OR 71/2011

Norsk institutt for luftforskning
Norwegian Institute for Air Research

Alternative approaches to standard toxicity testing

TQP ID 9 - OPTION - 257430181 - NILU

Mikael Harju, Solveig Ravnum, Elise Rundén Pran, Sonja Grossberndt,
Lise Marie Fjellsbø, Maria Dusinska and Eldbjørg S. Heimstad

Scientific report

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.

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. Amine – based solvent technology is one technology option for CO2 capture.

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 standard toxicity testing is one of several activities that has been launched for methods and tools development in the technology qualification phase of the CCM project development. This report is an continuation to report TQP ID 9 - 257430120 – NILU which included 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. Results / information gained by this option (TQP ID 9 – OPTION - 257430181 – NILU) are meant to be used in chemical risk assessment in the CCM project, and the report will be used as a reference.

Contents

Summary

The project report comprises an evaluation of a list of a new group of 13 substances, nitrosated and nitrated aminoacids, by literature and database searching, and thereby expanding the D1 spreadsheet with physicochemical data and (eco)toxicological endpoints for these substances. Furthermore clarifications of some general toxicological concepts and parameters, such as mutagenic potency, DNEL/DMEL and TD50/T25, have been provided. An extended evaluation of use and validation of QSAR models have been carried out

We found human related toxicological data for 13 compounds, except two. Among them two even had TD50 values available in the CPBD database. Ecotoxicological data were found for a few compounds but at least predicted physicochemical data are available for most of them. The data we found were implemented into an excel spreadsheet.

Genotoxic carcinogens give non-threshold effects due to their potential to induce permanent genetic changes in cells even in low non-cytotoxic doses. There have been discussions whether mutagenic potency can be correlated to carcinogenic potency. Quantification and applicability of mutagenic potency was evaluated and discussed. There is no clear indication that mutagenic potency correlates quantitatively with carcinogenic potency. However, recent meta-analysis results from data on human studies give good evidence of a semi-quantitative correlation. Testing strategy approaches for applying genotoxic results into carcinogenic risks are discussed and several tiers strategy is suggested. There is no international consensus when it comes to human risk assessment of genotoxic substances. Low-dose extrapolation is assessed by different approaches in different countries. We discuss several available methods and suggest a strategy for low-dose extrapolation of certain nitrosamines and nitramines for estimation of DMELs. The DMELs calculated in this report do not differ significantly from those that were reported by the NIPH recently. Also the DMELs for the nitrosamines and for the one nitramine calculated here are within the same range of concentration. Therefore it must be possible to extrapolate the risks in-between these compounds, if no other data are available. Furthermore, if the exposure level is well below the anticipated risk level, we recommend that only *in vitro* and *in silico* tests should be carried out from the suggested testing strategy; initially a **1st Tier** the (Q)SARs and the Ames test with two short term *in vitro* genotoxicity mammalian tests, and then a **2nd Tier** with the *in vitro* cell transformation tests.

Current status on the use of non-test methods in REACH dossiers with respect to the implementation of an (Q)SAR approach within REACH registration was made by ECHA in 2010. Results of the summary stated that the registry of compounds within REACH mainly used existing animal studies, read-across and weight of evidence in the REACH registration dossiers to fulfil the information requirements based on Annex IX (Regulation (EC) No 1907/2006, Directive 67/548/EEC). Only in a few instances have a (Q)SAR approach been used in the dossiers. Many of the QSAR approaches, made in the dossiers, was flawed and the model and prediction was not reported in detail as is demanded from the QMRF and QPRF formats and would not be valid in its current form.

Furthermore, there was generally limited information about the (Q)SAR model (e.g. version unclear, data on the model not transparent/ available), the scientific validity of model was not always demonstrated, applicability domain of the model was not or only partially analysed and results was not relevant for regulatory purposes and as such the endpoint predicted was not suitable to meet the information requirements of REACH. The conclusion is that there is still gaps in the REACH guidance documentation as there is no formal validation and adoption procedures for (Q)SAR models and detailed criteria for assessing the adequacy of (Q)SAR predictions is lacking also more examples is needed to illustrate how to demonstrate adequacy of a (Q)SAR model and how to successfully implement (Q)SARs for REACH information requirements.

As part of the ITS approach the feasibility for the use of QSAR to predict toxicity was evaluated. The QSAR information requirements were evaluated in accordance to REACH Annex VII-X. Available toxicity information and data were compiled with the use of the CPDB database for 94 nitrosamine compounds and the data was evaluated before the QSAR approach was undertaken.

Evidence exists that rodent carcinogenicity data can be modelled efficiently through a QSAR approach when qualitatively and quantitatively adequate data is available. Examples of successful QSAR models are abundant. The success of these models is mostly due to the use of appropriate sets of chemicals, belonging to the same class and acting through the same mechanism of action, an approach which is the most powerful for the creation of a predictive QSAR model. This is an approach which is fundamental for the applicability of *in silico* techniques such as (Q)SARs within REACH. Approaches which have been successful in QSAR modelling have been based on data for which a specific protocol for the rodent bioassay has been used (e.g. through the U.S: National Toxicology Program) while the use historic data of cancerogenic potency in rat shows some limitations on the quality and/or accuracy of the data.

This new QSAR model is an evaluation and development of the preliminary model created in the report TQP ID 9 - 257430120 – NILU. Results of the preliminary model were scrutinized based on the predictivity and normal distribution of TD_{50} data. It was discovered that the preliminary model was based on inaccurate data from a peer-reviewed publication and this model was discarded. For this new model we aimed at selecting a new set of additional *N*-nitroso compounds through the Berkley database and using the reported harmonic mean TD50 values of rat. We selected compounds which are assumed to have the same mechanism of action and are assumed to require activation by metabolization to the active mutagen. As the metabolization step is assumed to be the rate limiting step a QSAR model that would focus on this would be beneficial.

Unfortunately we were not able to create a valid model; the use of historical data such as the TD_{50} value in the Berkeley database is problematic due to unknown factors that will influence the assignment of harmonic mean TD_{50} values. *In vivo* bioassays on rat have been performed with non-standardised protocols, in many different laboratories, with different administration routes (through the food, gavage, water, and intravenous). Solubility of the compounds and efficacy of absorption through the gut into the bloodstream will be influenced by the route

but also on ADME properties. For some of the substances the results might be based on only a few animals which suggest that the "real" harmonic mean, if a protocol and enough animals would have been studied, might have been significantly improved.

A study of a congeneric set of chemicals and the use of a standardized *in vivo* rat protocol would improve the possibility of creating a valid QSAR model with a real predictive ability. A congeneric set of chemicals will have higher degree of close correlating properties such as solubility and lipophilicity as to have linearly correlating ADME properties and also to have a similar mechanism of action.

Abbreviations

3R	Reduce, refine and replace the use of animals for toxicity testing
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.
AF	Assessment Factor
ADI	Acceptable Daily Intake
ADME	Adsorption Distribution Metabolism Effect
ALARA	As Low As Reasonably Achievable
BMD	Benchmark Dose
CA	Chromosomal Abbreviations
CAS	Unique numerical identifiers for chemical elements, compounds, polymers, biological sequences, mixtures and alloys
CPDB	Carcinogenic Potency Database
CSA	Chemical Safety Assessment
CTA	Cell Transformation Assay
DMEL	Derived-Minimal-Effect-Level
DNEL	Derived-No-Effect-Level
DNA	Deoxyribonucleic acid: a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms
EBA	Exposure Based Adaptation
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
FDA	Food and Drug Administration (US regulation)
GLP	Good Laboratory Practice
HtLF	High to Low dose extrapolation factor
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.
LED	**Lowest Effective Dose**
LOAEL	**Lowest Observable Adverse Effect Level**
LMS	**Linearized multistage**

LSER Linear Solvation Energy Relationship

MN Micronucleus

MLR Multiple Linear Regression

MOE Margin of exposure

NIOSH National Institute for Occupational Safety and Health

NO(A)EL No Observable (Adverse) Effect Level

NTP National Toxicology Program

OECD Organisation for Economic Co-operation and Development

OSHA Occupational Safety and Health Administration

PCA Principal Component Analysis

PBMK Pharmacokinetic modelling

PNEC Predicted No Effect Concentration

PoD Point of Departure

(Q)SAR **(**Quantitative) Structure-Activity Relationship

REACH Registration, Evaluation, Authorisation and Restriction of Chemical

SA Structural Alerts

SHE Syrian Hamster Embryo

SMILES simplified molecular input line entry specification. SMILES is a specification for unambiguously describing the structure of chemical molecules using short ASCII strings

STT Short Term Tests

T25 Chronic dose rate which will give 25% of animals tumour at specific tissue site, after correction for spontaneous incidence, within the standard life-time of that species

TCC Threshold of toxicological concern

TDL Toxic Dose Level

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.

WHO **World Health Organization**

Alternative approaches to standard toxicity testing
TQP ID 9 - OPTION - 257430181 - NILU

1 Introduction

This option is a continuation of the contract ”CCM TQP Amine 9 - Alternative approaches to standard toxicity testing" from 2010. Results / information gained by this option are meant to be used in chemical risk assessment in the CCM project, and the report will be used as a reference.

The original aim of this study (“CCM TQP Amine 9 - Alternative approaches to standard toxicity testing" from 2010) was to verify (validate) the ITS approach.

2 Objectives

The frame agreement states the Service of the Tenderer to comprise the following objectives defined below:

Subtask 1: Expansion of the spreadsheet D1

- Further development and expansion of the spreadsheet D1 for the following list of substances, if available: Dinitrosopiperazine (CAS no 140-79-4), Nitrosated and Nitrated amino acids.

Subtask 2: General toxicology

- Quantification of mutagenic potency - estimating the carcinogenic potency of a substance based on mutagenicity test data.

- Evaluation of the interface between REACH requirements and concentration in relation to calculation of DMEL

- Evaluation of the correlation between TD50 and T25 and their applicability for derivation of DMELs and for use in risk assessment.

Subtask 3: QSAR as an alternative approach to toxicity testing

- The current status of QSAR as an applicable method for registering chemicals within REACH
- Estimation of uncertainty in the predicted TD50 values
- Improvement of the developed QSAR model (see original report)
- Training of Company personnel in the OECD QSAR Toolbox

3 Evaluation of the toxic properties of nitrated and nitrosated amino acids

3.1 Summary

Further development and expansion of the spreadsheet D1:
We have made an extensive summary spreadsheet with physicochemical, toxicological and ecotoxicological properties of a list of nitrosamines, nitramines, and nitrated and nitrosated amino acids (see below), defined by CCM, based on literature and databases search. We used ChemIdplus/TOXNET and RTECS databases to find toxicological data, the SciFinder database to find physicochemical data, and the EPI-Suite database to find ecotoxicological data. The work has been carried out without physical experiments or programming.

Toxicological, ecotoxicological and physicochemical endpoints have been included, when available, for the following compounds:

1. Nitrosamines and nitramines:
    - Dinitrosopiperazine (CAS no 140-79-4)

2. Nitrosated or nitrated amino acids
    - Glycine
    - Lysine
    - Taurine
    - β-alanine
    - L-proline
    - Sarcosine

All data in CTR1 are compiled in the spreadsheet D1 that can be found in Appendix A.

4 Evaluation of toxicological concepts related to genotoxic non-threshold substances

4.1 Scope and objectives

Part A
Genotoxic carcinogens give non-threshold effects due to their potential to induce permanent genetic changes in cells. In the Carcinogenic Potency Database (CPDB), the cancer potency of substances is expressed as TD50 values. It would be of interest to get a quantification of the mutagenic potency, e.g, by reporting the dose at which substances have shown to be mutagenic or the number of mutations at fixed doses. Correlation between mutagenic and carcinogenic potency is therefore a highly debated topic. We discuss the possibility of how the data obtained by mutagenicity testing can be used to estimate the carcinogenic potency of a substance and applied in risk assessment.

Part B
Human health risk assessment of non-threshold toxic substances is a heavily debated topic, and no consensus methodology exists internationally. Extrapolation modeling from high-dose animal exposures to low-dose human exposures can be crucial for the final risk calculation. Different extrapolation models are discussed, and suggestions on applications are given. Preferred methods for calculating derived minimal effect level (DMEL) are discussed and exemplified with selected nitrosamines and nitramines.

Evaluation of the correlation between the dose descriptors TD50 and T25, and their applicability:

- Evaluation of the possible correlation between TD50 and T25
- Discuss the applicability of TD50 and T25 for use in a risk assessment and for DMEL derivation specifically

Evaluation of the interface between REACH requirements and concentration levels in relation to calculation of DMEL:

- Evaluation of the term “well below" as defined by REACH, preferably expressed quantitatively
- Evaluation of what is valid if the exposure levels are found to be well below calculated DMELs, and which tests should be performedfrom the Integrated Testing Strategy (ITS), if possible
- Derivation of DMEL values are of interest to CCM. We will calculate DMELs for 5 nitrosamines and 2 nitramines according to ECHA/REACH if possible (ECHA/REACH_Chapter R8). Where REACH suggests different methods, the choice of method and assessment factors will be discussed.
    1. **Nitramines:** Methylnitramine and Dimethylnitramine (mg/kg/d to ng/m^3 & ng/l)
    2. **Nitrosamines:** 62-75-9, 55-18-5, 100-75-4, 59-89-2, and 140-79-4.

4.2 Background

Risk assessment of any substance consists of several steps such as hazard identification, hazard characterization, exposure assessment and risk characterization. Risk characterization consists of integrating information from the available data on hazard characterization and exposure assessment into an advice that can be used in decision-making. Several approaches are currently in use for risk characterization of substances with genotoxic and carcinogenic properties, within the EU and globally (ECHA R8 2008, EFSA 2005, EPA 2005). Genotoxic substances have the potential to directly or indirectly interact with DNA, induce permanent genetic changes in cells and cause cancer. It is widely accepted that any exposure of such substances should be avoided since non-threshold effects might occur. Thus, even low exposure levels exert a risk (EFSA 2005).

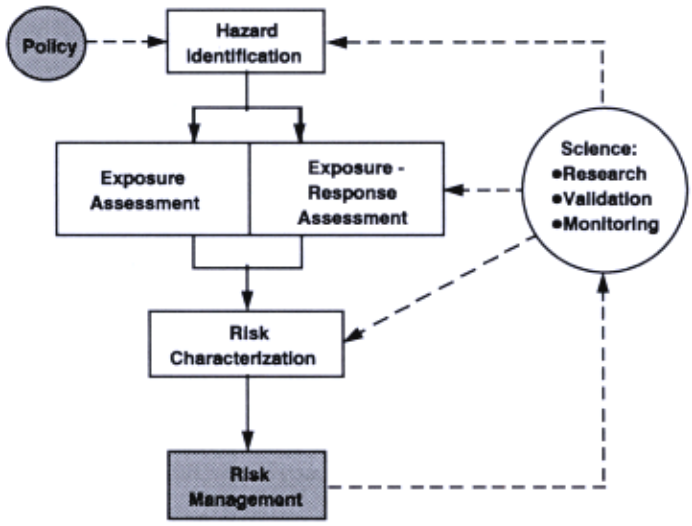


Figure 4.1 The process of risk assessment.

The carcinogenic process
Normally cells divide and die in a very controlled fashion. Cancer cells develop when these control system fails, and the cells divide and grow unregulated. They can get malignant and form tumors if not recognized and destroyed by the immune system in the body. Normal cells develop into cancerous cells by loss of genomic stability and gradual acquisition of genetic changes, called mutations (Loed and Loeb 2000, Gray and Collins 2000, Eyfjord 2005). Major mutational targets have been found in proto-oncogenes and tumor-suppressor genes (Bishop 1991 and Weinberg 1991).

Carcinogens
Carcinogens are classified into genotoxic carcinogens and non-genotoxic (epigenetic) carcinogens:

1) Genotoxic carcinogens induce cancer by causing irreversible genetic changes. They can interact with DNA directly or indirectly, either in original form or after metabolic transformation. The resulting DNA damage can be manifested into mutations if not repaired. This event (initiation) is one of the first steps in development of cancer (Arcos 1995). In general there is a threshold for significant biological effects to occur due to homeostatic and cryopreservative mechanisms. Thus, a certain dose is needed at the cellular target to achieve a toxicological effect (Dybing 2002). However, there is no indication that a threshold level has to be exceeded for manifestation of effects of genotoxic carcinogens. Many experimental studies show that covalent binding of genotoxic and carcinogenic substances to DNA exhibit a linear dose-response relationship also in the low-dose range (Neuman 1980, Dunn 1983, Lutz 1987, Beland 1988). Thus, there is no dose without a potential effect of genotoxic carcinogens, and thus a No Observable Effect Level (NOEL) cannot be postulated.

2) Non-genotoxic (epigenetic) carcinogens induce cancer through other mechanisms than induction of mutations. They act through a large variety of

different and specific epigenetic mechanisms and operate largely as promoters of cancer. In contrary to the genotoxic carcinogens, epigenetic effects can be reversible. These compounds are usually negative in standard mutagenicity assays (Woo 2003).

4.3 Part A - Quantification and Applicability of Mutagenic Potency

4.3.1 Introduction

There has been much discussion in the literature from the 1970 on whether mutagenic potency can be correlated to carcinogenic potency. It would be very useful if one could extrapolate carcinogenic potency from standard mutagenicity/genotoxicity assays, and thereby be able to grade the substance according to different levels of exposure. The need for chronic animal studies would also be greatly reduced, in accordance with REACH and the three R´s (reduce, refine, replace). In reality it is not as simple as that. There is no consensus in the literature of a good potency correlation between results from mutagenic assays and carcinogenicity studies. This could be due to the complex process of carcinogenesis and tumor formation, as well as the diversity of the adverse effects that carcinogenic compounds cause. However, recent publications indicate a correlation between the frequencies of genotoxic scores and risk of cancer (Bonassi et al., 2008, 2011).

4.3.2 Test methods and correlation between mutagenic and carcinogenic properties

Mutagenicity-based tests to predict carcinogenicity have generated useful results only for DNA-reactive chemicals, which are able to induce a wide spectrum of mutations. One of the most predictive mutagenicity-based assays so far is the prokaryotic Ames test, which shows good correlation between mutagenic and carcinogenic properties of a compound. Thus, a chemical that is found to be mutagenic in Salmonella has, together with its structural alerts, a high probability (around 80%) of being carcinogenic in animals (Zeiger 1987, Zeiger 1990, Benigni and Bossa 2011). However, the Ames test has several limitations; for example it does not detect mutagens that induce large malformations at the chromosomal level.

Recently, data from several OECD recommended mammalian tests and also newer promising *in vitro* genotoxicity mammalian tests showed a correlation between mutagenic and carcinogenic properties. On the other hand, for non-genotoxic (epigenetic) carcinogens, no reliable tests have been available to assess carcinogenic properties, due to the wide diversity of underlying mechanisms of action. However, the recent *in vitro* cell transformation assays (CTA) (OECD 2007, OECD Draft 31, EU B.21) can detect both genotoxic and non-genotoxic carcinogens. These CTAs are short-term tests not directly based on the concept of genetic mutation, but on cell transformation, mimicking the first stages of *in vivo* carcinogenesis. In these assays, carcinogenicity of test substances is determined by measuring phenotypic changes such as cell morphology, colony growth patterns and cell adhesion induced by chemicals in mammalian cell cultures. The main *in vitro* CTAs are the Syrian Hamster Embryo cell [SHE], the BALB/c 3T3 [Balb], especially the clone Bhas42 and the C3H10T1/2 [C3H] assays. The SHE assay is believed to detect early steps of carcinogenesis, and the Balb/c and C3H10 assays

detect later carcinogenic changes (TQP ID 9 2010, Sasaki et al., 2010, 2011, OECD, 2007, OECD Draft 31, EU B.21).

4.3.3 Correlation between mutagenic and carcinogenic potency

The potency of a compound is defined as the amount required to produce an effect of a given intensity. Mutagenic potency is defined as mutation frequency per µmole of mutagen tested. Carcinogenic potency is defined as the probability of developing tumors. Chemical carcinogenesis, the process by which normal cells are transformed into cancer cells, is a multistage, multifactorial process consisting of three stages: 1) initiation (include a mutational event and is the result of permanent genetic change), 2) promotion (involves clonal expansion, cell proliferation, inhibition of programmed cell death, persistent chronic inflammation, inhibition of terminal differentiation, or loss of growth control), and 3) progression (may involve a second mutation event, loss of tumor suppressor gene, impairment of immune surveillance, and acquisition of the ability to metastasize) (Woo 2009). It is largely this disjunction between the initiation step and the whole pathway leading to cancer that contributes to the lack of correlation between mutagenic and carcinogenic potencies. Ames test detects compounds that trigger some but not all of compounds that can induce the first stage of carcinogenesis (initiation), but not the last two stages (promotion and progression). There are several mechanisms that might result in genetic change, as point mutations, deletions or insertion, large chromosomal aberration and change in ploidy that may lead to cancer. However, Ames test detects only point mutations and small deletions. Mutagenic potency of indirect mutagens will depend upon metabolic transformation by cellular metabolic pathways. Metabolites can have distinct mutagenic potencies, depending on mechanisms of mutagenesis or how they are metabolized, and whether the necessary enzymes for metabolic activation are present in the mutagenicity assay used (Colvin 1988). Thus many indirect mutagens that need metabolic activation may not be detected in Ames test even external metabolic activation is used.

Fetterman analyzed the relationship between mutagenic potency in Ames test and rodent cancer potency. Eight measures of potency were defined and used to analyze a 73-chemical dataset in the NTP database, and then validated against a 42-chemical dataset. The study showed that no matter how mutagenic potency was measured or summarized, only qualitative mutagenicity is useful for the prediction of qualitative rodent carcinogenicity (Fetterman 1997). Benigni and Bossa stated that the correlation between positive Ames test and carcinogenicity is valid only at a qualitatively level of mutagenic and carcinogenic properties (yes/no), whereas mutagenic and carcinogenic potencies are uncorrelated (Benigni and Bossa, 2010). Piegorsch and Hoel demonstrated that the correlation between mutagenic and carcinogenic potencies could be affected by the chemical structural subsets analyzed (Piegorsch and Hoel 1988), and Hatch showed a higher correlation when calculations were limited to specific structural classes of chemicals (Hatch 1992).

Recent meta-analyses of human biomonitoring studies using chromosomal aberration (CA) as a biomarker showed good correlation between mutagenic and carcinogenic risk. Bonassi *et al.* performed a pooled analysis of original data from 11 national cohort studies from Europe that were followed up for cancer

incidence. The measured CA frequency showed association with the cancer risk, (Bonassi 2008). Similarly, Bonassi e*t al.,* (Bonassi 2011) demonstrated in a large international cohort study (results from 10 countries and more than 6000 subjects) a significant association between micronucleus (MN) frequency in healthy subjects and cancer risk. Cancer incidence was significantly higher in groups with medium and high MN frequency. This provides preliminary evidence that MN frequency in peripheral blood lymphocytes is predictive of cancer risk, suggesting that increased MN formation is associated with early events in carcinogenesis. Recent published data also show correlation between mutagenicity (frequency of micronuclei) and gene expression in pathways involved in process of carcinogenesis *(Hebels et al., 2011).* Goel et al and Aires et al also suggest using the MN assay as a potential biomarker for cancer (Goel 2011and Aires 2011). Additionally, a similar recent initiative to carry out meta-analysis of human biomonitoring data on the comet assay which can detect DNA strand breaks and different oxidized lesions has started. It can bring new information about correlation between the level of DNA damage and cancer risk but the results are not known yet. So far, it was shown at human population level that both CA and MN can be considered as markers of risk of cancer. Thus the biomarkers that show prediction for cancer risk in humans performed *in vitro* on human or mammalian cells can give valuable results that could be extrapolated into human risk estimates.

