European Union Risk Assessment Report

2,4-DINITROTOLUENE

CAS No: 121-14-2

EINECS No: 204-450-0

RISK ASSESSMENT

FINAL APPROVED VERSION

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RISK ASSESSMENT

Final report, February 2008

SPAIN

FINAL APPROVED VERSION

Rapporteur for the risk assessment of 2,4-Dinitrotoluene is Spain

Contact points:

Fernando Carreras Vaquer
Ministry of Health
Paseo del Prado 18-20
28071 Madrid (Spain)
Fax: +34 91 3601341
Email: sgsasl@msc.es

Ana Fresno
Ministry of Environment
Pza. San Juan de la Cruz s/n
28071 Madrid (Spain)
Fax: +34.91.534.05.83
e-mail: afresno@mma.es

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Foreword

We are pleased to present this Risk Assessment Report which is the result of in-depth work carried out by experts in one Member State, working in co-operation with their counterparts in the other Member States, the Commission Services, Industry and public interest groups.
The Risk Assessment was carried out in accordance with Council Regulation (EEC) 793/93[1] on the evaluation and control of the risks of "existing" substances. "Existing" substances are chemical substances in use within the European Community before September 1981 and listed in the European Inventory of Existing Commercial Chemical Substances. Regulation 793/93 provides a systematic framework for the evaluation of the risks to human health and the environment of these substances if they are produced or imported into the Community in volumes above 10 tonnes per year.
There are four overall stages in the Regulation for reducing the risks: data collection, priority setting, risk assessment and risk reduction. Data provided by Industry are used by Member States and the Commission services to determine the priority of the substances which need to be assessed. For each substance on a priority list, a Member State volunteers to act as "Rapporteur", undertaking the in-depth Risk Assessment and recommending a strategy to limit the risks of exposure to the substance, if necessary.
The methods for carrying out an in-depth Risk Assessment at Community level are laid down in Commission Regulation (EC) 1488/94[2], which is supported by a technical guidance document[3]. Normally, the "Rapporteur" and individual companies producing, importing and/or using the chemicals work closely together to develop a draft Risk Assessment Report, which is then presented at a meeting of Member State technical experts for endorsement. The Risk Assessment Report is then peer-reviewed by the Scientific Committee on Health and Environmental Risks (SCHER) which gives its opinion to the European Commission on the quality of the risk assessment.
If a Risk Assessment Report concludes that measures to reduce the risks of exposure to the substances are needed, beyond any measures which may already be in place, the next step in the process is for the "Rapporteur" to develop a proposal for a strategy to limit those risks.
The Risk Assessment Report is also presented to the Organisation for Economic Co-operation and Development as a contribution to the Chapter 19, Agenda 21 goals for evaluating chemicals, agreed at the United Nations Conference on Environment and Development, held in Rio de Janeiro in 1992 and confirmed in the Johannesburg Declaration on Sustainable Development at the World Summit on Sustainable Development, held in Johannesburg, South Africa in 2002.
This Risk Assessment improves our knowledge about the risks to human health and the environment from exposure to chemicals. We hope you will agree that the results of this in-depth study and intensive co-operation will make a worthwhile contribution to the Community objective of reducing the overall risks from exposure to chemicals.

1 O.J. No L 084, 05/04/199 p.0001 – 0075

2 O.J. No L 161, 29/06/1994 p. 0003 – 0011

3 Technical Guidance Document, Part I – V, ISBN 92-827-801 [1234]

Contact Details of the Rapporteur(s)

Environment

Rapporteur
Ana Fresno
Spanish Environmental Competent Authority
Ministry of Environment
San Juan de la Cruz s/n
28071-Madrid (Spain)
afresno@mma.es

Scientific Experts: The scientific work has been prepared by the National Institute for Agricultural and Food Research and Technology (INIA) by order of the rapporteur.

Effect assessment: José Vicente Tarazona, Mª Milagrosa Vega [(1)] and Elena Suárez
Exposure assessment: José Luis Tadeo and Lorena González

INIA - Department of Environment
Carretera de la Coruña, Km. 7.5
28040-Madrid (Spain)

Health

Rapporteur
Fernando Carreras Vaquer
Spanish Health Competent Authority
Ministry of Health
Paseo del Prado 18-20
28071-Madrid (Spain)
sgsasl@msc.es

Scientific Experts: The scientific work has been prepared by the Ministry of Health in collaboration with the Miguel Hernández University of Elche (Alicante).

Effect assessment: Carmen Barrueco, Covadonga Caballo, Raquel Fernández and Ana Vega [(2)] (Ministry of Health); Javier Esteban and Eugenio Vilanova (Miguel Hernández University).
Occupational exposure assessment: Esther Martín (Ministry of Health).

Ministry of Health
Paseo del Prado, 18-20
28071-Madrid (Spain)

Universidad Miguel Hernández
Avda. Universidad, s/n
03202-Elche-Alicante (Spain)

[(1)] Current address:
ERA Consult; (vega@era-consult.com)
C/ Palencia 15, 2ºB
28020-Madrid (Spain)

[(2)] Current address:
TRAGSATEC (avr@tragsatec.es)
C/ Velázquez 36, Bajo
28001-Madrid (Spain)

0 OVERALL RESULTS OF THE RISK ASSESSMENT[4]

CAS Number: 121-14-2
EINECS Number: 204-450-0
IUPAC Name: 2,4-dinitrotoluene

Environment

Conclusion (i) There is a need for further information and/or testing.

No conclusion (i) has been assigned.

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Conclusion (ii) applies to the aquatic and sediment compartment at continental and regional level and for sites A, D and E.

Conclusion (ii) applies to the marine compartment.

Conclusion (ii) applies to the STP compartment.

Conclusion (ii) applies to the terrestrial compartment.

Conclusion (ii) applies to the atmospheric compartment.

Conclusion (ii) applies to the secondary poisoning according to the low bioaccumulation potential and the rapid elimination of this compound in fish and mammals, a low risk for secondary poisoning on birds and mammals is expected from this substance.

Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

Conclusion (iii) applies to the need of risk reduction measures for the aquatic compartment and for sediment-dwelling organisms for one site at local level (site B). Nevertheless, it is expected that any risk reduction measure for surface water will also reduce the risks for sediments.

4 Conclusion (i) There is a need for further information and/or testing.
Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.
Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

Human health

Human health (toxicity)

Workers

Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

This conclusion is reached because of:

- concerns for carcinogenicity and mutagenicity as a consequence of inhalation and dermal exposure arising from all worker scenarios.
- concerns for repeated dose toxicity and toxicity for reproduction (fertility) as a consequence of dermal exposure arising from manufacture and use of explosives (worker scenarios 2 and 3).
- concerns for repeated dose toxicity and toxicity for reproduction (fertility) as a consequence of inhalation arising from manufacture of explosives (worker scenario 2).

Conclusion (**ii)** There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

This conclusion applies to acute toxicity by inhalation and dermal route; irritation/corrosivity for skin and eyes; sensitisation; repeated dose toxicity and toxicity for reproduction (fertility) for two worker scenarios (1 and 3) by inhalation and for one worker scenario (1) by dermal route, because these endpoints are of no concern.

Consumers

Conclusion (**ii)** There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

This conclusion applies because exposure of consumers is not assumed to exist.

Humans exposed via the environment

Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

This conclusion is reached because of:

- concerns for carcinogenicity and mutagenicity as a consequence of oral exposure arising from the local site B**.**

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

This conclusion is reached for repeated dose toxicity and toxicity for reproduction (fertility) because the calculated MOS for oral exposure of man via the environment in both local and regional scales are judged to be enough for these endpoints.

Combined exposure

The risk to human health under conditions of combined exposure is dominated by occupational exposure.

Human health (physico-chemical properties)

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

This conclusion is reached because the risk assessment shows that risks are not expected, and risk reduction measures already being applied are considered sufficient.

CONTENTS

TABLES

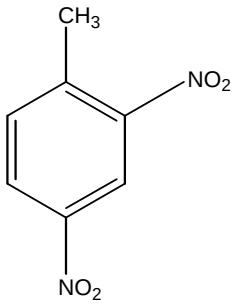
EUSES Calculations can be viewed as part of the report at the website of the European Chemicals Bureau: http://ecb.jrc.it

1 GENERAL SUBSTANCE INFORMATION

1.1 IDENTIFICATION OF THE SUBSTANCE

CAS Number: 121-14-2
EINECS Number: 204-450-0
IUPAC Name: 1,3-Dinitro-4-methylbenzene
Molecular formula: $C_7H_6N_2O_4$

Structural formula:



Molecular weight: 182.14
Synonyms: 1-Methyl-2,4-dinitro-benzene
1-Methyl-2,4-dinitro-benzol
2,4-dinitro-1-methylbenzene
2,4-dinitrotoluene
2,4-dinitrotoluol
Benzene, 1-methyl-2,4-dinitro
2,4-DNT
DNT
Dinitrotoluole
m-dinitrotoluol
Toluene, 2,4-dinitro-

1.2 PURITY/IMPURITIES, ADDITIVES

Purity: ≥ 99%
Impurity There are no data available.

1.3 PHYSICO-CHEMICAL PROPERTIES

2,4-dinitrotoluene is a yellow solid with slight odour which appears in rhombic needles or monoclinic prisms. It is stable under normal laboratory conditions but it may react violently in the presence of a base, oxidant and reducing agent or when heated to the boiling point. It is slightly soluble in water, soluble in ether, benzene and acetone, and very soluble in chloroform and toluene.

Melting point

Only one value is reported in IUCLID Data Set, referred to the BUA report, 1987. The indicated melting point of 69.9 ºC, matches with the reported by Bayer AG, and it has been used in the assessment report.

Boiling point

IUCLID Data Set indicates values of c.a.300 ºC (BUA report, 1987) and 319.5 ºC (Bayer AG), and this second value will be considered in the assessment.

Relative density

Roembke J., 1995, as well as Bayer AG, give a value of 1.326 g/cm^3 at 70 ºC. Another reference of Bayer AG, referred to 110 ºC, is 1.286 g/cm^3.

Vapour pressure

The measured value of vapour pressure of 2,4-dinitrotoluene quoted in IUCLID, 7.9 10^{-5} hPa at 20 ºC (Bayer AG), has been considered in the calculations.

Water solubility

IUCLID Data Set indicates two values from Bayer AG: 0.3 g/l at 23 ºC and 166 mg/l at room temperature. This last value will be used for the modelling processes.

Partition coefficient n-octanol/water (log value)

The value of 1.98, experimentally determined and included in THOR database, will be taken into account. Other calculated values are indicated in IUCLID, like 1.8 (Leo, A, 1991) and 2 (Bayer, 1986).

Granulometry

No data have been supplied in the IUCLID Data Set.

Conversion factors

No data have been supplied in the IUCLID Data Set.

Flash point

Bayer AG points out a value of 169 ºC, measured in close cup with DIN 51758. This will be considered in the risk assessment.

Autoflammability

The ignition temperature measured with DIN 51794 for 2,4-dinitrotoluene is 300 ºC, as indicated by Bayer AG.

Flammability

No data appear in the IUCLID Data Set for this property.

Explosive properties

Bayer AG has indicated that below 150 ºC 2,4-dinitrotoluene does not show a propagation in the 2” steel tube test.

Oxidizing properties

2,4-dinitrotoluene is not an oxidising agent on the basis of its chemical structure.

Viscosity

Property not applicable to solids.

Henry's constant

A value of 0.0870807 $Pa.m^3/mol$ is reported in the IUCLID Data Set. The experimental dimensionless value given by Altschuh, J. *et al.* (1999), $2.2 \cdot 10^{-6}$ will be used after conversion to the units needed by means of the equation 22 of the new Technical Guidance Document (TGD), giving a value of $5.45 \cdot 10^{-3}$ $Pa.m^3/mol$.

Surface tension

No data appear in the IUCLID Data Set for this property.

Table 1.1 Summary of physico-chemical properties

Property	Value	
Physical state	Solid	-
Melting point	69.9ºC	Bayer AG
Boiling point	319.5ºC	Bayer AG
Relative density	1.286 g/cm^3 at 110ºC 1.3206 g/cm^3 at 70ºC	Bayer AG Roembke J, (1995).
Vapour pressure	7.9 10^{-3} Pa at 20ºC	Bayer AG
Water solubility	166 mg/l at room temperature	Bayer AG
Partition coefficient n-octanol/water (log value)	1.98	Chemical Information Systems, Inc. Irvine CA USA (1991)
Granulometry	-	-
Conversion factors	1 ppm = 7.45 mg/m^3 at 25ºC	EPA (1999)
Flash point	169ºC	Bayer AG
Autoflammability	Auto ignition temperature: c.a. 400º C	Bayer AG
Flammability	-	-
Explosive properties	Under 150º C does not show a transmission of detonation	Bayer AG -
Oxidizing properties	-	-
Viscosity	Not applicable (solid)	-
Henry's constant	5.45·10^{-3} Pa-m^3/mol*	Calculated from an experimental dimensionless Henry's law constant value reported by Altschuh, J. (1999)
Surface tension	-	-

1.4 CLASSIFICATION

1.4.1 Current classification

2,4-Dinitrotoluene is included in Annex 1 Directive 67/548/CEE (29th ATP):

Carc.Cat. 2; R45: May cause cancer

Muta. Cat. 3; R68: Possible risk of irreversible effects.

Repr. Cat. 3; R62: Possible risk of impaired fertility

T; R23/24/25: Toxic by inhalation, in contact with skin and if swallowed.

Xn; R48/22: Harmful, danger of serious damage to health by prolonged exposure if swallowed.

N; R51-53: Dangerous for the environment. Toxic to aquatic organisms/May cause long-term adverse effects in the aquatic environment.

1.4.2 Proposed classification

It is proposed to keep the same classification as currently:

Carc.Cat. 2; R45: May cause cancer

Muta. Cat. 3; R68: Possible risk of irreversible effects.

Repr. Cat. 3; R62: Possible risk of impaired fertility

T; R23/24/25: Toxic by inhalation, in contact with skin and if swallowed.

Xn; R48/22: Harmful, danger of serious damage to health by prolonged exposure if swallowed.

N; R51-53: Dangerous for the environment. Toxic to aquatic organisms/May cause long-term adverse effects in the aquatic environment.

2 GENERAL INFORMATION ON EXPOSURE

2.1 PRODUCTION

2.1.1 Production processes

A typical sulphuric acid/nitric acid nitrating mixture reacting with toluene yields 78 wt% 2,4-dinitrotoluene, 18 wt% 2,6-dinitrotoluene, 2.5 wt% 3,4-dinitrotoluene, 1 wt% 2,3-dinitrotoluene and 0.5 w% 2,5-dinitrotoluene. If the single 2,4-isomer is required, the nitration is stopped at the mono-stage and pure p-nitrotoluene is obtained by crystallization. Subsequent nitration of the p-nitrotoluene yields only 2,4-dinitrotoluene. Furthermore 2,4-dinitrotoluene is also formed as an impurity during manufacture of 2,4,6-trinitrotoluene, by the dinitration of toluene, and must be removed by mechanical means.

2.1.2 Production capacity

According to available data, the production of 2,4-dinitrotoluene in Europe is located in Italy, France and Germany, and this substance is produced as a mixture of isomers, c.a. 80% of 2,4-dinitrotoluene and c.a. 20% of 2,6-dinitrotoluene. In addition, an amount from 11,025 to 50,025 kg/year is imported for use in explosives, according to data reported by a Spanish company. The resultant quantity of 2,4-dinitrotoluene produced and imported in the EU is 503,970 - 504,009 tons/year, as can be seen in Table 2.1:

Table 2.1: Volume produced or imported in metric tons/year

Country	Volume of 2,4 dinitrotoluene	Total volume (2,4 dinitrotoluene + 2,6 dinitrotoluene)
Italy	87,159 [a]	108,949
France	112,800 [b]	141,000
Germany	304,000 [c]	380,000
Spain	11 - 50 [d]	-
Total	503,970 - 504,009	629,949

[a] Production of 2002

[b] Production of 2000

[c] Production of 2004

[d] Imported

2.2 USES

2.2.1 Introduction

2,4-dinitrotoluene is primarily used as a chemical intermediate in the production of toluene diisocyanate (4-methyl-m-phenylenediisocyanate), also named toluene-2,4-diisocyanate (TDI), from toluene-diamine (4-methyl-m-phenylenediamine) also named toluene-2,4-diamine (TDA). This application uses about 99% of 2,4-dinitrotoluene production (Chemical Marketing Reporter, 1983). 2,4-dinitrotoluene is hydrogenated to yield toluene-2,4-diamine (TDA) and this diamine is reacted with phosgene to yield toluene-2,4-diisocyanate, which is used to make flexible polyurethane foams.

According to bibliography, 2,4-dinitrotoluene has several other minor uses. It is used as gelatinizing-plasticizing agent in both commercial and military explosive compositions (Sears and Touchette, 1982; SRI, 1976). It may also be a component of specialty explosive compositions and it is as well used as an intermediate in the production of dyes and dyestuffs (Dunlap, 1981: SRI, 1976). It has been used too as an intermediate to make rubber chemicals (ACS, 1983) and in automotive airbags (Ellenhorn, 1997). The oldest commercial uses of 2,4-dinitrotoluene involve dyes and explosives.

More recent and accurate data reported has been gathered from industry related to the breakdown of 2,4-dinitrotoluene uses. Data of 2004 from Germany indicate that all 2,4-dinitrotoluene was directly used as intermediate for the production of TDI over at least the past decade. The other two industries that have reported information on the use of 2,4-dinitrotoluene, one from France and another one from Italy, declared that all of it is transformed to 2,4/2,6-TDA.

Table 2.2: Industrial and use category according to TGD

Industry category	Use category	Quantity used (metric tons)	Percentage of total use
3. Chemical industry	33. Intermediate	503,959	99.99
15. Other industry		50	0.01
Total		504,009	100

2.2.2 Scenarios

The substance is produced and largely used in closed systems as an intermediate for further synthesis. 2,4-dinitrotoluene is mainly synthesised by the dinitration of toluene and once manufactured is used on site for obtaining TDA. However, 2,4-dinitrotoluene has had other minor uses, including its use as an additive in the production of explosives, though very scarce information has been provided. Concerning explosives, information has only been obtained from a Spanish industry, for an imported amount of 50 tons of 2,4-dinitrotoluene. Emissions of 2,4-dinotrotoluene can also take place during the manufacture of TNT, because this compound is present as an impurity, and information from a TNT manufacturing site located in United Kingdom has been provided. However, the TNT production in this site is now very infrequent and may cease in the near future.

Therefore, only the Spanish site has been considered in the present risk assessment report. Other minor uses of 2,4-dinitrotoluene have not been taken into account, as no information on processing sites and amounts used has been obtained.

The main industrial and use categories with the quantity of 2,4-dinitrotoluene used, considering the total amount produced and imported in the EU, have been summarised in Table 2.2. Nevertheless, as there are several places of production and processing of 2,4-dinitrotoluene, five different sites will be considered: four of them (Sites A to D) with the fraction of the tonnage of the substance corresponding to production and processing of TDA, and another one (site E) for the use of 2,4-dinitrotoluene as an additive in explosives, as can be seen in Table 2.3:

Table 2.3: Site specific information of the volume used of 2,4- dinitrotoluene (metric tons/year)

Scenario	Country	Volume
Site A	Italy	87,159
Site B	France	67,200
Site C *	France	45,600
Site D	Germany	304,000
Site E	Spain	50

* This site will not be considered in the calculations because it has been closed in December 2005.

On the other hand, four companies have reported data of the emissions, therefore the five scenarios will be brought up using these data.

