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Nitramine analysis procedures development and screening toxicity study

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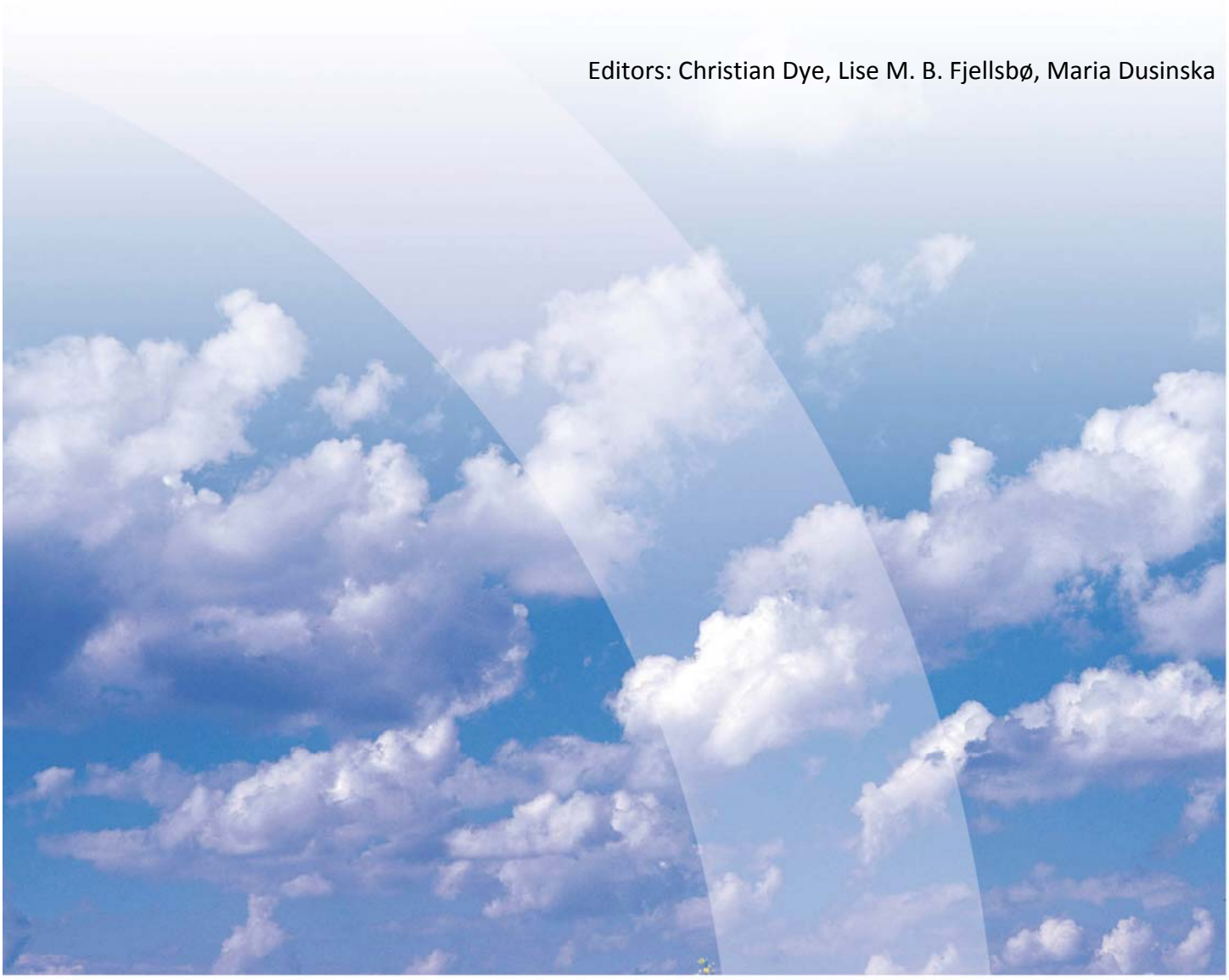

Scientific report

Preface

The CO_2 Capture Mongstad (CCM) Project is in an early phase of project development. The project is at the moment organized as a joint effort by Gassnova SF and Statoil, and is funded by the Norwegian government. All Frame Agreements and subsequent Call-Offs will be entered into with Gassnova SF. The purpose of the project is to plan and build a large scale CO_2 capture plant (the CCP). The facility will be situated next to the Mongstad Refinery on the Mongstad industrial site north of Bergen on the west coast of Norway.

"Nitramine analysis procedures development and screening toxicity study" is one of several H&E TQP Amine activities that will be launched for the CCP development in the technology qualification phase.

This report includes a description of nitramine synthesis and analysis procedures. It also contains a literature review to find available data on the toxicity of six nitramines, and toxicity studies including nine studies for human toxicology in addition to four studies for ecotoxicology.

NILU, Norwegian Institute for Air Research is the project leader with eight subcontractors:

- NTNU, Norwegian University of Science and Technology, Trondheim, Norway
- SINTEF, Trondheim, Norway
- SMU, Slovak Medical University, Bratislava, Slovakia
- UMB, Norwegian University of Life Sciences, Aas, Norway
- VITO/CARDAM, Brussels, Belgium
- Chemring Nobel AS
- Norwegian Defence Research Establishment
- Dangerous Goods Management AS

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Executive summary

Five nitramines have been synthesized, and nitramine analysis procedures have been developed and validated. Analyses of water wash samples show presence of nitramines. Mild acute toxicity was observed in four nitramines, while one nitramine is considered either mild or non-toxic. No skin irritation/corrosion/sensitisation was observed, but all tested nitramines were irritants to the eye. Results indicate three of the nitramines should be considered as mutagenic compounds category 3: Substances which cause concern for man owing to possible mutagenic effects. Any conclusion could not be made for evaluation of genotoxicity of the two other nitramines (further testing required). No major ecotoxicological effects were observed for the five nitramines. The acute ecotoxicity was low to moderate to organisms from two different trophic levels, and estimations of bioaccumulation potential showed that none were bioaccumulating. None of the nitramines were ready biodegradable. However, two were more biodegradable than the others.

The following five nitramines have been synthesized with an estimated purity better than 99%: ethanolnitramine (MEA-NO2), methylnitramine (MA-NO2), dimethylnitramine (DMA-NO2), N-nitropiperazine (PZ-NO2) and 2-methyl-2-(nitroamino)-1-propanol (AMP-NO2).

An important aim in this work has been, if possible, to develop three specialized chemical analysis methods with following properties:

a. A quantitative method for the specific nitramine, with best possible sensitivity.
b. A screening method where all nitramines in the sample will be detected.
c. A group method giving the total amount of the nitramine-compounds.

Based on the obtained results, it is difficult to come up with three analytical methods strictly according to the categories given above. The main explanation is that the chemical properties of the five target nitramines are too diverse for such classification, and several instrumental methods need to be used within each category. A sample preparation method for aqueous samples (water wash and stack emission impinger solution) has been developed for use as a platform for chemical analysis within all three categories. Depending on the scientific aim, several analytical instruments may be used in the chemical analysis. In this work the nitramines have been separated, identified, and measured by use of high performance liquid chromatography (HPLC) combined with UV-spectroscopy and high resolution mass spectrometry (HRMS).

The analytical method has been validated for water wash samples and aqueous impinger solutions. When known amounts of the five nitramines are added to real water wash samples, the recovery range is 15-80% with method detection limits in the range 0.5-40 µg/L. An inter-laboratory validation of the method has been performed with excellent results. Six water wash samples from carbon capture processes based on ethanolamine were provided by Company. MEA-NO2 was found in the range 0-26 µg/L in these samples, no other nitramines were identified. The major short-coming in the analytical method development is the lack of isotope labeled nitramines in the quantitative work.

The method should also be tested and validated with real stack emission sampling. Further method development is recommended.

In toxicology part, firstly, a review was performed to evaluate available knowledge regarding toxicity and to estimate acceptable environmental concentration values of six selected nitramines. As information was scarce or missing completely, a screening program was initiated, performing *in vitro* and *in vivo* experiments to identify possible toxicological effects due to exposure of five selected nitramines: MA-NO2, DMA-NO2, MEA-NO2, PZ-NO2, AMP-NO2. The human toxicity screening included testing for acute oral toxicity, cytotoxicity, skin irritation, skin and eye corrosion, sensitisation, mutagenicity and genotoxicity. The screening for ecotoxicity included biodegradation, bioaccumulation and acute toxicity on both aquatic phytoplankton and aquatic invertebrate. For each of these tests, 2-5 of the nitramines have been tested.

Results from acute oral toxicity studies conclude that all five nitramines induce low or very mild toxicity and can be considered harmful if swallowed. However, AMP-NO2 might be non toxic (LD_{50}>1600).

Cells exposed to DMA-NO2 and AMP-NO2 show low cytotoxicity and no signs of skin corrosion or irritation. Exposure to DMA-NO2 induced slight response to eye corrosion, and can be considered a mild eye irritant while AMP-NO2 was OECD classified as ocular corrosive or severe irritant to eye. DMA-NO2 was considered mutagenic by the mammalian gene mutation assay, but found to be negative in the Ames test. This result was surprising, as positive response was reported in previous studies. Negative results have been obtained by the comet assay. For AMP-NO2, only Ames test was performed to test for potential genotoxicity, and results were negative. Also PZ-NO2 was found negative by Ames test, but one test is not enough to draw any conclusion on genotoxicity.

MA-NO2 and MEA-NO2 induced strong cytotoxic effects at concentrations of 5.5 and 3.7 mg/ml respectively. No signs of toxicity were observed for skin corrosion/irritation/ sensitisation, however both test substances were considered as very severe eye irritants. Both are also considered mutagenic by Ames test, and MEA-NO2 is also considered mutagenic by gene mutation assay (MA-NO2 was not tested). No significant evidence was found using the comet assay, though exposure to MEA-NO2 showed slight increase in DNA strand breaks in a dose dependent matter.

No major exotoxicological effects were observed after exposure to the five nitramines. The acute toxicity of all nitramines was low to moderate to organisms from two different trophic levels. None of the nitramines were ready biodegradable, however, the MA-NO2 and MEA-NO2 were more biodegradable than the other nitramines.

This study indicates that MA-NO2, DMA-NO2 and MEA-NO2 might cause mutagenic effects on mammals and/or bacteria and should be considered as mutagenic compounds category 3: Substances which cause concern for man owing to possible mutagenic effects. We strongly recommend further testing where information is still missing to be able to draw final conclusions, and also to clarify where results are conflicting.

Objective and scope

Description of scope in tender

Part A: Methods for chemical analysis of nitramines

1. Synthesis of 4-6 individual nitramine substances
2. Establish analytical methods for the nitramine substances in different matrixes
3. Testing of the analytical methods established on samples
    a. Provided from Company, and or
    b. Provided from a 3rd party, and or
    c. Sampled by Contractor, on facilities made available by Company

The matrixes will be various solvents/amines for CO2 capture, examples of which are given in the tender, wash water and gaseous samples where the analyte(s) are collected on solid or liquid sorbents. The service will be based on available literature, standard methods and/or in-house development.

The proposed procedures will, if possible, include the following 3 approaches:

I. A quantitative method for the specific nitramine, with best possible sensitivity.
II. A screening method where all nitramines in the sample will be detected.
III. A group method giving the total amount of the nitramine-compounds.

Part B: Toxicity screening study of nitramines

The Service includes experimental toxicity studies of single nitramine substances which will be specified at contract award. Studies will be performed according to the requirements in SoW App B (screening set), or according to alternative methods proposed by Contractor.

For the alternative methods proposed, an approach that provides the key toxicological and exotoxicological information without, or with reduced use, of laboratory animals (in vivo tests) will be used, to avoid unnecessary suffering for animals and to reduce time and cost. The methods proposed should enable estimation of acceptable environmental concentration levels (tolerance criteria). Quantitative outcomes (i.e. NOAEL, LOAEL, T25, TD50) to be used in risk assessments are desirable. For quantitative outcomes, tolerance criteria will be recommended.

Organization into subtasks

Sub-task 1. Synthesis of 4-6 individual nitramine substances (Chapter A)
Sub-task 2. Establish basic analytical information as building blocks for complete methods (Chapter B)
Sub-task 3. Integrate building blocks into defined analytical methods (Chapter B)
    a. A quantitative method for the specific nitramine, with best possible sensitivity.

b. A screening method where all nitramines in the sample will be detected.
c. A group method giving the total amount of the nitramine-compounds.

Sub-task 4. Basic validation of the analytical methods according to ISO 17025 (Chapter B)

Sub-task 5. Testing and further validation of the analytical methods in co-operation with SINTEF (sub-contractor) (Chapter B)

Sub-task 6. Testing of the analytical methods established on samples (Chapter B)
a. Provided from Company, and or
b. Provided from a 3rd party, and or
c. Sampled by Contractor, on facilities made available by Company

Sub-task 7. Literature review (Chapter C)

Sub-task 8. Human toxicity (Chapter E)
a. Acute studies for health effects
b. In vitro studies for genotoxic (mutagenic) effects

Sub-task 9. Ecotoxicity (Chapter F)
a. Acute ecotoxicity
b. Biodegradation
c. Bioaccumulation

Comments and amendments to scope of work

Supporting analysis for toxicity experiments

It was decided to include additional analysis of the nitramines to measure concentrations in media before and after treatment. The aim was to identify if nitramines would "stick" to walls and thus reduce concentration in exposure media. In addition, these measurements were used to control if nitramines would be transferred into nitrosamines during time of exposure. This work was performed by the chemical lab at NILU.

Synthesis

Due to HSE aspects and the available time frame diethanolnitramine was deleted from the scope during the project progress.

Toxicity studies

Two important amendments were made in human toxicity studies.

1. Inclusion of S9-fraction to Comet Assay and Gene Mutation assay:
   The literature review revealed that these compounds might need metabolic activation for some *in vitro* assays. To avoid false negative results, it was

suggested to use S9-fraction on all genotoxicity/mutageniticy assays. This doubled the work required for each compound, but it was considered preferable to reduce the number of test substances and test them thoroughly. Otherwise these tests might have to be repeated at a later stage.

2. The reproductive toxicity study was removed:

   This study lasts for several months and as project was delayed from start it was not possible to finish this study in time. Also it is a rather expensive study, and it was decided to prioritize other tests at this stage. In addition it would be beneficial to wait until we know more about the potential genotoxicity of the test substances. This would give a better basis to select which nitramine(s) to investigate.

A Chapter A – Synthesis of 4-6 individual nitramine substances

Yngve Stenstrøm, UMB

A.1 Summary

Five nitramines (ethanolnitramine, methylnitramine, dimethylnitramine, N-nitro-piperazine, 2-methyl-2-(nitroamino)-1-propanol) have been synthesized with an estimated purity >99%.

Chapter A will be public available after publication in peer review journals.

B Chapter B - Nitramine analysis procedures development

Christian Dye, Arve Bjerke, Henriette Leknes, Stein Manø, Gro Hammerseth, May-Britt Pedersen, Marit Vadset, Kolbjørn Zahlsen, Martin Schlabach, Ole-Anders Braathen

B.1 Introduction

An important aim in this work has been, if possible, to tailor three analytical approaches with following properties:

a. A quantitative method for the specific nitramine, with best possible sensitivity.

b. A screening method where all nitramines in the sample will be detected.

c. A group method giving the total amount of the nitramine-compounds.

Based on the results obtained in this work, it is difficult to come up with three analytical methods strictly according to the categories as given above. The five target nitramines comprises acidic, neutral and basic compounds which need at least 2 different ionization modes in LC/MS applications, a negative mode for the acids and a positive mode for the basic and neutral nitramines. The generation of intense fragment ions which is suited for MS/MS is hard to obtain due the small molecules, which in turn makes it necessary to use the $[M-H]^-$ or $[M+H]^+$ ions to obtain adequate sensitivity. GC/MS has insufficient sensitivity for trace analysis of nitramines. GC/TEA has adequate sensitivity, but is not exclusively detecting nitramines – presence of nitrosamines may act as interference. Depending on the scientific aim, GC/TEA could be considered as complementary to the LC/MS analysis. HPLC combined with high resolution time of flight mass spectrometry (HRMS-TOF) turned out as the most powerful single tool for the analytical methods in this project.

An important technical characteristic of HPLC/HRMS-TOF is the possibility to do retrospective analysis of the acquired data, e.g an open search (or screening) for compounds outside the target compounds. This implies that although a compound is outside the five target nitramines in this project, the possible presence and semi-quantitative concentration is still technically possible to obtain for other nitramines, as long as the nitramine generates an ion which matches the instrumental ionization mode. This instrument feature is associated with the manner of operation of high resolution TOF instruments with continuous detector scanning. In addition, extracted ion chromatograms of less than 30 mDa have often strongly improved signal-to-noise ratio compared with chromatograms obtained by extracting unit masses. This highly selective and improved signal-to-noise ratio makes HRMS-TOF well suited for ultra trace analysis.

B.2 Best available method; scope and application

The method provides procedures for the determination of nitramines in water wash samples and impinger samples based on water as absorber medium. The method may be applicable to samples of water-diluted CCS solvent, but it has not been evaluated for this use. The method is applicable to water soluble nitramines that are efficiently partitioned from the water phase onto an activated carbon solid phase extraction (SPE) sorbent. Less water soluble nitramines may be included in the method by using additional SPE sorbent steps, but such sorbents steps have not been evaluated. The method includes the following compounds:

Table B.1: Examples of nitramines that can be measured by the method

Analyte	Chemical Abstract Services (CAS) Registry number
Dimethylnitramine	4164-28-7
Methylnitramine	598-57-2
Ethanolnitramine	74386-82-6
2-methyl-2-(nitroamino)-1-propanol	1239666-60-4
1-nitro-piperazine	42499-41-2

The method limit of detection (LOD) for a 30 mL water wash sample ranges from 0.3 - 40 µg/L. The potential for reaching LOD in the range of some tens of ng/L is good, but sample clean-up steps need to be developed.

This method should be performed only by or under the supervision of analysts with experience in solid phase extractions and LC/MS analyses.

B.3 Overview of the method

Gaseous and aerosol-adsorbed nitramines are sampled isokinetically from an emission source and collected by an impinger-train filled with MQ water as absorber solution.

Water wash samples are prepared according to the method as the samples are received.

Nitramines are extracted by passing a 30 mL water sample (water wash or impinger solution) through a solid phase extraction (SPE) cartridge containing 1 g of activated charcoal. The nitramines are eluted from the solid phase with a tailored blend of organic solvents.

The organic solvent extract is concentrated by evaporation. The nitramines are separated, identified, and measured by use of HPLC/HRMS. Nitramines eluting from the HPLC column are identified by comparing their high resolution mass spectra and retention times to reference spectra and retention times obtained by analytical standards. The concentration of each identified nitramine is measured by the standard addition

method until isotope labeled standards are available for use in the less time-consuming and preferred internal standard method.

B.4 Saftety

Each nitramine should be treated as a potential health hazard, and exposure to nitramines should be minimized. Pure standard materials and stock standard solutions of these compounds should be handled with suitable protection of skin and eyes.

B.5 Apparatus and materials

B.5.1 Sampling equipment

The equipment for isokinetic sampling, with configuration and dimensions, is not described in detail at this stage in the method development. However, the aim is to integrate the present method with standardized sampling equipment configuration and protocols.

B.5.1.1 Stack emission impinger sampling

Due to the high water solubility, MQ water is used as an efficient absorber solution (appendix B, table 16) of the impinger.

B.5.2 Glassware

All glassware must be cleaned by washing with detergent and water and rinsed with water and distilled water. Amber bottles are recommended as a precaution to protect the nitramines from light. Clear glass bottles may be used if they are wrapped in foil, or samples are stored in boxes that prevent exposure to light. Class A volumetric glass flasks should be used for preparation of standards.

B.5.3 Chemicals and reagents

HPLC gradient grade acetonitrile (ACN), methanol (MeOH), and tetrahydrofuran (THF) were obtained from Merck (Darmstadt, Germany). Ethylacetate and n-Pentane (SupraSolv) were obtained from Merck. The water used for HPLC analysis and sample preparation was purified by Waters MilliQ system. The chemicals used for Time-of-Flight (TOF) mass calibration were analytical grade. The nitrosamines were obtained by Supelco (EPA-8270 and Chiron, Trondheim). The glassware at the laboratory was cleaned by soaking it in a RBS-25 cleaning solution (R. Borghgraef s.a.- n.v., Brussels) for 24 hours with a subsequent MilliQ water-rinse procedure. The nitramines (MEA-NO2, MA-NO2, AMP-NO2, DMA-NO2, PZ-NO2) were synthesized by the Norwegian Institute for Life Science, UMB, Ås, Norway. Stock solutions of the nitramines were prepared in water and stored at -18 °C until use. Working standards were prepared by

diluting stock solutions in water, and the standards should be prepared fresh for each sequence of chemical analysis.

B.5.4 Solid phase extraction

The following SPE cartridges should be used: J.T Baker Bakerbond Carbon, 6mL, 1000mg, No 7575-07. It is recommended that the extraction procedure is accomplished by use of a 12 port vacuum extraction manifold equipped with flow/vacuum control (Supelco cat. #57275 or equivalent).

B.6 Sample handling and sample preparation methods

B.6.1 Sample handling

As a general guideline, the water wash samples or impinger solutions should be treated as fresh samples, i.e. the time between sampling and chemical analysis should be as short as possible. The samples should be stored or shipped cool (4 °C) and dark. The samples may also be stored frozen to improve the storage stability. An example of suited sample bottle for water wash storage is the amber glass bottles provided by Schott Duran.

B.6.2 Sample work up

Fill a measuring cylinder to the 30 mL mark with the sample and transfer the sample to a 250 mL flask. Adjust the pH to 6-6.5 by adding a known amount of 2M HCl in water, shake the sample. A suitable pH strip which may be used is Neutralit from Merck (1.09533.0001). Prepare a blank sample according to the same procedure. If needed, pH adjustment of pure MQ-water may be done by addition of aqueous Na_2CO_3 solution. Wait 30 minutes and re-measure the pH to make sure that the pH is 6-6.5.

Carbon adsorbents (Bakerbond Carbon, 7575-07) are conditioned with sequential drop wise wash with 5mL n-pentan, 5mL ethyl acetate, 2x5mL methanol, 100 mL MQ. Make sure that the adsorbent is wet during the preparation. Apply drop wise the water wash sample on to the carbon adsorbent. Finally wash the adsorbent with 2x3 mL MQ-water. Drain the adsorbent 5 seconds by drawing air through the adsorbent. The nitramines are eluted off the adsorbent by using the following procedure: Eluate with 3x5 mL methanol (5min. break keeping wet) +3x5 mL THF (5min. break keeping wet), 3x7 mL 50% ethyl acetate/50% methanol (5 min. break drain the adsorbent). Add 500 µl MQ to the sample extract as an evaporation keeper, evaporate to 0.5 mL by gently blowing purified N_2 at 30°C,. Re-suspend to 1.5 mL water and the sample is ready for analysis by HPLC/MS. Instrumental conditions are given in section B.1 and B.2.

B.7 Instrumental analysis

The following two instruments are suited for analysis of nitramines.

"LCT-Classic" : Agilent 1100 liquid chromatography system (Agilent Technologies, Waldbronn, Germany), equipped with an Agilent 1100 auto-sampler, a Agilent 1100 quaternary pump, an Agilent 1100 on-line degassing system and a Agilent 1100 diode array detector (UV). The analytical detector is a Micromass LCT orthogonal-acceleration time-of-flight (TOF) mass spectrometer (MS) equipped with a Z-spray electrospray ion source and a 4 GHz time to digital converter (TDC). Example of instrumental settings is given in appendix D.

Chromatographic columns:

Atlantis dC18 150x2.1mm, 3 µm, by Waters, No.186001299, has been used for MEA-NO2, MA-NO2, DMA-NO2 and AMP-NO2

Atlantis T3 150x2.1mm, 3 µm, by Waters, No 186003719, has been used for PZ-NO2

Table B.2: Recommened mobile phase gradient for the Atlantis columns

Time minutes	ACN %	Water %	Flow mL/min
0,0	2	98	0.2
2	2	98	0.2
10	40	60	0.2
16	100	0	0.45
23	100	0	0.45
23.1	2	98	0.45
31.5	2	98	0.45
32	2	98	0.2

"LCT-Premier": Waters UPLC liquid chromatography system combined with a Waters LCT PremierXE orthogonal-acceleration time-of-flight (TOF) mass spectrometer (MS) equipped with a Z-spray electrospray ion source. Example of instrumental settings is given in appendix D.

Chromatographic column:

Acquity UPLC, HSS T3, 1.8 µm, 150x2.1mm, No 186003540, by Waters has been used for PZ-NO2

Table B.3: Recommened mobile phase gradient for the HSS T3 column.

Time minutes	ACN %	Water %	Flow mL/min
0,0	0.1	99.9	0.35
7	1	99	0.35
8	1	99	0.35
8.1	99.9	0.1	0.35
11	99.9	0.1	0.35

B.7.1 Detection

Table B.4: Recommened monitoring parameters for detection of nitramines with HPLC/HRMS

Analyte	Molecular weight*	Monit. Ion $[M-H]^-$	Monit. Ion $[M+H]^+$	Ionisation mode
DMA-NO2	90.0429		91.0508	APCI+
MA-NO2	76.0273	75.0195		ES-
MEA-NO2	106.0378	105.0300		ES-
AMP-NO2	134.0691	133.0613		ES-
PZ-NO2	131.0695		132.0773	ES+

*: Monoisotopic

B.7.2 Quantification by the standard addition method

It is recommended to add a known amount of the analytical standard to an aliquot of the sample. The results obtained by the external standard method are corrected according to the response obtained by the standard added to the sample. This procedure enables good control of the combined effect of recovery loss and potential ion suppression or signal boosting due to matrix constituents. The main disadvantage of this method is the increased work load.

B.8 Method validation

B.8.1 Sample work up linearity and recovery

A 1:1 mixture of the CCM water wash samples ID K and ID L was added a range of nitramines (from 16 to 160 µg/L nitramines). A fixed sample volume of 50 mL for each spiking level was worked up according to the procedure given in B.7.2. The recovery from the experiment is shown in table B5.

Table B.5: Recovery % obtained at variable spiking levels in the real water wash samples

Exp. No.	Spiking level µg/L	Recovery % MEA-NO2	Recovery % MA-NO2	Recovery % DMA-NO2	Recovery % AMP-NO2	Recovery % PZ-NO2
1	0	-	-	-	-	-
2	16	45	18	58	81	NA*
3	32	34	17	51	81	NA*
4	64 ref level	35	14	56	77	NA*
5	128	35	14	52	68	NA*
6	160	33	14	58	67	NA*

*: Not analysed, sample application at pH 6 reduces the elution efficiency.

A 1:1 mixture of the CCM water wash samples ID K and ID L was added nitramines to a concentration of approximately 30 µg/L. A range of sample volumes from 10 mL to 100 mL were worked up according to the procedure given in B.7.2. The recovery from the experiment is shown in table B.6.

Table B.6: Recovery % obtained by spiking the real water wash samples to 30 µg/L and work up variable sample volumes.

Exp. No.	Sample volume	Recovery % MEA-NO2	Recovery % MA-NO2	Recovery % DMA-NO2	Recovery % AMP-NO2	Recovery % PZ-NO2
1	50 (unspiked)	-	-	-	-	-
2	10	49	31	82	81	NA*
3	30	50	19	67	56	NA*
4	50	37	17	63	56	NA*
5	70	29	11	61	55	NA*
6	100	26	7	67	55	NA*

*: Not analysed, sample application at pH 6 reduces the elution efficiency.

The linearity experiments results provided in table B.5 and B.6 reveal some recovery losses during sample work up, which makes it necessary to use the standard addition method in the quantification work. Preferably, isotope labeled standards should be implemented. The linearity experiments with variable sample volumes show reduced recovery with increased sample volumes, and analyses of the water sample passing through the SPE cartridge show no presence of nitramines. This implies that increased

sample volume causes increased ion suppression. The ion suppression can be handled by not exceeding 30 mL sample volume or by introducing additional clean up procedures as a part of the SPE extraction procedure.

B.8.2 Repeatability

A tap water sample was spiked with the 5 nitramines to approximately 80 µg/L of each compound. The sample was divided in two equal volumes, and one part was added 1M HCl (aq) in small portions in order to reach pH 3 using a pH strip. The two samples were further divided in 5 times 100 mL, and each of the sample aliquots were SPE extracted according to the given procedure. The sample extracts were resuspended in 10 mL to avoid overloading the mass spectrometer. The recovery results are given in table B.7.

Table B.7: Recovery % obtained by 5 parallel work up of nitramine spiked tap water

Exp. No.	pH	Recovery % MEA-NO2	Recovery % MA-NO2	Recovery % DMA-NO2	Recovery % AMP-NO2	Recovery % PZ-NO2
1	6	49	21	51	99	-
2	6	56	20	50	91	NA*
3	6	58	23	48	94	NA*
4	6	45	23	50	91	NA*
5	6	49	22	50	97	NA*
Mean	6	51	22	50	94	
RSD	6	11	6	3	4	NA*
1	3	69	38	47	71	23
2	3	71	37	52	83	23
3	3	75	33	49	77	29
4	3	78	32	46	83	26
5	3	80	29	48	81	24
Mean	3	75	34	48	79	25
RSD	3	6	11	5	7	10

As a comparison with the repeatability experiment, the instrumental contribution is given in table B.8 where the repeated injection of an analytical standard (200 µg/L) is shown.

Table B.8: Statistics of the software integration units obtained by repeated injections of an analytical standard

Exp. No.	Area units MEA-NO2	Area units MA-NO2	Area units DMA-NO2	Area units AMP-NO2	Area units PZ-NO2
1	340	179	48785	3768	-
2	318	179	44652	4225	NA*
3	328	193	46960	4087	NA*
4	332	182	46745	3985	NA*
5	394	207	48871	4316	NA*
Mean	342.4	188	47202.6	4076.2	
RSD	9	6	4	5	

*: Analysed in another instrument

B.8.3 Reproducibility

Five nitramines were added a 1:1 mixture of ID K and ID L. The spiked samples were sent to SINTEF laboratory for chemical analysis, and SINTEF used a different method than the NILU method. The SINTEF method is described as follows:

Nitramines (MEA-NO2, AMP-NO2 and PZ-NO2) were analyzed by liquid chromatography – triplequadrupole mass spectrometry (LC-MS-MS-QQQ). Measurements were made by direct injection of sample diluted 1:10 in water or undiluted on a Supelco Discovery HS F5 (15cm x2,1 mm, 3um) column, with 50/50 methanol/ 0.1% ammonium acetate as mobile phase. The following transitions were used for quantitation (target ion) and identification (qualifier ion):

Compound	Target ion (m/z)	Qualifier (m/z)
MEA-NO2	105-43	105-46
AMP-NO2	133-46	133-61
PZ-NO2	132-86	132-44

Table B.9: SINTEF analysis results of spiked samples analysed by direct injection

Sample	Spike level µg/L	MEA-NO2 µg/L	PZ-NO2 µg/L	AMP-NO2 µg/L	MEA-NO2 recovery %	PZ-NO2 recovery %	AMP-NO2 recovery %
A	0	22.1	Nd*	Nd*			
B	4.2	28.7	4.3	3.9	158	103	93
C	8.4	33.0	8.6	7.8	130	102	93
E	33.6	60.5	27.5	31	114	82	92
D	16.8	40.7	14.5	14.7	111	86	87
G	16.8	44	14	15.2	131	83	90
H	16.8	39.4	13.6	14.5	103	81	87
I	16.8	40.1	13.8	15.2	107	82	90
J	16.8	41.7	14.5	15.3	117	86	91
Average D,G,H,I,J		41.18	14.08	14.98			
RSD %		4	3	2			

*: Detection limits (LOD) were 1 µg/L for all compounds.

The unspiked sample is reported to 22.1 µg/L MEA-NO2, wheras a 1/10 dilution of the unspiked sample was measured to 26.7 µg/L. The NILU method has previously reported 24 and 26 µg/L MEA-NO2 in ID K and ID L. The composite sample (1:1 mixture of ID K and ID L) should then have an average of 25 µg/L as unspiked sample. The agreement between the SINTEF method and the NILU method is excellent for the unspiked sample. The precision of the SINTEF method (5 parallel samples) is better than the NILU method because the SPE cartridges contributes to a higher RSD. However, due to lack of instrumental sensitivity MA-NO2 and DMA-NO2 could not be reported. The advantage with the NILU-method is the enrichment step which provides

lower detection limits. In addition, the NILU method enables sample clean-up and makes it possible to do complementary analysis with GC/TEA.

B.8.4 Interferences

The nitramine recovery from the water wash samples is optimized by extracting (SPE) at pH 3. At this low pH a potential interference is observed for MEA-NO2 in some samples. At pH 6 this potential interference is eliminated. However, the recovery of all nitramines are reduced and PZ-NO2 sticks to the SPE surface with no recovery as a result. Clean-up steps needs to be developed to avoid this potential interference.

B.8.5 Method limit of detection

The method limit of detection (minimum reporting level) in water samples comprises the instrumental sensitivity combined with correction of recovery losses.

Table B.10: Method limit of detection for the five nitramines in 50 mL water samples.

MEA-NO2 (µg/L)	MA-NO2 (µg/L)	DMA-NO2 (µg/L)	AMP-NO2 (µg/L)	PZ-NO2 (µg/L)
0.5	2.1	40	0.3	0.5

By assuming an impinger volume of 100 mL and a LOD similar to the water wash sample, we need 50 ng in 100 mL to reach the level of 500 ng/L. If 50 ng is sampled from a 100 L air sample, the LOD of the emissions can be estimated to 500 ng/m^3 air sample or 116 ppt at STP for MEA-NO2.

Note! The extrapolation needs to be confirmed by experiments.

Table B.11: Method limit of detection for the five nitramines in emission samples.

MEA-NO2 (µg/m^3)	MA-NO2 (µg/m^3)	DMA-NO2 (µg/m^3)	AMP-NO2 (µg/m^3)	PZ-NO2 (µg/m^3)
0.5	2.1	40	0.3	0.5

The method limits of detection have a potential of reduction of one order of magnitude. This may be achieved by introduction of clean-up steps and improved instrumental sensitivity.

B.8.6 Testing of analytical methods on real samples

The method has been tested on 6 different water wash samples (MEA based technology) provided by Company (ID J, K,L,R,S and T). The method has also been tested on tap water, synthetic water wash samples and MQ water. Several of the validation experiments have been accomplished with some of the samples provided by the Company (ID K and L). The method seems robust and without any major deviation from the validation results given above. Figure B1 show the extracted ion chromatograms obtained from the analysis of ID K and ID L.

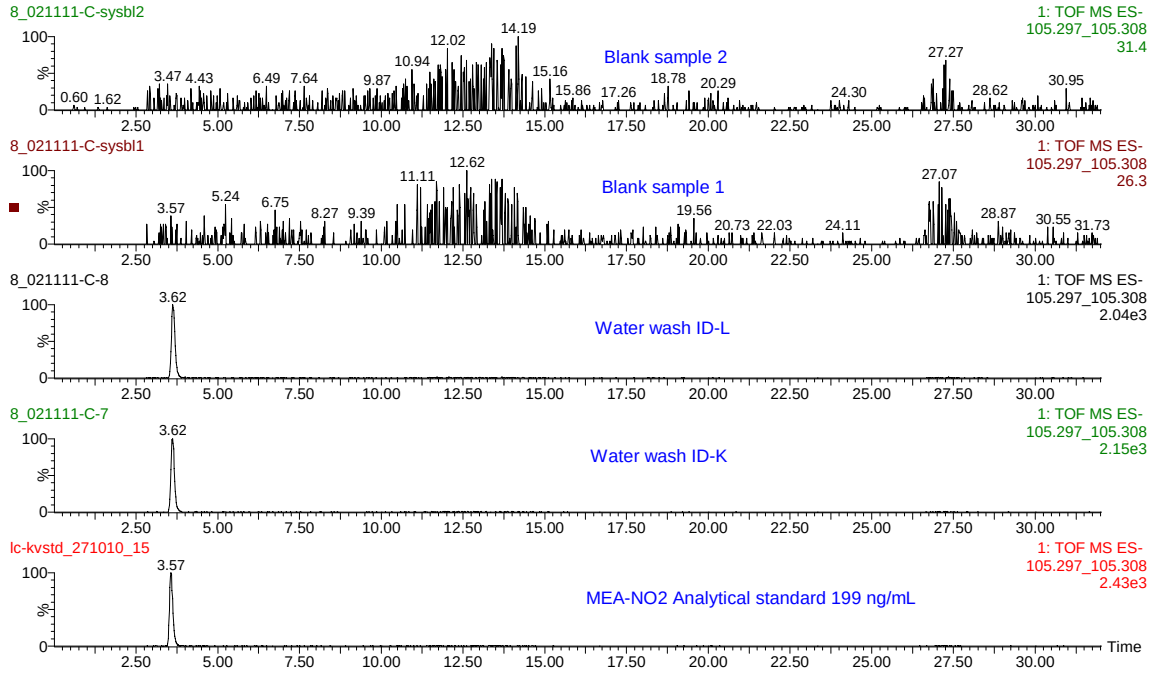

Figure B.1: Extracted ion chromatograms (10 mDa) from HPLC/HRMS analysis of real samples (ID-K and ID-L), blank samples and analytical standard. The concentration of MEA-NO2 is 26µg/L in ID K and 24 µg/L in ID L.

MEA-NO2 is the only nitramine identified in the samples (ID-J 275 ng/L, ID-K 26 µg/L, ID-L 24 µg/L, ID-R 27 µg/L, ID-S 24 µg/L and ID-T <100 ng/L).

B.8.7 Qualitative analysis confidence

How sure can we be that we have a correct identification of a nitramine?

By increasing the cone voltage (CV), the molecule fragment ion yield is increased. This technique should be used to improve the identification confidence. In figure B2 this is exemplified by comparison of the extracted ion chromatograms of MEA-NO2 in a standard and a sample. The three lower chromatograms show the standard chromatograms at high and low CV and the three upper chromatograms show the sample at the same high and low CV. The extracted ion accurate masses and the ion ratios are similar for the sample and the standard. In addition, the retention times matches – the small observed shift to higher retention times in the samples is caused by the matrix constituents (verified by the standard addition method)

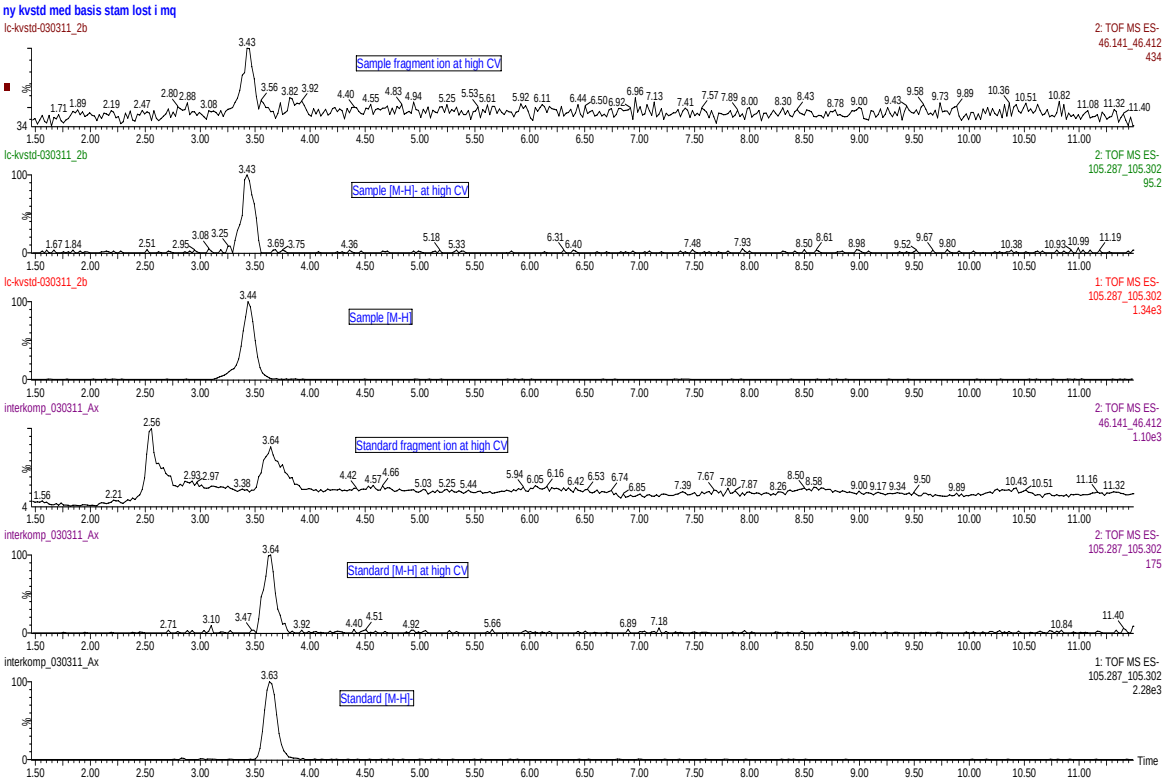

Figure B.2: Extracted ion chromatograms of an analytical MEA- NO2 standard and a water wash sample. The chromatograms are extracted at high and low cone voltage.

The corresponding mass spectra behind the chromatograms in figure B.2, is given in figure B3. The measured monoisotopic mass in the standard (lower mass spectrum) and the sample (upper mass spectrum) is 105.0319, which is 1.9 mDa higher than the MEA-NO2 theoretical $[M-H]^-$ value of 105.0300. The mass deviation should be less than 2 mDa.

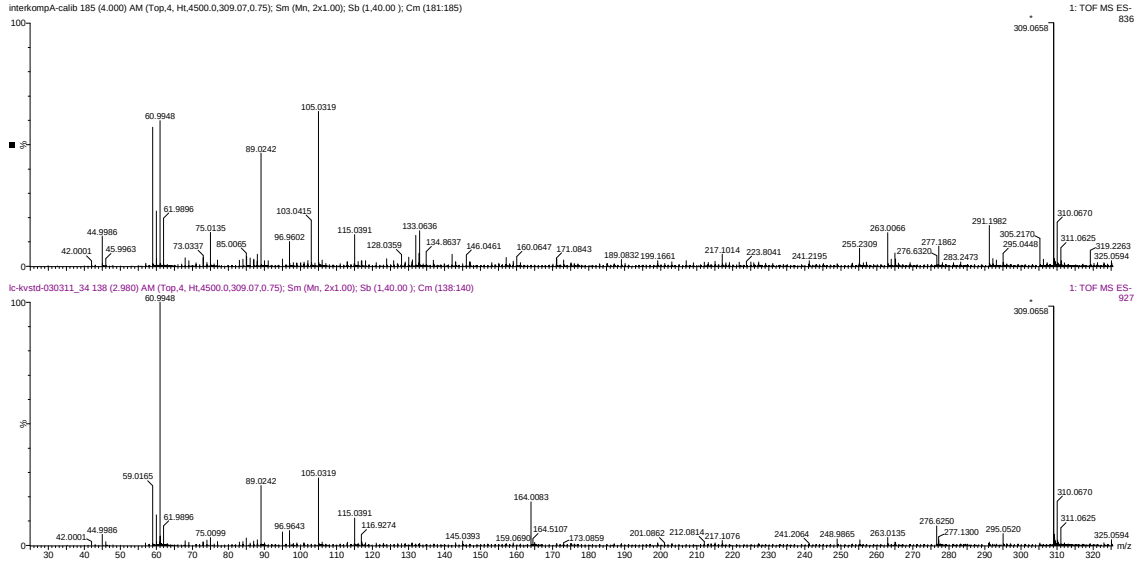

Figure B.3: Accurate mass measurement of an analytical MEA- NO2 standard and a MEA-NO2 candidate peak in a water wash sample.

The identification can also be supported by use of a different analytical tool, e.g. LC/MS/MS.

C Chapter C – Literature review of human toxicity studies

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C.1 Introduction

A thorough literature review has been performed to evaluate recent knowledge, identify knowledge gaps on human safety and to estimate acceptable environmental concentration levels of 6 nitramines. In this way an overview of knowledge is provided showing where information is lacking or missing altogether, and if experimental studies need to be performed.

By searching in SciFinder, Science Direct, PubMed, and using the databases CPDB, RTECS, IUCLID, GESAMP, IRIS, Toxnet and in available internal reports and relevant documents we reviewed toxicological data of DMA-NO2 (4164-28-7), MA-NO2 (598-57-2), Diethanolnitramine (13084-48-5), MEA-NO2 (74386-82-6), N-nitro-piperazine (42499-41-2), AMP-NO2 (1239666-60-4) for acute and chronic (long term) exposures effects.

Data on long term toxicity of DMA-NO2 and MA-NO2 show that both compounds are carcinogenic with DMA-NO2 being more potent (based on TD50). While DMA-NO2 is clearly mutagenic, data on mutagenicity of MA-NO2 are not convincing. In the above mentioned databases, no data were available on Diethanolnitramine, MEA-NO2, N-nitro-piperazine, AMP-NO2 for acute or chronic (long term) exposures effects (mutagenicity / carcinogenicity) found. None from 6 compounds had data available on reproductive toxicity.

We estimated acceptable environmental concentration levels of DMA-NO2 and MA-NO2 following REACH guideline and recommendations and using non-threshold approach and semi-quantitative reference value Derived Minimal Effect Level (DMEL). DMEL or minimal effect dose (acceptable level) was provisionally estimated as 0.547×10^{-5} mg/kg and 17.4×10^{-5}mg/kg, respectively. As no information was found for toxicological data of Diethanolnitramine, MEA-NO2, N-nitro-piperazine, AMP-NO2 it was not possible to estimate acceptable environmental concentrations for these compounds.

As there is lacking information on general as well as specific (mutagenic /reproductive) toxicity we suggest further investigation of all 6 compounds.

C.2 Method for information gathering

Chemical hazard summary sheets, provided generously by NTNU, were modified to this study. All relevant information from the collected literature was included to these sheets (Appendix E). In this way a structured overview of toxicity and hazard effects from each nitramine would be provided. CAS numbers have been used for searching correct chemical (except from PubMed and Science Direct, where name of chemical was used).

The results from the summary sheets were included to a summary table, and conclusions were drawn based on this summary. More detailed explanation of how to use the summary table can be found in the subchapters below.

Chemicals
Table C.1 shows the list of 6 nitramines which was provided by CCM.

Table C.1: List of selected nitramines

Name of the chemical	Abbreviation	CAS Numbers
Dimethylnitramine	DMA-NO2	4164-28-7
Methylnitramine	MA-NO2	598-57-2
Diethanolnitramine		13084-48-5
Ethanolnitramine	MEA-NO2	74386-82-6
N-nitropiperazine	PZ-NO2	42499-41-2
2-methyl-2-(nitramino)-1-propanol	AMP-NO2	1239666-60-4

Quality Assurance
This work was performed by two separate institutions; NILU and NTNU. The responsibility was shared by endpoints, but to ensure a high quality, the two groups have carefully reviewed each others' work. If anything was not consistent, this was discussed in detail until we made sure that all experts agreed on the final results.

C.2.1 Qualitative analysis confidence

The work has been carried out by reviewing literature, using search engines such as SciFinder, Science Direct, PubMed, and using the databases: CPDB, RTECS, IUCLID, GESAMP, IRIS and Toxnet. In addition, available internal reports and relevant documents have been reviewed.

Links to following databases:

- Scifinder: http://www.cas.org/products/scifindr/index.html
- Science Direct: http://www.sciencedirect.com/
- Pubmed: http://www.ncbi.nlm.nih.gov/pubmed
- RTECS: http://ccinfoweb.ccohs.ca/rtecs/search.html

- IUCLID data sheet: http://ecb.jrc.ec.europa.eu/esis/
- GESAMP-list: http://www.imo.org/includes/blastDataOnly.asp/data_id%3D25672/Report-BLGCirc.29annex6doc.pdf
- GESAMP background info: http://www.gesamp.org/publications/publicationdisplaypages/rs64
- CPDB: http://potency.berkeley.edu/chemicalsummary.html
- EPA-IRIS: http://www.epa.gov/ncea/iris/index.html
- Toxnet: http://toxnet.nlm.nih.gov/index.html

C.2.2 Method for evaluation of available information

Explanation for classification of long term health hazards is adopted form GESAMP-EHS: http://www.gesamp.org/publications/publicationdisplaypages/rs64

Carcinogenic

The term carcinogenic denotes substances or mixtures that are presumed to induce cancer or to increase its incidence in humans. Evidence to substantiate the notation "carcinogenic" should be available from epidemiological studies and/or from well conducted studies in experimental animals. On a case by case basis, scientific judgment may warrant a decision of presumed human carcinogenicity (C) derived from studies showing limited evidence in humans with limited evidence in experimental animals.

Mutagenic

A mutation is a permanent change in the amount or structure of the genetic material in a cell. The term mutation applies to genetic changes both for somatic cells and for germ cells that may give rise to subsequent adverse changes at the phenotypic level. The term mutagenic denotes substances or mixtures that can give rise to an increased occurrence of mutations *in vivo*, in populations of cells and/or organisms. Evidence to substantiate a notation of "mutagenicity" (M) is normally provided from studies conducted *in vivo* on mammalian somatic cells or germ cells. It is recognized that genetic events are central in the overall process of cancer development. Therefore, evidence of mutagenicity indicates that a substance has a potential to induce carcinogenic effects.

Reprotoxic

Reproductive toxicity includes adverse effects on sexual function and fertility in adult males and females or on the development of the offspring. The notation "reprotoxic" (R) includes substances for which there is reliable evidence from human experience or from experimental animals of an adverse effect on reproductive ability, capacity, or on development of the offspring in the absence of other toxic effects.

