain	Positive (1)	ND	ND
ND	Uncertain	ND	ND	ND
ND (might be possible to determine)	Positive	Positive (6) No concl 1)	ND	ND
ND (might be possible to determine)	Positive	Positive (3)	ND	ND
8.78[m,n]	Positive	Positive(4)	ND	ND
ND	Positive* (1 pos/inhalation, 3 equivocal tumorigen)	Positive (11) No concl (1)	ND	ND
0.109[m]	Positive (2 inhalation studies)	Positive(18+)/No concl.	ND	ND
0.799[m,P]	Positive	Positive	ND	ND
1.43[m]	Positive (1 inhalation study)	Positive (2 No concl.)	ND	classified as a reproductive effector (RTECS criteria)
3.17[m,v]	Positive	Pos(17)/neg(8)/No concl.	ND	ND
1,29	positive	Positive(2)	ND	ND
0.457[m,P,v]	Positive	Pos(13)/neg(3)/no concl(1).	ND	classified as a reproductive effector (RTECS criteria)
0.798[m] **(hamster)**	Positive	Positive(2)	ND	ND
ND	Uncertain	Positive (1)	ND	ND
ND (might be possible to determine)	Positive	Positive (2)	ND	ND
ND	ND	ND	ND	ND

Nitramines

The Carcinogenic Potency Database (CPDB)[f] (accessed 30th, June 2010)	RTECS[e] and Toxnet[g] databases (accessed 12th, August 2010)			
	Carcinogenicity	Mutagenicity	Reproductive data[e]	
TD50 oral rat (mg/kg/day)	Result	Result	TDLo (rat, oral, mg/kg body weight)	Effect
0.547[m,v]	Positive	Pos (3)/neg (1)	ND	ND
ND	Uncertain (1 test)	Pos (2)/neg (1)	ND	ND
ND	ND	ND	ND	ND
ND	ND	ND	ND	ND
ND	ND	ND	ND	ND
ND	ND	Positive (2)	ND	ND
ND	ND	ND	ND	ND
17.4[m]	Positive	Neg (2)/pos (1)	ND	ND
ND	ND	Positive (2)	ND	ND
ND	ND	ND	ND	ND
ND	ND	ND	ND	ND
ND	ND	ND	ND	ND
ND	ND	ND	ND	ND
ND	ND	Positive (2)	ND	ND
ND	ND	ND	ND	ND