2.3 TRENDS

The IUCLID Data Set of 2001 from a German industry indicates that its production volume in 1992 was 14,000 tons, which were used as additive in explosive preparations (3%) and as an intermediate for the production of toluylen-diisociante. In 2001, precise information about 2,4-dinitrotoluene production volume in 2000 was provided. In that year, three plants were in operation, two of them in Germany (with a production of 48,000 and 75,200 tons, respectively) and another in Belgium (30,400 tons). Recently, the situation has changed. The up to now existing production facilities have been closed and a new world-scale unit replaced them since 2003 in Germany. The new production site has started its orderly production in 2005, with a final production volume capacity of 304,000 tons. Regarding to the breakdown of uses, current information indicates that all 2,4-dinitrotoluene is used as an intermediate in the production of toluylenediamine and further toluylene diisocianate.

Regarding industry of site A, information on the production is available only for two years: 113,300 tons in 2001 and 87,159 tons in 2002.

Concerning site C, recent information from industry states than the plant placed there was closed the 31st of December 2005.

Finally, a Spanish industry has supplied information from a 2,4-dinitrotoluene processing site. The amount imported has followed a decreasing trend from 50,025 kg in 1998 to 11,025 kg in 2000. Since no current data have been provided, the amount of 50 tons has been taken as worst case processing volume for site E.

2.4 LEGISLATIVE CONTROLS

There are several legal regulations related to 2,4-dinitrotoluene in different countries. In Germany, the Administrative Rules Concerning Substances Hazardous to Water of 1990 (Verwaltungsvorschrift Wassergefaehrdende Stoffe) sets up a classification as the basis for water-protection requirements for industrial plants in which water-hazardous substances are handled. 2,4-dinitrotoluene is classified as very hazardous to water.

In Czech Republic 2,4-dinitrotoluene was classified as a poison in the Government Provision No. 192 on Poisons and Another Substances Harmful to Human Health. Besides, a TWA of 1.0 mg/m^3 and a CLV of 2.0 mg/m^3 are given by the Hygienic Regulations of Ministry of Health

In India, the Manufacture, Storage and Import of Hazardous Chemicals Rules, of 1989, applies to 2,4-dinitrotoluene.

In Japan, the Poisonous and Deleterious Substances Control Law of 1991 designates 2,4-dinitrotoluene and its preparations as deleterious.

In Canada, the Occupational Safety and Health Regulations prescribes a Time Weighted Average (TWA) of 1.5 mg/m3 for skin absorption. The Workplace Hazardous Materials Information System (WHMIS), which imposes standards on employers for the use, storage and handling of controlled products, states in the Ingredient Disclosure List that a concentration equal to or greater than 1% weight/weight of 2,4-dinitrotoluene must be disclosed in the Safety Data Sheet.

In the USA, the Clean Air Act, 112 National Emission Standards For Hazardous Air Pollutants of 1985 establish a list of pollutants judged to be hazardous for which emission standards will be developed. This list includes 2,4-dinitrotoluene. This substance is as well included on a list required by the Clean Water Act section 304 of conventional pollutants requiring maximum daily effluent limitations. Releases of this hazardous substance, in quantities equal to or greater than 10 LBS (4.54 kg), are subject to reporting to the national response centre under the comprehensive environmental response, compensation, and liability act, and for purposes of section 311 of the Clean Water Act 2,4-dinitrotoluene shall not be discharged into or upon the navigable waters of the United States or adjoining shorelines, waters of the contiguous zone, or outer deep waters which may affect natural resources belonging to the United States.

Finally, two other international legal references can be mentioned. The International Maritime Dangerous Goods Code of 1991 includes 2,4-dinitrotoluene in the Hazard Class 6.1, as a poisonous substance, and in the packing group II. In the Recommendations on the Transport of Dangerous Goods, of 1993, the Hazard Class 6.1, toxic substance, the packing group II, medium danger, and the packing method M, apply to 2,4-dinitrotoluene.

3 ENVIRONMENT

3.1 ENVIRONMENTAL EXPOSURE

3.1.1 General discussion

The releases of 2,4-dinitrotoluene to the environment and its behaviour once in each compartment will be discussed in the following sections, using the methods and calculations described in the Technical Guidance Document (TGD) for the risk assessment of new and existing substances.

3.1.2 Environmental releases

2,4-dinitrotoluene may be released into the environment via the air and waste water during its production, processing and formulation.

The companies settled in Italy (site A) and France (site B) have reported data of emissions to air and water, and they are shown in table 3.1.

The German industry (site D) has reported data on 2,4-dinitrotoluene concentration in the waste water treatment plant (WWTP) effluent; consequently, the emissions have been estimated from that concentration considering the information on the effluent discharge rate of the WWTP provided by industry. Regarding the air compartment, industry has indicated that no emissions of 2,4-dinitrotoluene to the atmosphere occur in this site.

For site E, the available information on emissions to waste water is related to the concentration in the effluent of the plant, which was always lower than the detection limit, 1 mg/l. The releases have been estimated from that value considering the information on the effluent flow of the plant provided by industry. Regarding emissions to air, industry has indicated that there is no release of 2,4-dinitrotoluene to this compartment.

3.1.2.1 Release from production and processing

2,4-dinitrotoluene is produced at three sites in the EU by the dinitration of toluene and site-specific information on releases of 2,4-dinitrotoluene to air and water during production has been provided by French (1 site) and Italian (1 site) manufacturers and processors, while the emission to water of the German industry has been calculated from the information on the effluent concentration supplied, and it confirmed no emissions to air. Consequently, an environmental exposure assessment based on site specific data provided by industry will be carried out for all sites.

In addition, information provided by industry from site A indicates that this site is located in a coastal zone. Therefore, the risk for the marine environment will be also assessed for that site in this report.

The local site emission to each compartment, in kg/day, are summarised in Table 3.1:

Table 3.1: Release of 2,4- dinitrotoluene in the local environment during production and processing (kg/day)

Site	Compartment	
	Air	Water
A	0	0.22
B	0.	16
D	0	0.64*

* Data calculated from information on concentration of wastewater effluent provided by industry.

According to the default emission factor to air indicated in A-tables of new TGD, which is zero, the information provided by industry indicates that in all of the sites, there is no release of 2,4-dinitrotoluene to air.

3.1.2.2 Release from formulation

A minor use of 2,4-dinitrotoluene is as an additive in explosives. Information on this use has only been obtained from a Spanish company, which processes about 50 tons/year. Information from industry indicates a concentration in the effluent lower than the detection limit (1 mg/l) and a plant effluent flow of 12,000 m^3/year, which is treated then in a WWTP of an effluent flow of 250,000 m^3/year. Moreover, this industry has indicated that there is no release of 2,4-dinitrotoluene to air. With this information, the emissions to the environment calculated for formulation are the following:

Table 3.2: Release of 2,4- dinitrotoluene in the local environment during formulation (kg/day)

Site	Compartment	
	Air	Water [a]
E	0	0.02

[a] Calculated from information provided by industry.

3.1.2.3 Release from disposal

Most of 2,4-dinitrotoluene is used as an intermediate in the production of TDA and release from disposal is not expected.

3.1.2.4 Summary of releases

Table 3.3. summarizes the local site emissions to each compartment, in kg/day:

Table 3.3: Summary of local release estimates of 2,4-DNT (kg/day)

Scale		Air	Water
Local	Site A	0	0.22 [a]
	Site B	0[a]	16 [a]
	Site D	0 [a]	0.64 [b]
	Site E	0 [a]	0.02 [c]

[a] Emission data provided by industry

[b] Value calculated from data provided by industry for the new production plant

[c] Value calculated from data provided by industry

For regional level, according to TGD, it can be assumed that 10% of the amount that is produced and used in the EU is produced/used within a region, unless specific information on use or emission is available. However, in this case the industry of each site has provided information on the production volume and consequently, it is more appropriate to use specific values from the region with the highest production volume as input for the regional model. Site D will be considered as the worst case for regional calculations, because it is in the region with the highest production volume. Moreover, this site has an emission factor similar to that of site A and both sites represent about 90% of the EU production. Site C had not been used for this purpose because this industry ceased its production in 2005. Finally, the continental emissions will be calculated using the total amount produced and used in the EU minus the regional amount.

Table 3.4: Summary of regional and continental release estimates of 2,4-DNT (kg/day)

	Compartment	
Scale	**Air**	**Waste water**
Regional	0	0.53
Continental	0	0.34

3.1.3 Environmental fate

2,4-dinitrotoluene may be released into the environment during its production, processing and formulation and emissions to water are expected to be the most important entry routes of 2,4-dinitrotoluene to the environment. General characteristics of 2,4-dinitrotoluene which are relevant for the exposure assessment are discussed in the following sections.

3.1.3.1 Degradation in the environment

3.1.3.1.1 Atmospheric degradation

If released to air, a vapour pressure of $7.9 \cdot 10^{-3}$ Pa at 20ºC indicates that 2,4-dinitrotoluene will exist as a vapour in the ambient atmosphere. 2,4-dinitrotoluene will be degraded in the atmosphere by reaction with photochemically produced hydroxyl radicals and the estimated rate constant of $2.253 \cdot 10^{-13}$ cm^3/molecule/sec for this gas-phase reaction corresponds to a half-life of 71 days at an average atmospheric hydroxyl radicals concentration of $5 \cdot 10^5$ molec/cm^3 (OECD, 1997).

I.A. Al-Ghusain and C.F. Lin developed a study to evaluate several processes for treating 2,4-dinitrotoluene, one of them the wet air oxidation. The experiment was conducted at 260ºC in a 2-L pressure reactor (Parr Instruments) containing one litre of 100 mg/l 2,4-dinitrotoluene. A determined amount of oxygen (P_{O_2} = 69 kPa at 25ºC) was discharged into the reactor, the heater turned on and the reactor content was mixed at 200 rpm. It took about 30 minutes to reach the desired temperature at the end of a 1-hour reaction period. Then the heater was turned off and the reactor content was cooled by circulating tap water through the cooling coil. The results indicated that 2,4-dinitrotoluene concentration in the treated solution was below the detection level after 1-hour wet air oxidation reaction at 260ºC, which shows that wet air oxidation is an effective process in removing 2,4-dinitrotoluene (Al-Ghusain, Cheng, 1994).

3.1.3.1.2 Aquatic degradation (incl. sediment)

<u>Abiotic</u>

Although scarce references have been found regarding to abiotic degradation in water, 2,4-dinitrotoluene may be degraded in water through several mechanisms, including photolysis, ozonation and chlorination, and oxidation by strong oxidants such as hydrogen peroxide, ozone or oxone (Andrews and Osmon 1977; Bausum *et al*. 1992; Bradley *et al*. 1995; Freedman *et al*. 1996; Ho 1986; Noguera and Freedman 1996; Roth and Murphy 1979).

2,4-dinitrotoluene is expected to adsorb moderately to suspended solids and sediment and volatilisation from water surfaces is not expected based upon its Henry´s Law constant, vapour pressure and water solubility. Photolysis is probably the most significant removal process for 2,4-dinitrotoluene in water, while hydrolytic degradation is not to be expected. Ho (1986) studied photooxidation of 2,4-dinitrotoluene in aqueous solution in the presence of hydrogen peroxide and suggested that the degradation pathway was, although it includes other intermediates, as follows: 2,4-dinitrotoluene → 1,3-dinitrobenzene → hydroxynitrobenzene derivatives + carboxylic acids → CO_2 H_2,O and HNO_3. Oxidation of aqueous 2,4-

dinitrotoluene with hydrogen peroxide or UV irradiation alone was very slow and elimination was not complete. Dillert *et al*. (1995) reported that degradations of 2,4-dinitrotoluene and several other nitroaromatics were accelerated in irradiated TiO_2 suspensions, and that the degradation rates were dependent on time, solution pH and light intensity. Spanggord *et al*. (1980) reported that the photolytic half-lives for 2,4-dinitrotoluene in river, bay and pond waters are 2.7, 9.6 and 3.7 h respectively, and 43 h in distilled water, but these data have not been corroborated with the original report of the study.

On the contrary, a well documented study of I.A. Al-Ghusain and C.F. Lin about photocatalytic oxidation was available. An aqueous TiO_2/DNT solution (1 g TiO_2 and 65 mg/l 2,4-dinitrotoluene) was placed in a 500 ml round bottom flask and mixed with a magnetic stirrer. The solution was internally recirculated (Cole-Parmer Masterflex 7553-10) at the rate of 100 ml/min through a plastic tube; a portion of the tube was enclosed inside a 50 cm long and 1.9 cm diameter Pyrex tube, which in turn was exposed to the UV lamp. Oxygen was bubbled into the solution mixture and samples were periodically withdrawn to evaluate 2,4-dinitrotoluene disappearance rate. The first-order rate constant was approximately 1.8 h^{-1}, a relative slow process compared to other compounds. The extent of the oxidation was also slow; the removal efficiency after 110 minutes was only 60% or 26 mg/l remaining.

Another study, carried out through the year under full exposure to sunlight and surface conditions at 40ºN latitude by Simmons and Zeep (1985), determined the photodegradation rates of several nitroaromatic compounds. Saturated solutions of 2,4-dinitrotoluene were made up in distilled water (organic-free water) and were centrifuged at 15.000 rpm for 30 min. The supernatant was removed carefully, this stock solution was diluted to concentration levels of 10^{-6} – 10^{-5} M and then exposed to mid-day sunlight. The kinetic results for 2,4-dinitrotoluene indicated a half-life of 0.96 days. The Handbook of Estimation Methods for Chemicals (eds Boethling and Mackay, Lewis Publishers) suggests a diffuse attenuation coefficient of 0.1 cm^{-1} as an average. For a depth of 3 metres as in the TGD regional model, the average rate over this depth would be 1/30th of the rate at the surface. Considering then that only 3.3% of the solved substance is exposed to photodegradation, an effective half-life of 29 days is estimated. The corresponding $K_{photowater}$ of $9.96 \cdot 10^{-4}$ h^{-1} has been used in the risk assessment.

The effect of humic substances in natural waters on photolysis was investigated in the same study and it was shown that they enhanced the sunlight-induced photodegradation rates compared to distilled water results. The rate of photolysis was found to increase at a factor of 2.4 - 4.6 in the presence of this dissolved humic material. The photolysis of dinitrotoluene isomers was found to be rapid in natural waters, although the 2,4-isomer photolyses more slowly than the 2,6-isomer. This finding is consistent with field studies of Hasimoto *et al*. (1982), in Dokay Bay, Japan, which showed that the 2,6-isomer is more photoreactive than the 2,4-isomer, especially with humic substances present in the water.

Finally, 2,4-dinitrotoluene may be also degraded by ozonation and chlorination. Lee and Hunter (1985) reported that both ozone and chlorine produced about 35% and 60% reduction respectively. Contact time did not appear to have any impact on the reduction rates.

Biodegradation

The MITI (1992) conducted a biodegradation test in accordance with the "Biodegradation test of chemical substance by microorganisms" stipulated in the Order Prescribing the Items of the Test Relating to the New Chemical Substance (1974, Order of the Prime Minister, the Minister of Health and Welfare, the Prime Minister of International Trade and Industry Nº 1).

This guideline corresponds to "301C Ready Biodegradability: modified MITI test". 0% degradation of 2,4-dinitrotoluene was observed after 14 days. So, it can be classified as **non-ready biodegradable**. A well-documented report is provided.

Bradley *et al*., (1997) demonstrated the degradation and subsequent mineralisation of dinitrotoluene compounds by aquifer microorganisms. Indigenous microorganisms were collected from a) a shallow aquifer, which underlies an explosives-contaminated site (2,4-dinitrotoluene concentrations as high as 3 mg/kg) at an abandoned munitions washhouse and, b) surface soil at an adjacent site with no detectable contamination and no history of nitroaromatic exposure. The TNT concentration of the contaminated surface-soil samples was approximately 0.5 μmoles/kg dry soil, and concentrations of dinitrotoluene were less than the analytical detection of 0.01 μmoles/kg dry soil. Experimental microcosms were prepared aerobically by placing 3 g saturated aquifer material or soil in 30 ml serum vials incubated statically in the dark at room temperature for 28 days. The microcosms were amended with 0.5 ml of an aqueous solution containing approximately $4 \cdot 10^5$ dpm of uniformly ring-labelled 2,4-dinitrotoluene. The microbial community indigenous to the shallow aquifer degraded 2,4-dinitrotoluene. The concentration was reduced by about 80% within 30 days. This reduction was attributed to biological activity, since no decrease in concentration occurred in sterilized control treatments. Approximately 28% of the 2,4-dinitrotoluene was recovered as CO_2 within 28 days. The aquifer material contained less than 0.01% by weight of organic carbon and carbonate carbon. The concentration of 2,4-dinitrotoluene sorbed onto the collected aquifer material was less than 0.01 μmoles/kg dry material. The ground water at the site was aerobic (dissolved oxygen concentrations ≥ 1.0 mg/l). Same authors (Bradley *et al*., 1997), also prepared an additional aquifer microcosms using unlabeled substrates in order to quantify changes in the 2,4-dinitrotoluene and the degradation intermediates. The results of the unlabeled aquifer study indicate that approximately 20% of the added 2,4-dinitrotoluene remained undegraded, but the decrease in the concentration of 2,4-dinitrotoluene was accompanied by accumulation of monoamine–nitrotoluene compounds. 2,4-dinitrotoluene was transformed, primarily, to 4-amino-2-nitotoluene (22.3% final concentration) and, to a lesser extent, 2-amino-4-nitrotoluene (6.3% final concentration).

Freedman *et al*., (1996), developed an enrichment inoculum from a munitions wastewater treatment plant, that consumed 2,4-dinitrotoluene when they were provided as sole organic substrate (average 160 mg/l) or when ethanol (600 mg/l) or ether (142 mg/l) were added as a co-substrate. The cultures were incubated at room temperature in the dark (to minimize the possibility of photodegradation of the aromatic compounds). Following several days of incubation, the concentration of aromatic compound remaining in the mixed liquor was measured by HPLC at 254 nm. The enrichment cultures were able to grow on 2,4-dinitrotoluene provided as sole organic substrate and in the presence of ethanol (600mg/l) as cosubstrate. Low effluent chemical oxygen demand (COD) levels are registered after c.a. 50 days, suggesting that the aromatic was mineralised, rather than simply transformed to other possibly hazardous compounds. When 2,4-dinitrotoluene was added to enrichment cultures as sole substrate, nitrite was released stoichiometrically. When ethanol was also added, nitrite released was only 59% of the maximum, indicating that most of the 2,4-dinitrotoluene (82%) was reduced prior to ring cleavage. However, the addition of ether as a co-substrate did inhibit the consumption of 2,4-dinitrotoluene. When provided to enrichment cultures at the sole substrates, the observed yield coefficients for 2,4-dinitrotoluene ranged from 0.10 to 0.28 mg of volatile suspended solids per mg COD.

In another study, Valli *et al*., (1992) presents the capability of the white rot fungus *Phanerochaete chrysosporium* in degrading 2,4-dinitrotoluene. White rot basidiomycetous

fungi are primarily responsible for the initiation of the depolymenrization of lignin in wood. The best-studied white rot fungus *P. chrysosporium*, degrades lignin during secondary metabolic growth. Under lignolytic conditions, *P. chrysosporium* secretes two heme peroxidases (a lignin peroxidase and a manganese peroxidase) in addition to a H_2O_2-generating system. The peroxidase system, which was purified from the extracellular medium of an acetate-buffered agitated cell extracts, is able to mineralise 2,4-dinitrotoluene. Three main aromatic products were identified as metabolites: 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene and 2,4-diaminonitrotoluene. After 24-days incubation period, approximately 34% of the substrate was mineralised to $^{14}CO_2$ in nitrogen-limited (1.2 mM ammonium tartrate) cultures, whereas only about 7% of the substrate was mineralised in nitrogen-sufficient (12 mM ammonium tartrate) cultures. As metabolites were detected: 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene and 2,4-diaminotoluene. 2-amino-4-nitrotoluene was found in the highest yield and was further metabolised to 4-nitrocatechol.