Sensitiser

The term sensitising denotes substances or mixtures, which can induce a condition of hypersensitivity in individuals following inhalation (respiratory sensitiser) or skin contact (contact sensitiser). Evidence to substantiate a notation of "sensitising" (S)

should be available from human experience and/or from appropriate studies using experimental animals. The term photosensitising (Sp) denotes substances or mixtures that require light to become active and may subsequently induce a condition of contact sensitivity. Evidence to substantiate the notation of "photosensitizing" should be available from human experience and/or from appropriate studies using experimental animals.

C.2.3 Chemical classification criteria

The summary table, Table C.4 condenses the results from the databases which have been examined. The detailed chemical hazard sheets can be found in Appendix E. For classification and numerical rating the definitions used by GESAMP/EHS have been used. GESAMP is an organization for cooperation between several UN organizations (UNEP, FAO, UNESCO, IOC, WHO, WMO, IMO, IAEA). GESAMP/EHS was established in 1974 and have carried out detailed examination on roughly 3000 compounds and products carried at sea. The GESAMP classification is carried out by a team of international experts on chemistry, marine ecotoxicity and human health hazard assessment. The experts are invited by IMO (International Maritime Organization) on behalf of GESAMP. Their classification has been published as the GESAMP Composite list by IMO. The GESAMP/EHS review is based on public records as well as confidential company information. In some cases laboratory reports from the toxicity studies have been examined. The files supporting the decisions of the GESAMP/EHS group is located at IMO, London, UK.

Although the GESAMP/EHS profile is generated in order to regulate the safe transport of chemicals at sea, the information on human health can be used also for other purposes.

There are two internationally recognized hazard classification systems available; GESAMP-EHS and GHS (Global Harmonized System). Both systems are endorsed and supported by the UN. The first is primarily for maritime transport of chemicals while the last forms the basis of the UN system for recognition of transport hazards as well as R/S-sentences. GESAMP is the oldest system and classifies chemicals for environmental fate, ecotoxicology and human hazards. The GESAMP and GHS classifications for mammalian health hazards are almost identical, the main differences being:

- The classification numbering is opposite (a rating of 4 is the most toxic in GESAMP while it is the least toxic in GHS)
- There are minor differences in cut-off values for acute toxicity
- There are some compounds classified as slightly irritant to skin and eye which is not classified as irritants in GHS
- There are a few compounds classified as C, M or R in GESAMP without similar rating in GHS

The GESAMP classification system was selected for this report as one member of the project team was member of the GESAMP group of experts for 24 years and have extensive experience with this system. The outcome of the hazard evaluation for the chemicals listed in Amine 5 would be the same regardless of whether GESAMP or GHS is used.

C.2.1.1 Principles of evaluation

In the present project exposure to humans may occur in an occupational setting or as a result of chemicals being dispersed to the neighbourhood of the production plant. Acute (4 hours) inhalation data will of course be important in any setting where atmospheric exposure is the prime source. However, acute short term exposure at relatively high concentrations may not be a good indicator of health hazards which may occur after low level and long term exposure.

At low level and long term exposure the following health hazards will be of prime interest when regulations are set for permissible exposure to population at or near a plant:

- Carcinogenicity (C)
- Mutagenicity (M)
- Reproductive effects (R)
- Sensitization, primarily by inhalation (S)

The data regarding important long term health issues is categorized according to the following criteria:

C	Shown to induce or increase cancer in animals or man
M	Shown to cause increased incidence of permanent changes in the amount or structure of the genetic material
R	Shown to cause adverse effects on reproductive ability or capacity, or the development of offspring
S	Shown to be a sensitizer (skin or respiratory)

C.2.4 Method for estimation of acceptable environmental concentration levels

Here we consider DNEL (the Derived No-Effect Level) and DMEL (derived Minimal-Effect Level) as estimated acceptable levels, e.g. the highest safe level of an introduced substance or the maximum level in the environment at which the substance poses no or minimal health hazards to human. The establishment of 'safe exposure levels' is done using risk assessment techniques that take into account the inherent toxicity of a substance as well as the type and degree of exposure. The risk models are designed to assure the outcome is protective of health. Thus they incorporate assumptions that will nearly always over predict, but rarely if ever under predict, health risks.

REACH: NOEL and DNEL approach for risk assessment

Risk assessment is usually derived, if possible, from long-term animal studies. First, the NOEL is determined, which is the highest dose with no toxic effects. A large safety factor is then added – usually by dividing the level in animals by 100 – to arrive at a safe level for humans. For example, if the no effect level in animals is found to be 100 mg/kg, then the human acceptable environmental concentration would be set at 1mg/kg.

REACH (Annex I, 1.0.1) defines the DNEL, i.e. the level of exposure above which humans should not be exposed. In the risk characterisation, the exposure of each human population known to be, or likely to be exposed is compared with the appropriate DNEL. The risk to humans can be considered to be adequately controlled if the exposure levels estimated do not exceed the appropriate DNEL. The safety (assessment) factor is built in partly to account for the differences between animals and humans, and also to allow for the variability between different population, and individual variations among people, such as age, health and how well nourished they are. The safety factor account for many uncertainty factors, such as the variability in the experimental information and or inter and intra-species variation (including genetic predisposition); the nature and severity of the effect; the sensitivity of the human (sub-) population to which the quantitative and/or qualitative information on exposure applies, etc. DNELs must consider populations (workers, consumers, general population), exposure routes (inhalation, dermal/eye, oral), duration of exposure: a) Long-term DNEL (or DNEL chronic); b) Short term DNEL (or DNEL acute) expressed as 15'value (where relevant), systemic and local effects.

For mutagens and genotoxic carcinogens, non-threshold mode of action is suggested. Thus, instead of DNEL, DMEL approach is taken with adequate animal cancer data of a (semi)quantitative reference value (DMEL). This implies the use of endpoint-specific large assessment factor (AF), i.e. 10,000 to ensure that the exposure causes a minimal risk. The specific dose descriptor BMDL10 is divided by that AF.

The BMDL10 is defined as the lower 95% confidence dose of a Benchmark-dose representing a 10% tumor response upon lifetime exposure, i.e. the lower 95% confidence dose of a BMD10. The use of the BMDL10, rather than the BMD10, is recommended if one wants to reflect the uncertainties and statistical errors in the available cancer dose-response data.

C.2.5 Method for reviewing literature for eco-toxicological effects of the selected nitramines

It is part of procedure to do a quick literature search before starting toxicity testing experiments. This was done by SINTEF who searched the databases:
IUCLID, ECOTOX (EPA), BIODEG (Syracuse Research Corporation) in addition to Web of Science.

Table C.2: Databases and explanation of health hazard terms used in Table C.4

Column heading	Explanation
Data bases	A "+" indicates that information was found. A "-" indicates that a search was done without finding any relevant information. Scifinder: http://www.cas.org/products/scifindr/index.html Science Direct: http://www.sciencedirect.com/ PubMed: http://www.ncbi.nlm.nih.gov/pubmed CPDB: http://potency.berkeley.edu/chemicalsummary.html RTECS: http://ccinfoweb.ccohs.ca/rtecs/search.html IUCLID data sheet: http://ecb.jrc.ec.europa.eu/esis/ GESAMP-list: http://www.imo.org/includes/blastDataOnly.asp/data_id%3D25672/Report-BLGCirc.29annex6doc.pdf GESAMP background info: http://www.gesamp.org/publications/publicationdisplaypages/rs64 EPA-IRIS: http://www.epa.gov/ncea/iris/index.html Toxnet: http://toxnet.nlm.nih.gov/index.html
Oral, acute	**Oral toxicity LD50 rating codes** 0: >2000 1: 300-2000 2: 50-300 3: 5-50 4: <5 mg/kg bw
Percutaneous, acute	**Percutaneous toxicity LD50 rating codes** 0: >2000 1: 1000-2000 2: 200-1000 3. 50-200 4: <50 mg/kg bw
Inhalation, acute	**Inhalation toxicity LC50 4 hours exposure rating codes** 0: >20 1: 10-20 2: 2-10 3: 0.5-2 4: <0.5 mg/l (4hrs)
Long term	Full description of rationale for rating given at bottom of table. Short form rating code: C: Shown to induce or increase cancer in animals or man M: Shown to cause increased incidence of permanent changes in the amount or structure of the genetic material R: Shown to cause adverse effects on reproductive ability or capacity, or the development of offspring S: Shown to be a sensitizer
Comments	A summary expert opinion on the chemical is given in the comments column. For oral/dermal/inhalation the numbers in respective columns indicate: • Negligible toxicity: 0 • Slight toxicity: 1 • Moderate toxicity: 2 • Moderately high toxicity: 3 • High toxicity: 4 • - No data available Ratings in brackets: Provisional ratings based on limited or no data. Expert judgment. OEL: Occupational exposure level – TWA will be used if available. TWA: time weight average (of exposure for 8 hours) ***Conclusions and recommendations written in italic bold***

C.3 Results

The information obtained from the databases was used to prepare a summary sheet for each compound for estimation of acceptable environmental concentration levels of nitramines where possible. In Table C.3 an overview of the findings from all databases and search engines can be found. The detailed information for each nitramine has been saved in the chemical hazard summary sheets in Appendix E.

However, in the selected databases, only data on DMA-NO2 and MA-NO2 were found (Appendix E). The summary sheets contain information about acute toxicity (oral, percutaneous, inhalation), mutagenicity, carcinogenicity and other long term effects, and the results from these were extracted to summary table (Table C.4).

C.3.1 Dimethylnitramine

Data on acute toxicity, mutagenicity/carcinogenicity and other long term toxicity studies have been found in RTECS, Toxnet and CPDB databases and in SciFinder, Science Direct and PubMed. DMA-NO2 causes serious long term effects (Toxicology review, 2005, for details see Appendix E). Data on acute toxicity reported by RTEC orally in rat show LD50 1095 mg/kg (Andersen and Jenkis, 1978), LD50 after intravenous application in rats is 600mg/kg and after intraperitoneal administration 897mg/kg. LD50 in mice intraperitoneally is 399 mg/kg (Andersen and Jenkis, 1978).

Data on both male and female rats show carcinogenic and tumorigenic potential (by RTECS criteria, CPDB/ TD50 = 0.547 mg/kg bw/day in both male and female rats). Liver and nasal cavity tumors were also reported in mice (Goodall and Kennedy, 1976) as well as male and female rats (Scherf et al., 1989) (http://potency.berkeley.edu/chempages/DIMETHYL-NITRAMINE.html). Numbers of tumors in liver, lung, kidney, malignant lymphoma, lung, duodenum, atriocaval were also found (Mirvish et al., 1980). Hassel et al. (1987) found positive-Aesthesineuroeptheliomas and neurogenic tumours of the lumbar region of the spine in rats. Goodal et al, (1976) observed renal adenocarcinomas in mice as well as hepatocelluar carcinomas in mice and rats.

Urinary bladder, liver and kidney tumors were observed in various animal species (Pliss et al., 1982). Gastrointestinal toxicity such as induction of hemorrhagic foci in the lining of the stomach and intestine were observed after single dose of DMA-NO2 (Andersen and Lenkins, 1978)

Various studies on mutagenicity using Ames test show positive effect (Khudoley et al., 1981, Frei et al., 1984, Pool et al., 1984 and 1986). However, on mammalian system only single strand breaks by Alkaline elution were performed showing induction of breaks (Pala et al., 1982, Pool et al., 1986). Using fluorometric Alkaline elution method, Frei et al. (1986) shows no effect of DMA-NO2 on DNA breaks but its monoalkyl metabolite (MA-NO2) was positive.

No available data were found on sensitisation, irritation or reproductive toxicity, RTECS has reported no data on reprotoxicity and the chemical was not found in IUCLID.

C.3.2 Methylnitramine

Data on acute and long term mutagenicity/carcinogenicity studies have been found in RTECS, Toxnet and CPDB databases and in Science Direct and PubMed. MA-NO2 causes serious long term effects (Toxicology review, 2005, see Appendix E). LD50 on mice administrated intraperitonealy is 500mg/kg exposure (Pharmaceutical Chemistry Journal, 1976). Several carcinogenicity studies are reported in Toxnet and CPDB and the chemical is considered as carcinogen and tumorigen by RTECS criteria. Nervous system cancers in rats were reported in cancer databases. (CPDB, TD50 = 17.4 mg/kg bw/day in both male and female rats) (http://potency.berkeley.edu/chempages/METHYLNITRAMINE.html). Hassel et al. (1987) found number of tumors in nasal cavity, spinal cord, spinal and peripheral nerves and other sites. Similarly Scherf et al., (1989) reported tumors in spinal cord and spinal nerve in both male and female rats when administered by gavage, with males being more susceptible.

However, data on mutagenicity are not convincing. Results on Ames test were negative (Pool et al., 1984, 1986, Malaveille et al., 1983). MA-NO2 induced DNA damage in various mammalian cells but only DNA strand breaks using Alkaline elution were investigated (Frei et al., 1986, Pool et al., 1986).

No available data were found on sensitisation or reproductive toxicity, RTECS has reported no data on reprotoxicity and the chemical was not found in IUCLID.

C.3.3 Diethanolnitramine, Ethanolnitramine, N-nitro-piperazine and 2-methyl-2-(nitramino)-1-propanol

No information has been found for these nitramines in RTECS, IUCLID or other toxicological databases about acute or chronic toxicity.

C.3.4 Eco-toxicological effects

A search on the databases IUCLID, ECOTOX and BIODEG showed that these databases did not contain any information on the nitramines relevant for this project. The ECOTOX and BIODEG databases included information for the nitramines RDX (1,3,5-trinitro-1,3,5-triazine) and HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) which all are cyclic or aromatic explosives. Also the Web of Science contained environmental information about these compounds. However, the structures of these chemicals indicate that they are not relevant for the nitramines associated with this project.

Table C.3: **Results from screening databases.** ***Yes: Information available; -: Chemical is not listed and no data available.***

Name	CAS	SciFinder	Science Direct	PUBMED	CPDB	RTECS	IUCLID	GESAMP	IRIS	Toxnet
DMA-NO2	4164-28-7	Yes	Yes	Yes	Yes	Yes	-	-	-	Yes
MA-NO2	598-57-2	-	Yes	Yes	Yes	Yes	-	-	-	Yes
Diethanolnitramine	13084-48-5	-	-	-	-	-	-	-	-	-
MEA-NO2	74386-82-6	-	-	-	-	-	-	-	-	-
N-nitro-piperazine	42499-41-2	-	-	-	-	-	-	-	-	-
AMP-NO2	1239666-60-4	-	-	-	-	-	-	-	-	-

Table C.4: **Summary table for DMA-NO2 and MA-NO2**

Chemical group	Name of the chemical	CAS Number	Data bases examined	Oral, acute	Percutaneous acute	Inhalation, acute	Long Term	Comment on human health
Nitramines	Dimethyl-nitramine	4164-28-7	Toxnet+ CCRIS+ RTECS+ IUCLID- GESAMP- CPDB+ IRIS- Pubmed+ Scifinder+	1	-	-	C M	***Confirm C*** ***Confirm M*** ***No data on reprotox.*** Slight oral toxicity No OEL/TWA available ***Serious long term effects.*** ***Candidate for testing for R*** ***Candidate for testing as positive control for C and M studies***
	Methyl-nitramine	598-57-2	Toxnet+ CCRIS+ RTECS+ IUCLID- GESAMP- CPDB+ IRIS- Pubmed+	-	-	-	C M?	***Confirm C*** ***M: Need more data*** ***No data on reprotox.*** Slight oral toxicity No OEL/TWA available ***Serious long term effects.*** ***Candidate for general testing and R studies*** ***Candidate for testing as positive control for C studies***

C.3.5 Example of estimation of acceptable environmental concentration levels of nitramines

As both DMA-NO2 and MA-NO2 were found to be carcinogenic, a non-threshold and non-linear mode of action can be suggested (ECHA 2010). Thus acceptable environmental concentrations or DMEL can be provisionally estimated. There is not enough data for relevant dose descriptor, therefore we had to modify it. As we had information only on TD50 we used it as base for provisional dose descriptor TD10 (carcinogenic potency – dose which induces tumors in 10% of animals). We assume there is no linearity, therefore we included safety factor 10 to estimate TD10. Further we followed 'Large assessment factor' approach by including assessment factor 10,000. Thus overall assessment factor is 100,000. TD50 for DMA-NO2 is 0.547 mg/kg bw/day and for MA-NO2 17.4 mg/kg bw/day thus estimated DMEL or minimal effect dose (acceptable level) is provisionally estimated as 0.547×10^{-5} mg/kg bw/day and 17.4×10^{-5} mg/kg bw/day, respectively. For Diethanolnitramine, MEA-NO2, N-nitro-piperazine and AMP-NO2 no data were found in the literature sources, and thus provisional estimation is difficult to establish.

C.4 Discussion and recommendations for experimental work within this project

While no data were found in the literature sources on MEA-NO2, DMA-NO2, PZ-NO2 and AMP-NO2, data on long term toxicity of DMA-NO2 and MA-NO2 show that both compounds are carcinogenic with DMA-NO2 being more potent (based on TD50). While DMA-NO2 is clearly mutagenic, data on mutagenicity of MA-NO2 are not convincing (bacterial mutation assay was negative). Available data on mammalian systems, though showing DNA damage in various cell cultures are not sufficient. Therefore further investigation is needed to understand the mode of action and to evaluate genotoxicity of MA-NO2.

No information has been found on sensitisation. However, our main concerns regarding the selected chemicals, based on typical exposure, are on mutagenicity, carcinogenicity and reproductive toxicity. It could still be useful to perform a screening on sensitisation, as this information is completely lacking. By a simple screening of 4-5 nitramines, as described in contract, this could give an indication if there is a need for more investigation.

The tender called for experimental toxicity studies to be executed for 4-6 chemicals and that in vitro testing is to be preferred following REACH recommendation. The development of NOELs (no observable effect level) as well as DMEL depends on quantitative or semi-quantitative data from primarily animal tests. It was very difficult to find/estimate these levels for selected nitramines. Available data allowed us to make estimations of DMEL only for DMA-NO2 and MA-NO2.

It is a challenge to transform *in vitro* data to animal based risk assessments. A recommended and often used approach is to include a control substance or reference standard, having the same chemically functional group and where toxicity data obtained from animals already are available. In the present case it would be necessary to test these chemicals in order to establish a basis for comparing the substances and prepare a provisional risk assessment. In this particular case we suggest DMA-NO2 as positive control.

Within the current project we recommend to continue with the original work plan, with main focus on mutagenicity. This means that for sensitivity/irritation/corrosion testing we suggest to test 4 nitramines, and for the genotoxicity tests we would recommend to screen all. For reproductive toxicity there are no available data. But due to long test period and high costs, we recommend starting with one nitramine, as proposed in tender. We therefore propose an extension of the current contract in order to establish a basis for comparing the substances and prepare a provisional risk assessment.

D Chapter D Analytical support for toxicity experimental work

Christian Dye, Arve Bjerke, Lise M. B. Fjellsbø, Mária Dušinská

Little is known about the test substances, and to be sure to use correct concentrations when performing toxicity tests it was decided to perform analytical measurements of the stock solutions in different conditions. These measurements were performed for three main reasons:

- Identify if nominal concentration was equal to measured concentrations
- Measure if nitramines would "stick" to walls and thus reduce concentration in exposure media
- Control if nitramines would be transferred to nitrosamines during time of exposure

D.1 Preparation of solutions for toxicity testing and concentration measurements

Due to HSE aspects and the need for UN-classification of dangerous goods, it was decided to ship the nitramines to the toxicity laboratories as water solutions. The nitramines were weighed and dissolved in water. Unfortunately, the initial solutions were made with too low concentrations for the toxicity tests, and enrichment at 50°C with N_2 flow was necessary to evaporate the water (Zymark Vaporator Station).

Aliquots of the nitramine solutions were taken for chemical analysis of the purity and correct concentration. The quantitative analysis showed that the extensive handling with enrichment had caused losses in some bottles. Evaporative loss is suspected to be the most important mechanism with possible formation of azeotrop mixtures. The chemical analysis did not show any significant presence of degradation products.

DMA-NO2 and PZ-NO2 were prepared without the need for extensive water evaporation, and hence no losses were observed for these nitramines.

The variability in losses from one laboratory to another is explained by the difference in need for stock solution handling and difference in laboratory procedures. In addition, none of the stock solution containers have been subjected to exactly the same handling or evaporation degree.

Two laboratories returned some aliquots of the nitramines for chemical analysis after completing the experiments. This was done to assure the quality of the toxicity test results.

Table D.1: Quantitative analysis of the nitramine solutions used for toxicity tests

Nitramine	Concentrations (g/L)	SMU	VITO	SINTEF	NILU
MEA-NO2	Initial	238	201	43	163
MEA-NO2	Corrected	238	195	43	124
MA-NO2	Initial	194	205	28	163
MA-NO2	Corrected	166	195	28	180
DMA-NO2	Initial	77	77	77	77
DMA-NO2	Corrected	77	77	77	77
AMP-NO2	Initial	85	240/240	28	219
AMP-NO2	Corrected	68	192/225	28	240
PZ-NO2	Initial	97	97	97	97
PZ-NO2	Corrected	97	97	97	97

Table D.2 Quantitative analysis of the nitramine solutions returned after completed experiments

Nitramine	Concentrations (g/L)	SMU	VITO
MEA-NO2	Corrected		195
MEA-NO2	Returned		159
MA-NO2	Corrected	166	195
MA-NO2	Returned	166	166
DMA-NO2	Corrected	77	77
DMA-NO2	Returned	77	48
AMP-NO2	Corrected	68	192
AMP-NO2	Returned	68	187
PZ-NO2	Corrected		97
PZ-NO2	Returned		77

The nitramine concentration is somewhat lower in the solutions from VITO after completion of the experiments. The VITO nitramine solutions were highly concentrated and a possible explanation is losses due to precipitation caused by storage at low temperature. The chemical analysis (HPLC/UV/HRMS) did not show any significant presence of degradation products in the returned solutions.

D.2 Control for equipment and containers used during experiments

Knowledge about the nitramine compatibility with reaction tubes, wells and reagents is important in evaluation of the quality of the toxicity experiments. In Table D.3 supporting results for the NILU experiments are provided. Firstly, concentrations before and after bacterial filtration were measured, no significant loss was observed. Secondly concentration before and after a typical exposure condition (using either Falcon-tube or 6-well plate) were measured to check if the nitramines sticks to the material used. The results in Table D.3 how good compatibility with chemicals and equipment used at NILU.

Table D.3 Chemical analysis of nitramines exposed to chemicals and equipment used in toxicity tests at NILU

		before filt	after filt	HI	THI	HO	THO	LO	TLO	LI	TIL
		mg/mL	mg/mL	mg/mL	mg/mL	mg/mL	mg/mL	ug/mL	ug/mL	ug/mL	ug/mL
A	DMA-NO2	3.84	3.91	3.53	3.72	3.74	3.83	0.343	0.345	0.337	0.342
B	MEA-NO2	3.78	3.89	3.98	3.32	4.18	3.01	0.035	0.035	0.048	0.062
C	MA-NO2	5.50	5.54	4.44	4.94	7.16	4.76	0.037	0.039	0.082	0.156
D	AMP-NO2	5.45	5.18	5.18	5.16	5.22	5.33	0.457	0.415	0.459	0.405

H: High concentration
L: Low concentration
O: 0 hours, before treatment
I: 3 hours, after treatment
T: Tube
No upper marking: 6 well

The corresponding check was performed for the VITO material and reagents (Table D.4). The results are given as the recovery per cent of the added nitramine. The compatibility is in general very good. The high recovery for AMP-NO2 for "phos-buff" and "dc-medium" is explained by the presence of media matrix compounds which boost the MS-signal.

Table D.4 Chemical analysis of nitramines exposed to chemicals and equipment used in toxicity tests at VITO

	Microplate Recovery %	well-cultcluster Recovery %	phos-buff Recovery %	dc-medium Recovery %	top-agar Recovery %
MEA-NO2	103	92	81	97	120
MA-NO2	105	95	84	89	114
DMA-NO2	96	97	103	114	118
AMP-NO2	73	89	156	175	119

The OECD experiments were also tested for nitramine losses and possible degradation to nitrosamines. The authentic toxicity mixtures were not available for chemical analysis and new solution were prepared at NILU according to procedures from SINTEF and with media nutritients and reagents provided by SINTEF. The nitramine recovery is given in table 5, and no significant losses are observed with time. The deviation from 100 % recoveries is explained by possible glass wall adsorption.

Table D.5 Chemical analysis of nitramines exposed to chemicals used in OECD test. The results are given as per cent recovered nitramine

	24 hours	24 hours	24 hours	48 hours	48 hours	48 hours	21 days	21 days	21 days
	OECD 201	OECD 202	OECD 301	OECD 201	OECD 202	OECD 301	OECD 201	OECD 202	OECD 301
MEA-NO2	99	91	90	94	72	86	92	94	92
MA-NO2	69	53	75	77	60	68	123	106	95
DMA-NO2	88	97	86	99	101	100	96	104	96
AMP-NO2	99	111	125	115	127	109	95	97	98

D.3 Control for degradation

The possible degradation of nitramines to nitrosamines in the media used for toxicity test was checked. Table D.6 shows that no nitrosamines have been detected in the tested samples.

Table D.6 Chemical analysis of nitrosamines in nitramine-spiked toxicity test media

Nitro-samine ng/mL	OECD 201	OECD 202	OECD 301	VITO Top-agar	VITO Fosph-buff	VITO DC medium	NILU PBS	NILU DMEM	NILU S9 mix
NDELA	<20	<20	<20	<20	<20	<20	<20	<20	<20
NPZ	<20	<20	<20	<20	<20	<20	<20	<20	<20
NDMA	<20	<20	<20	<20	<20	<20	<20	<20	<20
NMEA	<15	<15	<15	<15	<15	<15	<15	<15	<15
NDEA	<15	<15	<15	<15	<15	<15	<15	<15	<15
NPIP	<15	<15	<15	<15	<15	<15	<15	<15	<15
NDPA	<15	<15	<15	<15	<15	<15	<15	<15	<15
NDBA	<15	<15	<15	<15	<15	<15	<15	<15	<15

D.4 Considerations of importance for toxicity testing

The results from measured concentration vs nominal values were not available before the experiments were finalized, and therefore all experiments initially used nominal values, which later were recalculated. In this report, only recalculated (corresponding to the analytically measured) values are used. Nominal (initial), as well as correct (analytically measured) values are stated in Table D.1.

For VITO/CARDAM and SMU, analytical measurements were also performed on stock solutions which were returned to NILU. For SMU no changes in concentrations were found. For VITO/CARDAM the concentrations were lower for DMA-NO2, MA-NO2 and MEA-NO2. The most probable reason for the loss was filtration of the stock solutions, which was performed due to visible precipitation of the nitramines. We therefore assume that the measured concentrations after the samples return are the most correct values. However, some uncertainty must be taken into consideration for tests performed at VITO/CARDAM with DMA-NO2, MA-NO2 and MEA-NO2.

For assays which require sterile conditions, the test substances were filtered before use. Analytical measurements showed no loss of test substance after bacterial filtration.

No conversion to nitrosamines was observed, and we may assume that the toxicological effects therefore are due to exposure to the nitramine.

E Chapter E – Acute toxicity, cytotoxicity, irritation, sensitization and corrosion

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E.1 Introduction

This chapter gives an overview of acute oral toxicity and cytotoxicity of the test substances. A comparison of *in vivo* and *in vitro* results is performed. In addition, all experimental results from irritation-, sensitization- and corrosion tests are gathered in this chapter. Hence, this chapter will give the reader a picture of the human effects which can be expected after exposure to eye and skin and after intake.

E.2 Experimental Section

The experimental work has been performed in three different laboratories; Slovak Medical University, SMU (Slovakia), VITO/CARDAM (Belgium) and NILU (Norway). Six different methods have been performed to assess acute oral toxicity *in vivo* and cytotoxicity, sensitization, corrosion and irritation *in vitro*. For each of these assays, 2-5 of the test substances (see Table E.1) have been tested. It will be specified in each subchapter which test substances has been tested, and a total summary of results is given in Chapter 0.This report gives a short description of each assay. For more details regarding the experimental setup and results, please see Annex Study Reports.

Table E.1 List of test substances

Name of test substance	Abbreviation	CAS Numbers
Dimethylnitramine	DMA-NO2	4164-28-7
Methylnitramine	MA-NO2	598-57-2
Ethanolnitramine	MEA-NO2	74386-82-6
2-methyl-2-(nitramino)-1-propanol	AMP-NO2	1239666-60-4
Piperazine nitramine	PZ-NO2	42499-41-2

Important notice: All concentrations used are based on analytically measured values, which give a unique control of the exposure level compared to using nominal values. Some uncertainty must still be considered for tests performed at VITO/CARDAM with

DMA-NO2, MA-NO2 and MEA-NO2. More information regarding concentration analysis is given in Chapter D.

For experiments requiring sterile conditions, bacterial filtration of the stock solution was performed.

E.2.1 Acute oral Toxicity

This study has been performed at SMU, with all five test substances; Dimethylnitramine (DMA-NO2), Methylnitramine (MA-NO2), Ethanolnitramine (MEA-NO2), 2-methyl-2-(nitramino)-1-propanol (AMP-NO2) and Piperazine nitramine (PZ-NO2). The objective was to examine acute oral toxicity and the study was performed in rats according to OECD TG 425 Acute oral toxicity in the rat: Up and down procedure (OECD, 2008) (OECD, 2000a) (OECD, 2000b). Experiments were performed according to GLP.

Method

Limit test

Preceding the main experiment, a limit test was performed. This is a sequential test that uses a maximum of 5 animals per test substance.

One animal is administered a dose of 2000 mg/kg body weight. If the animal dies, the main test is conducted to determine the LD_{50}, which is the median lethal dose estiamted to kill half of the test animals that figures as an indicator of the acute toxicity of a test substance. If the animal survives, four additional animals are dosed sequentially so that a total of five animals are tested. However, if three animals die, the limit test is terminated and the main test is performed. If three of more animals survive, LD_{50} is set to > 2000 mg/kg body weight (bw), and the test substance is considered to be non-toxic.

Main experiment

Based on the results from the limit test, groups of fasted female rats were administered the test substances at dose levels following standard table (Annex Study Reports). Each test substance was given orally by gavage in water solution as a single application. Dosage was performed sequentially.

The rats were inspected twice daily for symptoms of toxicity. Clinical signs and bodyweight was monitored during the study. Each animal was weighed 48 hours after arrival, on the day before treatment, and thereafter weekly and at death. Any deviations from control animals were recorded for each animal in respect of nature and severity, date and time of onset, duration and progress of the observed response. Moribund animals were isolated to prevent cannibalism and observed carefully at least twice daily to be subjected to necropsy as soon as possible after death.

The animals were observed for a period of 14 days after exposure and sacrificed at assigned terms of the observation period. All animals were subjected to gross necropsy,

including those which died during the test or were removed from the study due to animal welfare reasons, and gross pathological changes were recorded for each animal. For animals that survived at least 24 hours after exposure and had gross pathological changes, microscopic examination of the organs were considered.

A commonly used *classification* of substances according to their LD_{50} is:

Classification	LD_{50} orally to rat (mg/kg body weight)
Very toxic	< 25
Toxic	25 – 200
Harmful	200 – 2000
Non toxic	> 2000

Result

All animals (including those which died during the test) were subjected to gross necropsy. Microscopic examination of organs in animals surviving 48 hours or more after the initial dosing was not done. No gross pathological changes were recorded.

Statistics were performed using SAS and BMDP Statistics Software (SAS Institute, 1990) (Dixon et al, 1990). Statistical estimates based on long term outcomes are summarized in Table E.2, while more detailed results regarding the study can be found in Annex Study Reports.

As stated previously in this report, the concentrations were corrected based on analytical measurements of stock solutions after treatment. For AMP-NO2, further testing was terminated after the limit test, as it was originally considered nontoxic. However, due to the corrections in concentration, we now know that the dose used in the limit test was too low to make this assumption (the measured dose for limit test was found to be 1600 and not 2000 mg/kg bw). A detailed LD_{50} value has therefore not been determined for AMP-NO2, and though it is > 1600, it cannot be considered as a nontoxic compound.

Table E.2 LD_{50} values for all test substances determined from OECD TG 425 Oral Toxicity up and down procedure.

Test substance	LD_{50} (mg/kg bw)	95 % CI
DMA-NO2	770	519 - 997
MA-NO2	834	659 - 951
MEA-NO2	970	609 - 1230
AMP-NO2	> 1600	
PZ-NO2	1750	1547 - 1970

Conclusion

According to the results obtained PZ-NO2, DMA-NO2, MA-NO2 and MEA-NO2 are harmful if swallowed and should be labeled accordingly. The order of toxicity (from

highest to lowest) of the test substances is DMA-NO2 > MA-NO2 > MEA-NO2 > PZ-NO2. AMP-NO2 is considered either harmful or non-toxic, but due to recalculation of concentration this cannot be decided within this project.

E.2.2 Cytotoxicity - Plating Efficiency assay

In this study, Dimethylnitramine (DMA-NO2), Methylnitramine (MA-NO2), Ethanolnitramine (MEA-NO2) and 2-methyl-2-(nitramino)-1-propanol (AMP-NO2) were investigated for their cytotoxic potential in a mammalian cell line. The experimental work was performed at NILU. Plating efficiency assay (PE), also called Clonogenic assay, is used to quantify colony formation in mammalian cells, and gives the overall toxicity by measuring survival or cell death. Normally, stable cell lines are used for *in vitro* toxicity testing, either in suspension or growing attached to the surface (Dušinská and Slameňova, 1982) (Mather and Roberts, 1998).

Plating efficiency assay is scientifically accepted as one of the most reliable assays for mammalian cytotoxicity testing (Dušinská and Slameňova, 1982), (Slameňová et.al., 1990), (Slameňová et.al., 1994). The assay is also implemented into OECD TG 476 (Aaron, 1994), (Abbondandolo, 1977), (Li A. C., 1987), (Slameňová et.al., 1990), (Slameňová et.al., 1992) for determination of mutant frequency for determination of both cytotoxicity as well as viability of cultured cells. Though following GLP procedures, the method is not accredited as such.

Method

Plating efficiency assay is based on plating cells in small inoculums in Petri dishes or 6 well-plates. The assay was performed on mammalian cells growing in monolayer attached to a surface. Normally, only a hundred or a few hundred cells are inoculated. Each viable cell will grow and form a colony. After a suitable incubation time (ca. 5-8 days, depending on cell line), colonies are stained and counted manually. PE (viability) is calculated as % of colonies from all seeded cells. As clonogenic activity of V79 cells may vary from experiment to experiment, cytotoxicity is always related to the viability of control culture. Cytotoxicity is determined by measuring the relative cloning efficiency (number of cell colonies) after the treatment with compound compared with cloning efficiency (number of colonies) of control cells. Small colonies indicate reduced cell viability.

Mammalian V79 cells (from hamster) in monolayer culture were exposed to three concentrations of each of the test substances dissolved in PBS, in addition to negative and positive controls, for 3 hrs. As positive control methyl methane sulphonate (MMS) (0.3 mM), an alkylating agent, was used. Concentration range of test substances was established with regards to expected cytotoxicity (pilot experiments), solubility in the test system and changes in pH or osmolarity.

After the treatment, cells were trypsinized to dissociate the cells from the surface of petri dish and from each other, diluted into a suspension of 1000 cells/ml, plated in

small inoculums of 100 cells per dish and cultivated in culture medium for 5-6 days. By this time, each viable cell had grown and created a colony. The survival was determined based on number of colonies versus number of inoculated cells. One experiment and one repeat were performed for each test substance. In each experiment there were 6 parallel dishes per concentration.

Plating efficiency (PE) (viability) is expressed as number of colonies (in %) from all seeded cells following formula:

PE (%) = (Colonies Counted / Cells Inoculated) x 100

Cytotoxicity is determined by expressing the PE of treated cells relatively to PE of control cells, where PE of control cells is set to 100%. A test substance is classified as cytotoxic if the cell viability is reduced by at least 20% compared to control.

Results

The clonogenic activity of control culture in two independent experiments varied but overall hamster V79 cells exhibited high viability with PE over 90%. Treatment of cells with the alkylating agent MMS, as a positive control, resulted in PE at about 17 %, indicating strong cytotoxicity and confirming that the cells are responding to the toxic compound. Both experiments gave conclusive results. All four test substances showed mild cytotoxicity (60-75% PE) in concentrations range up to around 500 µg/ml. DMA-NO2 and AMP-NO2 appeared to be the least toxic as about 60% of the cells were able to create colonies after treatment with the highest concentration at 5456 µg/ml or 3840µg/ml, respectively. MA-NO2 and MEA-NO2 caused strong cytotoxic effect at the highest concentrations. Exposure of cells with the 3769.6 µg/ml of MEA-NO2 reduced clonogenic ability to 25% whereas exposure with 5456 mg/ml of MA-NO2 fully inhibited plating of cells (Table E.3, Figure E.1, Figure E.2). Cytotoxicity is already considered below PE 80% of control, The cytotoxicity between 60%-40% relates to strong cytotoxicity. When cytotoxicity is below 40%,compound is extremely toxic. In the case of MEA-NO2, results in two independent experiments show viability 47% in first and 2.7% in the second experiment, respectively. This discrepancy is most likely due to the narrow margine of effect of MEA-NO2, but in both experiments the response is clearly cytotoxc and we can conclude that overall response is strong.

Table E.3: **Plating efficiency of V79 cells after 3hrs exposure to DMA-NO2, MA-NO2, MEA-NO2 and AMP-NO2. Two independent experiments performed in different time are included (I and II) and the average is calculated. Each experiment represents mean values of 6 independent dishes (parallels) with SEM (standard errors of the mean). Cytotoxicity is expressed as Plating efficiency (PE) in % of exposed cells compared to control (unexposed) cells (set to 100 %). The differences in concentrations between test substances are due to solubility (DMA-NO2) and minor corrections of concentrations after analytical measurement of stock solutions.**

DMA-NO2	I	PE (%)	SEM	PE to 100 % Control	II	PE (%)	SEM	PE to 100 % Control		Average PE (%)
control		93.83	3.79	100.00		110.00	3.18	100.00		100.00
50 µg/ml		80.33	4.31	85.61		55.33	2.96	50.30		67.96
500 µg/ml		67.67	2.64	72.11		66.33	4.80	60.30		66.21
3840 µg/ml		43.50	3.00	46.36		75.50	2.83	68.64		57.50
MMS 0.1mM		20.17	1.01	21.49		12.83	1.45	11.67		16.58

MA-NO2	I	PE (%)	SEM	PE to 100 % Control	II	PE (%)	SEM	PE to 100 % Control		Average PE (%)
Control		93.83	3.79	100.00		110.00	3.18	100.00		100.00
55.06 µg/ml		79.50	1.96	84.72		71.17	2.65	64.70		74.71
550.6 µg/ml		71.33	3.15	76.02		80.83	4.44	73.48		74.75
5506 µg/ml		0.00	0.00	0.00		1.00	0.26	0.91		0.45
MMS 0.1mM		20.17	1.01	21.49		12.83	1.45	11.67		16.58

MEA-NO2	I	PE (%)	SEM	PE to 100 % Control	II	PE (%)	SEM	PE to 100 % Control		Average PE (%)
Control		93.83	3.79	100.00		110.00	3.18	100.00		100.00
37.7 µg/ml		72.00	2.14	76.73		60.00	3.15	54.55		65.64
377 µg/ml		81.83	0.75	87.21		44.83	3.05	40.76		63.98
3770 µg/ml		45.00	2.56	47.96		3.00	0.77	2.73		25.34
MMS 0.1mM		20.17	1.01	21.49		12.83	1.45	11.67		16.58

Table D.3 (Continued)

AMP-NO2	I	PE (%)	SEM	PE to 100 % Control	II	PE (%)	SEM	PE to 100 % Control		Average PE (%)
control		93.83	3.79	100.00		110.00	3.18	100.00		100.00
54.56 µg/ml		66.00	2.73	70.34		65.00	3.54	59.09		64.71
545.6 µg/ml		57.83	3.62	61.63		71.17	2.77	64.70		63.17
5456 µg/ml		79.00	2.68	84.19		51.33	3.59	46.67		65.43
MMS 0.1mM		20.17	1.01	21.49		12.83	1.45	11.67		16.58

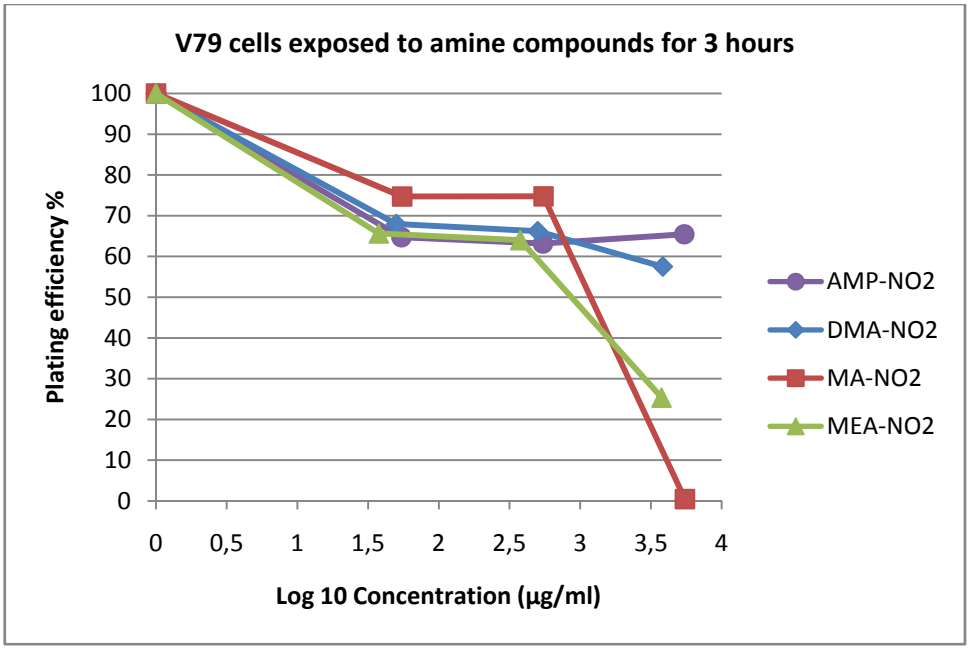


Figure E.1: **Plating efficiency of V79 cells after 3hrs exposure to DMA-NO2, MA-NO2, MEA-NO2 and AMP-NO2 compared to control (100%). Average from 2 experiments, each in 6 parallels.**

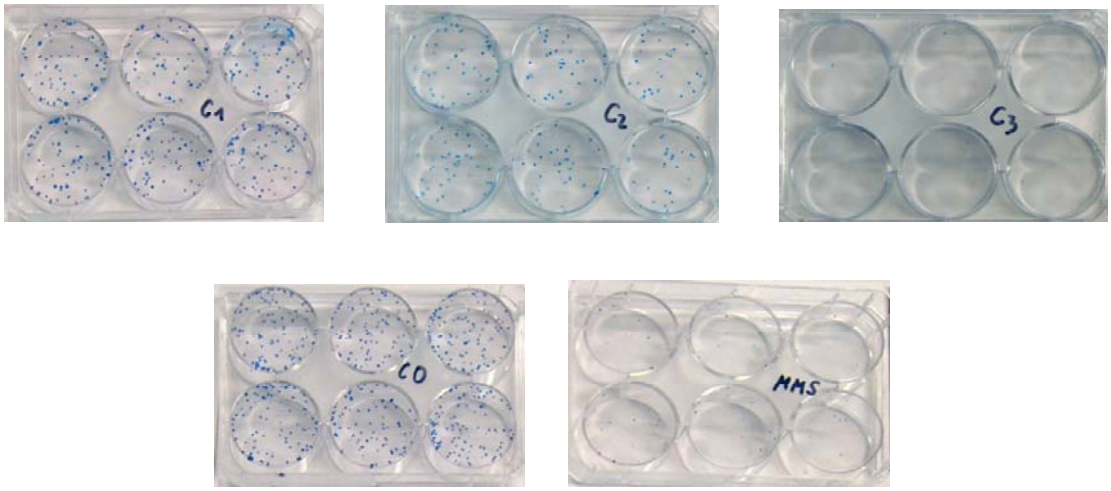


Figure E.2: **Plating efficiency of V79 cells with compound C (MEA-NO2, three different concentrations C1, C2 and C3), negative control (C0) or positive control (MMS)**

Conclusion

All tested compounds show low toxicity up to 500 µg/ml. AMP-NO2 and DMA-NO2 induce low cytotoxic effect for all concentrations tested whereas MA-NO2 and MEA-NO2 show dose dependent cytotoxicity, with high toxicity at the highest concentrations tested.

E.2.3 Skin irritation – Reconstructed Human Epidermis (RHE) Assay

The objective of this study was to predict and classify the skin irritating potential of Dimethylnitramine (DMA-NO2), Methylnitramine (MA-NO2), Ethanolnitramine (MEA-NO2) and 2-methyl-2-(nitramino)-1-propanol (AMP-NO2) solutions on a reconstructed human epidermis model (RHE) of SkinEthic Laboratories. The SkinEthic Laboratories RHE assay is one of the three validated methods in OECD guideline No. 439 for *In Vitro* Skin Irriation: Reconstructed human Epidermis (RhE) Test Methods (OECD, 2010). The work was performed at CARDAM and the assay is GLP compliant.

Method

Skin irritation is a local, reversible inflammatory response on normal living skin to direct injury caused by the application of an irritating compound. The principle of the RHE model test is based on the premise that irritant chemicals are able to penetrate the stratum corneum by diffusion and are cytotoxic to the cells in the underlying layers. Cell viability is measured by dehydrogenase conversion of the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; European Inventory of Existing Commercial Substances number 206-069-5, CAS number 298-93-1)], into a blue formazan salt that is quantitatively measured after extraction from tissues (Mosmann, 1983); (INVITTOX, 1990). Irritant test items are identified by their ability to decrease cell viability below defined threshold levels.

The test substances were applied as provided (dissolved in water) topically to a 3-dimensional RHE model. Concentrations are listed in Table E.4. The RHE model is comprised of normal, human-derived epidermal keratinocytes, which have been cultured for 17 days on an inert 0.5 cm^2 polycarbonate filter at the air-liquid interface, to form a multilayered, highly differentiated model of the human epidermis (Rosdy et.al., 1990) (Rosdy et.al., 1993). On day 17, the cultures consist of organized basal, spinous and granular layers, and a multilayered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found in *in vivo* human tissues (Fartasch and Ponec, 1994) (Kandárová H, 2006). The RHE model presents a histological morphology comparable to the *in vivo* human tissue (Doucet O, 1998). On culture day 19, the test substance is applied topically to the epidermis cells and cell viability is measured.

Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (DPBS) was used as negative control. As positive control sodium dodecyl sulphate (SDS) 5 % (w/v aqueous solution) was used.

Table E.4: Concentrations for each test substance applied to RHE test

Test item	Conc. Tested (% w/v)
Dimethyl-nitramine	7.7
Methyl-nitramine	19.5
MEA-NO2	19.5
AMP-NO2	19,2

The test substance is considered to be a skin irritant or non-irritant if:

Criteria for *in vitro* interpretation	**Classification**	
	EU label	***UN GHS* in EU: CLP label***
Mean tissue viability is ≤ 50 % viability of the DPBS treated negative control	R38, Irritant	Cat. 2
Mean tissue viability is > 50 % viability of the DPBS treated negative control	No label, Non-irritant	No Cat.

* United Nations Globally Harmonized System of Classification and Labelling of Chemicals: category 2 = irritants

(ECVAM, Performance Standards for Applying Human Skin Models to In Vitro Skin Irritation Testing, 2007) (ECVAM, 2009) (Tornier C, 2010) (OECD, 2010)).

Results

Figure E.3 shows the mean tissue viability after treatment with DMA-NO2, MA-NO2, MEA-NO2 and AMP-NO2. The viability is > 80 % for all test substances, which is far from the threshold level, indicating that all test substances are non-irritants.

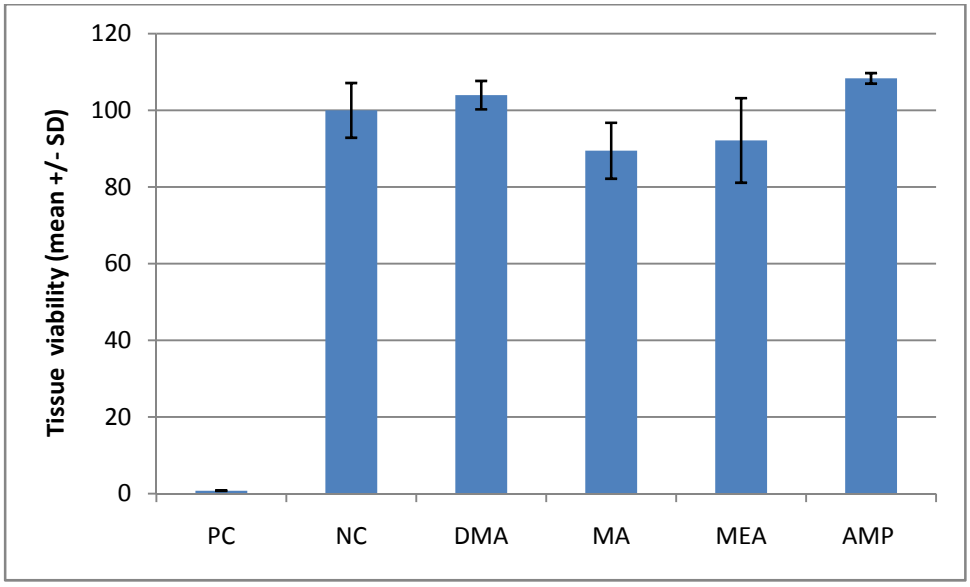


Figure E.3: **Tissue viability of epidermis cell after topical treatment with all four test substances. PC: positive control (SDS), NC: negative control (DPBS), DMA-NO2: dimethylnitramine, MA-NO2: methylnitramine, MEA-NO2: ethanolnitramine, AMP-NO2: 2-methyl-2-(nitramino)-1-propanol**

Conclusion

According to OECD TG 439, all tested substances can be labelled non-irritants for skin.