a	Subcutaneous						
b	Very toxic: LD50 <= 25 mg/kg body weight	Reference:					
	Toxic: 25 < LD50 <= 200 mg/kg body weight	Commision directive 67/548/EEC Annex V, Commision directive 2001/59/EC)					
	Harmful: 200 < LD50 <= 2000 mg/kg body weight						
	No classification: 2000 mg/kg body weight < LD50						
c	CLASSIFICATION IN EU (http://ecb.jrc.ec.europa.eu/esis/)						
d	Two reports on LD50						
e	Canadian Centre for Occupational Health and Safety (CCOHS)- **RTECS** database, available at http://www.ccohs.ca/products/rtecs/ (accessed 12th, August 2010)						
f	The Carcinogenic Potency Database (CPDB) available at http://potency.berkeley.edu/(accessed 30th, June 2010)						
	The Carcinogenic Potency Database, developed at the University of California, Berkeley, and Lawrence Berkeley Laboratory, provides standardized analyses						
	of the results of 6540 chronic, long-term animal cancer tests that have been conducted since the 1950's and reported in the general published literature or						
	by the National Cancer Institute and the National Toxicology Program.						
g	United States National Library of Medicine - Toxnet (CCRIS database) available at http://toxnet.nlm.nih.gov/ (accessed 12th, August 2010)						
h	U.S. Environmental Protection Agency - ESTIMATES of CANCEROGENIC RISK to human - Integrated Risk Information System (IRIS) database available at http://www.epa.gov/IRIS/ (accessed 24th, June 2010)						
i	The WHO International Agency for Research on Cancer (IARC) CLASSIFICATION OF CANCEROGENICITY available at http://monographs.iarc.fr/ENG/Classification/index.php (accessed 24th, June 2010)						
	IARC Classification						
	Group 1	Carcinogenic to humans					
	Group 2A	Probably carcinogenic to humans					
	Group 3	Possibly carcinogenic to humans					
	Group 4	Not classifiable as to its carcinogenicity to humans					
	Group 5	Probably not carcinogenic to humans					
j	European Commision - Joint Resarch Centre - European Substances Information System (ESIS) database available at http://ecb.jrc.ec.europa.eu/esis/ (accessed 24th, June 2010)						
LD50	Lethal dose, 50 percent kill						
LC50	Lethal concentration, 50 percent kill						
LCLo	Lowest published lethal concentration						
TD50	Daily dose rate in mg/kg body weight/day to induce tumors in half of test animals that would have remained tumor-free at zero dose (http://potency.berkeley.edu/index.html)						
TDLo	Lowest published toxic dose						
LC	Lethal concentration						
ppm	Part per million						
IP	Intraperitoneal						
IV	Intravenous						
Sub	Subcutaneous						
m	There is more than one positive experiment in the species, and TD_{50} values from each positive experiment are used in the calculation of the reported Harmonic mean of TD_{50}.						
v	Variation is greater than ten-fold among statistically significant (two-tailed p <0.1) TD_{50} values from different positive experiments.						
ND	No data found/available						
*	Only one study shows positive for inhalation						

Ref 1	Group 2A: The agent is probably carcinogenic to humans	IARC. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Geneva: World Health Organization, International Agency for Research on Cancer, 1972-PRESENT. (Multivolume work). Available at: http://monographs.iarc.fr/index.php p. S7 67 (1987)] **PEER REVIEWED**
Ref 2	Hazardous Substances Data Bank (HSDB)	[NIOSH. NIOSH Pocket Guide to Chemical Hazards. DHHS (NIOSH) Publication No. 94-116. Washington, D.C.: U.S. Government Printing Office, June 1994., p. 232] **PEER REVIEWED**
		[Budavari, S. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. Whitehouse Station, NJ: Merck and Co., Inc., 1996., p. 1139] **PEER REVIEWED**
		[IARC. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Geneva: World Health Organization, International Agency for Research on Cancer, 1972-PRESENT. (Multivolume work). Available at: http://monographs.iarc.fr/index.php p. V17 221 (1978)] **PEER REVIEWED**
		[IARC. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Geneva: World Health Organization, International Agency for Research on Cancer, 1972-PRESENT. (Multivolume work). Available at: http://monographs.iarc.fr/index.php p. V17 177 (1978)] **PEER REVIEWED**
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Version		September 06 2010