Sanner and Dybing have compared the carcinogenic potency with *in vivo* genotoxic potency estimates (Sanner and Dybing 2005). Several methods for quantitative hazard risk characterization have been applied in the regulation of carcinogens (e.g. the linearized multistage (LMS) model (US EPA 1986), the LED10 method (US EPA 1996) and the T25 method (Sanner 2001) (See table 4.1 for method descriptions). At present, no quantitative or semi-quantitative method has been accepted for regulatory purposes of mutagens. Sanner and Dybing demonstrated a linear correlation between the lowest effective dose (LED) for *in vivo* genotoxicity after oral administration and the lowest dose descriptor T25 forinhalation exposure in rats of 34 genotoxic substances. The correlation coefficient (R^2) was 0.71. Further they demonstrated that the numerical value of LED is similar to the numerical value of T25 within a factor of 5-10. The authors suggest that LED can be used as a bias for regulation of mutagens in cases where a threshold cannot be demonstrated or inferred, and where the substance has not been studied in long-term carcinogenicity studies. In such cases LED divided by a specified assessment factor may represent a virtually safe level or a tolerable risk level for a possible carcinogenicity effect.

4.3.4 Available testing strategies

A wide range of different *in vitro* and *in vivo* mutagenicity tests is important, as no single mutagenicity test is able to detect all possible genotoxic events. These tests have been developed and adopted internationally to fulfill the new regulatory requirements from REACH. The testing strategies should take into consideration several aspects and may vary depending on e.g. the type of chemicals, intended use, production levels, and also upon the regulatory authority.

Different strategies have been applied to evaluate the correlation between mutagenic and carcinogenic potency. A theory that “mutagens=cancer” was raised

by James and Elisabeth Miller in the 1970s (Miller and Miller 1981). The large databases of accumulated experiments (Zeiger 2004) gave support to the theory in approximately 80% of the cases (Benigni 2011). Another approach is based on the formalization of the structure-activity relationship (SAR) concepts, which also Miller applied for revealing the underlying mechanisms of chemical carcinogenicity (Ashby 1988 and 1985). A recent progress with *in silico* has resulted in quantitative structure-activity relationships (Q)SARs approaches. Different ways to improve the testing strategies have been suggested. Miller's work with electrophilic, DNA-reactive chemical carcinogens gave focus to mutation-based *in vitro* short-term tests (STTs) (Zeiger 1994, Benigni 2010). Today it is generally recognized that prokaryotic Ames test and SARs are able to efficiently detect DNA-reactive genotoxic carcinogens, thus these tests indicate a good correlation between mutagenicity and carcinogenicity. However, *in vitro* mammalian mutagenicity assays are highly preferred in order to mimic eukaryotic conditions.

The dominant trend is to use a two-tiered integrated testing approach with use of *in silico* and *in vitro* assays. The first tier includes inexpensive and fast *in vitro* and *in silico* tests (e.g. Ames test and SARs), and the second tier involves use of short-term transformation *in vitro* assays, e.g. CTA (OECD 2007, (OECD Draft 31, EU B.21) (Benigni 2011). The main CTAs, Bhas assay (Sasaki et al 2011) and the SHE test, are both very promising assays as they are sensitive to both genotoxic and non-genotoxic chemicals, and only 5-10% of the initial number of carcinogens tested were found to be undetected. It was shown that application of a tiered strategy enabled identification of up to 90% of the carcinogens (Benigni 2011). Kirkland *et al.,* suggested to use a core *in vitro* genotoxic battery of Ames test for the first step,and the *in vitro* MN test as the second step to be able to detect rodent carcinogens and *in vivo* genotoxins (Kirkland 2011). Costa *et al.,* proposed a combination of MN and comet assay to screen for carcinogenic exposure risks (Costa S 2011). Matthews *et al.* concluded that the chromosomal aberration (CA) test and the SHE cell transformation assay were the best tests to predict carcinogenicity (Matthews E J 2006). The FDA regulatory battery for approval of food additives consists of four genotoxic tests to predict carcinogenicity: Ames test, *in vivo* MN test, mouse lymphoma gene mutation test, and *in vitro* CA test (USFDA 2004).

We already recommended in the testing strategy for nitrosamines and nitramines in TQP ID 9 (2010) an approach where a compound should be tested in at least 3 different *in vitro* genotoxicity tests. Hence, in addition to Ames test, two other short term genotoxicity mammalian tests (CA, MN, mammalian HPRT gene mutation assay or comet assay) should be included in order to pick up genotoxic carcinogens (OECD Guideline tests 476, 473, 487). The gene mutation assay for mammalian cells (OECD Guideline test 476) has been performed to examine potential genotoxicity of two nitrosamines (CAS 62-75-9, 59-89-2). The comet assay was performed for detection of DNA damage. CA (OECD Guideline test 473) additionally can detect large chromosomal mutation and clastogenic effects and was already performed for one nitrosamine (CAS 59-89-2). The MN assay (OECD Guideline test 487) is an alternative method for detecting both mutagenicity and clastogenicity. Mammalian genotoxicity tests such as comet assay, gene mutation, CA and MN assays, can be used across species *in vivo* as

well as *in vitro*. *In vitro* assays with human cells are very promising and though they do not cover the higher organisiation level of a living system, the use of same markers and endpoints. Also, mammalian (preferably human) cells allow direct extrapolation to humans.

For concluding upon gonotoxicity of a tested compound, at least two out of the three *in vitro* genotoxicity tests should be positive. Thus, then the compound is regarded as genotoxic and further testing for genotoxicity *in vivo* may be recommended.

4.3.5 Results

Correlation between mutagenic and carcinogenic potency
Mutagenic and carcinogenic properties of DNA-reactive chemicals were shown to have a high correlation. A qualitative good correlation between mutagenic and carcinogenic potency was found for Ames test as well as for several mammalian cell based tests. Bacterial system does not cover the complexity of human and animal species, and thus mammalian genotoxicity assays (comet assay, gene mutation, MN and CA assays) should strongly be taken into consideration when selecting testing strategy. CA and MN performed on human blood cells demonstrated that these endpoints are good predictors of cancer risks based upon epidemiological studies. Thus results from human studies with CA and MN show a correlation between genotoxic frequencies and cancer risks.

Applicability of mutagenic potency for estimating carcinogenicity and for risk assessment
A two-tiered testing strategy approach based upon fast and inexpensive *in vitro* and *in silico* tests (Ames test, SARs) in the first tier and *in vitro* transformation CTA test in the second tier was suggested by Benigni *et al* (Benigni 2011). We agree on applying Ames and/or in silico modeling for the first step. However, we suggest that additionally in the first tier, to include two short term genotoxicity mammalian tests in order to better pick up genotoxic carcinogens. This could be e.g. CA (OECD 473), MN (OECD 487), gene mutation (OECD 476) or comet assay. Consistency with Benigni (2011) we suggest to include the carcinogenicity (cell transformation) *in vitro* test in tier 2. The cell transformation tests (OECD Draft 31, EU B.21), including SHE and Balbc (Bhas 42) assays (Sasaki et al., 2011), are the only available CTA tests at this time.

Depending on outcome of the tests of this two-tiered strategy, a third tier involving *in vivo* study for genotoxicity/carcinogenicity testing should be considered case by case. The decision on this third tier will largely depend on life cycle of compound, extend of exposure, exposed population, amount produced and used, etc. Suggestion of strategy for *in vivo* study was given in TQP ID 9 (2010).

In vivo genotoxicity data could be important for estimating carcinogenicity when no *in vivo* carcinogenicity data are available as suggested by Dybing et al (2005).

4.4 Part B - Human Health Risk Assessment of Genotoxic Non-threshold Substances

4.4.1 Introduction

In human health risk assessment the determination of adverse effects and the relationship with exposure is one of the major steps. A large number of steps are involved in between the administration of the external dose and the final toxic effect. Assessment of adverse effects starts with evaluation of non-human and human data. Non-human data include animal data, *in vitro* data and *in silico* data, such as QSARs. Evaluation of these data results in identification of the hazard. Together with exposure estimation a risk characterization can be performed to find the probability and consequences of harm, as a base for risk management (see figure 4.1). A no-effect or acceptable effect level for humans is calculated, by applying one or more steps of extrapolation. Several modeling approaches for extrapolation exists, and in this report we consider approaches for risk assessment of genotoxic non-threshold substances.

4.4.2 Dose descriptors and extrapolation models

Many models exist for extrapolation from a high dose in animal carcinogenicity studies to lower doses to which humans are exposed, from simple linear extrapolation (Linear low dose extrapolations using BMDL10 or T25) to very complex models (LMS=Linearized multistage models). The outcome depends on the model used. Hence differing conclusions can be achieved for the same substance by distinct models. It is not known whether the model truly reflects the underlying biological process. Table 4.1 summarizes the most common dose descriptors used in risk assessment, the corresponding different existing extrapolation models and the different human risk estimates that can be applied from the extrapolations.

Table 4.1 Definition of different dose descriptors, extrapolation models and risk estimates that can be applied in risk assessment.

Dose descriptors	T25	T25 is a carcinogenicity potency estimate that is defined as the chronic dose rate which will give tumors to 25% of the animals at a specific tissue site, after correction for spontaneous incidence, within the standard life-time of that species (Dybing 1997 and Sanner 2001).
	TD50	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 the 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.
	BMD	BMD, the benchmark dose, is defined as the dose of a substance that is expected to result in a pre-specified level of effect (IPCS 2004).

	BMDL10	BMDL10 is defined as the lower 95 % confidence limit for benchmark dose (BMD) which gives rise to a 10 % response
	NOAEL	NOAEL is defined as No Observable Adverse Effect Level
	LOAEL	LOAEL is defined as Lowest Observable Adverse Effect Level
	LED	LED is the lowest effective dose/LOEL – lowest observable effect level or LOAEL – lowest observable adverse effect level. LED/LOEL or LOAEL can be obtained only from in vivo genotoxicity studies.
Extrapolation models- Qualitative	ALARA	The ALARA principle means as low as reasonable achievable.
Extrapolation models - Quantitative	LED/T25	Using the correlation between *in vivo* genotoxic potency and carcinogenic potency, e.g. comparison of the lowest effective dose (LED) giving response in genotoxicity tests with a carcinogenic dose descriptor (T25) (Sanner and Dybing 2005).
	TTC	TTC is the threshold of toxicological concern, and it means the level of human intake or exposure that is considered to be of negligible risk, despite the absence of chemical-specific toxicity data. The method use data on other compounds and the uncertainty is balanced against the low level of exposure, below the level for significant risk to human health (Kroes 2004, Munro et al., 2008).
	LMS	LMS is a linearized multistage model (US EPA 1986).
	Linear low dose extrapolation	Based on a linear dose response relationship, which is incorporated in a high to low dose assessment factor. T25 should be selected as the default dose descriptor in relation to linear extrapolation. The BMDL10 should be used sometimes together with the T25.
	The MOE approach/the Large assessment factor approach	Based on a linear dose response relationship, which is incorporated in a high to low dose assessment factor (EFSA 2005). BMDL10 is preferred as the dose descriptor.
Human risk estimates	MOE	MOE is the margin of exposure. This method is useful for assessing exposures in risk characterization of genotoxic carcinogens. The approach combines information on potency in animal models and human exposure, and it can

		be used to indicate levels of concern and also the ranking between various agents. For exposures to mutagens lacking carcinogenicity data, it is suggested to use the MOE approach rather than LED/LOEL. Both T25 and BMDL10 dose descriptors may be used as a reference point. MOE = BMDL10/human exposure. The larger the MOE, the smaller the risk of exposure. This method has successfully been demonstrated for risk assessment of six genotoxic carcinogens of dietary exposure (Dybing, 2008).
	DNEL	DNEL is the derived no effect level and is defined by REACH as a human health-based limit value for threshold substances (ECHA 2008). The NOAEL dose descriptor may be used as a reference point.
	DMEL	DMEL is the derived minimal effect level; and is used for non-threshold substances (ECHA 2008). Both the T25 and BMDL10 dose descriptors may be used as a reference point.

4.4.3 Dose-response assessment – Hazard characterization

4.4.3.1 Human health-based limit values

An important criterion is to determine if a carcinogenic compound is genotoxic to be able to select between a non-threshold (genotoxic) and a threshold (non-genotoxic) risk assessment approach. Mechanisms of genotoxicity can be obtained from standard *in vitro* and *in vivo* genotoxicity assays, or from mode of action studies by which the compounds interact with DNA. Mechanistic data can also be obtained from assessment of structure-activity relationship, and comparison with genotoxic carcinogens known to interact with DNA (Dybing 2008). For non-genotoxic compounds, DNEL is defined by REACH as a human health-based limit value (ECHA 2008), while for genotoxic compounds DMEL should be used. However, there is no international consensus on how to assess the risk of substances that are both genotoxic and carcinogenic (Barlow 2006, EFSA 2005, O'Brien 2006).

4.4.3.2 Dose-response evaluation for non-genotoxic carcinogens - threshold effects

The "NOAEL approach" is the standard approach for evaluating dose-effect data for threshold effects. NOAEL is the highest dose at which no adverse effects could be observed in animal studies. This dose is often called the "Reference Point" (RP) or the "Point of Departure" (PoD). Non-genotoxic carcinogens are considered to have threshold effects, and NOAEL could thus be applied for derivation of DNEL and risk assessment of these compounds.

4.4.3.3 Dose-response evaluation for genotoxic compounds - non-threshold effects

Carcinogens that act via a genotoxic mechanism are considered to cause a non-threshold effect. It is assumed to be a risk associated with any dose, so only estimation of a dose where the risk is acceptable small could be calculated, e.g. one in a million (10^{-6}) for the public, meaning one extra case of cancer in a million people. Due to the lack of a dose-threshold, the NOAEL approach is not considered suitable for genotoxic carcinogens. A biological threshold for cancer may exist, but this threshold cannot be inferred from a NOAEL on a dose-response curve. Thus extrapolation models and reference doses need to be determined. Most commonly the T25 or the BMDL10 reference doses are used for derivation of DMELs.

4.4.4 Risk characterization: Available approaches

The low-dose extrapolation is solved by different methods in different countries. Some countries tend to regard the extrapolation of risk levels observable in animal studies to human risk levels as impossible. They exclude any quantitative evaluation of tumor incidence data and use the ALARA (as low as reasonably achievable) principle instead. **The ALARA method** is regarded as unnecessarily weak, since it treats all genotoxic carcinogens equally, and is a more qualitative approach. It does not distinguish between high and low potency carcinogens, and does not relate potential hazard to exposure (Dybing 2008). In the US, **the LMS model** (linearized multi-stage) is used at the other extreme; the default procedure is to fit a dose-response model to the tumor incidence data, and to use a fitted curve to estimate the dose at a specific low dose level (10^{*-6}), which is then called the VSD ("virtually safe dose"). The LMS method is recently considered as an unwarranted extrapolation method.

For genotoxic compounds where carcinogenicity data are missing, "Threshold of toxicological concern – **TTC**" can be applied. Another approach is to use correlation between *in vivo* genotoxic potency and carcinogenic potency, e.g. comparison of lowest effective dose (**LED**) giving response in genotoxicity tests together with a carcinogenic dose descriptor, as T25 (Sanner and Dybing 2005).

There are two main pathways to proceed from the BMD approach for quantitative risk characterization: 1) The Linear extrapolation approach and 2) the Margin of Exposure (MOE) approach/the Large assessment factor approach. In **the Linear extrapolation approach** it is anticipated that the tumor probability is proportional to the dose (number of molecules) in the low dose region. However, there are various biological processes that make the dose-response curve more sigmoid (O'Brien 2006). A linear extrapolation from a reference point, such as T25 and BDML10, is a conservative approach that is simple to apply. However, the result should be considered as an upper limit of the risk based upon rodent data, and not a real risk estimate for humans (Dybing 2008). In **the MOE approach/the Large assessment factor approach** (the EFSA approach) the estimated human exposure is divided by the reference point, usually the BMDL10, and the resulting ratio reflects the interval between the human exposure and the "known" risk level, thus a dose leading to tumor formation in animal studies. The method combines

information on animal potency and human exposure. EFSA consider a MOE of 10.000 or higher, based upon BDML10, as generally being of low concern. Newer data from Dybing indicate that T25 and BMD are equally good reference points for calculating MOE (Dybing 2008).

4.4.4.1 Human risk estimates

REACH has defined a four step procedure for deriving DNEL/DMEL and/or arriving at other human risk estimates:

1. Derivation of dose descriptor(s) relevant for the concerned endpoint: N(L)OAEL, BMD, LD50, LC50, T25, BMD(L)10, from all relevant available studies.
2. Modification of dose descriptor(s) to the correct starting point (i.e. the unit of exposure).
3. Application of assessment factors to the correct starting point to obtain DNEL(s) specific for the relevant endpoint and exposure pattern.
4. Selection of the leading health effect(s) and the corresponding qualitative/semi-quantitative description.

4.4.4.2 Derivation of DNEL for non-genotoxic threshold effect compounds

Derivation of DNEL for non-genotoxic compounds can be done by extrapolating from the dose descriptors NOAEL, LOAEL or BMD, by using the overall assessment factor approach (ECHA/REACH Chapter R8).

Assessment factors for derivation of DNEL:

- *Interspecies differences* - The toxicokinetic behavior in the test animal can be extrapolated to humans by allometric scaling of the critical dose. The generally equitoxic doses, expressed in mg per kg bw per day, scale with body weight to the power of 0.75. This gives different allometric scaling factors for different animal species when compared to humans. There exist default allometric assessment factors (AFs) for common experimental animals, for example; mouse = 7 and rat = 4. Toxicodynamic differences and intrinsic susceptibility between the test animal and humans is generally expressed with an AF of 2.5. In case of the rat **the overall AF** = 4 (toxicokinetic differences) x 2.5 toxicodynamic differences) = 10.

- *Intraspecies differences* - It is generally assumed that the default assessment factor of 10 covers the vast majority of the human population. It has been suggested that the AF should be divided into two default AFs, each with a value of 3.16. One of these covers toxicokinetics and one cover toxicodynamics.

- *Overall assessment factor* - The overall AF for deriving DNEL is obtained by multiplication of the individual AFs, as shown in the following equation:

    - **DNEL = (overall NOAEL or BMD) / (AF1xAF2x…x AFn).**

Table 4.2 Assessment factors for DNEL calculations

Assessment factors for derivation of DNEL from threshold substances	**Explanation**	**DNEL = NOAEL or BMD/AF1xAF2x…AFn**
Interspecies differences	Toxicokinetic differences x Toxicodynamic differences (e.g. 4 x 2.5 from rat to human due to interspecies differences in sensitivity)	AF1 = 10
Intraspecies differences	Toxicokinetic differences x Toxicodynamic differences (e.g. 3.16 x 3.16 from one individual to another due to polymorphism)	AF2 = 10
Differences in duration of exposure	Allowing for differences in experimental exposure duration.	AF = 2-6
Dose-response relationship	To give consideration to uncertainties in the dose descriptor as a surrogate for the true starting point.	AF = 3-10
Quality of whole database	To compensate for the potential remaining uncertainties in the derived DNEL.	AF = 1 (>1 in some cases)
Endpoint-specific issues	Application of AFs for acute toxicity, irritation/corrosion, sensitization, and reproductive toxicity	See appendix R.8-8 to 8-12 .
Overall assessment factor	Multiplication of the individual AFs	AF1xAF2x …x AFn

4.4.4.3 Derivation of DMEL for genotoxic non-threshold effect compounds

DMEL for genotoxic carcinogens can be derived by The "Linearized" approach or the MOE/"Large Assessment Factor" approach as summarized below (ECHA/REACH Chapter R8). The Linearized approach gives DMEL values that represent exposure levels where the probability that effects are avoided is high, thus of very low concern. The Large Assessment Factor approach is similar to the overall assessment factor approach that it is used for threshold effects for deriving DNEL, and gives DMEL values that imply exposure levels with high probability that effects are avoided and thus of very low concern. Both formats either uses T25, BMD10 or BMDL10 as dose descriptors, thereby applying the same principal elements of risk extrapolation and risk evaluation.

1) Linearized approach

This approach is based on an assumption of a linear dose relationship between tumor formation and exposure, which is incorporated into a high to low dose extrapolation assessment factor:

- T25 should be selected as the default dose descriptor in relation to linear extrapolation. The BMD10 should be used sometimes together with the T25 when data are adequate for modeling purposes.
- The relevant dose descriptor should be modified, when necessary, to the correct starting point.
- The DMEL should be derived from the correct starting point, for the relevant exposure pattern, by linear high to low dose extrapolation, and by application of assessment factors.

Assessment factors considered for the linearized approach of DMEL derivation:

- *Interspecies differences* - Only an assessment factor for differences in metabolic rate is applied for non-threshold effects (allometric scaling).
- *Intraspecies differences* – No assessment factor is to be applied for this extrapolation step for non-threshold effects.
- *Differences in duration of exposure* – No assessment factor is to be applied for this extrapolation step for non-threshold effects.
- *Issues related to dose-response* – Uncertainties related to the observable region of dose response curve for non-threshold effects.
- *Quality of the whole database* – An assessment factor should be applied, if justified.

High to low dose risk extrapolation factor (HtLF):

- The preceding steps result in human equivalent lifetime daily doses HT25 (Human T25) and HBM10 (Human BMD10), that represent human daily exposures associated with tumor incidences of 25% and 10%.
- Risk at very low concern is decided at policy level. There is no EU legislation for this. Cancer risk levels of 10^{*-5} and 10^{*-6} can be seen as indicative tolerable risk levels when setting DMELs for workers and the general population, respectively.
- When using BMD10 the HtLF is: ($\underline{10^{-5}}$/0.10) = 1/10 000 for the worker population and ($\underline{10^{-6}}$/0.10) = 1/100 000 for the general population.
- When using T25 the HtLF is: ($\underline{10^{-5}}$/0.25) = 1/25 000 for the worker population and ($\underline{10^{-6}}$/0.25) = 1/250 000 for the general population.

Equations for calculating DMEL

DMELs are then finally calculated by dividing the modified dose descriptor by the product of all assessment factors and the high to low risk extrapolation

factor:

- **DMEL at a 10^{-5} (10^{-6}) risk= BMD10/AFx10 000(workers) or BMD10/AFx100 000(general population)**
- **DMEL at a 10^{-5} (10^{-6}) risk= T25/AFx25 000(workers) or T25/AFx250 000 (general population)**

Table 4.3 Assessment factors for DMEL calculation with the Linearized approach at a cancer risk levels of 10^{*-6}

Assessment factors for derivation of DMEL from non-threshold substances	**Linearized Approach**	**DMEL at 10^{-6} risk= BMDL10/AF1x100000 or T25/AF1x250000**
Interspecies differences	Only an assessment factor for differences in metabolic rate is to be applied for non-threshold substances (allometric scaling)	AF1 = 4 (from rat to human)
Intraspecies differences	No assessment factor is to be applied for non-threshold substances	AF2 = 1
Differences in duration of exposure	No assessment factor is to be applied for non-threshold substances	AF3 = 1
Issues related to dose-response	Uncertainties related to the observable region of dose response curve for non-threshold substances	AF4 = not specified by REACH
Quality of the whole database	An assessment factor should be applied, if justified	AF5 = not specified by REACH
High to low dose extrapolation factor (HtLF)		10^{-5} (workers) or 10^{-6} (general population)

2) Large assessment factor approach / The MOE approach (the EFSA approach)

This approach to characterise and evaluate carcinogenic risks involves the application of several assessment factors to the starting point rather than linear extrapolation of the dose descriptor:

- BMDL10 is preferred as the dose descriptor (EFSA 2005). This value is the lowest statistically significant increased incidence that usually can be measured, also the method requires little or no extrapolation outside

observed experimental data.
- The relevant dose descriptor should be modified, when necessary, to the correct starting point.
- The DMEL should be derived from the correct starting point, for the relevant exposure pattern, by linear high to low dose extrapolation, and by application of assessment factors.