E.2.4 Skin corrosion: Corrositex

The objective of this study was to evaluate the corrosive potential of Dimethylnitramine (DMA-NO2), Methylnitramine (MA-NO2), Ethanolnitramine (MEA-NO2) and 2-methyl-2-(nitramino)-1-propanol (AMP-NO2). The assay was performed at CARDAM, and is compliant with GLP.

This Corrositex test is an *in vitro* toxicology test, an acellular (no living tissues involved) barrier model (Barratt, 1998) (Stobbe, 2003). The kit was supplied by InVitro International (www.invitrointl.com) and distributed by Res Pharma (www.respharma.com) for Europe. The test method utilizes a synthetic membrane (Biobarrier) designed to respond to corrosive substances in a manner similar to animal skin *in situ*. This test replaces the rabbit test of dermal corrosivity by providing a reliable means of mimicking this test.

The test is based on OECD guideline 435: In Vitro Membrane Barrier Test Method for Skin Corrosion (OECD, 2006a). It is considered scientifically valid by ECVAM (Barratt M.D., 1998), (Fentem J.H., 1998), (ECVAM, 2000) (INVITTOX, 2008), (ICCVAM, 1999) and (ICCVAM, 2003).

Method

The Corrositex testing system consists of a glass vial filled with a chemical detection fluid capped by a proprietary Biobarrier membrane, which is designed to mimic the

effect of corrosives on living skin. The Biobarrier consists of a hydrated collagen matrix and supporting filter membrane. The Biobarrier covers a compartment filled with a Chemical Detection System (CDS= 2 pH dye indicator; one for acids, one for bases).

As soon as the corrosive test substance destroys this Biobarrier, the fluid in the compartment below changes colour or texture. The time it takes for the test item to break through the Biobarrier – seen as a color change in the pH sensitive compartment- is a measure for the degree of corrosiveness of the test substance. The recorded Corrositex time is governed by three factors:

- The strength of the acid or base capacity of the test substance
- the rate of diffusion of the test substance
- For very corrosive substances, the rate of destruction of the Biobarrier

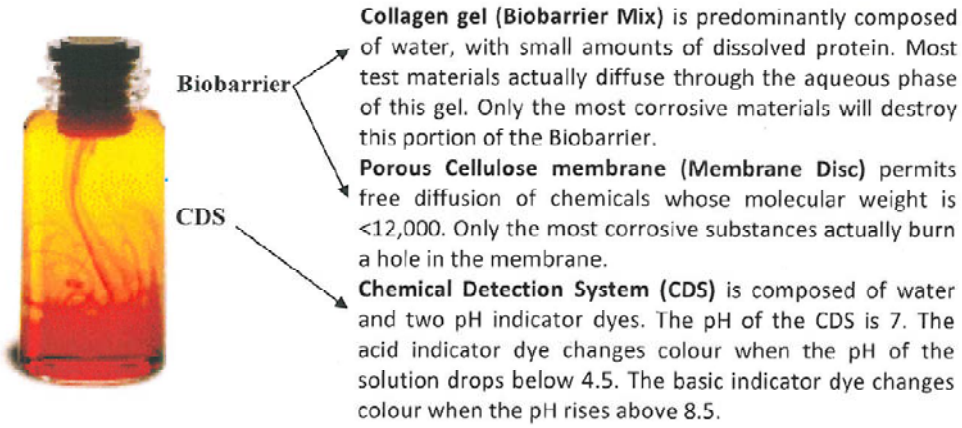


Figure E.4: **Illustration of the Corrositex kit, with a Biobarrier that covers a compartment filled with a Chemical Detection System. The time it takes for the test item to break through the barrier (seen as a color change in the pH sensitive compartment) is a measure for the corrosiveness of the test substance.**

The test is divided into three steps. The test substances were applied as dissolved in water.

Step 1: Qualify = the test substance Compatibility Test

This step ensured that the test substance was compatible with the Corrositex system prior to running step 3. The test substance solution was added to the quality test tube and if a change in color or consistency in CDS was observed the test was continued to step 2. If the CDS did not detect the solution, this Corrositex system was considered not suitable for evaluating the potential corrosivity of the test substance.

Step 2: Categorize = the test item Categorization Test

The test substance was subjected to a categorization test, i.e. a screening test to distinguish between either category 1 (high acidity/alkalinity) or category 2 (low acidity/alkalinity). Two different breakthrough timescales were used for determining corrosivity, based on the acid or alkali reserve of the test substance solution.

Step 3: Classify = the test substance and control items Classifying Test

This step determines for all test substance solutions and control items the appropriate U.N. Packing Group, and is a ranking tool for corrosivity. The number of replicates for was four.

The Biobarrier was positioned in a vial containing the CDS. The test substances or control items were evenly applied onto the top of the Biobarrier and the timer was instantly started. The vials were observed for 3 minutes and the time of any colour change was noted. If no colour change had occurred, the vials were observed at regular intervals until a change did occur, to a maximum of 4 hours for category 1 and 1 hour for category 2 test substances. The time of the color change was recorded.

This Classifying step was repeated for the remaining vials, staggering each start time by a minimum of one minute. The start time difference for each vial was subtracted from the final time to determine the net response time.

The test substance is considered to be skin corrosive or non-corrosive in the study if:

	CORROSITEX Time (min)			
Category 1	0 to 3 min	>3 to 60 min	>60 to 240 min	>240 min
Category 2	0 to 3 min	>3 to 30 min	>30 to 60 min	>60min
	⇩	⇩	⇩	⇩
	Packing group I	Packing group II	Packing group III	No Classification
	Strong corrosive	corrosive	weak corrosive	non corrosive

Results

All experiments were performed according to plan except from DMA-NO2, which was not compliable with the Corrositex system due to its pH. The pH of DMA-NO2 was 6.8 and aqueous test items with pH in the range of 4.5 to 8.5 often fail to provoke a colour change. An overview of Corrositex Breakthrough (CB) time for all nitramines can be found in Table E.5.. For all three nitramines which were able to determine CB (MA-NO2, AMP-NO2, MEA-NO2), no corrosivity was found, as they all had mean CB of > 60 min.

Conclusion

MA-NO2, AMP-NO2 and MEA-NO2 are categorized in category 2 and can be assigned to Packing Group No classification = non-corrosives. DMA-NO2 was not compliable with the Corrositex system, and no classification is retrieved from this test.

Table E.5: **Corrositex Breakthrough time. MCB: Mean Corrositex Breakthrough, SD: Standard Deviation, corr: corrosive, Positive control: Sulfuric acid (95-97%), Negative control: Propionic acid (6%), Q = Qualified, NQ = Not Qualified**

Test item	Conc. Tested (% w/v)	Qualified (Q or NQ)	Category (1 or 2)	MCB time ± SD (min)	Pos control MCB time ± SD (min)	Neg control MCB time ± SD (min)	Corrosive potential
Dimethyl-nitramine	7.7	NQ	-	-	-	-	-
Methyl-nitramine	19.5	Q	2	> 60	0.90	61.48	Non-corr
MEA-NO2	19.5	Q	2	> 60	1.10	> 60	Non-corr
AMP-NO2	19.2	Q	2	> 60	1,05	61	Non-corr

E.2.5 Eye corrosion: Bovine Corneal Opacity and Permeability (BCOP) test

The objective of this study was to evaluate the potential ocular irritancy of Dimethylnitramine (DMA-NO2), Methylnitramine (MA-NO2), Ethanolnitramine (MEA-NO2) and 2-methyl-2-(nitramino)-1-propanol (AMP-NO2) solutions as measured by its ability to induce opacity and increase permeability in isolated calf corneas. This study provides a rational basis for risk assessment in man as ocular contact is one of the probable routes of human exposure. The experimental work was performed at CARDAM, and is compliant with GLP.

As an *in vitro* (*ex vivo*) alternative to the *in vivo* Draize eye irritation test (Draize JH, 1944), the Bovine Corneal Opacity and Permeability (BCOP) assay using isolated bovine/calf corneas was developed (Gautheron P, 1992) (Vanparys P, 1993). The test is described in OECD TG 437 (OECD, 2008), and the detection of ocular corrosives and severe irritants are defined by the United States Environmental Protection Agency (EPA, 1996), the European Union (EU, 2001) and in the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN, 2003).

Method

The potential ocular irritancy is measured by decreased light transmission through the cornea (opacity) and increased passage of sodium fluorescein dye through the cornea (permeability). The opacity and permeability assessments of the cornea following exposure to test substance solutions (Table E.6) was considered individually, and also combined to derive an *In Vitro* Irritation Score (IVIS), which was used to classify the irritancy level of the test substance.

Retrieving and handling of cornea

A slaughterhouse veterinarian excised the calf eyes as soon as possible after slaughter, and the eyes were used within 3 hours after slaughtering. Before dissection, all eyes were carefully examined, and those presenting defects, such as neovascularization, pigmentation or scratches were discarded. During dissection great care was taken to avoid damage of corneal surfaces. Corneas were mounted in vertical position in holders,

and Eagle Minimal Essential Medium solution (EMEM) was added to two compartments (posterior and anterior of the cornea). Background opacity was measured with an opacitometer (T0).

Treatment of corneas and opacity measurements

Three corneas were treated with each test substance solution. In addition, three corneas were treated with the concurrent positive control and three corneas were treated with the concurrent vehicle/solvent control.

The test substances or controls were applied as a solution in water with concentrations for each test substance given in Table E.7 (values reflect analytical measured concentrations) and incubated in horizontal position (anterior side upwards) for 240 ± 10 minutes. After incubation, the EMEM solution of both compartments was replaced, and the corneal opacity was measured (T240). Special attention was taken to observe dissimilar opacity patterns, tissue peeling, or residual test substance.

Permeability determinations

Permeability was measured quantitatively as the amount of sodium fluorescein dye that passes across the full thickness of the cornea, as detected in the medium in the posterior chamber. Permeability determination was performed immediately after the final measurement of opacity. The medium was removed from the anterior compartment and replaced by 1 mL of a 0.5 % sodium fluorescein solution. Corneas were incubated in a horizontal position for 90 ± 5 minutes. After incubation, medium from the posterior chamber was removed and its optical density (OD) determined with a UV/VIS spectrophotometer at 490 nm.

Data recording

Results from the two test method endpoints, opacity and permeability, were combined in an empirically derived formula that generates an IVIS for MA-NO2 solution.

Opacity measurement

The change of opacity value of each treated cornea or positive or vehicle/solvent control corneas were calculated by subtracting the initial basal opacity (T0) from the post-treatment opacity reading (T240), for each individual cornea. The average change in opacity of the vehicle/solvent control corneas was calculated and this value subtracted from the change in opacity of each treated cornea or positive control to obtain a corrected opacity. The mean corrected opacity value of each treatment group was then calculated from the individual corrected opacity values of the treated corneas for each treatment condition.

Permeability determination

The corrected OD490 value of each treated cornea or positive control corneas was calculated by subtracting the average vehicle control cornea value from the original

permeability value for each cornea. The mean corrected permeability values of each treatment group were calculated from the individual corrected permeability values of the treated corneas for each treatment condition.

In vitro irritancy score

The following formula was used to determine the *in vitro* score:

IVIS = mean opacity value + (15 x mean OD490 value)

Depending on the score obtained, the test substance will be classified into one of the following categories (Vanparys P, 1993):

IVIS:

3.0 = non eye irritant
3.1 to 25.0 = mild eye irritant
25.1 to 55.0 = moderate eye irritant
55.1 to 80.0 = severe eye irritant
≥ 80.1 = very severe eye irritant

Results

For each of the test substances and each exposure condition, three corneas were visually inspected and showed no abnormalities.

For each of the test substances, three corneas were selected and treated for 240 minutes. Table E.6 summarizes the opacity score, permeability and IVIS for all test substances, positive and negative control. According to OECD TG 437, DMA-NO2 is considered as a mild eye irritant though it is not OECD classified as ocular corrosive or severe irritant. MA-NO2, MEA-NO2 and AMP-NO2 are classified as very severe eye irritants, and are all OECD classified as ocular corrosives or severe irritants.

Table E.6: **Summary results table BSOP tests. All data recorded in mean ± standard deviation. IVIS: In vitro irritancy score, PC: Positive Control (imidazole, 20% solution), NC: Negative Control (sterile deionized water 100% pure), DMA-NO2: Dimethylnitramine, MA-NO2: Methylnitramine, MEA-NO2: Ethanolnitramine, AMP-NO2: 2-methyl-2-(nitramino)-1-propanol**

	PC	NC	DMA-NO2	MA-NO2	MEA-NO2	AMP-NO2
Opacity score	48.7 ± 2.7	2.6 ± 1.1	4.2 ± 3.2	187.7 ± 5.1	84.9 ± 3.1	109.9 ± 18.6
Perme-ability	3.060 ± 1.079	0,036 ± 0.051	0.064 ± 0.095	0.018 ± 0.052	0.031 ± 0.056	0.145 ± 0.051
IVIS	94.6 ± 18.8	3.1 ± 1.8	5.2 ± 4.6	187.9 ± 4.4	85.4 ± 3.2	112.1 ±17.9

Table E.7: Classification of test substances

Test substance	Concentration (% w/V)	In vitro score	IVIS category	OECD classified as ocular corrosive or severe irritant
DMA-NO2	7.7	5.2	Mild eye irritant	No
MA-NO2	19.5	187.9	Very severe eye irritant	Yes
MEA-NO2	19.5	85.4	Very severe eye irritant	Yes
AMP-NO2	19.2	112.1	Very severe eye irritant	Yes

Conclusion

In conclusion, all test substances showed irritation to the eye. MA-NO2, MEA-NO2 and AMP-NO2 are all classified as ocular corrosive or severe irritants (Table E.7).

E.2.6 Skin sensitization: VITOSENS

The objective of the study is to evaluate the potential sensitization capacity of Dimethylnitramine (DMA-NO2), Methylnitramine (MA-NO2), Ethanolnitramine (MEA-NO2) and 2-methyl-2-(nitramino)-1-propanol (AMP-NO2) solutions as measured by gene expression in human dendritic cells. This study indicates if the test substances should be classified as sensitizing or non-sensitizing chemicals. The experimental work was performed at CARDAM.

The OECD guidelines (406, 429) originally use guinea pig tests to assess skin sensitization. Later on the local lymph node assay in mouse was accepted as a new assay that has the potential to reduce the number of animals required (Gerberick GF., 2007). The purpose of the VITOSENS assay is the replacement of existing *in vivo* regulatory toxicological tests for skin sensitization. The VITOSENS assay as conducted in this study has been designed as a dichotomous classifier. VITOSENS classifies a test substance as sensitizing or non-sensitizing (Hooyberghs J., 2008).

Method

VITOSENS is based on cell culture of human CD34+ progenitor-derived dendritic cells (CD34-DC) from cord blood. In the immunological cascade these antigen-presenting cells play an essential role in the sensitization phase. In the *in vitro* VITOSENS assay the response of CD34-DC to chemical exposure is assessed on the level of gene expression. VITOSENS is based on real time quantitative polymerase chain reaction (RT-qPCR) that measures the fold change in expression for a set of genes. This fold change is induced in the exposed sample *versus* its solvent control sample. At present the expression of the genes CCR2 and CREM is used after exposing CD34-DC for 6h to a chemical concentration that yields around 20% cell damage (IC_{20}). These fold changes are combined by a weighted average into a predictor variable that should be positive for

sensitizing compounds and negative for non-sensitizers. The experiments were repeated on 2 cell cultures, each from a different cord blood donor.

Preceding the main study, the IC_{20} was assessed by propidium iodide (PI) staining using flow cytometry. Cytotoxicity of each test substance was assessed on 2 different CD34-DC cultures from different cord blood donors, first to perform a range finding test, and a second for a final dose range of the test substance. Sterile water was used as solvent.

Results

Cytotoxicity
After exposure of CD34-DC, an IC_{20} (concentration which gives 20% reduction of cell viability) was determined using GraphPad Prism software. Table E.8 gives a summary of the IC_{20} values per test substance. Test item solutions DMA-NO2 solution and AMP-NO2 showed an extremely high IC20 based on the calculated concentration values. Therefore these test items were not assessed in the predictive second step of the assay. Note here, that due to the filtration that was performed on the test item solutions prior to the start of the experiments, the concentration was lowered. During the experimental phase of the study we did not have access to the correct numerical values of these post-filtration concentrations. Therefore the decision to exclude the 2 compounds was based on the assumption that filtration had no effect on concentration; with hindsight this exclusion was not necessary.

Table E.8: **IC_{20} values of test substances based on PI staining using flow cytometry.**

Test substance	IC_{20} (mg/ml)
DMA-NO2	1.39
MA-NO2	0.33
MEA-NO2	0.39
AMP-NO2	2.25

Gene expression
Gene expression changes were analyzed after exposure of CD34-DC to the final IC_{20} value of test items MA-NO2 and MEA-NO2. Gene expression data are presented as the fold change of the expression induced by exposure *versus* their respective solvent Table E.9).

Table E.9: **Gene expression changes in methylnitramine (MA-NO2) and ethanolnitramine (MEA-NO2), presented as the fold change of the expression induced by exposure versus their respective solvent**

Test substance	Gene	Cell culture 1	Cell culture 2
MA-NO2	CCR2	1.17	1.12
	CREM	0.92	0.86
MEA-NO2	CCR2	1.25	0.89
	CREM	0.93	1.06

Predictor variable
The test substance is considered to be sensitizing if the predictor variable is positive (>0) in each biological donor experiment. The test item is considered to be non-sensitizing if the predictor variable is negative (<0) in each biological donor experiment. If the predictor variables are not consistent, the majority rule is applied. Table E.10 shows predictor variables and prediction for MA-NO2, MEA-NO2 and positive control (PC), indicating that both test substances can be considered non-sensitizing.

Table E.10: Predictor variables and prediction: + = sensitizer, - = non-sensitizer. PC= positive control = DNFB (2 µg/ml)

Test substance	Predictor variable		Test substance prediction
	Experiment 1	Experiment 2	
MA-NO2	**-1.45**	**-1.50**	**-**
MEA-NO2	**-1.42**	**-1.06**	**-**
PC	**1.24**	**1.50**	**+**

Conclusion

MA-NO2 and MEA-NO2 are predicted to be non-sensitizing by the VITOSENS model. For test compounds DMA-NO2 and AMP-NO2, no prediction was made since we at the time of experiment believed that these compounds induced 20% cell death at extremely high concentrations.

E.3 Discussion

According to common classification, a chemical is considered as very toxic by swallowing if LD_{50} in rats is below 25 mg/kg bw of oral dose, toxic if LD_{50} is between 25-200mg/kg bw and harmful if LD_{50} is between 200-2000mg/kg bw.

In this study, all 5 test substances were tested at the same test facility with exactly the same procedure. The literature review (Chapter 0) shows that only DMA-NO2 has been tested for oral toxicity in rat prior to this experiment, giving LD_{50} value of 1095 mg/kg bw. Other exposure routes gave LD_{50}s (mg/kg bw): 399 (mice, intraperitoneal i.p.); 600 (rat, intravenous); 897 (rat, i.p.) (Andersen & Jenkins, 1978). These results are in agreement with the LD_{50} value we obtained for oral administration of DMA-NO2 (770mg/kg bw). For MA-NO2, one previous study has been performed previously in 1967 on mice with intraperitoneal exposure, giving an LD_{50} value of 500 mg/kg bw. Our results on rats show LD_{50} at 970 mg/kg bw after oral administration. It should be kept in mind that different administration routes can influence upon toxicity as well as metabolism. Generally, systemic administration induces toxicity at lower doses compared to oral administration, where the test substance first has to be taken up into the blood by uptake from the gut, where some metabolism also could occur.

According to OECD TG 425 we conclude that PZ-NO2, DMA-NO2, MA-NO2 and MEA-NO2 belong to the category "Harmful if swallowed", and should be labeled accordingly. DMA-NO2 was found to be most toxic, followed by MA-NO2, MEA-NO2 and the slightly toxic PZ-NO2. Unfortunately, no conclusion could be made for AMP-NO2 due to recalculation of the concentration. However, it can be considered either as a non-toxic compound or "Harmful if swallowed" by oral exposure.

Plating efficiency assay showed that all test substances had very low toxicity up to 500 µg/ml. AMP-NO2 and DMA-NO2 was only slightly toxic even at the highest tested concentration (5450 and 3840 µg/ml respectively), whereas MA-NO2 and MEA-NO2 were highly cytotoxic at the highest concentrations tested and inhibited viability and cell proliferation. Thus plating efficiency showed a dose-response relationship.

Compared with cytotoxicity results obtained from two other assays performed within this project, the same trends are visible. Similar to PE, Propium Iodide (PI) -stain (VITOSENS) indicate a stronger cytotoxic effect in MA-NO2 and MEA-NO2 compared to the other test substances. Also results from Growth Activity (Comet Assay, Chapter E) show cytotoxic response when exposed to MA-NO2. Cells exposed to DMA-NO2 and AMP-NO2 showed some cytotoxicity at highest concentration in plating efficiency, and this corresponds well with what is measured by PI-stain. For growth activity, no cytotoxicity was observed for DMA-NO2 or MEA-NO2, though it must be taken into consideration that the highest concentrations tested were 1920 mg/ml and 1880 µg/ml respectively

Table E.11: Comparison of cytotoxicity from plating efficiency, growth activity and PI-stain

Test substance	PlatingEfficiency (V79 cells)		Growth activity (TK6 cells)		PI-stain (CD34-dendritic cells)
	Conc. (µg/ml)	% viability	Conc. (µg/ml)	% viability	IC20 (µg/ml)
DMA-NO2	3840	57.50	1920	>100	1390
MA-NO2	5500	0.45	2770	50	330
MEA-NO2	3770	25.34	1900	95	310
AMP-NO2	5450	66.43	-	-	2250

No previous publications have been found for irritation/corrosion/sensitization. The outcome of this project is therefore important results for risk assessment and labeling of the test substances. MA-NO2 and MEA-NO2 were found to be non-sensitizers to skin and none of the test substances were found to be irritants or corrosive for skin. DMA-NO2 was found to be a mild eye irritant. However MA-NO2, MEA-NO2 and AMP-NO2 were found to be very severe eye irritants and should be labeled accordingly. One reason for the mild level of irritancy of DMA-NO2 could be due to the low

concentration applied (7.7 %), compared with the others (~19 %). The difference in concentrations was due to low solubility of DMA-NO2.

Thus for handling of all test substances, eye protection is important to avoid serious eye damage in case of contact with the compounds. This is particularly important for MA-NO2, MEA-NO2 and AMP-NO2, which all have corrosive potential in the eye. All test compounds do not pose a risk for irritation or sensitization of the skin, however it should be kept in mind that dermal uptake and acute toxicity by dermal contact has not been tested so far.

F Chapter F Genotoxicity

Lise M. B. Fjellsbø, Zuzana Magdolenova, Solveig Ravnum, Elise Rundén Pran, Mária Dušinská

F.1 Introduction

This chapter provides an overview of *in vitro* testing for genotoxicity of the test substances. Two OECD validated methods, the Ames test (OECD 471) and Mammalian Gene Mutation assay (OECD 476) have been used. Additionally, the Comet assay, also known as the Single Cell Gel Electrophoresis assay, was used for measurement of DNA damage. This method is widely used as a screening test for potential genotoxicity and is presently under validation by ECVAM/JaCVAM. All three tests are sensitive to mutagenic substances. The potential genotoxicity and mutagenicity is detected by different endpoints and the tests are performed in different biological models.

Ames test is performed in bacteria, and is recommended as first screening test for mutagenicity, although extrapolation to humans has some limitations. Five strains of bacteria *Salmonella typhimurium* have been used for investigating of induction of point mutations by the test substances, either base substitutions or frameshift mutations (deletion or insertion of one or a few bases).

The Comet assay is used as a first screening to detect DNA damage. Normally, only DNA strand breaks are measured, but in this study we also detected specific DNA lesions such as oxidized purines (especially 8-oxoGuanine). If damaged DNA is not repaired, it could lead to mutations and therefore the assay is considered to be reliable for testing of potential genotoxicity. Only a small amount of human or mammalian cells are needed, thus the assay is used in many applications *in vitro*, *in vivo* as well as on humans (usually blood cells).

The Mammalian Gene mutation assay was performed for detection of mutation in the HPRT gene. This assay is used in regulatory toxicology both *in vitro* and *in vivo* for mutagenicity testing of substances.

Depending on the mechanism of action mutagenic compounds can induce different DNA lesions such as DNA breaks, oxidised bases, DNA alkylation, bulky DNA adducts, large DNA rearrangements, etc. Consequently, many mutagenic events can happen resulting in point gene mutations, deletions, large chromosomal abnormalities, clastogenic or aneugenic effects. The three tests described here are designed to detect several endpoints (mutations in different genes, DNA breaks, oxidised DNA lesions) and are performed on different biological systems (human, hamster and bacteria). As there might be different mechanisms involved in mutagenesis of chemicals they do not necessary appear positive in all tests. If positive response is found in at least two from 3 tests, it shows clear mutagenicity. However, even if only one test (especially at the

mammalian system) shows positive response, the compound can be considered as harmful and potentially mutagenic.

F.2 Experimental Section

The experimental work has been performed in two different laboratories; VITO/CARDAM (Belgium) and NILU (Norway). Three different methods have been performed to assess genotoxicity of tested substances *in vitro*. For each of these assays, 2-5 of the test substances (see Table F.1) have been tested. The experimental approach, methodology and results are described in each subchapter, and a total summary is given in Chapter 0.

This report gives a short description of each assay. For more details regarding the experimental setup and all results, please see Annex: Study Report.

Metabolic activation system
Bacteria as well as human and mammalian cells in culture do not contain the enzyme systems which in mammals are known to metabolize pro-mutagens into mutagenic metabolites. To overcome this, an exogenous metabolic activation system, a rat liver post-mitochondrial fraction (S9) was included to each test system. Bacteria or human and mammalian cells were treated with test substances in the presence and absence of S9 mix.

Table F.1: List of test substances and assays for testing

Name of test substance	Abbreviation	CAS Numbers	Test method
Dimethylnitramine	DMA-NO2	4164-28-7	Ames test Comet assay HPRTgene mutations
Methylnitramine	MA-NO2	598-57-2	Ames test Comet assay
Ethanolnitramine	MEA-NO2	74386-82-6	Ames test Comet assay HPRT gene mutations
2-methyl-2-(nitramino)-1-propanol	AMP-NO2	1239666-60-4	Ames test
Piperazine nitramine	PZ-NO2	42499-41-2	Ames test

Important notice: All concentrations used are based on analytically measured values, which give a unique control of the exposure level compared to using nominal values. Some uncertainty must still be considered for tests performed at VITO/CARDAM with DMA-NO2, MA-NO2 and MEA-NO2. More information regarding concentration analysis is given in Chapter D.

For assays which require sterile conditions, the test substances were filtered before use.

F.2.1 Bacterial Reverse Mutation Test (Ames test)

The study followed the procedures indicated by the international accepted guidelines and recommendations: OECD Guideline 471: Genetic Toxicology: Bacterial Reverse Mutation Test (OECD 1997a) and International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH 1996, ICH 1997). The tests were performed at CARDAM, and are GLP compliant.

For the Ames test, which is a Bacterial Reverse Mutation Test (Ames et al, 1975) (Maron and Ames, 1983) histidine-requiring strains of *Salmonella typhimurium* are commonly used to measure the frequency of spontaneous or chemical-induced point mutations. In this study we used five strains designed for base pair substitution (TA100, TA102 and TA1535) and frameshift mutations (insertion or deletion of one or a few DNA base pairs) (TA98, TA1537). This Bacterial Reverse Mutation Test detects mutations restoring the functional capability of the bacteria to synthesize histidine. Reverted bacteria are easily detected as they recover the ability to grow in the absence of histidine. The mutagenic frequency (induction factor = IF) of the test substances is calculated by dividing the mean number of revertant colonies (colonies growing on histidine poor medium) by the mean number of revertant colonies found in controls (spontaneous revertants). This test has been shown to identify a wide range of chemical mutagens (McCann and Ames,1976) (McCann et al, 1975). The bacteria used in the Ames test are suitable to detect mutagenic effects induced by genotoxic compounds, according to OECD guideline 471 (OECD 1997a).

The histidine-requiring *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 have also the rfa mutation which causes partial loss of the lipopolysaccharide barrier, and thus increase penetration through the bacterial cell wall by large test substances. All strains, except TA102 contain the UvrB mutation that causes a reduction in the DNA excision repair activity, resulting in an increased sensitivity for detection of mutagens. The TA98, 100 and TA102 are strains with R-factor which contain the plasmid pKM 101, an ampicillin resistance marker. This plasmid increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system normally present in *Salmonella typhimurium*. The strain TA102 also contains the plasmid pAQ1 which gives resistance to tetracycline.

Method

Genotoxic potential of five test substances (DMA-NO2, MA-NO2, MEA-NO2, PZ-NO2 and AMP-NO2) and their metabolites were evaluated for induction of reverse mutations in five *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 in the absence and presence of a rat liver metabolic activation system (S9). The study followed the procedures indicated by the international accepted guidelines and recommendations: OECD Guideline 471: Genetic Toxicology: Bacterial Reverse Mutation Test (OECD 1997) and International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH 1996, ICH 1997). For each test, fresh cultures were prepared. The test substances were

applied in water as solvents. Negative (untreated) and positive control (sodium azide, 4-NQO, 9-AAC and 2-AA) plates were included.

The experiment was considered valid if: a) the mean number of spontaneous revertants and solvent control revertants fall within the normal historical control range; b) the positive control induced a biological significant increase in the number of revertant colonies; c) the highest concentration was tested. The highest concentration was determined in preliminary toxicity range-finder study either as the highest water-soluble concentration, the concentration which induced toxic effects or the concentration which showed limited solubility in the solvent.

Toxicity range-finder experiment – plate incorporation method
A selection of an adequate range of doses was based on a toxicity range-finder experiment with the strain TA100 using 10 concentrations. The highest concentration was the highest water-soluble concentration. Untreated, negative (solvent/vehicle), and positive control plates were included. Suspensions of bacterial cells were exposed to the test substances in the presence and absence of S9. The plate incorporation method was used as described by Ames et al. (1975) and updated by Maron and Ames (Maron and Ames, 1983). The test solution was mixed in triplicates with the strain TA100, the sterile buffer (-S9) or the metabolic activation system (+S9) and with the overlay (histidine-containing) agar containing small amount of L-histidine and biotine to allow a few cell divisions. If at least five concentrations were scored, the counting of the number of revertant colonies were considered valid and used as the first Reverse Mutation Experiment.

Reverse Mutation Experiment 1 – plate incorporation method
The test substances were tested for reverse mutations in the *Salmonella typhimurium* strains TA98, TA102, TA1535, and TA1537. TA100 was not re-tested. Seven concentrations, separated by a factor of 2, were tested in triplicates in the absence and in the presence of rat liver S9 mix. Untreated controls, negative controls, and positive controls were included. The highest dose level was selected on basis of criteria specified in international regulations and on basis of data generated in the preliminary toxicity range-finder study.

Reverse Mutation Experiment 2 – pre-incubation method
If the first Reverse Mutation Test gave clearly negative results, a second Mutation Experiment was performed. This included a pre-incubation step, where the bacteria were incubated with the test substances in the presence and absence of S9 mix for 30 minutes at 37 +/- 1 °C. Thereafter 2 ml of molten top agar (+/- 45 °C) was added, and the test performed as described above for plate incorporation method.

Results

A test substance was considered to be mutagenic in the study if: a) the study is considered valid (acceptance criteria are met); b) a concentration related increase in the number of mean revertant colonies was observed with at least a 2-fold increase with the strains TA98, TA100, and TA102, and at least a 3-fold increase with the strains TA1535 and TA1537 in the absence and/or in the presence of rat liver S9 mix.
The concurrent positive controls demonstrated the sensitivity of the assay and the metabolizing activity of the S9 mix. After a toxicity range-finder experiment, two independent reverse mutation tests were performed. The first was a standard plate incorporation assay and the second involved a pre-incubation stage when negative results were obtained in the first Reverse Mutation Experiment. When positive results were obtained in the first plate incorporation assay, the second Reverse Mutation Experiment was a plate incorporation assay to confirm the findings.

Results of the Ames tests are summarised in Table F.2. In the toxicity range-finder experiment, no substantial increases in revertant colony numbers over solvent control counts were obtained with strain TA100 following exposure to any of the test substances at 10 selected concentrations up to a concentration of 4.50 mg/plate for DMA-NO2, 18.70 mg/plate for AMP-NO2 7.70 µg/plate for PZ-NO2, 15.50 mg/plate for MA-NO2 and 15.90 µg/plate for MEA-NO2 in either the presence or absence of S9 mix. As sufficient concentrations could be scored, these data were used for the actual mutagenicity data of the first Reverse Mutation Experiment. The top concentrations for the plate incorporation test with the strains TA98, TA102, TA1535, and TA1537 in the absence and in the presence of S9 mix were the same as for the toxicity range-finder experiment with the strain TA100. The concentrations for the plate incorporation study were 0.07, 0.14, 0.28, 0.56, 1.13, 2.25 and 4.50 mg/plate for DMA-NO2, 0.29, 0.58, 1.17, 2.34, 4.68, 9.35 and 18.7 mg/plate for AMP-NO2, 0.12, 0.24, 0.48, 0.96, 1.93, 3.85 and 7.70 mg/plate for PZ-NO2, 0.24, 0.48, 0.97, 1.94, 3.88, 7.75 and 15.50 mg/plate for MA-NO2 and 0.25, 0.50, 0.99, 1.99, 3.98, 7.95 and 15.90 mg/plate for MEA-NO2.

No evidence of mutagenic activity was seen at any concentration of DMA-NO2, AMP-NO2 or PZ-NO2 with any of the *Salmonella typhimurium* strains in the plate incorporation test as well as in the pre-incubation test in the absence and presence of S9 mix. A clear mutagenic response was observed at five concentrations of MA-NO2 0.48, 0.97, 1.94, 3.88, 7.75 mg/plate (the highest concentration indicated bacteriotoxicity) and at all concentrations of MEA-NO2 with the *Salmonella typhimurium* strain TA102 in the plate incorporation test in the first and second Reverse Mutation Experiment both in the absence and in the presence of S9 mix. A clear mutagenic response was also observed at the top concentrations of MEA-NO2 without S9 mix and for four concentrations of MEA-NO2 with S9 mix with the strain TA1535 in the plate incorporation test and in the first and second Reverse Mutation Experiment. No evidence of mutagenic activity was seen at any concentration of MEA-NO2 and MA-

NO2 with TA98, TA100, and TA1537 and in case of MA-NO2 also with TA1535 strains in the plate incorporation test or in the pre-incubation test in the absence and in the presence of S9 mix.

In some of the treated strains, reversion rate was decreased. This indicates induction of toxicity towards the bacteria (bacteriotoxicity), and was seen with all test substances with and without S9 mix.

Table F.2: Summary table showing the genotoxic response of test substance in Salmonella typhimurium strains with and without S9 mix in two independent experiments

Test substance	TA98	TA100	TA102	TA1535	TA1537
Reverse mutation exp 1 – plate incorporation method (PI)					
DMA-NO2	Negative	Negative	Negative	Negative	Negative
MA-NO2	Negative	Negative	Positive	Negative	Negative
MEA-NO2	Negative	Negative	Positive	Positive	Negative
AMP-NO2	Negative	Negative	Negative	Negative	Negative
PZ-NO2	Negative	Negative	Negative	Negative	Negative
Reverse mutation 2 – PI or pre-incubation method (PRE)					
DMA-NO2	Negative	Negative	Negative	Negative	Negative
MA-NO2	Negative	Negative	Positive	Negative	Negative
MEA-NO2	Negative	Negative	Positive	Positive	Negative
AMP-NO2	Negative	Negative	Negative	Negative	Negative
PZ-NO2	Negative	Negative	Negative	Negative	Negative

Conclusion

DMA-NO2, AMP-NO2 and PZ-NO2 show no evidence of mutagenic activity towards the *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 at the tested concentrations. Exposure to MA-NO2 induced mutagenicity towards the strain TA102. On the other hand, no mutagenic activity was found in the strains TA98, TA100, TA1535, and TA1537 at the tested concentrations. A clear mutagenic response was seen in the strains TA102 and TA1535 after the treatment with MEA-NO2, both with and without S9 mix. However, no mutagenic activity was found in TA98, TA100 and TA1537 strains with MEA-NO2 at the concentrations tested.

F.2.2 Comet Assay

The objective of this study was to investigate potential genotoxicity of Dimethylnitramine (DMA-NO2), Methylnitramine (MA-NO2) and Ethanolnitramine (MEA-NO2) solutions on human lymphoblastoid cells *in vitro* by detecting DNA strand

breaks and oxidized DNA lesions using the Comet assay. The exposure was performed in the presence and absence of external metabolic activation system S9.

The Comet assay (also called Single cell gel electrophoresis assay) is a sensitive method for detection of DNA damage and for potential genotoxicity. All mutagenic compounds induce different DNA damage that can manifest into a mutation and further to cells death if not repaired. Comet assay is able to pick up mutagenic compounds causing strand breaks and/or apurinic/apyrimidinic (AP) sites. In combination with lesion specific enzymes, different types of base DNA damage can also be determined.

The Comet assay is widely used for genotoxicity testing, and have been standardized within European Commission (EC) Framework Programme (FP) 4 project 'Standardisation and validation of the Comet assay for genotoxicity and human biomonitoring', EC FP5 project ESCODD and EC FP6 project Comics. The assay is recently under validation by ECVAM/ JaCVAM both *in vitro* and *in vivo*. The work has been performed by NILU. Though following the principles for GLP, the results from this assay is not accredited as such.

Method

Human lymphoblastoid TK6 cells were exposed for 3 hrs to 3 concentrations of the test substances + negative control.

After exposure, the cells were embedded in agarose on a microscope slide, and lysed with detergent and high concentration of salt (NaCl), which removes membranes, cytoplasm, and most of the nuclear matrix. DNA remains in a series of loops. The loops are supercoiled, because although the histones have been removed, the winding of the DNA (formerly around the nucleosomes) remains. When DNA is subjected to an electrophoretic field, it tends to move towards the anode. In the case of gel- embedded nucleoids, movement of intact supercoiled DNA loops is very limited, as the DNA is so compact. However, if a single break is present in the loop, the supercoiling is relaxed and the loop is free to extend under the electrophoretic field and move towards the anode. When stained and examined microscopically, images resembling comets are seen, the tail consists of loops of DNA with breaks that have moved out from the head of supercoiled DNA. The amount of DNA in the tail reflects the number of relaxed loops, and therefore the number of breaks in the DNA. Over a certain range of damage (including the levels of damage cells are likely to be exposed to), there is a close to linear relationship between the proportion of DNA in the tail and the number of breaks.

Formamidopyrimidine DNA glycosylase (FPG) was used to detect oxidized purines in DNA. FPG cleaves damaged bases, leaving apurinic sites in DNA that can be detected as additional break by the Comet assay. Thus by applying FPG, strand breaks (standard comet protocol) can be distinguished from oxidized bases sensitive to FPG.

Human TK6 cells have limited metabolic capacity to metabolize pre-mutagenic compounds to mutagenic metabolites. Therefore all tests were performed with and without S9 fraction.

At the end of the exposure period, cells were washed, counted and sub-cultured to determine survival rate 48 hours after the treatment for determination of relative growth activity (RGA). If RGA becomes less than 80 %, a compound can be considered cytotoxic.

Results

The % tail DNA for control (untreated cells without FPG) is in the range 2-4%. This represents the background level of strand breaks or the limit of sensitivity of the assay. (An increase in % tail DNA of 4% is typically equivalent to about 150 breaks per 10^{12} Da of DNA) (Collins et al., 2008).

The results for DMA-NO2 and MEA-NO2 (means of 2-3 independent experiments) with or without S9 and MA-NO2 with S9 are within or close to this control range, and so we can conclude that they do not directly cause DNA strand breaks. Nevertheless, there is a tendency for the highest dose of MEA-NO2 to produce rather more breaks than lower doses, indicating slight genotoxicity. Results for MA-NO2 without S9 show significant increase of DNA strand breaks for the highest concentration, though this might result from cytotoxicity..

The % tail DNA for control, untreated cells with FPG is around 10-12%, representing an increase over the basal level of strand breaks of about 8% tail DNA (around 300 breaks per 10^{12} Da of DNA). The levels of net FPG-sensitive sites (i.e. the increases over strand break levels) in samples treated with DMA-NO2, MA-NO2 or MEA-NO2, with or without S9, mostly fall in the range from 6 to 10%, which is very close to control values.

Positive control for detection of oxidised purines photosensitiser ROXX exposed in the presence of visible show clear induction of oxidised base (up to 40%, details in Annex: Study Reports). However, B(a)P in the presence of S9 mix failed to show induced DNA damage most likely due to precipitation of B(a)P in solvent (DMSO) and lack of time for the optimalisation of treatment with S9 mix. Nevertheless, we consider the results with S9 mix valid, as all other controls were in the expected range of damage, and the activity of S9 enzymes was measured by provider. However, we would recommend additional experiment with optimal conditions to verify this.

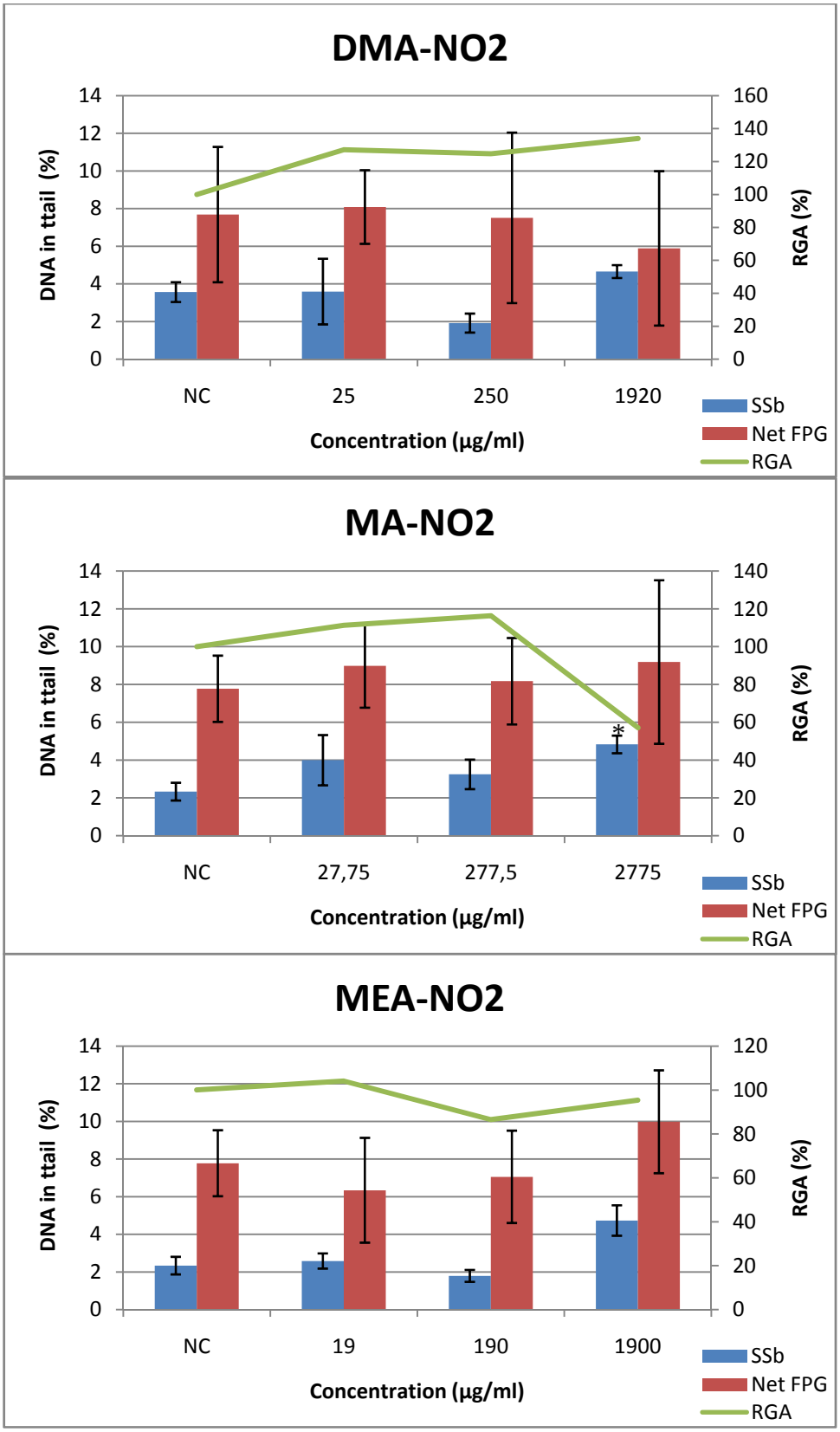


Figure F.1: **TK6 cells were exposed for 3 hrs to DMA-NO2, MA-NO2 and MEA-NO2. The graphs shows DNA in tail (%) for single breaks (SBs), net FPG-sensitive sites (net FPG) with SEM. The right axis shows relative growth activity (RGA (%)) 48 hours after exposure, using control as 100 %. Columns represent mean values from means of 2-3 independent repeats. * = mean values are significantly different compared to control at 0.05 level using t-test.**

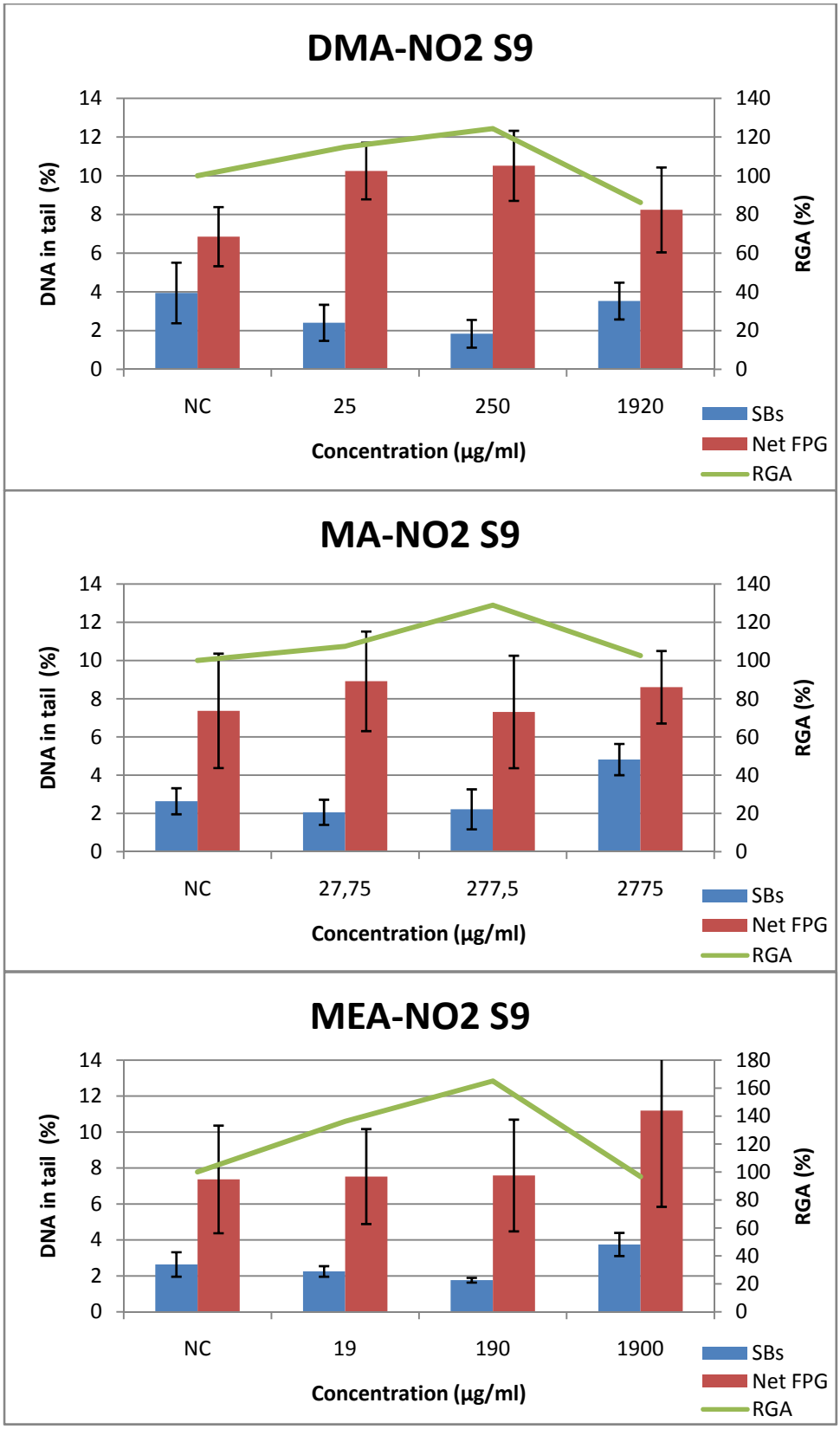


Figure F.2: **TK6 cells were exposed for 3 hrs with S9 to DMA-NO2, MA-NO2 and MEA-NO2. The graphs shows DNA in tail (%) for single breaks (SBs), net FPG-sensitive sites (net FPG) with SEM. The right axis shows relative growth activity (RGA (%)) 48 hours after exposure, using control as 100 %. Columns represent mean values from means of 2-3 independent repeats. For RGA only results from one experiment is included.**

Conclusions

All % tail DNA data (without FPG) are within or close to the control range of values, indicating no significant genotoxic effect in terms of strand breakage (with the possible exception of the highest concentrations of MEA-NO2, where a tendency to higher DNA break levels is seen). For MA-NO2 significant increase in DNA breaks was observed, but this is most likely due to cytotoxicity.