Nishino *et al*., (2000) reported the isolation of additional strains of bacteria *Burkholderia sp*. with the ability to mineralise 2,4-dinitrotoluene through an oxidative pathway. They are represented by the strain *Alcaligenes denitrificans* and the strain *Alcaligens xylosoxidans* isolated from soil and ground water samples from a number of sites contaminated by 2,4-dinitrotoluene, and activated sludges 2,4-dinitrotoluene containing waste streams. 2,4-dinitrotoluene disappearance accompanied by accumulation of nitrite in enrichment cultures began after several days to several weeks of incubation with 2,4-dinitrotoluene provided as the sole source of carbon. 2,4-dinitrotoluene remained in all cultures provided with 200 μM or more 2,6-dinitrotoluene while cultures without added 2,6-dinitrotoluene completely removed 2,4-dinitrotoluene within 1 to 2 days. *A. xylosoxidans* exhibited particularly rapid growth on 2,4-dinitrotoluene. The third cluster, represented by *B. cepacia* strain, exhibited particularly rapid growth on 2,4-dinitrotoluene. Depletion of 2,4-dinitrotoluene took place in 24 h compared to 2 to 5 days for the other strains. There are indications on the way of isolations but no culture procedures are indicated. This assay will be considered as additional information.

Other authors, Davies *et al*., (1981), compared the capability of degradation of 2,4-dinitrotoluene by a microbial population of industrial wastewater treatment units, which received eight organics, including 2,4-dinitrotoluene, to municipal activated sludge organisms. The industrial microbial population (industrial seed) from a wastewater treatment unit, was comprised of four bacterial genera (*Acinetobacter, Alcaligenes, Flavobacterium* and *Pseudomonas*) and one yeast (*Rhodotorula*). A conventional activated sludge is indicated for the municipal sludge. Impairment or simulation of respiration rates using Warburg analyses were conducted at 28ºC. Respiration stimulation was regarded as a statistically significant uptake rate at 3 h above the control valve, and a similar decrease represented inhibition. At 50 mg/l dose, 2,4-dinitrotoluene degraded rapidly, about 80%, within 2 days, in the presence of the industrial seed, remaining the residual amount from 2 to 7 days. At higher concentrations, i.e. 200 mg/l, the response was extremely slow for the first 100 min. Then, the remaining 80 min, a rapid uptake rate occurred. Municipal seed showed inhibition at all test concentrations (i.e. 200 down to 10 mg/l). Mass-spectrometry analyses confirmed the presence of 4-methyl-3-nitroaniline as metabolite.

Tabak *et al*., (1981) presented a well documented report in which explained a static culture biodegradability screening procedure and, a culture enrichment technology utilizing a synthetic medium, containing 5 mg yeast extract per litre, a 7-day static incubation at 25ºC in the dark, followed by 3 weekly subcultures and settled domestic wastewater as microbial inoculum. The biodegradability test method used in the studies was a static culture flask

screening procedure according to Bunch and Chambers (1967) . 5 and 10 mg/l concentrations of 2,4-dinitrotoluene were used as test compound. A stock solution was prepared in absolute alcohol as 10% solution. Fine suspensions of 2,4-dinitrotoluene in the synthetic medium was prepared by adding appropriate amounts of the ethanolic stock solutions of these compounds to pre-chilled synthetic media, and subsequent blending. 2,4-dinitrotoluene exhibited initially a significant bio-oxidation activity with gradual adaptation and induced enzyme formation, followed however, by a deadaptative process due to loss of the metabolically efficient microbial population or due to the gradual built-up of toxicity of either the parent substrate or metabolic products to microbiota. A gradual acclimation process with significant bio-oxidation of the substrate was observed with 2,4-dinitrotoluene. Subsequent subcultures demonstrated a reduction of the biodegradation rate (35% and a 46% using 5 and 10 mg/l, respectively) with the test compound. According to the authors, the gradual reduction of biodegradation activity may be due to a possible loss of synergistic activity on the substrate exhibited by the original heterogenous microbial population as a result of the subculture method, or due to the retardation of the adaptative (induced) enzyme process because of possible accumulation of toxic by-products of metabolism. This study was considered as additional information.

Haidour and Ramos (1996) grown *Pseudomonas sp*. clone cells for 1-7 days under aerobic conditions at 30ºC in M8 minimal medium with fructose (0.5% wt/vol). As N-source 0.1-2 g/l TNT or 2,4-dinitrotoluene and 2,6-dinitrotoluene were supplied. A. *Pseudomonas sp*. Clone was able to growth with 2,4- and 2,6-dinitrotoluene as a N-source in addition to trinitrotoluene. Bacteria grew with a generation time of 12 h. The use of 2,4-dinitrotoluene as a N-source by *Pseudomonas sp*. clone A involves the removal of one or both groups. In addition to removing nitro groups, the authors indicated the reduction of 2,4-dinitrotoluene to amino derivatives, (2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene), which were not used as N-source. From these cultures three azoxytoluenes derived from 2,4-dinitrotoluene were synthesised, namely 4-4'-dinitro-2,2'-azoxytoluene; 2-2'-dinitro-4,4'-azoxytoluene and 2-4'-dinitro-2'-4-azoxytoluene. The biological reduction of 2,4-dinitrotoluene to recalcitrant amino derivatives and the production of more recalcitrant azoxytoluenes represent a serious obstacle to the process. The information is considered as not assignable for the assessment but included as additional information.

Bausum *et al.* (1992) conducted an aerobic study to examine the disappearance of 2,4-dinitrotoluene upon incubation with an enrichment culture isolated from natural surface waters from an ammunition plant. A degradation of 45-64% of the compound was resulted by $^{14}CO_2$ measurement. This information was extracted by the IUCLID database, but the original report was not available for validation, and so, was included as not assignable for the assessment, but included as additional information.

It was also included information form the USEPA (1999), in which it is indicated a removal rate of 99.15% at an water waste treatment plant using industrial activated sludge. Although the original reference was not available for validation, it was included in the assessment due to the quality of the institution that performed the assessment.

Liu *et al*. (1984) indicated that no breakdown of 2,4-dinitrotoluene was observed in aerobic fermentors after 14 days of incubation in the presence of a fresh sample of municipal activated sludge 10-fold diluted.

Jackson *et al*., (1999) conducted a study to examine whether the disappearance of 2,4-dinitrotoluene occurred upon incubation with *P. chrisosporium* cultures grown under non-lignolytic conditions as well as under lignolytic conditions. In nature, *P. Chrysosporium*

thrives on woody substances and degrades lignin. 100 ppm of 2,4-dinitrotoluene were added two-day old cultures grown in the medium or six cultures in low-nitrogen (LN) medium. Samples were taken out each day for eight days after addition of the chemical. When *P. chrisosprium* was grown in either malt extract (ME) medium and LN medium containing 0.14Mm 2,4-dinitrotoluene as substrate, completely disappearance of the substance occurred by day six. These results indicate that biotransformation of 2,4-dinitrotoluene by this fungus occurs under both, lignolytic and non-lignolytic grown conditions. This information is considered as not assignable for the assessment, but it is included as additional information.

There have also been provided indications of biotransformation under anaerobic conditions:

Liu *et al*. (1984) indicated that the reduction of aromatic nitro groups to amino groups preceded through nitroso and hydroxyamino intermediates. Whereas the isolation and characterisation of nitroso intermediates have only been reported from the reduction of 2,4-dinitrotoluene. According to these authors, the intermediates of microbial transformation of 2,4-dinitrotoluene by a mixed bacterial culture derived from activated sludge were identified as 2-amino-4-nitrotoluene (2-NH_2-4-NT), 4-amino-2-nitrotoluene (4-NH_2-2-NT), 2-nitroso-4nitrotoluene (2-NO-4NT) and 4-nitroso-2-nitrotoluene (4-NO-2NT). The biotransformation of 2,4-dinitrotoluene occurred only under anaerobic conditions with and exogenous carbon source. The two nitroso compounds were unstable and could be observed at the early stage of 2,4-dinitrotoluene anaerobic transformation. A fresh sample of municipal activated sludge 10-fold diluted with distilled water was maintained in cyclone fermentors. One ml of benzene was added periodically as the carbon source, causing heavy bacterial growth. This inoculum was used in the initial experiments on 2,4-dinitrotoluene transformation (5 to 25 mg 2,4-dinitrotoluene/l). The fermentors were purged with a flow of air (aerobic) or nitrogen (anaerobic) 1 h before introducing the test compound. A gas flow rate of 20 ml/min was then maintained for each fermentor throughout the exposure time of 14 days. On the contrary, 2,4-dinitrotoluene was extensively biotransformated under the anaerobic conditions, with concurrent accumulation of three intermediates, i.e., 2-NO-4-NT, 2-NH_2-4-NT and 4-NH_2-2-NT. The nitroso compound, 2-NO-4-NT, could only be detected at an early stage of 2,4-dinitrotoluene biotransformation, usually *ca*. 2 to 3 days after anaerobic incubation. The two amino intermediates (4-NH_2-2-NT and 2-NH_2-4-NT) were more stable and lasted almost throughout the entire 14 days of incubation. At higher concentrations (25 mg 2,4-dinitrotoluene/ml of ethanol) a new biotransformation product, which had not been observed before, was found in the anaerobic fermentor broth. This new intermediate was tentatively identified as 4-nitroso-2-nitrotoluene (4-NO-2NT). This information is considered as additional information for the assessment.

Noguera and Freedman (1997) examined the anoxic biotransformation of 2,4-dinitrotoluene using two denitrifying enrichment cultures. In one, the inoculum was acclimated to treatment of munitions wastewater that contained 2,4-dinitrotoluene (RAAP); the other was derived from activated sludge that was routinely exposed to nitroaromatics (URB). Microorganisns were grown at room temperature in a mineral medium. Ethanol (34Mm) was the primary electron donor. 2,4-dinitrotoluene was added to the cultures at concentrations between 0.25 and 0.6 Mm. In URB culture, 2,4-dinitrotoluene was consumed completely by day 24 by both cultures only when ethanol was provided as a primary substrate, with the rate of transformation twice as fast in the acclimated culture in which all the 2,4-dinitrotoluene was transformed by day 11. A negligible amount of the 2,4-dinitrotoluene was mineralised. The main initial biotransformation pathway involved reduction of 2,4-dinitrotoluene to aminonitrotoluene isomers and DAT, which subsequent disappeared with subsequent incubation. Although the initial transformation of 2,4-dinitrotoluene and the RAAP cultures

was similar, a significant difference emerged after the dissapearance of DAT. Metabolites in the acclimated culture were mainly solvent extractable versus highly hydrophilic in the unacclimated culture. The principal initial biotransformation pathway reduction of 2,4-dinitrotoluene to aminonitrotoluene (6-nitroindazole, 2-nitrotoluene, and 4-nitrotoluene, which have not been previously as metabolites of 2,4-dinitrotoluene). Acetilation at the para-position was another important transformation pathway in the acclimated culture, resulting in the accumulation of 4-acetamide-2-nitrotoluene and 4-acetamidetoluene. The diversity and characteristics of these metabolites indicates that biotransformation of 2,4-dinitrotoluene under anoxic conditions does not necessarily eliminate the toxicological hazards associated with the parent compound. According to Noguera and Freeman (1997), from a toxicological stand point, the identification of transformations products from a nitroaromatic compounds is essential, because the may pose a similar or greater toxicological hazard that the parent compounds. This is considered not assignable for the assessment but included as additional information.

Hughes *et al*., (1999) studied the potential of nitroaromatic transformation under anaerobic fermentative conditions by *Clostridium acetobutylicum* (ATCC 824), due to their ability to reduce rapidly aryl nitro groups. During the log-growth phase, the cultures were spiked with 2,4-dinitrotoluene (5 mg dissolved in 0.5 ml of methanol). Nearly all the test compound was transformed in 2 h, forming one product 2,4-diaminotoluene with a yield of 92% and 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene with a yield of about 5% each.

Davidova and Suflita (1997) investigated the transformation of 2,4-dinitrotoluene by a variety of anaerobic bacteria including the sulphate producer *Desulfomonile tiedei*, the methanogen *Methanobacterium thermoautotrophicum*, the homoacetogen *Eubacterium limosum* and the fermentative organisms *Clostridium bifermentants*. It is indicated that all bacteria reduced 2,4-dinitrotoluene to 2,4-diaminaotoluene. Unfortunately, only an abstract has been provided and so, there has been no possibility of validating the information. However, this information is included in the table as additional information.

3.1.3.1.3 Degradation in soil

The estimated soil adsorption coefficient (Koc) for 2,4-dinitrotoluene of 118.74 indicates that it is slightly mobile in soil, so there is not a significant potential for geoaccumulation. Aromatic nitro compounds are not susceptible to hydrolysis, and photolysis should not be an important process in soil. Volatilisation from moist soil surfaces is not expected to be an important fate process based upon a Henry´s Law constant of $5.45 \cdot 10^{-3}$ Pa-m^3/mol. Little information was found regarding transformation of 2,4-dinitrotoluene in soil; however, based on aqueous biodegradation test, some biodegradation may occur in both aerobic and anaerobic zones of soil. Microorganisms indigenous to surface soils collected at a munitions-contaminated site were reported to transform 2,4-dinitrotoluene to amino-nitro intermediates within 70 days (Bradley *et al*. 1994).

Bringmann and Kühn, (1971) carried out another biodegradation study using *Azotobacter agilis*, from soil compost previously adapted to 2,4,6-trinitrotoluene. The assay was performed adding 146 mg/l of 2,4-dinitrotoluene to the bacteria culture medium. 100% of reduction of 2,4-dinitrotoluene was measured. Duration of the exposure is not indicated. So, the data will be included as additional information.

Brodsky *et al*.(1997) developed a test strategy for the assessment of the ecotoxicological hazard potential of existing chemicals in the terrestrial environment, the soil degradation and

leaching of several substances, 2,4-dinitrotoluene between them. To achieve comparability of test results on the degradation, a German soil of standardised composition were used (Lufa St. 2.2), within the range common for central European arable soils, in line with the specifications prescribed by the German Federal Research Centre for Agriculture and Forestry guidelines (1986) regarding Aethe pesticide registration process. The data on the concentration of the substance were used to calculate the degradation of 50% (DT_{50}, estimated in 7 days) and DT_{90}, estimated in 128.5 days of a initial applied amount. of 5 mg/kg Nevertheless, Brodsky *et al*. (1997) indicate that a decrease in the concentration of a chemical is not the same as total degradation and that test results may also be distorted through volatility or a strong bonding of the test substance to soil particles. It seems that the study indicated a disappearance rate, rather than a degradation rate, since no distinction could be made between degradation, sorption and volatilisation. This may falsely imply that degradation has occurred, and so, the proposed half-life of 7 days has been considered as "disappearance half-life". Therefore, the results from that study have not been considered for the assessment of the degradation.

Table 3.5: Concentration of 2,4-dinitrotoluene in the standard soil at different sampling points after days of application

Days	0	2	4	8	16	32	64	100
Concentration (mg/kg)	4.60	2.16	2.06	2.04	1.55	1.32	0.86	0.55

The low DT values of 2,4-dinitrotoluene indicate that it is a non-persistent substance. Besides, the degradation slows down sharply, resulting in a significantly higher DT_{90} value.

Table 3.6: DT-values (in days) of 2,4-dinitrotoluene

	DT_{50}	**DT_{90}**
Measured	7.0	128.5
From literature	≈ 4	-

Other literature references regarding soil degradation for 2,4-dinitrotoluene show that adapted microorganisms from a soil polluted with Trinitrotoluene transformed 99% of the 2,4-dinitrotoluene in 14 days (Neumeier *et al*., 1989). Fungi do not appear to make much of a contribution to degradation (only 5 of the 190 cultures tested had this capacity (Rippen, 1986)).

According to equilibrium partitioning equations, degradation half-life for (bulk) soil, partly based on Kp_{soil} has been followed. Measured Kp_{soil} values are preferred, but as not available, a calculated Kp_{soil} = 2.37 l/kg for 2,4-dinitrotoluene (see Section 3.1.3.2.1) will be used for estimations. And so, according to the Technical Guidance Document, the half-life for the soil compartment will be assumed to be 300 days.

Regarding the degradability on sediment compartment, and following the equilibrium partitioning method according to the Technical Guidance Document equations:

$$Kbio_{sed} = Ln\ 2 \cdot Faer_{sed} / DT_{50}\ bio_{soil}$$

$DT_{50}\ bio_{soil}$ half-life for biodegradation in bulk soil (days).
$Kbio_{sed}$ first order rate constant for degradation in bulk sediment ($days^{-1}$).
$Faer_{sed}$ fraction of the sediment compartment that is aerobic (0.10)

$$Kbio_{sed} = Ln\ 2 \cdot 0.1 / 300 = 2.3 \cdot 10^{-4}\ days^{-1}$$

This implies that the total half-life for the sediment compartment will be a factor of ten higher than the half-life in soil. That means $DT_{50}\ bio_{sed}$ = 3,014 days (8.3 years).

Regarding this information, and in terms of the environmental modelling of the risk assessment, the biodegradation rate in sediment and soil compartments given in the TGD has been considered in calculations.

In the Table 3.7, the information regarding biodegradation assays is compiled.

Table 3.7: Different biodegradation test results for 2,4-dinitrotoluene

Type	Source of microorganisms	Method	Result	Detection	Comments	Ref
Aerobic	Activated sludge	MITI (OECD 301C)	0% after 14 days	O_2 comsumption	V	MITI, 1992
Aerobic	Aerobic microorganisms Inoculum form aquifer sediment	Other	80% after 30 days	Degradation determined $^{14}CO_2$ measurement	V	Bradley *et al*., 1997
Aerobic	Aerobic microorganisms, Inoculum from uncontaminated surface soil	Other	28% after 28 days	Mineralisation determined by $^{14}CO_2$ measurement	I (documentation insufficient)	Bradley *et al*., 1997
Aerobic	Industrial sewage adapted	Other	Growth of the culture	4DNT disappearance	NA	Freeman *et al*., 1996
Aerobic	Funghi (*Phanerochaaete chrysosporium*)	Other	7%-34% after 24 days incubation	Mineralisation determined by $^{14}CO_2$ measurement	VWR	Valli *et al*., 1992
Aerobic	Bacteria (*Burkholderia cepacia, Alcligenes denitrificans and Alcaligenes xylosoxidans*)	Other	100% after 1-5 days	DNT disappearance/nitrite enrichment	Additional information	Nishino, SF *et al*., 2000.
Aerobic	Soil microbial population	German pesticide registration	50% after 7 days 90% after 128 days	DNT disappearance	Additional information	Brodsky *et al.,* 1997
Aerobic	Enrichment culture from compost soil (*Azotobacter agilis*)	Other (2 stage model by Bringmann and Kühn	96-99% primary degradation	DNT disappearance	Additional information	Bringmann and Kühn, R. 1971
Aerobic	Industrial waster water containing four bacteria genera (*Acinetobacter, Alcaligenes, Flavovacterium* and *Pseudomonas*) and one yeast (*Rhodotorula*)	Other, (Umbreit, 1972)	80% after 2 day	O_2 comsumption	VWR	Davies *et al*., 1981
Aerobic	Municipal sewage	Other, (Umbreit, 1972)	0% after 14 days	O_2 comsumption	V	Davies *et al*., 1981
Aerobic	Settled domestic waste water	Other (Bunch and Chambers, modified)	47% after 7 days	O_2 comsumption	Additional information	Tabak, *et al*., 1981

Type	Source of microorganisms	Method	Result	Detection	Comments	Ref
Aerobic	Domestic activated sludge	Other	0% after 14 days	DNT dissapearance	V	Liu *et al*., 1984.
Aerobic	Bacteria (*Pseudomonas sp*)	Other: Growth of the culture 2,4 and 2,6-DNT as source of N	-	Appearance of metabolites	NA	Haidour and Ramos, 1996.
Aerobic	Enrichment culture form natural surface water	Other	45-64%	$^{14}CO_2$ measurement	NA	Bausum *et al*., 1992 (cited in IUCLID)
Anaerobic	Domestic activated sludge	Other	100% after 14 days	DNT disappearance	V	Liu *et al*., 1984.
Anaerobic	Non adapted activated sludge	Other	In the presence of ethanol, 100% after 24 days	DNT disappearance	NA,additional information	Noguera and Freeman, 1997
Anaerobic	Adapted industrial sewage	Other	In the presence of ethanol, 100% after 11 days. Mineralization negligible	DNT disappearance	NA, additional information	Noguera and Freeman, 1997
Anaerobic	Domestic anaerobic sludge	Other	0% after 56 days	CO_2 and methane	V	Bayer, AG 1991.
Anaerobic	Anaerobic bacteria (*Clostridium accertobutylicum*)	Other	Almost 100% after 2 hours	-	VWR	Hughes *et al*., 1999.
Anaerobic	Anaerobic bacteria (*Desulfominilie tiedjei, Methonobacterium thermoautotrophicum, Eubacterium limosum and Clostridium bifermentans*)	Other-	Were able to reduce 2,4-DNT to 2,4-diaminotoluene (2,4-DAT). Only *C.bifermentants* and *E.limosum* were able to further metabolise 2,4-DAT	-	NA (only abstract available)	Davidova,and Suflita, 1997.
-	Industrial activated sludge	Other	Removal at one WWTP 99.15%	-	NA (original reference not available)	USEPA. 1999.
-	Funghi (*Phanerochaete chrysosporium*)	Other	100% after 6 days	Disappearance of DNT	VWR	Jackson *et al*., 1999.
	Adapted microorganisms, from TNT contaminated soil	Other	99% after 14 days	-	NA	Neumeier *et al*., 1989

[1] IUCLID Data set.; V: Valid; VWR: Valid With Restrictions; NA: Not Assignable; I: Invalid

3.1.3.1.4 Summary of environmental degradation

The available biodegradation data show that 2,4-dinitrotoluene can undergo primary biodegradation to form several products. Furthermore, on the light of the available information 2,4-dinitrotoluene should be degraded by biological sewage treatment when suitable acclimation is provided to the cultures, so it can be classified as inherent biodegradable with adapted inoculum (non-ready biodegradable).