Assessment factors considered for the linearized approach of DMEL derivation:

- *Interspecies and intraspecies differences* – The usual default factor of 100 for non-genotoxic substances represents the product of two 10-fold factors (WHO 1987 and 1994). The factors allow for physiological and metabolic differences. The factors can be reduced or increased when appropriate data is available (WHO/IPCS 2005). The assessment factor for interspecies differences[1)] may be reduced due to additional route-to-route extrapolation and exposure group extrapolation. The assessment factor for intraspecies differences[2)] is set to 10 for the general population and 5 for workers.

- *The nature of the carcinogenic process* – The probability of genetic alterations at sensitive targets during exposure to genotoxic substances may be affected by the efficiency of DNA damage repair and the cell cycle control. Polymorphism in candidate genes such as TP53, p21, and cyclin D1 have been associated with increased susceptibility of cancer (Powell 2002, Wang 2002, Quiling 2003). EFSA SC considers that a default factor of 10 covers this area of uncertainty.

- *The reference point on the animal dose-response curve is not a NOAEL*- EFSA SC considers that a default factor of 10 covers this area of uncertainty.

- *Using T25* – Since T25 is considered to be less conservative than the BMDL10 this needs to be considered when defining an exposure level regarded as "a low priority of risk management". An additional factor of 2.5 is applied.

Equations for calculating DMEL

DMELs are then finally calculated by dividing the modified dose descriptor by the product of all assessment factors:

- **DMEL 10^{-5} (10^{-6}) risk= BMDL10/AF1[1)] xAF2[2)] x…xAFn**
- **DMEL 10^{-5} (10^{-6}) risk= T25/AF1[1)] xAF2[2)] x…xAFn**

Table 4.4 Assessment factors for DMEL calculation with the Large Assessment factor approach at a cancer risk levels of 10^{-6}*

Assessment factors for derivation of DMEL from non-threshold substances	**Large Assessment Factor Approach (EFSA)**	**DMEL at 10^{-6} risk= BMDL10 or T25/AF1xAF2x…AFn= BMDL10/10000 or T25/25000**
Interspecies differences	Physiological and metabolic differences	AF1 = $10^{1)}$
Intraspecies differences	Physiological and metabolic differences	AF2 = $10^{2)}$
The nature of the carcinogenic process	Due to polymorphism	AF3 = 10
The reference point on the animal dose-response curve is not NOAEL	Uncertainty	AF4 = 10
When using T25	An assessment factor should be applied, if justified	AF5 = 2.5
High to low dose extrapolation factor (HtLF)		Not applied

Alternatives to the conventional extrapolation procedures
PBPK modeling is an alternative to the approaches described above to derive DMEL.

4.4.4.4 Derivation of DMEL for non-threshold effect substances without existing adequate cancer data

Risk characterization as described above is not possible in some cases, for example in the absence of carcinogenicity data. In such cases the following approaches can be explored to derive a DMEL:

- *Read across*
- *Use of sub-chronic studies*
- *Use the threshold for toxicological concern (TTC) concept*

4.4.4.5 Qualitative approach

When no dose descriptor is available for an endpoint, a more qualitative approach can be followed. This may apply for acute toxicity, irritation/corrosion, sensitization, and mutagenicity/carcinogenicity.

4.4.5 Adaptation of information requirements as defined by REACH

There are recommendations by ECHA/REACH of exposure based adaptation in situations where human or environmental exposure is absent or very low in "Guidance on information requirements and chemical safety assessment, Chapter R.5: Adaptation of information requirements" (ECHA/REACH_Chapter R5). Exposure based adaptation (EBA) is defined by REACH as a deviation from the standard information requirement at the actual tonnage level based on exposure arguments. In CCM this may be relevant in situations where exposure is "not significant", meaning that the (predicted) exposure level is well below the calculated DNEL/DMEL. ECAH/REACH require exposure to be 'absent' or 'not significant', and also a demonstration that the predicted exposure is always well below a relevant DNEL/PNEC (predicted no effect concentration).

4.4.6 Results

4.4.6.1 T25/TD50 correlation

Dybing (1997) gave several specific examples of how to calculate T25 from different studies and compared the results for 110 substances with the corresponding TD50 values calculated by Gold *et al.,* for their Carcinogenicity Potency Database (Gold 1984, 1986). Dybing et al stated that even if the T25 index is a more crude estimate of potency than the TD50 index, since they do not take into account possible non-linearity in dose-response, the evaluation for correlation of T25 with TD50 values for the 110 carcinogens were well correlated (R^2=0.96). The result indicated that the use of the T25 index is an acceptable parameter when compared to TD50 in describing carcinogenicity potency. Using T25 as a dose descriptor for carcinogenicity is well accepted, even if some authorities prefer other dose descriptors (EFSA 2005). REACH makes use of T25 as a typical dose descriptor when deriving DMELs (ECHA/REACH_Chapter R8). T25 fulfills the 3Rs (reduce, refine, replace) in order to reduce animal experiments.

4.4.6.2 Using TD50 for calculating DMELs?

It was pointed out at the joint EFSA/WHO/ILSI European Conference in 2005 (Barlow 2006, EFSA 2005, O'Brien 2006) that the linear extrapolation of TD50 can seriously under- or overestimate the true risk. When starting with a dose causing 50% effect, the uncertainties derived when extrapolating to the lower doses will be much larger than when starting with a dose causing 25% effect. Dose descriptors used for derivation of DMELs, should be derived from exposures to lower doses that will affect as few as possible in the population, e.g. T25, BMD10, BMD05.

Calculation of T25 is done as follows:

$$T25 = (f)^2 \times d,$$

where f= duration of exposure when 25% tumors and d= dose. In an experiment that is terminated before the standard lifespan, the number of tumours found will be reduced, and the dose rate d needed to give 25% of the animals tumours (after correction for spontaneous incidence) will be greater than the true T25. For this reason, the true T25 is estimated as $(f)^2 \times d$, where f = (duration of experiment)/(standard lifespan) (Peto et al., 1984).

TD50 is calculated by a much more complicated procedure using a software. It takes into account a possible non-linearity in the dose-response. Also TD50 can be calculated both for a particular category of neoplastic lesion or for all tumors. T25 is calculated for tumors only at a specific tissue site. Therefore, a TD50 derived for all tumors can be smaller than the corresponding T25. This is true, since the calculated linear slope is 1.72 in a plot of T25 versus TD50 (Dybing 1997). Even if the average of the 110 carcinogens analyzed by Dybing *et al.,* correlated quite well in a log-log plot, but when examining the single compounds of thework, hardly any of the TD50 values seems to be simply a doubling of the T25 value (Dybing et a 1997).

4.4.6.3 Calculations of DMELs, which method preferred

The BMDL approach is considered the most appropriate reference point for calculating MOE and DMEL, better than T25 (Barlow 2006, EFSA 2005, O'Brien 2006, Landigham and Crump 2001, US EPA 1996 and 2004). In the case of linear or close to linear dose response relationship the results of the two procedures are virtually identical. However, for a sub-or supralinear dose response relationship will T25 under- or overestimate the true risk (Landigham and Crump 2001). If data are not sufficient to derive a BMDL10, T25 is an alternative option. Further, in some cases where data are very limited, the ALARA approach is the only feasible (Barlow 2006, EFSA 2005, O'Brien 2006). Newer data from Dybing 2008 say that T25 and BMD are equally good methods to obtain MOE (Dybing 2008). The T25 values are not corrected for intercurrent mortality, e.g. premature death due to toxicity. This correction of T25 for non-neoplastic effects can be done by using the Kaplan-Meier Adjustment described in Appendix R.8-6 in ECHA/REACH_Chapter R8, but this adjustment should not be necessary where the dose do not materially affect non-neoplastic causes of death (ECHA/REACH_Chapter R8).

4.4.6.4 Summary for selection of reference dose and extrapolation method for non-threshold genotoxic carcinogens

Genotoxic carcinogens are considered not to have a threshold level for effect to occur. This means that NOAEL or LOAEL are not suitable to use for finding the risk that gives acceptable increase in the incidence of cancer in the population. Risk is usually considered to be tolerable in the range from 10^{-6} (general population) to 10^{-5} (occupational exposure) levels, meaning 1 or 10 additional incidence of cancer in a population of one million, respectively.

It is much more difficult to establish an acceptable risk level for genotoxic carcinogens than for non-genotoxic carcinogens, which are assumed to have a threshold level for manifestation of effects. The best approach for genotoxic compounds is to use DMEL as a risk estimate, in contrast to DNEL for non-genotoxic compounds. Another estimate that can be used is MOE. For both methods different dose descriptors can be used, as well as different ways of extrapolating from exposure to high doses in animal studies to low exposure levels in humans. Thus the different approaches apply different assessment factors for covering the uncertainty in the extrapolations. Often scant information on exposure is a major source of uncertainly, especially for genotoxic carcinogens.

According to the REACH guideline (ECHA/REACH_Chapter R8), assessment factors should be applied for non-threshold effects only for interspecies toxicokinetics differences when using the Linearized approach. Also allometric scaling for distinct metabolic rates is used. Assessment factor for intraspecies differences is as a default not applied for non-genotoxic compounds, in contrast to genotoxic compounds. This is due to the linear model for high-to-low dose extrapolation, which is assumed to be conservative enough to account for differences in human sensitivity. Linear extrapolation for low-dose exposure also takes into accounts both animal data and human exposure when quantifying the estimated risk.

4.4.6.5 Calculation of DMELs for selected nitrosamines and nitramines

Five nitrosamines and two nitramines were chosen for derivation of DMELs. Available dose descriptors for the selected compounds are listed in table 4.5.

Table 4.5 Available dose descriptors for the nitrosamines and nitramines mentioned in the report. All TD50 data are from rat (from mouse).*

Compound	Name	CAS nr	TD50 (mg/kg bw/day)	T25 (mg/kg bw/day)	Reference
Nitrosamines	N-Nitrosodimethylamine (NDMA)	62-75-9	0,0959	0.150	CPDB, NIPH report 2011
	N-Nitrosodiethylamine (NDEA)	55-18-5	0,0265		CPDB
	N-Nitrosomorpholine	59-89-2	0,109		CPDB
	N-Nitrosopiperidine (NPIP)	100-75-4	1.43		CPDB
	Dinitrosopiperazine (DNP)	140-79-4	3.6*		CPDB
Nitramines	N-Methylnitramine (NTMA)	598-57-2	17,4		CPDB
	Dimethylnitramine (NDTMA)	4164-28-7	0,54		CPDB

DMEL calculations using T25 estimations based on available raw data.

T25 calculations have been performed according to Dybing (Dybing 1997) assuming a linear dose response relationship, by estimating the dose in mg/kg bw/day by the amount of tumors received in 25% of the animals in a specific tissue (Peto 1991 and Goodall 1976). We used the average T25 calculated from three different doses if available, with close to 25% tumor incidents (see table 4.6). T25 = $(f)^2$ x d = (duration of exposure when 25 % tumors)^2 x dose = (duration of exposure) x (duration of observation) x (dose when 25% tumor incidents).

Table 4.6 Calculation of T25 for 4 nitrosamines and 1 nitramine from raw data (Peto et al., 1991, Goodall et al., 1976, Druckery et al.,1967 and Eisenbrand et al., 1980).

CAS registry	Dose (mg/kg bw/day)	Exposure & Observation	% Tumors	T25 (mg/kg bw/day)	Average T25
62-75-9 (NDMA) (Peto *et al.,)*	0.131	33 months exposure, 33 years observation	14/60=23.33%	(33/24)x(33/24)x(25/23.33)x0.131= 0.229	
	0.174	25.44 months of exposure, 24 months of observation	19/60= 31.67%	(25.44/24)x(24/24)x(25/31.67)x 0.174 = 0.145	
	0.109	30.48 months of exposure, 24 months of observation	13/60=21.67%	(30.48/24)x(24/24)x(25/21.67)x 0.109 = 0.159	
					0.178 mg/kg bw/day
55-18-5 (NDEA) (Peto *et al.,)*	0.061	29.04 months of exposure, 33 months of observation	18/60=30.00%	(29.04/24)x(33/24)x(25/30)x 0.061 = 0.102	
	0.082	28.08 months of exposure, 24 months of observation	10/60=16.67%	(28.08/24)x(24/24)x(25/16.67)x0.082 = 0.096	
	0.102	23.3 months of exposure, 24 months of observation	21/60=35%	(23.3/24)x(24/24)x(25/35)x 0.102 = 0.099	
					0.100 mg/kg bw/day
100-75-4 (NPIP) Eisenbrand *et al.,)*	3.00	392 days exposure and 392 days of observation.	11/34=32%	(392/730)x(392/730)x(25/32) x 3.00 = 0.675	
	0.60	816 days exposure and 795 days of observation.	16/34=47%	(816/730)x(795/730)x(25/47) x 0.60 = = 0.387	
	0.12	800 days exposure and 746 days of observation.	6/75=7%	(800/730)x(746/730)x(25/7) x 0.12 = 0.480	
					0.514

CAS registry	Dose (mg/kg bw/day)	Exposure & Observation	% Tumors	T25 (mg/kg bw/day)	Average T25
					mg/kg bw/day
140-79-4 (DNP) (Druckery *et al.,*)	4.00	466 days exposure and 466 days of observation	5/31=16.1%	(466/730)(466/730) (25/16.1) x 4.00 = 2.531	2.531 mg/kg bw/day *
4164-28-7 (NDTMA) (Goodall *et al.,)*	1.83	330 days of exposure and 730 days of observation	8/10=80%	(330/730)x(730/730)x(25/80) x 1.83/365 = 0.709	0.709 mg/kg bw/day

* This number contains a level of uncertainty as it originates from work with limited data.

The following DMEL calculations below using the estimated T25 values are done according to REACH. To extrapolate from high to low dose we choose the Linearized approach for the T25 values since a linear dose relationship is assumed for the T25 values. To adjust for route of exposure we choose the "Default sequence of extrapolation" since it is preferred to the "Modified sequence of extrapolation"/"10m^3 Approach" (Appendix R.8-2 in ECHA/REACH_Chapter R8).

Calculations of DMEL:

DMEL (mg/kg bw/day) calculation (according to Table R.8-7, R.8.5.2.1 in ECHA/REACH_Chapter R8):

- We use the estimated T25 as the dose descriptor
- We use the Linearised approach and multiply the T25 with a HtLF of 1/250 000.

DMEL (ng/m^3) calculation (according to Example B.3, Appendix R.8-2 in ECHA/REACH_Chapter R8):

- We use an additional adjustment aactor for route of exposure: from rat oral exposure (in mg/kg/d for 6 hours) to human inhalational exposure (in m^3/min/kg bw for 24h) = 1/1.15 m^3/kg bw.

Summary calculations of DMEL:

Step 1) Calculation of DMEL can be done by first extrapolating the dose descriptor = T25 / 250 000= DMEL in mg/kg bw/day

Step 2) Calculation of a modified/corrected dose descriptor relevant for the concerned endpoint in, this case air, can further be done= DMEL(mg/kg bw/day) x 1/1.15 m3/kg bw/24h = DMEL mg/m^3

Example of calculation of DMEL for NDMA:
Step 1) DMEL in mg/kg bw/day is calculated by dividing the T25 by the assessment factors: (T25 / 250 000) = (T25 / 250 000) = 0.178 mg/kg bw/day / 250 000 = 0.712×10^{-6} mg/kg bw/day

Step 2) DMEL in mg/m^3 is calculated by correcting the T25 to the relevant endpoint: (T25 / 250 000) x (1/1.15 m3/kg bw/24h) = (0.178 mg/kg bw/day / 250 000) x (1/1.15 m3/kg bw/24h) = 0.6194×10^{-6} mg/m^3 = 0.6194 ng/m^3.

Table 4.7 Calculation of DMELs for 4 nitrosamines and 1 nitramine from the rat oral route to the human inhalational route by using the estimated T25 values from raw data, at a 10^{-6} risk (Peto et al 1991, Goodall et al 1976, Druckney et all 1967 and Eisenbrand et al 1980).

CAS registry	TD50 oral rat (mg/kg bw/day) (data from CPDB)	T25 oral rat (mg/kg bw/day) (Estimations from NILU)	Step1) DMEL (mg/kg bw/day) 10^{-6} risk	Step2) DMEL (mg/m^3) 10^{-6} risk	Step2) DMEL (ng/m^3) 10^{-6} risk
62-75-9	0.0959[m,v]	0.178	0.712×10^{-6}	0.619×10^{-6}	0.62
55-18-5	0.0265[m,v]	0.100	0.400×10^{-6}	0.348×10^{-6}	0.35
100-75-4	1.43	0.514	2.056×10^{-6}	1.789×10^{-6}	1.79
140-79-4	3.6	2.531	10.13×10^{-6}	8.809×10^{-6}	8.81*
4164-28-7	0.547[m,v]	0.709	2.836×10^{-6}	2.467×10^{-6}	2.47

* This number contains a level of uncertainty due to a limited data available

DMEL calculations carried out by using TD50 instead of T25, when raw data for T25 calculations are not available.

The following DMEL calculations below are done using the available TD50 values. This method shown as an example, but we do not recommend this method. To extrapolate from high to low dose we chose the Large assessment factor approach is chosen, since the TD50 values do not anticipate a linear relationship, and we assume more uncertainty than with the T25 data. To adjust for route of exposure we choose the "Default sequence of extrapolation" since it is preferred to the "Modified sequence of extrapolation"/"10m^3 Approach" (Appendix R.8-2 in ECHA/REACH_Chapter R8).

Calculations of DMEL:
DMEL (mg/kg bw/day) calculation

- We use TD50 as a base for the provisional dose descriptor TD10, assume no linearity and use a safety Factor = 10
- We use the Large assessment factor approach and include the assessment factors (10x10x10x10 = 10.000). AF for Interspecies extrapolation= 10; Af for intraspecies extrapolation= 10; Af for nature of carcinogenic process= 10; Af for point of comparison = 10.

DMEL (ng/m^3) calculation

- We use an additional adjustment factor for route of exposure: from rat oral exposure (in mg/kg/d for 6 hours) to human inhalational exposure (in m^3/min/kg bw for 24h) = 1/1.15 m^3/kg bw.

Summary calculations of DMEL:
Step 1) Calculation of DMEL by extrapolating the dose descriptor with assessment factors = TD50/AFs = TD50 / 10x10x10x10x10= DMEL in mg/kg bw/day

Step 2) Calculation of a modified/corrected dose descriptor relevant for the concerned endpoint in, this case air = DMEL(mg/kg bw/day) x 1/1.15 m3/kg bw/24h = DMEL mg/m^3

Table 4.8 Calculation of DMELs for 5 nitrosamines and 2 nitramines from the rat oral route to the human inhalational route by using the Safety Factor calculation, at a 10^{-6} risk.

CAS registry	TD50 oral rat (mg/kg bw/day) (data from CPDB)	1) DMEL (mg/kg bw/day) 10^{-6} risk	2) DMEL (mg/m^3) 10^{-6} risk	2) DMEL* (ng/m^3) 10^{-6} risk
62-75-9	0.0959[m,v]	0.0959x10-^{5}	0.0834x10^{-5}	0.834
55-18-5	0.0265[m,v]	0.0265x10^{-5}	0.0230x10^{-5}	0.230
59-89-2	0.109[m]	0.109x10^{-5}	0.0948x10^{-5}	0.948
100-75-4	1.43[m]	1.43x10^{-5}	1.2441x10^{-5}	12.44
140-79-4	3.6 [m]	3.6x10^{-5}	3.132x10^{-5}	31.32
4164-28-7	0.547[m,v]	0.547x10^{-5}	0.4759x10^{-5}	4.759
598-57-2 and 113282-39-6	17.4[m]	17.4x10^{-5}	15.138x10^{-5}	151.38

* These numbers all have high uncertainty due to using TD50 as a dose descriptor for calculations

4.4.6.6 How to interpret "Well below" in ECHA Chapter R5?

In situations where human or environmental exposure is absent or very low and substances are not released to the environment, the standard information requirements from REACH can be adapted and reduced. In CCM this may be relevant in situations where exposure is "not significant", meaning that the

(predicted) exposure level is well below the calculated DNEL/DMEL. Exposure well below the calculated risk level is defined in ECHA_Chapter_R5 as exposure that is absent, not significant, strictly controlled, not expected in any life cycles stages, or with no release/waste or expected secondary exposure. ECHA/REACH require exposure to be 'absent' or 'not significant', and also a demonstration that the predicted exposure is always well below a relevant DNEL/PNEC (predicted no effect concentration).

If the exposure level is well below the anticipated risk level, we recommend that only *in vitro* and *in silico* tests to be carried out from the suggested testing strategy and that all *in vivo* tests can be omitted. We recommend initially a 1st Tier with the Ames test, two short term *in vitro* genotoxicity mammalian tests, and the (Q)SARs and then a 2nd Tier with the *in vitro* cell transformation tests.

4.4.7 Conclusions

Our results for the DMELs calculated from our estimated T25 values in this report (table 4.7) do not differ significantly from those that were recently calculated by NIPH (NIPH report 2011). Our calculated DMELs for nitrosamines range from 0.35 ng/m^3 – 8.81 ng/m^3 and we get 2.47 ng/m^3 for one of the nitramines. The NIPH reported a DMEL of 0.52 ng/m^3 for all nitrosamine and nitramine compounds as a group, but the calculations are based on data from only one nitrosamine; oral exposure of rat with NDMA (Peto et al 1991). The NIPH also derived a DMEL of 0.3 ng/m^3 for NDMA based on inhalational exposure data of rat (Klein 1991). We based our calculations on oral exposure, and recalculated the numbers to inhalational exposure in humans, since there is more data available on nitrosamines and nitramines through oral exposure than inhalational exposure, and we wanted to calculate the risk for more compounds, not only NDMA. In addition to our DMEL calculations of NDMA and NDEA that are very low (0.62 and 0.35 ng/m^3) we also calculated DMELs for two other nitrosamines: NPIP (1.79 ng/m^3) and DNP (8.81 ng/m^3). In general all the calculated DMELs from us, for the nitrosamines and the one nitramine, and the data from the NIPH, are in the same range of concentrations. We therefore conclude that it should be possible to extrapolate the calculated risks of these compounds among each-other, if no other data are available.

Furthermore, if the exposure level is well below the anticipated risk level, based on the definitions in ECHA_Chapter_R5 and on our recommended testing strategy in Part A in this report, we recommend that only *in vitro* and *in silico* tests should be carried out from the suggested testing strategy; initially a **1st Tier** the (Q)SARs and the Ames test with two short term *in vitro* genotoxicity mammalian tests, and then a **2nd Tier** with the *in vitro* cell transformation tests.