Similarly, net FPG-sensitive sites are within or very close to the control range and so we can conclude that there is no significant induction of base oxidation from these chemicals.

F.2.3 Mammalian HPRT Gene Mutation test

The objective of this study was to evaluate the potential mutagenicity of Dimethylnitramine (DMA-NO2) and Ethanolnitramine (MEA-NO2) and their metabolites on mammalian V79 cells *in vitro* by determination of mutations in hypoxanthine guanine phosphoribosyl transferase (HPRT) gene in the absence and presence of a rat liver metabolic activation system (S9).

A specific protocol was established and an OECD guideline was developed and adopted 21st July 1997 (OECD TG 476). Thus the test has been used for regulatory genotoxicity testing for more than 13 years (Aaron, 1994)(Abbondandolo, 1977) (Li A. C., 1987) (Li et al, 1988) (Slameňová et al, 1990) (Slameňová et al, 1992a) (Slameňová et al, 1992b) (Slameňová et al, 1983a) (Slameňová et al, 1983b) (Dušinská & Slameňová, 1990) (Slameňová et al, 1994). The work was performed by NILU. Though following the principles of GLP the results are not accreditet as such.

Method

HPRT mutation assay detects gene mutations, e.g. base change (one from 4 bases-adenine, thymine, guanine, uracil). This mutation results in amino acid change in the enzyme HPRT which metabolites precursors of purine bases. HPRT enzyme converts free purine bases into the corresponding nucleotide, thus bringing them back into the cellular pool. HPRT gene is X-linked so only 1 allele has to be inactivated to affect the phenotype. HPRT is a non-essential enzyme for the cell so mutant cells survive.

Selection of mutants is based on the selective toxicity of the purine analogue 6-Thioguanine (6-TG). 6-TG is a base analogue of the purine precursors that can be metabolized by HPRT enzyme through phosphorylation and reduction to nucleosid triphosphate. This metabolite intercalates into DNA causing inhibition of DNA replication and thus toxicity resulting in cell death. While 6-TG kills normal cells, $HPRT^-$ (mutant) cells survive, grow and form colonies which are detected visually. Mutants are identified by loss of activity of the purine salvage enzyme HPRT. Cells without a mutation are poisoned by 6-TG. Thus in the media with 6-TG, normal cells die, but cells with mutation in HPRT gene survive due to non-functional HPRT enzyme.

Those cells that are able to form colonies are mutant cells resulting from either a spontaneous mutation or from an induced mutation caused by the test substance.

Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent 6-TG to detect mutant cells, and 100 or few hundred cells in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time (ca. 5-6 days for PE and 7-10 days for mutations), colonies are stained and counted.

The test was performed according to OECD TG 476 on stable V79 cell line (derived from lung of Chinese hamster). V79 cells in monolayer culture were exposed to the test substances for 3 hrs in the presence and absence of metabolic activation S9 from rat liver (Elliott, 1992) (Slameňová et al, 1984) and thereafter sub-cultured (at least 10^6 cells) for 6-8 days to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. After the treatment, cells from each culture (concentration) were trypsinized and plated in small inoculums (100 cells/dish on 6 dishes) to measure cytotoxicity. Cytotoxicity is determined by measuring the relative plating efficiency (survival) of the cultured cells immediately after the treatment period. Frequency of spontaneous as well as induced mutations was determined from the number of mutant colonies in selective medium related to the number of viable cells grown as colonies in non-selective medium.

Step 1: Exposure

Mammalian V79 cells in monolayer culture were exposed to three analyzable concentrations of the test substance, in addition to control, for 3 hrs in phosphate buffered saline (PBS) with Ca^{++} and Mg^{++} in the presence and absence of S9 mix (metabolic activation S9 from rat liver with cofactors (Glucose-6-phosphate and NADP). Final theoretical exposure concentrations for DMA-NO2 were 50, 500 µg/ml and the highest concentration was 1920 µg/ml due to lower solubility). For MEA-NO2 the concentrations were 18.85µg/ml, 188.465µg/ml and 1884.46 µg/ml.

Step 2: Determination of cytotoxicity

After treatment cells from each culture (concentration) were trypsinized, plated in small inoculums (100 cells per dish) and incubated for about 6 days to determine cytotoxicity. Cytotoxicity was determined by measuring the relative plating efficiency (survival) of the cultured cells (see chapter D).

Step 3: Determination of gene mutation

After treatment cells from each culture (concentration) were cultivated and sub-cultured for a period of 6-8 days to allow phenotypic expression of mutations prior selection of mutants (at least 10^6 cells in each subculture). Mutant frequency was determined by seeding known numbers of cells in medium containing the selective agent 6-TG for detection of mutant cells (colonies), and in medium without 6-TG to determine the cloning efficiency (viability). Plating efficiency in non-selective medium was

determined after 5-6 days of culturing, mutant colonies in selective medium with 6-TG were determined after 7-10 days of culturing. Frequency of spontaneous as well as induced mutations was determined related to corresponding PE which is expressed in % following formula:

PE (%) = (Colonies Counted / Cells Inoculated) x 100

The mutant frequency for the treated and control cultures were calculated as number of mutant cells (colonies) per 100 000 of surviving cells (colonies) following formula:

Mutation frequency (%) = (Mutant Colonies / surviving inoculated Cells) x 100

A result is classified as positive if there is a concentration-related or reproducible increase in mutant frequency observed. Positive results for the HPRT-mutation assay indicate that the test substance induces gene mutations in the cultured cells used. A positive concentration-response that is reproducible is most meaningful.

Results

Mutagenicity of DMA-NO2 and MEA-NO2 in mammalian V79 cells was investigated after 3h treatment with three noncytotoxic concentrations. Two independent experiments with two mutation samplings were performed. Mutant frequency (calculated as number of mutant colonies per surviving cells) in both DMA-NO2 and MEA-NO2 treated cells, in the absence of metabolic activation S9, shows that both compounds are mutagenic. The level of spontaneous mutants was 13 per million cells. Summary results are presented in Figure F.3 (average mutant frequency from 2-4 mutation samplings) and in Table F.3.

In control samples with S9 mix, (cells cultivated in the presence of S9 mix) we detected relatively high levels of spontaneous mutations indicating the toxic effects of S9. (See Annex: Study Report) and therefore it was not possible to conclude whether metabolites of DMA-NO2 and MEA-NO2 are genotoxic. However, without metabolic activation there was a clear dose response in the occurrence of mutant colonies. Both DMA-NO2 and MEA-NO2 in all three concentrations induced mutations in the HPRT gene. The frequency of DMA-NO2-induced mutations was 2-3 fold higher compared with the background mutation level found in untreated cells. Treatment with MEA-NO2 caused a 2-fold increase in mutations compared to the control. Treatment with the positive mutagen MMS resulted in a similar mutant frequency to that seen after treatment with DMA-NO2. The frequency of MMS-induced mutants was on average 34 per million cells whereas in DMA-NO2-treated cells mutation frequency was on average between 29-38 per million cells. Figure F.4 shows mutant colonies from control (positive and negative) as well as DMA-NO2-treated plates (three concentrations A1, A2, A3). DMA-NO2 in the absence of S9 mix appeared to be a stronger mutagen than MEA-NO2.

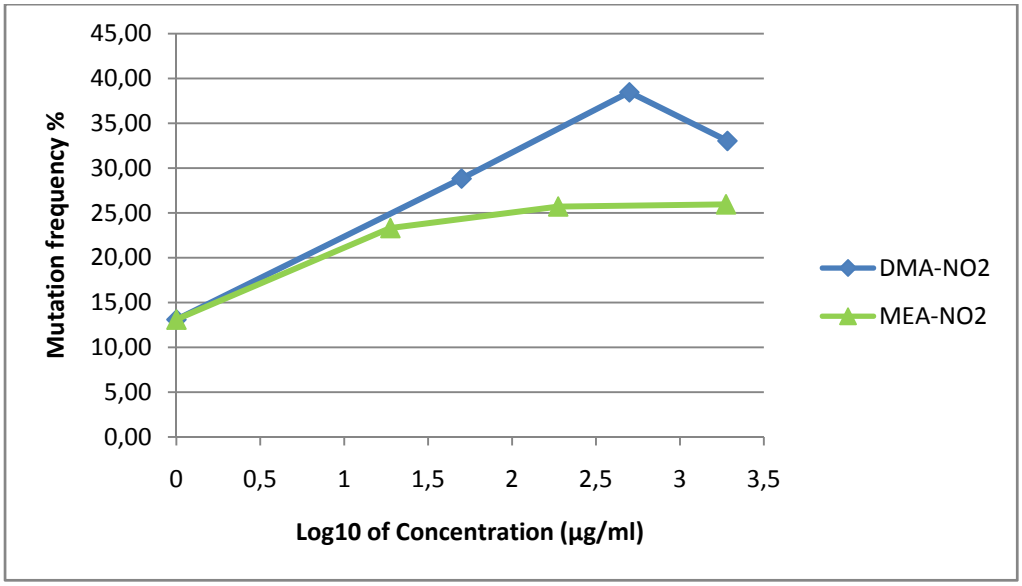


Figure F.3: **Induction of HPRT mutations after 3hrs exposure of V79 cells with DMA-NO2 and MEA-NO2 in the absence of S9 mix)**

Table F.3: **Induction of HPRT mutations (frequency of mutation per 1milion of cells) after 3hrs exposure of V79 cells with DMA-NO2 and MEA-NO2 in the absence of S9 mix in two independent experiments I and II. In each experiment two mutation harvests (A and B) has been performed. As negative control cells cultivated 3hrs in PBS were used. As positive control alkylating agent methyl methanesulfonate (MMS) was used (30 min treatment).**

Test substance	Mutation Experiment I		Mutation Experiment II		Conclusion
	A	B	A	B	
Control (PBS)	12.07	13.10	5.66	9.41	Negative
MMS 0.1M	36.85	56.34	25.49	21.16	Positive
DMA-NO2 50 µg/ml	26.09	31.58			Positive
DMA-NO2 500 µg/ml	37.17	27.88	55.48	33.33	
DMA-NO2 1920 µg/ml	21.38	32.64	60.53	17.62	
MEA-NO2 18.85 µg/ml	15.50	11.74	40.35	25.67	Positive
MEA-NO2 188.45 µg/ml	22.42		26.69	28.06	
MEA-NO2 1884.46 µg/ml	22.83	21.57	29.34	30.11	

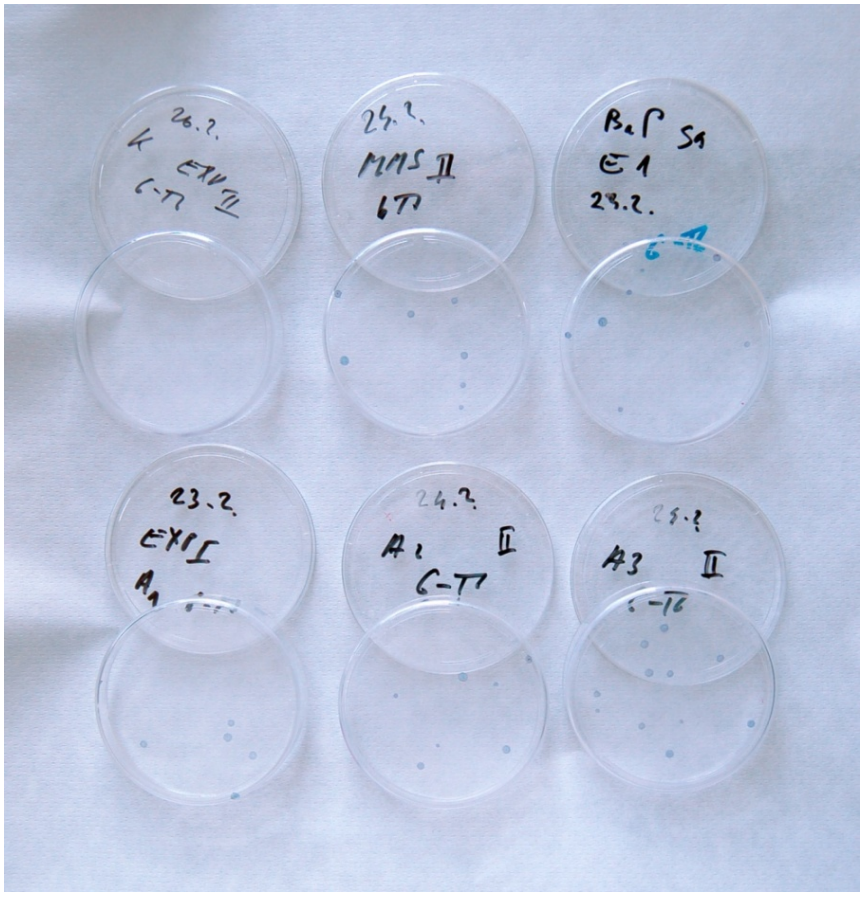


Figure F.4: **Representative plates with mutant colonies of V79 cells in negative control (K), positive control MMS, and BaP and in three different concentrations of DMA-NO2 A1 (50 µg/ml), A2 (500 µg/ml) and A3 (1920 µg/ml)**

Conclusion

Our results show that both DMA-NO2 and MEA-NO2 induced mutations in HPRT genes in mammalian cells with DMA-NO2 to be more potent.

F.3 Discussion

Mutagenic substances are classified using the criteria according to Annex VI to Directive 67/548/EEC into three categories: Category 1: Substances known to be mutagenic to man. There is sufficient evidence to establish a causal association between human exposure to a substance and heritable genetic damage; Category 2: Substances which should be regarded as if they are mutagenic to man. There is sufficient evidence to provide a strong presumption that human exposure to the substance may result in the development of heritable genetic damage, generally on the basis of appropriate animal studies or other relevant information; Category 3: Substances which cause concern for man owing to possible mutagenic effects. There is evidence from appropriate mutagenicity studies, but this is insufficient to place the substance in Category 2. To

conclude an appropriate classification and labelling position with regard to mutagenicity, the available data should be considered.

In our study three genotoxicity tests have been performed; Ames test (OECD 471), comet assay and mammalian HPRT gene mutation assay (OECD 476). Five compounds (DMA-NO2, MA-NO2, MEA-NO2, AMP-NO2 and PZ-NO2) have been tested using the Ames test, three (DMA-NO2, MA-NO2 and MEA-NO2) in the comet assay and two compounds (DMA-NO2 and MEA-NO2) using HPRT gene mutation assay. Results are summarized in Table H.1.

In the Ames test, the results are positive when an induction factor more than 2-fold is obtained for strains TA98, TA100, and TA102 and when an induction factor more than 3-fold is obtained for strains TA1535 and TA1537. For genetic toxicology, there is no acceptance of thresholds, and therefore in the Ames test, the answer 'yes or no' is used in terms of mutagenic potential. However, when an induction factor of e.g. 2-3-fold is obtained for the strains TA98, TA100, and TA102 then this can be described as a weak mutagenic response compared to an induction factor of 5-fold or even higher, which is a stronger response.

Except for DMA-NO2 and MA-NO2, there are no available data on genotoxicity of these compounds in the literature. In our study AMP-NO2 and PZ-NO2 tested in the Ames test show negative results. Similarly negative results were obtained after treatment with DMA-NO2, though DMA-NO2 was shown in previous studies to be positive in the Ames test (Khudoley et al., 1981, Frei et al., 1984, Pool et al., 1984 and 1986). The fact that DMA-NO2 is negative in the Ames test could be related to the lower concentration tested (DMA-NO2 was tested as a 4.5 % concentration) in this study.

Exposure to MA-NO2 induced mutagenicity towards the strain TA102 in contrast to previously published negative Ames test data (Pool et al., 1984, 1986, Malaveille et al., 1983).

MEA-NO2 also showed a mutagenic response in the Ames test as it can induce base pair substitutions in the *Salmonella typhimurium* strains TA102 (MA-NO2) and TA1535. Compared to MA-NO2, MEA-NO2 showed a higher induction factor in TA 102 at comparable tested concentrations. MEA-NO2 was also shown to be bacteriotoxic at the highest tested concentration and induced a week mutagenic response in strain TA1535. For both MA-NO2 and MEA-NO2, the induction factors in TA 102 are high, so the response could be considered as a ‘strong’ mutagenic response. There was also a dose-dependent increase in mutation for both test compounds with increasing concentration.

There are no data available on the comet assay with DMA-NO2, MA-NO2 or MEA-NO2. However, DMA-NO2 has been shown to induce strand breaks by alkaline elution (Pala et al., 1982, Pool et al., 1986) but negative results were obtained using the

fluorometric alkaline elution method. MA-NO2 induced DNA strand breaks in various mammalian cells using alkaline elution (Frei et al., 1986, Pool et al., 1986).

The negative results obtained in our study for DMA-NO2, MA-NO2 and possibly MEA-NO2 with the comet assay (no increase in strand breaks or damaged bases) should be seen in perspective. FPG, a bacterial repair enzyme, is primarily involved in removing oxidised purines and then breaking DNA at the site of the baseless sugar (AP-site). It will also recognise AP-sites caused by other means. But it is not able to recognise all damaged bases, and so we cannot exclude the possibility that these agents are inducing, for example, alkylation damage or so-called bulky adducts.

In the mammalian HPRT gene mutation assay results are classified as positive if a concentration-related or reproducible increase in mutant frequency is observed. In our study both DMA-NO2 and MEA-NO2 have shown dose-dependent increases in mutant frequency. DMA-NO2 appeared to be more mutagenic compared to MEA-NO2 and induced mutations in a similar range to the positive control MMS.

It is not surprising that different assays give diverging results, since they target different genetic end-points. However, we can only compare DMA-NO2 and MEA-NO2 results as these compoundes were tested in all three tests. MEA-NO2 gave postitive results in Ames and mammalian HPRT mutation assay and was equivocal in the comet assay thus gave the most meaningful answer. DMA-NO2 was clearly positive in the mammalian gene mutation but not in other two assays though there are positive results in Ames test known from literature. MA-NO2 was tested only in two assays, Ames test (positive) and the comet assay (negative) and unfortunately not in gene mutation assay which could clarify this discrepancy, However, we consider it as harmful compound. Finally, not much can be concluded from testing AMP-NO2 and PZ-NO2 as only Ames test with negative results was performed and clearly further testing is needed.

Three tests we used for potential genotoxicity and mutagenicity testing detect several endpoints (mutations in different genes, DNA breaks, oxidised DNA lesions) and are performed on different biological systems (human, hamster and bacteria). It should be also noted that our panel does not include all possible mutagenic endpoints and thus larger panel of genotoxicity tests and endpoints is recommended especially for AMP-NO2, PZ-NO2, and MA-NO2 but might be needed possibly also for MEA-NO2 and DMA-NO2 especially for understanding of mode of action.

G Chapter G – Ecotoxicity

Odd Gunnar Brakstad, Bjørn Henrik Hansen, Kristin Bonaunet and Siv-Hege Hansen

G.1 Introduction

Standard ecotoxicity testing includes a number of bioassays with organisms representing different trophic levels. For aquatic organisms these include phytoplankton (primary producers), invertebrates and vertebrates. In addition organisms representing the other compartments (soil, sediments, and air) may be included. OECD Guidelines are available for all these ecotoxicity tests. Typical acute aquatic ecotoxicity tests are described in OECD Guideline 201 (green algae), 202 (*Daphnia* sp.) and 203 (fish species) (OECD 2006b).

Biodegradation is measured by a number a standard methods, as described in different OECD Guidelines, including Guideline 301 (Ready biodegradability) OECD 302 (inherent biodegradability).

Previous search in the public databases or scientific literature showed that no ecotoxicity or biodegradability data were available for any of the nitramines included in the current study.

G.2 Experimental Section

The following ecotoxicological studies of Dimethylnitramine (DMA-NO2), Methylnitramine (MA-NO2), Ethanolnitramine (MEA-NO2), 2-methyl-2-(nitramino)-1-propanol (AMP-NO2) and Piperazine nitramine (PZ-NO2) were conducted:

- Determination of EC-values with the aquatic phytoplankton *Pseudokirchneriella subcapitata*
- Determination of EC-values with the aquatic invertebrate *Daphnia magna*.
- Ready biodegradability in an water with non-adapted bacterial consortia
- Bioaccumulation

The methods and the results of the tests are described below, while reports for the individual tests are enclosed in Annex: Study Reports.

Important notice: All concentrations used are based on analytically measured values, which give a unique control of the exposure level compared to using nominal values. More information regarding concentration analysis is given in Chapter D.

G.2.1 Ecotoxicity tests with aquatic phytoplankton

Methods

A phytoplankton test was conducted with the unicellular photosynthesising green algae *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*). The test was performed according to an OECD Guideline 201 (Freshwater Alga and Cyanobacteria, Growth Inhibition Test) (OECD 2006b).

Stock cultures of the green algae *P. subcapitata*, purchased from the culture collection of the Norwegian Water Research Institute (NIVA), was kept at 20± 2°C and reinoculated twice every week (every Tuesday and Friday) in the OECD TG201 medium described in the OECD Guideline 201 (OECD 2006b).

The nitramines were diluted in an algal culture medium in several concentrations to create a concentration gradient. The choice of test concentrations was based on a preliminary test in which ten-fold dilutions of test substance were tested (1 to 1000 mg/L final concentrations). All nitramines were treated as water-soluble substances, and algal cultures were directly inoculated to each amine test concentration. The inoculation was performed from an algal pre-culture in exponential growth (retrieved from the stock culture) to the different exposure concentration and controls (without amines). The volume of algal culture inoculated was sufficient to ensure an accepted growth limit for the control cultures of at least 0.92 day^{-1}, corresponding to an exponential biomass increase of 16. All test tubes were incubated with agitation under constant light intensity (60-120 fμE/sec/m^2) at a temperature of 20± 2°C for 72 hours.

In vivo chlorophyll fluorescence was measured daily in a filter fluorometer (model TD-700, Turner Designs, Sunnyvale, CA, USA). Growth parameters calculated included:

- Daily growth rates (μ), determined by linear regression,
- Biomass integral (A), determined as the area under the growth curves

The effective concentrations (EC) of each nitramine inhibiting the algal *in vivo* photosynthesis were estimated. These concentrations were determined as the concentrations (mg/L) inhibiting algal photosynthesis by 50 % (EC_{50}), by 10 % (EC_{10}), and by 90 % (EC_{90}) relative to the control cultures. These results were calculated by 95 % confidence interval (calculations are performed with the computer program Toxedo (Water Quality Institute, Denmark).

Results

The algal growth curves for controls and exposed cultures are shown in Figure G.1. For most of the nitramines we were able to generate growth curves representing a span from non-inhibited curves (close to the control culture curve profiles) to growth curves with high degree of inhibition. The exception was DMA-NO2, where all curves were close to the control cultures.

Results for linear regression analyses of growth inhibition are shown in Figure G.2. These figures show growth inhibition between close to zero to 48-77 %, except for the DMA-NO2, which was not inhibited. Linear regression analyses

The algal EC-values for the nitramines are shown in Table G.1 and visualised in Figure G.3. These values were calculated as the concentrations of chemicals inhibiting the algal growth rates and biomass integrals by 10, 50 and 90 % (EC-10, EC-50 and EC-90) by 95 % confidence intervals. The EC-50 values varied from 430 mg/l for the most toxic nitramine, PZ-NO2, to > 2000 mg/l for the least toxic compound, DMA-NO2.

Conclusions

The results from the ecotoxicity studies with the green alga *Pseudokirchneriella subcapitata* showed that all the acute toxicity of the nitramines tested were moderate to low.

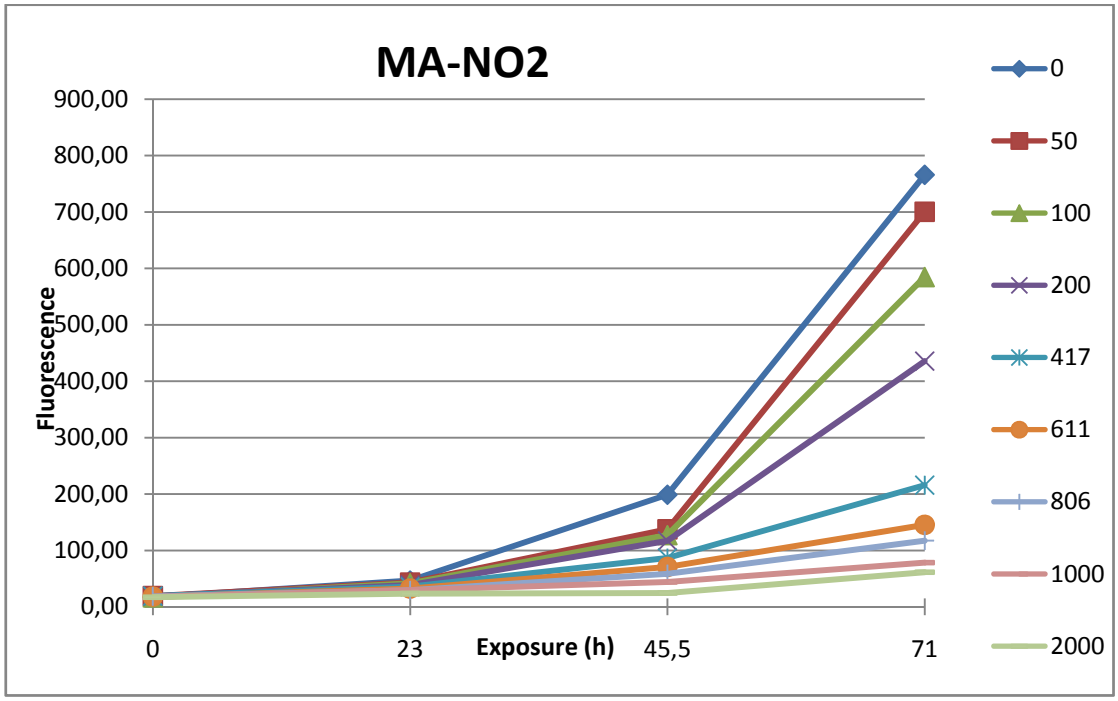


Figure G.1: **Growth curves for cultures exposed to different concentrations of nitramines and control cultures (0). The curves represent mean of the controls (n=6) and exposed (n=3) cultures.**

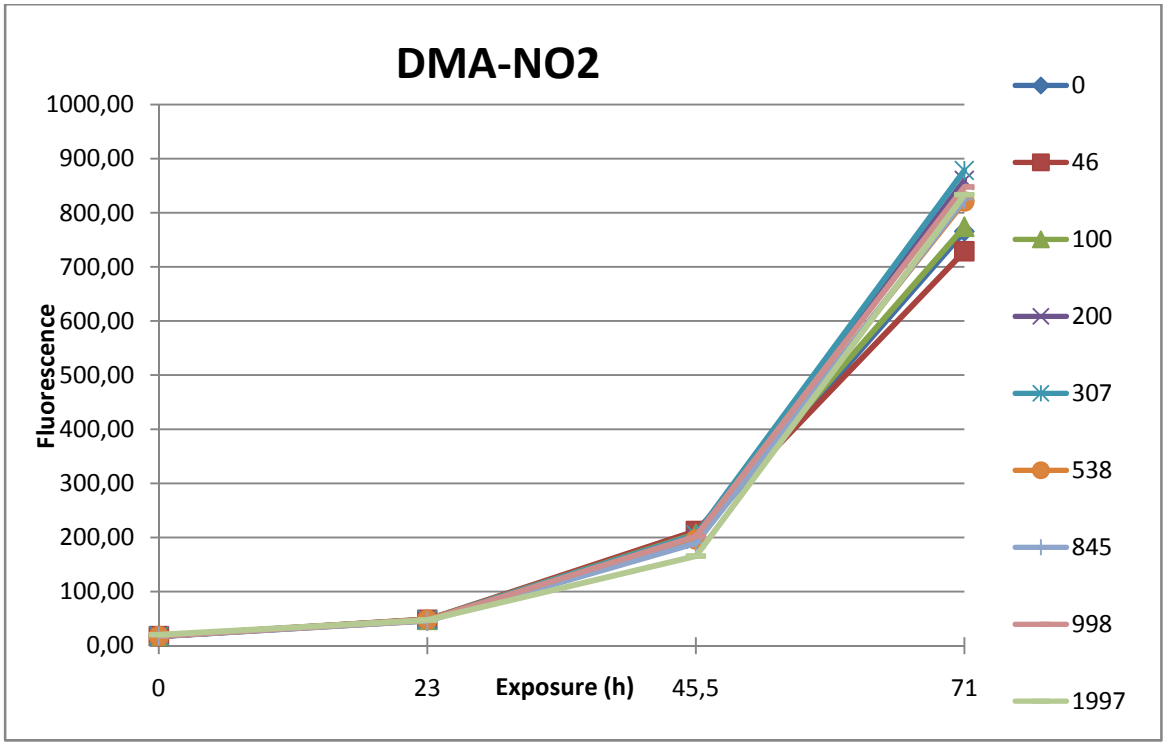


Figure G.1 **Continued.**

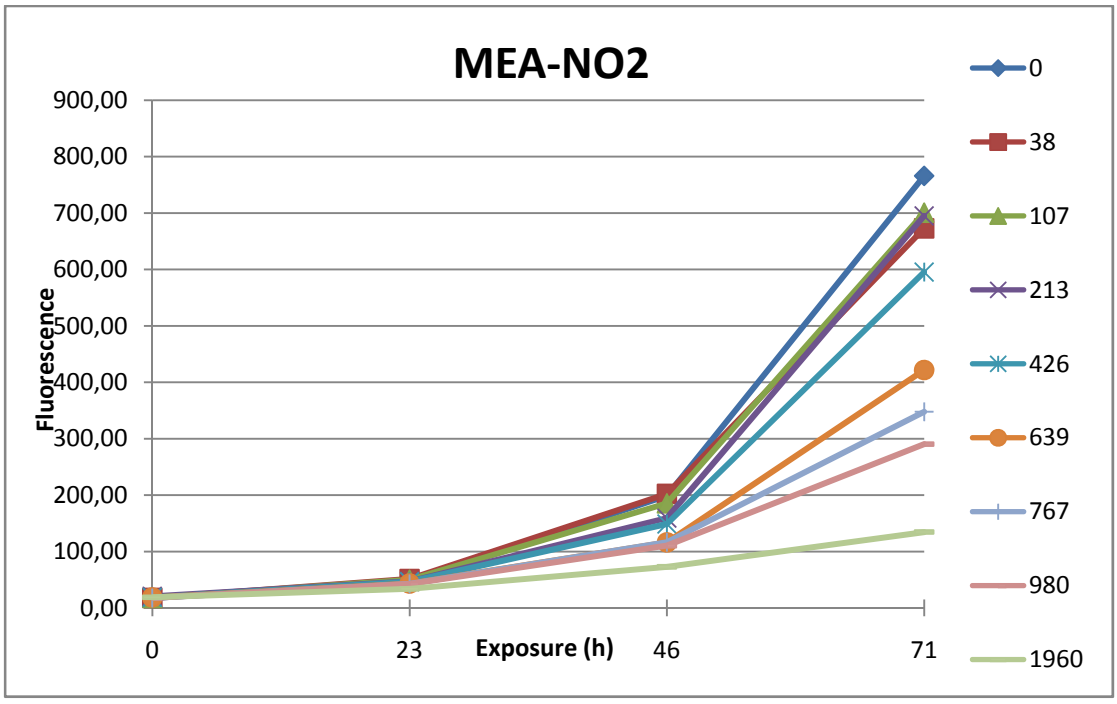


Figure G.1 **Continued.**

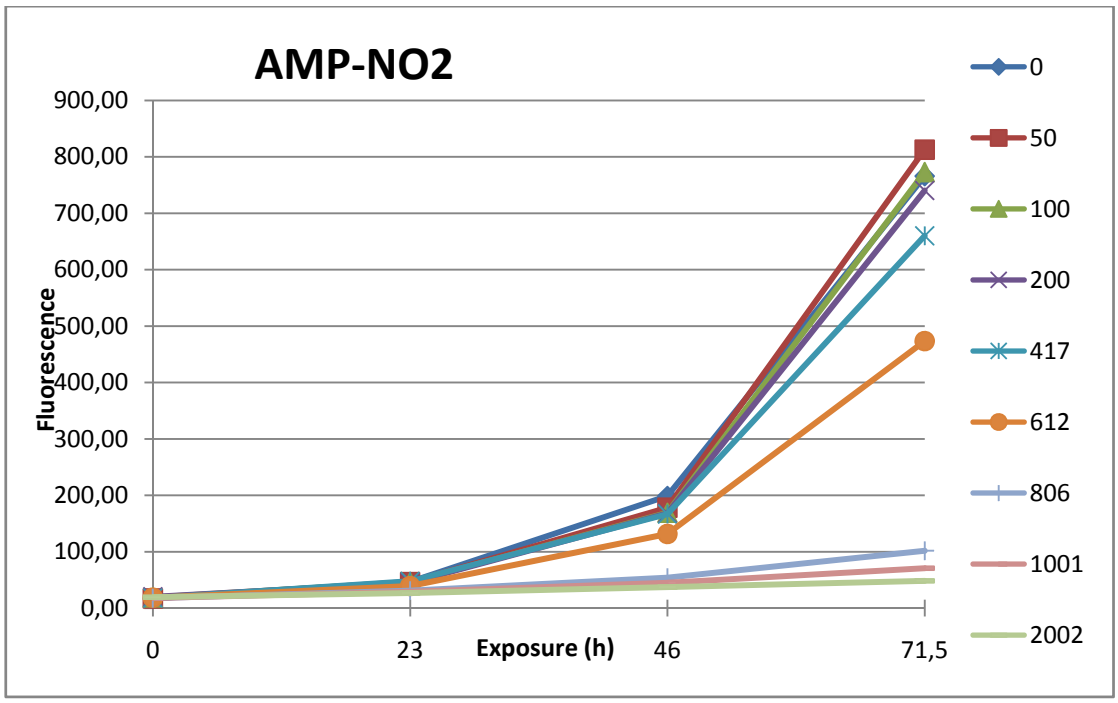


Figure G.1 Continued.

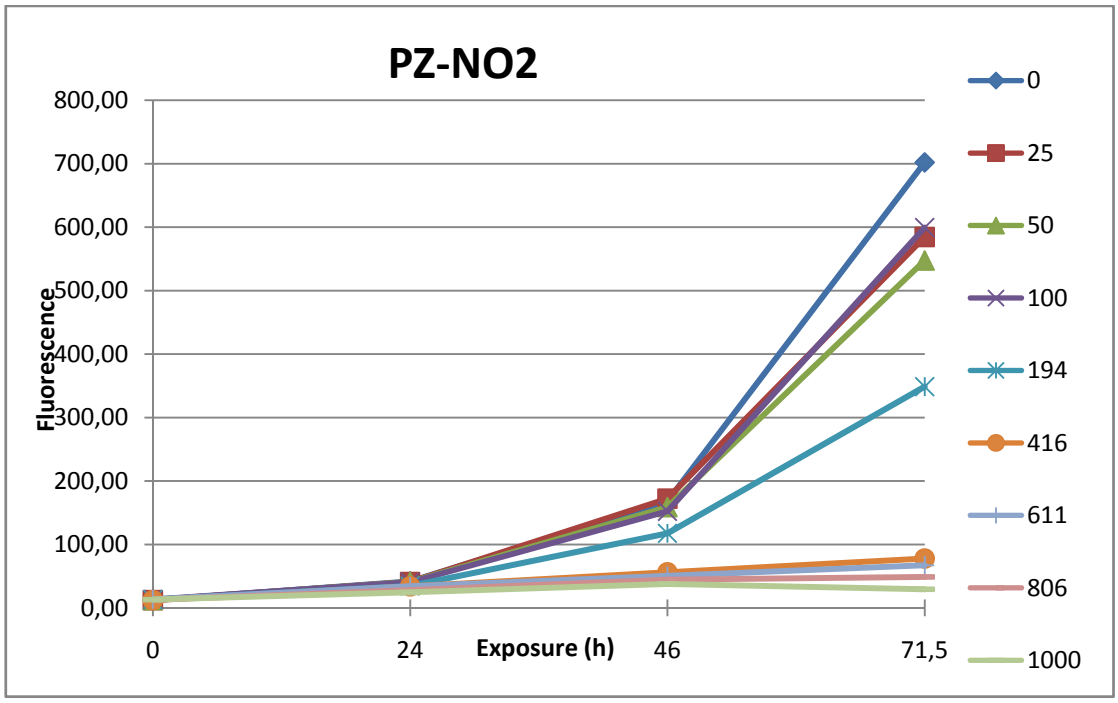


Figure G.1 Continued.

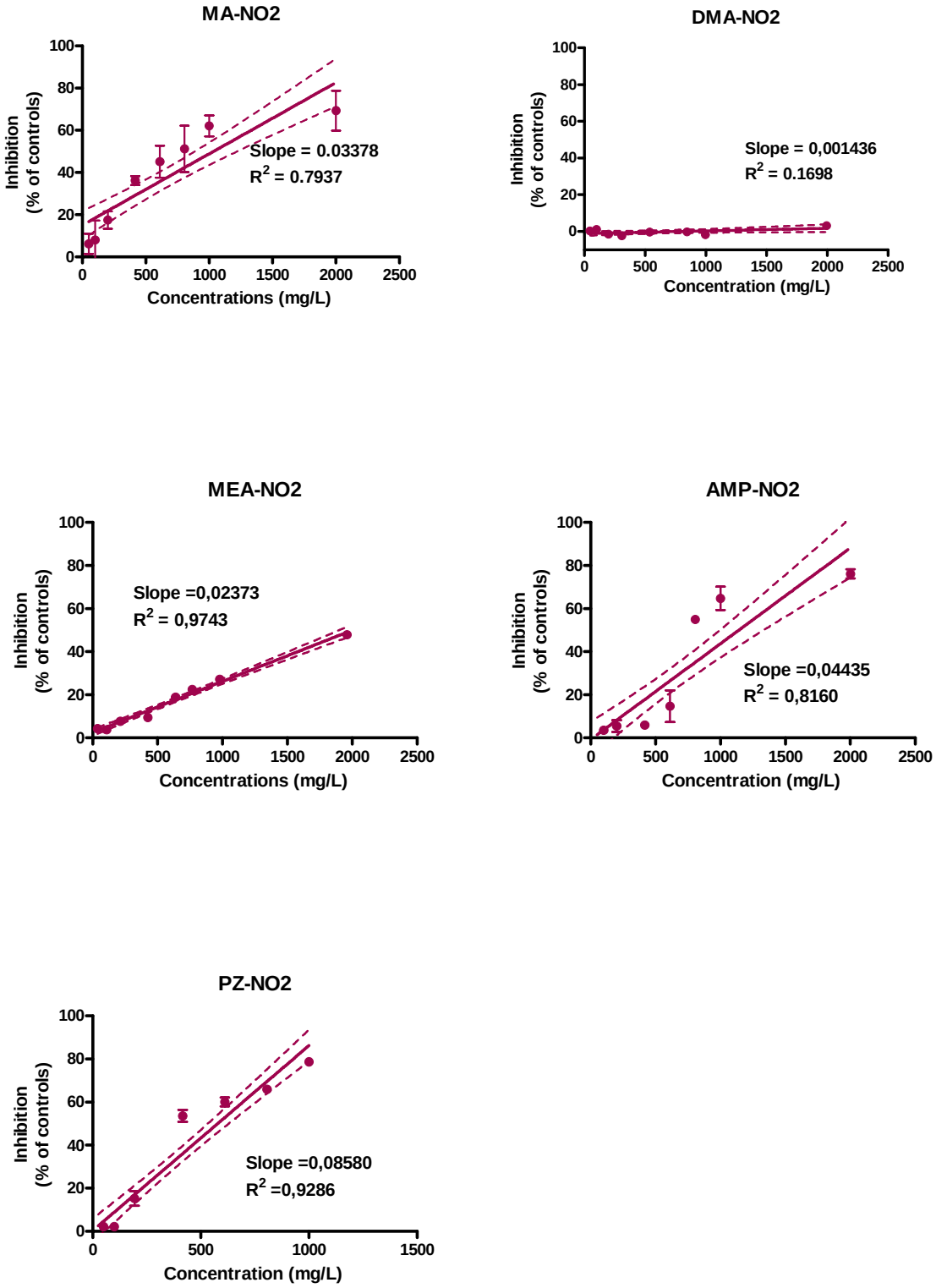


Figure G.2: **Linear regression analyses of the inhibition of green algae by the nitramines. The measuring points are shown with standard error, while the lines are shown with 95 % confidence intervals (95 % CI). The curve slopes and goodness of fits (R^2) are presented for the individual curves.**

Table G.1: **Effective concentrations (EC) of nitramines inhibiting algal growth rates (μ) by 10 % (EC-10), 50 % (EC-50) and 90 % (EC-90) are shown with 95 % confidence intervals (CI). The results are based on the calculations of growth rates (μ).**

Nitramine Growth rates (μ)	Effective concentrations (EC) (mg/L)		
	EC-10 (± 95 % CI)	EC-50 (± 95 % CI)	EC-90 (± 95 % CI)
MA-NO2	98 (82 - 115)	754 (709 – 803)	5777 (4862 - 7063)
*DMA-NO2DMA-NO2	>2000	>2000	>2000
MEA-NO2	292 (260 .. 323)	2535 (2308 .. 2820)	22033 (17329 ..29159)
AMP-NO2	270 (254 - 287)	871 (850 - 891)	2803 (2659 - 2968)
PZ-NO2	101 (88 - 115)	430 (408 - 453)	1827 (1646 - 2061)

** No inhibition at the highest concentration tested (2000 mg/L)*

Table G.2: **Effective concentrations (EC) of nitramines inhibiting algal biomass integral (A) by 10 % (EC-10), 50 % (EC-50) and 90 % (EC-90) are shown with 95 % confidence intervals (CI).**

Nitramine Biomass (A)	Effective concentrations (EC) (mg/L)		
	EC-10 (± 95 % CI)	EC-50 (± 95 % CI)	EC-90 (± 95 % CI)
MA-NO2	36.6 (30.2 - 43.3)	250.0 (231.4 - 269.3)	1707.2 (1490.6 - 1992.6)
*DMA-NO2DMA-NO2	>2000	>2000	>2000
MEA-NO2	144 (120 - 167)	875 (817 - 942)	5340 (4433 - 6669)
AMP-NO2	236 (219 - 253)	644 (620 - 668)	1754 (1644 - 1885)
PZ-NO2	39 (34 - 44)	213 (200 - 226)	1159 (1050 - 1291)

** No inhibition at the highest concentration tested (2000 mg/L)*

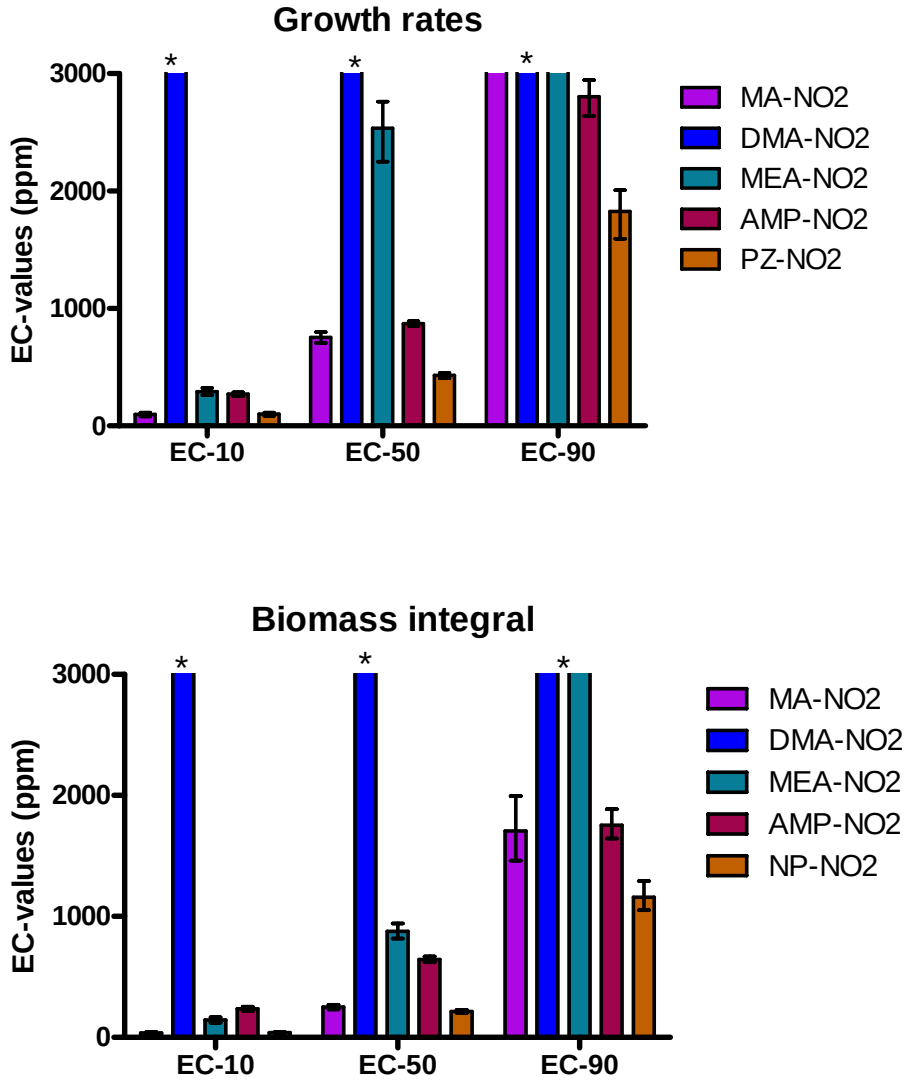


Figure G.3: **EC-values for growth rates and biomass integrals. The error bars represent 95 % confidence intervals. Only the EC-levels < 2000 mg/l are included.**

G.2.2 Ecotoxicity tests with the aquatic invertebrate *Daphnia magna*

Method

An invertebrate acute ecotoxicity test was conducted with the *Daphnia magna*. The method was performed according to OECD Guideline 202 (*Daphnia* sp. Acute Immobilisation Test).

A *Daphnia magna* start culture, purchased from Norwegian Institute of Water Research (NIVA), consisted of approximately 100 pregnant females which were transferred to M7 medium, as described in the OECD 202 Guideline. The start culture was kept for at

least three generations before neonates were used in exposure experiments testing single nitramines. The *Daphnia* culture was kept at 20-22°C with a light: dark-regime of 16:8 hours, and fed green algae (*P. subcapitata*) daily. The algal culture was kept under the same conditions as the daphnids.

Exposure experiments were conducted using neonates less than 24 hours old. In order to collect the neonates pregnant females were taken out of the culture into separate glasses with medium and left overnight. The following morning the medium was filtered and neonates picked individually and transferred to exposure solutions.

Stock solutions of the single nitramines were made at an appropriate stock concentration in M7 medium based on the amounts of nitramines available. From this stock solution, dilutions were made to exposure medium at seven different concentrations chosen based on EC50-values from the algae test. The exposure solutions (25 ml) were added to Erlenmeyer flasks, and 5 neonates were added. Neonates were not fed during the experiment which lasted for 48 hours with reading of dead daphnids at 24 and 48 hours. All exposure concentrations were performed with four replicates, and 8 controls containing only M7 medium were used.

After counting dead daphnids at the end of the experiment, exposure solutions were analyzed for O_2 and pH to verify that they were within the acceptable range reported in the OECD guideline. Results were loaded into GraphPad Prism 4.0 and analyzed statistically using Non-linear regression analyses and sigmoidal dose-response curve (variable slope). Values were reported as lethality curves as well as calculated LC50 concentrations.