Appendix I

Deliverable D.1 – Summary of Data Gap Analysis

Nitrosamines		8. Toxicological information	Annex VIII	Annex VIII		
		8.1 skin irritation or skin corrosion[l]	8.2 Eye irritation[l]	8.3 Skin sensitation[l]	8.4 Mutagenicity[j,l,m]	Annex VIII
CAS registry	RTECS record	Steps 1-4	Steps 1-3	Steps 1-2	8.4.1 In vitro gene mutation study in bacteria	8.4.2 In vitro cytogenicity in mammalian cells[a]
62-75-9	YES	ND	ND	ND	2A, regarded as carcinogenic to humans[j]	2A
55-18-5	YES	ND	ND	ND	2A, regarded as carcinogenic to humans[j]	2A
10595-95-6	YES	ND	ND	ND	2B, regarded as carcinogenic to humans[j]	2B
924-46-9	YES	ND	ND	ND	5 pos in RTECS	ND
621-64-7	YES	ND	ND	ND	2B, regarded as carcinogenic to humans[j]	2B
601-77-4	YES	ND	ND	ND	1 pos in RTECS	ND
924-16-3	YES	ND	ND	ND	2B, regarded as carcinogenic to humans[j]	2B
997-95-5	YES	ND	ND	ND	1 pos in RTECS	ND
16339-04-1	YES	ND	ND	ND	ND	ND
7068-83-9	YES	ND	ND	ND	4 pos in RTECS, 1 pos 1 no concl in GENE-TOX	ND
4549-44-4	YES	ND	ND	ND	1 pos in RTECS	ND
5632-47-3	YES	ND	ND	ND	3 pos in RTECS, 1 pos in GENE-TOX	ND
16339-07-4	YES	ND	ND	ND	5 pos in RTECS, 1 pos 1 no concl in GENE-TOX	ND
59-89-2	YES	ND	ND	ND	2B, regarded as carcinogenic to humans[j]	2B
930-55-2	YES	ND	ND	ND	2B, regarded as carcinogenic to humans[j]	2B
100-75-4	YES	ND	ND	ND	2B, regarded as carcinogenic to humans[j]	2B
1116-54-7	YES	ND	ND	ND	2B, regarded as carcinogenic to humans[j]	2B
26921-68-6	YES	ND	ND	ND	ND	ND
3817-11-6	YES	ND	ND	ND	2 pos in RTECS, 3 pos 3 neg in CCRIS	2 pos in RTECS, 1 neg GENE-TOX
39884-52-1	YES	ND	ND	ND	2 pos in RTECS	ND
35627-29-3	YES	ND	ND	ND	1 pos in RTECS	ND
35631-27-7	YES	ND	ND	ND	2 pos in RTECS	ND
39884-58-7	NO	ND	ND	ND	ND	ND

Nitramines						
CAS registry						
4164-28-7	YES	ND	ND	ND	2 pos in RTECS, 1 pos 1 neg in CCRIS	ND
7119-92-8	YES	ND	ND	ND	2 pos in RTECS, 1 neg in CCRIS	ND
4164-29-8	YES	ND	ND	ND	ND	ND
108249-27-0	NO	ND	ND	ND	ND	ND
13084-48-5	NO	ND	ND	ND	ND	ND
4164-32-3	YES	ND	ND	ND	2 pos in RTECS	ND
42499-41-2	NO	ND	ND	ND	ND	ND
598-57-2 and 11328	YES	ND	ND	ND	2 neg in CCRIS	ND
19091-98-6	YES	ND	ND	ND	1 pos in RTECS, 1 pos in CCRIS	ND
627-07-6	NO	ND	ND	ND	ND	ND
74386-82-6	NO	ND	ND	ND	ND	ND
?	NO	ND	ND	ND	ND	ND
51883-27-3	NO	ND	ND	ND	ND	ND
32818-80-7	YES	ND	ND	ND	1 pos in RTECS, 1 pos in CCRIS	ND
42499-46-7	NO	ND	ND	ND	ND	ND