5 Status with respect to the use of QSAR in REACH

For QSAR methods to be acceptable to ECHA for the registration of chemicals into REACH multiple criteria needs to be fulfilled. For the toxicological endpoint a clear mechanism of activity for the set of compounds under investigation must be presented, the set of compounds involved in the creation of the QSAR must support this mechanism reasoning. Examples of important mechanisms are those

assigned in the OECD QSAR Toolbox such as protein binding, DNA binding, and estrogen receptor binding. The models needs to be scientifically valid and compounds incorporated in the model should be within the applicability domain.
The QSAR model preferably needs to have a simple equation with variables/physicochemical properties that support the mechanistic reasoning or are easily understood and well described as properties of importance. ECHA and national legislators (scientific officers) needs to understand why these properties were selected and what they mean. In general the QSAR model should be transparent and well documented using the QSAR model reporting format (QMRF) and results reported in the QSAR Prediction Reporting Format (QPRF). The results should be adequate for classification and labeling (C&L) and risk assessment (RA). (Personal communication with Dr. Emil Rorije, RIVM, The Netherlands and Dr. Evelin Fabjan, ECHA).
It is clear that regulatory bodies nationally and within ECHA points to a need for an weight of evidence (WoE) approach in combination to any use of (Q)SAR model prediction. This also points to instances where WoE is lacking or needs strengthening that the use of an integrated testing strategy (ITS) in combination with chemical categorization and (Q)SAR might be beneficial.

5.1 Conclusions made by ECHA of the suggested use of non-test methods in REACH dossiers

ECHA expects higher use of non-test methods for 2013 and 2018 and only a small number of dossiers have been evaluated so far.

General observations made in registration dossiers was that (Q)SAR predictions have been used both as key studies and as supporting evidence, and that read-across was used more frequently.
“In certain cases, (Q)SAR models fulfilled the conditions outlined in REACH Annex XI, either as stand alone for the prediction of certain properties or as part of supporting evidence in hazard assessment. In other cases, data generated by (Q)SAR were considered inadequate as they did not provide sufficient information for predicting the presence or absence of certain properties, e.g. long term toxicity.”

Shortcomings observed in dossiers on points to limited information about (Q)SAR model (e.g. version unclear, no QMRF, data model not transparent/available). The scientific validity of models was not always demonstrated and the applicability domain of the models often not or only partially analyzed.

Conclusions by ECHA are that in many cases (Q)SAR models, based on the shortcomings above, was not relevant for regulatory purpose (e.g. the endpoint predicted is not suitable to meet the information requirements of REACH) and that the documentation was limited. Further, ECHA implies that lacking or limited documentation of (Q)SAR predictions and models used can lead to additional uncertainties for authorities (ECHA 2010a, Hirmann 2010).

5.2 Recommendations from ECHA in the use of (Q)SAR models under REACH

ECHA recommends that the information on the (Q)SAR model should be provided in the (Q)SAR Model Reporting Format (QMRF) and the (Q)SAR Prediction Reporting Format (QPRF), reports which is deemed to be necessary for assessing the validity of any (Q)SAR model. The QSAR model should also be submitted for peer review to the JRC (Q)SAR Model Database (QMDB) (http://qsardb.jrc.it/qmrf/search_catalogs.jsp).

"The use of (Q)SAR models as supportive evidence in hazard assessment is recommended. Information generated by expert systems on the presence or absence of alerts may provide valuable information in the overall of test data."

"QSAR model predictions may be used in a weight-of-evidence approach, in correlation to test data, in order to develop and support justification for read-across and grouping approaches."

"QSAR model predictions can often help in deciding on integrated testing strategy (ITS) when examining chemical categories." (ECHA 2010a, Hirmann 2010)

5.3 ECHA support of regulatory use of (Q)SAR methodology

ECHA support the use of the QSAR Toolbox as a valuable tool (albeit still under development/refinement) is a software to help registrants and authorities to use (Q)SAR methodologies to group chemicals into categories and to fill data gaps by read-across, trend analysis and (Q)SARs for assessing (eco)toxicity hazards of chemicals under REACH, and thus to help saving costs and the need for testing on animals (ECHA 2010a, Hirmann 2010).

5.3.1 Grouping of substances and read-across approach

Grouping and read-across approaches provide a suitable basis for data gap filling for regulatory purposes providing that certain conditions are satisfied. This avoids the need to test every substance for every endpoint.
In 2010, ECHA evaluated several read across approaches and in certain cases the read-across approach was used adequately in order to fulfil the information requirements, both for vertebrate and non animal testing and was deemed appropriate for the purposes of classification and labelling and for risk assessment.

The following recommendations are made with regard to the use of read-across and grouping approaches under REACH (ECHA 2010a):

- Results from the read-across approach should be adequate for the purpose of classification and labelling and/or risk assessment.
- Accurate data on the substance(s) composition shall be provided.
- Reliable information on the physicochemical properties that is relevant for biological effects shall be provided.
- Preferably the physical-chemical information used in order to support a

read-across approach shall be generated using a test method as specified in the Test Method Regulation (EC) No 440/2008 or OECD Guidelines.

- The documentation must detail which hazard end-points are covered by the read-across.
- The read-across hypothesis and justification for it must be detailed in the dossier.
- Studies on toxicokinetics may improve the robustness of the read-across hypothesis. Theoretical assumptions based on robust criteria together with modelling approaches are considered useful in the overall evaluation.
- Analysis of the test data together with predictive properties generated by QSAR tools (e.g OECD QSAR Toolbox) is essential for providing good justification for read-across approaches.
- Consideration of mode of action or other mechanistic information need to be provided when the data available allows for doing so.
- Assessment of the overall data should be done in a Weight of Evidence approach to allow sound conclusions as to which endpoints are covered by read-across/grouping.

Further information can be found in the Practical Guide 6: How to report read-across and categories (ECHA 2010b).

5.3.2 The use of alternatives to testing on animals for the REACH regulation in view of the use of read-across and (Q)SAR methods

5.3.2.1 Repeated dose toxicity

Repeated dose toxicity cannot be predicted by an QSAR approach. Alternative methods are therefore mainly other prediction methods (read-across and grouping), Weight of Evidence approaches.

For phase-in substances at or above 1 000 tpa: Read-across approaches have been used in 28.1 % of the ESRs. Weight of evidence was flagged by the registrants in 6.6 % while QSAR predictions are not relevant to these endpoints and correspondingly have been used only in 0.1 % of the cases (ECHA 2011).

5.3.2.2 Genetic toxicity

The aims of testing for genetic toxicity (genotoxicity) are to assess the mutagenic potential of substances, i.e. their ability to induce genotoxic effects which may lead to cancer or cause heritable damage in humans. Information is required on the capability of substances capability to induce gene mutations, structural chromosome aberrations (clastogenicity) and numerical chromosome aberrations (aneugenicity). To obtain such information, many *in vitro* and in *vivo* test methods officially adopted by the EU or the OECD are available. Non-testing options, for example (Q)SAR and the use of read-across approaches, may also provide

information on the mutagenic potential of chemical substances.

For genetic toxicity *in vitro* endpoint. For phase-in substances at or above 1 000 tpa: In 12.1 % of theses entries Weight of Evidence approach was flagged by the registrant and read-across approaches have been flagged in 22.0 % of ESRs. QSAR was used in 5 ESRs (0.05%).

For phase-in substances produced at 100 – 1 000 tpa: No significant difference from results shown above.

For the non-phase-in substances produced at or above 100 tpa: the read-across approaches have been flagged in only 10.3 % of the ESRs and Weight of Evidence has been only flagged in 2.8 % of all ESRs.

Genetic toxicity *in vivo* studies for phase-in substances manufactured or imported at or above 1 000 tpa: In 11.0 % of these entries a Weight of Evidence approach was flagged by the registrant and read-across approaches have been flagged in 24.8 %. Registrants have not used QSAR predictions for this endpoint.

For the dossiers of phase-in substances produced at 100 – 1 000 tpa: No significant difference from results shown above.

For the non-phase-in substances at or above 100 tpa: Read-across approaches have been flagged in only 5.3 % of the ESRs and only one ESR has been flagged as Weight of Evidence. Registrants have not used QSAR predictions for this endpoint (ECHA 2011).

5.3.2.3 Carcinogenicity

The objective of carcinogenicity studies on chemical substances is to identify potential human carcinogens, their mode(s) of action and their potency. Human data are available for only a few substances; therefore animal tests are generally used for detecting such a property.

Once a substance has been identified as a carcinogen, the next step is to assess whether a known carcinogen is directly genotoxic or not. Exposure conditions are utmost important as the hazard and a mode of action of a carcinogen may be highly dependent on, for example, the route of exposure.

Based on the complexity and length of the process of carcinogenesis, complex biological interactions and many different modes of action involved, even for the same substance, it is not possible to date to get a full understanding and complete mimicking by the use of alternative, non-animal tests. The 2-year cancer assay in rodents, usually the rat or mouse, is typically conducted to evaluate the cancer hazard and potency of a substance. Standard information requirements for carcinogenicity endpoint under REACH are laid down in Annex X, thus they are applicable for the highest tonnage substances (at 1 000 tpa or above).

For phase-in substances at 1 000 tpa or above: in 27.9 % registrants chose read-

across approach and 12.2 % of the entries a Weight of Evidence approach was flagged by the registrant. Two testing proposals on carcinogenicity have been submitted. QSAR predictions have been proposed seven times.

For the dossiers of phase-in substances produced at 100 – 1 000 tpa: A read-across approach was selected in 22.2 % of the cases and only 29 ESRs for non-phase-in substances at or above 100 tpa have been found (ECHA 2011).

5.3.3 Summary and conlusions by ECHA on the use of alternative methods and the implementation of (Q)SAR and read-across within REACH

Registry of compounds to be done within REACH mainly used existing animal studies (conducted before REACH), read-across and weight of evidence in the REACH registration dossiers to fulfil the information requirements based on Annex IX (Regulation (EC) No 1907/2006, Directive 67/548/EEC). Only in a few instances have a QSAR approach been used in the dossiers and for what (Q)SAR endpoints have been used, have not been reported. Personal communication and presentation by ECHA representative (Dr. Evelin Fabjan) point to that many of the QSAR approaches is flawed and the model and prediction is not reported in detail as is demanded from the QMRF and QPRF formats and would not be valid in its current form. There was generally limited information about the (Q)SAR model (e.g. version unclear, data on the model not transparent/ available), the scientific validity of model was not always demonstrated, applicability domain of the model was not or only partially analysed and results was not relevant for regulatory purposes and as such the endpoint predicted was not suitable to meet the information requirements of REACH. Further, those QSARs suggested to be used within REACH registration are based on prediction of other chemical properties such as environmental endpoints and are not directly related to (eco)toxicity.

Conclusions drawn from experience of the JRC (Dr. Andrew Worth) states that in principle, (Q)SAR estimates to be used within REACH could be used as direct replacements for test data, but in practice, use in weight-of-evidence assessments is more likely. There is still gaps in the REACH guidance documentation as there is no formal validation and adoption procedures for (Q)SAR models and detailed criteria for assessing the adequacy of (Q)SAR predictions is lacking also more examples is needed to illustrate how to demonstrate adequacy of a (Q)SAR model and how to successfully implement (Q)SARs for REACH information requirements.

6 QSAR model development

In NILU's final report (Alternative approaches to standard toxicity testing
TQP ID 9 - 257430120 - NILU) the developed QSAR model was a preliminary model that needed further validation prior to the potential use for risk assessment. The model was based on the publications by Helguera et al. (2008a,b) who published their results in a peer reviewed paper. As a final investigation we evaluated the normal distribution of the TD50 data which was evaluated not to fulfil the requirements for a normally distributed data. In addition we predicted

TD50 values for some selected new nitrosoamines and the model did not perform well suggesting that the QSAR model should be improved or discarded.
Helgurera et al. (2008a) used the lowest TD50 values tested on rats which were fed by the gavage route but they also reported data considering compounds added to drinking water in another publication. We suggest to only use the harmonic mean of the most potent TD50 value (identified as positive for cancerogenicity by the published author) for which some deviances exists compared to the TD50 used by Helguera et al. (2008b), one example is a compound for which they used the lowest TD50 but for which there is no verified cancerogenic effect and as such is set as no positive in the CPDB database.

The conclusion of the QSAR model assessment/validation of the preliminary model suggested that an improved/new QSAR model needed to be created. An estimation of the TD50 uncertainties within a 95% confidence interval should be determined and the validity of this model would need to be statistically acceptable and validated based on the OECD criteria (OECD, 2004).

6.1 Selection of an representative dataset (N-nitroso compounds)

The 12 nitrosamine compounds for which QSAR prediction was aimed for, was for the prediction of genotoxic potency (TD_{50}) data in rat collected from the CPDB database (http://potency.berkeley.edu/). These nitrosamines were combined with available data of other *N*-nitrosamines comprising in total 92 compounds, also collected from the CPDB database and a comparison to the selected nitrosamines made within the Orchestra FP7 project (Fjodorova et al. 2010). All TD_{50} data was transformed to the unit µmol/kg body wt/day (see Appendix B) and converted to the negative logarithm ($-\log_{10}(TD_{50})$) before model development.

6.2 Generation of molecular properties

The structures of the 92 nitrosoamine compounds was downloaded from ChemIDplus advanced (http://chem.sis.nlm.nih.gov/chemidplus/) and the structures was cleaned up and geometry optimized using CaChe Worksystems Pro (v7.5, Fujitsu Inc) with the semiempirical AM1 formalism. Structures were also checked for discrepancies based on CAS numbers through the SciFinder CAS databases (https://scifinder.cas.org/).
Physicochemical properties and 2-dimensional (2D) and 3-dimensional (3D) structural descriptors was calculated from the software ADMEWORKS ModelBuilder and CaChe Worksystems Pro/ProjectManager (using AM1 single point calculations), 350 descriptors in total.

6.2.1 Applicability domain (AD)

94 compounds with available TD_{50} values and their corresponding calculated properties was the basis for evaluating the applicability domain.
Evaluation of the applicability domain was done by Principal Component Analysis (PCA) with the software SIMCA P+ (v. 11.5, Umetrics Inc.). The main tool for evaluating the physicochemical and 2D/3D structural domain is to use the PCA in combination with the 95% confidence interval of Hotelling´s T^2 (Hotelling´s T^2 is the multivariate generalization of Student's t distribution but instead of studying univariate differences the multivariate differences is studied).

The PCA plot (Figure 6.1a) 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 350 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 *N*-nitroso compounds, 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 *N*-nitroso compounds of the physicochemical and 2D/3D descriptors used in this project and defines the different properties of the obsevations. Simca-P draws the tolerance ellipse based on Hotelling‘s T2 (with a 95% confidence interval). *N*-nitroso compounds outside of the Hotelling´s T^2 ellipse might be considered outliers. Four outliers was identified; 2-(3-(2-chloroethyl)-3-nitrosoureido)-D-glucopyranose (CAS 54749-90-5), 2-Deoxy-2-[[(methylnitrosoamino)carbonyl]amino]-D-glucose (CAS 18883-66-4), *N*-methyl-*N*-[4-(2-quinolin-4-ylethenyl)phenyl]nitrous amide (CAS 16699-10-8) and *N*-Methyl-*N*-nitroso-1-tetradecanamine (CAS 75881-20-8).

CAS 54749-90-5 and CAS 18883-66-4 could be considered to be real outliers in our dataset due to their structures and properties are deviating a great deal compared from the major set of compounds under study which mostly consists of nitrosoalkylamines and heterocyclic nitrosoalkylamines while these are D-glucose based nitrosoureas. Also CAS 16699-10-8 might be considered as an outlier due to the high molecular weight and multiple aromatic structures. CAS 75881-20-8 on the other hand have a relatively high molecular weight but is an alkylnitrosoamine and propably have the same toxic mechanism and as such was not excluded from the dataset.

In the PCA plot the slightly different *N*-nitroso compounds are visualized. The class of nitrosourea compounds are well separated from the compound classes nitrosoalkylamines, heterocyclic nitrosoalkylamines and aromatic nitrosoamines.
Also the alkohol/ketone/allyl nitrosoalkylamines are mostly separated from this group. Evaluation of the loadings plot (Figure 6.1b) shows the correlation in the X-space consisting of the physicochemical and 2D/3D variables. Comparison of both plots gives information which variables have most or less importance for the grouping of nitrosoamines 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. The positioning of the large and lipophilic *N*-nitroso compounds is located at the far left hand side of the scatter plot (Fig. 6.1a) correlating to size related properties and a high lipophilicity (high log P) on the left hand side of the loadings plot (Figure 6.1b), and as such variables or molecular properties that are located on the left hand side

far from the center of the plot have the strongest positive correlation with compounds located at the same side (see arrows in Figure 6.1a and b).

Based on the PCA, the Applicability Domain (AD) for the structural and physicochemical properties is clearly defined, while the response space (Y-data) is more clearly defined by a histogram (Figure 6.2). By binning the nitroso compounds based on activity, the normal distribution of the response (TD_{50}) data is visualized. Figure 6.3 shows the histogram of the TD_{50} for 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 which is shown in this case. We have compiled additional data compared to the original report (TQP ID9 – 257430120 - NILU) 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 our selection of nitroso compounds the –logTD50 values is within 3.5 orders of magnitude.

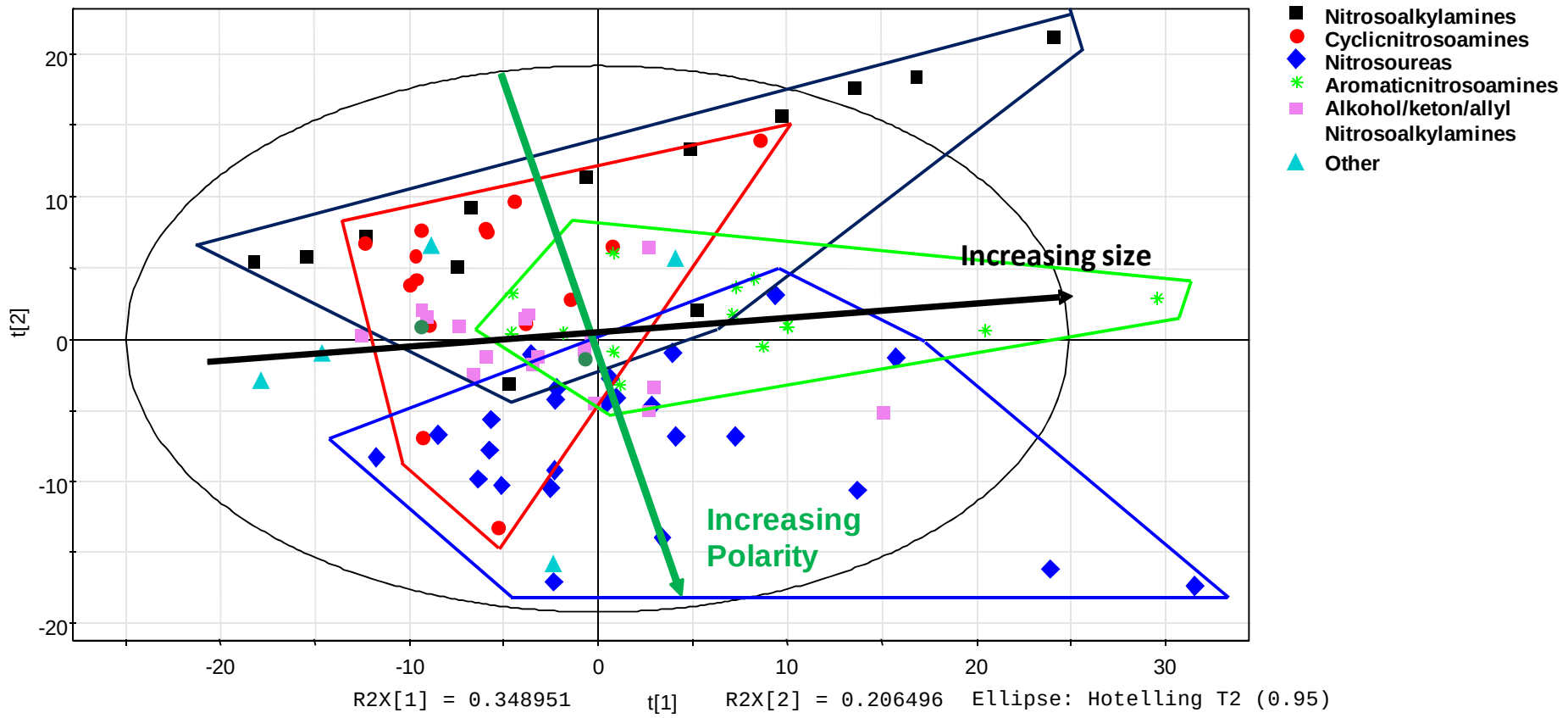


Figure 6.1a *Principal Component Analysis (PCA) plot: Scores scatter plot with the two first Principal Components, t[1] and t[2] describing 52% of the variation of the N-nitroso compounds in the physicochemical and 2D/3D structural space. Six outliers identified, which are outside of the Hotelling T2 (95% confidens inteval).*

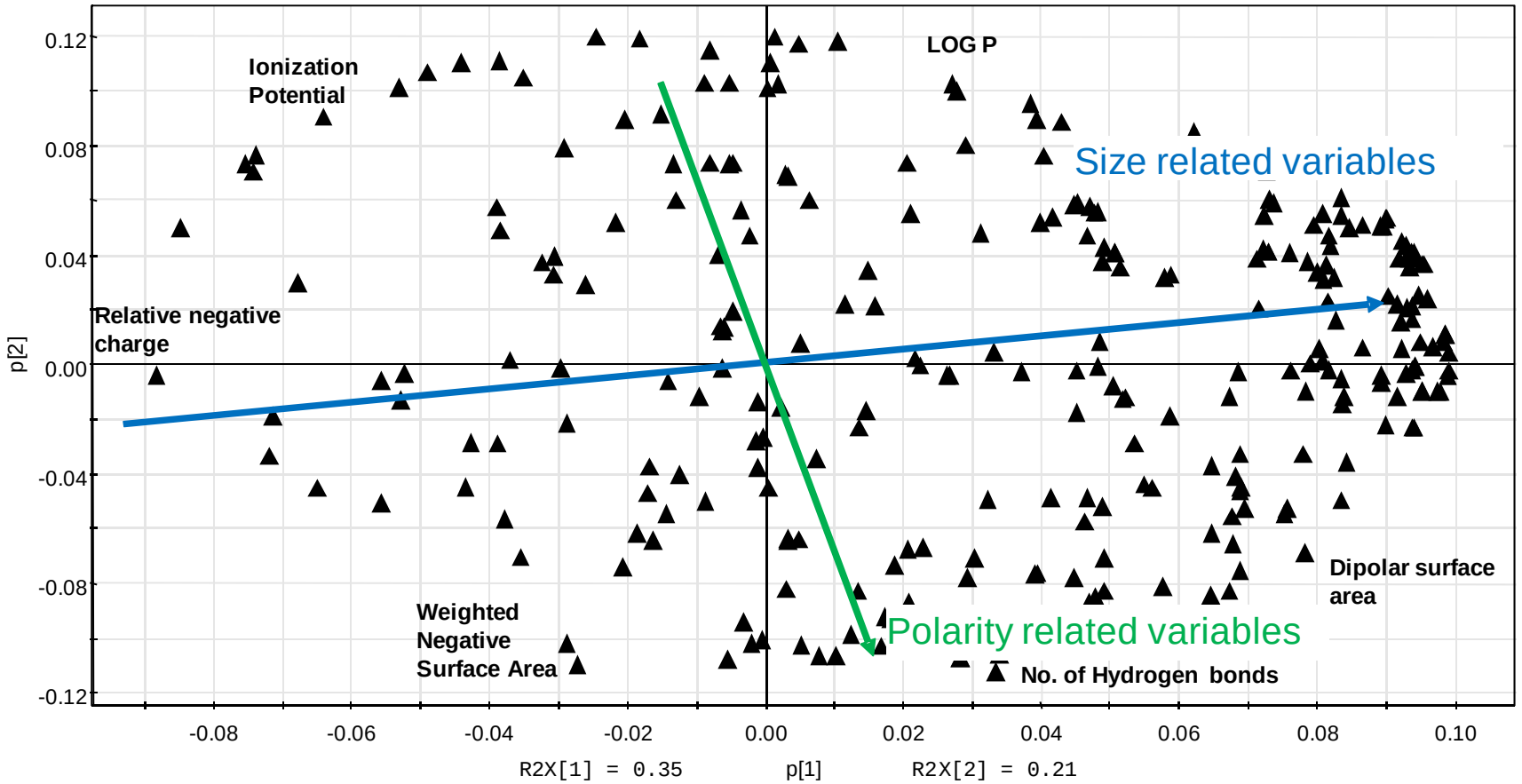


Figure 6.1b *Principal Component Analysis (PCA) plot: Loadings plot containing the correlation structure of the X-space (physicochemical and 2D/3D variables). Comparison of both plots 6.1a and 6.1b gives information which variables have most or less importance for the grouping of N-nitroso compounds in the physicochemical/2D/3D domain.*