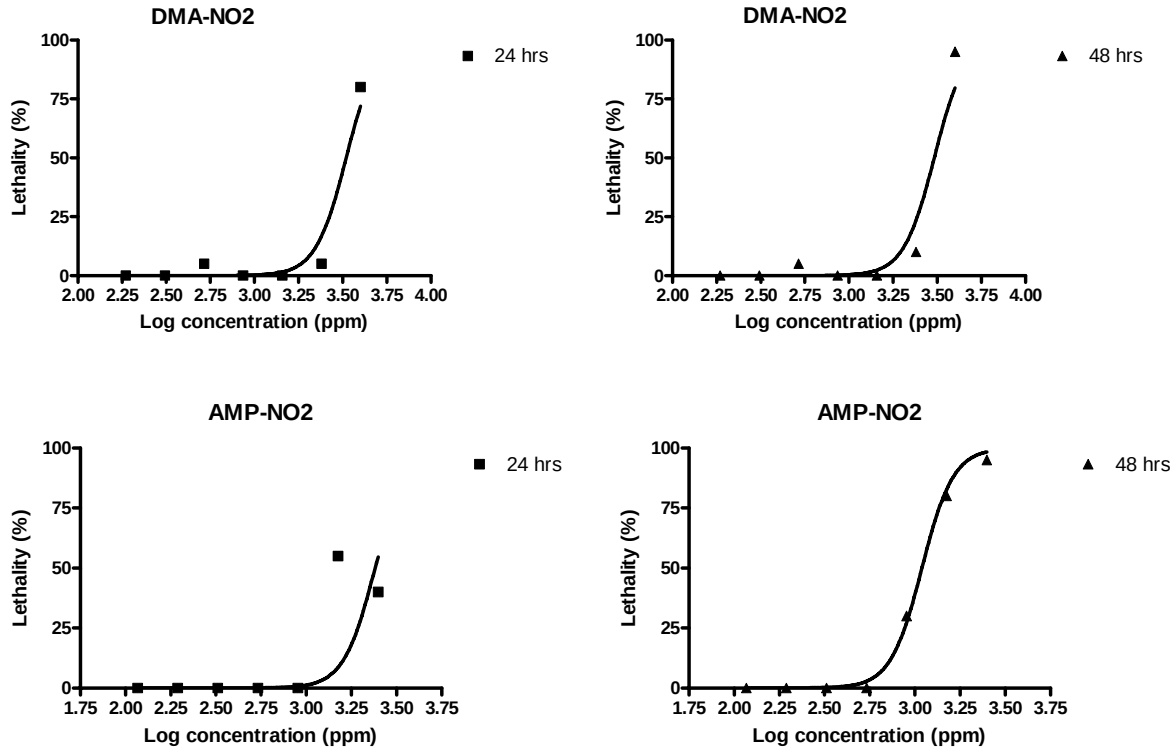
Result

For three of the nitramines (DMA-NO2, PZ-NO2 and AMP-NO2) tested on *D. magna* we were able to generate a sigmoid lethality curve yielding EC50-concentrations for 24 and 48 hrs (Figure G.4). For DMA-NO2 the calculated LC50-concentrations after 24 and 48 hrs were 3314 and 3042 mg/L, respectively. For PZ-NO2 the calculated LC50-concentrations after 24 and 48 hrs were 1423 and 1031 mg/L, respectively. Finally, for AMP-NO2 the calculated LC50-concentrations after 24 and 48 hrs were 2408 and 1095 mg/L, respectively. Note that after 24 hrs 100% mortality was not observed for DMA-NO2 and AMP-NO2.

For the remaining two (MA-NO2 and MEA-NO2), LC50-concentrations were not possible to calculate from the experiments. The data are given in Figure G.5. For MEA-NO2, the highest concentration (2500 mg/L) did not result in 100% mortality during the 48 hrs and should therefore be tested with higher concentrations. This was not possible in the current study because of limited supply of nitramines for toxicity testing. For MA-NO2, the highest concentration resulted in only 95% mortality, but exposure to the different concentrations did not result in a dose-dependent mortality. In fact, after 48 hrs exposure, the 25% mortality was observed in the 540 mg/L exposure and 85% mortality was observed in the 194 mg/L exposure. Lack of dose-response relationships invalidates

the test, and the calculated LC50-data should not be used. If the three lowest concentrations were removed from calculations the 24-hour LC50 value was 1623 mg/L (95 % confidence interval 1074-2452 mg/L), while 48-hour LC50 value was 1426 mg/L (95 % confidence interval 810-2512 mg/L). Although the data for MA-NO2 was used to calculate LC50 using the non-linear regression analyses and LC50-concentrations are given in Table F.3, this nitramine needs to be re-tested in order to generate reliable LC50-data. The reason for the lack of dose-response relationship for MA-NO2 is uncertain, but may be caused by photoxidation of nitramines during the 48 hrs exposure. We would consider doing re-testing in the dark.

The *D. magna* LC50-concentrations are given in Table G.3. These values were calculated as the concentrations of nitramines that caused 50% mortality after 24 and 48 hrs.



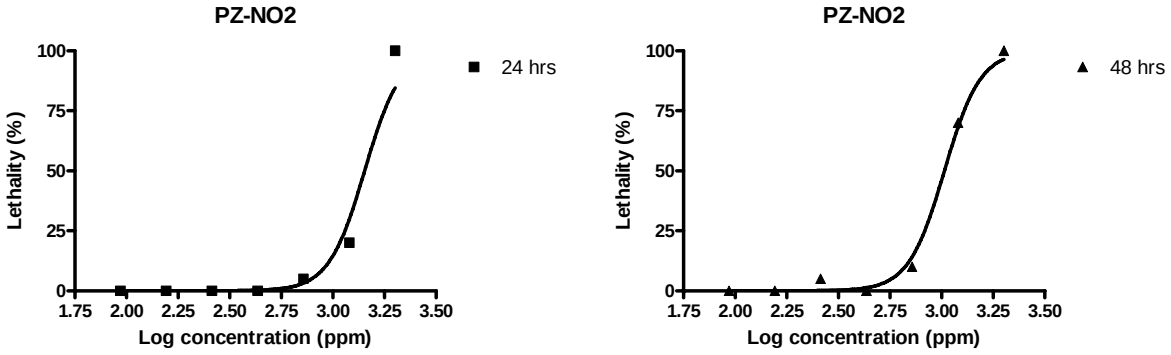


Figure G.4: **Lethality curves for the individual nitramines tested. Lethality (in % of controls) is plotted as a function of exposure concentration (in LOG mg/L). The sigmoid dose-response curves are given for data at 24 hrs (left) and 48 hrs (right).**

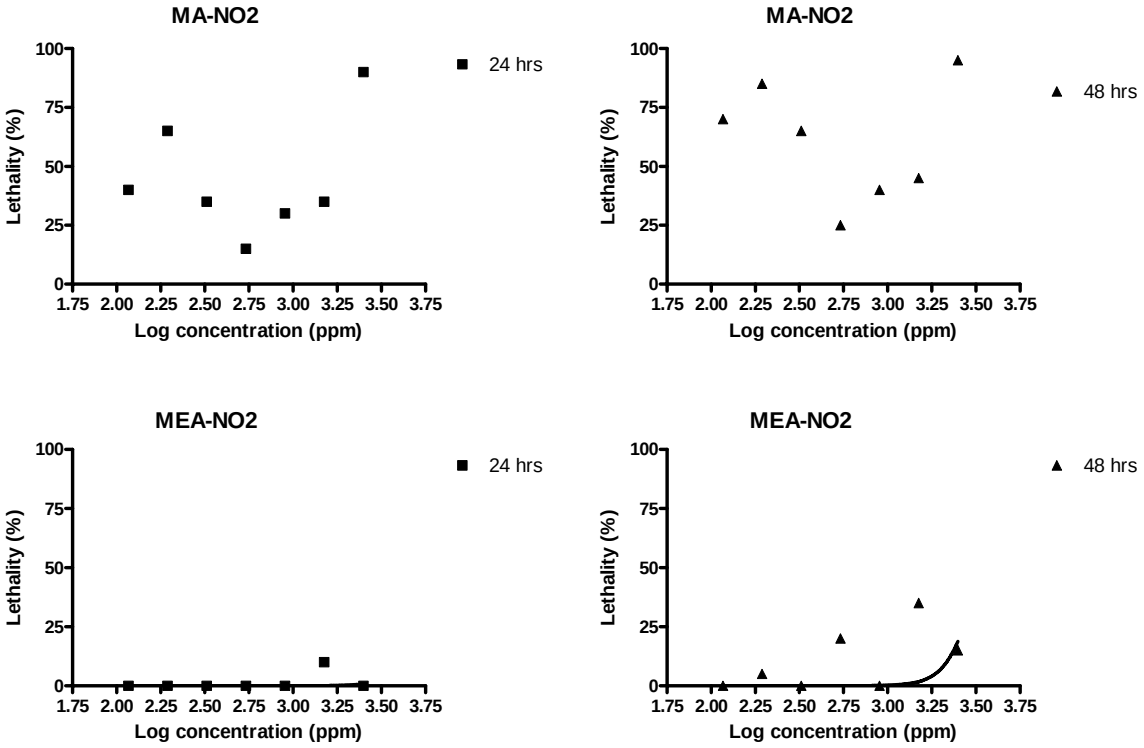


Figure G.5: **Lethality (in % of controls) as a function of exposure concentration (in LOG mg/L) at 24 hrs (left) and 48 hrs (right). Sigmoid dose-response curve is only given for MEA-NO2 after 48 hrs exposure.**

Conclusions

The results from the ecotoxicity studies with Daphnia magna showed that all the acute toxicity of the nitramines tested were moderate to low for diemthylnitramine, AMP-NO2 and PZ-NO2, and that DMA-NO2 was the least toxic of the three. The remaining two nitramines (MA-NO2 and MEA-NO2) needs to be re-tested.

Table G.3: **Lethal concentrations (LC50) of nitramines causing mortality to D. magna after 24 and 48 hrs are shown with 95 % confidence intervals (CI).**

Nitramine	Lethal concentrations (LC50) (mg/L)	
	LC-50 (± 95 % CI) 24 hrs	**LC-50 (± 95 % CI) 48 hrs**
[A)] *MA-NO2*	[B)] *1623 (1074-2452)*	[B)] *1426 (810-2512)*
DMA-NO2	3314 (2931-3747)	3042 (2557-3618)
[A)] *MEA-NO2*	*>2500*	*>2500*
AMP-NO2	2408 (1657-3501)	1095 (1043-1149)
PZ-NO2	1423 (1227-1650)	1031 (970-1096)

[A)] *These amines need to be re-tested in order to provide valid data.*
[B)] *Data based on calculation without the three lowest test concentrations included*

G.2.3 Ready biodegradability test

Methods

Biodegradability testing of the single chemicals was performed according to OECD Guideline 301D (Ready Biodegradability – Closed Bottle Method). The methods were performed briefly as described below:

The test principles are based on the measurements of biological oxygen demand (BOD) over a period of 28 days, and the determination of the percentage BOD related to theoretical oxygen demand (ThOD) if the chemical is completely oxidized to CO2 and water. When nitrogen atoms are present these will end up as ammonium (NH3).

Surface water was collected in a small lake close to Trondheim (Hauklivatnet and a local river Nidelva) well upstream of influence from seawater tide. The water from the two sources were mixed in equal volumes and aged for 8-10 days at room temperature in darkness, One litre of the water was subjected to bacterial enrichment in a filter inside an aquarium pump, and with continuous circulation of the water through the pump. At the end of the aging period the accumulated bacteria in the filter of the aquarium pump were applied to the rest of the aged water, and this enriched water was used as inoculum during the biodegradation testing. The inoculum was added 2 mg/L of each nitramine and distributed in 275-ml BOD bottles in duplicate. Water without chemicals was distributed in BOD bottles as blank solutions. A reference solution of 2 mg/L aniline was included. Essential nutrients for metabolic activities were added to the solution and the nitramine-solutions (test solutions) were incubated for 7 to 28 days at 20 ± 2°C. In each series bottles were removed for measurements at day 0, 7, 14, 21 and 28 during the incubation period.

The dissolved oxygen concentrations (DO) of the test bottles were measured with a BOD probe connected to a dissolved oxygen meter. The BOD values were calculated as the difference in DO between solution with blank (water) and test solutions.

The nitramines analysed were pure chemicals with known empirical formulas, and the ThOD was calculated as described in the Guideline:

$$ThOD_{NO2} = \frac{16\left[2c + 1/2(h - cl) + 3s + 3/2n + 5/2p + 1/2na - o\right]}{MW}$$

where *c*, *h*, *cl*, *n*, *s*, *p*, *na* and *o* are the numbers of carbon, hydrogen, chlorine, nitrogen, sulphur, phosphorous, sodium and oxygen atoms present in the formula and MW is the molecular weight of the substance.

The final results were determined as percentage BOD relative to the calculated ThOD values. The reference chemical (aniline) should have a biodegradability of > 60 % of its ThOD value.

Results

Figure G.6 shows the measured BOD-curves for all the tested nitramines and for the reference substance aniline. The results for all the nitramines were considerably lower than for the reference substance. In Figure G.7 the BOD after 28 days are compared for all the nitramines, showing that MEA-NO2, MA-NO2 and AMP-NO2 were more biodegradable in this test than DMA-NO2 and PZ-NO2.

The biodegradabilities as % BOD of the calculated ThOD are shown in Table G.4 and illustrated in Figure G.8 for respiration curves and in Figure G.9 for biodegradabiity after 28 days. The biodegradabilities varied from 3 % for PZ-NO2 and DMA-NO2 to 33-34 % for MA-NO2 and MEA-NO2. The results for the reference substance showed nearly full ultimate biodegradation after 28 days. All the nitramine curves showed a peculiar reduction at the 14-days measurements, except the curve for PZ-NO2. All the nitramines showing this reduction were analysed in the same experiment, and whether this was caused by measuring errors at day 7 or 14, or some other reason, was not clarified from the experiments.

Inhibition experiments were also conducted as part of the biodegradation experiments. In these experiments the individual nitramines were mixed with the reference substance, and inhibition of the reference substance respiration (BOD) was determined. The results are shown in Figure G.10. These results showed that only DMA-NO2 and PZ-NO2 inhibited the aniline respiration moderately. The other nitramines stimulated the respiration and had therefore no toxic effects on the microbial consortia.

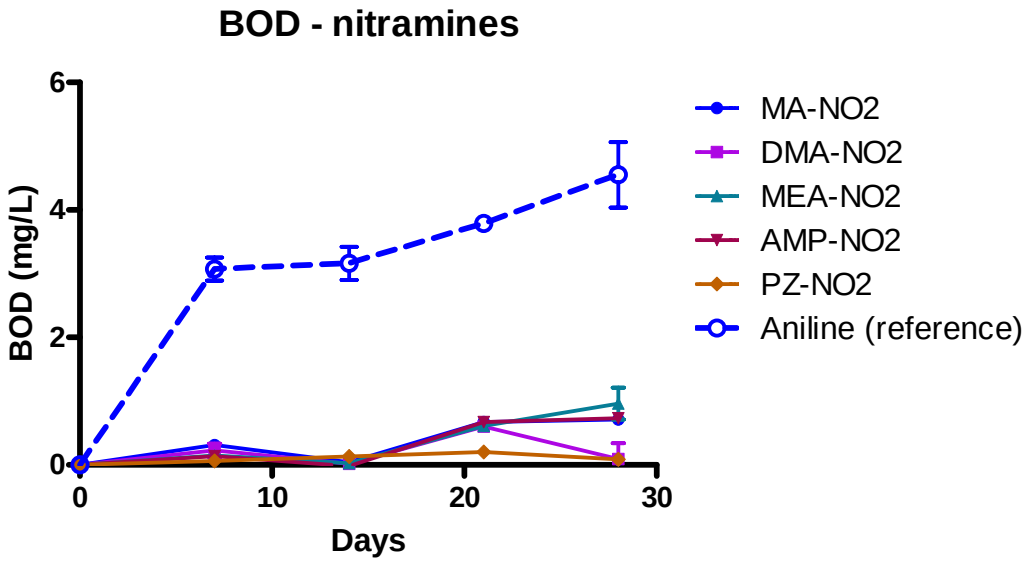


Figure G.6: **Biochemical oxygen demand in the nitramines and reference substance during the biodegradation period.**

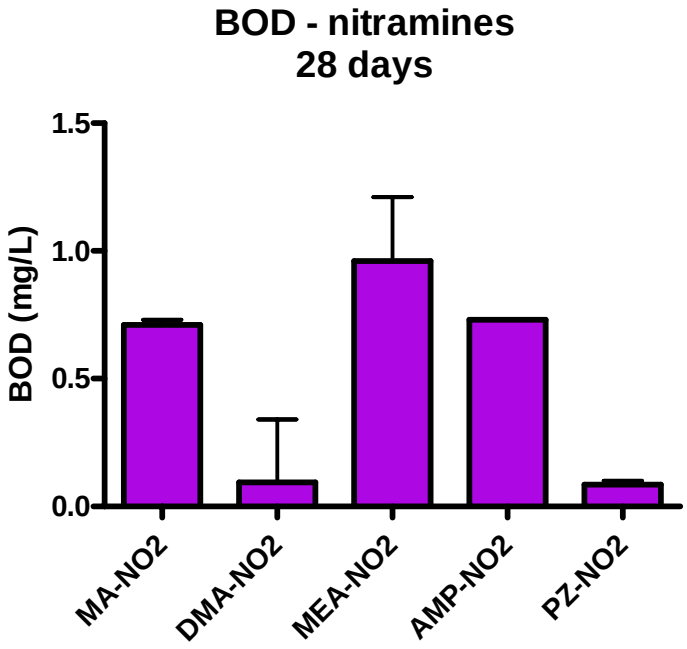


Figure G.7: **Biochemical oxygen demand in the nitramines after 28 days of biodegradation.**

Table G.4: **BOD- values for test and reference solutions (average of duplicate measurements) and ThOD calculations related to the amount of substance (mg BOD or ThOD/g test- or reference substance).**

Test solution	ThOD	Incubation (days)			
		7	14	21	28
Reference (exp.	2.41	62,96	64,91	77,75	93,36
MA-NO2	1.05	14,52	1,90	31,67	33,57
DMA-NO2	1.42	7,76	0,71	20,99	3,35
MEA-NO2	1.43	4,89	1,05	21,13	33,36
AMP-NO2	1.85	3,51	-0,68	17,97	19,59
PZ-NO2	1.53	1,96	4,25	6,54	2,78

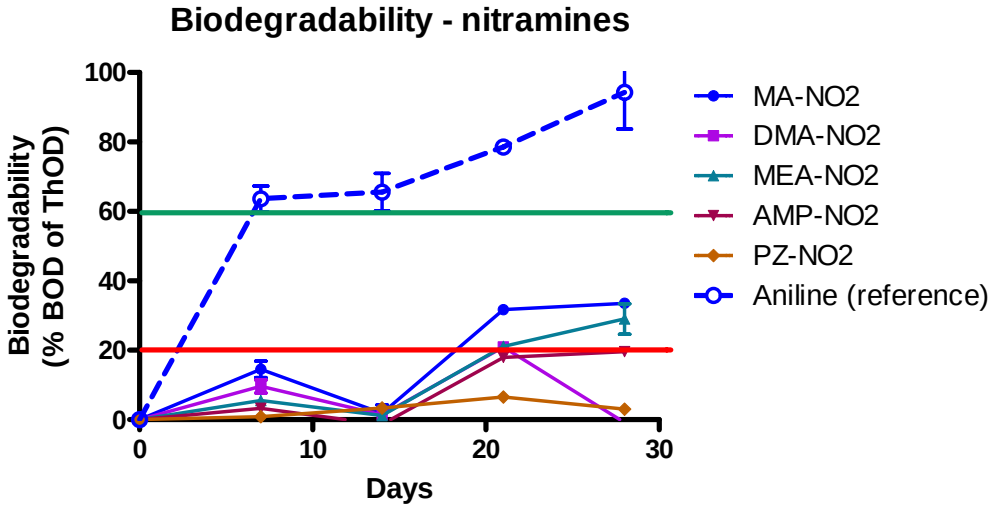


Figure G.8: **Biodegradabiity of nitrosamines and reference substance during the biodegradation period. The results are shown at % BOD of ThOD.**

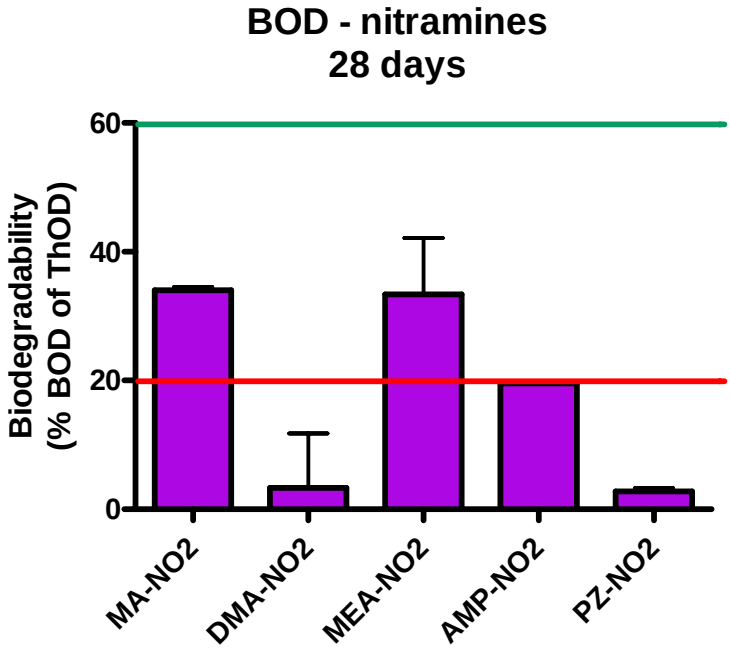


Figure G.9: **Biodegradabiity of nitrosamines and reference substance after 28 days. The results are shown at % BOD of ThOD.**

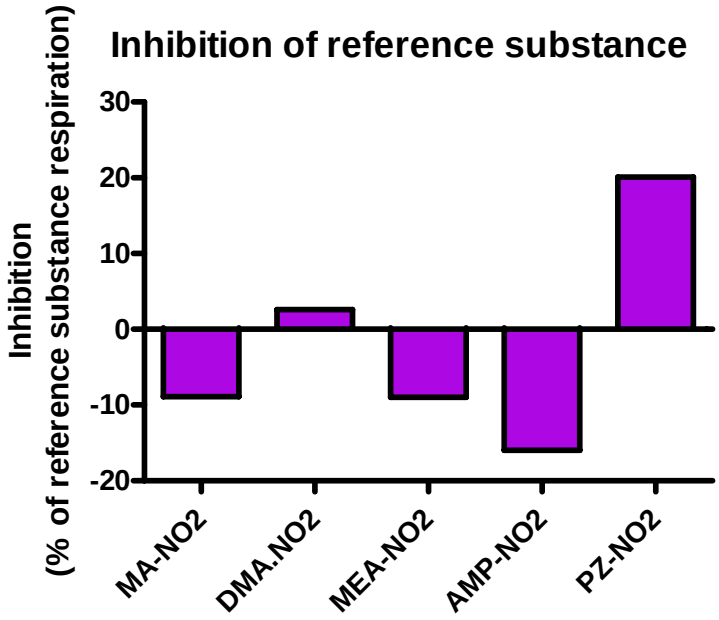


Figure G.10: **Inhibition of reference substance respiration caused by the individual nitramines. Values > 0 represent true inhibition, while values <0 represent stimulation of the reference substance respiration.**

Conclusions

If a test substance should be defined as ready biodegradable in the OECD 301 test a biodegradability of 60 % or more should be determined. Thus, none of the nitramines tested were defined as ready biodegradable.

G.2.4 Bioaccumulation

Bioaccumulation potential was estimated the log partition coefficient between octanol and water (logPow) by a structure-activity relationship (SAR) method, the KOWWIN version 1.67 of the Estimation Programs Interface (EPI) SuiteTM (US Environmental Protection Agency's Office of Pollution Prevention). CAS numbers or SMILES notation were used as input data for the estimations. The results are shown in Table G.5. Chemicals are defined as bioaccumulating if the logPow is higher than 3. However, the logPow of the nitramines ranged from -0.52 to -1.51, and none of the chemicals were therefore defined as bioaccumulating.

Table G.5: Estimated data for bioaccumulation of nitramines, based on calculations in EPISUITE (KOWWIN-program). Input data were either CAS-numbers or SMILES notation.

Nitramine	CAS no.	Log Kow	Input data
MA-NO2	598-57-2	-1.51	CAS no.
DMA-NO2	4164-28-7	-0.52	CAS no.
MEA-NO2	74386-82-6	-1.70	SMILES
AMP-NO2	1239666-60-4	-0.83	SMILES
PZ-NO2	42499-41-2	-1.15	SMILES

H Summary and Conclusion

H.1 Synthesis

Five nitramines; ethanolnitramine (MEA-NO2), methylnitramine (MA-NO2), dimethylnitramine (DMA-NO2), N-nitropiperazine (PZ-NO2) and 2-methyl-2-(nitroamino)-1-propanol (AMP-NO2) have been synthesized with an estimated purity >99%.

H.2 Chemical analysis

A method for determination of nitramines in water samples has been developed. Gaseous and aerosol-adsorbed nitramines are sampled isokinetically from an emission source and collected by an impinger-train filled with MQ water as absorber solution.

Water wash samples are prepared according to the method as the samples are received.

Nitramines are extracted by passing a water sample through a solid phase extraction (SPE) cartridge. The nitramines are eluted from the solid phase with a tailored blend of organic solvents.

The organic solvent extract is concentrated by evaporation. The nitramines are separated, identified, and measured by use of HPLC/HRMS. Nitramines eluting from the HPLC column are identified by comparing their high resolution mass spectra and retention times to reference spectra and retention times obtained by analytical standards. The concentration of each identified nitramine is measured by the standard addition method.

The method has been validated for water wash samples and aqueous impinger absorber solution. When known amounts of the five nitramines are added to real water wash samples, the recovery range is 15-80% with the following method limits of detection; MEA-NO2 0.5 µg/L, MA-NO2 2.1 µg/L, DMA-NO2 40 µg/L, AMP-NO2 0.3 µg/L, PZ-NO2 0.5 µg/L. The potential for recovery and sensitivity improvement is promising. An inter laboratory validation of the method has been performed with excellent results.

Six water wash samples provided by Company (ID-J,K,L,R,S,T) have been analysed with respect to the nitramines. The results obtained with the method show the presence of MEA-NO2 in five of the samples. The concentrations are measured to 26 µg/L for ID-K and 24 µg/L for the ID-L sample.

Simulated stack emissions of nitramines have been collected by impinger sampling with recovery in the range of 87-120%.

H.3 Toxicity data review

Toxicological data of six nitramines (DMA-NO2, MA-NO2, MEA-NO2, AMP-NO2, PZ-NO2 and diethanolnitramine) for acute and chronic exposure effects have been reviewed. Data on long term toxicity of DMA-NO2 and MA-NO2 showed that both compounds were carcinogenic with DMA-NO2 being more potent (based on TD50). No data were found on Diethanolnitramine, MEA-NO2, PZ-NO2 and AMP-NO2 for acute or chronic (long term) exposures effects (mutagenicity/carcinogenicity). None from 6 compounds had data available on reproductive toxicity.

Acceptable concentration levels of DMA-NO2 and MA-NO2 were estimated as Derived Minimal Effect Levels (DMELs), following REACH guideline and recommendations, and using non-threshold approach and semi-quantitative reference value. DMEL was provisionally estimated as 0.547x10-5 mg/kg for DMA-NO2 and 17.4x10-5mg/kg for MA-NO2. However, no information was found for toxicological data of Diethanolnitramine, MEA-NO2, PZ-NO2 and AMP-NO2 and thus no estimation of acceptable levels for these compounds could be made.

Due to the lacking information on general as well as specific (mutagenic /reproductive) toxicity we recommended that all chemicals should be thoroughly tested for reproductive toxicity, mutagenicity and carcinogenicity.

H.4 Acute toxicity, cytotoxicity, irritation, sensitization and corrosion

Six different methods were performed to assess acute oral toxicity *in vivo* and cytotoxicity, sensitization, skin and eye corrosion and irritation *in vitro* or *ex vivo*. For each of these assays, 2-5 of the test substances were tested. Four tests were performed in compliance with GLP.

Acute toxicity was performed according to OECD TG 425 Oral Toxicity up and down procedure on rats (OECD 2000, 2008) on all five test substances; DMA-NO2, MA-NO2, MEA-NO2, AMP-NO2 and PZ-NO2. Results showed that PZ-NO2, DMA-NO2, MA-NO2 and MEA-NO2 are harmful if swallowed. The order of toxicity (from highest to lowest) of the test substances is DMA-NO2 > MA-NO2 > MEA-NO2 > PZ-NO2. AMP-NO2 was considered either harmful or non-toxic (LD_{50} > 1600).

In vitro cytotoxicity was investigated using clonogenic (Plating efficiency) assay. Test is based on ability of viable cells to form colonies. All tested compounds showed low toxicity up to 0.3-0.5 mg/ml. AMP-NO2 and DMA-NO2 induced low cytotoxic effect for all concentrations tested whereas MA-NO2 and MEA-NO2 showed dose dependent cytotoxicity, with strong cytotoxic effect at concentrations of 5.5 and 3.7 mg/ml respectively.

Both acute toxicity as well as *in vitro* toxicity showed that the compounds had low cytotoxicity and can be considered as harmful. AMP-NO2 was the least toxic in both tests and MA-NO2 was more toxic than MEA-NO2. There was slight discrepancy in DMA-NO2 toxicity between *in vitro* and *in vivo* tests showing that DMA-NO2 had highest toxicity *in vivo* but not *in vitro*.

The skin irritation SkinEthic Laboratories RHE assay OECD TG439 showed that the mean tissue viability after treatment with DMA-NO2, MA-NO2, MEA-NO2 and AMP-NO2 was > 80 % for all test substances indicating that all test substances are non-irritants for skin.

The Corrositex test, an acellular (no living tissues involved) barrier model was also used as an *in vitro* toxicology test. The test is based on OECD guideline 435: *In Vitro* Membrane Barrier Test Method for Skin Corrosion (OECD, 2006a). The result showed that MA-NO2, AMP-NO2 and MEA-NO2 can be assigned to Packing Group as non-corrosives (category 2). DMA-NO2 was not compliable with the Corrositex system, and no classification is retrieved from this test.

Eye corrosion: Bovine Corneal Opacity and Permeability (BCOP) test was performed according to OECD TG 437 (2008) (OECD, 2009). The test was performed to evaluate the potential ocular irritancy of DMA-NO2, MA-NO2, MEA-NO2 and AMP-NO2 as measured by its ability to induce opacity and increase permeability in isolated calf corneas. All test substances showed irritation to the eye. DMA-NO2 was considered as a mild eye irritant. MA-NO2, MEA-NO2 and AMP-NO2 were all OECD classified as ocular corrosives or severe irritants.

Potential sensitization capacity of DMA-NO2, MA-NO2**,** MEA-NO2 and AMP-NO2, was evaluated by Skin sensitization test VITOSENS. Sensitization was measured by gene expression in human dendritic cells CD34-DC at IC 20 (toxicity of 20%). MA-NO2 and MEA-NO2 were shown to be non-sensitizing by this model. For test compounds DMA and AMP, no prediction was made since IC20 did not fulfill the criteria at time of experiment.

H.5 Genotoxicity and mutagenicity

In vitro genotoxicity/mutagenicity was investigated using two OECD validated methods; the Ames test (OECD 471) and Mammalian Gene Mutation assay (OECD 476). Additionally, the Comet assay, also known as the Single Cell Gel Electrophoresis assay, was used for measurement of DNA damage. This method is widely used as a screening test for potential genotoxicity and is presently under validation by ECVAM/JaCVAM. These tests detect several endpoints (mutations in different genes, DNA breaks, oxidised DNA lesions) and are performed on different biological systems (human, hamster and bacteria).

Ames test is performed in bacteria, and is recommended as first screening test for mutagenicity, although extrapolation to humans has some limitations. In our study DMA-NO2, AMP-NO2 and PZ-NO2 showed no evidence of mutagenic activity towards the *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 at the tested concentrations. Exposure to MA-NO2 induced mutagenicity towards the strain TA102. On the other hand, MA-NO2 did not induce mutagenic activity in the strains TA98, TA100, TA1535, and TA1537 at the tested concentrations. A clear mutagenic response was seen in the strains TA102 and TA1535 after the treatment with MEA-NO2, both with and without S9 mix. However, no mutagenic activity was found in TA98, TA100 and TA1537 strains with MEA-NO2 at the concentrations tested.

The Comet assay normally measures only DNA strand breaks, but in this study we also detected specific DNA lesions such as oxidized purines (especially 8-oxoGuanine) after exposure to DMA-NO2, MA-NO2 AND MEA-NO2. Our results indicated no significant genotoxic effect in terms of strand breakage (with the possible exception of the highest concentrations of MEA-NO2, where a tendency to higher DNA break levels was seen). For MA-NO2 significant increase in DNA breaks was observed, but this is most likely due to cytotoxicity. Similarly, no significant induction of base oxidation from these chemicals was found. For DMA-NO2, no genotoxic effects were observed.

The Mammalian Gene mutation assay (OECD TG 476) (OECD, 1997) was performed for detection of mutation in the HPRT gene in mammalian cells V79 after exposure to DMA-NO2 and MEA-NO2. In our study both test substances showed dose-dependent increases in mutant frequency. DMA-NO2 appeared to be more mutagenic compared to MEA-NO2.

It is not surprising that different assays give different results, since they target different genetic end-points. However, we can only compare DMA-NO2 and MEA-NO2 as these compounds were tested in all three tests. MA-NO2 was tested only in two assays, Ames test (positive) and the comet assay (negative). Finally, not much can be concluded from testing AMP-NO2 and PZ-NO2 as only Ames test with negative results was performed and clearly further testing is needed.

Based on our results DMA-NO2, MA-NO2 and MEA-NO2 should be considered as mutagenic compounds category 3: Substances which cause concern for man owing to possible mutagenic effects. We recommend further testing to confirm these findings and for more precise assessment of hazard. Any conclusion could not be made for evaluation of genotoxicity of AMP-NO2 and PZ-NO2 as results from one test do not provide sufficient information. Therefore further testing is recommended with larger panel of tests.

H.6 Ecotoxicity

Standard ecotoxicity testing includes a number of bioassays with organisms representing different trophic levels. For aquatic organisms these include phytoplankton (primary producers), invertebrates and vertebrates. Biodegradation is measured by a number a standard methods, as described in different OECD Guidelines, including Guideline 301 (Ready biodegradability) OECD 302 (inherent biodegradability).

Previous search in the public databases or scientific literature showed that no ecotoxicity or biodegradability data were available for any of the nitramines included in the current study.

The ecotoxicity testing of the nitramines into organisms from two different trophic levels showed that the acute toxicity of all the nitramines were low or moderate. The phytoplankton species *P. subcapitata* was more sensitive to the nitramines than the invertebrate *D. magna*. However, the latter test showed poor dose-response curves for two of the nitramines (MA-NO2 and MEA-NO2). However, due to the lack of available test substances it was not possible to re-test these nitramines.

The biodegradability tests showed that none of the nitramines were ready biodegradable. However, the MA-NO2 and MEA-NO2 was more biodegradable than the other nitramines, in accordance with the simple linear structures of these compounds.

Estimations of bioaccumulation potential showed that none of the nitrosamines were bioaccumulating.

Table H.1 Overview of all human/mammalian related toxicity tests

Lab	Assay	Toxicity	Validation	Endpoint/ Quantification	DMA-NO2	MA-NO2	MEA-NO2	AMP-NO2	PZ-NO2
SMU	Acute oral toxicity	Survival	OECD 425	LD 50	770	834	970	> 1600	1750
NILU	Plating efficiency	Cyto-toxicity	Based on OECD 476	Cytotoxicity at ~ 50 µg/ml	low	low	low	low	-
				Cytotoxicity at ~ 500 µg/ml	low	low	low	low	-
				Cytotoxicity at ~ 5000 µg/ml	low	high	high	low	-
VITO	RHE assay	Skin irritation	OECD 439	EU label (irritant or no irritant)	No	No	No	No	-
VITO	Corrositex	Skin corrosion	OECD 435	Corrosive potential	N/A	No	No	No	-
VITO	BCOP	Eye corrosion	OECD 437	OECD classified as ocular corrosive or severe irritant	No	Yes	Yes	Yes	-
VITO	Vitosens	Cyto-toxicity (PI staining)		IC 20	1.39	0.33	0.39	2.25	-
		Sensitisation		Sensitizer	N/A	No	No	N/A	-
VITO	Ames test	Muta-genicity	OECD 471	Mutagenic for TA 198	No	No	No	No	No
				Mutagenic for TA 100	No	No	No	No	No
				Mutagenic for TA 102	No	Yes	Yes	No	No
				Mutagenic for TA 1535	No	No	Yes	No	No
				Mutagenic for TA 1537	No	No	No	No	No
NILU	Comet assay	Geno-toxicity		DNA strand breaks and oxidised bases – S9	No	No	Equi vocal	-	-
		Genotoxicity		DNA strand breaks and oxidised bases + S9	No	No	Equi vocal	-	-
NILU	Mammal-ian Gene Mutation	Muta-genicity	OECD 476	Mutations - S9	Yes	-	Yes	-	-
				Mutations + S9	?	-	?	-	-

I Future testing

I.1.1 Chemical Analysis

The following points need to be followed up in further method development:

- Isotope labelled nitramines should be implemented
- The method should be tested and validated with real stack emission sampling
- The nitramine stability and sample integrity should be tested in real samples
- The potential interference at low pH during sample work up needs to be further explored
- The sample work up at low pH is preferred due to no recovery of PZ-NO2 at pH 6. How can PZ-NO2 be recovered in a further developed method?
- More sample clean-up steps should be implemented to reduce instrumental ion suppression and improve the detection limits.

I.1.2 Toxicity Testing

Although this study has provided a large amount of toxicological data, there is still a lot of information missing to be able to make any conclusions.

1. Genotoxicity/mutagenicity
    a. Finalisation: Most importantly, we would recommend finalising the screening for genotoxicity/ mutagenicity, which was started in this project, to have a complete set of at least four nitramines. In the future we consider it useful to have such a set of reference data to compare with, for any nitramine which might occur in a CCS process.
    b. Additional test: Additionally to the tests which has been performed in this project we propose further focus on OECD or ECVAM validated *in vitro* human/mammalian cells assays (such as *in vitro* micronucleus test, point mutation OECD 479, *in vitro* mammalian chromosome aberration test (OECD 473), additional specific DNA lesions (such as DNA adducts) by the comet assay and *in vitro* cell transformation assay (draft guideline EU B21) as alternatives to carcinogenicity assay (OECD 453), etc.).
    c. Photogenotoxicity: Literature reviews have shown that these compounds are likely to release photooxidative products; therefore this point should be addressed and the compounds should be tested for photogenotoxicity.
2. Cytotoxicity
    a. Estimation of LD50, LC50 using OECD validated NRU method and compare with Clonogenic and cell proliferation assays as well as to compare results with existing *in vivo* data.
3. Reproductive toxicity
    a. No data are available for reproductive toxicity on nitramines and therefore we recommend performing combined repeated dose toxicity (OECD 422) on 1-2 suitable nitramine(s).

J List of Abbreviations

AF	Assessment factor
AMP-NO2	2-methyl-2-(nitramino)-1-propanol
AP	apurinic/apyrimidinic
APCI (+)	Atmospheric Pressure Chemical Ionistaion in positive mode
BCOP	Bovine Corneal Opacity and Permeability
BMD	The Benchmark dose; BMD concept involves fitting a mathematical model to dose-response data. The BMD is defined as the dose causing a predetermined change in response.
BMD10	The Benchmark-dose associated with a 10% response (for tumours upon lifetime exposure after correction for spontaneous incidence, for other effects in a specified study)
BMDL10	Defined as the lower 95% confidence dose of a Benchmark-dose representing a 10% tumor response upon lifetime exposure, i.e. the lower 95% confidence dose of a BMD10.
BOD	Biological Oxygen Demand
CAS	Chemical Abstract Services
CB	Corrositex Breakthrough
CCM	CO2 Capture Mongstad
CCP	CO2 Capture Plant
CCS	CO2 Capture Storage
CDS	Chemical Detection System
DMA-NO2	Dimethylnitramine
DMEL	Derived Minimal-Effect Level; For non-threshold effects, the underlying assumption is that a no-effect-level cannot be established and a DMEL therefore expresses an exposure level corresponding to a low, possibly theoretical, risk, which should be seen as a tolerable risk.
DMEL	Derived Minimal Effect Levels
DNA	Deoxyribonucleic acid
DNEL	The derived no-effect level (DNEL) is the level of exposure to a substance above which humans should not be exposed
DO	dissolved oxygen concentrations
DPBS	Dulbecco's Phosphate Buffered Saline

EC	effective concentrations
EMEM	Eagle Minimal Essential Medium solution
EPI	Estimation Programs Interface
ES (-)	Electrospray Ionization in negative mode
ES (+)	Electrospray Ionization in positive mode
FP	Framework Programme
FPG	Formamidopyrimidine DNA glycosylase
GC/MS	Gas Chromatography combined with Mass Spectrometry
GC	Gas Chromatography
GLP	Good Laboratory Practice
HPLC/HRMS	Liquid chromatography combined with High Resolution Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HPRT	hypoxanthine guanine phosphoribosyl transferase
IVIS	*In Vitro* Irritation Score
LC50	Median lethal concentration of a toxic substance or radiation is the concentration required to kill half the members of a tested population after specified test duration
LD50	Median lethal dose of a toxic substance or radiation is the dose required to kill half the members of a tested population after specified test duration
logPow	log partition coefficient between octanol and water
MA-NO2	Methylnitramine
MCB	Mean Corrositex Breakthrough
MEA-NO2	Ethanolnitramine
MeOH	Methanol
MMS	methyl methane sulphonate
MS	Mass Spectrometry
MTT	3-(4,5-Dimethylthiazol-Z-yl)-2,5-diphenyltetrazolium bromide
NaCl	Common salt, Sediumchloride
NC	Negative Control
NOAEL	No Adverse Effect Level (NOAEL) - the highest dose with no toxic effects

OD	Optical density
OEL	Occupational exposure level
PC	Positive Control
PE	Plating efficiency assay
PI	propidium iodide
PZ-NO2	Piperazine nitramine / 1-nitroPZerazine/N-nitropiperazine
RGA	relative growth activity
RHE	Reconstructed human Epidermis
RT-qPCR	real time quantitative polymerase chain reaction
SAR	structure-activity relationship
SD	Standard Deviation
SDS	sodium dodecyl sulphate
SPE	solid phase extraction
TD10	The median toxic dose of a drug or toxin is the dose at which toxicity occurs in 10% of cases
TD50	The median toxic dose of a drug or toxin is the dose at which toxicity occurs in 50% of cases
TEA	Thermal Energy Analyser
ThOD	theoretical oxygen demand
TOF	Time-of-Flight
TWA	Time weight average
UPLC	Ultra-high Pressure Liquid Chromatography

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Appendix A

Examples of chromatograms, mass spectra and UV spectra

Examples of chromatograms, mass spectra and UV spectra

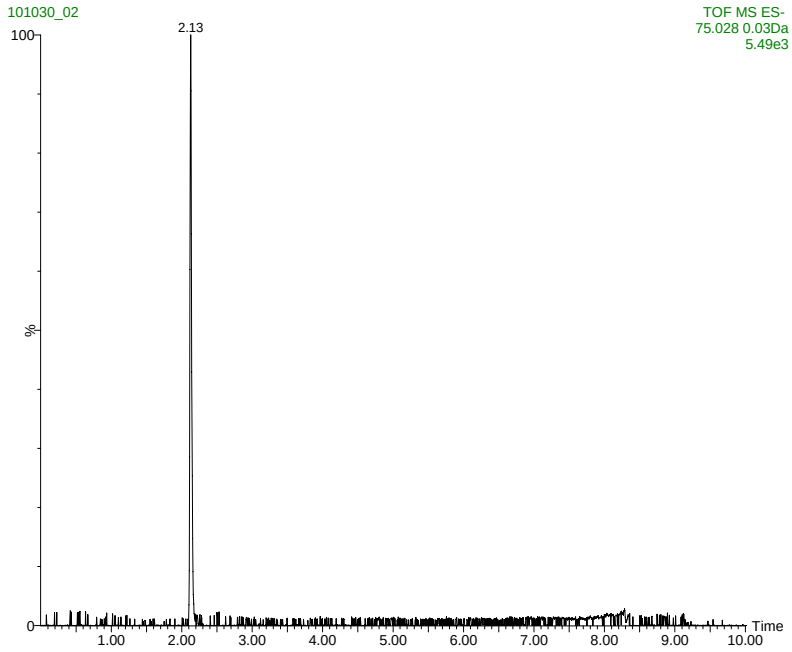


Figure 1: ES- chromatogram of MA-NO2 (0.2 ng injected) on Acquity HSS T3, 150 × 2.1 mm, 1.8 µm particle size.

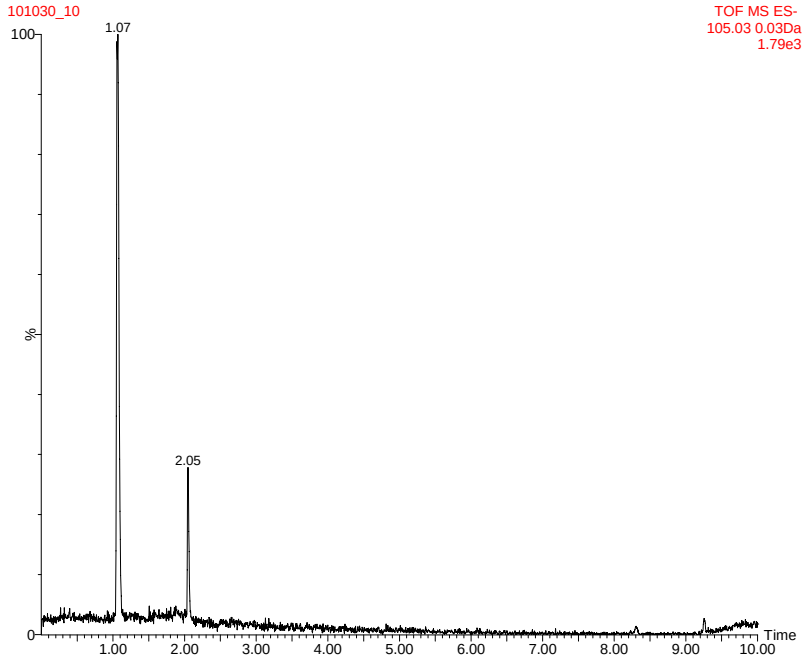


Figure 2: ES- chromatogram of MEA-NO2 (0.002 ng injected) on Acquity HSS T3, 150 × 2.1 mm, 1.8 µm particle size. Rt = 2.05 min.