	Annex VIII
	Annex IX
	Annex X
	Data is missing/further testing is needed
	Data may not be reliable/further studies nessary
	No furter testing necessary
ND	No data available. Annex endpoints not found in available litterature or databases
a	No cytogenicity test are usually needed if the substance is known to be carcinogenic category 1 or 2 or adequate data from an in vivo cytogenicity test are available
b	Need to be done if the results from annex VII 8.4.1 and VIII 8.4.2 are negative
c	In vivo somatic cell genotoxicity study need to be performed according to annex IX.
d	Specific rules of adaptation states that in adition to the oral route at least one addition route of exposure, i.e. inhalation needs to be tested for acute toxicity.
e	No testing needed if available information on related substances(read-across), (Q)SAR estimates or in vitro methods that the substance may be a developmental toxicant. or the substance is known to be a genotoxic carcionogen, germ cell mutagen or there are available pre-natal developmental toxicicity studies or a two generation reproductive toxicity study.
f	If available data supports these compound to be genotoxic carcionogen or germ cell mutagens, no screening for developmental toxicity is needed.
g	Most appropriate route of adminsitration is through the inhalation route on female and male rats when regarding the likely route of exposure. Sub-chronic toxicity testing may be avoided if the substance showes severe toxicity effect (classified as R48) for which the observed NOAEL-28 days may allow an extrapolation to a NOAEL-90 days.
h	May be proposed by registrant or agency. See annex X.
i	May be proposed by registrant or agency, based on long-term human exposure or compound beeing classified as mutagen category 3. Carcinogenicity testing may not be required if the substance is a mutagen category 1 or 2.
j	The WHO International Agency for Research on Cancer (IARC) CLASSIFICATION OF CANCEROGENICITY available at http://monographs.iarc.fr/ENG/Classification/index.php (accessed XXth, June 2010) IARC Classification Group 1 Carcinogenic to humans Group 2A Probably carcinogenic to humans Group 3 Possibly carcinogenic to humans Group 4 Not classifiable as to its carcinogenicity to humans Group 5 Probably not carcinogenic to humans
k	If there is a positive result in any of the in vitro genotoxicity studies and there are no results available from an in vivo study already, an appropriate in vivo somatic cell genotoxicity study shall be propo All historical data, in vivo studies and QSAR models should be taken under consideration before the specific rule under Annex IX takes under consideration
l	Canadian Centre for Occupational Health and Safety (CCOHS)- RTECS database, available at http://www.ccohs.ca/products/rtecs/ (accessed 12th, August 2010)
m	United States National Library of Medicine - Toxnet (CCRIS database) available at http://toxnet.nlm.nih.gov/ (accessed 12th, August 2010)

Annex VIII	Annex IX	Annex X	8.5 Acute toxicity[j]	Annex VIII
8.4.3 In vitro gene mutation study in mammals[b]	8.4 Specific rules[k]	8.4 Specific rules (quality and relevance of available data)	8.5.1 By oral route	8.5.2 By inhalation
2A	2A	2A	Available (7 oral + many additional studies)	Available
2A	2A	2A	Available (5 oral + many additional studies)	ND[d]
2B	2B	2B	Available (1 study)	ND[d]
1 pos in RTECS, 2 pos in CCRIS	Positive for carcinogenicity	Positive for carcinogenicity	subcutaneous (3 studies)	ND[d]
2B	2B	2B	Available (2 oral + 4 additional studies)	ND[d]
ND	ND[c] (QSAR possibility? See anne	ND[c] (QSAR possibility? See annex XI)	Available (1 study)	ND[d]
2B	2B	2B	Available (2 oral + 3 additional studies)	ND[d]
ND	Uncertain in vivo results, additio	Uncertain in vivo results, additional studies/QSAR suggested	sub, hamster (1 study)	ND[d]
ND	Uncertain in vivo results, additio	Uncertain in vivo results, additional studies/QSAR suggested	Available (1 study)	ND[d]
ND	Positive for carcinogenicity	Positive for carcinogenicity	Available (2 oral + 3 additional studies)	ND[d]
2 pos in RTECS	Positive for carcinogenicity	Positive for carcinogenicity	Available (1 oral + 1 additional studies)	ND[d]
ND	Positive for carcinogenicity	Positive for carcinogenicity	Available (1 study)	ND[d]
3 pos in RTECS, 1 pos GENE-TOX	Positive for inhalation (1 study	Positive for inhalation (1 study only)	Available (1 study)	ND[d]
2B	2B	2B	Available (2 oral + 5 additional studies)	inhalation-mouse
2B	2B	2B	Available (3 oral + 1 additional studies)	ND[d]
2B	2B	2B	Available (2 oral + 4 additional studies)	inhalation-mouse
2B	2B	2B	Available (1 oral + 1 additional studies)	ND[d]
2 pos in RTECS	Positive (1 study) for carcinogen	Positive (1 study) for carcinogenicity	ND	ND[d]
5 pos in RTECS, 1 pos 1 no concl GENE-TOX	Positive for carcinogenicity	Positive for carcinogenicity	Available (1 oral + 1 additional studies)	ND[d]
ND	Positive for carcinogenicity	Positive for carcinogenicity	Available (1 study)	ND[d]
ND	Positive (1 study, high concentra	Positive (1 study, high concentration) for carcinogenicity	Available (1 study)	ND[d]
ND	Positive for carcinogenicity	Positive for carcinogenicity	ND	ND[d]
ND	ND[c] (QSAR possibility? See anne	ND[c] (QSAR possibility? See annex XI)	ND	ND[d]