Final conclusions on degradability would have to be done on a weight of evidence basis, since, under certain anaerobic conditions, may pose the formation of recalcitrant amino derivatives and the production of more recalcitrant azoxytoluenes, among other compounds. The relevance of formation of recalcitrant metabolites under realistic environmental conditions is not known.

According to previous information, 2,4-dinitrotoluene was considered no-biodegradable in water, on the one hand, and inherent biodegradable in sediment and soil, on the other hand, because of the different residence times in the different compartments. The adaptation of the microbial population is expected for sediment and soil. However, in running waters, the water column microbial population has a very high spatial and temporal variability, and the condition of continuous exposure of the same population allowing its acclimatization cannot be guaranteed even for continuous point emissions.

According to the TGD procedures, the biodegradation rate of 2,4-dinitrotoluene by soil microorganisms would correspond to a half-life of 10 months in soil and more than 8 years in sediments. There are additional data indicating a much more rapid degradation in soil, including soils preexposed to TNT, however, as these data are based on dissipation or primary degradation, the conservative value estimated by the TGD will be used.

Related to abiotic degradation, the gas-phase reaction with photochemically produced hydroxyl radicals has an estimated rate constant of $2.253 \cdot 10^{-13}$ cm^3/molecule/sec, which corresponds to a half-life of 71 days. In water, the log octanol-water partition coefficient is sufficiently large to indicate adsorption to soil organic matter. The relatively low volatility and high solubility of 2,4-dinitrotoluene indicate that it will tend to remain in water for long periods of time, unless acted upon by light, oxygen or biota. As a result, 2,4-dinitrotoluene can be transported to groundwater or surface waters. Photolysis is probably the most significant removal process, and the half life estimated from the information of bibliography is 29 days.

The ultimate biodegradation rate constants and half-lives that will be used in the environmental modelling are summarized in Table 3.8:

Table 3.8: Environmental degradation

Compartment		Half-life
Atmospheric		71 days
Aquatic	Abiotic degradation	29 days
	Biodegradation	∞ days
Sediment		3,014 days
Soil		300 days

Values utilised in EUSES calculations

3.1.3.2 Distribution

Based on its physico-chemical properties, the preferred environmental compartment of 2,4-dinitrotoluene is the hydrosphere. According to the distribution Mackay Fugacity Model Level 1, 98,40% of 2,4-dinitrotoluene emitted into the environment will be found in the hydrosphere at an equilibrium distribution.

Table 3.9: Level I environmental partitioning of 2,4-dinitrotoluene

Compartment	Percentage
Air	0.16
Water	98.40
Soil	0.74
Sediment	0.69
Suspended sediment	0.001
Biota	0.0005

Data from IUCLID Data Set, 2001

The IUCLID Data Set indicates that the input parameters and the physico-chemical properties used in the Mackay Fugacity Model Level 1 are the following:

- molar mass: 182.14 g/mol
- log Kow: 1.98
- vapour pressure: 0.0079 Pa
- water solubility: 0.3 g/l
- melting point: 69.9ºC
- volumes used (m^3):
  - air: $6 \cdot 10^9$
  - water: $7 \cdot 10^6$
  - soil: $4.5 \cdot 10^4$
  - sediment: $2.1 \cdot 10^4$
  - susp. sediment: 35.0
  - biota (fish): 7.00
  - aerosol: 0.120

3.1.3.2.1 Adsorption

According to the TGD equation for the chemical class of nitrobenzenes (log Koc = 0.77·log Kow + 0.55) and considering the measured log Kow 1.98, the result obtained for Koc is 118.74 l/kg. This Koc value is within the range of the value calculated with the empirical regression equation by Briggs (1981) published in BUA (1990) which is 117.48 l/kg, obtained from log Koc = 0.45·log Pow + 1.17. Therefore, a Koc of 118.74 l/kg will be considered in all model calculations of this report. This K_{oc} value suggests that 2,4-dinitrotoluene will have a slight tendency to sorb to sediments, suspended solids and biota.

The K_{oc} value can be used to derive the solid-water partition coefficient (K_p) of 2,4-dinitrotoluene for each compartment, soil, sediment and suspended matter (Equation 23, Chapter 3 of the new TGD). These may also be expressed as dimensionless partition coefficients (Equation 10, Chapter 24 of the new TGD):

Partition coefficient organic carbon-water	K_{oc} = 118.74 l/kg
Partition coefficient solids-water in suspended matter	$K_{p\ susp}$ = 11.9 l/kg
Partition coefficient solids-water in sediment	$K_{p\ sed}$ = 5.94 l/kg
Partition coefficient solids-water in soil	$K_{p\ soil}$ = 2.37 l/kg
Soil-water partitioning coefficient	K_{soil_water} = 3.76 m^3/m^3
Suspended matter-water partitioning coefficient	K_{susp_water} = 3.87 m^3/m^3
Sediment-water partitioning coefficient	K_{sed_water} = 3.77 m^3/m^3

The adsorption of 2,4-dinitrotoluene from aqueous solution to various natural clay minerals was investigated by Haderlein *et al.*(1996) in batch experiments in the presence of K^+, Na^+ or Ca^{2+} electrolytes. The adsorption coefficient to clay minerals (K^+-Montmorillonite) obtained was 7400 l/kg, and it indicates a tendency to adsorb specifically to clay minerals. Therefore, if released into water, 2,4-dinitrotoluene is expected to adsorb moderately to suspended solids and sediment.

3.1.3.2.2 Precipitation

The percentage in atmospheric compartment according to the distribution model by Mackay is rather low but the lifetime in the atmosphere of 71 days may lead to a certain removal of 2,4-dinitrotoluene from the atmosphere by precipitation. The concentrations due to precipitation of 2,4-dinitrotoluene from the atmosphere are expected to be greatest near the point of emission.

3.1.3.2.3 Volatilisation

The volatilisation rate constant for 2,4-dinitrotoluene from a body of water was estimated as $6.6 \cdot 10^{-5}$/h (half-life = 438 days), which indicates that volatilisation of 2,4-dinitrotoluene from waters will probably not be a significant transport process. Unfortunately, it has not been possible to find the reference of this information, so it only can be taken as illustrative.

On the other hand, the volatilisation of 2,4-dinitrotoluene from surface water to air may be estimated by the Henry's Law constant, which is $5.45 \cdot 10^{-3}$ Pa-m^3/mol and the air-water partitioning coefficient ($K_{air\text{-}water}$) derived from it, calculated as $2.3 \cdot 10^{-6}$ $m^3.m^{-3}$. Both low

values suggest that volatilisation is unlikely to be a significant removal mechanism for 2,4-dinitrotoluene from water systems, as it has been said before.

3.1.3.2.4 Distribution in wastewater treatment plants

The distribution of 2,4-dinitrotoluene in wastewater treatment plants has been obtained using EUSES. The calculation has been done assuming inherently biodegradable conditions for 2,4-dinitrotoluene in the plant, despite the fact that this compound is non-ready biodegradable in the environment, as it was said in 3.1.3.1.2.

According to previous information, 2,4-dinitrotoluene was considered no-biodegradable in water, on the one hand, and inherent biodegradable in sediment and soil, on the other hand, because of the different residence times in the different compartments. The adaptation of the microbial population is expected for sediment and soil. However, in running waters, the water column microbial population has a very high spatial and temporal variability, and the condition of continuous exposure of the same population allowing its acclimatization cannot be guaranteed even for continuous point emissions.

Table 3.10: Estimated distribution in a STP

Fraction of emission directed to air (%)	0.1
Fraction of emission directed to water (%)	58.4
Fraction of emission directed to sludge (%)	1.3
Fraction degraded (%)	40.2

3.1.3.3 Accumulation and metabolism

Fish (experimentally):

Lang *et al*., in 1997 conducted an experiment to investigate the bioconcentration curves of 2,4-dinitrotoluene in carps (whole fish, liver, intestine and muscle) using a semi-static system. 200 fish, ranging from 5 to 18 g, were exposed to 2,4-dinitrotoluene which had two different aqueous concentrations: 100 fishes, 9.35% average lipid contents, were exposed at 613 µg/l and another 100 fishes, 7.08% average lipid content, were exposed at 75 µg/l. Dechlorinated tap water was used in the assay, with a pH value of 7.6 ± 0.2, a temperature of 15 ± 1 ºC and the content of dissolved oxygen maintained at 9.51 ± 0.40 mg/l. Half of the contaminated water was replaced once a day and the fish were fed daily with a commercial fish food. During the accumulation phase, fish (whole fish, liver, intestine and muscle) and water samples were periodically analysed. After 40 days the exposure was stopped. The elimination of 2,4-dinitrotoluene was studied for 8 days after 10 days' or 40 days' uptake. During the elimination phase, half of the water was replaced once a day. The bioconcentration factors (BCF) in the whole fish during the first and second steady state were 9.15 and 4.15, respectively (97.86 and 44.39, based on lipid content). The peak of muscle was milder than that of the whole fish. The bioconcentration curves of liver and intestine show two major peaks probably as an indication of the metabolism taken place in those tissues. The corresponding peaks in whole fish resulted from the biotransformation in liver and intestine. According to the two steady states observed, two BCF were determined: 9.15 for the first equilibrium given and 4.15 for the second one. The elimination curve was a biphasic course, estimating the elimination rates to 3.6 and 0.1 d^{-1}. Two major metabolites 4-amino-2-

nitrotoluene (4A2NT) and 2,4-diamino-toluene (2,4-DAT), were found in liver extracts of 2 days and 15 days after the fish were exposed to 1 mg/l of 2,4-dinitrotoluene. The mechanism of metabolism was reduction step by step.

In several independent experiments carried out by Liu *et al*. (1983), different test organisms (*Lepomis macrochirus*, *Daphnia magna*, *Lumbriculum variegates* and *Selenastrum capricornutum*) were exposed to ^{14}C-2,4-dinitrotoluene for 4 days. The BCFs were based on wet weight estimations, by dividing the radioactivity/gram of organism by the radioactivity/gram of water. The exploratory 4-days bioconcentration tests indicated that the ring-labelled ^{14}C-2,4-dinitrotoluene is not rapidly or extensively taken up from water by invertebrates or fish, and is apparently excreted rapidly by fish (with a fat content about 8%). However, it was extensively sorbed by algae. The 4-day BCF for 2,4-dinitrotoluene in algae exceeded 2000. In one series tests, bluegills (12 juveniles/30L of 2,4-dinitrotoluene solution), *D. magna* (100 adults/2L), *L. variegatus* (50 adults/2L), and *S. capricornutum* (initially about 10,000 cells/0.1L) were exposed to solutions containing 1 mg/l of ^{14}C-2,4-dinitrotoluene. To prepare the standards the flasks were wrapped in aluminium foil to prevent photodecomposition. The amount of radioactivity in the test medium were determined at the beginning of the test and in the whole bodies of cells of the invertebrates and algae after 4-day exposure period. Aquatic species were subjected to an exposure phase only and were sampled and radioanalyzed on day 4. The blue gills were subjected to a 10-day depuration phase, and individuals were removed and radioanalyzed on 2-day and 4-day of the exposure phase and on day 3 and 10 of the depuration phase. However, with the blue gills, radioactivity measurements were performed on samples of the viscera (gills and kidneys excluded) and dorsolateral muscle on the second and fourth days of exposure, and 3 and 10 days after the exposed fish had been transferred to clean flowing water. The depuration data obtained with the bluegill tissues indicates that 2,4-dinitrotoluene or its metabolites are rapidly excreted. Nearly 100% of the radioactivity found in the tissues of the bluegill on day-4 had disappeared from the tissues three days after the fish were placed in clean water. The alga sorbed a considerable amount of labelled 2,4-dinitrotoluene. The 4-day BCFs obtained with the alga were 2507 for 2,4-dinitrotoluene. During the 4-day exposure period, only the algae concentrated 2,4-dinitrotoluene extensively. It is indicated by the authors that the compound was not sorbed extensively by the animal species, and the amount sorbed by the bluegills was excreted. These observations suggest that the bioconcentration potential of 2,4-dinitrotoluene vary largely between taxonomic groups, the bioaccumulation is large for algae but much lower for aquatic animals including fish and invertebrates. It should be noted that the experimental approach did not distinguish if the bioaccumulation in algae was related to uptake by the cytoplasm, absorption in the algae walls, or adsorption on the algal surface. However, as bioaccumulation is not used as a measure of toxicity/effects but as an indication of potential for secondary poisoning, this aspect is not relevant as the risk for secondary poisoning is leaded by the concentration in food items, not the bioaccumulation mechanism in the prey. The concentration used by Liu (1 mg/l) in the bioconcentration test, would be close to the recommended OECD highest concentration (one-tenth of the incipient LC_{50}), according to the information collected on the growth rate effects (see Table 3.28). Moreover, the average 96h-EC_{20} on algae has been determined as 1.6 mg/l (ranged within 1.16 - 2.18 mg/l) (see section 3.2.1.1.3.). The EC_{20} effect percentile is within the range of variation of controls, and so, it has been considered as a no effect concentration. The concentration used by Liu *et al* (1983) in the bioconcentration test (1 mg/l), for the same exposure period of 96 hours, is even lower than the lower range of this interval (1.16 mg/l), and so it was considered that the concentration used by Liu (1 mg/l) in the bioconcentration test would not exert any toxic effect on the algae. So, even although this BCF study on algae can be considered a non-

standard test, this study provides useful information regarding bioadsorbance capacity, and so, it has been considered valid for the assessment.

In another experiment (Wang *et al*., 1999), 2,4-dinitrotoluene was selected as a model compound for laboratory continuous-flow-experiment where the uptake of the compound by goldfish (*Carasius auratus*) and triolein SemiPermeable Membrane Device (SPMD) was followed. 40 goldfish and the SPMDs were placed in an exposure chamber. A concentrated mixture of 9 nitroaromatics was added at a constant rate (1 ml/min) into the main water flow to the chamber by a peristaltic pump. The experiment was conducted for 20 days with a concentration of 0.39 mg/l of 2,4-dinitrotoluene in the exposure chamber. Measured, BCF was determined as 191.3 and 275.1 for fish and SPMDs, respectively. The dialysis recovery for the nitroaromatic compound from goldfish in recovery studies were from 57% to 71%, and the deviation of the averaged recovery did not exceed 16%. Based on the K_{ow} and the uptake rate constant, Wang *et al*., (1999) estimated the time to reach 90% steady-state concentration, in SPMD and in goldfish, in 0.5608 and 4.86 days, respectively. The elimination rate in 4.09 (hours-1) and0.47 (hours-1) for SPMDs and goldfish, respectively. The assay has been carried out with a concentrated mixture of nitroaromatic compounds, and so, it has been considered as not assignable for the assessment, but included as additional information.

Another study, (MITI, 1992), presented a well documented study to estimate the BCF on fish, conducted in accordance with Japanese Industrial Standard (JIS K 0102-1986-71) titled "Testing methods for industrial waste water, corresponding to "305C-Bioaccumulation: Degree of bioconcentration in fish" stipulated in the OECD Guidelines for testing of chemicals (1981). According to the information provided, the test was conducted on orange-red killifish (*Oryzias latipes*) for 8 weeks, until the BCF reached to an equilibrium. Two exposure concentrations were assayed, 0.25 and 0.025 mg/l resulting in BCFs of 1.75 (0.6-2.9) and 12.2 (3.2-21.2), respectively. Both average values have been included as valid information, considering the BCF of 12.2 much more appropriated for the assessment.

According to Hartley (1981) 2,4-dinitrotoluene was rapidly absorbed (24-96 hours), by fish *Lepomis macrochirus* reached relatively low bioconcentration levels and was rapidly eliminated (24-72 hour) when placed in a 2,4-dinitrotoluene free environment. Unfortunately, it has been no possibility to go trough the original report, just a short abstract. However, this data has been included as additional information.

Fish (calculated):

The log K_{ow} (1.98) for 2,4-dinitrotoluene indicates weak bioaccumulation potential. According to the TGD, BCF data for fathead minnows (*P. promelas*) for substances with log $K_{ow} < 6$ can be estimated following the equation:

$$\text{Log BCF} = 0.85 * \log K_{ow} - 0.70 = 0.85 * 1.98 - 0.70 \qquad \text{BCF} = 7.65$$

Table 3.11: Different bioaccumulation studies results for 2,4-dinitrotoluene

Specie	Exp. period (days)	Exp. Conc. (mg/l)	BCF	Elimination rate ($days^{-1}$)?	Comments	Reference
Cyprinus carpio	40	0.613	Two steady states: 9.15 and 4.15	Biphasic course: 3.6 and 0.1	V	Lang *et al*., 1997
Cyprinus carpio	56	0.25	1.75 (0.6-2.9)		V	MITI, 1992
Cyprinus carpio	56	0.025	12.2 (3.2-21.2)		V	MITI, 1992
Cassarius auratus	20	0.39	191.3	0.47	NA	Wang *et al*., 1999
Lepomis macrochirus	4	1	4-78 (in viscera and muscle)	0.34 (Nearly 100% in 4 days)	V	Liu *et al*., 1983
Lepomis macrochirus	-	-	-	0.7-0.23	Additional information	Hartley, 1981
Daphnia magna	4	1	13	-	V	Liu *et al*., 1983
Lumbriculus variegatus	4	1	58		V	Liu *et al*., 1983
Selenastrum capricornutum	4	1	2507		V	Liu *et al*., 1983

V: Valid; VWR: Valid With Restrictions; NA: Not Assignable; I: Invalid.

Summary of bioaccumulation data

The BCF for 2,4-dinitrotoluene has been experimentally determined to be between 0.6 and 2500 for aquatic organisms in general and between 0.6 and 191 for fish. Only one fish BCF is above 100 and considering the rapid elimination rate it should be concluded that 2,4-dinitrotoluene will not bioaccumulate significantly in fish.