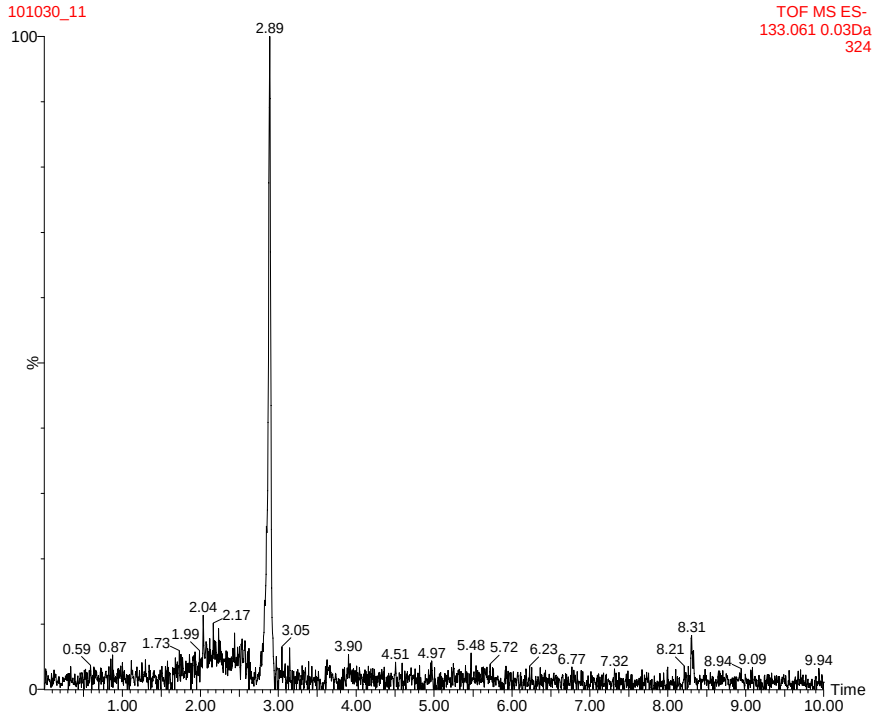


Figure 3: **ES- chromatogram of AMP-NO2 (0.001 ng injected) on Acquity HSS T3, 150 × 2.1 mm, 1.8 µm particle size.**

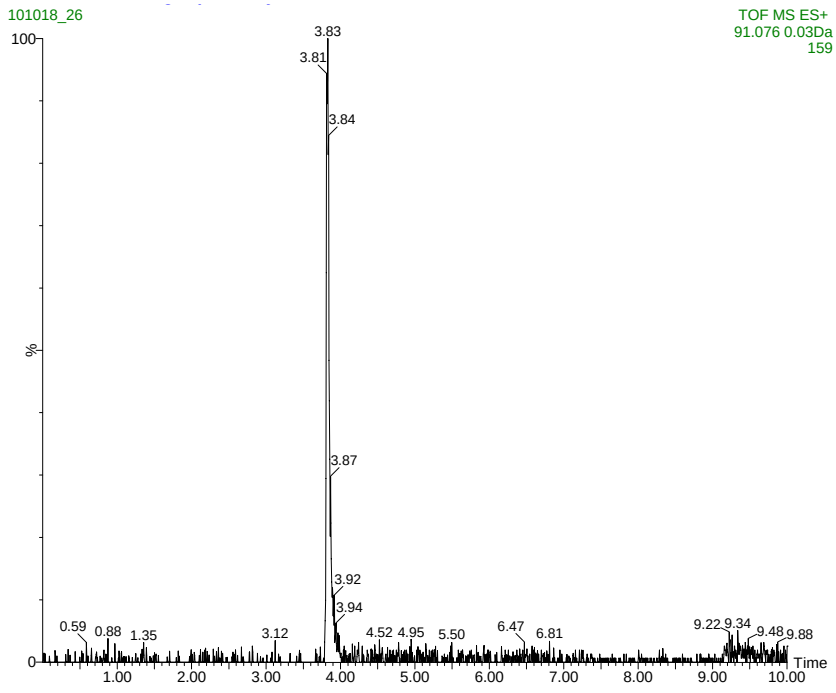


Figure 4: **ES+ chromatogram of DMA-NO2 (20 ng injected) on Acquity HSS T3, 150 × 2.1 mm, 1.8 µm particle size.**

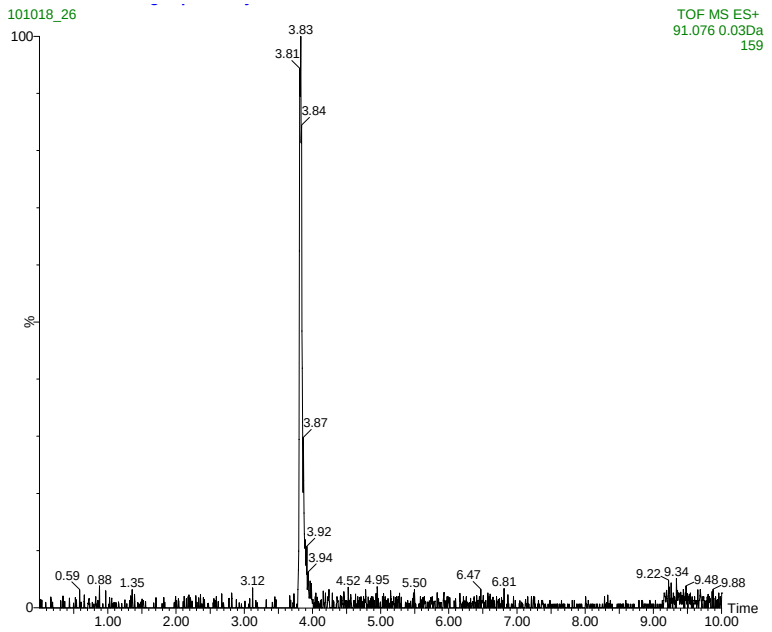


Figure 5: **ES+ chromatogram of DMA-NO2 (20 ng injected) on Acquity HSS T3, 150 × 2.1 mm, 1.8 µm particle size.**

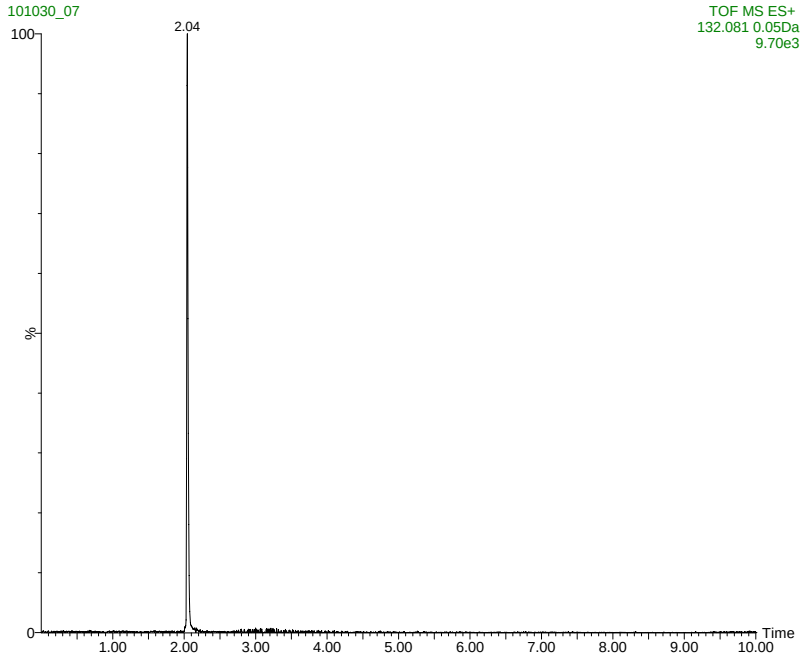


Figure 6: **ES+ chromatogram of PZ-NO2 (0.2 ng injected) on Acquity HSS T3, 150 × 2.1 mm, 1.8 µm particle size.**

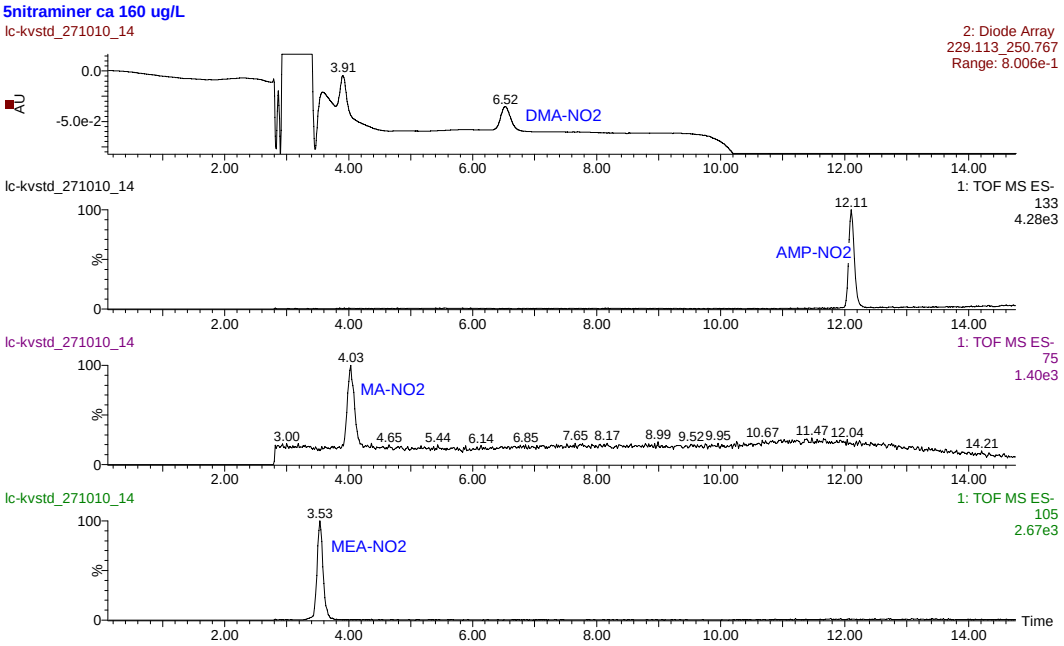


Figure 7: **Separation of the nitramines by the HPLC column Waters Atlantis dC18, 3µm, 2.1x150mm, and a binary water/acetonitrile gradient.**

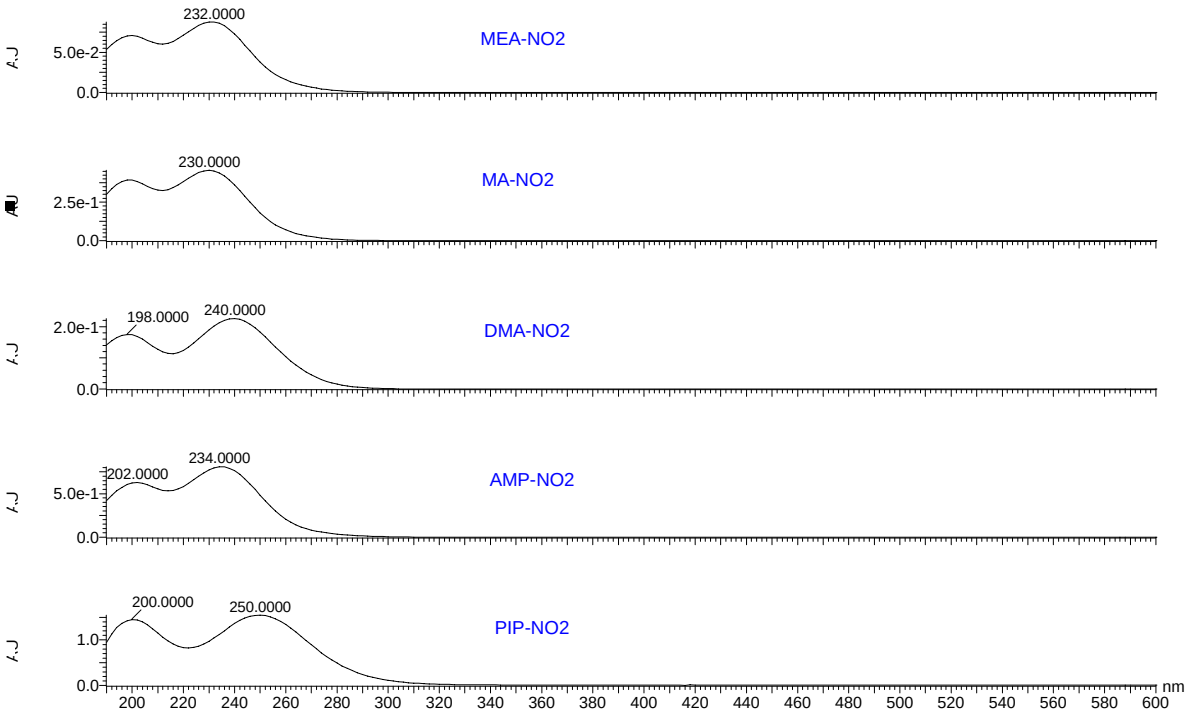


Figure 8: UV-spectra of the nitramines obtained by HPLC/UV.

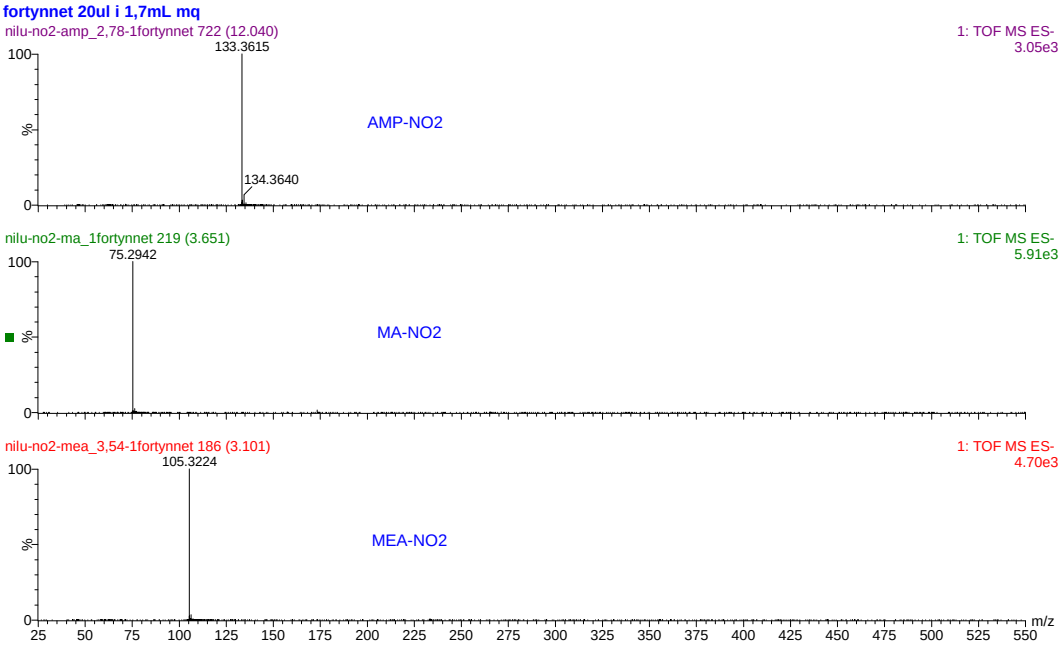


Figure 9: **HRMS spectra of the ions $[M-H]^-$ of MEA-NO2, MA-NO2 and AMP-NO2 obtained by ES- (LCT-Classic).**

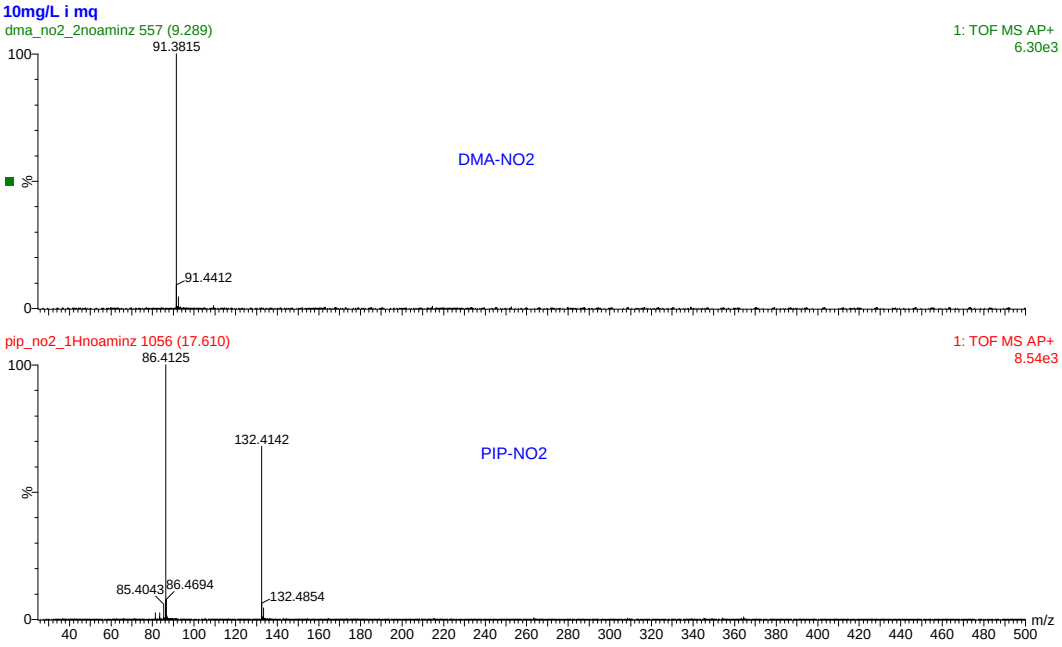


Figure 10: **HRMS spectra of the ions $[M+H]^+$ of PZ-NO2 and DMA-NO2 obtained by APCI+ (LCT-Classic). The m/z 86 is generated by NO2 loss from PZ-NO2.**

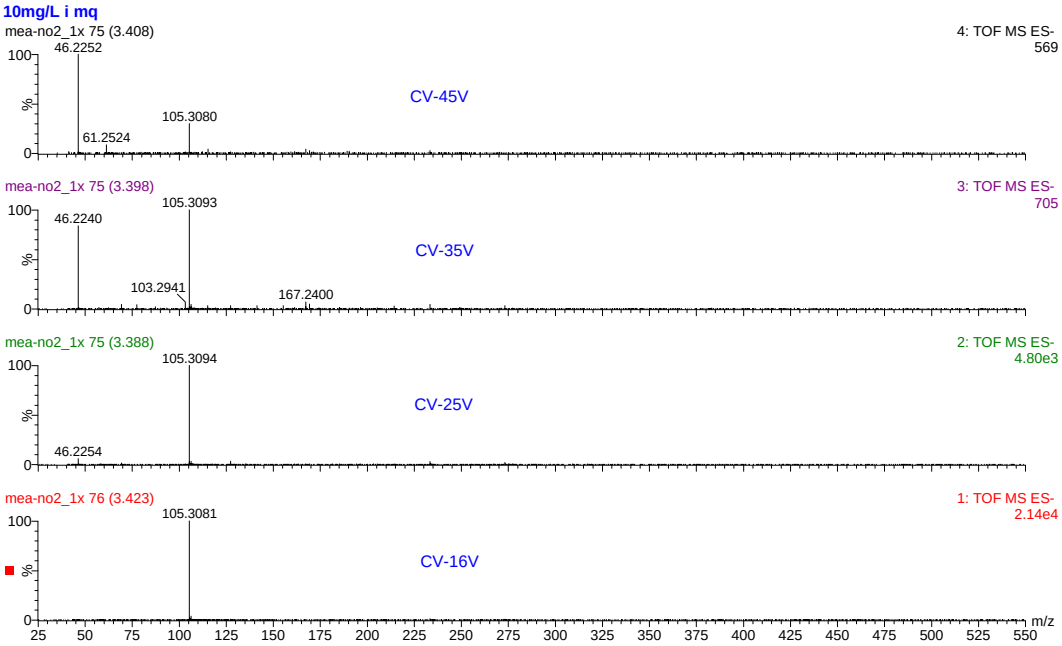


Figure 11: **Fragmentation pattern of HRMS spectra of $[M-H]^-$ of MEA-NO2 at denoted cone voltages obtained by LCT-Classic. The m/z 46 is generated by loss of the NO2 with increasing CV.**

Appendix B

Basis experiments in the method development

Basis experiments in the method development

Detection of nitramines

The sensitivity of relevant analytical detectors has been tested; e.g mass spectrometry (MS) combined with GC and LC. The Thermal Energy Detector (TEA) combined with GC has been tested. UV/visible spectroscopy has been tested. Four ionization modes in LC/MS have been tested, i.e. atmosperic pressure chemical ionization APCI (positive and negative mode) and electrospray ionization ES (positive and negative mode).

Table 1: The sensitivity of relevant analytical detectors. The useful techniques are highlighted with green color.

	ng inj/(S/N)-	ng inj-S/N)	ng inj-S/N)	ng inj-S/N)	ng inj-S/N)	Instrumentation	Chromatography
	MA-NO2	MEA-NO2	AMP-NO2	DMA-NO2	PZ-NO2	LCT-Premier	Waters, HSS T3
ES- $[M-H]^-$	0.2 200	0.002 15	0.001 30	20 ND	20 ND	LCT-Premier	Waters, HSS T3
ES+ $[M+H]^+$	20 ND	20 ND	20 ND	20 36	0.2 430	LCT-Premier	Waters, HSS T3
APCI+ $[M+H]^+$ $[M+ACN]^+$	20 ND	20 ND	20 ND	20 30	20 58	LCT-Premier	Waters, HSS T3
APCI- $[M-H]^-$	20 180	0.2 3	0.1 11	20 ND	20 ND	LCT-Premier	Waters, HSS T3
ES- $[M-H]^-$	0.18/3	0.03/3	0.01/3	IS	IS	LCT-Classic	Waters, Atlantis
ES+ $[M+H]^+$	IS	IS	IS	IS	IS	LCT-Classic	Waters, Atlantis
APCI+ $[M+H]^+$	IS	IS	IS	1/3	PP	LCT-Classic	Waters, Atlantis
APCI- $[M-H]^-$	ND	ND	ND	ND	ND	LCT-Classic	Waters, Atlantis
UV*	0.05/3	0.05/3	0.05/3	0.05/3	PP	LCT-Classic	Waters, Atlantis
GC/TEA	L	L	L	L	L		
GC/MS(EI)	IS	IS	IS	IS	IS		

*:(220-240nm)-(300-400nm)

ND: Not detected
IS: Inadequate sensitivity
PP: Inadequate sensitivity due to poor peakshape. Adequate instrumental sensitivity.
L: Promising, some issues needs to be sorted out.

Experimental results:

LC/MS is an analytical tool with adequate sensitivity for the services in this project. The physical and chemical propertiea of the nitramines make it necessary to use one application for the nitramines of primary amines (negative mode), and one application for the nitramines of secondary amines (positive mode).

GC/TEA is a complementary but less specific technique, and more columns need to be tested. It looks like the high injector temperature may introduce some issues, on-column injection should be explored. GC/MS with EI is a less sensitive method, chemical ionisation should be explored.

To avoid chemical analysis overloading (due to the need for several detection methods) the experiments which are intended to provide basic analytical data will be accomplished by MS detection in negative mode (primary amines) combined with the UV-trace for dimethylnitramine and PZ-NO2.

Chromatograpic separation of nitramines

The chromatographic properties of nitramines have been explored by testing several columns in high performance liquid chromatography (HPLC) comprising both reversed phase and normal phase. Capillary gas chromatography (GC) has also been tested.

Table 2: **The chromatographically properties of selected columns The useful columns are highlighted with green color.**

	Rt./symmetry	Rt./symmetry	Rt./symmetry	Rt./symmetry	Rt./symmetry	
Column	**MA-NO2**	**MEA-NO2**	**AMP-NO2**	**DMA-NO2**	**PZ-NO2**	Gradient
HSS T3 10 cm	1.18 / fronting, broad	1.01 / fronting, broad	1.91 / tailing, narrow	1.58 / symmetrical, narrow	1.06 / Tailing, broad	UPLC, Water/ACN 98/2
HSS T3 15 cm	2.13 Symmetrical, narrow	2.05 / symmetrical, narrow	2.89 / symmetrical, narrow	3.83 / tailing, narrow	2.04 / symmetrical, narrow	UPLC, Water/ACN 99.9/0.1
BEH Shield RP18	0.67 / tailing, broad	0.46 / tailing, broad	1.20 / Peak split, broad	1.15 / Symmetrical, narrow	1.31 / symmetrical, narrow	UPLC, Water/ACN 98/2
BEH Phenyl	0.58 / tailing, broad	0.56 / symmetrical, broad	1.45 / Fronting, broad	1.29 / Symmetrical, narrow	2.44 / Symmetrical, broad	UPLC, Water/ACN 98/2
BEH HILIC	0.61 / tailing, narrow	0.64 / tailing, narrow	0.63 / tailing, narrow	NA	NA	UPLC, Water/ACN 5 / 95
ATLANTIS,d-C18	4.0/ symmetric	3.5 symmetric	12.1 symmetric	6.5 symmetric	11.9 Fronting,tailing, broad	HPLC Water/ACN
ATLANTIS,C18-T3	Scheduled for testing	Scheduled for testing	Scheduled for testing	Scheduled for testing	Scheduled for testing	HPLC Water/ACN
ZB-1,				4.34 symmetric		GC/TEA
DB-5	11.2 Some tailing	6.1 Some tailing	13.5 Some tailing	5.8 symmetric	12.5 Some tailing	GC/FID

ND: Not detected. NA: Not applicable

Examples of chromatograms are given in appendix A.

Experimental results:

UPLC/HRMS: A 15 cm HSS T3 column gave best retention and peak shape for all investigated compounds. Maximum sensitivity was achieved in negative electrospray (ES-) mode for MA-NO2, MEA-NO2 and AMP-NO2, and positive electrospray (ES+) mode for PZ-NO2. DMA-NO2 gave very low sensitivity in all ionization modes.

HPLC/HRMS: A 15 cm Waters Atlantis gave retention on MEA-NO2, MA-NO2, DMA-NO2 and AMP-NO2. The peak shape of PZ-NO2 was poor, and a new column will be tested. As a consequence, some of the least critical experiments will not include PZ-NO2. It is possible to derivatize the amino-group of PZ-NO2 and to obtain nice peak shape.

GC chromatography needs more testing to sort out some issues.

Solid phase (SPE) extraction of nitramines

Several experiments have been performed to test the retention properties of SPE adsorbents as a tool for water wash sample work up. The following SPE-adsorbents have been tested:

- IST Isolute ENV+, 6mL, 200mg, No. 915-0020-C.
- Phenomenex Strata-X, 6mL, 200mg, No. 8B-S100-FCH
- J.T.Baker Bakerbond Carbon, 6 mL, 1000mg, No. 7575-07
- Waters Oasis HLB, 6mL, 200mg, No. WAT106202

SPE experiment 1

In the first experiment the adsorbents were conditioned in a classic manner by a sequential wash with 5 mL methanol and 5 mL water (or 5 mL acetonitrile and 5 mL water). Two samples were prepared, each with a nitramine concentration of ~800 µg/L. One of the samples was prepared with pure MQ water and the other sample was prepared with acidified water (0.001M HCl). 10 mL of the sample was drawn through the adsorbent, and the SPE effluent water was collected for later analysis. The nitramines were eluted off the adsorbent by 2x3 mL of the solvent which was used for conditioning the adsorbent. The solvent was evaporated to dryness by gently blowing purified N_2 at 30°C. The samples were re-suspended in 10 mL water before analysis. The analysis results are given as per-cent of the initial nitramine concentration, and the residue on the adsorbents are estimates based on the nitramine mass balance.

Table 3: Results obtained by the SPE-experiment 1.

		mea-no2	ma-no2	dma-no2	amp-no2
			ACN for prep		MQ as Solvent
Carbon	Breakthrough	49	65	0	34
	Recovery	12	7	52	40
	Estim. residue on ads.	39	28	48	26
Strata X	Breakthrough	138	127	1	0
	Recovery	6	29	82	152
	Estim. residue on ads.	0	0	17	0
HLB	Breakthrough	140	125	0	0
	Recovery	15	55	9	112
	Estim. residue on ads.	0	0	91	0
ENV+	Breakthrough	73	35	0	0
	Recovery	100	130	60	139
	Estim. residue on ads.	0	0	40	0
			MeOH for prep		MQ as Solvent
Carbon	Breakthrough	43	40	0	11
	Recovery	16	17	11	42
	Estim. residue on ads.	41	43	89	47
Strata X	Breakthrough	86	100	0	0
	Recovery	6	27	69	115
	Estim. residue on ads.	8	0	31	0
HLB	Breakthrough	124	92	0	0
	Recovery	15	55	72	125
	Estim. residue on ads.	0	0	28	0
ENV+	Breakthrough	57	28	0	0
	Recovery	73	97	73	110
	Estim. residue on ads.	0	0	27	0
			ACN for prep		HCl(aq)pH3
Carbon	Breakthrough	64	104	0	38
	Recovery	18	7	51	38
	Estim. residue on ads.	18	0	49	24
Strata X	Breakthrough	208	178	0	0
	Recovery	21	64	81	156
	Estim. residue on ads.	0	0	19	0
HLB	Breakthrough	201	172	0	0
	Recovery	26	58	70	125
	Estim. residue on ads.	0	0	30	0
ENV+	Breakthrough	142	81	0	5
2	Recovery	97	125	88	141
	Estim. residue on ads.	0	0	12	0
			MeOHfor prep		HCl(aq)pH3
Carbon	Breakthrough	87	87	0	36
	Recovery	14	16	1	44
	Estim. residue on ads.	0	0	99	20
StarataX	Breakthrough	122	141	0	0
	Recovery	11	32	70	93
	Estim. residue on ads.	0	0	30	7
HLB	Breakthrough	211	142	0	0
	Recovery	24	51	74	104
	Estim. residue on ads.	0	0	16	0
ENV+	Breakthrough	97	47	2	1
	Recovery	45	63	62	69
	Estim. residue on ads.	0	0	36	30

Experimental results from SPE-experiment 1:

The most promising adsorbent is activated carbon combined with a neutral aqueous sample. The nitramine retention needs to be improved. All adsorbents except carbon seem to introduce positive artefacts by boosting the MS-signal.

SPE experiment2

In order to optimise the nitramine retention the SPE conditioning procedure was modified. The new procedure for the carbon adsorbent was sequential wash with 5mL n-pentan, 5mL ethyl acetate, 2x5mL methanol, 4x5mL tap water, and the new procedure for the HLB adsorbent was sequential wash with 2mL n-pentan, 2mL ethyl acetate, 2x5 mL methanol, 2x5mL tap water. 10mL water sample was applied (~800µg/L), and the adsorbent was washed with 1 mL MQ water. The nitramines were eluted off the adsorbent by using 2x0.5 mL methanol and then 3x5 mL dichloromethane. The solvent was evaporated to dryness by gently blowing purified N_2 at 30°C, and the sample was re-suspended in 10mL water before analysis.

Table 4: Results obtained by the SPE-experiment 2.

		mea-no2	ma-no2	dma-no2	amp-no2
				MQ as solvent	
Carbon	Breakthrough	7	9	0	2
	Recovery	0	0	95	5
	Est.residue on ads.	93	91	5	93
				HCl as solvent	
Carbon	Breakthrough	9	13	0	2
	Recovery	1	0	84	4
	Est.residue on ads.	90	87	16	94
				MQ as solvent	
HLB	Breakthrough	87	86	1	9
	Recovery	0	3	95	77
	Est.residue on ads.	12	11	4	14
				HCl as solvent	
HLB	Breakthrough	66	46	0	0
	Recovery	19	8	0	85
	Est.residue on ads.	15	46	100	15

Experimental results from SPE-experiment 2:

A significant improvement in the nitramine retention was achieved for the carbon adsorbent. However, the solvent elution strength needs to be improved. The HLB adsorbent is not suited for adsorbtion of nitramines.

SPE experiment 3

Five carbon columns were conditioned with sequential wash with 5 mL n-pentan, 5 mL ethyl acetate, 2x5 mL methanol, 4x5 mL tap water. 10 mL acidified water sample (0.001M HCl) was applied (~800 µg/L), and the adsorbent was washed with 2x3 mL MQ water. The nitramines were eluted off the adsorbent by using the following procedures:

Method 1. Dry the adsorbent by drawing air for 15 minutes. Eluate with 3x3 mL methanol (5min. break keeping wet) +3x5mL DCM (5+3+1 min. break keeping wet). Add 500 µl MQ, evaporate to 0.5 mL by gently blowing purified N_2 at 30°C,. Re-suspend to 10 mL with water. Analyse.

Method 2. Dry the adsorbent by drawing air for 15 minutes. Eluate with 3x3 mL methanol (5min. break keeping wet) +3x5mL ethyl acetate (5+3+1 min. break keeping wet). Add 500 µl MQ, evaporate to 0.5 mL by gently blowing purified N_2 at 30°C,. Re-suspend to 10 mL with water. Analyse.

Method 3. Dry the adsorbent by drawing air for 15 minutes. Eluate with 3x3 mL methanol (5min. break keeping wet) +3x5mL ethyl acetate:DCM 1:1 (5+3+1 min. break keeping wet). Add 500 µl MQ, evaporate to 0.5 mL by gently blowing purified N_2 at 30°C,. Re-suspend to 10 mL with water. Analyse.

Method 4. Dry the adsorbent by drawing air for 15 minutes. Eluate with 3x3 mL methanol (5min. break keeping wet) +3x5mL 95% DCM5% methanol (5+3+1 min. break keeping wet). Add 500 µl MQ, evaporate to 0.5 mL by gently blowing purified N_2 at 30°C,. Re-suspend to 10 mL with water. Analyse.

Method 5. Dry the adsorbent by drawing air for 15 minutes. Eluate with 3x3 mL methanol (5min. break keeping wet) +3x5mL 95% ethyl acetate5% methanol (5+3+1 min. break keeping wet). Add 500 µl MQ, evaporate to 0.5 mL by gently blowing purified N_2 at 30°C,. Re-suspend to 10 mL with water. Analyse.

Table 5: Recovery results obtained in SPE experiment 3, using five different elution strengths of the solvents

	mea-no2	ma-no2	dma-no2	amp-no2
Method 1	7	0	44	59
Method 2	8	4	43	72
Method 3	6	4	39	72
Method 4	9	5	40	59
Method 5	10	5	42	88

Experimental results from SPE-experiment 3:

The best elution strength is obtained by method 5. More experiments are needed to improve the solvent strength.

Reactivity of nitramines

An experiment for testing the nitramine reactivity in aqueous systems towards potential matrix compounds such as alcohols, amines, carbonyls and carboxylic acids, has been initiated. The specific matrix compounds and concentrations are given in the table footer. The nitramine concentration in the experiments is ~80 µg/L.

Table 6: Reactivity vs matrix compounds in MQ-water. Nitramine level is given in % of the first day theoretical value.

Matrix compounds	Nitramine	Rel. resp. day1	Rel. resp. day4	Rel. resp. Day14	Rel. resp. Day 42
Alcohols	MA-NO2	142	88	75	68
Carbonyls		144	94	74	75
Carb. acids		98	84	61	58
Amines 1		131	83	63	51
Amines 2		175	111	90	73
All in mix		131	104	104	74
Alcohols	MEA-NO2	96	108	63	99
Carbonyls		89	98	86	85
Carb. acids		63	64	50	54
Amines 1		91	81	63	67
Amines 2		2	8	49	90
All in mix		2	2	0	0
Alcohols	AMP-NO2	125	90	91	110
Carbonyls		132	94	96	118
Carb. acids		123	104	95	117
Amines 1		132	85	96	108
Amines 2		119	81	81	106
All in mix		76	77	82	105
Alcohols	DMA-NO2	107	91	68	23
Carbonyls		109	91	69	22
Carb. acids		110	98	68	23

Matrix compounds	Nitramine	Rel. resp. day1	Rel. resp. day4	Rel. resp. Day14	Rel. resp. Day 42
Amines 1		105	94	24	22
Amines 2		110	92	29	22
All in mix		108	92	intf	23

Alcohols: Methanol, Ethanol (200mg of each/L I MQ)
Carbonyls: Formaldehyde, acetaldehyde, acetone (200mg of each/L I MQ)
Carb. Acids: Formic acid, acetic acid (200mg of each/L I MQ)
Amines 1: methylamine, dimethylamine, (200mg of each/L I MQ)
Amines 2: MEA, AMP, MDEA, PZerazin (200mg of each/L I MQ)
All in mix: A mixture (1:1:1:1:1) of the above mentioned matrix solutions, each at a concentration of 40mg/L.

Table 7: **Reactivity vs matrix compounds in impinger solution. Nitramine level is given in % of the first day theoretical value.**

Matrix compounds	Nitramine	Rel. resp. day1	Rel. resp. day14	Rel. resp. Day 42
Alcohols	MA-NO2	31	72	
Carbonyls		0	56	
Carb. acids		0	49	
Amines 1		0	78	
Amines 2		23	74	
All in mix		9	56	
Alcohols	MEA-NO2	0	10	Intf
Carbonyls		0	6	Intf
Carb. acids		6	7	Intf
Amines 1		14	8	Intf
Amines 2		18	9	Intf
All in mix		11	7	Intf
Alcohols	AMP-NO2	119	75	
Carbonyls		102	79	
Carb. acids		114	88	
Amines 1		108	88	
Amines 2		106	94	
All in mix		103	79	
Alcohols	DMA-NO2	25	0	Intf
Carbonyls		86	63	Intf
Carb. acids		106	72	Intf
Amines 1		0	70	Intf
Amines 2		95	68	Intf
All in mix		98	67	Intf

Alcohols: Methanol, Ethanol (200mg of each/L I impingerløsning)
Carbonyls: Formaldehyde, acetaldehyde, acetone (200mg of each/L I impingerløsning)
Carb. Acids: Formic acid, acetic acid (200mg of each/L I impingerløsning)
Amines 1: methylamine, dimethylamine, (200mg of each/L I impingerløsning)
Amines 2: MEA, AMP, MDEA, PZerazin (200mg of each/L I impingerløsning)
All in mix: A mixture (1:1:1:1:1) of the above mentioned matrix solutions, each at a concentration of 40mg/L.

Experimental results from the reactivity experiments:

Typical CCS matrix compounds seem to impair the storage stability of nitramines. CCS samples need to be handled as fresh samples.

pH stability of nitramines

An experiment for testing the stability of the nitramines as a function of pH and time has been initiated. A known amount of the nitramines (~400 µg/L) were prepared at the given pH values and the solutions were analysed at day 1 and later at the given day number. The given pH was prepared as follows: 0.01M HCl in water (pH 2.07), 0.1% acetic acid in water (pH 3.13), MQ water (pH 6.00), 0.1% sodiumhydrogencarbonat in water (pH 8.47), 0.1% ammoniumhydroksid in water (pH 10.55).

Table 8: Reactivity vs pH in MQ-water. Nitramine level is given in % of the first day theoretical value.

pH*	Nitramine	Rel. resp. day1	Rel. resp. day 6	Rel. resp. day 13	Rel. resp. Day 42
10.55	MA-NO2	0*	1*	52*	56
8.47		0*	0*	0*	0
6.00		102	99	59	83
3.13		90	83	55	85
2.07		94	56	43	50
10.55	MEA-NO2	1*	0*	54*	70
8.47		0*	0*	0*	0
6.00		78	94	79	90
3.13		33	40	28	36
2.07		68	54	43	56
10.55	AMP-NO2	89	57	84	89
8.47		100	0?	73	86
6.00		108	76	80	94
3.13		111	73	81	107
2.07		104	76	77	119
10.55	DMA-NO2	100	92	70	88
8.47		100	91	69	85
6.00		104	74	68	83
3.13		107	91	68	86
2.07		108	74	70	88

*: Analytical disturbances for early eluting peaks

Experimental results from the pH experiments:

Low pH may impair MEA-NO2 and MA-NO2. A general tendency is that storage degrades the nitramines.

Storage stability of nitramines

An experiment for testing the storage stability of nitramines have been initiated. The nitramines are dissolved (~800µg/L) in the following solvents; water, dichloromethane, impinger solution, acetonitrile and methanol. The nitramine solution is stored at -18°C, 4°C and 24°C, and aliquots of the samples are analysed as a function of time (days). The results are given in per cent of the calculated concentrations of day 1.

Table 9: Stability vs temperature in MQ-water

Temperature	Nitramine	Rel. resp. day1	Rel. resp. day14	Rel. resp. Day42
-18°C	MA-NO2	103	92	77
4°C		103	75	71
24°C		103	70	71
-18°C	MEA-NO2	108	99	102
4°C		108	94	99
24°C		108	89	10
-18°C	AMP-NO2	110	100	139
4°C		110	87	128
24°C		110	93	114
-18°C	DMA-NO2	102	*Intf	96
4°C		102	71	87
24°C		102	71	96

*: Interference due to UV disturbance.

Table 10: Stability vs temperature in DCM

Temperature	Nitramine	Rel. resp. day1	Rel. resp. day14	Rel. resp. Day42
-18°C	MA-NO2	65	23	21
4°C		65	20	18
24°C		65	19	17
-18°C	MEA-NO2	98	77	83
4°C		98	81	86
24°C		98	80	88
-18°C	AMP-NO2	96	84	113
4°C		96	83	104
24°C		96	98	114
-18°C	DMA-NO2	*intf	*intf	*intf
4°C		*intf	*intf	*intf
24°C		*intf	*intf	*intf

*: Interference due to UV disturbance.

Table 11: Stability vs temperature in impinger

Temperature	Nitramine	Rel. resp. day1	Rel. resp. day14	Rel. resp. Day42
-18°C	MA-NO2	80	56	58
4°C		80	45	50
24°C		80	51	43
-18°C	MEA-NO2	48	51	79
4°C		48	32	65
24°C		48	29	49
-18°C	AMP-NO2	109	83	129
4°C		109	75	120
24°C		109	81	110
-18°C	DMA-NO2	107	67	Intf.
4°C		107	67	96
24°C		107	69	91

Table 12: Stability vs temperature in acetonitrile

Temperature	Nitramine	Rel. resp. day1	Rel. resp. day14	Rel. resp. Day42
-18°C	MA-NO2	91	54	78
4°C		91	45	75
24°C		91	46	86
-18°C	MEA-NO2	96	61	95
4°C		96	69	95
24°C		96	69	91
-18°C	AMP-NO2	114	88	117
4°C		114	87	111
24°C		114	95	115
-18°C	DMA-NO2	86	*intf	101
4°C		86	59	90
24°C		86	56	90

*: Interference due to UV disturbance.

Table 13: Stability vs temperature in methanol

Temperature	Nitramine	Rel. resp. day1	Rel. resp. day14	Rel. resp. Day42
-18°C	MA-NO2	106	59	66
4°C		106	57	79
24°C		106	61	69
-18°C	MEA-NO2	129	69	94
4°C		129	73	97
24°C		129	74	94
-18°C	AMP-NO2	107	92	100
4°C		107	92	108
24°C		107	84	115
-18°C	DMA-NO2	86	56	80
4°C		86	57	78
24°C		86	56	71

Experimental results from the storage stability experiments:

A general tendency is that storage impairs the nitramines for all the tested solvents. MQ water seems to be the best suited solvent for storage with the frozen sample (-18°C) as the most stabilizing temperature. However, the results are not unequivocal. No degradation products have been observed so far in this work (table D.6), and some stock solutions have also been analyzed with respect to occurrence of the corresponding amine - without any positive identification. This leaves adsorption to the glass wall as the most likely explanation of the observed effects. The glass wall effect hypothesis is supported by the abrupt loss in the first day(s) of the experiments. In table 8 the low values for the primary nitramines may be explained by the high pH which will cause early eluting peaks to flush through the column with the void volume. Glass wall effects are unpredictable and may be more pronounced in clean samples (i.e. standards) than real samples having matrix constituents that may serve as protecting agents.

Liquid/liquid extraction of nitramines

A water sample (MQ) was prepared by adding nitramines to a concentration of 1mg/L. The spiked 10 mL water sample was transferred to a separation funnel. Liquid/liquid extraction was performed by adding 2.5 mL organic solvent and shaking the mixture vigorously for one minute. After separating the phases (30 minutes) the extraction process was repeated twice. After three extractions the water phase was analyzed with respect to the remaining nitramine concentration. The result obtained by liquid/liquid extraction with four different organic solvents are given in table 14.

Table 14: Liquid/liquid extraction of nitramines in a selection of solvent/water systems.

Exp. No.	Organic solvent	Remaining MEA-NO2 in water (%)	Remaining MA-NO2 in water (%)	Remaining DMA-NO2 in water (%)	Remaining AMP-NO2 in water (%)	Remaining PZ-NO2 in water (%)
1	Dichloromethane	95	87	4	94	39
2	Diethylether	99	85	133	95	65
3	Ethylacetat	71	56	24	50	63
4	Hexane	92	85	101	117	86

Experimental results: None of the tested solvents are suitable for liquid/liquid extraction of the nitramines due to poor extraction efficiency. Hexane may possibly be used to clean out lipid molecules from the aqueous phase.

Nitramine solubility in water

A saturated solution of the nitramin was prepared by adding the crystalline nitramine to water in great excess and carefully heating the solution with stirring to 40°C. The solution was stored in room temperature (24°C) over night and an aliquot of the liquid phase was diluted in water for chemical analysis. The concentration of the saturated solution is given in table 15.

MEA-NO2 is an oily liquid at room temperature with high water solubility (>200g/L), thus the maximum solubility was difficult to obtain.

The equilibrium between solid state and dissolved MA-NO2 was difficult to establish. The color of the concentrated solution turned yellowish while the crystalline is white. The water solubility is high (>200g/L) but a reliable limit was difficult to obtain for MA-NO2.

Table 15: The measured water solubility of nitramines in water

Nitramine	MEA-NO2 (g/L)	MA-NO2 (g/L)	DMA-NO2 (g/L)	AMP-NO2 (g/L)	PZ-NO2 (g/L)
Water solubility	>200	>200	77	238	128

Sampling strategies of nitramines

Impinger sampling with absorption solution will have the first priority as given by CCM.

Two impinger bottles (500 mL) were filled with 380 mL MQ water and 20 mL CCM water wash ID-L. The bottles were connected in series and placed in an ice bath. A syringe with 250 µL nitramine solution (80mg/L in methanol) was connected to a syringe pump and the needle was placed into the impinger inlet. Close to (and beneath) the impinger inlet a beaker with hot water was placed on a heater and covered with aluminum foil so that the water vapor was easily mixed with the impinger air sample. A pump with a gas meter was connected

downstream to the impinger. The sampling was performed at an air flow rate of 3.4 L/min with the syringe pump set to 4 µL/min. The sampling duration was 90 minutes. The recovered nitramine concentration in the first bottle is given in table 16.

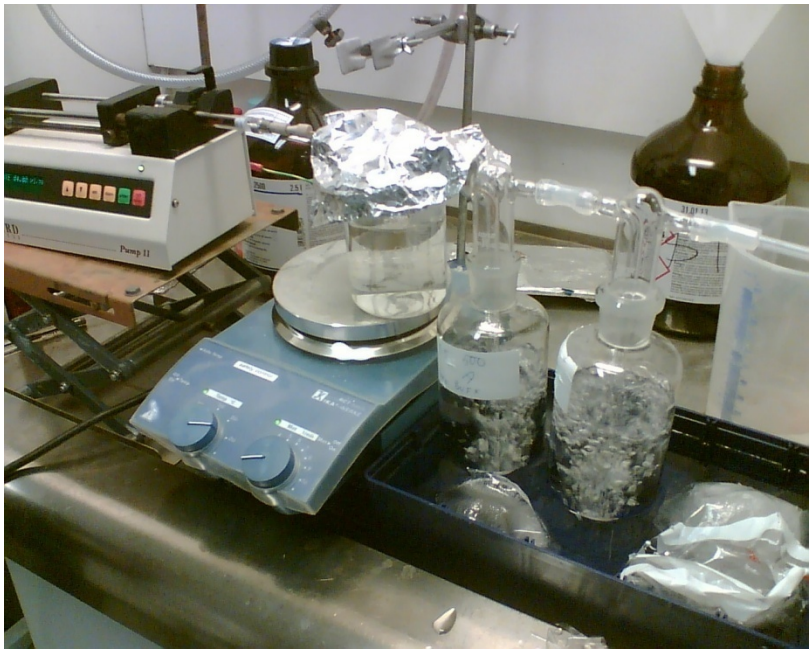


Figure 1: Impinger sampling set up in the spiking experiments

Table 16: Per cent recovered nitramines in the first impinger bottle.

Exp. No.	MEA-NO2 (µg/L)	MA-NO2 (µg/L)	DMA-NO2 (µg/L)	AMP-NO2 (µg/L)	PZ-NO2 (µg/L)
Spiked level	51	65	48	50	48
1	49	64	57	92	66
2	41	68	51	68	40
3	51	87	39	48	37
4	36	57	38	49	32
Average	44	69	46	64	44
% RSD	16	19	32	32	35
Recovery %	87	107	96	128	92

Experimental results: 100 % of the recovered nitramines was found in the first impinger. The recovery results show that water is an efficient absorber solution for nitramines.

The method has been tested for robustness against nitration of amines during sample work up. Table 17 show the experimental set-up. Experiment 1 is a synthetic sample containing 100 mg/L each of $NO2^-$/$NO3^-$ at pH 3. The sample has been worked up and analysed, and in

the two right columns the observation has been noted. The other experiments (2-14) given in the table follows the same pattern.

Table 17: Experimental set-up and observations in the nitration experiment.

Experiment No.	Amine mixture	$NO2^-/NO3^-$ (mg/L each)	pH	Potential Interference*	Retention area
1	1	100	3	Yes/ H	MEA-NO2
2	2	100	3	Yes/ L	MEA-NO2
3	1	100	7	Yes/ L	MEA-NO2
4	2	100	7	Yes/ M	MEA-NO2
5	3	10	3	Yes/ M	MEA-NO2
6	4	10	3	Yes/ M	MEA-NO2
7	3	10	7	Yes/ M	MEA-NO2
8	4	10	7	Yes/ M	MEA-NO2
9	5	0	3	Yes/ M	MEA-NO2
10	6	0	7	Yes/ L	MEA-NO2
11	7	99	7	Yes/ L	MEA-NO2
12	7	99	3	Yes/ H	MEA-NO2
13	7	9.9	7	Yes/ L	MEA-NO2
14	7	9.9	3	Yes/ L	MEA-NO2

*: H means high intensity signal, M means medium intensity signal, L means low intensity signal
Amine 1: MEA (114mg/L) ,DEA (114mg/L),MDEA (114mg/L), AMP (114mg/L), PZ (114mg/L),MA (656mg/L),DMA (640mg/L)
Amine 2: MEA (266mg/L), DEA (266mg/L), MDEA (266mg/L)
Amine 3: MEA (140mg/L) ,DEA (140mg/L),MDEA (140mg/L), AMP (140mg/L), PZ (140mg/L),MA (804mg/L),DMA (784mg/L)
Amine 4: MEA (326mg/L), DEA (326mg/L), MDEA (326mg/L)
Amine 5: MEA (143mg/L) ,DEA (143mg/L),MDEA (143mg/L), AMP (143mg/L), PZ (143mg/L),MA (820mg/L),DMA (800mg/L)
Amine 6: Amine 4: MEA (326mg/L), DEA (326mg/L), MDEA (326mg/L)
Amin 7: EPA 8270 Nitrosamine mix at 2 mg/L of each nitrosamine

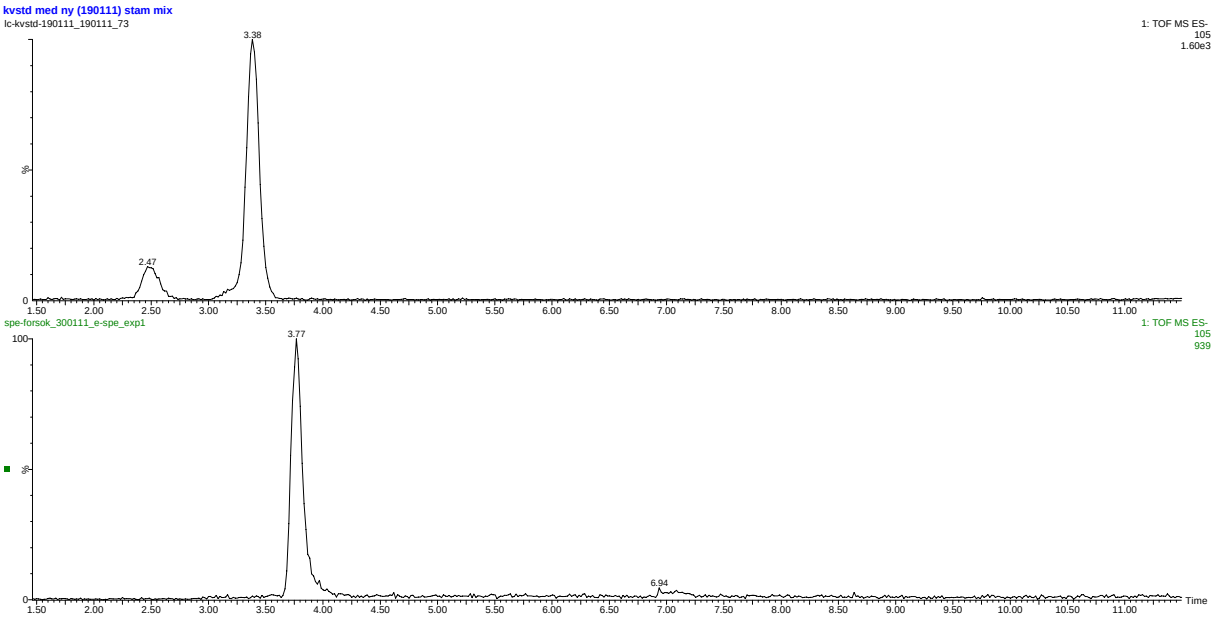


Figure 2: Extracted ion chromatograms (nominal masses) showing MEA-NO2 in a standard in the upper chromatogram, and the observed interference in experiment 1 in the lower chromatogram.