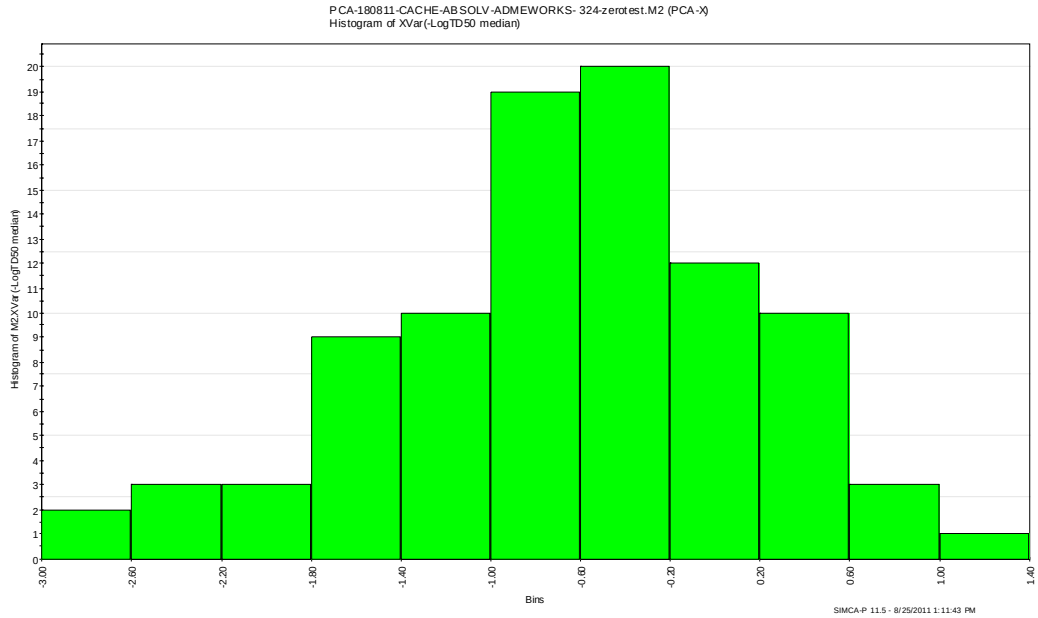


Figure 6.2 Histogram of the carcinogenic potency TD_{50} ($-Log_{10}(TD50)$) of the 92 selected N-nitrosamines. The harmonic mean using the TD_{50} values from the most potent target site in each positive experiment. The TD_{50} values are binned (x-axis) and the total numbers of binned N-nitroso compounds are shown (Y-axis).

6.2.2 Defining the "domain of applicability" – OECD principles

Accordingly we wanted to use compounds in this model which was relevant for this study and which follows the OECD principles for the validation of QSARs (OECD, 2007 and Amine 9 report: TQP ID9 – 257430120 - NILU):

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

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.

Of the basis of 92 collected *N*-nitroso compounds, 28 compounds was not selected for QSAR modelling due to them being irrelevant for this study such as the *N*-nitrosoureas, urethanes or guanidine as these compounds might decompose before metabolism and may elicit tumors at more remote sites (Rao et al. 1984).
In addition, five compounds was removed, the *N*-Nitrosodiphenylamine (CAS 86-30-6) as it is assumed that the mechanism follows a S_N1 route and degrades to N=O+ (OECD 2010a), Pyridine, 3-(1-nitroso-2-pyrrolidinyl)-, 1-oxide (CAS 78246-24-9) which have the possibility of two routes of mechanism (Alert SA_21

and SA_26), N-ethyl-2,2,2-trifluoro-N-nitrosoethanamine (CAS 82018-90-4) and 4,4,4-trifluoro-N-nitroso-N-(4,4,4-trifluorobutyl)-1-Butanamine (CAS 83335-32-4) as they are highly fluorinated dialkyl *N*-nitrosoamines for which their solubility and ADME properties might be significantly different from the main group of dialkyl *N*-nitrosoamines.

The final set of 58 compounds was the basis for QSAR modelling follows the Benigni/Bossa rulebase (Benigni et al. 2007 and 2008) as having the alert molecular substructures alkyl and aryl *N*-nitroso groups (Alert SA_21). The selected substance groups are either *N*-nitrosoalkylamines with or without hydroxyl and/or ketone groups or heterocyclic *N*-nitrosoalkylamines or aryl *N*-nitrosoalkylamines. To our knowledge all these compounds needs to be metabolically activated through the MFO metabolic system for which CYP 2E1, CYP3A6 and CYP2B4 are reported to be the major metabolization enzymes (Sulc et al. 2010).

6.2.3 Training and test set selection using SOM

The aim was to have a representative training and test set selected from the set of 58 preselected structures and selecting ~30% as a test set would achieve a balanced and representative test set which would give a sound statistical foundation for the evaluation the QSAR model. ¨

Accordingly we follow the definition in the OECD guidance document on the validation of (quantitative) structure-activity relationships (Q)SAR models (OECD (2007)).

When performing statistically designed external validation, the goal is to ensure that: a) the training and test sets separately span the whole descriptor space occupied by the entire data set; and b) the structural domains in the two sets are not too dissimilar. It is important that the training set contains compounds that are informative and good representatives of many other similar compounds: a) representative points of the test set must be close to points in of the training set; b) representative points of the training set must be close to points in the test set; and c) the training set must be diverse. These criteria have been proposed to ensure that the similarity principle can be adopted when predicting the test set.

We used a self-organizing map (SOM) to select the training and test set. SOM is a neural network based on unsupervised learning (Kohonen, 1995), and is a visualization tool for the classification of components based on property similarities. Components allocated in the same box have similar structures and physicochemical properties. The SOM was used in this case for compound classification and for selection of 41 training set compounds and 17 test set compounds (Figure 6.3). N-nitrosamines allocate to the upper-left-hand side are smaller molecules of alkyl and heterocyclic *N*-nitrosamines and on the lower-left-hand side is larger alkyl and heterocyclic *N*-nitrosamines. On the upper-right-hand side we have polar alkyl and heterocyclic *N*-nitrosamines while we have aromatic N-nitrosamines on the lower-right-hand side.

1,2,25, 26, 36, 57	14,30,31	15,28,29, 39,40		16,19,20, 22
7,17,35	10	51,52	3,44,47, 53	54
13,56	41	8,9,27,34		23,32,48, 49,50,55
5,21	18	6, 46,58		
24,38,42, 43		4	12,37,45	11,33

Figure 6.3 Self-organizing map (SOM) based on an unsupervised neural network (Kohonen). N-nitrosamines marked in black is the training set compounds and numbers marked in red are the test set compounds. Reference to the numbers is found in Appendix C.

6.2.4 Quality assessment and data pre-treatment of variable data

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 and Particle Swarm Optimization (PSO) 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 6.0 (Heimstad et al., 2009).

6.2.5 Variable selection procedure

Standard procedures for removing correlated descriptors were made based on a maximum of 90 % correlation between variables. This procedure removed of a total of 350 variables leaving 135 variables and was followed by a variable selection procedure with the use of Genetic Algorithm or PSO to find the best descriptor set (with lowest number of descriptors) with highest correlation to -$LogTD_{50}$. The next step is the regression of 40 training compounds made with Interactive MLR in ADMEWORKS ModelBuilder with quality parameters for the regression such as Adjusted R^2, Cross-validated (leave-many-out) R^2CV (LMO) and prediction Q^2_{ext} for a test set of 17 compounds.

Commonly a variable selection procedure is used for variable datasets which are large and usually a genetic algorithm procedure or SVM-based variable selection is used. In this case we used a variable selection procedure using Particle Swarm Optimization (PSO algorithm). The PSO algorithm starts with the creation of a population of randomly generated Parameter Sets - individuals. This population is

called an intelligent swarm. Individuals are then compared according to Objective Function (based on MLR model). The form of objective function favors sets that have the R^2 as high as possible, while minimizing the number of Parameters used as Descriptors. The best individual is called Leader. The parameter set is like a location in parameter space – leader is the individual which is as close to the best location as possible. Leader can change in time because swarm is on the move. PSO finishes when each individual from the swarm is as close to the leader as possible (ADMEWORKS Model Builder Reference guide).
The best population from the PSO algorithm was selected with an R^2 over 0.7 and limited to maximum of 6 variables which is adequate to the MLR rule of a parameter to compound ratio of minimum 6 to 1, in this case 40 compounds (excl 1 outlier)(see Table 6.1). A QSAR model has acceptable predictive power if the following conditions are satisfied: $R^2CV > 0.5$, $R^2 > 0.6$ (Golbraikh et al. 2003 and http://www.epa.gov/nrmrl/std/cppb/qsar/testuserguide.pdf, accessed 21.09.2011).

Table 6.1 Variable selection and interactive MLR for the creation of a QSAR model. The five most optimal models are presented for genetic algoritm (GA) and Particle Swarm Optimisation (PSO).

No.	Variable selection method[a]	R^2	$R^2CV(LMO)$[b]	Q^2_{ext}[c]
1	GA	-	-	-
2	PSO	0.63	0.50	-0.64
3	PSO	0.66	0.58	-1.50
4	PSO	0.72	0.60	-0.69

a) Variable selection method using Particle Swarm Optimization (PSO) and genetic algorithms (GA). GA generated too many variables to be useful in this QSAR model training set.

b) Leave-many-out (LMO) cross validation is based on a 7 fold split of the data and an iterative prediction of the 7 blocks of data in turn to get the R^2CV (similar to Q^2). The R^2CV parameter represents the goodness of fit of the jackknifed predicted values vs. the original values.

c) External validation (Q^2ext) using the test set of compounds which was not used for creating the QSAR model.

6.2.6 Applicability domain of the test set

To verify that we have a test set of compounds which lies within the QSAR applicability domain, we use a PLS on the selected variables used for the QSAR model and plot the DmodX for each test set compound. A DmodX bar plot (Figure 6.4) is a multivariate statistic that visualises the absolute distance of an observation to the current QSAR prediction model based on the trainingset. A compound with a value higher than the D-criterion (95% confidence interval), RED line might be considered as an outlier. In this case all the test set compounds are within the criterion and are valid for the creation of a model.

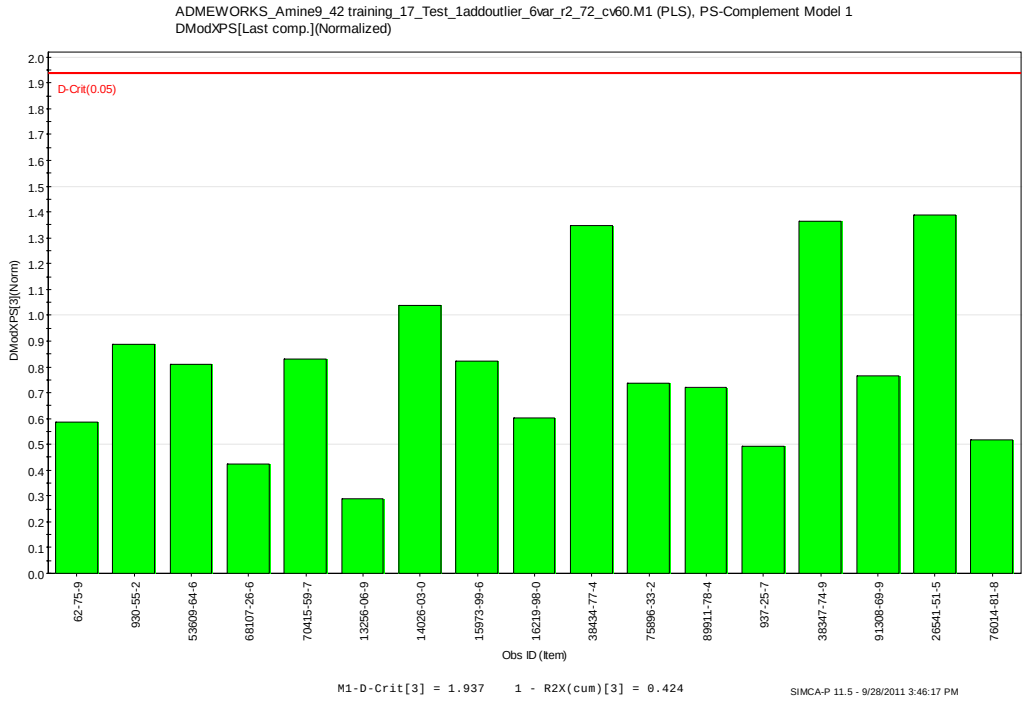


Figure 6.4 DmodX plot (Absolute distance to the model of an observation in the test set). Visualizes the absolute distance of an observation to the current QSAR prediction model based on the trainingset. A compound with a value higher than the D-criterion (95% confidence interval), RED line might be considered as an outlier.

6.2.7 QSAR model based on 40 training and 17 test compounds

Figure 6.5 shows the correlation between observed and predicted -Log(TD_{50}) values for the training (n=40, 1 outlier exluded) and the test set (n=17). Experimental and predicted values, for each chemical, are given in Appendix C.

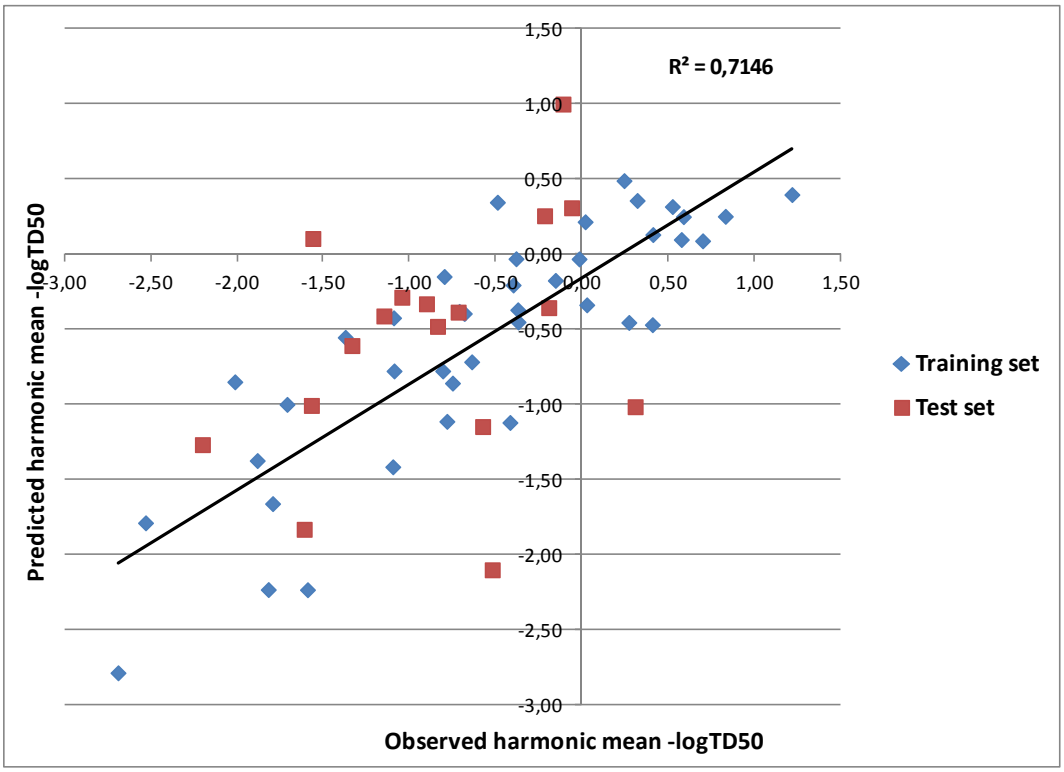


Figure 6.5 QSAR using Multiple Linear Regression. The training set which was used to train the QSAR is assigned blue diamonds while the test set (not used in model development) assigned red squares is used to verify the accuracy of the model.

The regression equation is given in table 6.2 and the importance of descriptors to the regression is decreasing when going from left to right in the equation:

Table 6.2 QSAR model summary based on the optimal model achieved during variable selection procedure (see Table 6.1). 40 training set compounds (for building the model) and 17 test set compounds (for validation of the model)

Number of descriptors	Training set n=40 -Log(TD_{50})	R^2	Test set n=17 Q^2_{ext}	R^2CV (LMO)
6	-0.584 +0.365**(Pi2(S))** +0.705**(V3C)** -0.928**(V6CH)** - 0.732 **(QPOS)** -0.576**(GEOM3)** - 0.496**(PND2)** Mean square error (MSE)= 0.297 F-statistic = 14.7 p-value = 0.0000	0.71 Adj. 0.67	-0.69 (MSE=0.214)	0.60

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

Pi2(S):	Polarizability (ADME property based on the Abraham LFER)
V3C:	3rd order cluster MC Valence
V6CH:	6th order chain MC Valence
GEOM3:	Mass weighted Thickness
PND2:	Superpendentivity index Carbons only
QPOS:	Charge of the most positive atom

6.2.8 QSAR model results

Results shown in table 6.1 and 6.2 prove that it is possible to generate a seemingly valid model if we would base the results only on the 40 training set compounds. We have generated a model with a correlation coefficient R^2 better than 0.6 and a R^2CV better than 0.5 (as suggested by Golbraikh et al. 2003 and http://www.epa.gov/nrmrl/std/cppb/qsar/testuserguide.pdf), in this case we achieved the best model with an R^2 of 0.72 with a cross-validated R^2CV result of 0.6 which is good enough to be usable for prediction purposes. To determine the validity of the QSAR model we used the 17 test set compounds. The test set is not used for the creation of the model, and as such, are valuable in the quality assessment of the model. By using this test set and predicting values using the QSAR model we have a way of estimating the "real" predictivity of the model. Unfortunately, the model fails based on the test set (Q^2ext = -0.69) suggesting that the model created is inadequate. The model might be over-predictive, which is a pitfall in MLR model generation and too much random correlation of the input variables to the response data (TD50). Evaluating the input variables of the model shows that these variables selected are not optimal (skewness of data).

6.2.9 Uncertainties in the predicted TD50 values

The Company states that the contractor should give an estimate of the uncertainty of the TD50 values for the 12 nitrosamines (Appendix C, (see prediction header)) that were going to be predicted based on this (see above) improved/new QSAR model. An estimation of the uncertainties within a 95% confidence interval of these predicted TD50 values would have been determined based on this model and the validity of this model needs to be statistically acceptable and validated based on the OECD criteria (OECD, 2004). The conclusion is that we are not able to make such a prediction due to the failure of having a statistically valid model and as such these predicted values are left as not determined (ND).

6.3 Discussion

The condition of applicability of QSAR is that a sufficient number of representative congeneric (chemicals belonging to the same class of the chemical that is to be predicted) has been previously tested experimentally for the activity to be predicted (Benigni & Zito, 2004). The author argues that based on current evidence and recent publications that QSAR has provided successful models for a wide range of different biological endpoints with different degrees of complexity by pointing to and describing the rate limiting step of the study under investigation. Evidence exists that rodent carcinogenicity data can be modelled efficiently through a QSAR approach when qualitatively and quantitatively adequate data is available. Examples of successful QSAR models are the model for skin carcinogenicity of the non-heterocyclic aromatic hydrocarbons (Zhang et al., 1992) and for the rodent carcinogenicity of the aromatic amines (Benigni & Passerini, 2002). The success of these models is mostly due to the use of appropriate sets of chemicals, belonging to the same class and acting through the same mechanism of action, an approach which is the most powerful for the creation of a predictive QSAR model. The QSAR model of the aromatic amines where 2/3 of the compounds was based on the *in vivo* rodent bioassays done through the aegis second U.S: National Toxicology Program comparative exercises on the prediction of rodent cancerogenicity and as such follows a specific protocol and the use of Hansch analysis for the aromatic amine structures (Kubinyi, 1993).

Similarly, such an approach has been aimed for in this work. We have selected compounds from the CPDB database of *N*-nitroso compounds which are assumed to have the same mechanism of action, based on the screening of compounds which follows the Benigni-Bossa rules of having the molecular substructures alert: alkyl and aryl *N*-nitroso groups (Alert SA_21). In addition, we have selected compounds which needs to be activated by metabolization through a similar mechanism to the active mutagen. *In vitro* studies have shown that the rate limiting step in *N*-nitrosamine carcinogenesis is the *in vitro* metabolism to mutagens (Guttenplan, 1987). They showed by using deuterium labelling in alpha-position of a few *N*-nitrosamines that cleavage of a carbon-hydrogen bond in the alpha position was a rate-limiting step in carcinogenesis. Results was inconclusive for longer chained *N*-nitrosalkylamines and dinitroso-2,6-dimethylpiperazine suggesting that oxidation at the alpha carbon might not be a rate-limiting step for these molecules (Lijinsky, 1986). Accordingly, a QSAR model that would focus on the rate limiting step would be beneficial.

Pitfalls in QSAR are: inappropriate biological data, wrong scaling of biological data, data from different labs, different binding modes, mixed data (e.g. oral absorption and bioavailability), different mechanism of action, too few data points, too many single points, lack of chemical variation, clustered data, small variance of y values (activity), systematic error/s in y, too large errors in y values, outliers / wrong values and wrong model selection (Kubinyi, 2010, http://www.kubinyi.de/Rhodos-09-10.pdf).

Unfortunately many of these pitfalls are evident in this dataset. The use of historical data such as the TD_{50} value in the CPDB database is problematic due to unknown factors that will influence the assignment of harmonic mean TD_{50}

values. *In vivo* bioassays on rat have been performed with non-standardised protocols, at many different laboratories, with different ways to deliver the substances to the rat: through the food, gavage, drinking water, and intravenous. Solubility of the compounds and efficacy of absorption through the gut into the bloodstream will be influenced by the type of delivery but also on ADME properties. For a few of the substances there is only one reported study of TD_{50} which suggests that the harmonic mean, if a standardized protocol would have been studied, might have produced significantly different and better models.
A study of a congeneric set of chemicals and the use of a standardised *in vivo* rat protocol would improve the possibility of creating a valid QSAR model with a real predictive ability. A congeneric set of chemicals will have higher degree of close correlating properties such as solubility and lipophilicity as to have linearly correlating ADME properties and also to have a similar mechanism of action.

6.4 Conclusion

The conclusion is that we are not able to make a valid QSAR prediction based on the current set of input data. The QSAR model fail in giving a statistically valid model and the QSAR model should be discarded.