Annex VIII	Annex IX	Annex X	8.5 Acute toxicity[j]	Annex VIII
8.4.3 In vitro gene mutation study in mammals[b]	8.4 Specific rules[k]	8.4 Specific rules (quality and relevance of available data)	8.5.1 By oral route	8.5.2 By inhalation
ND	Positive for carcinogenicity	Positive for carcinogenicity	Available (1 oral + 2 additional studies)	ND[d]
ND	Uncertain in vivo results, additio	Uncertain in vivo results, additional studies/QSAR suggested	IP, mouse (1 study)	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]
1 pos in RTECS	Positive for carcinogenicity	Positive for carcinogenicity	IP, mouse (1 study)	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]
ND	ND[c]	ND[c]	ND	ND[d]

Annex VIII	Annex VIII 8.6 Repeated dose toxicity[j]	Annex IX	Annex X	Annex X
8.5.3 By dermal route	8.6.1 Short-term repeated dose toxicity study (28 days)	8.6.2 Sub-chronic toxicity study (90-day)[g]	8.6.3 Long-term repeated toxicity study (12-months)[h]	8.6.4 Further studies[h]
Not relevant	Available (8 studies with 28-d or more-TDLo)	Available (3 studies with 90-d or more-TDLo)	ND	ND
Not relevant	Available (6 studies with 28-d or more-mostly TDLo)	Available (2 studies with 90-d or more-TDLo, 3 studies with	Available (2 studies with 12-month-TDLo)	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	Available (3 studies with 28-d or more-TDLo)	"Available" (2 studies with 49-56-d TDLo, extrapolation?)	ND	ND
Not relevant	Available (1 study with 28-d or more-TDLo)	Available (1 study with 90-d or more-TDLo)	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	Available (3 studies with 28-d or more-TDLo)	Available (3 studies with ~90-d or more-TDLo)	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND

Annex VIII	Annex VIII 8.6 Repeated dose toxicity[j]	Annex IX	Annex X	Annex X
8.5.3 By dermal route	8.6.1 Short-term repeated dose toxicity study (28 days)	8.6.2 Sub-chronic toxicity study (90-day)[g]	8.6.3 Long-term repeated toxicity study (12-months)[h]	8.6.4 Further studies[h]
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND
Not relevant	ND	ND	ND	ND

Annex VIII		Annex IX	Annex VIII
8.7 Reproductive toxicity (RTECS database, accessed online 13t	Annex IX	Annex IX	8.8 Toxicokinetics
8.7.1 Screening for reproductive/developmental toxicity[a]	8.7.2 Pre-natal developmental toxicity[a]	8.7.3 Two-generation reproductive toxicity study[a]	8.8.1 Assessment of the toxicokinetic behaviour
2A[e,f]	2A[e,f]	2A[e,f]	Detailed assessment of available long term/chronic to:
2A[e,f]	2A[e,f]	2A[e,f]	Detailed assessment of available long term/chronic to:
2B[e,f]	2B[e,f]	2B[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
2B[e,f]	2B[e,f]	2B[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
2B[e,f]	2B[e,f]	2B[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
2B[e,f]	2B[e,f]	2B[e,f]	Detailed assessment of available long term/chronic to:
2B[e,f]	2B[e,f]	2B[e,f]	Detailed assessment of available long term/chronic to:
2B[e,f]	2B[e,f]	2B[e,f]	ND
2B[e,f]	2B[e,f]	2B[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	Detailed assessment of available long term/chronic to:
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND