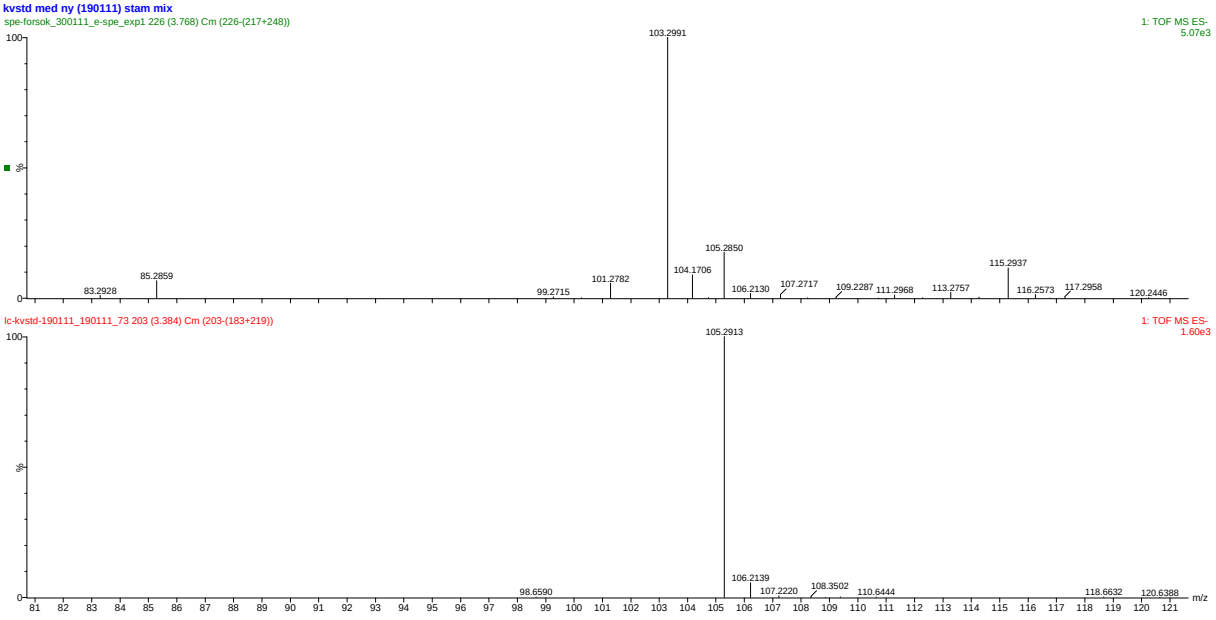


Figure 3: Background corrected mass spectra of the peaks in figure B.16, with the standard in the lower spectrum and the synthetic sample from experiment 1 in the upper spectrum.

Based on the observations above a new experiment was carried out. The set-up and observations are given in table 18. The water wash sample ID K was worked up at pH 3 and 7, and the results are given in table 18 and figures 4 and 5.

Table 18: Experimental set-up and observations in the nitration experiment

Experiment No	Sample	pH	Potential Interference*	Retention area
1	ID-K	3	Yes/ H	MEA-NO2
2	ID-K	7	Yes/L	MEA-NO2
-	-	-	-	-
4	1	3	Yes/H	MEA-NO2
5	2	5.6	No	
6	3	3	No	

*: H means high intensity signal, M means medium intensity signal, L means low intensity signal
Sample 1: 100 mL 0.001 M HCl with 100 mg/L $NO2^-$ and 100 mg/L $NO3^-$
Sample 2: 100 mL MQ water with 100 mg/L $NO2^-$ and 100 mg/L $NO3^-$
Sample 3: 100 mL 0.001 M HCl

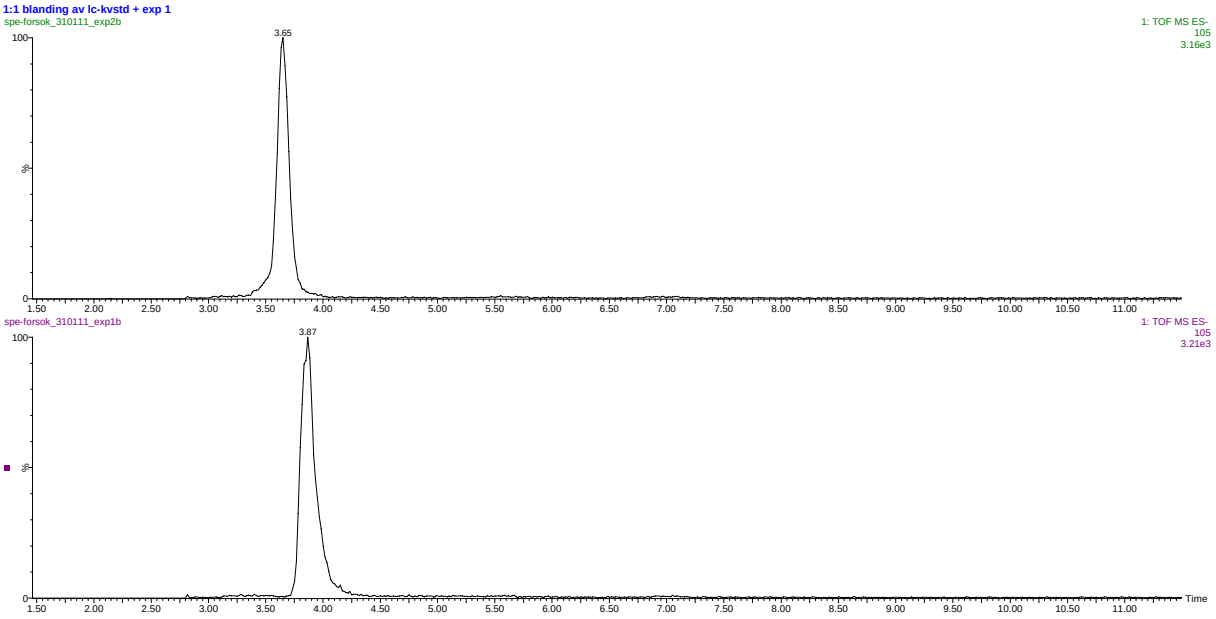


Figure 4: Extracted ion chromatograms (nominal masses) showing m/z 105 for the water wash sample ID K at pH 7 in the upper chromatogram and pH 3 in the lower chromatogram.

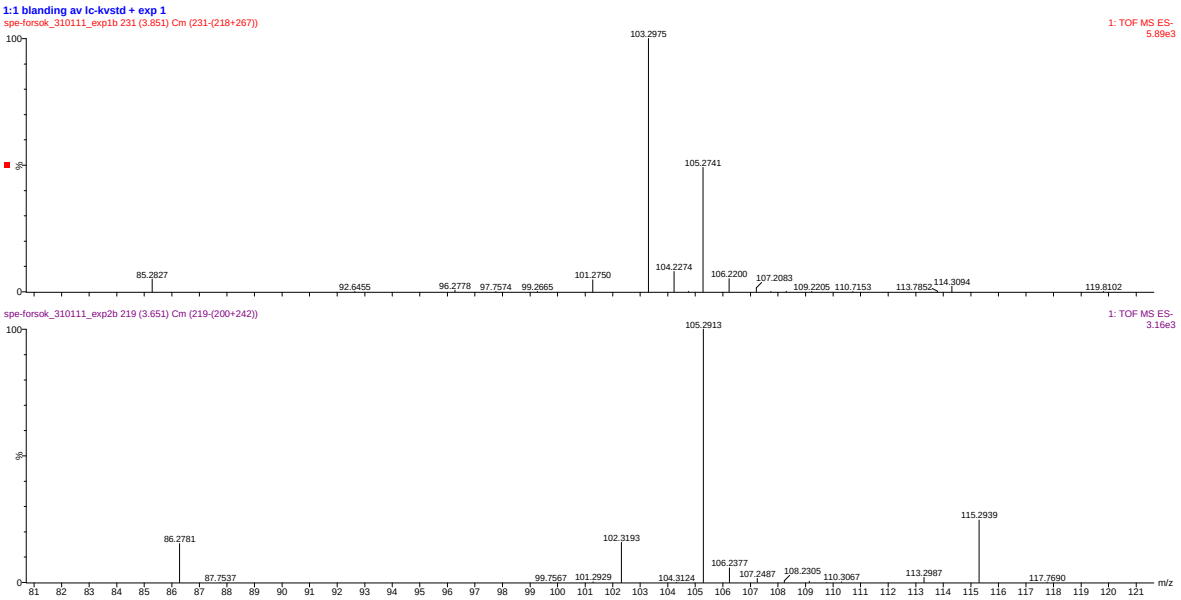


Figure 5: Mass spectra of the peaks in figure B.18, were the lower spectrum corresponds to the pH 7 and the upper spectrum corresponds to pH 3.

The conclusion so far is that the nominal mass m/z is not MEA-NO2. At high concentration of $NO2^-/NO3^-$, combined with the presence of HCl at low pH, the m/z 105 peak appear together with the ions 101, 103 and 104. The observed signal reported in table 18 is the ^{13}C-isotope of a m/z 104 peak. At lower concentrations of $NO2^-/NO3^-$ the problem is strongly reduced as seen in table B.19.

Table 19: Experimental set-up and observations in the nitration experiment

Experiment No.	Amine mixture	$NO2^-/NO3^-$ (mg/L)	pH	Potential interference	Retention area
1	5	17.5/1.47	3.14	Y/L	MEA-NO2
2	5	17.5/1.47	3.14	Y/L	MEA-NO2
3	5	17.5/1.47	4.10	Y/L	MEA-NO2
4	5	17.5/1.47	4.10	Y/L	MEA-NO2
5	5	17.5/1.47	5.00	Y/L	MEA-NO2
6	5	17.5/1.47	5.00	Y/L	MEA-NO2
7	5	17.5/1.47	6.30	Y/L	MEA-NO2
8	5	17.5/1.47	6.30	Y/L	MEA-NO2
9	5	17.5/1.47	7.24	Y/L	MEA-NO2
10	5	17.5/1.47	7.24	Y/L	MEA-NO2

*: H means high intensity signal, M means medium intensity signal, L means low intensity signal
Amine 5: MEA (143mg/L) ,DEA (143mg/L),MDEA (143mg/L), AMP (143mg/L), PZ (143mg/L),MA (820mg/L),DMA (800mg/L)

Conclusion:

No nitration is observed in the experiments. However, possible artifacts can be present at low pH combined with high concentration of $NO2^-/NO3^-$. Clean-up steps should be explored such that the work-up can be performed at pH3 which improves the recovery, especially for PZ-NO2.

Appendix C

Evaluation of the nitramine purity

Evaluation of the nitramine purity

Accomplishment

The purity estimates are based on chemical analysis using complementary methods such as GC/MS and LC/MS. The nitramines were dissolved in methanol for GC analysis and water for LC analysis.

Case: "Do we have possible nitramine impurities?"

The search for potential nitrosamines in DMA-NO2 is used as an example of how the purity is estimated. DMA-NO2 is selected because this nitramine is best suited for GC chromatography, which for demonstration purpose provides a less complex picture when the purity is evaluated.

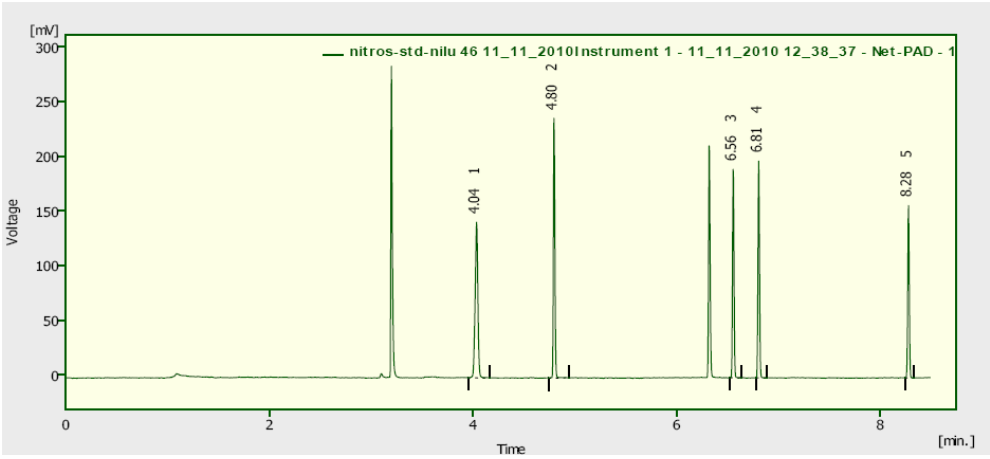


Figure 1: **GC/TEA chromatogram of EPA 512 nitrosamine standard (1 mg/L). Column: Phenomenex, ZB-1, 30 m, 0,25 mmID, 0,25 µm. NO-DMA is eluting at Rt 3.2 min.**

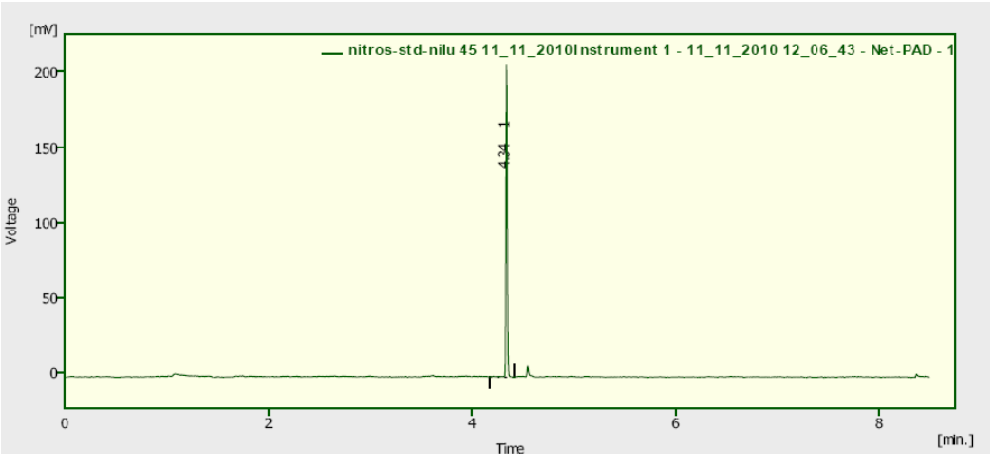


Figure 2: **GC/TEA chromatogram of a nitramine standard mixture with MEA-NO2, MA-NO2, DMA-NO2, AMP-NO2, PZ-NO2 (8 mg/L).The chromatographic peak at 4.35 min. is generated by DMA-NO2. Column: Phenomenex, ZB-1, 30 m, 0,25 mmID, 0,25 µm.**

The chromatogram in figure 2 shows no presence of the EPA 512 nitrosamines seen in the figure 1.

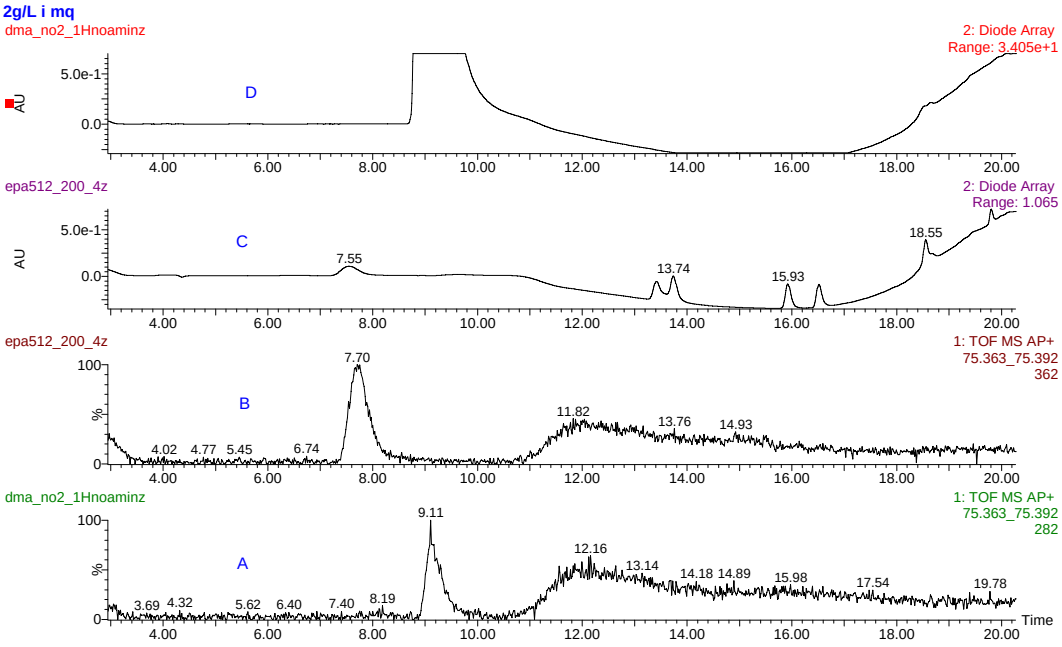


Figure 3: **HPLC/UV/HRMS chromatograms. A is the extracted ion chromatogram of a DMA-NO2 stock-solution; no NO-DMA is seen at Rt 7.7. B is the analytical standard of NO-DMA. C is the UV trace of EPA-512 nitrosamine standard. D is the UV trace of DMA-NO2 stock solution. Column: Waters Atlantis dC18, 3µm, 2.1x150mm.**

The A chromatogram of NO2-DMA standard in figure 3 shows no presence of NO-DMA at Rt 7.7, which is seen in chromatogram B of the analytical standard EPA 512 nitrosamines. The UV-trace of NO2-DMA in chromatogram D shows no peak match with the UV-trace of the EPA-512 nitrosamines in chromatogram C.

Conclusion of the case: "possible nitramine impurities"

None of the analysis (figure 1-3) shows presence of nitrosamines in the NO2-DMA stock solution in water (2g/L).

GC/FID is used for general identification of organic compounds. In figure 4 a GC/FID chromatogram is shown for pure methanol, and in figure 5 the GC/FID chromatogram is given for a NO2-DMA stock solution in methanol. For purity evaluation the chromatogram in figure 4 is subtracted from the chromatogram in figure 5.

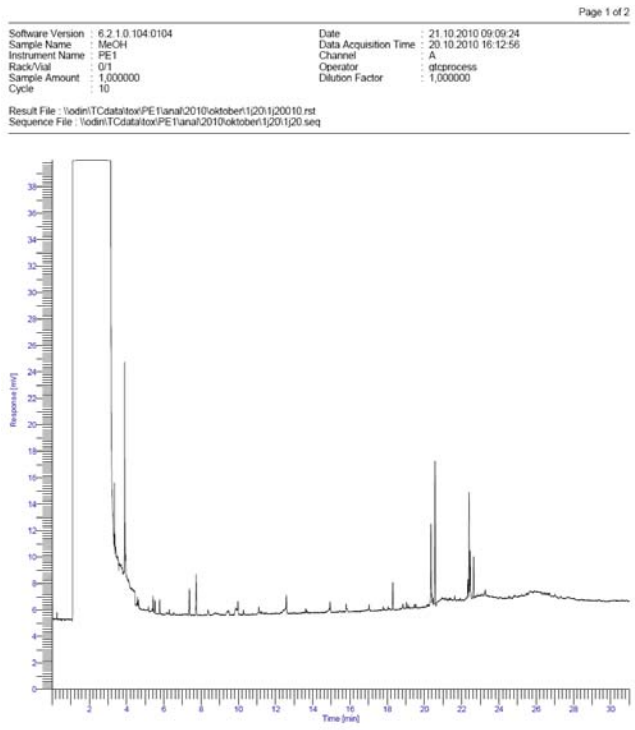


Figure 4: **GC/FID signal of pure methanol which is used as a solvent for the nitramines. Column: J&W Scientifi, DB-5, 30 m, 0,25 mmID, 0,25 µm.**

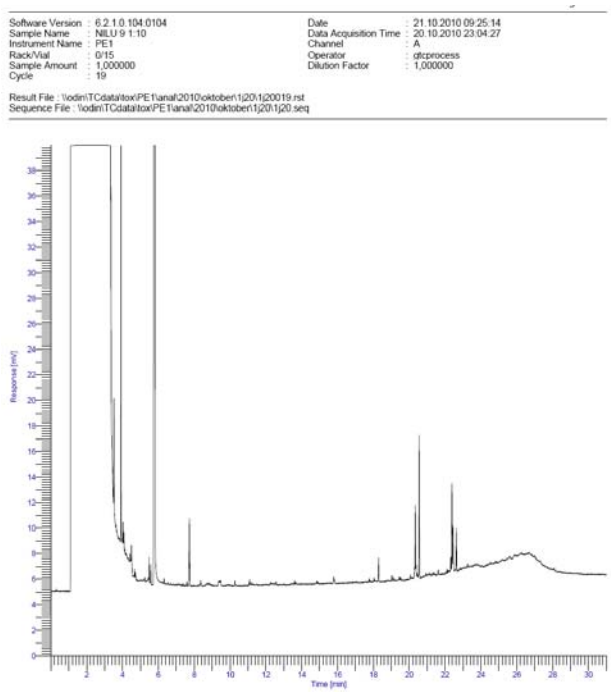


Figure 5: **GC/FID signal of DMA-NO2 dissolved in methanol. The large peak at Rt 5.8 min. is DMA-NO2. Column: J&W Scientifi, DB-5, 30 m, 0,25 mmID, 0,25 µm.**

Table 1: Estimates of the purity of the synthesized nitramines.

	Nitramine >99%	Nitrosamines* >99%	UV >99%	GC/TEA >99%	GC/FID >99%	C-NMR >99%	H-NMR	IR
MA-NO2	>99.5	Not indentified	>99.5	>99.5	>99.5	>99.5		ok
MEA-NO2	>99.5	Not indentified	>99.5	>99.5	>99.5	>99.5		ok
AMP-NO2	>99.5	Not indentified	>99.5	>99.5	>99.5	>99.5		ok
DMA-NO2	>99.5	Not indentified	>99.5	>99.5	>99.5	>99.5		ok
PZ-NO2	>99.5	Not indentified	>99.5	>99.5	>99.5	>99.5		ok

*: EPA mix 8270

Conclusion:

The purity of the synthesized nitramine batches have been analysed by several complementary analysis methods. The nitramines are cleaner than 99%.

GC/FID Chromatogram of the nitramines

Software Version	: 6.2.1.0.104:0104	Date	: 17.02.2011 16:37:13
Sample Name	: NILU MeOH	Data Acquisition Time	: 11.02.2011 20:14:16
Instrument Name	: PE1	Channel	: A
Rack/Vial	: 0/20	Operator	: Miljo
Sample Amount	: 1,000000	Dilution Factor	: 1,000000
Cycle	: 1		

Result File : \\odin\TCdata\tox\PE1\anal\2011\februar\1b11\1b11004.rst
Sequence File : \\odin\TCdata\tox\PE1\anal\2011\februar\1b11\1b11.seq

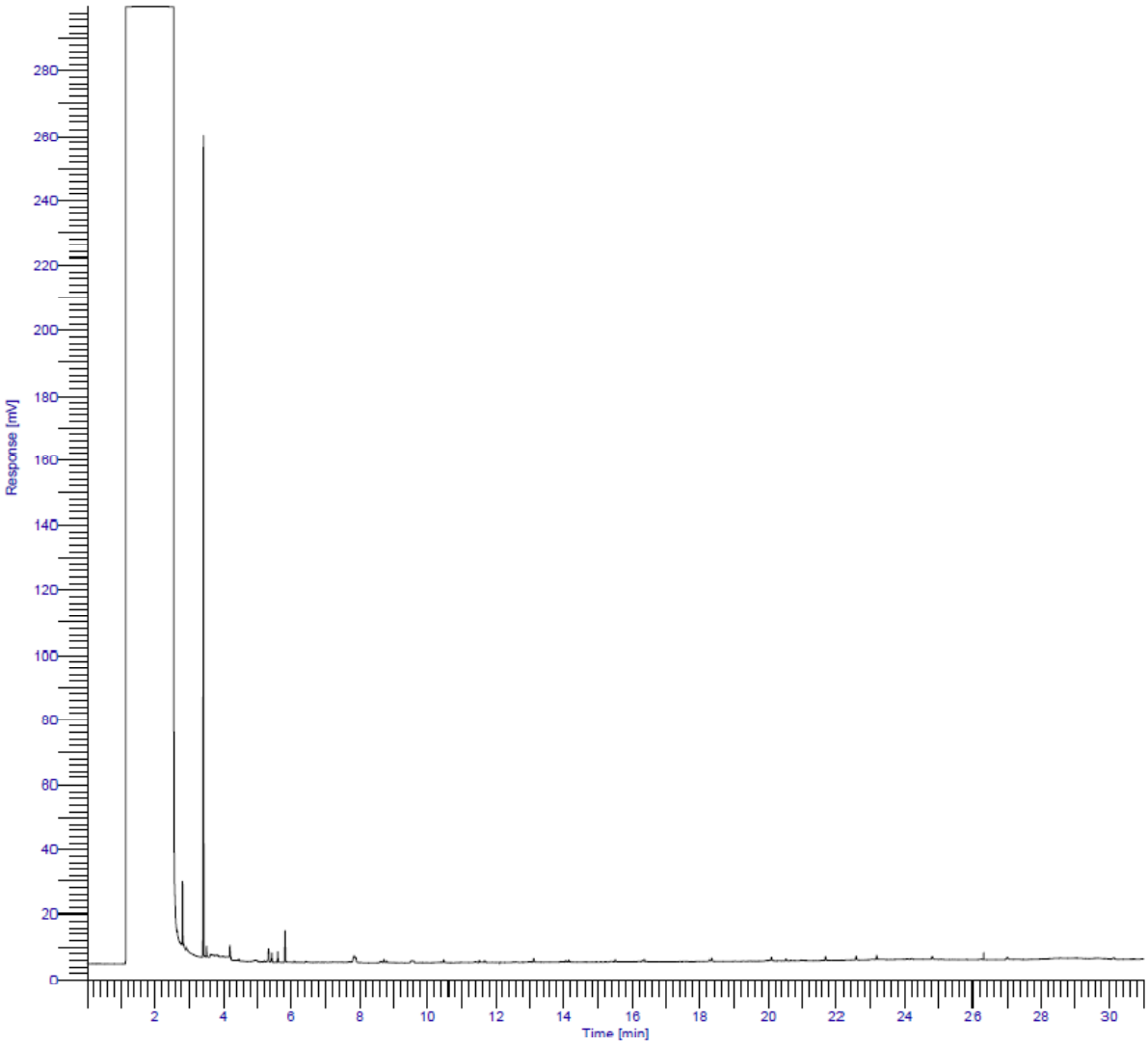


Figure 6: **GC/FID signal of methanol**

Software Version	: 6.2.1.0.104:0104	Date	: 17.02.2011 16:20:21
Sample Name	: NILU 2	Data Acquisition Time	: 12.02.2011 14:16:11
Instrument Name	: PE1	Channel	: A
Rack/Vial	: 0/37	Operator	: gtcprocess
Sample Amount	: 1,000000	Dilution Factor	: 1,000000
Cycle	: 3		

Result File : \\odin\TCdata\tox\PE1\anal\2011\februar\1b11\1b11022.rst
Sequence File : \\odin\TCdata\tox\PE1\anal\2011\februar\1b11\1b11.seq

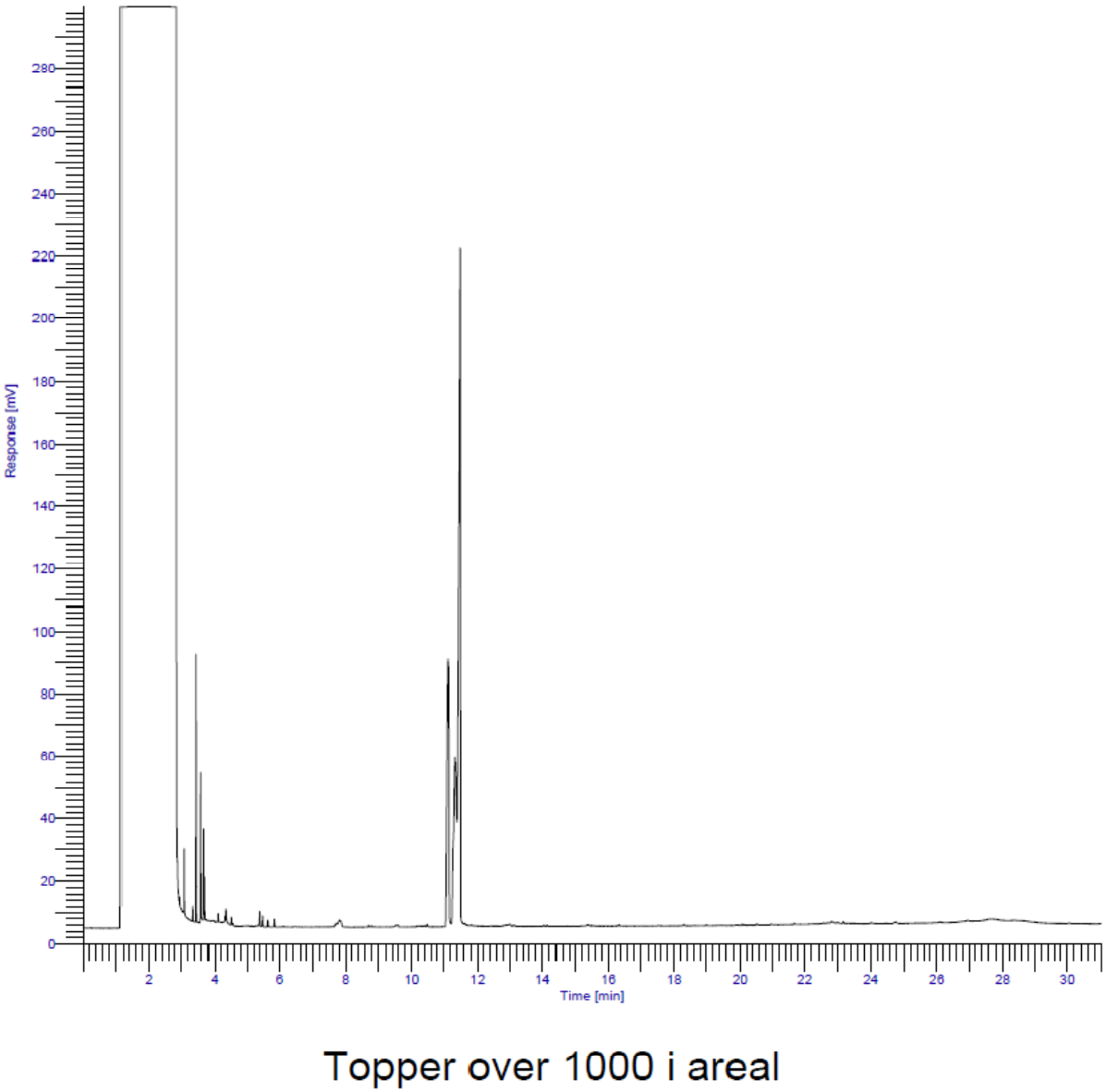


Figure 7: **GC/FID signal of AMP-NO2**

Software Version	: 6.2.1.0.104:0104	Date	: 17.02.2011 16:22:24
Sample Name	: NILU 3	Data Acquisition Time	: 12.02.2011 15:03:34
Instrument Name	: PE1	Channel	: A
Rack/Vial	: 0/38	Operator	: gtcprocess
Sample Amount	: 1,000000	Dilution Factor	: 1,000000
Cycle	: 4		

Result File : \\odin\TCdata\tox\PE1\anal\2011\februar\1b11\1b11023.rst
Sequence File : \\odin\TCdata\tox\PE1\anal\2011\februar\1b11\1b11.seq

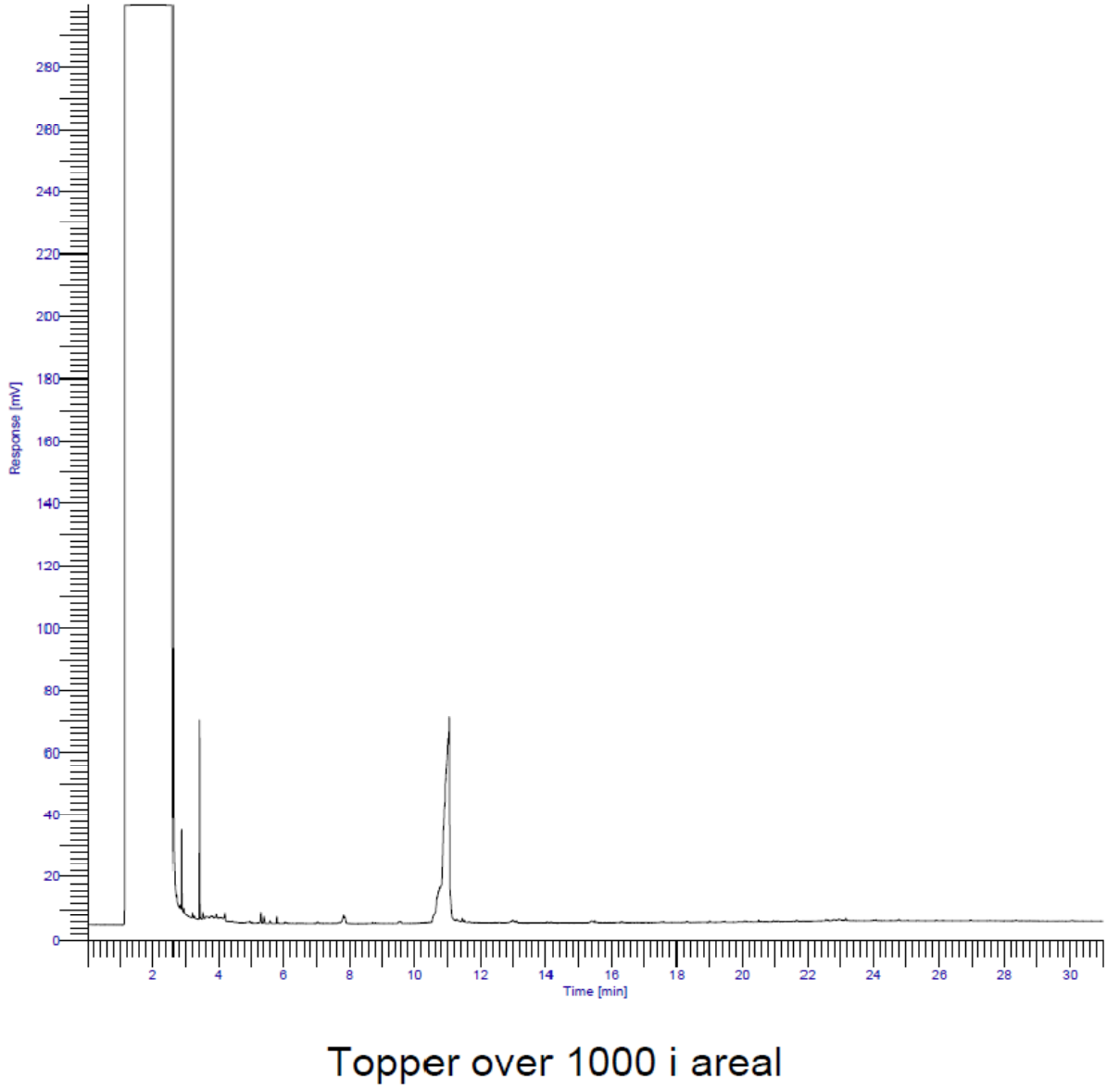


Figure 8: **GC/FID signal of MEA-NO2**

Software Version	: 6.2.1.0.104:0104	Date	: 17.02.2011 16:23:34
Sample Name	: NILU 4	Data Acquisition Time	: 12.02.2011 15:50:57
Instrument Name	: PE1	Channel	: A
Rack/Vial	: 0/39	Operator	: gtcprocess
Sample Amount	: 1,000000	Dilution Factor	: 1,000000
Cycle	: 5		

Result File : \\odin\TCdata\tox\PE1\anal\2011\februar\1b11\1b11024.rst
Sequence File : \\odin\TCdata\tox\PE1\anal\2011\februar\1b11\1b11.seq

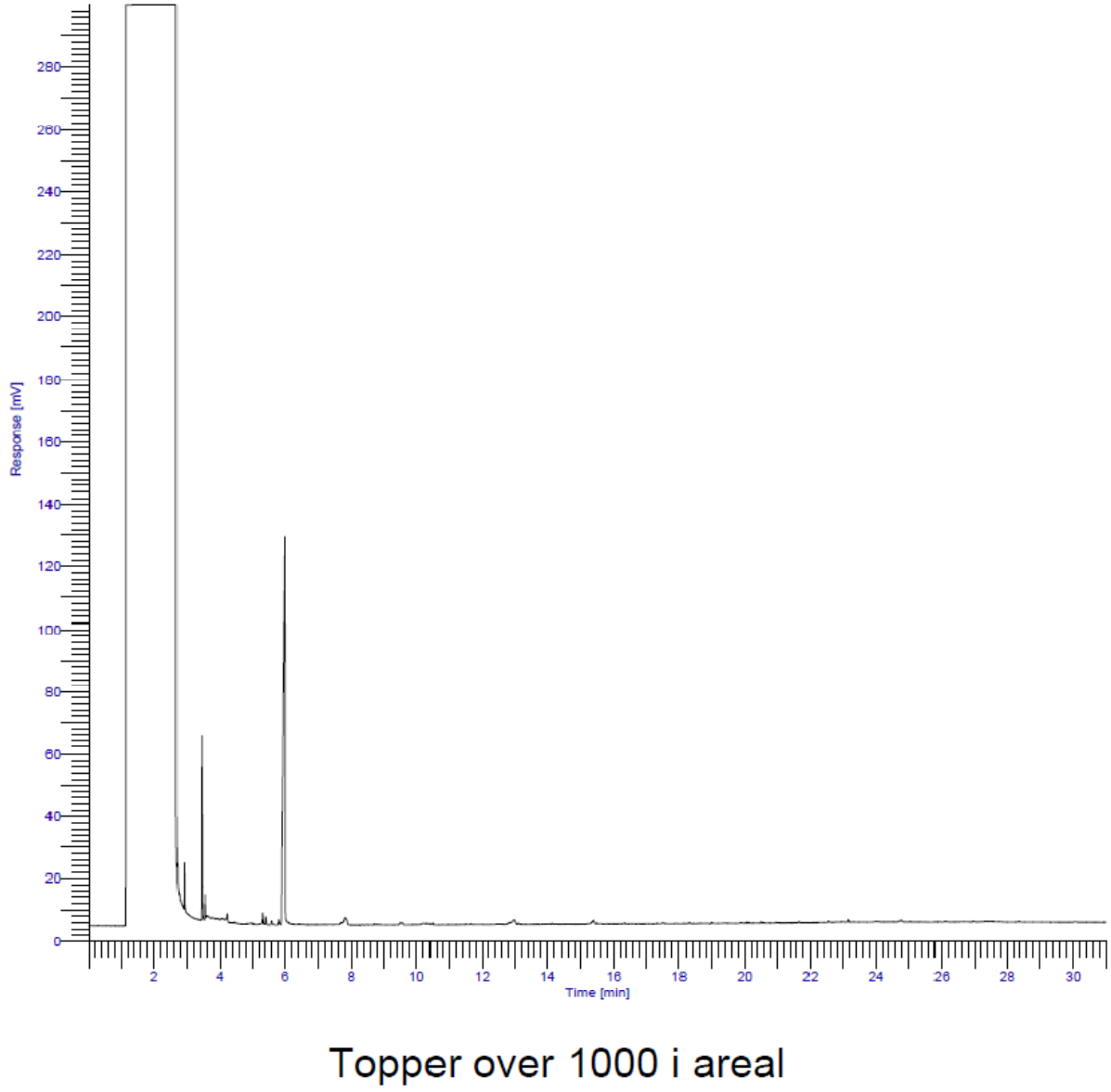
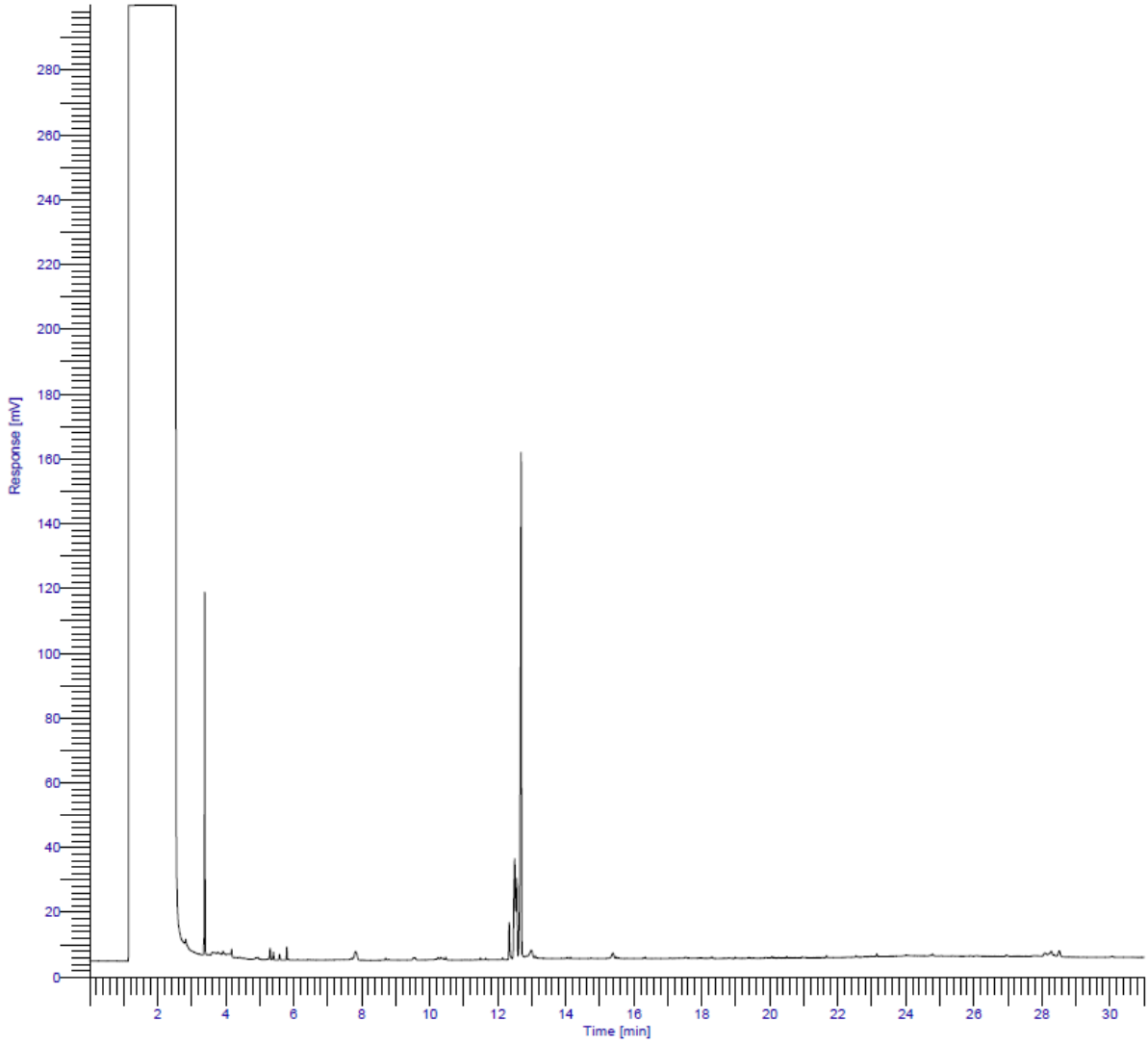


Figure 9: **GC/FID signal of MA-NO2**

Software Version	: 6.2.1.0.104:0104	Date	: 17.02.2011 16:27:40
Sample Name	: NILU 7	Data Acquisition Time	: 12.02.2011 18:12:37
Instrument Name	: PE1	Channel	: A
Rack/Vial	: 0/42	Operator	: gtcprocess
Sample Amount	: 1,000000	Dilution Factor	: 1,000000
Cycle	: 8		

Result File : \\odin\TCdata\tox\PE1\anal\2011\februar\1b11\1b11027.rst
Sequence File : \\odin\TCdata\tox\PE1\anal\2011\februar\1b11\1b11.seq