7 References

Aires, G.M., Meireles, J.R., Oliveira, P.C., Oliveira, J.L., Araújo, E.L., Pires, B.C., Cruz, E.S., Jesus, N.F., Pereira, C.A., Cerqueira, E.M. (2011) Micronuclei as biomarkers for evaluating the risk of malignant transformation in the uterine cervix. *Genet Mol Res. 2011;10(3):1558-64. doi: 10.4238/vol10-3gmr1156*.

Arcos, J.C., Argus, M.F. (1995) Multifactor interaction network of carcinogenesis- a “Tour Guide”. In: Arcos, J.C., Argus, M.F., Woo, Y.T., editors, Chemical induction of cancer. Modulation and combination effects. Birkhauser. Boston. P. 1-20.

Ashby, J. (1985) Fundamental structural alerts to potential carcinogenicity or noncarcinogenicity. *Environ Mutagen.*;7(6):919-21.

Ashby, J., Tennant, R.W. (1988) Chemical structure, Salmonella mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogenesis among 222 chemicals tested in rodents by the U.S. NCI/NTP. *Mutat Res. 1988 Jan;204(1):*17-115. Review.

Barlow, S., Renwick, A.G., Kleiner, J., ,Bridges, J.W., Busk, L., Dybing, E., Edler, L., Eisenbrand, G., Fink-Gremmels, J., Knaap, A., Kroes, R., Liem, D., Müller, D.J., Page, S., Rolland, V., Schlatter, J., Tritscher, A., Tueting, W., Würtzen, G. (2006) Risk assessment of substances that are both genotoxic and carcinogenic report of an International Conference organized by EFSA and WHO with support of ILSI Europe. *Food Chem Toxicol.* 2006 Oct;44(10):1636-50. Epub 2006 Jul 8.

Beland, F.A., Fullerton, N.F., Kinouchi, T., Poirier, M.C. (1988) DNA adduct formation during continuous feeding of 2-acetylaminofluorene at multiple concentrations. *IARC Sci Publ.* 1988;(89):175-80.

Benigni, R., Bossa, C. (2011) Alternative strategies for carcinogenicity assessment: an efficient and simplified approach based on in vitro mutagenicity and cell transformation assays. *Mutagenesis*. 2011 May;26(3):455-60. Epub 2011 Mar 11.

Benigni, R., Bossa, C., Tcheremenskaia, O., Giuliani, A. (2010) Alternatives to the carcinogenicity bioassay: in silico methods, and the in vitro and in vivo mutagenicity assays. *Expert Opin Drug Metab Toxicol.* 2010 Jul;6(7):809-19. Review.

Benigni, R., Bossa, C., Jeliazkova, N., Netzeva, T., Worth, A. (2008) The Benigni / Bossa rulebase for mutagenicity and carcinogenicity - a module of Toxtree (European Commission report: EUR 23241 EN – 2008, http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology/doc/EUR_23241_EN.pdf)

Benigni, R., Bossa, C., Netzeva, T., Rodomonte, A., Tsakovska I. (2007) Mechanistic QSAR of aromatic amines: new models for discriminating between mutagens and nonmutagens, and validation of models for carcinogens. *Environ mol mutag* 48:754-771.

Benigni, R., Zito, R. (2004) The second National Toxicology Program comparative exercise on the prediction of rodent cancerogenicity: definitive results. *Mutation Research* 566: 49-63.

Bishop, J.M. (1991) Molecular themes in oncogenesis.Cell. 1991 Jan 25;64(2):235-48. Review.

Bonassi, S., Norppa, H., Ceppi, M., Strömberg, U., Vermeulen, R., Znaor, A., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Gundy, S., Hansteen, I.L., Knudsen, L.E., Lazutka, J., Rossner, P., Sram, R.J., Boffetta, P. (2008) Chromosomal aberration frequency in lymphocytes predicts the risk of cancer: results from a pooled cohort study of 22 358 subjects in 11 countries. *Carcinogenesis.* 2008 Jun;29(6):1178-83. Epub 2008 Mar 19.

Bonassi, S., El-Zein, R., Bolognesi, C., Fenech, M. (2011) Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis.* 2011 Jan;26(1):93-100.

Colvin, M.E., Hatch, F.T., Felton, J.S. (1998) Chemical and biological factors affecting mutagen potency. *Mutat Res.* 1998 May 25;400(1-2):479-92. Review.

Costa, S., Pina, C., Coelho, P., Costa, C., Silva, S., Porto, B., Laffon, B., Teixeira, J.P. (2011) Occupational exposure to formaldehyde: genotoxic risk evaluation

by comet assay and micronucleus test using human peripheral lymphocytes. *J Toxicol Environ Health A.* 2011 Aug;74(15-16):1040-51.

Crump, K.S. (1984) An improved procedure for low-dose carcinogenic risk assessment from animal data. *J Environ Pathol Toxicol Oncol.* 1984 Jul;5(4-5):339-48.

Druckrey, H., Preussmann, R., Ivankovic, S., Schmähl, D. [Organotropic carcinogenic effects of 65 various N-nitroso- compounds on BD rats]. *Z Krebsforsch.* 1967;69(2):103-201.

Dunn, B.P. (1983) Wide-range linear dose-response curve for DNA binding of orally administered benzo(a)pyrene in mice. *Cancer Res.* 1983 Jun;43(6):2654-8.

Dybing, E., O'Brien, J., Renwick, A.G., Sanner, T. (2008) Risk assessment of dietary exposures to compounds that are genotoxic and carcinogenic--an overview. *Toxicol Lett.* 2008 Aug 15;180(2):110-7. Epub 2008 May 23. Review.

Dybing, E., Sanner, T., Roelfzema, H., Kroese, D., Tennant, R.W. (1997) T25: a simplified carcinogenic potency index: description of the system and study of correlations between carcinogenic potency and species/site specificity and mutagenicity. *Pharmacol Toxicol.* 1997 Jun;80(6):272-9.

Dybing, E., Doe, J., Groten, J., Kleiner, J., O'Brien, J., Renwick, A.G., Schlatter, J., Steinberg, P., Tritscher, A., Walker, R., Younes, M. (2002) Hazard characterisation of chemicals in food and diet. dose response, mechanisms and extrapolation issues. *Food Chem Toxicol.* 2002 Feb-Mar;40(2-3):237-82. Review.

Dybing, E., Sanner, T., Roelfzema, H., Kroese, D., Tennant, R.W. (1997) T25: a simplified carcinogenic potency index: description of the system and study of correlations between carcinogenic potency and species/site specificity and mutagenicity. *Pharmacol Toxicol.* 1997 Jun;80(6):272-9.

ECHA (2011): The Use of Alternatives to Testing on Animals for the REACH Regulation (2011). (ECHA-11-R-004.2-EN) Http://echa.europa.eu/doc/117reports/alternatives_test_animals_2011_en.pdf

ECHA (2010a): Evaluation under REACH, Progress Report 2010. (ECHA-11-R-001-EN) http://echa.europa.eu/doc/evaluation_under_reach_progress_report_2010.pdf

ECHA (2010b): Practical guide 6: How to report read-across and categories. (ECHA-10-B-11-EN) Http://echa.europa.eu/doc/publications/practical_guides/pg_report_readacross_categ.pdf

ECHA/REACH R5 2008

http://guidance.echa.europa.eu/docs/draft_documents/26102010%20final%20draft_Inforeq_CSR_R5_clean.pdf

ECHA/REACH R8 2008
http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_r8_en.pdf

EC Regulation No 1907/2006, Directive 67/548/EEC:

Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency. *OJEC* L396, 30.12.2006, pp. 1–849.

(http://www.reach-compliance.eu/english/legislation/docs/launchers/launch-2006-1907-EC-06.html).

Edler, L., Poirier, K., Dourson, M., Kleiner, J., Mileson, B., Nordmann, H., Renwick, A., Slob, W., Walton, K., Würtzen, G. (2002) Mathematical modelling and quantitative methods. *Food Chem Toxicol.* 2002 Feb-Mar;40(2-3):283-326. Review.

Efron, B. and Gong, G. (1983). A Leisurely Look at the Bootstrap, the Jack-knife, and Cross-validation, *American Statistician,* 37, 36-48.

Eisenbrand, G., Habs, M., Schmähl, D., Preussmann, R. (1980) Carcinogenicity of N-nitroso-3-hydroxypyrrolidine and dose-response study with N-nitrosopiperidine in rats. *IARC Sci Publ.* 1980;(31):657-66.

EFSA 2005 Opinion of the Scientific Committee on a request from EFSA related to harmonized approach for risk assessment of substances which are both genotoxic and carcinogenic (Request N. EFSA-Q-2004-020). Adopted on 18 October 2005. http://www.efsa.europa.eu/en/scdocs/doc/619.pdf

EPA (US) (2004) Benchmark Dose Software (BMDS) Version 1.3.2 National Center for Environmental assessment. U.S. enviromnetla Protection Agency. http://www.epa.gov/ncea/bmds.htm

EPA (US) (1996) Proposed Guidelines for carcinogenic risk assessment. Federal Register, 17960-18011.

EPA (US) (1995) The use of the benchmark dose approach in health risk assessment. EPA/630/R-94/007. Risk Assessment Forum, Washington DC.

Eyfjord, J.E., Bodvarsdottir, S.K. (2005) Genomic instability and cancer: networks involved in response to DNA damage. *Mutat Res.* 2005 Dec 30;592(1-2):18-28. Epub 2005 Jul 5. Review.

FDA (US) (2006) Guidance for Industry and Review Staff Recommended

Approaches to Integration of Genetic Toxicology Study Results. http://www.fda.gov/ohrms/dockets/98fr/04d-0493-gdl0002.pdf

Fetterman, B.A., Kim, B.S., Margolin, B.H., Schildcrout, J.S., Smith, M.G., Wagner, S.M., Zeiger, E. (1997) Predicting rodent carcinogenicity from mutagenic potency measured in the Ames Salmonella assay. *Environ Mol Mutagen.* 1997;29(3):312-22. Review.

Fjodorova, N., Vračko, M., Novič, M., Roncaglioni, A., Benfenati, E. (2010) New public QSAR model for carcinogenicity. *Chem Cent J. 4*(Suppl 1): S3.

Goel, S., Bhatia, A., Dey, P. (2011) Spontaneously Occurring micronuclei in infiltrating ductal carcinoma of breast: A potential biomarker for aggressive phenotype detection? *Diagn Cytopathol.* 2011 Oct 11.

Goodall, C.M., Kennedy, T.H. (1976) Carcinogenicity of dimethylnitramine in NZR rats in NZOmice. *Cancer Lett.* 1976 May;1(5):295-8.

Gold, L.S., Sawyer, C.B., Magaw, R., Backman, G.M., de Veciana, M., Levinson, R., Hooper, N.K., Havender, W.R., Bernstein, L., Peto, R., et al. (1984). A carcinogenic potency database of the standardized results of animal bioassays. *Environ Health Perspect.* 1984 Dec;58:9-319.

Gold, L.S., de Veciana, M., Backman, G.M., Magaw, R., Lopipero, P., Smith, M., Blumenthal, M., Levinson, R., Bernstein, L., Ames, B.N. (1986/1993) Chronological supplement to the Carcinogenic Potency Database: standardized results of animal bioassays published through December 1982. *Environ Health Perspect.* 1986 Aug;67:161-200. Review. Erratum in: *Environ Health Perspect* 1993 Apr;100:69-70.

Gray, J.W., Collins, C. (2000) Genome changes and gene expression in human solid tumors. *Carcinogenesis.* 2000 Mar;21(3):443-52. Review.

Gray, R., Peto, R., Brantom, P., Grasso, P. (1991) Chronic nitrosamine ingestion in 1040 rodents: the effect of the choice of nitrosamine, the species studied, and the age of starting exposure. *Cancer Res.* 1991 Dec 1;51(23 Pt 2):6470-91.

Guttenplan, J.B. (1987) *N*-nitrosamines: bacterial mutagenesis and in vitro metabolism, Mutation Research/Reviews in *Genetic Toxicology,* 186 (2), 81-134

Hatch, F.T., Knize, M.G., Moore, D.H. 2nd, Felton, J.S. (1992) Quantitative correlation of mutagenic and carcinogenic potencies for heterocyclic amines from cooked foods and additional aromatic amines. *Mutat Res.* 1992 Jun;271(3):269-87.

Hebels, D.G., Jennen, D.G., van Herwijnen, M.H., Moonen, E.J., Pedersen, M., Knudsen, L.E., Kleinjans, J.C., de Kok, T.M. (2011) Whole-genome gene

expression modifications associated with nitrosamine exposure and micronucleus frequency in human blood cells. *Mutagenesis.* 2011 Nov;26(6):753-61. Epub 2011 Jul 1.

Hirmann, D. (2010) Presentation. http://www.orchestra-qsar.eu/sites/default/files/3_Hirmann.pdf, accessed 10.10.2011.

Kirkland, D., Reeve, L., Gatehouse, D., Vanparys, P. (2011) A core in vitro genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and in vivo genotoxins. *Mutat Res.* 2011 Mar 18;721(1):27-73.

Klein, R.G., Janowsky, I., Pool-Zobel, B.L., Schmezer, P., Hermann, R., Amelung, F., Spiegelhalder, B., Zeller, W.J. (1991) Effects of long-term inhalation of N-nitrosodimethylamine in rats. *IARC Sci Publ,* 1991; (105):322-8.

Kohonen, T. (1995) *Self-organizing maps*, vol 30 of springer series in information sciences. Springer, Berlin, Heidelberg.

Kroes, R., Renwick, A.G., Cheeseman, M., Kleiner, J., Mangelsdorf, I., Piersma, A., Schilter, B., Schlatter, J., van Schothorst, F., Vos, J.G., Würtzen, G. (2004) European branch of the International Life Sciences Institute. Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. *Food Chem Toxicol.* 2004 Jan;42(1):65-83.

Landigham, Allen, Ship and Crump (2001) Comparison of the EU T25 Single Point Estimate Method with Benchmark Dose Response Modeling for Estimating Potency of Carcinogens. *Risk Analysis, Volume 21, Issue 4,* pages 641–656.

Lijinsky, W. (1986) Deuterium isotope effects in carcinogenesis by *N*-nitroso compounds. *J Cancer Res Clin Oncol.* 112(3):229-39.

Loeb, K.R., Loeb, L.A. (2000) Significance of multiple mutations in cancer.Carcinogenesis. 2000 Mar;21(3):379-85. Review.

Lutz, W.K. (1990) Dose-response relationship and low dose extrapolation in chemical carcinogenesis. *Carcinogenesis*. 1990 Aug;11(8):1243-7.

Lutz, W.K. (1987) Quantitative evaluation of DNA-binding data in vivo for low-dose extrapolations. *Arch Toxicol Suppl.* 1987;11:66-74.

Malling, H.V., Frantz, C.N. (1973) In vitro versus in vivo metabolic activation of mutagens. *Environ Health Perspect.* Dec;6:71-82.

Matthews, E.J., Kruhlak, N.L., Cimino, M.C., Benz, R.D., Contrera, J.F. (2006) An analysis of genetic toxicity, reproductive and developmental toxicity, and carcinogenicity data: I. Identification of carcinogens using surrogate endpoints. *Regul Toxicol Pharmacol.* 2006 Mar;44(2):83-96.

Miller. E.C., Miller. J-A. (1981) Mechanisms of chemical carcinogenesis. *Cancer. Mar 1*;47(5 Suppl):1055-64.

Munro. I.C., Renwick, A.G., Danielewska-Nikiel, B. (1980) The Threshold of Toxicological Concern (TTC) in risk assessment. Toxicol Lett. 2008 Aug 15;180(2):151-6.Neumann HG. Dose-response relationship in the primary lesion of strong electrophilic carcinogens. *Arch Toxicol Suppl.* 1980;3:69-77.

O'Brien, J., Renwick, A.G., Constable, A., ,Dybing, E., Muller, D.J.G., Schlatter, H., Slob, W., Tueting, W., van Benthem, J., Williams, G.M. and Wolfreys, A. (2006) Approaches to the risk assessment of genotoxic carcinogs in food. *Food and chemical Toxicology;* 44 (10): 1613-1625.

OECD (2010a) Series of testing and assessment, Number 120: Report on the expert consultation on scientific and regulatory evaluation of organic chemistry mechanism-based structural alerts for the identification of DNA binding chemicals. ENV/JM/MONO(2010)8/Part1

OECD (2010b) Series of testing and assessment, Number 120: Report on the expert consultation on scientific and regulatory evaluation of organic chemistry mechanism-based structural alerts for the identification of DNA binding chemicals. ENV/JM/MONO(2010)8/Part2

OECD (2007) Guidance document on the validation of (quantitative) structure-activity relationships [(Q)SAR] models (ENV/JM/MONO(2007)2) http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2007)2&doclanguage=en)

OECD (2004) Principles for the Validation, for Regulatory Purposes, of (Quantitative) Structure-Activity Relationship Models

Peto, R., Gray, R., Brantom, P., Grasso, P. (1984) Nitrosamine carcinogenesis in 5120 rodents: chronic administration of sixteen different concentrations of NDEA, NDMA, NPYR and NPIP in the water of 4440 inbred rats, with parallel studies on NDEA alone of the effect of age of starting (3, 6 or 20 weeks) and of species (rats, mice or hamsters). *IARC Sci Publ.* 1984;(57):627-65.

Peto, R., Pike, M.C., Bernstein, L., Gold, L.S., Ames, B.N. (1984) The TD50: a proposed general convention for the numerical description of the carcinogenic potency of chemicals in chronic-exposure animal experiments. *Environ Health Perspect.* 1984 Dec;58:1-8.

Peto, R., Gray, R., Brantom, P., Grasso, P. (1991) Dose and time relationships for tumor induction in the liver and esophagus of 4080 inbred rats by chronic

ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine. *Cancer Res 51*(23 Pt 2): 6452-69.

Piegorsch, W.W., Hoel, D.G. (1988) Exploring relationships between mutagenic and carcinogenic potencies. *Mutat Res.* 1988 Sep;196(2):161-75.

Powell, B.L., Iris, L., van Staveren, I.L., Rooske, P., Grieu, F., Berns, E.M.J.J., Iacopetta, B. (2002) Associations between common polymorphisms in TP53 and p21WAF1/Cip1 and phenotypic features of breast cancer. *Carcinogenesis 23,* 311-315.

QSAR (1993): Hansch analysis and related approaches. by Hugo Kubinyi. Published by VCH in Weinheim, New York.

Quiling, S., Yuxin, Z., Suhua, Z., Cheng, X., Shuguang, L., Fengsheng, H. (2003) Cyclin D1 gene polymorphism and susceptibility to lung cancer in a Chinese population. *Carcinogenesis* 24, 1499-1503.

Sanner, T. and Dybing, E. (2005) Comparison of carcinogenic and in vivo genotoxic potency estimates. *Basic Clin Pharmacol Toxicol.* 2005 Feb;96(2):131-9.

Sanner, T., Dybing, E., Willems, M.I., Kroese, E.D. (2001) A simple method for quantitative risk assessment of non-threshold carcinogens based on the dose descriptor T25. *Pharmacol Toxicol.* 2001 Jun;88(6):331-41.

Sulc, M., Hodek, P., Stiborová, M. (2010). The binding affinity of carcinogenic N-nitrosodimethylamine and N-nitrosomethylaniline to cytochromes P450 2B4, 2E1 and 3A6 does not dictate the rate of their enzymatic N-demethylation. *Gen Physiol Biophys.,* 29(2):175-85.

Wang, L., Habuchi, T., Takahashi, T., Mitsumori, K., Kamoto, T., Kakehi, Y., Kakinuma, H., Sato, K., Nakamura, A., Ogawa, O., Kato, T. (2002) Cyclin D1 gene polymorphism is associated with an increased risk of urinary bladder cancer. *Carcinogenesis* 23, 257-264.

Weinberg, R.A. (1991) Tumor suppressor genes. *Science*. 1991 Nov 22;254(5035):1138-46. Review.

Woo, H.G., Park, E.S., Lee, J.S., Lee, Y.H., Ishikawa, T., Kim, Y.J., Thorgeirsson, S.S. (2009) Identification of potential driver genes in human liver carcinoma by genomewide screening. *Cancer Res.* 2009 May 1;69(9):4059-66. Epub 2009 Apr 14.

Woo, Y.T. and Lai, D.Y. (2003) Mechanisms of action of chemical carcinogens, and their role in Structure-Activity Relationships (SAR) analysis and risk assessment. In: Quantitative Structure-Activity Relationship (QSAR) Models of Mutagens and Carcinogens (R Benigni, ed). Boca Raton: CRC Press: 41–80.

Zhang, L., Sannes, K., Shusterman, A.J., Hansch C. (1992) The structure-activity relationship of skin carcinogenicity of aromatic hydrocarbons and heterocycles. *Chem Biol Interact.* Jan;81(1-2):149-80.

Zeiger, E. (2004) History and rationale of genetic toxicity testing: an impersonal, and sometimes personal, view. *Environ Mol Mutagen.* 2004;44(5):363-71.

Zeiger, E., Haseman, J.K., Shelby, M.D., Margolin, B.H., Tennant, R.W. (1990) Evaluation of four in vitro genetic toxicity tests for predicting rodent carcinogenicity: confirmation of earlier results with 41 additional chemicals. *Environ Mol Mutagen.* 1990;16 Suppl 18:1-14.

Zeiger, E. (1994) Series: 'Current issues in mutagenesis and carcinogenesis.' No. 42. Strategies and philosophies of genotoxicity testing: what is the question? *Mutat Res.* 1994 Jan 16;304(2):309-14.

Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., Mortelmans, K., Speck, W. (1987, 1988) Salmonella mutagenicity tests: III. Results from the testing of 255 chemicals. Environ Mutagen. 1987;9 Suppl 9:1-109. Erratum in: *Environ Mutagen 1988*; 11 Suppl 12:158.