BCFs were less than 100 for two invertebrates, *D. magna* and *L. variegatus*, after short exposure and with no information regarding if concentrations had reached or not steady-state conditions. High values ranging from 2100 to 2500 have been determined in the alga *S. capricornutum*. Therefore, a potential risk for biomagnification in the lower part of the food-chain should be considered.

A toxicokinetic model (adapted from Carbonell *et al*., 2000) has been employed for comparing the bioconcentration results in *D. magna* and fish; the model outcome suggests an elimination rate in invertebrates one order of magnitude lower than the value calculated for fish. This difference can be explained by the role of cytochrome P-450 in the metabolism of 2,4-dinitrotoluene; which has been confirmed in mammals. A similar value is obtained from the comparison of fish and SPMD. Studies of the metabolism of 2,4- DNT presented in the Human Healht part indicates that the substance is converted to a dinitrobenzyl alcohol in a cytochrome P-450-dependent process, being the alcohols then conjugated with glucuronic acid and excreted in the bile and urine (Rieckert *et al*., 1986; Rieckert, 1987; Shoji *et al*., 1987). Other authors also state that multiple P450 isoforms may contribute to the side-chain oxidation of DNT (ATSDR, 1998). According to the BUA (1989), differences in metabolism could not be attributed to microsomal mechanisms, but rather to differing reaction rates with glucuronyl transferase.

Recent studies on the metabolism of cetaceans indicate that these marine mammals´ detoxifying capacity is limited. In fact, these animals have a low capacity for degradation of

certain compounds due to a specific mode of their cytochrome P450 enzyme system (Tanabe and Tatsukawa, 1992). Significant differences in Mixed Function Oxidase Activity (BPMO) levels have been found in some marine mammals (Fossi *et al*., 2000; Fossi *et al*., 2001).

These metabolic disadvantages could make invertebrates and marine mammals more susceptible to the bioaccumulation of certain contaminants including 2,4-dinitrotoluene. The rapporteur, considering this potential, has conducted a preliminary assessment of this potential for bioaccumulation in some organisms; data suggest that differences of about one order of magnitude in the elimination rate due to reduced metabolisms are not sufficient for considering a concern for secondary poisoning as even the higher elimination rate will still be of few hours as observed in the SPMD experiment.

Therefore, a low potential for bioaccumulation is expected in all organisms except algae.

3.1.4 Aquatic compartment (incl. sediment)

3.1.4.1 Calculation of predicted environmental concentrations (PEC_{local})

3.1.4.1.1 Calculation of PEC_{local} for production and processing

Releases from production are considered altogether with releases from processing, because production and processing happen one just after the other in the same place in sites A, B and D. Therefore, only a PEC_{local} for production and processing is calculated with EUSES for these sites, using the equations given in the TGD and assuming the information provided by industry.

$$PEC_{local(water)} = C_{local(water)} + PEC_{regional(water)}$$

$$C_{local(water)} = C_{eff} / (1+K_{p\ susp} \cdot C_{susp} \cdot 10^{-6}) \cdot D$$

$$C_{eff} = W \cdot (100 – P) / 100 \cdot Q$$

where:

$C_{local(water)}$ = local concentration in surface water during emission episode
$PEC_{regional(water)}$ = regional concentration in surface water
C_{eff} = concentration of the chemical in the WWTP effluent
$K_{p\ susp}$ = solids-water partitioning coefficient of suspended matter = 11,9 l/kg
C_{susp} = concentration of suspended matter in the river = 15 mg/1
D = dilution factor
W = emission rate (kg/day)
P = percentage removal in the WWTP (%) = 41.6
Q = volume of waste water

Industry of site A has indicated that about 40% of the waste water is treated in a WWTP, which has a discharge flow of 37,152 m^3/d. After treatment, waste waters are discharged into a lagoon, and as no dilution factor has been provided, the default factor of 10 given in the TGD has been used. Due to the fact that the place is located at a costal zone, the marine

exposure will be assessed and therefore, the PEC in the aquatic compartment has been calculated for both fresh and marine water.

Concerning the site located in France, information on the flow of the effluent, after physico-chemical treatment has been provided and the value is 8,115.35 m^3/h in site B. The 10%ile of the river flow has been supplied as well, being 34,7 m^3/s. This value gives a dilution factor of 15.4 for site B.

In site D, the information on the flows on the WWTP effluent (0.74 m^3/s) and the receiving river (1,050 m^3/s) make it possible to estimate a dilution factor of 1,418. As indicated in the TGD, in case of site-specific assessments the dilution factor that is applied for calculation of the local concentration in surface water should not be greater than 1000. Therefore, 1000 has been set as dilution factor for site D.

Table 3.12: PEC_{local} calculated for aquatic environment

Site	Release to wastewater (kg/day)	$C_{local,water}$ (µg/l)	$PEC_{local,water}$ (µg/l)
A	0.22 [a]	0.346	0.363
B	16 [a]	5.25	5.28
D	0.64 [b]	$5.85 \cdot 10^{-3}$	0.022

[a] Emission data provided by industry

[b] Value calculated from data provided by industry for the new production plant

Regarding the marine environment, the default emission factors proposed in the TGD have been applied for site A, which is located at a coastal zone, and the resulting PEC in sea water is 0.06 µg/l.

The PEC_{local} for sediment has been calculated as well with the equation given in the TGD, using the suspended matter-water partitioning coefficient:

$$PEC_{local,sed} = (K_{susp,water} / RHO_{susp}) \cdot PEC_{local,\ water} \cdot 1{,}000$$

Table 3.13: PEC_{local} calculated for sediment

Site	$PEC_{local,water}$ (µg/l)	$PEC_{local,sediment}$ (µg/kgwwt)
A	0.363	1.22
B	5.28	17.8
D	0.022	0.08

Regarding the marine environment, the resulting PEC in marine sediment for site A, located at a coastal zone, is 0.201 µg/kg.

The ground water levels resulting from infiltration of rainwater or from infiltration of river water to ground water can be considered the same as the concentration in porewater of agricultural soil, according to the TGD.

Table 3.14: PEC_{local} calculated for ground water (μg/l)

Site A	$1.63 \cdot 10^{-3}$
Site B	$1.63 \cdot 10^{-3}$
Site D	$1.63 \cdot 10^{-3}$

To assess the risk for microorganisms in the STP, PEC_{stp} has been taken as equivalent to the $C_{local,eff}$

Table 3.15: PEC_{local} calculated for microorganisms in the STP (μg/l)

Site A	3.46
Site B*	-
Site D	5.85

3.1.4.1.2 Calculation of PEC_{local} for formulation

In this case, the PEC_{local} for water has been calculated considering 250,000 m^3/year as the effluent discharge rate of a STP that collects effluents of several industries apart from the one considered in this assessment. The flow of the receiving river (10%) is 153,600 m^3/day, as industry has indicated. The estimated dilution factor is 224 and the results obtained are:

Table 3.16: PEC_{local} calculated for different compartments

Surface water	0.142 (μg/l)
Sediment	0.479 (μg/kgwwt)
Ground water	$1.63 \cdot 10^{-3}$ (μg/l)
Sewage treatment plant	28 (μg/l)

3.1.4.2 Measured levels

The available monitoring data provide a base for comparing the calculated exposure values obtained with EUSES. Most of these monitoring data from surface water refer to the river Elbe and they have been reported by the German company. Another data from Rhine and Main rivers come from the IUCLID Data Set, but they are older than the other ones.

These monitoring data are relatively old and, as no information on the conditions of the measurements and their proximity to the production and processing sites has been found, they cannot be assigned to the local emission sites. Even so these data provide a basis for comparing the calculated exposure data with the orders of magnitude which are to be expected.

Finally, measured 2,4-dinitrotoluene values in sediment and pore water of sediment only have been found from the Grand Calumet River-Indiana Harbour (USA), where the concentration of 2,4-dinitrotoluene was determined from 10 samples taken from 10 different locations between October 1988 and May 1990. No data on measured levels in ground water have been

found, and related to the concentration in the STP, a reference from the IUCLID Data Set has been considered, but it has not been possible to go back to the original publications to confirm this value.

Table 3.17: Measured levels in aquatic compartments

Compartment	Site	Concentration	Year
Surface water	Netherlands (Rhein River)	0.3 µg/l[a]	1979
	Main River	2.0 µg/l[a]	July 1984 - December1987
	Zehren; Germany (Elbe River)	0.51 µg/l [b] 0.41 µg/l [b]	1997
	Schmilka; Germany (Elbe River)	0.42 µg/l [b] 0.41 µg/l [b] 0.44 µg/l [b]	1997
	Dommitzsch; Germany (Elbe River)	0.33 µg/l [b] 0.33 µg/l [b]	1997
	Schnackenburg; Germany (Elbe River)	0.1 µg/l [b]	1997
Sediment	Indiana, USA (Grand Calumet River)	< 0.01 – 0.07 mg/kg dry wt) [a]	Oct. 1988 – May 1990
Pore water of sediment	Indiana, USA (Grand Calumet River)	0.1 – 1.7 µg/l	Oct. 1988 – May 1990
Sewage treatment plant	USA	0.19 mg/l [a]	-

[a] Data from IUCLID Data Set

[b] Data fro m Industry

3.1.4.3 Comparison between predicted and measured levels

Comparing monitored and predicted local concentrations in surface water, the predicted values for sites A, D and E, which range between 0.024 and 0.364 µg/l, are similar to the measured levels, between 0.1 and 2.0 µg/l. The value for site B, 5.28 µg/l, is somewhat higher. Nevertheless, they cannot be assigned to the local scale because no information on the place of measurement was available. For this reason, measured levels should rather be compared to $PEC_{regional,}$ which is 0.0178 µg/l (see Table 3.25).

Regarding to the sediment compartment, local PECs calculated for all sites are lower than the measured reference level . Concerning PEC_{local} in ground water, no information on measured levels was found and no comparison can be done.

Finally, the local PECs for microorganisms in the STP, ranging from 3.46 to 5.85 µg/l, are lower than the measured level in the USA (0.19 mg/l).

3.1.5 Terrestrial compartment

3.1.5.1 Calculation of PEC_{local}

3.1.5.1.1 Calculation of PEC_{local} for production and processing

PECs have been calculated with EUSES for agricultural soil and grassland, considering only the atmospheric deposition, since no spreading of sludge from industrial sewage treatment plants has been assumed in any site.

$$PEC_{local,soil} = C_{local,soil} + PEC_{regional,natural,soil}$$

Table 3.18: PEC_{local} calculated for terrestrial compartment (µg/kgwwt)

	Site A	Site B	Site D
Soil (total) averaged over 30 days	$3.61 \cdot 10^{-3}$	$3.61 \cdot 10^{-3}$	$3.61 \cdot 10^{-3}$
Agricultural soil (total) averaged over 180 days	$3.61 \cdot 10^{-3}$	$3.61 \cdot 10^{-3}$	$3.61 \cdot 10^{-3}$
Grassland (total) averaged over 180 days	$3.61 \cdot 10^{-3}$	$3.61 \cdot 10^{-3}$	$3.61 \cdot 10^{-3}$

The PEC_{local} for pore water of soil has been calculated from the soil/water partition coefficient, using the equation in the TGD:

$$PEC_{local,pore\ water} = PEC_{local} \cdot RHO_{soil} / K_{soil,water} \cdot 1{,}000$$

where:

RHO_{soil} = bulk density of wet soil
$K_{soil,water}$ = soil/water partition coefficient

Table 3.19: PEC_{local} calculated for agricultural soil pore water (µg /l)

	Site A	Site B	Site D
Pore water of agricultural soil	$1.63 \cdot 10^{-3}$	$1.63 \cdot 10^{-3}$	$1.63 \cdot 10^{-3}$
Pore water of grassland	$1.63 \cdot 10^{-3}$	$1.63 \cdot 10^{-3}$	$1.63 \cdot 10^{-3}$

3.1.5.1.2 Calculation of PEC_{local} for formulation

Table 3.20: PEC_{local} calculated for terrestrial compartment (µg /kgwwt)

Soil (total) averaged over 30 days	$3.61 \cdot 10^{-3}$
Agricultural soil (total) averaged over 180 days	$3.61 \cdot 10^{-3}$
Grassland (total) averaged over 180 days	$3.61 \cdot 10^{-3}$

Table 3.21: PEC_{local} calculated for agricultural soil pore water (µg /l)

Pore water of agricultural soil	$1.63 \cdot 10^{-3}$
Pore water of grassland	$1.63 \cdot 10^{-3}$

3.1.5.2 Measured levels

Concentration of 2,4-dinitrotoluene in soil was measured at a former ammunition site in North Rhine-Westphalia (Germany) in order to assess the exposure via plants. Levels between 0.03 and 1.50 mg/kg were detected and in five soil samples 2,4-dinitrotoluene could not be found (Schneider, K., 1996). Soil samples were removed from vegetable beds of a contaminated area, from a depth of 0 – 0.3 m, 0.3 – 0.6 m and 0.6 – 1 m using a Pürckhauer probe. The samples were sieved (< 8 mm), homogenized, dried with sodium sulphate and sonicated after the addition of toluene. Toluene extracts were directly analysed by GC-MS (single determinations). The main contaminant in soil was 2,4,6-trinitrotoluene, whereas dinitrotoluenes (2,4-dinitrotoluene and 2,6-dinitrotoluene) were present only in trace amounts.

3.1.5.3 Comparison between predicted and measured levels

The local PEC calculated for soil of sites A to D is $3.61 \cdot 10^{-3}$ µg/kg, and represents the contamination of agricultural soil due to atmospheric deposition, since no spreading of sludge from industrial sewage treatment plants has been assumed. Therefore, this predicted local concentration in soil of sites A to D is not comparable with the monitoring data provided in section 3.1.5.2., which comes from a former ammunition site. These data could only be compared with $PEC_{local,soil}$ calculated for site E, in which explosives manufacture takes place. In this way, it can be said than the value calculated for this site, $3.61 \cdot 10^{-3}$ µg/kg, is lower than the measured levels range in samples with detected residues, which is 0.03 – 1.50 mg/kg. However, in five soil samples no 2,4-dinitrotoluene was detected and an explanation to the high levels found in the ammunition site is that they could have been caused by direct contamination with explosives.

3.1.6 Atmosphere

3.1.6.1 Calculation of PEC_{local}

The PEC_{local} is calculated with the method described in the TGD Chapter 3, Section 2.3.8.2. For PEC_{local} calculation, emissions from point sources and sewage treatment plants need to be considered. The sewage treatment plant is assumed to be a point source and the concentration of the chemical is calculated at a 100 m distance from it. The indirect emission of a chemical from a sewage treatment plant to air is calculated from the fraction of emission directed to air by the sewage treatment plant. For 2,4-dinitrotoluene this is 0.1% (see Table 3.9).

The direct emission to air is multiplied by the concentration in air at a source strength of 1 kg/d ($Cstd_{air}$ $2.78 \cdot 10^{-4}$ mg/m^3) to give the local air concentration. The local concentration can be converted to an annual concentration if the number of emission days per year is known.

$$C_{local,air} = \max (E_{local,air}, Estp_{air}) \cdot Cstd_{air}$$

$$C_{local,air,ann} = C_{local,air} \cdot T_{emission} / 365$$

where:

$E_{local,air}$: Local direct emission rate to air during emission episode
$Estp_{air}$: Local indirect emission to air during emission episode
$T_{emission}$: Number of days per year that the emission takes place

No emissions to air are reported by industry for sites A and D and therefore only releases from WWTP are considered to obtain $C_{local,air}$, except for sites B , where there are not emissions to air, according to the industry. The regional PEC is then taken as a background concentration and added to the local concentration to give the PEC_{local}.

3.1.6.1.1 Calculation of PEC_{local} for production and processing

Table 3.22: PEC_{local} calculated for atmosphere

Site	Annual average total deposition flux (μg/m²/d)	$C_{local,air}$ (μg /m³)	$PEC_{local,air}$ (μg /m³)
A	$6.5 \cdot 10^{-6}$	$4.15 \cdot 10^{-6}$	$6.16 \cdot 10^{-6}$
B	0	0	$2.75 \cdot 10^{-6}$
D	$1.89 \cdot 10^{-5}$	$1.2 \cdot 10^{-5}$	$1.26 \cdot 10^{-5}$

3.1.6.1.2 Calculation of PEC_{local} for formulation

Table 3.23: PEC_{local} calculated for atmosphere

Site	Annual average total deposition flux (μg /m³/d)	$C_{local,air}$ (μg /m³)	$PEC_{local,air}$ (μg /m³)
E	$5.95 \cdot 10^{-7}$	$3.8 \cdot 10^{-7}$	$3.07 \cdot 10^{-6}$

3.1.6.2 Measured levels

No information on measured levels was found and no comparison can be provided.

3.1.7 Secondary poisoning

Assessment of biomagnification potential

According to the Technical Guidance Document, the risk characterisation for secondary poisoning is required if three specific situations are fulfilled:

- Indirect exposure likely
- Indication of bioaccumulation potential and,

- Potential to cause toxic effects if accumulated in higher organisms.

According to the TGD strategy for secondary assessment, for substances with a log K_{ow} < 4.5, the following equation will be used:

$$PEC_{oral,predator} = PEC_{water} \cdot BCF_{fish} \cdot BMF$$

It has been applied the more appropriate scenario were 50% of the diet comes from a local area, and the other 50% came from the regional area. So, the average PEC_{water} value has been considered within the assessment. $3.63 \cdot 10^{-4}$ mg/l, corresponding to the Site A, located in a coastal zone, and $1.78 \cdot 10^{-5}$ mg/l have been selected as the most realistic worst case scenario for the local PEC_{water} and the calculated $PEC_{regional}$, respectively.

$$PEC_{oral,predator} = [(3.63 \cdot 10^{-4}/2) + (1.78 \cdot 10^{-5}/2)] \cdot 12 \cdot 1 = 2.28 \cdot 10^{-3} \text{ mg/kg}$$

Considering the capacity of adsorption onto the surface of the algae, the possibility of indirect exposure to higher trophic level organisms, especially algae feeding invertebrates, has been assessed by using the long-term study on daphnia used for the PNEC derivation (see section 3.2.1.1.2.). In this study, the daphnids were already exposed to the substance, simultaneously via water and via food, considering that the algae used for the long-term feeding also adsorbed the substance.

So, according to the low bioaccumulation potential and the rapid elimination of this compound in fish, no secondary poisoning potential is expected from this substance. But, the calculated $PEC_{oral,predator}$, or measured, if available, should be compared with the no effect concentration for these predators once the assessment of the mammalian data for the draft Human Health risk Assessment is completed.

3.1.8 Calculation of $PEC_{regional}$ and $PEC_{continental}$

The calculations of PECs at a regional and continental scale were done using the EUSES model and the values obtained for the worst-case scenario of a regional production of 2,4-dinitrotoluene of 304,000 tonnes and the associated emissions, estimated with default values from EUSES, are shown below:

Table 3.24: Regional and continental PECs

Compartment	Regional	Continental
Surface water (total) (μg/l)	0.0178	$2.22 \cdot 10^{-4}$
Surface water (dissolved) (μg/l)	0.0178	$2.22 \cdot 10^{-4}$
Air (μg/l)	$2.75 \cdot 10^{-6}$	$9.14 \cdot 10^{-8}$
Agricultural soil (total) (μg/kg wet wt)	$2.4 \cdot 10^{-3}$	$6.65 \cdot 10^{-5}$
Pore water of agricultural soils (μg/l)	$1.09 \cdot 10^{-3}$	$3.01 \cdot 10^{-5}$
Natural soil (total) (μg/kg wet wt)	$3.6 \cdot 10^{-3}$	$1.2 \cdot 10^{-4}$
Industrial soil (total) (μg/kg wet wt)	3.21	0.0241
Sediment (total) (μg/kg wet wt)	0.0542	$7 \cdot 10^{-4}$

3.2 EFFECTS ASSESSMENT: HAZARD IDENTIFICATION AND DOSE (CONCENTRATION) - RESPONSE (EFFECT ASSESSMENT)

3.2.1 Aquatic compartment (incl. sediment)

The provided information includes a set of data on the toxicity of 2,4-dinitrotoluene only for fresh water organisms. No information has been provided regarding toxicity on marine organisms.