Topper over 1000 i areal

Figure 10: **GC/FID signal of PZ-NO2**

NMR spectra of the nitramines

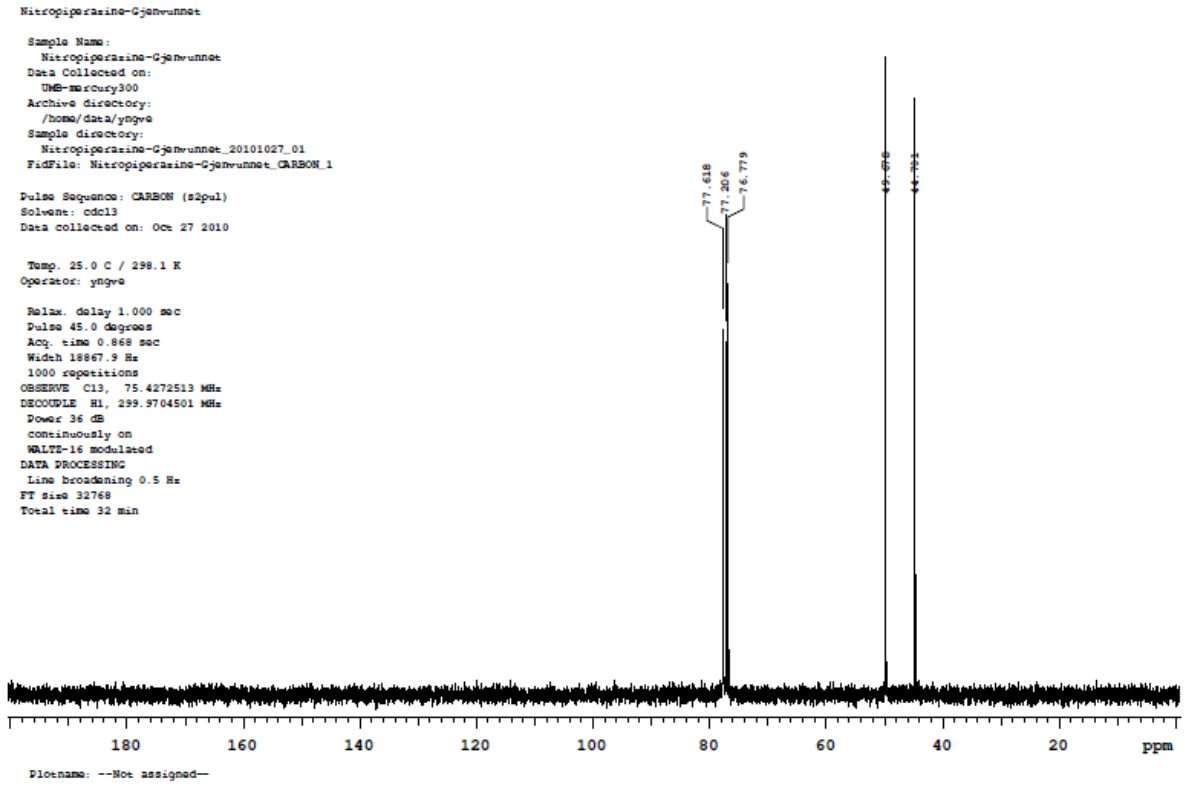


Figure 11: **13C-NMR of PZ-NO2**

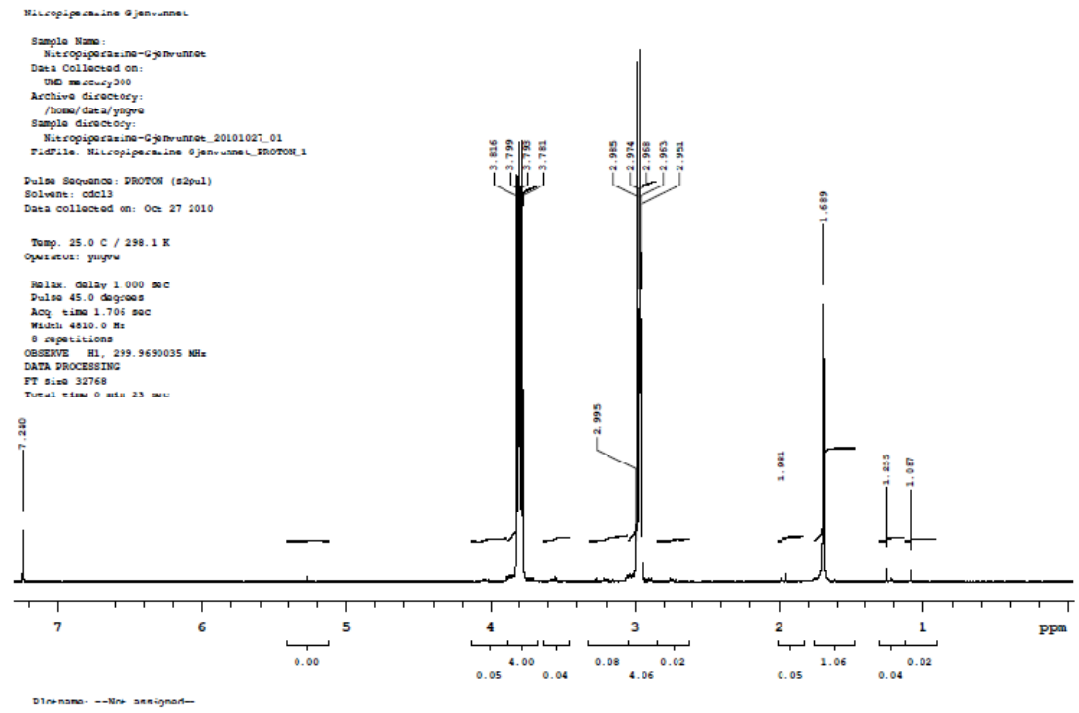


Figure 12: **H-NMR of PZ-NO2**

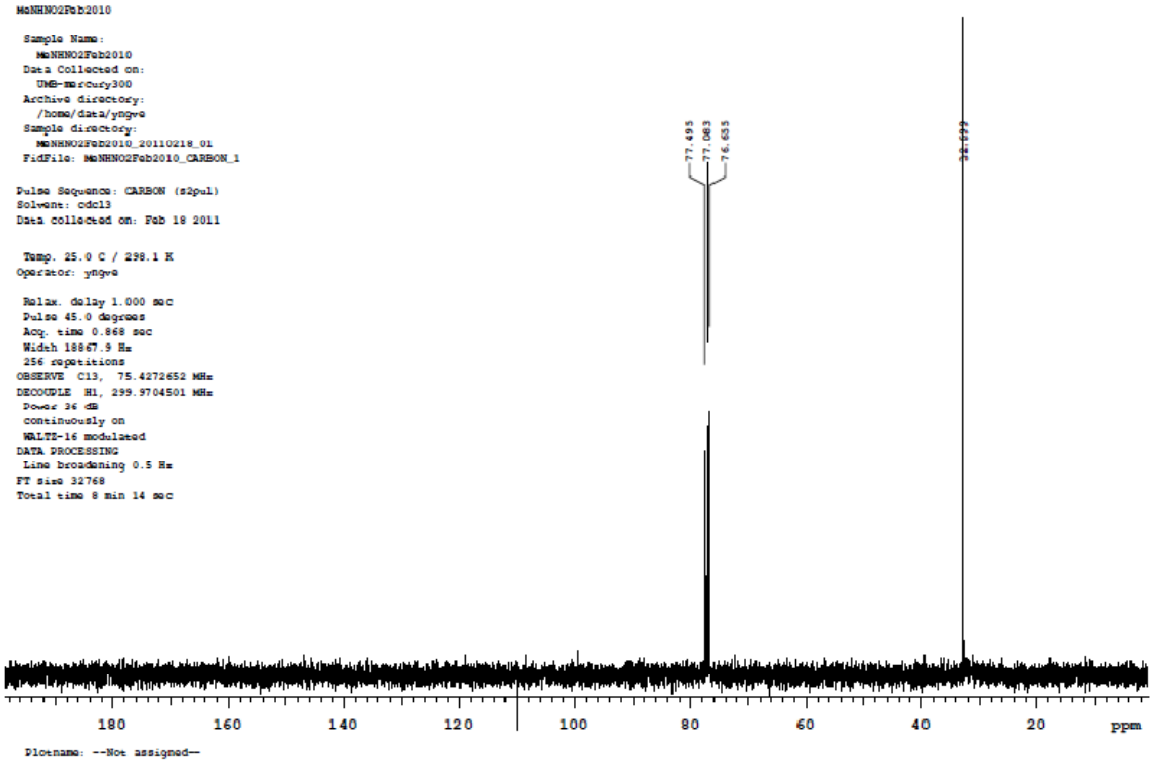


Figure 13: **13C-NMR of MA-NO2**

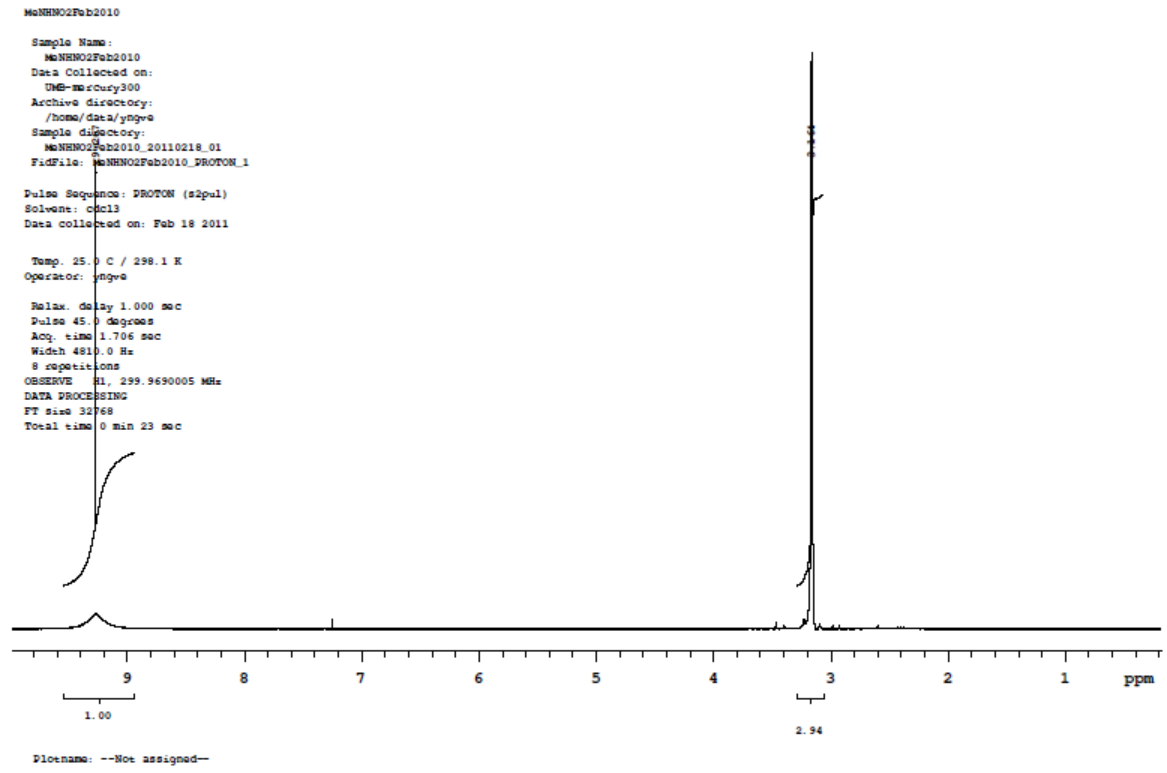


Figure 14: **H-NMR of MA-NO2**

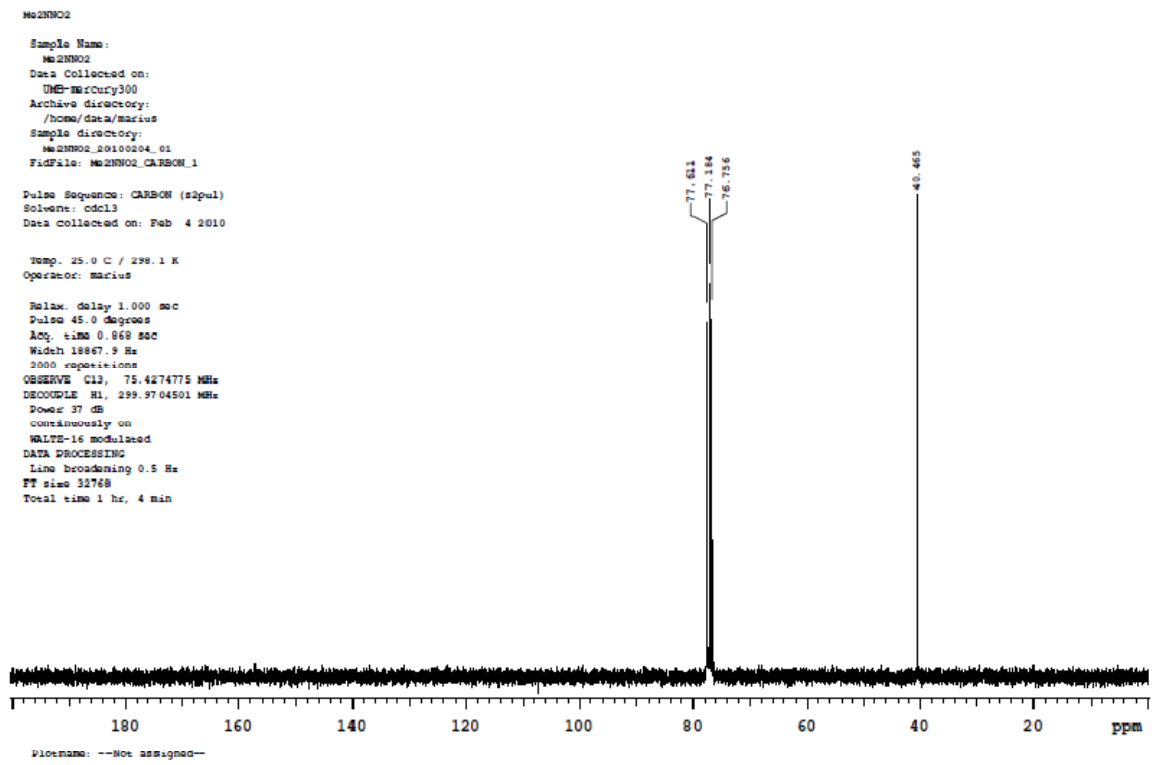


Figure 15: **13C-NMR of DMA-NO2**

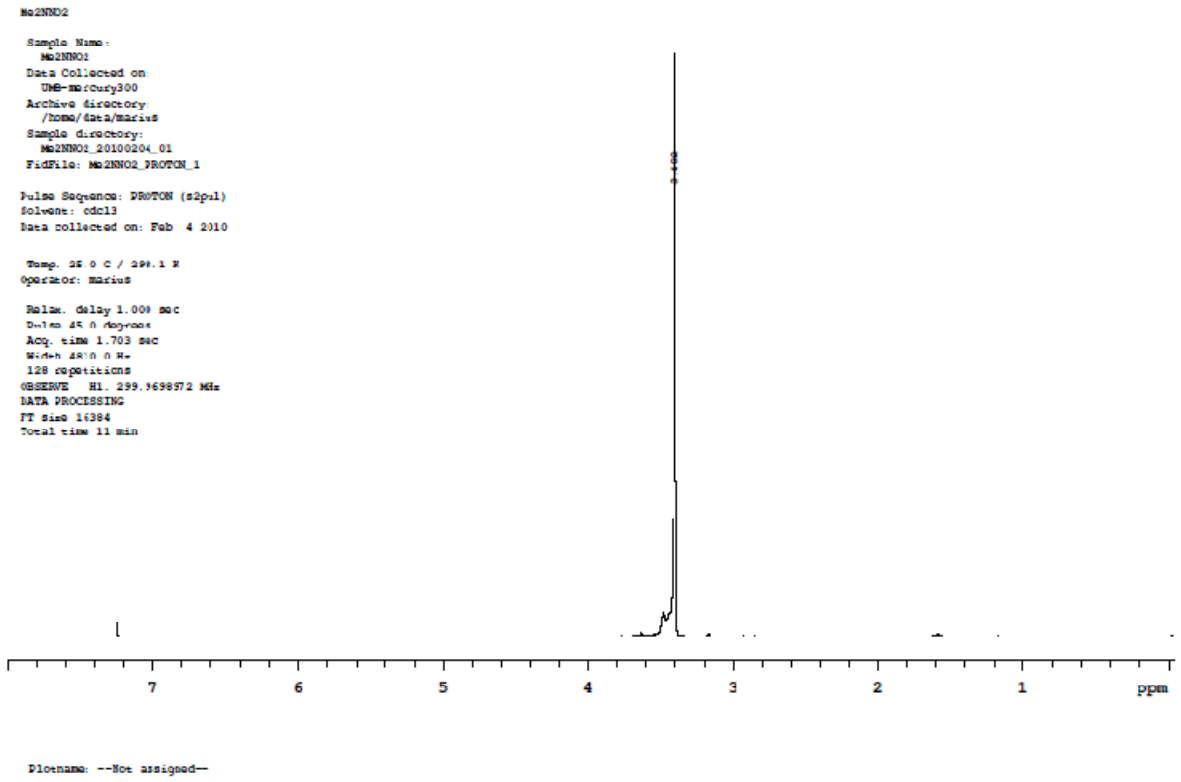


Figure 16: **H-NMR of DMA-NO2**

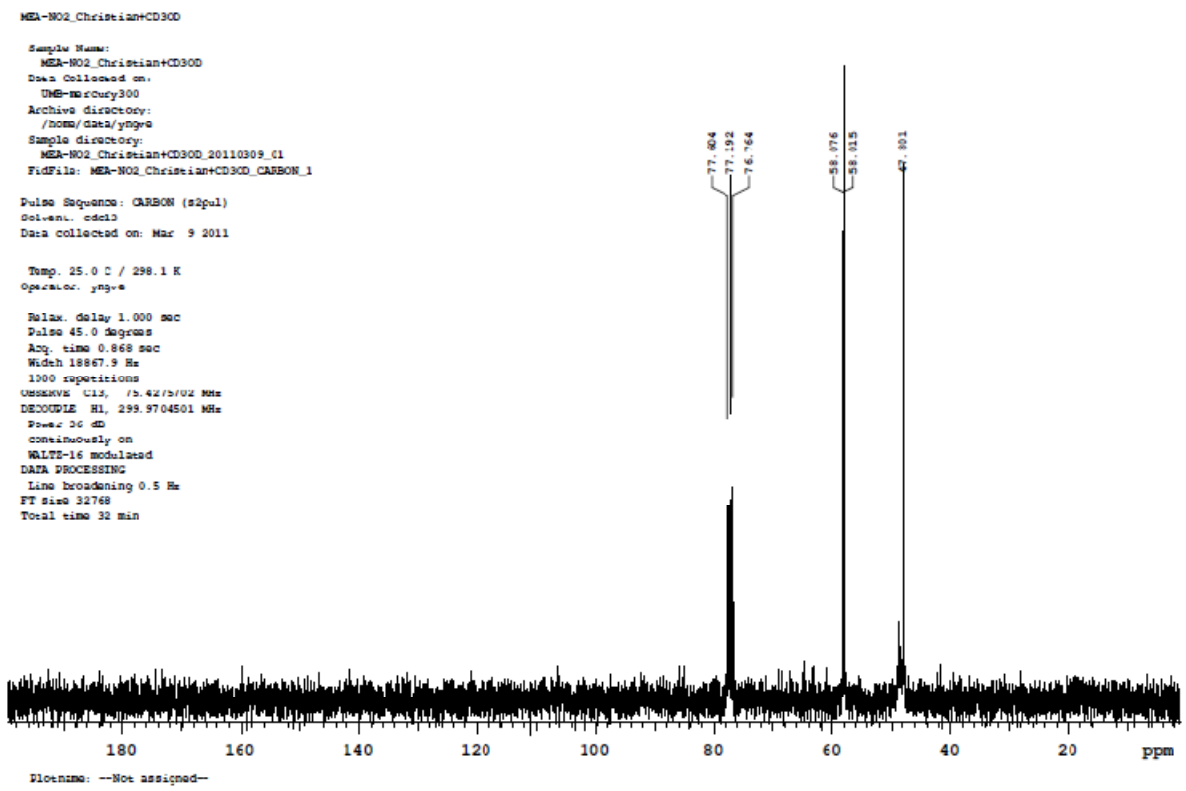


Figure 17: **13C-NMR of MEA-NO2**

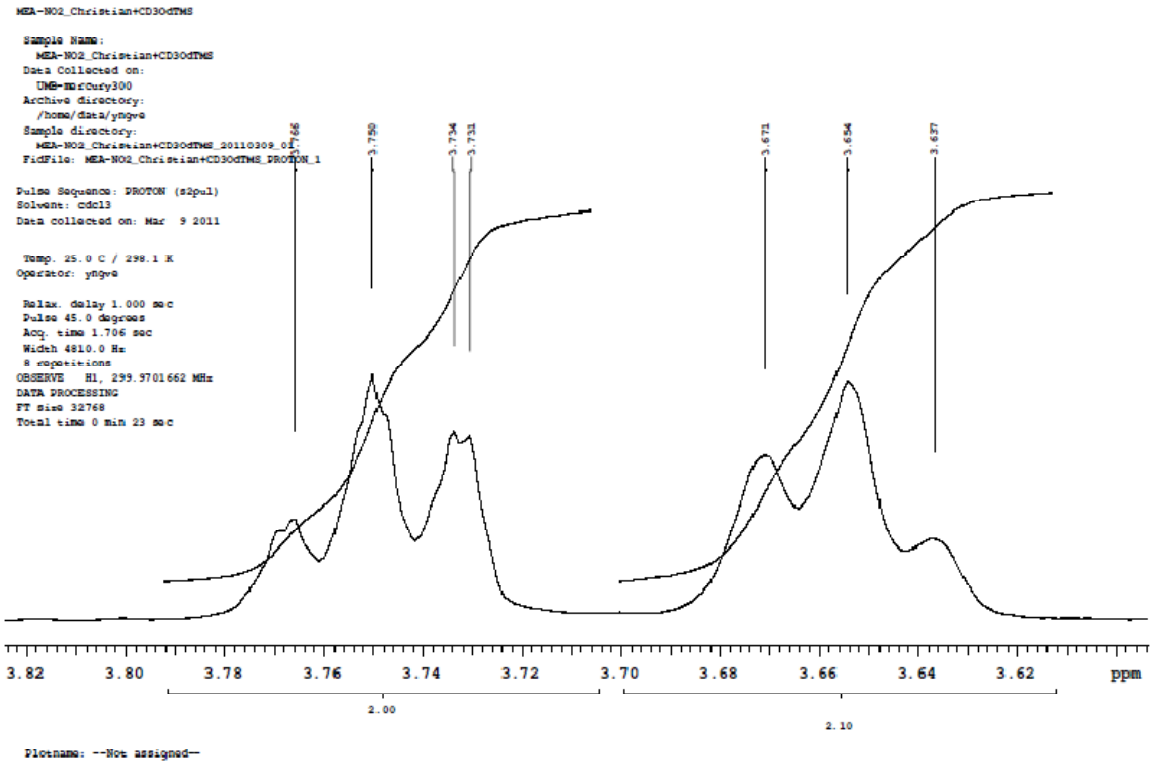


Figure 18: **H-NMR of MEA-NO2**

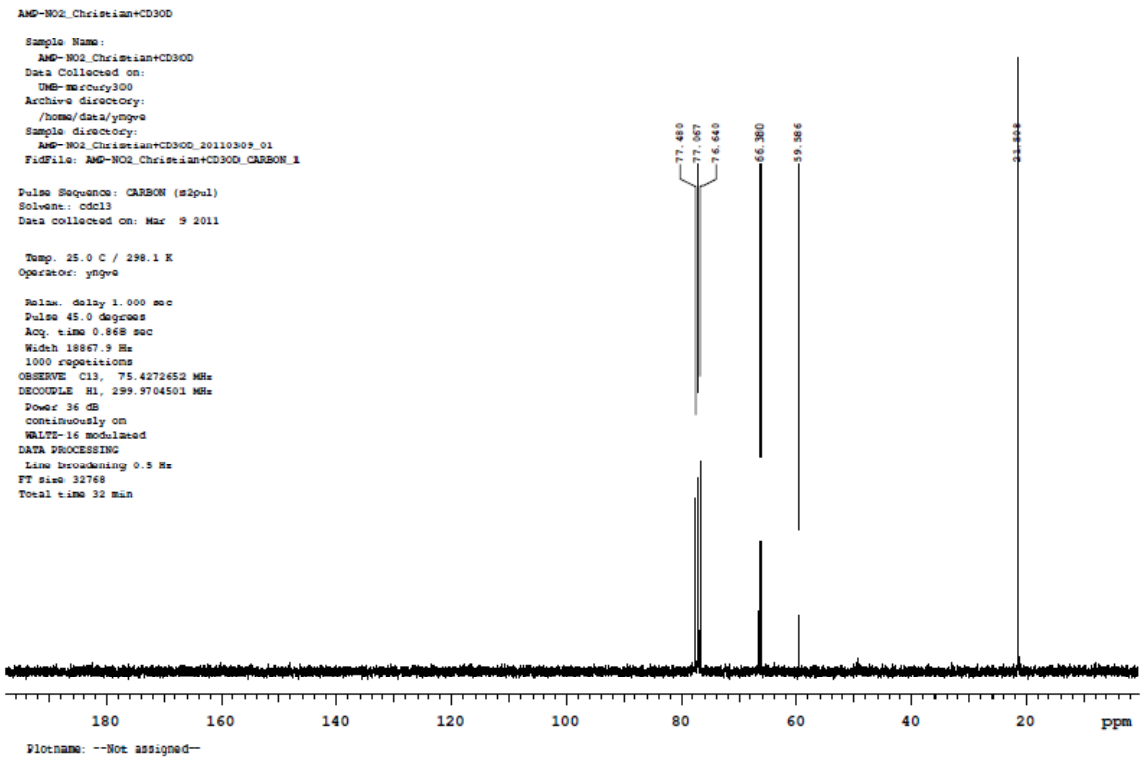


Figure 19: **13C-NMR of AMP-NO2**

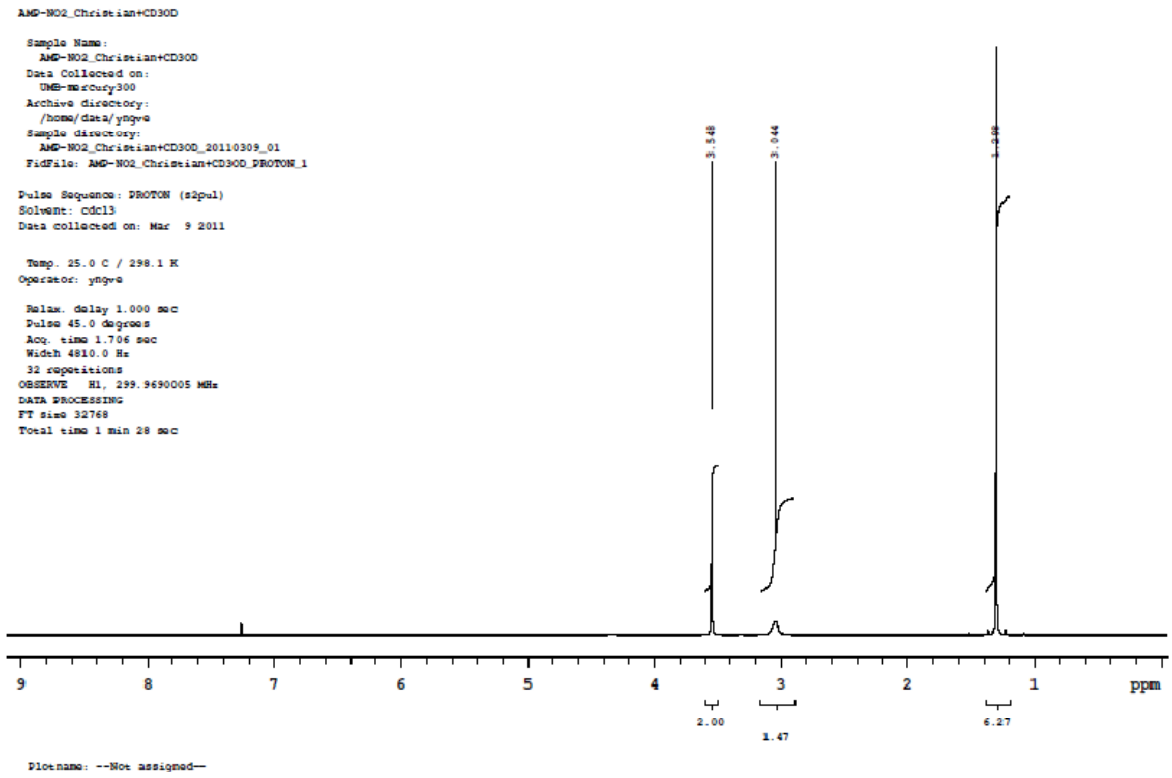


Figure 20: **H-NMR of AMP-NO2**

Appendix D

Examples of Instrumental settings

Instrumentsettings for UPLC/HRMS

cquisition Experiment Report
File: d:\projects\nitramin.pro\data\110130_24.raw

Header
Acquired File Name: 110130_24
Acquired Date: 30-Jan-2011
Acquired Time: 19:04:51
Job code: cd_300111
Task code:
User Name:
Laboratory Name:
Instrument: LCT Premier
Conditions:
Submitter:
SampleID: 270111_exp6
Bottle Number: 1:13
Description: 99.9:0.1

Instrument Calibration
Parameters
MS1 Static: None
MS1 Scanning: None
MS1 Scan Speed: None
MS2 Static: None
MS2 Scanning: None
MS2 Scan Speed: None
Calibration Time: 13:58
Calibration Date: 10/25/10
Coefficients
MS1 Static: None
MS2 Static: None
Function 1: 0.000000000238*x^5 + -0.000000027636*x^4 + 0.000001172982*x^3 + -0.000020935901*x^2 + 1.000236880760*x +-0.002461680846, Root Mass

Parameters for D:\Projects\Nitramin.PRO\ACQUDB\Espos_centr.EXP
Created by Masslynx V4.1

Use TTP 4GHz TDC YES
Dynamic Range Enhancement NO
Pirani Pressure(mbar) 2.01e0
Penning Pressure (mbar) 5.57e-7

Instrument Parameters - Function 1:
Polarity ES+

Analyser W Mode
Capillary (V) 3200.0
Sample Cone (V) 20.0
Desolvation Temp (C) 350.0
Source Temp (C) 120.0
Cone Gas Flow 10.0
Desolvation Gas Flow 750.0
Syringe Type SGE 250uL
Ion Guide One 1.0
Aperture 1 Voltage 8.0
Ion Energy (V) 34.0
Aperture 2 Voltage 3.0
Hexapole DC Voltage 2.0
Aperture 3 Voltage 3.0
Acceleration (V) 100.0
Y Focus (V) 0.0
Steering (V) 0.0
Tube Lens (V) 63.0
Attenuated Z Focus (V) 156.3
Normal Z Focus (V) 40.0
TOF Flight Tube (V) 7200.0
Reflectron (V) 1800.0
Pusher Voltage 893.0
Pusher Offset Voltage 0.95
Puller Voltage 733.0
Puller Offset Voltage 0.00
MCP Detector (V) 2350.0
Pusher Cycle Time Auto (62.0)
Pusher Frequency 16129.03
Pusher Width 4.00
Centroid Threshold 1.0
Min Points 4.0
Np Multiplier 0.70
Resolution 8500.0
Lteff 2225.0000
Veff 7185.2000
Trigger Threshold (mV) 600.0000
Signal Threshold (mV) 30.0000
Data Threshold 0.0000
DXC Temperature 0.0
IonGuide1InitialRF 50.0
IonGuide1FinalRF 50.0
IonGuide2InitialRF 100.0
IonGuide2FinalRF 100.0
Fixed Hexapole RF True
HexapoleRF 20.0
DRE Mass 0.0000 Setting 3.1000
DRE Mass 280.0000 Setting 31.0000
DRE Mass 1000.0000 Setting 31.0000
DRE Mass 2000.0000 Setting 31.0000

DRE Mass 3000.0000 Setting 31.0000

ACE Experimental Record

---------- Run method parameters ----------

-- PUMP --

Waters Acquity SDS
Run Time: 11.00 min
Comment:
Solvent Selection A: A1
Solvent Selection B: B1
Low Pressure Limit: 0 psi
High Pressure Limit: 14000 psi
Solvent Name A:
Solvent Name B:
Switch 1: No Change
Switch 2: No Change
Switch 3: No Change
Seal Wash: 3.0 min
Chart Out 1: System Pressure
Chart Out 2: %B
System Pressure Data Channel: No
Flow Rate Data Channel: No
%A Data Channel: No
%B Data Channel: No
Primary A Pressure Data Channel: No
Accumulator A Pressure Data Channel: No
Primary B Pressure Data Channel: No
Accumulator B Pressure Data Channel: No
Degasser Pressure Data Channel: No
[Gradient Table]
Time(min) Flow Rate %A %B Curve
1. Initial 0.350 99.9 0.1
2. 7.00 0.350 1.0 99.0 6
3. 8.00 0.350 1.0 99.0 1
4. 8.10 0.350 99.9 0.1 6
5. 11.00 0.350 99.9 0.1 1
Run Events: Yes

-- END PUMP --

-- AUTOSAMPLER --

Waters Acquity AutoSampler
Run Time: 3.00 min
Comment:
Load Ahead: Disabled
Loop Option: Partial Loop With Needle Overfill
LoopOffline: Disable
Weak Wash Solvent Name: Water/Acetonitrile
Weak Wash Volume: 1500 uL
Strong Wash Solvent Name: Water/Methanol
Strong Wash Volume: 500 uL
Target Column Temperature: 35.0 C
Column Temperature Alarm Band: Disabled
Target Sample Temperature: 5.0 C
Sample Temperature Alarm Band: Disabled
Full Loop Overfill Factor: Automatic
Syringe Draw Rate: 100
Needle Placement: Automatic
Pre-Aspirate Air Gap: 5.0
Post-Aspirate Air Gap: 5.0
Column Temperature Data Channel: No
Ambient Temperature Data Channel: No
Sample Temperature Data Channel: No
Sample Organizer Temperature Data Channel: No
Sample Pressure Data Channel: No
Switch 1: No Change
Switch 2: No Change
Switch 3: No Change
Switch 4: No Change
Chart Out: Sample Pressure
Sample Temp Alarm: Disabled
Column Temp Alarm: Disabled
Run Events: Yes
Needle Overfill Flush: Automatic
Sample Loop Size: 10.0

Sample Run Injection Parameter

Injection Volume (ul) - 2.00
-- END AUTOSAMPLER --

---------- oOo ----------

End of experimental record.

Function 1
Scans in function: 1919

Cycle time (secs): 0.310
Scan duration (secs): 0.30
Interscan delay (secs): 0.01
Retention window (mins): 0.000 to 10.000
Ionization mode: ES+
Data type:
Function type: TOF MS
Mass range: 40 to 500

Instrumentsettings for HPLC/HRMS (LCT-Classic)

Acquisition Experiment Report
File:c:\masslynx\ccm.pro\data\spe-forsok_270113_exp3.raw

Header
Acquired File Name: spe-forsok_270113_exp3
Acquired Date: 27-Jan-2011
Acquired Time: 20:53:53
Job code: NO2-forsok_270111
Task code:
User Name:
Laboratory Name:
Instrument: LCT
Conditions:
Submitter:
SampleID:
Bottle Number: 67
Description:

Instrument Calibration
Parameters
MS1 Static: None
MS1 Scanning: None
MS1 Scan Speed: None
MS2 Static: None
MS2 Scanning: None
MS2 Scan Speed: None
Calibration Time:
Calibration Date:
Coefficients
MS1 Static: None
MS2 Static: None
Function 1: None
Function 2: None

Parameters for C:\MassLynx\CCM.PRO\ACQUDB\CCM_no2_renhetstest.EXP
Created by Masslynx V4.1

TDC Gain Control 0.0
TDC Edge Control 0.0
Use 4GHz TDC YES
Use TTP 4GHz TDC NO
Source is Z Spray Mk2 YES
NO
Pirani Pressure(mbar) 1.80e0
Penning Pressure (mbar) 3.38e-7
Cone Gas Flow (L/hr) 36
Desolvation Gas Flow (L/hr) 780

Instrument Parameters - Function 1:

Polarity	ES-
Capillary (V)	3000.0
Sample Cone (V)	16.0
RF Lens (V)	80.0
Extraction Cone (V)	3.0
Desolvation Temp (C)	350.0
Source Temp (C)	120.0
RF DC Offset 1 (V)	6.0
RF DC Offset 2 (V)	10.0
Aperture (V)	0.0
Acceleration (V)	200.0
Focus (V)	0.0
Steering (V)	0.0
MCP Detector (V)	2805.0
Pusher Cycle Time	Auto (50.0)
Pusher Frequency	20000.00
Ion Energy (V)	38.0
Tube Lens (V)	0.0
Grid 2 (V)	66.0
TOF Flight Tube (V)	4640.0
Reflectron (V)	1796.0

Centroid Threshold	1.0
Min Points	2.0
Np Multiplier	1.00
Resolution	4000.0
Lock Mass	0.0000
Mass Window +/-	1.0000
Lteff	1243.0000
Veff	4600.0000
TDC Start (mV)	700.0000
TDC Stop (mV)	21.0000
TDC Threshold	0.0000

ACE Experimental Record

---------- Run method parameters ----------

HP1100 LC Pump Initial Conditions

Solvents	
A%	0.0
B%	0.0
C%	2.0
D%	98.0
Flow (ml/min)	0.200
Stop Time (mins)	32.0
Min Pressure (bar)	10
Max Pressure (bar)	400
Oven Temperature Left(°C)	30.0
Oven Temperature Right(°C)	30.0

HP1100 LC Pump Gradient Timetable

The gradient Timetable contains 8 entries which are :

Time	A%	B%	C%	D%	Flow (ml/min)	Pressure
0.00	0.0	0.0	2.0	98.0	0.200	400
2.00	0.0	0.0	2.0	98.0	0.200	400
10.00	0.0	0.0	40.0	60.0	0.200	400
16.00	0.0	0.0	100.0	0.0	0.450	400
23.00	0.0	0.0	100.0	0.0	0.450	400
23.10	0.0	0.0	2.0	98.0	0.450	400
31.50	0.0	0.0	2.0	98.0	0.450	400
32.00	0.0	0.0	2.0	98.0	0.200	400

HP1100 LC Pump External Event Timetable

The Timetable contains 7 entries which are :

Time	Column Switch	Contact 1	Contact 2	Contact 3	Contact 4
Initial	On	Off	Off	Off	Off
0.00	On	On	Off	Off	Off
0.10	On	Off	Off	Off	Off
2.50	On	Off	Off	Off	Off
3.00	On	Off	Off	Off	Off
22.00	On	Off	Off	Off	Off
32.00	On	Off	Off	Off	Off

Column Name

HP1100 PDA Spectrum

Storage type : All
Start Range (nm) 190
End Range (nm) 600
Range Interval (nm) 2.0
Threshold (mAU) 0.1

Using Stop Time
Stop Time (mins) 0.0

Pre Auto Balance is On
Post Auto Balance is Off
PeakWidth (mins) 0.2

HP1100 Autosampler Initial Conditions

Draw Speed 200.0
Eject Speed (µl/min) 200
Draw Position (mm) 0.00
Stop Time (mins) 32.00
Vial Number

Sample Run Injection Parameter

Injection Volume (ul) - 10.00
---------- oOo ----------

End of experimental record.

Function 1
Scans in function: 1919
Cycle time (secs): 1.000
Scan duration (secs): 0.90
Interscan delay (secs): 0.10
Retention window (mins): 0.000 to 32.000
Ionization mode: ES-
Data type: Enhanced Mass
Function type: TOF MS
Mass range: 25 to 550

Function 2
Scans in function: 4793
Function type: Diode Array
Wavelength range (nm) : 190 to 600

Appendix E

Chemical hazard summary sheets

CAS - No.	Name
4164-28-7	**Dimethylnitramine**

EU-Risk phrases	
Comments on chemical	
Comments on evaluation	
GESAMP/EHS file	**-**
RTECS file	IQ0450000; Last updated 200711
IUCLID file	**-**
REACH file	
Other sources	• **Toxnet** Literature references to studies and databases • **CCRIS** Carcinogenicity and mutagenicity studies • **CPDB** Liver and nasal cavity cancers in rats (http://potency.berkeley.edu/chempages/DIMETHYLNITRAMINE.html) • **SciFinder** • **PubMed**

Evaluation based on (if based on similar chemical)

No.	CAS - No.	chemical name	remark
1			

Column C1: Oral Toxicity

0: >2000 1: 300-2000 2: 50-300 3: 5-50 4: <5 mg/kg bw

Study no.	rating based on this study	LD_{50} value	animal species	Source or comment
1	**1**	1095 mg/kg	rat	Reliable data RTECS- Toxicology and Applied Pharmacology, 1975
2	**1**	1095 mg/kg	rat	(Andersen and Lenkins, 1978)
3			rat	LD50 i.v. 600 mg/kg (Andersen and Lenkins, 1978)
4			rat	LD50 i.p. 897 mg/kg (Andersen and Lenkins, 1978)
5			mice	LD50 i.p. 399 mg/kg (Andersen and Lenkins, 1978)

Column C2: Percutaneous Toxicity

0: >2000 1: 1000-2000 2: 200-1000 3. 50-200 4: <50 mg/kg bw

Study no.	rating based on this study	LD_{50} value	animal species	Source or comment

Column C3: Inhalation Toxicity

0: >20 1: 10-20 2: 2-10 3: 0.5-2 4: <0.5 mg/l (4hrs)

Study no.	rating based on this study	LC_{50} value exp. Time	animal species	Details, remarks, please indicate exposure time (hrs)
1				

mg/l = ppm x mw / 24 x 1000

Column D1: Skin Irritation / Corrosion

0: non-irritating 1: Mildly irritating 2: Irritating 3: Severely irritating or corrosive

3A: Corrosive >1 hr-4 hr 3B: Corrosive >3 min < 1 hr 3C: Corrosive < 3min

study no.	proposed rating	source / kind of study / animal species
1		

Column D2: Eye Irritation / Corrosion

0: Not irritating 1: Mildly irritating 2: Irritating 3: Severely irritating with irreversible corneal injury

study no.	proposed rating	source / kind of study / animal species
1		

Column D3: Other long term effects (indicate by appropriate letter in box)

Carcinogenic - C	**C**	Aspiration haz. - A		Neurotoxic - N	
Lung injury - L		Reprotoxic – R		Immunotoxic - I	
Mutagenic - M	**M**	Photosensitizer - P		Sensitizing - S	

Source/comment:

M: Mutagen by RTECS criteria

C: Carcinogen and tumorigen by RTECS criteria

C: CPDB/ TD50 = 0.547 mg/kg bw/day in both male and female rats. Reported to cause tumors in liver and nasal cavity. (Goodall and Kennedy, 1976). Endpoint: Number of tumors in liver, lung kidney, malignant lymphoma, lung, duodenum, atriocaval; Method: Full post-mortem and histologic examinations of tissues

Mutagenicity

M = Mutagenic

study no	proposed rating	source / kind of study / animal species
1	Positive	Khudoley et al., 1981/ Mutagenecity assays in TA 1535 and TA 100 (Liquid incubation assays, Host mediated assay) / Salmonella typhimurium TA1530 in a host-mediated assay in rats. Endpoint: Mutagenicity and mutation frequency
2	Positive	Frei E et al., 1984 / Ames test: Mutagenecity assays in TA 1535 and TA 100 (Plate incorporation assay and preincubation modification assay) / Salmonella typhimurium TA100. Endpoint: In vitro metabolism and mutagenicity
3	Negative	Frei E et al., 1986 / Alkaline elution method- fluorimetrically, Radioactivity-scintillation counting, Enzyme activites-biochemical method / Hepatocytes and SV 40-transformed chinsese hamster embryo cell lines. Endpoint: Induction of single strand breaks, selective DNA amplification and enzyme activities
4	Positive	Pala et al., 1982 / alkaline elution assay / Damage in mice liver DNA. Endpoint: DNA damage in vivo

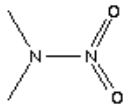
study no	proposed rating	source / kind of study / animal species
5	Positive	Pool BL et al., 1984 / Ames test: Positive mutagenic when pre-incubated with bacteria and a complete metabolizing mixture containing 9000 g liver supernatant and NADPH-regenerating cofactors
6	Positive	Pool BL et al., 1986 / DNA single-strand breaks in mammalian cells, amplified DNA sequence in cultured cells, Mutagenecity in S.typhimurium with or without metabolic activation Positive based on literature results from different assay systems. Endpoint: DNA damage, amplified DNA sequence and mutagenicity
7	Positive	Malaveille C. et al., 1983 / Ames test: Mutagenicity assay in TA100 strain/ with and without metabolic activation.

Carcinogenicity

1=Carcinogenic to humans 2 = Probably carcinogenic to humans 3 = Possibly carcinogenic to humans 4 = Not classifyable as a human carcinogen

study no	proposed rating	source / kind of study / animal species
1	Positive	Scherf HR et al., 1989: Positive- tumors in nasal cavity in both male and female rats when administered by gavage, with males being more susceptible. Endpoint: Number of tumors in nasal cavity, spinal cord, spinal and peripheral nerves, pituitary and mammary gland and other; Method: Full post-mortem and histologic examinations of tissues. TDLo - Lowest published toxic dose: 90 mg/kg/2Y (continuous).
2	Positive	Mirvish et al., 1980 / Full post-mortem and histologic examinations of tissues, LD50 determination / Positive- liver and nasal cavity tumors. Endpoint: Survival data, body weight, Number of tumors in respiratory tract, GIT, liver, kidney, nervous system, RES, endocrine and mammary glands, skin, soft tissue and other. Dosage: TD: 20 gm/kg/1Y (continuous).
3	Positive	Andersen and Lenkins, 1978 / Necroscopy / Induction of hemorrhagic foci in the lining of the stomach and intestine after single dose of dimethylnitramine. Endpoint: Gastrointestinal toxicity
4	Positive	Hassel et al. 1987 / Examination of tissues Positive- Aesthesineuroeptheliomas and neurogenic tumours of the lumbar region of the spine in rats. Endpoint: Number of tumors in nasal cavity, spinal cord, spinal and peripheral nerves and other sites
5	Negative	Pala et al., 1982 / light microscopy, histopathology No necrosis in liver. Endpoint: Liver necrosis
6	Positive	Pliss et al., 1982 / Full post-mortem and histologic examinations of tissues. Endpoint: Number of tumors in urinary bladder, liver and kidney in various animal species
7	Positive	Goodal et al, 1976 / The mice developed hepatocellular carcinomas and renal adenocarcinomas. The rats developed hepatocelluar carcinomas, some which metastasized. Statistically significant increases of other tumor types also occurred in mice.
8	Positive	TD: 40 gm/kg, Oral, Rat (NATWAY Naturwissenschaften. (Springer-Verlag, Heidelberger Platz 3, D-1000 Berlin 33, Fed. Rep. Ger.) V.1- 1913- Volume(issue)/page/year: 48,134,1961)
9	Positive	TD - Toxic dose (other than lowest). Rodent – rat. Oral. 34 gm/kg/82W (continuous). Tumorigenic - equivocal tumorigenic agent by RTECS criteria Liver - tumors Blood – leukemia. ZEKBAI Zeitschrift fuer Krebsforschung. (Berlin, Fed. Rep. Ger.) V.1-75, 1903-71. For publisher information, see JCROD7. Volume (issue)/page/year: 69,103,1967.
10	Positive	Goodall and Kennedy, 1976/ Reported to cause tumors in liver and nasal cavity. Endpoint: Number of tumors in liver, lung kidney, malignant lymphoma, lung, duodenum, atriocaval; Method: Full post-mortem and histologic examinations of tissues

Remarks

Chemical name	CAS.	Structure	Ref./ Comment	Data bases	Oral	Dermal	Inhal	Long term	Comment on human health hazard
Dimethyl nitramine	4164-28-7		-	Toxnet+ CCRIS+ RTECS+ IUCLID- GESAMP-CPDB+ IRIS- Pubmed+ Scifinder+	1	-	-	C M	***Confirm C*** ***Confirm M*** ***No data on reprotox.*** Slight oral toxicity No OEL/TWA available ***Serious long term effects.*** ***Candidate for testing for R*** ***Candidate for testing as positive control for C and M studies***

Serious long term effects
Toxicology Review (Mutation Research, 2005)
No data on reproductive effects or sensitization

GESAMP/EHS Marine transport

C1	C2	C3	D1	D2	D3	Date

CAS - No.	Name
598-57-2	**Methylnitramine**

EU-Risk phrases	
Comments on chemical	
Comments on evaluation	
GESAMP/EHS file	-
RTECS file	PF8870000; Last updated 200802
IUCLID file	-
REACH file	
Other sources	• **Toxnet** Literature references to studies and databases • **CCRIS** Carcinogenicity and mutagenicity studies **CPDB:** nervous system cancers http://potency.berkeley.edu/chempages/METHYLNITRAMINE.html • **Pubmed**

Evaluation based on (if based on similar chemical)

No.	CAS - No.	chemical name	Remark
1			
2			

Column C1: Oral Toxicity

0: >2000 1: 300-2000 2: 50-300 3: 5-50 4: <5 mg/kg bw

Study no.	rating based on this study	LD_{50} value	animal species	Source or comment
1	-	500mg/kg	Mouse	Intraperitoneal exposure. PCJOAU Pharmaceutical Chemistry Journal (English Translation). Translation of KHFZAN. (Plenum Pub. Corp., 233 Spring St., New York, NY 10013) No.1- 1967- Volume(issue)/page/year: 10,1504,1976. Data not applicable for GESAMP classification

Column C2: Percutaneous Toxicity

0: >2000 1: 1000-2000 2: 200-1000 3. 50-200 4: <50 mg/kg bw

Study no.	rating based on this study	LD_{50} value	animal species	Source or comment
1				

Column C3: Inhalation Toxicity

0: >20 1: 10-20 2: 2-10 3: 0.5-2 4: <0.5 mg/l (4hrs)

Study no.	rating based on this study	LC_{50} value exp. time	animal species	Details, remarks, please indicate exposure time (hrs)
1				

mg/l = ppm x mw / 24 x 1000

Column D1: Skin Irritation / Corrosion

0: non-irritating 1: Mildly irritating 2: Irritating 3: Severely irritating or corrosive

3A: Corrosive >1 hr-4 hr 3B: Corrosive >3 min < 1 hr 3C: Corrosive < 3min

study no.	proposed rating	source / kind of study / animal species
1		

Column D2: Eye Irritation / Corrosion

0: Not irritating 1: Mildly irritating 2: Irritating 3: Severely irritating with irreversible corneal injury

study no.	proposed rating	source / kind of study / animal species
1		

Column D3: Other long term effects (indicate by appropriate letter in box)

Carcinogenic - C	**C**	Aspiration haz. - A		Neurotoxic - N	
Lung injury - L		Reprotoxic – R		Immunotoxic - I	
Mutagenic - M	**M?**	Photosensitizer - P		Sensitizing - S	

Source/comment:

M: Mutagen by RTECS criteria

C: Carcinogen and tumorigen by RTECS criteria

C: CPDB/ TD50 = 17.4 mg/kg bw/day in both male and female rats. Reported to cause tumors in nervous system.

Mutagenicity

M = Mutagenic

study no	proposed rating	source / kind of study / animal species
1	Positive for DNA single strand breaks in hepatocytes.	Frei et al., 1986 / Alkaline elution method- fluorimetrically, Radioactivity-scintillation counting, Enzyme activites-biochemical method / Rat hepatocytes and SV 40-transformed Chinese hamster embryo cell lines / Endpoint: Induction of single strand breaks, selective DNA amplification and enzyme activities using hepatocytes and SV 40-transformed Chinese hamster embryo cell lines. Dosage: 12500 nmol/L.
2	Positive / negative	Pool et al., 1986 / positive for DNA single-strand breaks (ssb) in hepatocytes, amplified DNA sequence in cultured cells, Mutagenecity in S.typhimurium with or without metabolic activation /. Endpoint: DNA damage, amplified DNA sequence and mutagenicity. Negative in Ames test and ssb in Chinese hamster embryo cells
3	Negative	Frei E et al., 1984 / Ames test: Mutagenecity assays in TA 1535 and TA 100 (Plate incorporation assay and preincubation modification assay) / Salmonella typhimurium TA100. Endpoint: In vitro metabolism and mutagenicity
4	Negative	Pool BL et al., 1984 / Ames test: Mutagenicity assay in TA100 strain / with and without metabolic activation.
5	Negative	Malaveille C. et al., 1983 / Ames test: Mutagenicity assay in TA100 strain/ with and without metabolic activation.

Carcinogenicity

1=Carcinogenic to humans 2 = Probably carcinogenic to humans 3 = Possibly carcinogenic to humans 4 = Not classifyable as a human carcinogen

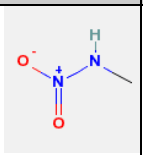
study no	proposed rating	source / kind of study / animal species
1	Positive	Scherf HR et al., 1989 / Full post-mortem and histologic examinations of tissues / tumors (neurinoma) in spinal cord and spinal nerve in both male and female rats when administered by gavage, 0.05 and 1 mmol/kg per week, with males being more susceptible. TDLo - Lowest published toxic dose: 76 mg/kg/2Y (continuous).
2	Positive	Hassel M et al. 1987 / Examination of tissues / Neurogenic tumours of the lumbar region of the spine in rats.

Remarks

Serious long term effects
Toxicology Review (Mutation Research, 2005)
No data on reproductive effects. No data on sensitization

GESAMP/EHS Marine transport

C1	C2	C3	D1	D2	D3	Date

Chemical name	CAS	Structure	Ref./ Comment	Data bases	Oral	Dermal	Inhal	Long term	Comment on human health hazard
Methyl - nitramine	598-57-2		-	Toxnet+ CCRIS+ RTECS+ IUCLID- GESAMP- CPDB+ IRIS- Pubmed+	-	-	-	C M?	***Confirm C M: Need more data*** ***No data on reprotox.*** Slight oral toxicity No OEL/TWA available ***Serious long term effects.*** ***Candidate for general testing and R studies*** ***Candidate for testing as positive control for C studies***

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KEYWORDS		
Nitramine	Toxicity	LC/MS

ABSTRACT
Five nitramines have been synthesized, and nitramine analysis procedures have been developed and validated. Analyses of water wash samples show presence of etanolnitramine. Mild acute toxicity was observed in four nitramines, while one nitramine is considered either mild or non-toxic. No skin irritation/corrosion/sensitisation was observed, but all tested nitramines were irritants to the eye. Two nitramines were positive in at least two genotoxicity/mutagenicity assays.

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

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