Appendix A

Expanded Spreadsheet D1

CAS no	Chemical name	Molecular formula	Molecular weight *)	Melting point *)	Boiling point (760 Torr, *)	Vapour pressure (25°C, *)	Density (20 °C; 760 Torr; *)	Mass solubility (pH7; 25°C; *)	pK_a value (25°C; *) #)	RTECS	CPDB	TOXNET	IARC	Mutagenicity (RTECS and GENETOX)		
														in vitro gene mutation study in bacteria	in vitro cytogenicity in mammalian cells	in vitro gene mutation/genotoxicity study in mammals
Taurine																
-		-	-	-	-	-	-	-	NA	-	-	-	-	-	-	-
Lysine																
-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	-
Beta-alanine																
79955-38-7	beta-Alanine, N-((2-chloroethyl)nitrosocarbamoyl)-	C6-H10-Cl-N3-O4	223.61	NA	NA	NA	1.51±0.1 g/cm3	very soluble (1000 g/L)	3.82±0.10; Most Acidic Temp: 25 °C -1.94±0.70; Most Basic Temp: 25 °C	Yes	No	No	NA	NA	NA	NA
102516-67-6	beta-Alanine, N-(2((5-diazo-4-oxopentyl)nitroa	C10-H16-N6-O7	NA	NA	NA	NA	NA	NA	NA	Yes	No	No	NA	NA	NA	NA
79448-03-6	beta-Alanine, N-(4hydroxybutyl)-N-nitroso-, methyl ester, acetate	C10-H18-N2-O5	246.26	NA	378.7±27.0 °C	6.17E-6 Torr	1.16±0.1 g/cm3	soluble (16 g/L)	Not applicable	Yes	No	Yes	NA	50 nmol/plate for salmonella typhimurium; 1 umol/plate for escherichia coli; 5 umol/plate for bacillus subtilis in RTECS.	NA	NA
62018-92-2	beta-Alanine, N-butyl-N-nitroso-	C7-H14-N2-O3	174.23	<25 °C (exp) §)	359.3±25.0 °C	3.89E-6 Torr	1.14±0.1 g/cm3	very soluble (1000 g/L)	4.54±0.10 most acidic	Yes	No	Yes	NA	30 umol/plate for salmonella typhimurium in RTECS. 1 pos in GENETOX.	NA	NA
70103-81-0	beta-Alanine, N-(hydroxymethyl)-N-nitroso-, methyl ester, acetate	C7-H12-N2-O5	204.18	NA	340.6±27.0 °C	8.52E-5 Torr	1.24±0.1 g/cm3	Soluble (65 g/L)	Not applicable	Yes	No	Yes	NA	50 nmol/plate for salmonella typhimurium; 1 umol/plate for escherichia coli; 100 umol/plate for bacillus subtilis in RTECS.	NA	NA
Sarcosine																

CAS no	Chemical name	Molecular formula	Molecular weight *)	Melting point *)	Boiling point (760 Torr, *)	Vapour pressure (25°C, *)	Density (20 °C; 760 Torr; *)	Mass solubility (pH7; 25°C; *)	pK_a value (25°C; *) #)	RTECS	CPDB	TOXNET	IARC	Mutagenicity (RTECS and GENETOX)		
														in vitro gene mutation study in bacteria	in vitro cytogenicity in mammalian cells	in vitro gene mutation/genotoxicity study in mammals
13256-22-9	Sarcosine, N-nitroso-	C3-H6-N2-O3	118.09	66-67 (exp) †)	353.1±25.0 °C	2.61E-03 mmHg;	1.35±0.1 g/cm3	very soluble (100 g/L)	3.40±0.10; Most Acidic Temp: 25 °C	Yes	No	Yes	2B	1 neg in GENETOX (+S9)	NA	NA
13344-50-8	Sarcosine, N-nitroso-, ethyl ester	C5-H10-N2-O3	146.14	NA	253.3±23.0 °C	0.0184 Torr	1.14±0.1 g/cm3	soluble (86 g/L)	Not applicable	Yes	No	Yes	NA	NA	NA	NA

CAS no	Chemical name	Molecular formula	Molecular weight *)	Melting point *)	Boiling point (760 Torr, *)	Vapour pressure (25°C, *)	Density (20 °C; 760 Torr; *)	Mass solubility (pH7; 25°C; *)	pK_a value (25°C; *) #)	RTECS	CPDB	TOXNET	IARC	Mutagenicity (RTECS and GENETOX)		
														in vitro gene mutation study in bacteria	in vitro cytogenicity in mammalian cells	in vitro gene mutation/genotoxicity study in mammals
Dinitrosopiperazine																
140-79-4	Piperazine, 1,4-dinitroso-	C4-H8-N4-O2	144.13	156-160 °C (exp) ‡)	406.1±38.0 °C	1.96E-6 Torr	1.53±0.1 g/cm3	very soluble (148 g/L)	Not applicable	Yes	3.6 mg/kg /day for mouse	Yes	NA	50 nmol/plate for bacteria - salmonella typhimurium; 16700 umol/L for bacteria - escherichia coli; 10 mg/kg in mouse/bacteria - salmonella typhimuriom (+S9) in RTECS. 2 pos in GENETOX (+S9).	50 mg/kg in mouse, ip in RTECS	95 umol/kg for drosophila melanogaster, o; 50 umol/L for saccharomyces cerevisiae; 100 umol/plate for human lung; 10 mmol/L for human lymphocyte; 50 mg/kg for rat, ip; 50 mg/kg for rat, sc; 50 mg/kg for rat, sc; 1 mg/L for mouse embryo; 20 gm/kg for mouse, ip in RTECS

CAS no	Chemical name	Molecular formula	Molecular weight *)	Melting point *)	Boiling point (760 Torr, *)	Vapour pressure (25°C, *)	Density (20 °C; 760 Torr; *)	Mass solubility (pH7; 25°C; *)	pK_a value (25°C; *) #)	RTECS	CPDB	TOXNET	IARC	Mutagenicity (RTECS and GENETOX)		
														in vitro gene mutation study in bacteria	in vitro cytogenicity in mammalian cells	in vitro gene mutation/genotoxicity study in mammals
L-proline																
96409-05-1	L-Proline, 1-(((2-chloroethyl)nitrosoamino)carbonyl)-4-hydroxy-, trans-	C8-H12-Cl-N3-O5	265.68	NA	464.7±55.0 °C	1.38E-10 Torr	1.71±0.1 g/cm3	Very Soluble (999 g/L)	2.35±0.40 at 25 °C	Yes	No	No	NA	NA	NA	NA
80687-32-7	Proline, N-((2-chloroethyl)nitrosocarbamoyl)-, L-	C8-H12-Cl-N3-O4	249.65	NA	406.2±55.0 °C	9.77E-8 Torr	1.58±0.1 g/cm3	Very Soluble (1000 g/L)	2.56±0.20	Yes	No	No	NA	NA	NA	NA
96409-03-9	Proline, 1-((2-chloroethyl)nitrosocarbamoyl)-, benzyl ester, L-	C15-H18-Cl-N3-O4	339.77	NA	450.3±55.0 °C	2.67E-8 Torr	1.35±0.1 g/cm3	Sparingly Soluble (0.099 g/L)	Not applicable	Yes	No	No	NA	NA	NA	NA
96409-04-0	Proline, 1-((2-chloroethyl)nitrosocarbamoyl)-4-	C15-H18-Cl-N3-O5	355.77	NA	494.0±55.0 °C	1.41E-10 Torr	1.43±0.1 g/cm3	Sparingly Soluble (0.20 g/L)	13.25±0.40 most acidic	Yes	No	No	NA	NA	NA	NA
122130-63-6	L-Proline, 1-(2-methyl-3-(nitrosothio)-1-	C9-H14-N2-O4-S	246.28	NA	470.9±55.0 °C	3.63E-10 Torr	1.47±0.1 g/cm3	Very Soluble (1000 g/L)	3.52±0.20 most acidic	Yes	No	Yes	NA	NA	NA	NA
Glycine																
NA	Glycine, hydrochloride, nitrosated	NA	NA	NA	NA	NA	NA	NA	NA	Yes	No	No	NA	100 ul/plate for salmonella typhimurium in RTECS.	NA	NA
56516-72-4	Glycine, N-nitroso-N-(phosphonomethyl)-	C3H7N2O6P	198.07	NA	634.1±65.0 °C	1.04E-17 Torr	1.97±0.1 g/cm3	Very Soluble (1000 g/L)	2.00±0.10 most acidic	Yes	No	Yes	NA	1 uL/plate for salmonella typhimurium in RTECS.	NA	NA
39978-27-3	Glycine, N-((5-nitro-2-thienyl)carbonyl)-, (3-(5-nitro-2-furanyl)-2-propenylidene)hydrazide	C14-H11-N5-O7-S	393.33	NA	NA	NA	1.62±0.1 g/cm3	Sparingly Soluble (1.5E-3 g/L)	11.67±0.46 most acidic; -0.82±0.70 most basic	Yes	No	No	NA	NA	NA	NA

CAS no	Chemical name	Molecular formula	Molecular weight *)	Melting point *)	Boiling point (760 Torr, *)	Vapour pressure (25°C, *)	Density (20 °C; 760 Torr; *)	Mass solubility (pH7; 25°C; *)	pK_a value (25°C; *) #)	RTECS	CPDB	TOXNET	IARC	Mutagenicity (RTECS and GENETOX)		
														in vitro gene mutation study in bacteria	in vitro cytogenicity in mammalian cells	in vitro gene mutation/genotoxicity study in mammals
Glycine																
39978-24-0	Glycine, N-(2-thienylcarbonyl)-, (3-(5-nitro-2-	C14-H12-N4-O5-S	348.33	NA	NA	NA	1.47±0.1 g/cm3	Sparingly Soluble (5.2E-3 g/L)	11.98±0.46 most acidic; -0.76±0.70 most	Yes	No	No	NA	NA	NA	NA
60391-92-6	Glycine, N-carbamoyl-N-nitroso-	C3-H5-N3-O4	147.11	NA	353.6±44.0 °C	5.99E-6 Torr	1.79±0.1 g/cm3	Very Soluble (1000 g/L)	3.59±0.10 most acidic; -0.92±0.70 most basic	Yes	4.31 mg/kg /day for rat	Yes	NA	5 mmol/L for escherichia coli in RTECS. No concl in GENETOX. 2 pos in CCRIS.	125 mg/L/48H for hamster (fibroblast); 50 mg/L for hamster (lung) (RTECS)	
25081-31-6	Acetic acid, nitrosiminodi-	C4-H6-N2-O5	162.10	NA	538.8±35.0 °C	4.85E-13 Torr	1.64±0.1 g/cm3	Very Soluble (1000 g/L)	3.70±0.10 most acidic	Yes	not positive for rat, no test for mouse	Yes	NA	25 umol/plate for salmonella typhimurium in RTECS. 1 pos and 10 neg in CCRIS.	NA	NA
25081-33-8	Acetic acid, nitroiminodi-	C4-H6-N2-O6	178.10	NA	574.7±35.0 °C	1.05E-14 Torr	1.715±0.06 g/cm3	Very Soluble (999 g/L)	2.71±0.10 most acidic	Yes	No	Yes	NA	NA	NA	NA
83472-50-8	Glycinamide, 1-(((2-chloroethyl)nitrosoamino)carbonyl)-L-prolyl-L-leucyl-N-(2-chloroethyl)-	C18-H30-Cl2-N6-O5	481.37	NA	NA	NA	1.42±0.1 g/cm3	Sparingly Soluble (0.10 g/L)	12.48±0.20 most acidic; -1.03±0.70 most basic	Yes	No	No	NA	NA	NA	NA
104639-53-4	Glycine, L-alanyl-L-arginyl-, nitrosated	NA	NA	NA	NA	NA	NA	NA	NA	Yes	No	No	NA	2500 umol/L for	NA	NA
61864-02-6	Glycine, N-butyl-N-nitroso-	C4-H8-N-O2.Cl-H.1/3(N-Na-O2)	160.17	61 °C (exp) §)	344.7±25.0 °C	1.15E-5 Torr	1.18±0.1 g/cm3	Very Soluble (999 g/L)	4.19±0.10 most acidic	Yes	No	Yes	NA	1 no concl GENETOX	NA	NA

CAS no	Chemical name	Molecular formula	Molecular weight *)	Melting point *)	Boiling point (760 Torr, *)	Vapour pressure (25°C, *)	Density (20 °C; 760 Torr; *)	Mass solubility (pH7; 25°C; *)	pK_a value (25°C; *) #)	RTECS	CPDB	TOXNET	IARC	Mutagenicity (RTECS and GENETOX)		
														in vitro gene mutation study in bacteria	in vitro cytogenicity in mammalian cells	in vitro gene mutation/genotoxicity study in mammals
NA	Glycine, N-(4-hydroxybutyl)-N-nitroso-, methyl ester, acetate	C9-H16-N2-O5	232.27	NA	NA	NA	NA	NA	NA	Yes	No	No	NA	1 umol/plate for salmonella typhimurium, 1 umol/plate for eschericia coli, 500 nmol/plate for baclus subtilis in RTECS.	NA	NA
57564-91-7	Glycine, N-(N-L-gamma-glutamyl-2-nitroso-L-cysteinyl)-	C10-H16-N4-O7-S	336.36	NA	NA	NA	NA	NA	NA	Yes	No	Yes	NA	5 umol/plate for salmonella typhimurium in RTECS.	NA	NA
70103-80-9	Glycine, N-(hydroxymethyl)-N-nitroso-, methyl ester, acetate	C6-H10-N2-O5	190.15	NA	323.1±27.0 °C	2.68E-4 Torr	1.28±0.1 g/cm3	Soluble (87 g/L)	Not applicable	Yes	No	Yes	NA	1 umol/plate for salmonella typhimurium; 1 umol/plate for escherichia coli; 1 umol/plate for bactillus subtilis in RTECS.	NA	NA
10339-31-8	Glycine, N-nitro-	C2-H4-N2-O4	120.06	106 °C (exp) ¬)	349.7±44.0 °C	8.00E-6 Torr	1.543±0.06 g/cm3	Very Soluble (1000 g/L)	0.78±0.10	Yes	No	Yes	NA	NA	NA	NA

CAS no	Chemical name	Molecular formula	Carcinogenicity (RTECS)	Acute toxicity (RTECS)			Ecotoxicity						
			TD50 animal route	LD50	inhalation, LC50	cytotoxicity, in vitro	$LogK_{ow}$ (=LogP; 25 °C; *)	BAF *)	BCF (pH7; 25 °C; *)	LC50 (mg/L; ppm)	EC50 (mg/L; ppm)	ChV (mg/L; ppm)	Ready biodegradability prediction [e)]
Taurine													
-		-	-	-	-	-	-	-	-	-		-	-
Lysine													
-	-	-	-	-	-	-	-	-	-	-		-	-
Beta-alanine													
79955-38-7	beta-Alanine, N-((2-chloroethyl)nitrosocarbamoyl)-	C6-H10-Cl-N3-O4	NA	50mg/kg for mouse, ip	NA	NA	NA	NA	1.0	NA	NA	NA	NA
102516-67-6	beta-Alanine, N-(2((5-diazo-4-oxopentyl)nitroa	C10-H16-N6-O7	NA	375mg/kg for mouse, ip	NA	NA	NA	NA	NA	NA	NA	NA	NA
79448-03-6	beta-Alanine, N-(4hydroxybutyl)-N-nitroso-, methyl ester, acetate	C10-H18-N2-O5	NA	NA	NA	NA	0.612±0.280	NA	1.72	NA	NA	NA	NA
62018-92-2	beta-Alanine, N-butyl-N-nitroso-	C7-H14-N2-O3	NA	NA	NA	NA	0.922±0.266	NA	1.0	NA	NA	NA	NA
70103-81-0	beta-Alanine, N-(hydroxymethyl)-N-nitroso-, methyl ester, acetate	C7-H12-N2-O5	NA	NA	NA	NA	0.371±0.442	NA	1.0	NA	NA	NA	NA

CAS no	Chemical name	Molecular formula	Carcinogenicity (RTECS)	Acute toxicity (RTECS)			Ecotoxicity						
			TD50 animal route	LD50	inhalation, LC50	cytotoxicity, in vitro	$LogK_{ow}$ (=LogP; 25 °C; *)	BAF *)	BCF (pH7; 25 °C; *)	LC50 (mg/L; ppm)	EC50 (mg/L; ppm)	ChV (mg/L; ppm)	Ready biodegradability prediction [e])
Sarcosine													
13256-22-9	Sarcosine, N-nitroso-	C3-H6-N2-O3	TDLo: 29gm/kg/41W(continuous) for rat, o; 118gm/kg/56W(continuous) for mouse, o	>5gm/kg for rat, o, 184mg/kg for mouse, ip	NA	NA	-0.429±0.358	NA	1.0	557.066 ppm Daphnia, 9816.545 ppm Fish, 10220.646 ppm Fish (SW), 566.555 ppm Mysid Shrimp (SW)	96.748 ppm Green Algae, 95.846 ppm Green Algae (SW)	133.597 ppm Fish, 0.262 ppm Daphnia, 80.325 ppm Green Algae, 133.597 ppm Fish (SW), 0.262 ppm Mysid Shrimp (SW), 70.190 ppm Green Algae (SW)	No

CAS no	Chemical name	Molecular formula	Carcinogenicity (RTECS)	Acute toxicity (RTECS)			Ecotoxicity						
			TD50 animal route	LD50	inhalation, LC50	cytotoxicity, in vitro	$LogK_{ow}$ (=LogP; 25 °C; *)	BAF *)	BCF (pH7; 25 °C; *)	LC50 (mg/L; ppm)	EC50 (mg/L; ppm)	ChV (mg/L; ppm)	Ready biodegradability prediction [8])
13344-50-8	Sarcosine, N-nitroso-, ethyl ester	C5-H10-N2-O3	TDLo: 4000mg/kg/16W(intermittent) for rat, o; 3900mg/kg/39W(intermittent) for rat, iv; 4000mg/kg/8W(intermittent) for rat, o; TD: 160 gm/kg/8W (intermittent) for rat, o; 9700 mg/kg/28W (continuous) for rat, o; 21 gm/kg/8W (intermittent) for rat, o; 7900 mg/kg/23W (continuous) for rat, o; 4250 mg/kg/17W (continuous) for rat, o	4gm/kg for rat, o; 4gm/kg for rat, iv	NA	NA	0.306±0.327	NA	1.01	27.575 Daphnia, 413.848 Fish, 433.825 Fish (SW), 27.523 Mysid Shrimp (SW)	6.165 Green Algae, 6.089 Green Algae (SW)	0.027 Daphnia, 6.016 Fish, 4.163 Green Algae, 6.016 Fish (SW), 0.027 Mysid Shrimp (SW), 3.724 Green Algae (SW)	No

CAS no	Chemical name	Molecular formula	Carcinogenicity (RTECS)	Acute toxicity (RTECS)			Ecotoxicity						
			TD50 animal route	LD50	inhalation, LC50	cytotoxicity, in vitro	$LogK_{ow}$ (=LogP; 25 °C; *)	BAF *)	BCF (pH7; 25 °C; *)	LC50 (mg/L; ppm)	EC50 (mg/L; ppm)	ChV (mg/L; ppm)	Ready biodegradability prediction [e])
Dinitrosopiperazine													
140-79-4	Piperazine, 1,4-dinitroso-	C4-H8-N4-O2	TDLo: 1040 mg/kg/1Y (intermittent) for rat, o; 1070 mg/kg/53W (intermittent) for rat, sc; 140 mg/kg for mouse, o; 1568 mg/kg/28W (continuous) for mouse, o; 720 mg/kg/72W (intermittent) for mouse, sc; TD: 1800 mg/kg/64W (continuous) for rat, o; 1100 mg/kg/110W (intermittent) for rat, sc; 2250 mg/kg/50W (intermittent) for rat, o; 7300 mg/kg/52W (continuous) for mouse, o; 560 mg/kg/10W	160 mg/kg for rat, o; 160 mg/kg for rat, sc; 100 mg/kg for mouse, ip	NA	Yes	-0.946±0.269	NA	1.0	63.851 Daphnia, 1112.849 Fish, 1159.200 Fish (SW), 64.855 Mysid Shrimp (SW)	11.283 Green Algae, 11.175 Green Algae (SW)	0.032 Daphnia, 15.214 Fish, 9.236 Green Algae, 15.214 Fish (SW), 0.032 Mysid Shrimp (SW), 8.084 Green Algae (SW)	No

CAS no	Chemical name	Molecular formula	Carcinogenicity (RTECS)	Acute toxicity (RTECS)			Ecotoxicity						
			TD50 animal route	LD50	inhalation, LC50	cytotoxicity, in vitro	$LogK_{ow}$ (=LogP; 25 °C; *)	BAF *)	BCF (pH7; 25 °C; *)	LC50 (mg/L; ppm)	EC50 (mg/L; ppm)	ChV (mg/L; ppm)	Ready biodegradability prediction [e])
L-proline													
96409-05-1	L-Proline, 1-(((2-chloroethyl)nitrosoamino)carbonyl)-4-hydroxy-, trans-	C8-H12-Cl-N3-O5	NA	>500 mg/kg for mouse, ip	NA	NA	-0.470±0.401	NA	1.0	NA	NA	NA	NA
80687-32-7	Proline, N-((2-chloroethyl)nitrosocarbamoyl)-, L-	C8-H12-Cl-N3-O4	NA	80 mg/kg for mo	NA	NA	0.134±0.345	NA	1.0	NA	NA	NA	NA
96409-03-9	Proline, 1-((2-chloroethyl)nitrosocarbamoyl)-, benzyl ester, L-	C15-H18-Cl-N3-O4	NA	160 mg/kg for m	NA	NA	2.189±0.393	NA	27.1	NA	NA	NA	NA
96409-04-0	Proline, 1-((2-chloroethyl)nitrosocarbamoyl)-4-	C15-H18-Cl-N3-O5	NA	250 mg/kg for mouse, ip	NA	NA	1.459±0.406	NA	7.57	NA	NA	NA	NA
122130-63-6	L-Proline, 1-(2-methyl-3-(nitrosothio)-1-oxopropyl)-, (S)-	C9-H14-N2-O4-S	TDLo: 2000 mg/kg for rat, o; 4.5 gm/kg/90D (continuous) for rat, o; 13.5 gm/kg/90D (continuous) for rat, o; 45 gm/kg/90D (continuous) for rat, o	2078 mg/kg for mouse, o; 674 mg/kg for mouse, ip;	NA	NA	0.936±0.693	NA	1.0	NA	NA	NA	NA

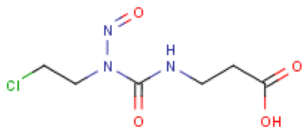
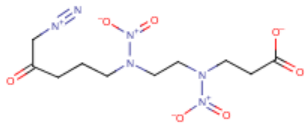
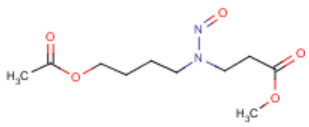
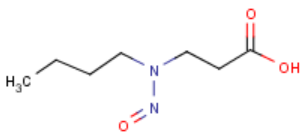
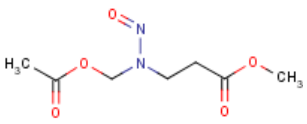
CAS no	Chemical name	Molecular formula	Carcinogenicity (RTECS)	Acute toxicity (RTECS)			Ecotoxicity						
			TD50 animal route	LD50	inhalation, LC50	cytotoxicity, in vitro	$LogK_{ow}$ (=LogP; 25 °C; *)	BAF *)	BCF (pH7; 25 °C; *)	LC50 (mg/L; ppm)	EC50 (mg/L; ppm)	ChV (mg/L; ppm)	Ready biodegradability prediction [e])
Glycine													
NA	Glycine, hydrochloride, nitrosated	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
56516-72-4	Glycine, N-nitroso-N-(phosphonomethyl)-	C3H7N2O6P	NA	NA	NA	NA	-0.679±0.599	NA	1.0	NA	NA	NA	NA
39978-27-3	Glycine, N-((5-nitro-2-thienyl)carbonyl)-, (3-(5-nitro-2-furanyl)-2-propenylidene)hydrazide	C14-H11-N5-O7-S	NA	>3200 mg/kg for mouse, o	NA	NA	1.268±0.599	NA	5.41	NA	NA	NA	NA
39978-24-0	Glycine, N-(2-thienylcarbonyl)-, (3-(5-nitro-2-	C14-H12-N4-O5-S	NA	>3200 mg/kg for mouse, o	NA	NA	1.405±0.580	NA	6.88	NA	NA	NA	NA
60391-92-6	Glycine, N-carbamoyl-N-nitroso-	C3-H5-N3-O4	TDLo: 6720 mg/kg/64W (continuous) for rat, o; TD: 12390 mg/kg/59W (continuous) for rat, o; 4 gm/kg/74W (intermittent) for rat, o	210 mg/kg for rat, ip	NA	NA	-0.632±0.301	NA	1.0	NA	NA	NA	NA