Provided data have been summarised in Tables 3.25, 3.26 y 3.27 for aquatic vertebrates, aquatic invertebrates and algae, respectively.

3.2.1.1 Toxicity test results

3.2.1.1.1 Fish

In this section it is presented the toxicity results on fish. Comments on tests have been focused especially on those ones considered for the assessment. Provided information has been summarized in Table 3.25.

Acute toxicity

A well documented report has been presented by Liu *et al*., (1983), who uses the Manual entitled "*Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians*" (EPA, 1975) as a guide for describing and conducting the acute toxicity tests with the animal species. Static techniques were used. Acceptable levels of mortality in the controls were 10% for fish. Standards were prepared by dissolving a known weight of the standard compound in reagent grade ethyl acetate in an appropriately sized glass volumetric flask. The flasks were wrapped in aluminium foil to prevent photodecomposition, since the toxicity of the compound is reduced by photodegradation. As additional information, exposure of 2,4-dinitrotoluene to 100% photolyzed by filtered UV light reduced the acute toxicity to fathead minnows by a factor of about 2, from an average EC_{50} of 31.4 (26.3-34.0) to 64.8 mg/l. Results of acute tests on different species, at different exposure periods and under static and flow-through conditions are presented in Table 3.25.

In another publication Liu *et al*. (1983b), provided an 96h-LC_{50} of 32.8 mg/l (ranging from 27.3 to 38.0 mg/l of 2,4-dinitrotoluene). Acute toxicity tests were performed on *P. promelas* under static conditions. Fish were immature and no food was provided to the organisms during the test.

Broderius *et al*., (1995) conducted 96-h acute toxicity test with 2,4-dinitrotoluene, on 26 to 34-d old laboratory-cultured juvenile fathead minnows (*Pimephales promelas*), according to the following test conditions procedures, exposures were conducted at 25ºC in continuous flow-through systems with minimum four toxicant concentrations and a control in duplicate for each LC_{50} determination. Toxicity tests were conducted without using solvent carriers. The dilution water had a water hardness, alkalinity, and pH of approximately 45 mg/l as Ca CO_3, 42 mg/l as $CaCO_3$, and 7.8, respectively. Toxicant concentrations in the test chambers were continuously

renewed and measured daily (HPLC and GC). Under these conditions a 96h-LC_{50} of 24.3 mg/l was provided.

Van den Dikkenberg *et al*., 1989 carried out a well-documented study in which a 96-h short term semi static (renewal once at 48h) toxicity test with young fish (4 to 5 weeks old) to determine LC_{50}, EC_{50}, NOLC (No Observed Lethal Concentration) and NOEC after 24-, 48-, 72 and 96-h, (mortality, swimming behaviour and appearance), in which 10 organisms were placed (in duplicate) in 1 litre of test volume in glass vessels (fish did not feed during the test period). Individuals, which were reared under standardized conditions, were kept in glass containers with fresh water at a temperature of 19±1ºC and long-day conditions (16h light/8h dark). The three-spined stickleback is very common in Europe, parts of Asia and Northern America, and occurs in a wide variety of water types in countries with a temperate climate. The fresh water from each aquarium is filtered constantly (carbon and perlon filters) and aerated. The levels of pH, NO_2 and total hardness (all checked weekly) were kept between 6.5 and 8.4, <0.1 mg/l and between 10 and 14ºDH, respectively. The temperature was checked daily and was kept between 18-20ºC. Only initial concentrations in the test chambers of the lowest and the highest test concentrations were confirmed by chemical analyses, showing that the test concentrations are at least 70% of the initial nominal concentration 72-h after preparation of the solutions. Under these conditions there were provided an LC_{50} of 6.3 mg/l; a EC_{50} of 1.3 mg/l; a NOLC of 1.8 mg/l and a NOEC of 0.98 mg/l with sticklerbacks.

Deneer *et al*., (1987) provided an LC_{50} = 12.60 mg/l after an exposure period of 14 days. Fish used in the toxicity experiments were male and female guppies, varying in age from two to three months, and in weight from 60 to 450 mg (mean fat content 8 ± 2%). Procedure for obtaining the 14d-LC_{50} values were according to Könemann (1981).

There is also very poorly detailed information provided by Bayer (1985), which has been included in the table as additional information but considered not assignable for the assessment.

Pearson *et al*., 1979 carried out an acute static toxicity tests on fathead minnow (*Pimephales promelas*). Tests were conducted according to procedures described elsewhere (EPA, 1975). The test and culture water was dechlorinated municipal water. The average hardness, alkalinity, and conductivity of the water were 26 ppm (as $CaCO_3$), and 103 µmhos·cm, respectively. The pH ranged from 7.2 to 8.6. The median effect levels (96h-LC_{50}) were determined, using the probit analysis. Results show an LC_{50} of 32.5 mg/l.

The following additional information may be found in the AQUIRE database:

- A 96h-LC_{50} of 22 mg/l (ranging from 19 to 25 mg/l) on *Jordanella floridae*, with a lifestage of 1-2 days, has been provided under the following test conditions: temperature = 23 °C, pH = 8.2, dissolved O_2 = 6.5 mg/l and hardness = 209.43 mg $CaCO_3$/l. Nominal results were obtained under renewal exposure conditions (Adema *et al*., 1981 in AQUIRE (2004)). This will be considered as additional information since no validation of the test was possible.

- A 96h-LC_{50} of 16 mg/l on *Oryzias latipes*, with a lifestage: 1-2 days, has been provided under the following test conditions: temperature = 23 °C, pH = 8.2, dissolved O_2 = 6.5 mg/l and hardness = 209.43 mg $CaCO_3$/l. Nominal results were obtained under renewal exposure conditions (Adema *et al*., 1981 in AQUIRE (2004)). This will be considered as additional information since no validation of the test was possible.

- A 96h-LC_{50} of 13 mg/l, (ranging from 11 to 16 mg/l), on *Danio rerio* with a lifestage of 1-2 days has been provided under the following test conditions: temperature = 23 °C, pH = 8.2, dissolved O_2 = 6.5 mg/l and hardness = 209.43 mg $CaCO_3$/l. Nominal results were obtained under renewal exposure conditions (Canton *et al*., 1984 in AQUIRE (2004)). This will be considered as additional information since no validation of the test was possible.

Long-term toxicity

Bailey *et al*. (1984), in a well-documented report, carried out several early life stage studies conducted on 2,4-dinitrotoluene with rainbow trout, channel catfish and fathead minnow. They were performed in 19-L aquaria containing 15 l of test solution. Fathead minnow and channel catfish eggs were exposed in eggs cups made from 5 cm diameter glass or PVC tubing with one end covered transferred to rearing chambers. Eggs from rainbow trout were simply placed on the bottoms of the aquaria during exposure. Dechlorinated tap water was used to culture and maintain the test animals, to prepare the stock solutions, and as the diluent for the flow-through tests. The laboratory tap water was a blend from different origins resulting in the following parameters: dissolved oxygen: 7.8-10.8 mg/l; pH 6.9-8.0; temperature: 11.8-13 ºC; hardness: 24-110 mg/l as $CaCO_3$; alkalinity: 20-100 mg/l as $CaCO_3$. The locations of the test aquaria were randomised within each replicate series. Nominal test temperatures for the early life stage studies were 12 ºC for rainbow trout, 25 ºC for channel catfish and fathead minnows.

Rainbow trout. Early life stage tests were initiated with 60 eggs per duplicate tank at each exposure level. The eggs were fertilized in the presence of toxicant and allowed to water-harden before they were transported to the laboratory. In the first series of test, a different female was used for each concentration; in the second test series, eggs from different females were randomised over the mixing containers before they were fertilized. Because of low overall fertility was observed in one of the tests in the second series, the test was restarted using eyed eggs obtained directly from the hatchery. The first series of the tests were terminated 30 days after hatching was complete, and the second series were terminated after a 60-day post hatch exposure period. Once the fry entered the swim-up stage, they were fed a combination on *Artemia nauplii*, dry trout food, and frozen adult brine shrimp three times a day. Throughout the tests, the tanks were inspected daily and dead eggs and fry were removed. At the end of the test, surviving fry were individually weighted and the total weight measured (total length). Chemical concentrations were determined weekly, alternating between the replicates. 2,4-dinitrotoluene, after an exposure period of 60 days, did not affect hatching success or fry survival within the range of concentrations tested (0.10 to 2.05 mg/l), although it must be considered that there were problems with non-randomization of gametes and hatching success and fry survival in the controls was low (25% at average). Although the overall density of fry in each tank was rather variable, the overall trends in growth effects as well as the magnitude of these effects suggests that, at least within the concentration range of 0.49 to 2.05 mg/l, the observed reductions in growth were toxicant-related, showing a NOEC of 0.23 mg/l and a LOEC of 0.49 mg/l for this parameter. 2,4-dinitrotoluene on rainbow trout during a 90 day exposure period produce no statistically significance effects on egg survival, number of deformed fry, or fry survival within the range of tested concentrations of 0.05 to 4.02 mg/l. However, it is indicated that all the fry in the highest concentrations (4.02 mg/l) were unable to swim and remained at the bottom of the aquaria. On the other hand, it is indicated that fry length and fry weight were reduced at all range concentrations (0.05 to 4.02 mg/l) after 90 days exposure period in one of the series. As it is indicated, there are

shortcomings in the performance of the study and great variability in the results and so, it has been included as additional information.

Channel catfish. Same tests were initiated with 30 eggs per treatment in each of the duplicate test series. However, some difficulties were encountered in separating the eggs because they were past the initial hardening stage and were easily damaged when handled. Therefore, a similar sized clumps of eggs from the original egg masses were cut off, counted the eggs in each mass, weighted them, and transferred them into the egg cups. In addition, because the tests were started with clump eggs, the initial number of eggs varied markedly among the treatment groups. Another problem using this approach was that, because the eggs were not separated, the fungus-affected eggs could not be removed to prevent the spread of disease to other eggs. To minimize problems with fungal infection, the eggs were flushed daily with malachite green up to the time of hatching. After hatching, the fry were transferred from the eggs cups into the aquaria for 30-day post-hatch exposure period. During this period, the fry were fed brine shrimp *nauplii*, frozen adult brine shrimp, and dried chow *ad libitum* three times a day. Excess of food and waste materials were siphoned from the bottom of the tanks as necessary. The surviving fry were measured (total length) at the end of the exposure period. Chemical concentrations were determined twice weekly, alternating between the replicates. Exposure concentrations ranged from 3.4 to 32.6 mg/l. The eggs were exposed for approximately 10 days and the fry for the remainder of the 30 days exposure period. Early life stage studies with channel catfish were not successful. The eggs could not be separated because of their advanced stage of development; heavy losses occurred due to fungal infectious in spite of the prophylactic treatment with malachite green. Because it had to start the test with clumps of eggs, the initial number of eggs varied markedly among the treatment groups. Under these conditions, data were highly variable. Dinitrotoluene appeared to reduce significantly hatching success at concentrations of 6.2 to 32.6 mg/l (100% of reduction); Referring to the controls the NOEC could be determined at 3.4 mg/l, although this value should be considered invalid, since controls registered a 61% of reduction in hatching (versus 55% of reduction for the NOEC). Fry survival seems to be affected as concentrations as low as 3.4 mg/l with an average percentage of survival of 25%. In both of these tests, the eggs were exposed for approximately 10 days and the fry for the remainder of the 30-day exposure period. Due to high uncertainties in test conditions, these results will be considered as no valid information.

Fathead minnows. Breeding stock was reared under flow-through conditions at 25ºC. The adult minnows were fed frozen adult brine shrimp, live daphnids and trout chow. Early life stage tests were initiated with 30 embryos (24 hours old) per egg cup. Two eggs cups were used per tank. The tests were generally terminated 30 days after initiation and in duplicate. After hatching, the fry were counted and transferred into larval rearing chambers. During the post-hatch exposure period, the fry were fed brine shrimp *nauplii* three times a day. Excess food and waste materials were siphoned from the bottom of the tanks as measurement at the end of the exposure period. Total fry length was determined photographically or by direct measurement at the end of the exposure period. Chemical concentrations were determined twice weekly, alternating between the replicates. It was observed a uniformly low hatching success (approximately 50-60%) even in the controls. Referring to the controls, 2,4-dinitrotoluene had no appreciable effects on egg hatchability and fry growth at concentration ranges (1.0 to 3.1 mg/l), except at the highest one (6.8 mg/l), which markedly reduced all parameters (compared to the controls). It will result in the following figures for fry survival and fry length: LOEC of 6.8 and a NOEC of 3.1 mg/l. The study will be considered no valid for the assessment.

A second chronic study, a full life-cycle, was initiated by Bailey *et al*. (1984) on fathead minnows by randomly distributing a minimum of 40 eggs to each of two eggs cups suspended in each tank testing a range of concentrations from 0.28 to 6.71 mg/l. The duplicate series were started approximately 1 week apart. During the period of embryo development, the egg cups were inspected daily and dead eggs were removed. Once the fry began to hatch, the cups were not disturbed except for the daily checks to determine whether hatching had been completed. If the hatching process took longer than 24 hours to complete, brine shrimp *nauplii* were added to the egg cup twice daily to ensure a food source for the hatched fry. When hatching was completed in all cups, deformed and normal fry were counted, and normal fry were transferred to rearing chambers. Fry were maintained in the rearing chambers for 90 days. During this period, they were fed a mixture of brine shrimp *nauplii*, dry trout food, and frozen adult brine shrimp four times a day. Food mixture varied depending on the size of the fry. On day 30, 60 and 90 post-hatch, the rearing chambers were removed from the tanks, placed on millimeter grid paper and photographed several times to determine the total length and survival of the fry. After the pictures were taken at 90 days, the fry were released from rearing chambers into the aquaria. After release, observations continued to check the fry for signs of developing breeding characteristics in the males, such as dark banding, blunt snouts, and tubercles. When several males were obvious in all the tanks, the fish were removed from the tanks and carefully segregated according to sex. Males were determined on the basis of banding, blunt snouts, or tubercles. Females were differentiated by the presence of urogenital papillas. A third category was reserved for fry whose sex could not be readily determined. Four males and four females were selected and randomly assigned as individual pairs in stainless-steel breeding cages. Spawning substrates were inspected daily for eggs. If eggs were present, the substrate was replaced with a clean one and counted. A minimum of 35 eggs were selected at random and placed in egg cups to determine hatchability. If the spawn did not contain at least 35 eggs, the eggs were counted and discharged. After the eggs hatched, the fry were counted and discarded or added to a rearing chamber if one was available. It was attempted to rear two batches of F1 fry to 30 days and two batches to 60 days in each tank. If possible, each set of fry selected for rearing was obtained from different spawning pair. In addition to hatchability and mortality, records of deformations and length and weights were taken for the F1 fry. Lengths and weights were taken at the end of the exposure period, and interim 30-days lengths were determined photographically on fry reared to 60 days. Each test was terminated when no spawns occurred in any concentration for 1 week. Data indicate that the survival of the spawning pairs was not significantly affected over the range of 179 days exposure period, although their ability to produce eggs was significantly reduced at concentrations as low as 1.31 mg/l. *c.a.*, 20% of mortality in the controls was considered acceptable. The effect of 2,4-dinitrotoluene on fry length indicates the reduction at a concentration of 1.31 mg/l was significant after 30, 60 and 90 days of exposure. In the pooled series, eggs per spawn were significantly reduced at 0.62 mg/l after 90 days of exposure. The data also suggest that the reduction in reproductive capability could be extended to the lowest concentration tested, which was 0.28 mg/l, since some reproductive parameters experimented decreasing nearing 20% at this concentration. Exposure on the F1 generation resulted in a statistically significant reduction in the egg-hatching success, at a concentration 2.69 mg/l. Survival of F1 fry reared to 30 days was significantly reduced at concentration of 0.62 mg/l. Survival of F1 fry raised to 30 days was also significantly reduced in the A series at a concentration of 2.69 mg/l. The study is considered valid for the assessment.

In another study, Hartley in 1981 exposed juvenile bluegills to 0.05- 8.0 mg/l of 2,4-dinitrotoluene for 56 days. Sublethal histological and growth responses of the fish to sub-acute concentrations of this compound were evaluated. The threshold concentration for significant

growth rate reduction was 0.05 mg/l. Unfortunately, only a short abstract is available. So, as the report could not be validated, it will not be assignable although it is included as additional information.

Van den Dikkenberg *et al*., (1989), carried out a well-documented study in which, *c.a.* 35-d early life stage tests on eggs (less than 6 hours old) were used to determine NOEC (embryogenic stage) and LC_{50}, EC_{50}, NOLC and NOEC (mortality, and embryogenic development of egg, and mortality, swimming behaviour, appearance and growth of the fry) after 4 weeks (fish stage). The semi-static flow conditions imply the renewal of the test medium 3 times a week. The adult individuals were fed with dry food and artemia. Both, the eggs and young fish used in experiments were at least of the 2^{nd} generation of fish (*G. aculeatus)* maintained under standard conditions. Individuals, which were reared under standardized conditions, were kept in glass containers with fresh water at a temperature of 19±1ºC and long-day conditions (16h light/8h dark). The three-spined stickleback is very common in Europe, parts of Asia and Northern America, and occurs in a wide variety of water types in countries with a temperate climate. The fresh water form each aquarium is filtered constantly (carbon and perlon filters) and aerated. The levels of pH, NO_2 and total hardness (all checked weekly) were kept between 6.5 and 8.4, <0.1 mg/l and between 10 and 14ºDH, respectively. The temperature was checked daily and was kept between 18-20ºC. Only initial concentrations in the test chambers of the lowest and the highest test concentrations were confirmed by chemical analyses, showing that the test concentrations are at least 70% of the initial nominal concentration 72-h after preparation of the solutions. Under these conditions, the provided NOLC (35 days) and NOEC (35 days) with sticklerbacks were 1.4 mg/l and 0.77 mg/l, respectively. Results regarding mortality have not been considered as chronic results, but have been included as additional information.