Annex VIII		Annex IX	Annex VIII
8.7 Reproductive toxicity (RTECS database, accessed online 13t	Annex IX		8.8 Toxicokinetics
8.7.1 Screening for reproductive/developmental toxicity[e]	8.7.2 Pre-natal developmental toxicity[e]	8.7.3 Two-generation reproductive toxicity study[e]	8.8.1 Assessment of the toxicokinetic behaviour
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND
ND[e,f]	ND[e,f]	ND[e,f]	ND

	(Q)SAR possibilities		
Annex X	Cancerogenic potency estimation	Acute toxicity	Short-term repeated dose toxicity (28 days)
8.9.1 Carcinogenicity study[i]	TD50 oral rat (mg/kg/day)	LD50 oral rat (mg/kg/day)	NOAEL (No Observed Adverse Effect level)
2A	Available data	Available data	Available data
2A	Available data	Available data	Available data
2B	Available data	Available data	QSAR estimation NOT possible
Positive for carcinogenicity/additional st	QSAR estimation possible	QSAR estimation possible	QSAR estimation NOT possible
2B	Available data	Available data	QSAR estimation NOT possible
ND (QSAR possibility? See annex XI)	QSAR estimation possible	Available data	QSAR estimation NOT possible
2B	Available data	Available data	QSAR estimation NOT possible
Uncertain in vivo results, additional stud	QSAR estimation possible	QSAR estimation possible	QSAR estimation NOT possible
Uncertain in vivo results, additional stud	QSAR estimation possible	Available data	QSAR estimation NOT possible
Positive for carcinogenicity/additional st	QSAR estimation possible	Available data	QSAR estimation NOT possible
Positive for carcinogenicity/additional st	QSAR estimation possible	Available data	QSAR estimation NOT possible
Positive for carcinogenicity/additional st	Available data	Available data	QSAR estimation NOT possible
Positive for inhalation (1 study only), su	QSAR estimation possible	Available data	QSAR estimation NOT possible
2B	Available data	Available data	Available data
2B	Available data	Available data	Available data
2B	Available data	Available data	QSAR estimation NOT possible
2B	Available data	Available data	QSAR estimation NOT possible
Positive (1 study) for cacerogenicity, sup	Available data	QSAR estimation possible	QSAR estimation NOT possible
Positive for carcinogenicity/additional st	Available data	Available data	Available data
Positive for carcinogenicity/additional st	Available data	Available data	QSAR estimation NOT possible
Positive (1 study, high concentration) fo	QSAR estimation possible	Available data	QSAR estimation NOT possible
Positive for carcinogenicity/additional st	QSAR estimation possible	QSAR estimation possible	QSAR estimation NOT possible
ND (QSAR possibility? See annex XI)	QSAR estimation possible	QSAR estimation possible	QSAR estimation NOT possible

	(Q)SAR possibilities		
Annex X	Cancerogenic potency estimation	Acute toxicity	Short-term repeated dose toxicity (28 days)
8.9.1 Carcinogenicity study[i]	TD50 oral rat (mg/kg/day)	LD50 oral rat (mg/kg/day)	NOAEL (No Observed Adverse Effect level)
Positive for carcinogenicity/additional st	Available data	Available data	QSAR estimation NOT possible
Uncertain in vivo results, additional stud	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
Positive for carcinogenicity/additiona st	Available data	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible
ND	QSAR estimation NOT possible	QSAR estimation NOT possible	QSAR estimation NOT possible

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