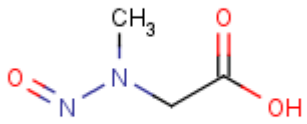
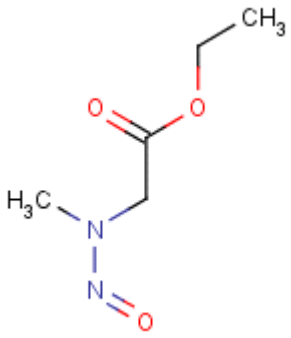
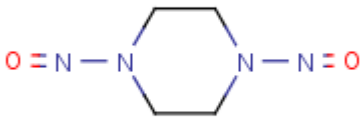
CAS no	Chemical name	Molecular formula	Carcinogenicity (RTECS)	Acute toxicity (RTECS)			Ecotoxicity						
Glycine			TD50 animal route	LD50	inhalation, LC50	cytotoxicity, in vitro	$LogK_{ow}$ (=LogP; 25 °C; *)	BAF *)	BCF (pH7; 25 °C; *)	LC50 (mg/L; ppm)	EC50 (mg/L; ppm)	ChV (mg/L; ppm)	Ready biodegradability prediction [e])
25081-31-6	Acetic acid, nitrosiminodi-	C4-H6-N2-O5	NA	NA	NA	NA	-0.082±0.480	NA	1.0	NA	NA	NA	NA
25081-33-8	Acetic acid, nitroiminodi-	C4-H6-N2-O6	NA	100 mg/kg for mouse, ip	NA	NA	-0.826±0.530	NA	1.0	NA	NA	NA	NA
83472-50-8	Glycinamide, 1-(((2-chloroethyl)nitrosoamino)carbonyl)-L-prolyl-L-leucyl-N-(2-chloroethyl)-	C18-H30-Cl2-N6-O5	NA	410 mg/kg for mouse, ip	NA	NA	0.970±0.620	NA	3.22	NA	NA	NA	NA
104639-53-4	Glycine, L-alanyl-L-arginyl-, nitrosated	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
61864-02-6	Glycine, N-butyl-N-nitroso-	C4-H8-N-O2.Cl-H.1/3(N-Na-O2)	NA	NA	NA	NA	1.100±0.358	NA	1.0	NA	NA	NA	NA

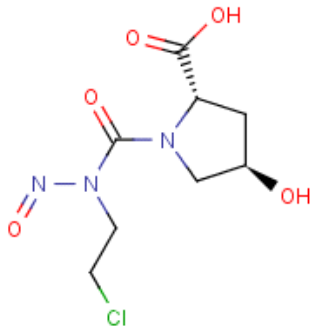
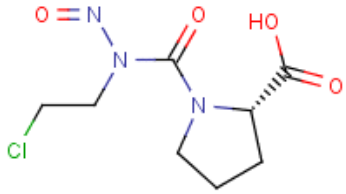
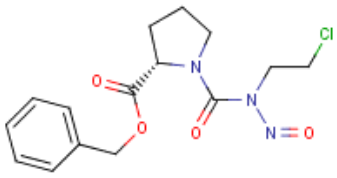
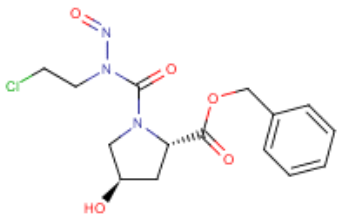
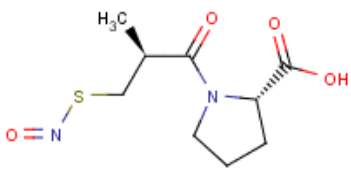
CAS no	Chemical name	Molecular formula	Carcinogenicity (RTECS)	Acute toxicity (RTECS)			Ecotoxicity						
Glycine			TD50 animal route	LD50	inhalation, LC50	cytotoxicity, in vitro	$LogK_{ow}$ (=LogP; 25 °C; *)	BAF *)	BCF (pH7; 25 °C; *)	LC50 (mg/L; ppm)	EC50 (mg/L; ppm)	ChV (mg/L; ppm)	Ready biodegradability prediction [e])
NA	Glycine, N-(4-hydroxybutyl)-N-nitroso-, methyl ester, acetate	C9-H16-N2-O5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
57564-91-7	Glycine, N-(N-L-gamma-glutamyl-2-nitroso-L-cysteinyl)-	C10-H16-N4-O7-S	TDLo: 8.1 ug/kg for rat, iv; 101 ug/kg for rat, iv; 336 mg/kg/1M for rat, iv; 35 mg/kg for dog; 50 mg/kg for dog	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
70103-80-9	Glycine, N-(hydroxymethyl)-N-nitroso-, methyl ester, acetate	C6-H10-N2-O5	NA	NA	NA	NA	0.371±0.442	NA	1.13	NA	NA	NA	NA

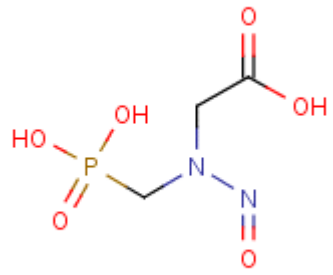
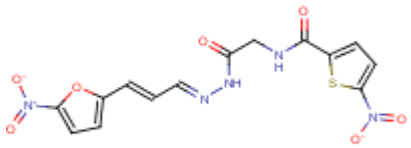
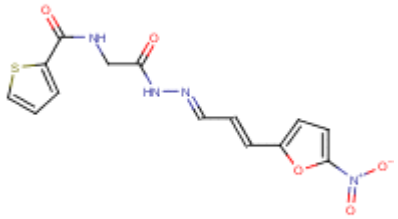
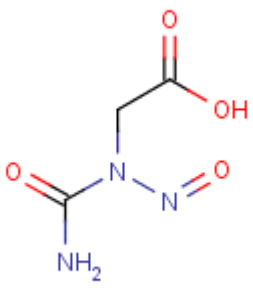
CAS no	Chemical name	Molecular formula	Carcinogenicity (RTECS)	Acute toxicity (RTECS)			Ecotoxicity						
Glycine			TD50 animal route	LD50	inhalation, LC50	cytotoxicity, in vitro	$LogK_{ow}$ (=LogP; 25 °C; *)	BAF *)	BCF (pH7; 25 °C; *)	LC50 (mg/L; ppm)	EC50 (mg/L; ppm)	ChV (mg/L; ppm)	Ready biodegradability prediction ℓ)
10339-31-8	Glycine, N-nitro-	C2-H4-N2-O4	NA	40 mg/kg for mouse, oral; 43 mg/kg for mouse, ip; 32 mg/kg for mouse, iv	NA	NA	-0.928±0.480	NA	1.0	NA	NA	NA	NA

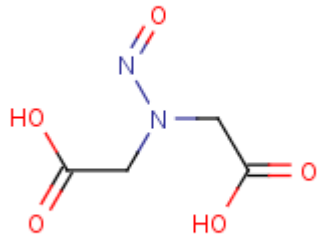
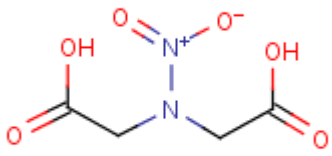
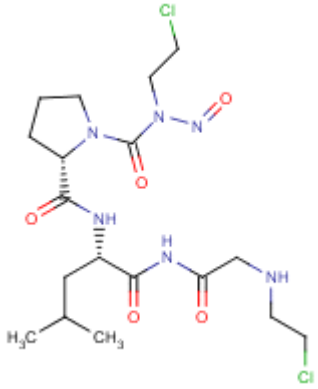
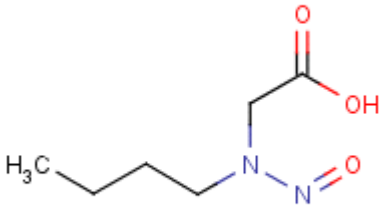
*) predicted values; Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2011 ACD/Labs)
§) experimental property - Okada, Masashi; Chemical & Pharmaceutical Bulletin 1978, V26(12), P3909-13CAPLUS
†) 'Hazardous Substances Data Bank' data were obtained from the National Library of Medicine (US)
‡) experimental property - Chehardoli, Gholamabbas; Journal of Chemical Sciences (Bangalore, India) 2009, V121(4), P441-447CAPLUS
¯) experimental property - Miyazaki, Yukio; Journal of Antibiotics 1968, V21(4), P279-82CAPLUS
#) As a pKa value below -2 is a strong acid all compounds with a pKa below -2 was removed and if they are a protonated compound, in water they will be dissociated. For many compounds pKa values are irrelevant as they will be neutral under most circumstances and that is also why the pKa values are so extremely low.
ℓ) Ready Biodegradability Prediction - NA = No CAS match found; No = the prediction is NO;
Criteria for the YES or NO prediction: If the Biowin3 (ultimate survey model) result is "weeks" or faster (i.e. "days", "days to weeks", or "weeks") AND the Biowin5 (MITI linear model) probability is >= 0.5, then the prediction is YES (readily biodegradable). If this condition is not satisfied, the prediction is NO (not readily biodegradable). This method is based on application of Bayesian analysis to ready biodegradation data.

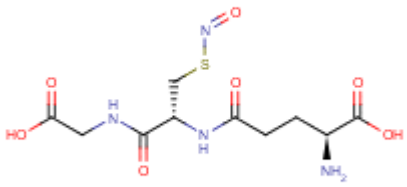
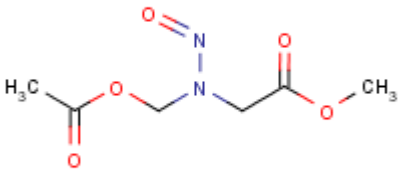
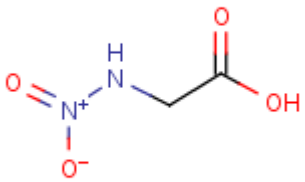
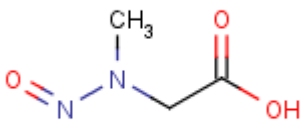
CAS no	
Taurine	
NA	
Lysine	
NA	
Beta-alanine	
79955-38-7	
102516-67-6	
79448-03-6	
62018-92-2	
70103-81-0	

Sarcosine	
13256-22-9	
13344-50-8	
Dinitrosopiperazine	
140-79-4	

L-proline	
96409-05-1	
80687-32-7	
96409-03-9	
96409-04-0	
122130-63-6	

Glycine	
NA	
56516-72-4	
39978-27-3	
39978-24-0	
60391-92-6	

Glycine	
NA	
25081-31-6	
25081-33-8	
83472-50-8	
104639-53-4	no structure
61864-02-6	
NA	

Glycine	
NA	
57564-91-7	
70103-80-9	
10339-31-8	
13256-22-9	

Appendix B

Collected data of 92 N-nitrosamines with harmonic mean TD50 available through the CPDB database

CAS no.	TD50 harmonic mean (µmol/kg bw/day)	-LogTD50	CAS no.	TD50 harmonic mean (µmol/kg bw/day)	-LogTD50
100-75-4	12.5	-1.097	**614-95-9**	0.62	0.208
10589-74-9	3.49	-0.543	**621-64-7**	1.43	-0.155
10595-95-6	0.57	0.243	**62-75-9**	1.29	-0.111
110559-84-7	1.05	-0.021	**63412-06-6**	19.7	-1.294
1116-54-7	23.6	-1.373	**63642-17-1**	3.61	-0.557
1133-64-8	62.2	-1.794	**64005-62-5**	5.37	-0.730
13010-07-6	7.48	-0.874	**64091-91-4**	0.48	0.317
13256-06-9	21.6	-1.334	**68107-26-6**	11.1	-1.045
13256-11-6	0.06	1.216	**684-93-5**	0.90	0.046
13743-07-2	1.83	-0.262	**69112-98-7**	0.92	0.034
14026-03-0	159	-2.201	**70-25-7**	5.46	-0.737
15973-99-6	1.15	-0.061	**70415-59-7**	14.1	-1.149
16219-98-0	1.56	-0.193	**71752-70-0**	6.65	-0.823
16338-97-9	269	-2.430	**75411-83-5**	0.39	0.407
16699-10-8	2.42	-0.383	**75881-18-4**	0.96	0.018
16813-36-8	0.69	0.163	**75881-20-8**	6.43	-0.808
17608-59-2	490	-2.690	**75881-22-0**	6.29	-0.799
18774-85-1	2.96	-0.471	**75896-33-2**	6.88	-0.838
18883-66-4	3.63	-0.560	**759-73-9**	8.10	-0.908
20917-49-1	0.27	0.575	**76014-81-8**	0.49	0.308
26541-51-5	40.8	-1.611	**760-56-5**	2.64	-0.422
26921-68-6	12.40	-1.093	**760-60-1**	32.6	-1.513
33868-17-6	5.64	-0.751	**78246-24-9**	4.53	-0.656
36702-44-0	103	-2.013	**816-57-9**	28.7	-1.458
3817-11-6	2.62	-0.418	**81795-07-5**	2.51	-0.400
38347-74-9	3.32	-0.521	**82018-90-4**	16.1	-1.207
38434-77-4	37.1	-1.569	**83335-32-4**	2.81	-0.449
38777-13-8	1.48	-0.185	**86-30-6**	842	-2.925
40580-89-0	51.3	-1.710	**86451-37-8**	4.82	-0.683
42579-28-2	339	-2.530	**869-01-2**	3.56	-0.551
51542-33-7	4.78	-0.679	**88208-16-6**	5.15	-0.712
53609-64-6	5.22	-0.718	**89911-78-4**	36.4	-1.561
54749-90-5	0.12	0.923	**89911-79-5**	0.30	0.523
55090-44-3	2.35	-0.371	**91308-69-9**	3.77	-0.576
55-18-5	0.26	0.587	**91308-70-2**	6.08	-0.784
55556-92-8	0.54	0.271	**91308-71-3**	2.36	-0.373
55557-00-1	0.39	0.410	**92177-49-6**	12.3	-1.09
55984-51-5	0.15	0.830	**92177-50-9**	0.20	0.699
56222-35-6	65.9	-1.819	**924-16-3**	4.37	-0.64
5632-47-3	76.3	-1.883	**930-55-2**	7.98	-0.902
56654-52-5	21.3	-1.328	**937-25-7**	1.65	-0.217
59-89-2	0.94	0.027	**96724-44-6**	3.24	-0.51

CAS no.	TD50 harmonic mean (µmol/kg bw/day)	-LogTD50	CAS no.	TD50 harmonic mean (µmol/kg bw/day)	-LogTD50
60391-92-6	29.3	-1.467	**96724-45-7**	3.49	-0.542
60599-38-4	3.10	-0.491	**96806-34-7**	1.82	-0.26
61034-40-0	39.1	-1.592	**96806-35-8**	4.16	-0.619
614-00-6	1.04	-0.017	**99-80-9**	7.87	-0.896

Appendix C

QSAR model development and prediction of cancerogenic potency TD_{50}

		Physchem						Exp.	Pred-icted
No	**Cas.no**	**Properties**						**-LogTD$_{50}$**	**-LogTD$_{50}$**
	Training set	Pi2 (S)	V3C	V6CH	QPOS	GEOM3	PND2		
1	100-75-4	1,11	0,10	0,08	0,05	0,11	0,00	-1,10	-1,41
2	10595-95-6	1,02	0,14	0	0,05	0,09	5,29	0,24	0,49
3	1116-54-7	1,46	0,10	0	0,30	0,20	0	-1,37	-0,55
4	1133-64-8	1,69	0,27	0,09	0,09	0,45	0	-1,79	-1,66
6	13256-11-6	1,48	0,26	0,03	0,08	0	6,63	1,22	0,40
10	16338-97-9	1,15	0,10	0	0,08	0,31	9,38	-2,43	Outlier
11	16699-10-8	2,53	0,49	0,07	0,12	0,15	11,87	-0,38	-0,03
12	17608-59-2	1,60	0,44	0,03	0,30	0,80	14,14	-2,69	-2,78
13	20917-49-1	1,12	0,10	0	0,05	0,39	0	0,58	0,10
15	26921-68-6	1,23	0,14	0	0,30	0	3,87	-1,09	-0,42
16	33868-17-6	1,56	0,10	0	0,41	0	3,32	-0,75	-0,86
17	36702-44-0	1,10	0,26	0,06	0,06	0,11	4,69	-2,01	-0,85
18	3817-11-6	1,31	0,10	0	0,30	0,15	7,48	-0,42	-1,12
21	40580-89-0	1,14	0,10	0	0,05	0,83	0	-1,71	-1,00
22	42579-28-2	1,84	0,19	0,05	0,46	0	0	-2,53	-1,79
24	55090-44-3	1,06	0,14	0	0,05	0	21,40	-0,37	-0,45
25	55-18-5	1,02	0,10	0	0,05	0,10	6,56	0,59	0,25
54	55556-92-8	1,21	0,10	0,05	0,08	0	0	0,27	-0,45
27	55557-00-1	1,95	0,20	0	0,06	0,82	0	0,41	0,13
28	55984-51-5	1,51	0,29	0	0,20	0,16	7,00	0,83	0,25
29	56222-35-6	1,31	0,23	0,08	0,30	0,07	0	-1,82	-2,23
30	5632-47-3	1,30	0,10	0,06	0,19	0,10	0	-1,88	-1,37
31	59-89-2	1,26	0,10	0,05	0,06	0,09	0	0,03	-0,34
32	60599-38-4	2,01	0,39	0	0,20	0,34	10,58	-0,49	0,35
33	61034-40-0	2,43	0,67	0,07	0,34	0,65	17,20	-1,59	-2,23
34	614-00-6	1,57	0,17	0,03	0,14	0	5,20	-0,02	-0,03
35	621-64-7	1,03	0,10	0	0,05	0,19	9,38	-0,16	-0,17
37	64091-91-4	2,11	0,30	0,02	0,19	0,06	8,43	0,32	0,36
40	75411-83-5	1,22	0,32	0	0,30	0,16	7,00	0,41	-0,47
41	75881-18-4	1,27	0,61	0,03	0,06	0,13	19,05	0,02	0,22
42	75881-20-8	1,07	0,14	0	0,05	0,01	25,85	-0,81	-0,78
43	75881-22-0	1,05	0,14	0	0,05	0	17,32	-0,80	-0,15
46	81795-07-5	1,37	1,26	0,10	0,08	0,51	16,25	-0,40	-0,20
47	86451-37-8	1,49	0,27	0	0,30	0,22	4,90	-0,68	-0,39
48	88208-16-6	1,55	0,23	0	0,30	0,14	6,56	-0,71	-0,38
50	89911-79-5	1,71	0,41	0	0,30	0,16	6,71	0,52	0,32
52	91308-70-2	1,28	0,28	0	0,30	0,29	10,00	-0,78	-1,11
53	91308-71-3	1,58	0,24	0	0,20	0,30	10,00	-0,37	-0,37
54	92177-49-6	1,73	0,24	0	0,30	0,41	5,66	-1,09	-0,78
55	92177-50-9	1,99	0,37	0	0,30	0,30	6,71	0,70	0,09
56	924-16-3	1,04	0,10	0	0,05	0,31	12,69	-0,64	-0,71

	Test set								
5	13256-06-9	1,05	0,10	0	0,05	0,16	16,43	-1,33	-0,61
7	14026-03-0	1,10	0,26	0,06	0,06	0,28	4,69	-2,20	-1,27
8	15973-99-6	1,95	0,20	0,05	0,07	0,17	0	-0,06	0,31
9	16219-98-0	1,63	0,16	0,02	0,23	0	5,20	-0,19	-0,35
14	26541-51-5	1,26	0,10	0,10	0,05	0,13	0	-1,61	-1,83
19	38347-74-9	1,63	0,11	0,04	0,49	0	0	-0,52	-2,10
20	38434-77-4	1,57	0,07	0	0,41	0	4,12	-1,57	-1,00
23	53609-64-6	1,44	0,47	0	0,30	0,29	10,58	-0,72	-0,38
36	62-75-9	1,01	0,20	0	0,05	0	4,24	-0,11	1,00
38	68107-26-6	1,06	0,14	0	0,05	0	19,31	-1,05	-0,29
39	70415-59-7	1,29	0,14	0	0,30	0	4,47	-1,15	-0,41
44	75896-33-2	1,45	0,28	0	0,30	0,24	5,66	-0,84	-0,48
45	76014-81-8	1,81	0,33	0,02	0,30	0,29	8,43	0,31	-1,01
49	89911-78-4	1,72	0,23	0	0,30	0,20	0	-1,56	0,11
51	91308-69-9	1,30	0,10	0	0,30	0,21	5,48	-0,58	-1,15
57	930-55-2	1,11	0,10	0,05	0,05	0	0	-0,90	-0,33
58	937-25-7	1,57	0,24	0,03	0,14	0	5,74	-0,22	0,26

	Prediction set								
	140-79-4	ND						ND	
	16339-04-1	ND						ND	
	16339-07-4	ND						ND	
	35627-29-3	ND						ND	
	35631-27-7	ND						ND	
	39884-52-1	ND						ND	
	39884-58-7	ND						ND	
	4549-44-4	ND						ND	
	601-77-4	ND						ND	
	7068-83-9	ND						ND	
	924-46-9	ND						ND	
	997-95-5	ND						ND	

NILU – Norwegian Institute for Air Research
P.O. Box 100, N-2027 Kjeller, Norway
Associated with CIENS and the Fram Centre
ISO certified according to NS-EN ISO 9001/ISO 14001

REPORT SERIES SCIENTIFIC REPORT	REPORT NO. OR 71/2011	ISBN: 978-82-425-2465-2 (print) ISSN: 0807-7207	
DATE 16/12-11.	SIGN.	NO. OF PAGES 96	PRICE NOK 150.-

TITLE Alternative approaches to standard toxicity testing TQP ID 9 - OPTION - 257430181 - NILU	PROJECT LEADER Mikael Harju NILU PROJECT NO. O-111085
AUTHOR(S) Mikael Harju, Solveig Ravnum, Elise Rundén Pran, Sonja Grossberndt, Lise Marie Fjellsbø, Maria Dusinska and Eldbjørg S. Heimstad	CLASSIFICATION * B CONTRACT REF. 257430181

QUALITY CONTROLLER: Eldbjørg S. Heimstad

REPORT PREPARED FOR
Gassnova SF, CO2 prosjekter
Postboks 1555, 7439 Trondheim
v/ Lars Bergersen

ABSTRACT
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 behaviour and potential effects of these chemical compounds. The substances produced can pose a risk to the environment and human health. This report is an option for the TQP ID 9 - 257430120 - NILU report (or 14/2011) and includes a review on nitrosated and nitrated amino acids with detail physicochemical data and (eco)toxicological endpoints for these substances. Furthermore evaluation of toxicological concepts and parameters related to risk assessment of these compounds, such as mutagenic potency, DNEL/DMEL and TD50/T25, has been provided. An evaluation of the use of a new developed QSAR model to predict TD50 of an extended list of nitrosamines was performed. In addition a current knowledge status on the use of (Q)SAR within REACH have been carried out.

NORWEGIAN TITLE

Alternative innfallsvinkler til standard toksisitetstesting
TQP ID 9 - OPTION - 257430181 – NILU

KEYWORDS		
CCM	ITS	(Q)SAR

** Classification*
A Unclassified (can be ordered from NILU)
B Restricted distribution
C Classified (not to be distributed)

REFERENCE: O-111085
DATE: DECEMBER 2011
ISBN: 978-82-425-2465-2 (print)
ISSN: 0807-7207

NILU – Norwegian Institute for Air Research is an independent, nonprofit institution established in 1969. Through its research NILU increases the understanding of climate change, of the composition of the atmosphere, of air quality and of hazardous substances. Based on its research, NILU markets integrated services and products within analyzing, monitoring and consulting. NILU is concerned with increasing public awareness about climate change and environmental pollution.