Table 3.25: Toxicity of 2,4-dinitrotoluene to fish

SPECIES	TEST TYPE	Measured/ Nominal	TEST Guideline	DURATION	TOXICITY END POINT (mg/l)	COMMENTS	REFERENCE
Fathead minnow (*P. promelas*)	Static	Nominal	EPA Guideline(1975)	96-h	LC_{50} 28.5 (26.3-32.5) LC_{50} 31.4 (29.3-34.0)	V	Liu *et al*., 1983
Fathead minnow (*P. promelas*)	Static Aerated	Nominal	-	96-h	LC_{50} 32.8 (27.3-38.0)	V	Liu *et al*., 1983b
Fathead minnow (*P. prormelas*)	Flow-through	Measured	EPA Guideline(1975)	24-h 48-h 96-h 14-d	LC_{50} 37.7 LC_{50} 37.7 LC_{50} 36.1 LC_{50} 26.0 (23.4-28.5)	V	Liu *et al*., 1983
Fathead minnow (*P. promelas*)	Static	-	EPA Guideline(1975)	96-h	LC_{50} 32.5	V	Pearson *et al*., 1979
Fathead minnow (*P. promelas*)	Flow-through	Measured	Test procedure not given in full details	96-h	LC_{50} 24.3	VWR	Broderius *et al*., 1995
Bluegill (*L. macrochirus*)	Static	Nominal	EPA Guideline(1975)	96-h	LC_{50} 13.5 (12.1-15.1)	V	Liu *et al*., 1983
Bluegill (*L. macrochirus*)	Flow-through	Measured	EPA Guideline(1975)	24-h 48-h 96-h 14-d	LC_{50} 30.2 LC_{50} 29.9 LC_{50} 16.0 LC_{50} 9.2 (8.5-10.4)	V	Liu *et al*., 1983
Channel catfish (*I. punctatus*)	Flow-through	Measured	EPA Guideline(1975)	24-h 48-h 96-h 14-d	LC_{50} 36.7 LC_{50} 33.8 LC_{50}32.0 LC_{50} 14.1-18.9	V	Liu *et al*., 1983
Channel catfish (*I. punctatus*)	Static	Nominal	EPA Guideline (1975) Tests solutions aerated	96-h	LC_{50} 24.8 (21.0-29.3)	V	Liu *et al*., 1983
Zebra fish (*B. rerio*)	Static	-	-	96-h	LC 0 23.7 LC_{50} 47.26 (calculated)	NA	Bayer, 1985

SPECIES	TEST TYPE	Measured/ Nominal	TEST Guideline	DURATION	TOXICITY END POINT (mg/l)	COMMENTS	REFERENCE
					LC 100 75		
Ide (*Leuciscus idus*)	Static	-	-	96-h	LC 0 23.7 LC_{50} 27.8 (calculated) LC 100 31.6	NA	Bayer, 1985
Rainbow trout (*O. mykiss*)	Static	Nominal	EPA Guideline(1975)	96-h	LC_{50} 13.6 (12.2-15.2)	V	Liu *et al*., 1983
Rainbow trout (*O. mykiss*)	Flow-through	Measured	EPA Guideline (1975)	24-h 48-h 96-h 14-d	LC_{50} 19.6 LC_{50} 19.3 LC_{50} 13.9 LC_{50} 6.3 (5.6-7.0)	V	Liu *et al*., 1983
Bluegill (*L. macrochirus*)	Static	Nominal	T° (24.5) pH (6.3) pH (7.1) pH (8.0) (Tests solutions aerated; EPA (1975)	96-h 96-h 96-h 96-h	LC_{50}7.8 (7.2-8.5) LC_{50} 8.4 (7.0-10.0) LC_{50} 12.8 (11.5-14.0) LC_{50} 9.4 (8.7-10.4)	V	Liu *et al*., 1983
Jordanella floridae	Renewal	Nominal	Other	96-h	LC_{50} 22 (19-25)	Additional information	Adema *et al*. 1981 (in AQUIRE database)
Oryzias latipes	Renewal	Nominal	Other	96-h	LC_{50} 16	Additional information	Adema *et al*. 1981 (in AQUIRE database)
Danio rerio	Renewal	Nominal	Other	96-h	LC_{50} 13 (11-16)	Additional information	Canton *et al*. 1984 (in AQUIRE database)
Guppy (*Poecillia reticulata*)	Semiestatic	Measured	Other	14 d	LC_{50} 12.60	V	Deener *et al*., 1987
Sticklerbacks (*Gasterosteus aculeatus*) (Estuary/marine fish)	Fresh water Semiestatic	Measured	Other; acc. to Adema *et al*.	96-h	LC_{50} 6.3 EC_{50} 1.3 NOLC 1.8 NOEC 0.98	V	Van den Dikkenberg *et al*., 1989

SPECIES	TEST TYPE	Measured/ Nominal	TEST Guideline	DURATION	TOXICITY END POINT (mg/l)	COMMENTS	REFERENCE
Long-term results							
Rainbow trout *(O. mykiss)*	Flow-through	measured	Other: early life stage test	60 days 60 days 90 days 90 days 90 days	NOEC fry growth 0.23LOEC fry growth 0.49 LOEC behaviour 0.49 LOEC < 0.05 LOEC < 0.05	VWR	Bailey *et al*., 1984
Channel fish (*Ictalurus punctatus*)	Flow-through	Measured	Other: early life stage test	30 days	NOEC hatchability 3.4	VWR	Bailey *et al*., 1984
Fathead minnow (*P. promelas*)	Flow-through	Measured	Other: early life stage test	30 days	LOEC fry survival/length 6.8 NOEC fry survival/length 3.1	VWR	Bailey *et al*., 1984
Fathead minnow *(P. promelas)*	Flow-through	Measured	Other: early life stage test	90-d	NOEC hatchability 0.28 LOEC hatchability 0.62	VWR	Bailey *et al*., 1984
Fathead minnow *(P. promelas)*	Flow-through	Measured	Other	179-d	NOEC reproduction 0.28 LOEC reproduction 0.62	VWR	Bailey *et al*., 1984
Bluegill *(Lepomis macrochiru)*	-	-	Other	56 days	TT growth 0.05	NA	Hartley, 1981
Sticklerbacks (*Gasterosteus aculeatus*) (Estuary/marine fish)	Fresh water semiestatic	Measured	Other; acc. to Adema *et al*. (early life stage test)	35-d	NOLC 1.4 NOEC mortality / sublethal 1.4 NOEC growth 0.77 LC_{50} 2.2 EC_{50} mortality / sublethal 2.2	V	Van den Dikkenberg *et al*., 1989
Sticklerbacks (*Gasterosteus aculeatus*)	Semiestatic	Measured	Other; acc. to Adema *et al*. (early life stage test)	35-d	NOEC embryogenic 2.5	V	Van den Dikkenberg *et al*., 1989

SPECIES	TEST TYPE	Measured/ Nominal	TEST Guideline	DURATION	TOXICITY END POINT (mg/l)	COMMENTS	REFERENCE
(Estuary/marine fish)							

V: Valid; VWR: Valid With Restrictions; NA: Not Assignable; NOLC (No Observed Lethal Concentration).

3.2.1.1.2 Aquatic invertebrates

In this section it is presented the toxicity tests results on aquatic invertebrates. Comments on tests have been focused especially on those ones considered for the assessment. Provided information has been summarized in Table 3.26.

Acute toxicity

Pearson *et al*., (1979) conducted acute static toxicity tests with the water flea (*Daphnia magna*). All tests were conducted according to procedures described elsewhere (EPA, 1975). The test and culture water was dechlorinated municipal water. The average hardness, alkalinity, and conductivity of the water were 26 ppm (as $CaCO_3$), and 103 µmhos/cm, respectively. The pH ranged from 7.2 to 8.6. The median effect levels (48h-EC_{50} for *daphnia*) were determined, using the probit analysis. Results show an EC_{50} of 35.0 mg/l .

Liu *et al*., (1983) used the EPA Guideline (1975) as a guide for describing and conducting several acute toxicity tests with on different animal species. Static techniques were used. Acceptable levels of mortality in the controls were 20% for the invertebrate tests. Acute results on different species at different exposure times are included in Table 3.26.

In another study, Liu *et al*., (1983b) performed a 48-h exposure static test. It is indicated that no food was provided to the organisms during the test, but no full description of the test procedure is shown. An average LC_{50} 47. 5mg/l (ranging from 29.5 to 99.7 mg/l) is provided.

Kühn *et al*., (1988), provided information on the effects of 2,4-dinitrotoluene (10% moistered) on *Daphnia magna*. The acute test was performed in accordance with national standard methods (DIN 36 412, part 11: 1981), after 24 and 48 hours, but only acute data regarding 24 hours exposure period appears in the report. An EC_0 of 13 mg/l and an EC_{50} of 38 mg/l were determined after 24 hours of exposure. These data were considered as not assignable for the assessment but included in the table as additional information.

In another study, Bringmann and Kühn (1977), determined the 24h-LC_{50} (22 mg/l) toxicity of 2,4-dinitrotoluene by means of a standardized procedure using 24h old animals from a clone of *Daphnia magna*. At the same time, the LC_0 (10 mg/l) and the LC_{100} (39 mg/l) was also determined. The test medium was tap water free from chlorine, saturated with oxygen, hardness 16º (German), pH 7.6-7.7, temperature 20-22 ºC. The data have been included in the table as additional information, but data obtained after a 24 hours exposure period was not considered in the assessment.

The following additional information can be found in the Aquire database:

- A 48h-EC_{50} (immobilisation) of 26.2 mg/l (ranging from 22.5 to 30.5 mg/l), on *Daphnia magna* with a lifestage: < 24 hours, has been provided under the following test condition: temperature = 22 °C, pH = 7.7 and hardness = 154.5 mg $CaCO_3$/l. Nominal results were obtained under static exposure conditions (Randall and Knopp, 1980, in AQUIRE database 2004)). This will be considered as additional information since no validation of the test was possible.

Long-term toxicity

Kühn *et al*., (1988) conducted long-term toxicity tests, to assay the potential effects of 2,4-dinitrotoluene (10% moistered) on *Daphnia magna* reproduction, over the 21-day test period. Testing was performed according to the preliminary method proposed by the Federal Environmental Agency (1984) using a semi-static procedure. The study was performed in accordance with the "Proposed Preliminary Testing method: Prolonged toxicity Test on *Daphnia magna* (determination of NOEC for reproduction rate, mortality and time of the first appearance of offspring; 21d) as of 1 January 1984, published as Recommendation of the Federal Environmental Agency for the Performance of Testing according to nº5, paragraph 1 Nº 3 of the Regulations on Documents to be submitted and Evidence of Testing under Chemical Act. A semi-static procedure was used as method with a pH 8± 0.2. Under these conditions after an exposure period of 21 days, a nominal NOEC of 0.04 mg/l was determined (bellow the detection limit of the analytical method: 0.05 mg/l), and so, the recovery rate found for higher tests concentrations was used to estimate the effective NOEC. With a recovery rate of 50% a NOEC of was 0.02 mg/l was estimated. This information will be considered for the assessment.

Bailey *et al*., 1984 also conducted a test on *Daphnia magna* in 80-L aquaria that contained approximately 28L of water. The breeding stock was reared under flow-through conditions at 20ºC. Adult daphnids were fed with *Selenastrum capricornutum* alone or in conjunction with a vitamin supplement. The photoperiod was set at 16 hours light (100 ft. candels) and 8 hours. Ten beakers were placed in each aquarium; seven of the 10 beakers received one daphnid each and the remaining three beakers received five daphnids each. Young daphnids were reared in a colony maintained under the same conditions as the test, except that individual beakers contained two adults each. Reproduction was carefully monitored in the colony to ensure that daphnids used in the tests did not come from the first brood. Twenty four hours before the test started, all young were removed from beakers in the rearing colony. On the day of the test, the new young were removed from the beakers and distributed randomly into the test beakers using a large replicated series. If a sufficient number of young was not available to initiate a test, this procedure was repeated until enough could be obtained within a 24-hour period. The beakers were inspected daily for mortality and young. Dead daphnids were removed and young individuals were removed and counted. The daphnids exposed to 2,4-dinitrotoluene were fed algae (*Selenastrum capricornutum*) twice daily at a rate of 30,000 cells/ml. The tests were terminated after 28 days of exposure, and surviving daphnids from the beakers that contained one individual were measured. No effect on survival was observed on the range of concentrations tested (0.07 to 1.78 mg/l). Regarding the number of young produced per individual female during the reproductive period, survival was significantly high at the lower concentrations, but this parameter does not follow a dose-response inverse relationship, and so an increase in the number of young is observed at the three higher concentrations and in replicates. This fact could be considered as methodological deficiencies or an early response. Under these conditions, and from a conservative point of view, the LOEC for reproduction, after an exposure period of 28 days, has been selected as 0.4 mg/l, and so the NOEC will be determined as the immediate lower concentration as 0.19 mg/l. This study was considered as valid for the assessment with some restrictions.

It has also been presented an unpublished information by Bayer (1986) on *Daphnia magna*, according to the OECD Guideline 202. Results indicate an EC_{50} reproduction rate *c.a.* 0.5 mg/l. This information will be considered as valid, since it was carried out according to a standardized procedure, although there was no possibility of validating the report due to the very short detailed information provided.

Table 3.26: Toxicity of 24-dinitrotoluene to aquatic invertebrates

SPECIES	TEST TYPE	Measured/ Nominal	TEST Guideline	DURATION	TOXICITY ENDPOINT (mg/l)	COMMENTS	REFERENCE
Dapnhia magna	-	-	DIN 36 412	24-h	EC_0 = 13 EC_{50} = 38	NA: additional information	Künh *et al*., 1988
Dapnhia magna	Static	Nominal	Other (Bringmann and Kühn) see text above	24-h	EC_0 = 10 EC_{50} = 32 EC_{100} = 39	NA, additional information	Bringmann, and Kühn, 1977
Daphnia magna	static	-	EPA-660/3-75-009 (1975)	48-h	EC_{50} = 35.0	V	Pearson *et al*., 1979
Daphnia magna	static	Nominal	EPA Guideline(1975)	48-h	LC_{50} 33.6-43.8	V	Liu *et al*., 1983
Daphnia magna	static	Nominal	Other	48-h	LC_{50} 26.2 (22.5-30.5)	Additional information	Randall and Knopp, 1980 (in AQUIRE database)
Daphnia magna	Static		Other exposure of first instars	48-h	LC_{50} = 47.7 (29.5-99.7)	NA Additional information	Liu *et al*. 1983b
Scud (*H. azteca*)	static	Nominal	EPA Guideline(1975)	48-h	LC_{50} > 83.2	V	Liu *et al*., 1983
Midge (*T. dissimilis*)	static	Nominal	EPA Guideline(1975)	48-h	LC_{50} 19.8-25.5	V	Liu *et al*., 1983
Worm (*L. variegates*)	static	Nominal	EPA Guideline(1975)	48-h	LC_{50} > 83.2	V	Liu *et al*., 1983
Daphnia magna	Flow-through	Measured	EPA Guideline (1975)	24-h 48-h 96-h 12-d	LC_{50} 31.2 LC_{50} 30.4 LC_{50} 23.9 LC_{50} 3.0-5.3	V	Liu *et al*., 1983
Aquatic worm (*L. variegaus*)	Flow-through	Measured	EPA Guideline(1975)	24-h 48-h 96-h 14-d	LC_{50} 99.5 LC_{50} 80.9 LC_{50} 47.2 LC_{50} 25.8-35.8	V	Liu *et al*., 1983
Long-term results							
Daphnia magna	-		OECD Guideline nº202	21-d	EC_{50} repro *c.a.* 0.5	V	Bayer, 1986
Dapnhia magna	Semi-static	Nominal	Other	21-d	reproduction NOEC = 0.02	V	Künh *et al*., 1988
Daphnia magna	-	Measured	Other	28-d	NOEC reproduction 0.19	VWR	Bailey *et al*., 1984

SPECIES	TEST TYPE	Measured/ Nominal	TEST Guideline	DURATION	TOXICITY ENDPOINT (mg/l)	COMMENTS	REFERENCE
					LOEC reproduction 0.4		

V: Valid; VWR: Valid With Restrictions; NA: Not Assignable; I: Invalid

3.2.1.1.3 Algae

In this section the toxicity tests results on algae are presented. Comments have been focused on those ones considered for the assessment. Provided information has been summarized in Table 3.27. When clearly stated, indication of the growth phase of the algae culture has also been included.

Acute toxicity

Künh and Pattard (1990) used a modified procedure described elsewhere by Bringmann and Kühn for testing water pollutants with regard to their harmful effect on the cell multiplication of *Scenedesmus subspicatus*, further developed by the Standards Committee on Water with the German Institute of Standardization in DIN 38412, Part 9 (1988). Modifications are related to the use of 250 ml bottles with ground glass stoppers and with and overall reduction of the test time of 48 h. Range of concentrations tested were from 0.80 to 100 mg/l of 2,4-dinitrotoluene. The inoculated flasks were placed on a white surface protected from daylight and exposed to constant lighting from two parallel fluorescent, at 24 ± 1 ºC and relative humidity of 50%. To maintain the test strain, fresh stock cultures were prepared at 10-day intervals. Cultures were in a process of logarithmic growth after 72 hours. The method selected to determine the biomass was measurement of optical density (measurement of turbidity), filter 578 nm. Cell multiplication inhibition test shows an E_bC_{10} and E_bC_{50} of 1.3 and 3 mg/l, respectively and growth rate based effect values of E_rC_{10} = 1.9 mg/l and E_rC_{50} = 6.3 mg/l.

In another report, Dodard *et al*. (1999), studied the chronic phytotoxic effects of 2,4-dinitrotoluene and their metabolites on the freshwater green algae *Selenastrum capricornutum* by using the 96-well microplate method, which is detailed elsewhere. Algal cells were used during their logarithmic growth phase. Briefly, algae were exposed to different dilutions of the test sample and the measurements on algal growth were taken after 96 ± 2 hours. The assays were performed at 25 ± 1 ºC under continuous light conditions (4000 ± 400 lux). The number of algal cells counted in the test sample at the end of the test was compared to that of the control (water with 0.25% DMSO vehicle) from the determination of algal growth. EC_{20} = 1.6 mg/l and EC_{50} = 2.6 mg/l parameters were determined, ranging the 95% confidence interval from 1.16-2.18 and 1.82-3.46, respectively.

Liu *et al*., 1983 carried out a 14-days static algal test following the EPA Guideline (1971) ("*Algal Assay Procedures: Bottle Test*") on four species of algae (*S. capricornutum, Anabaena flos-aquae, Microcystis aeruginosa* and *Navicula pelliculosa). S. capricornutum* showed significant inhibition of grown at all the tested concentrations (ranging from 0.9 to 94.4 mg-/l) after four days of exposure. The degree on inhibition increased from 37.4 to 98.1% at the consecutive concentrations of 0.9 and 4.7 mg/l, respectively. *A. flos-aquae* showed significant growth inhibition (from 23% to 98% at consecutive concentrations) at all tested concentrations (ranging form 0.9 to 99.4 mg/l). With this alga, cell counting was performed only on day 14; *N. pelliculosa* goes from about 5% growth stimulation at 4.9 mg/l to an inhibition growth of 91 % at the next concentration (9.8 mg/l). With this alga, cells counting were performed only after 14 days exposure period. In the test with *M. aeruginosa* all concentrations ranging from 0.5 to 10.0 mg/l caused statistically significant growth inhibition on days 4 and 14. The degree of inhibition increased with the concentration of 2,4-dinitrotoluene in the nutrient medium and ranged from 4.2 to 99.5% on day 14. For this alga IC_{50}s of 0.49 and 0.6 can be estimated after 4 and 14 days exposure periods, respectively. So the IC_{50}s of 0.49 mg/l estimated after 4 and 14 days on *Mycrocistic aeruginosa* are certainly below 1 mg/l. However taking into account the facts that,

(i) this is the only validated value below 1 mg/l over the 20 compiled EC_{50}s covering several taxonomic groups and therefore, representing a much larger data set than usually available for classification and (ii) there is another study on the same species, *Microcystis aeruginosa*, reporting a toxicity threshold for 1% effect at 0.13 mg/l, on the basis of the weight of evidence, which indicates that the acute toxicity of this chemical is within the 1-10 mg/l range for algae (several taxa); the 1-100 mg/l range for fish, and the 10-100 mg/l range for aquatic invertebrates, the substance can keep the current environmental classification of N; R51/R53.

The following additional information may be found in the AQUIRE database:

- A 96h-EC_{50} (growth) of 3.8 mg/l (ranging from 3.5 to 4.1 mg/l) on *Chlorella pyrenoidosa* (green algae), has been provided under the following static test conditions: temperature = 23 °C and hardness = 54.3 mg $CaCO_3$/l. Nominal results were obtained under a log phase with 10,000 cells per ml (Adema *et al*., 1981, in AQUIRE database). This will be considered as additional information since no validation of the test was possible.

- A 96h-EC_{50} (growth) of 6.2 mg/l (ranging from 5.8 to 6.8 mg/l) on *Stephanodiscus hantzschii* (diatom), has been provided under the following static test conditions: temperature= 17 °C and a pH ranging from 7.8to 7.9. Nominal results were obtained under a log phase with 40,000 cells per ml (Hanstveit *et al*., 1985, in AQUIRE database (2004)). This will be considered as additional information since no validation of the test was possible.

- A 96h-EC_{50} (growth) of 1.9 mg/l (ranging from 1.8 to 2.1 mg/l) on *Gomphonema parvulum* (diatom), has been provided under the following static test conditions: temperature = 23 °C and a pH ranging from 7.8 to 7.9. Nominal results were obtained under a log phase with 10,000 cells per ml, (Hanstveit *et al*., 1985, in AQUIRE database (2004)). This will be considered as additional information since no validation of the test was possible.

- A 96h-EC_{50} (growth) of 0.08 mg/l (ranging from 0.07 to 0.1 mg/l) on *Anacystis aeruginosa* (blue-green algae), has been provided under the following static test conditions: temperature = 23 °C and a pH = 7.8. Nominal concentrations were obtained under a log phase of 30,000 cells per ml (Adema *et al* 1981 in AQUIRE-2004). This will be considered as additional information since no validation of the test was possible.

- A 96h-EC_{50} (growth) of 0.44 mg/l (ranging from 0.36 to 0.53 mg/l) on *Oscillatoria agardhii* (cyanobacteria), has been provided under the following static test conditions: temperature = 23 °C and pH ranging from 7.8 to 7.9). Nominal concentrations were obtained under a log phase of 500,000 cells per ml (Hanstveit *et al*., 1985, in AQUIRE-2004). This will be considered as additional information since no validation of the test was possible.

Long-term toxicity

Bringmann (1975) determined that the Lowest Observed Effect Concentration (toxicity threshold) for the cell growth inhibition test on cyanobacterium/blue algae, *Mycrocistis aeruginosa,* after an exposure period of 8 days was 0.13 mg/l. As the toxicity threshold is defined by the authors as 1% effect this parameter is directly assumed as NOEC. Cultures were shaked daily and final turbidity was measured and compared to controls, using a Hg-lamp wavelength of 578 nm. Measure chamber 10 mm thickness.

Bringmann and Kühn (1977b), conducted a study on *Scenedesmus quadricauda*, after an exposure time of 8 days, the growth rate was determined by measuring the turbidity using a Hg-lamp wavelength of 578 nm. A toxicity threshold of 2.7 mg/l was determined.

Table 3.27: Toxicity of 2,4-dinitrotoluene to algae

SPECIES	TEST TYPE	Measured/ Nominal	TEST Guideline	DURATION	TOXICITY END POINT (mg/l)	COMMENTS	REFERENCE
Scenedesmus subspicatus	-	Nominal	Other: modified DIN 38412 biomass	48-h	E_bC_{10} = 1.3 E_rC_{10} = 1.9 E_bC_{50} = 3 E_rC_{50} = 6.3	V	Künh and Pattard, 1990
S. capricornutum	Static	-	Other: microplate method Growth rate inhibition	96-h	EC_{20} = 1.6 (1.16-2.18) EC_{50} = 2.6 (1.82-3.46)	V	Dodard *et al*., 1999
S. capricornutum	Static	Nominal	EPA (1971) Growth rate inhibition	4-d	37.4% inhibition at 0.9	additional information*	Liu *et al*., 1983
C. pyreinoidosa	Static	Nominal	Growth rate inhibition	4-d	EC_{50} = 3.8 (3.5-4.1)	additional information*	Adema *et al*., 1981 (in AQUIRE database-2004)
S. hantzschii	Static	Nominal	Growth rate inhibition	4-d	EC_{50} = 6.2 (5.8-6.8)	additional information*	Hanstveit *et al*., 1985 (in AQUIRE database-2004)
G. parvulum	Static	Nominal	Growth rate inhibition	4-d	EC_{50} = 1.9 (1.8-2.1)	additional information*	Hanstveit *et al*., 1985 (in AQUIRE database-2004)
O. agardhii	Static	Nominal	Growth rate inhibition	4-d	EC_{50} = 0.44 mg/l	additional information*	Hanstveit *et al*., 1985 (in AQUIRE database-2004)
A. aeruginosa	Static	Nominal	Growth rate inhibition	4-d	EC_{50} = 0.08 (0.07-0.1)	additional information*	Adema *et al*., 1981 (in AQUIRE database-2004)
M. aeruginosa	Static	Nominal	EPA (1971) Growth rate inhibition	4-d 14-d	IC_{50} 0.49 IC_{50} 0.6	V	Liu *et al*., 1983
A. flos-aquae	Static	Nominal	EPA (1971) Growth rate inhibition	14-d	23% inhibition at 0.9	V, additional information	Liu *et al*., 1983
N. pelliculosa	Static	Nominal	EPA (1971) Growth rate inhibition	14-d	91% inhibition at 9.8	V, additional information	Liu *et al*., 1983

SPECIES	TEST TYPE	Measured/ Nominal	TEST Guideline	DURATION	TOXICITY END POINT (mg/l)	COMMENTS	REFERENCE
Long-term results							
Blue-green algae *M. aeruginosa*	-	Nominal	Other: DEV L9 modified	8 d	TT (NOEC)= 0.13	V	Bringmann, 1975
S. quadricauda	-	-	Other	8 d	TT = 2.7	V	Bringmann and Kühn (1977b)

V: Valid; VWR: Valid With Restrictions; NA: Not Assignable; I: Invalid.

* Test not validated

3.2.1.1.4 Microorganisms

Bringmann and Kühn (1977b), used an analogous method of the cell growth inhibition test, the toxicity threshold for 2,4-dinitrotoluene was determined for bacteria (*Peseudomonas putida*), resulting a Toxicity Threshold concentration of 57 mg/l after 16 h exposure period at 25ºC. Cultures turbidity was measured and compared to controls, using a Hg-lamp wavelength of 436 nm. Measure chamber 10 mm thickness.

Dodard *et al*. (1999) determined the effect on the bacteria *Vibrio fisheri*, using the 15 min-Microtox test. IC_{20} and IC_{50} parameters were determined, ranging the 95% confidence interval from 4.0-12.18 and 36.0-73.14, respectively. These data have been included as additional information but considered not assignable for the assessment.

Drzyzga *et al*., (1995), subjected aqueous samples of 2,4-dinitrotoluene to the luminescent bacterium *Vibrio fischeri* NRRLB-11177, to determine its ecotoxicity potential. The marine luminescent bacteria strain (formally *Photobacterium phosphoreum*; DSM 7151) was used for the luminescence inhibition test system. In order to ensure optimal salt conditions for the bacteria, the samples must have added 2% NaCl in solid form. The LUMIStoxTM was used to determine the ecotoxicological potential of the substance after exposure periods of 30, 60 and 90 minutes giving EC_{50}s of 45.07 mg/l, 41.34 mg/l and 37.50 mg/l, respectively. These data have been included as additional information and not assignable for the assessment.

Bringmann and Kühn, (1981) gave the following Toxic Threshold Concentrations (EC5) measuring the cell growth inhibition rate of various protozoa by using analogous procedures of the cell multiplication inhibition test. The following EC5s are presented: 0.98 mg/l on *Entosiphon sulcatum* (bacteriovorous flagellate protozoa, consuming flagellate) after 72h exposure; 0.55 mg/l on *Uronema parduczi holozoic* (bacteriovorous ciliate protozoa, consuming ciliate) after 20h exposure and 13 mg/l on *Chilomonas paramecium* (saprozoic flagellate protozoa) after 48h exposure. Exposure times have been extracted from the IUCLID database.

The following additional information can be found in the Aquire database:

- A 96h-EC_{50} (growth) of 9.6 mg/l (ranging from 8.8 to 11 mg/l) on *Euglena gracilis* (flagellate), has been provided under the following static test conditions: temperature = 23 °C and hardness = 54.3 mg $CaCO_3$/l. Nominal concentrations were obtained under a log phase of 10,000 cells per ml (Adema *et al*., 1981, in AQUIRE database-2004). This will be considered as additional information since no validation of the test was possible.

Table 3.28: Toxicity of 2,4-dinitrotoluene to microorganisms

SPECIES	TEST TYPE	Measured/ Nominal	TEST	DURATION (Hours)	TOXICITY END POINT (mg/l)	COMMENTS	REFERENCE
Bacteria (*P. putida*)	Aquatic	Nominal	Bringmann and Kühn Inhibition of cell reproduction	16 h	TT = 57	V	Bringmann and Kühn (1977b)
Marine bacteria (*V. fischeri*)	Aquatic	-	Microtox	15 min	IC_{20}= 4.0-12.18 IC_{50} = 36.0-73.1	NA Additional information	Dodard *et al*., 1999
Marine bacteria (*V. fischeri*)	Aquatic	-	LumixTox, Bioluminescence inhibition test	0.5 1 1.5	EC_{50} = 45.07 EC_{50} = 41.34 EC_{50} = 37.50	NA Additional information	Drzyzga, *et al*., 1995
Additional species							
Protozoa (*U. parduzci*)	Aquatic	Nominal	Bringmann and Kühn Inhibition of cell reproduction	20 h	TT = 0.55	V	Bringmann and Kühn, 1981
Protozoa (*Chilomonas paramecium*)	Aquatic	Nominal	Bringmann and Kühn Inhibition of cell reproduction	48 h	TT = 13	V	Bringmann and Kühn, 1981
Protozoa (*Entosiphon sulcatum*)	Aquatic	Nominal	Bringmann and Kühn Inhibition of cell reproduction	72 h	TT = 0.98	V	Bringmann and Kühn, 1981
(Euglena gracilis)	Aquatic	Nominal	-	96-h	EC_{50} = 9.6 (8.8-11)	V, Additional information	Adema *et al*., 1981 (in Aquire database-2004)

V: Valid; VWR: Valid With Restrictions; NA: Not Assignable.

3.2.1.1.5 Amphibians

No data on amphibians have been provided.

3.2.1.2 Calculation of Predicted No Effect Concentration (PNEC)

Regarding the aquatic vertebrates there are available acute and long-term data. Lowest data validated for the assessment is a 90d-LOEC for fry growth of *Oncorhynchus mykiss* minor than 0.05 mg/l, which derivates a NOEC < 0.025 mg/l for fry growth.

Regarding aquatic invertebrates a 21d-NOEC for reproduction of *Daphnia magna* of 0.02 mg/l has been selected for the assessment.

In the case of algae, a 8d-Toxicity threshold of 0.13 mg/l on *M. aeruginosa* has been validated. This value is considered a NOEC but although it was divided by a factor of 2, to extrapolate the LOEC to a NOEC equal to 0.065 mg/l, it would not be either the most sensitive group. This value is in the same order of magnitude as the rest of the other aquatic groups.

There are long-term information on aquatic vertebrates, invertebrate and algae. The 21d-NOEC for reproduction on *Daphnia magna*, has been selected for the PNEC derivation. This value, which is more reliable than the 90d-LOEC for fry grown of *O. mykiss*, is quite similar to the concentration validated for the aquatic vertebrates. Therefore, according to the TGD, an assessment factor of 10 is applied:

$$PNEC_{aquatic\ organisms} = \text{lowest end chronic toxicity range} / 10 = 0.02 / 10 = 2\ \mu g/l$$

$$\mathbf{PNEC_{aquatic\ organisms} = 2\ \mu g/l}$$

Marine Compartment

For the estimation of the PNEC for the marine compartment following points have been taken into account:

- Chronic toxicity data for fresh water compartment are similar when comparing fish to aquatic invertebrates,
- Regarding aquatic invertebrates acute toxicity data are similar among different species, and,
- Toxicity information on marine fish is included in the same range of acute and chronic toxicity for fresh water fish.

In rapporteur's opinion, the sound PNEC for the seawater organisms can not be derived appropriately with the available information. However, following the agreement adopted by the TC NES II 2004, the PNEC for the marine environment has been derived according to the Technical Guidance Document, by applying an assessment factor of 100 to the lowest chronic toxicity data for fresh water

$$PNEC_{sea\_water} < \text{lowest end chronic toxicity range} / 100 = 0.02 / 100 = 0.2\ \mu g/l$$

$$\mathbf{PNEC_{sea\_water} = 0.2\ \mu g/l}$$

Sewage Treatment Plants

A datum on *U. parduczi* has been used for calculations. The toxicity threshold of 0.55 mg/l is an EC_5. This effect percentile is within the range of variation of controls, and so it has been considered as a NOEC. According to TGD an assessment factor of 1 is applied:

$$PNEC_{microorganisms} = \text{cell reproduction} / 1 = 0.55 / 1 = 0.55\ mg/l$$

$$\mathbf{PNEC_{microorganisms} = 0.55\ mg/l}$$

3.2.1.3 Toxicity test results for sediment organisms

No data have been provided regarding toxicity on freshwater nor marine sediment organisms.

3.2.1.4 Calculation of Predicted No Effect Concentration (PNEC) for sediment organisms

Taken into account the lack of data, and according to the Technical Guidance Document, the equilibrium partitioning method can be applied as a conservative calculation method to identify a potential risk to the soil compartment. Thus, the $PNEC_{sed}$ has been calculated using the equilibrium partitioning method with the PNEC for aquatic organisms.

$$\mathbf{PNEC_{sed}} = \frac{K_{susp\text{-}water}}{RHO_{susp}} \times PNEC_{water} \times 1000$$

where

$K_{susp\text{-}water}$ = suspended sediment-water coefficient = 3.87 m^3/m^3 for 2,4-dinitrotoluene
RHO_{susp} = bulk density of suspended sediment = 1150 kg/m^3.

$$PNEC_{sed} = 3.87\ m^3/m^3 \times 2\ \mu g/l \times 1000 / 1150\ kg/m^3 = 6.73\ \mu g/kg$$

$$\mathbf{PNEC_{sed} = 6.73\ \mu g/kg\ ww}$$

Following the same approach the PNEC for the marine sediment using the equilibrium partitioning method:

$$\mathbf{PNEC_{marine\_sed}} = \frac{K_{susp\text{-}water}}{RHO_{susp}} \times PNEC_{saltwater} \times 1000$$

$$\mathbf{PNEC_{marine\_sed} = 0.673\ \mu g/kg\ ww}$$

3.2.2 Terrestrial compartment

The provided information includes a set of data on the toxicity on 2,4-dinitrotoluene on terrestrial compartment, including plants, soil invertebrates and soil microorganisms.

3.2.2.1 Toxicity test results

3.2.2.1.1 Plants

In this section they are presented the results of the toxicity tests on plants. Comments on tests have been focused especially on those ones considered for the assessment. Provided information has been summarized in Table 3.29.

Acute toxicity

The toxicity of 2,4-dinitrotoluene was determined by Adema and Henzen (1989) on three types of plants, according to OECD Guideline 208 procedures. Three different species of test plants (tomato, *Lycopersicum esculentum*; oats, *Avena sativa*; and lettuce, *Lactuca sativa*) were cultured in two types of soil: humic sand (organic matter 3.7%, pH 5.1, silt 5.8%, sand 90.5%) and loam soil (organic matter 1.4%, pH 7.5, silt 17.1%, sand 71.7%). In the terrestrial tests the chemicals to be tested are usually added directly to a soil of a certain prescribed type. In the case of the OECD Guideline 208, this is loam-type soil. Soil tests were carried out according to OECD Guideline 208 (1984), the water content of the soils being 80% of their maximum water holding capacity. Test concentrations were spaced by a factor of 3.2 and expressed as milligrams per kilogram of dry soil. Five pots with 10 seeds inside were placed. The pots were covered with glass plates until germination of the plants. The plants were grown in a culture room at 25 ± 2ºC, 65% relative humidity, and 6500 lx from fluorescent tubes. Evaporation of water was accurately compensated daily by addition of distilled water; in this way, pots without holes could be used and cross-contamination or contamination of the surrounding would be avoided. Two weeks after germination the plants were cut off just above the soil and the wet weight was determined. Both tests, performed with different types of soils are considered as valid, as long as general requirements in relation to controls and OECD conditions were fulfilled.

Results presented by Bayer AG (1990) were conducted on *Brassica rapa* and *Avena sativa* with an exposure period of 14 days, according to a proposed guideline of BBA (1984) "*Phytotoxicity test on amonocotiledoneous plant*". It provided an EC_{50} of 65 and 6.5 mg/kg on *Avena sativa* and *Brassica rapa*, respectively, after a two weeks exposure period, regarding growth reduction. These values are included as additional information since the original report could not be validated.

On a second approach, the toxicity of 2,4-dinitrotoluene was determined by Adema and Henzen (1989) on three types of plants on a soil-less culture (hydroponic test). Three different species of test plants (tomato, *Lycopersicum esculentum*; oats, *Avena sativa*; and lettuce, *Lactuca sativa*) were tested on a complete aqueous nutrient solution according to Steiner (1968) (information not included). Test with soil-less culture (hydroponic tests) were carried out in 10-L aquaria. The water was aerated and recirculated. Plants were shown as precultures in perlite soaked with the test solution (nutrient solution with the test chemical concentration) to be tested in the aquaria. Two to three days after germination the young plants were taken from the pots with perlite and placed with their roots in the test solution in the aquaria. Test

concentrations were spaced by a factor of 3.2 and expressed in milligrams per litre of nutrient solution. One aquarium, with 20 plants, was used for each test concentration. The test conditions and measurements were as far as possible analogous to those in the soil tests following the OECD 208 Guideline; in addition, the oxygen content of the test solutions was monitored during the test. The concentration of 2,4-dinitrotoluene in nutrient solutions at the end of the test was about the same as those at the start. Two weeks after exposure the plants were cut off just above the soil and the wet weight was determined. These results are summarized in Table 3.29.

Long-term toxicity

No data on long-term toxicity on plants has been provided.

Table 3.29: Toxicity of 2,4-dinitrotoluene to plants

SPECIES	TEST TYPE	Measured/ Nominal	COMMENTS	DURATION (Days)	TOXICITY END POINT (mg/kg dry weight)	COMMENTS	REFERENCE
Avena sativa	Loam soil	Nominal	OECD 208 guideline	14	EC_{50}growth 46	V	Adema and Henzen, 1989
	Humic sand		Other: following OECD procedure		EC_{50} 35	V	
Avena sativa	-	-	Other: BBA (1984)	14	EC_{50} 65	VWR; Additional information	Bayer AG, 1990 (IUCLID dataset)
Lactuca sativa	Loam soil	Nominal	OECD 208 guideline	14	EC_{50} 5.8	V	Adema and Henzen, 1989
	Humic sand		Other: following OECD procedure		EC_{50} 13	V	
Licopersicum esculentum	Loam soil	Nominal	OECD 208 guideline	14	EC_{50} 4.9	V	Adema and Henzen, 1989
	Humic sand		Other: following OECD procedure		EC_{50} 10	V	
Brassica rapa	-	-	Other: BBA(1984)	14	EC_{50}growth red 6.5	VWR; Additional information	Bayer AG, 1990 (IUCLID dataset)
Avena sativa	Hydroponic	Nominal	Aquatic test	14	EC_{50} 5.3 (*)	NA	Adema and Henzen, 1989
Lactuca sativa	Hydroponic	Nominal	Aquatic test	14	EC_{50} 2.1 (*)	NA	Adema and Henzen, 1989
Licopersicum esculentum	Hydroponic	Nominal	Aquatic test	14	EC_{50} 2.1 (*)	NA	Adema and Henzen, 1989

V: Valid; VWR: Valid With Restrictions; NA: Not Assignable; (*) mg/l

3.2.2.1.2 Earthworm

Acute toxicity

Heimbach (1990) carried out the testing of a mixture of dinitrotoluenes (80% of 2,4-dinitrotoluene and 20% of 2,6-dinitrotoluene) on *Eisenia foetida*. The assay was performed according to the OECD Guideline nº 207 following GLP-regulations. Earthworms were exposed to different concentrations of dinitrotoluenes mixture in an artificial soil consisting of sand, clay mineral and peat. Test substrate consists of 69% fine quartz (84% o f the sand had a particle size of 0.06 – 0.2 mm), 10% dried, finely ground peat (pH 2 – 4), 20% kaolin to adjust the pH value to 6±0.5. Test conditions were 20±1ºC; 70-90% relative humidity, and constant light (400-800 lux). The animals were not fed during the test. After 14 days, the number of surviving animals and their weight alteration during the test period was determined. Mortality rate in the control was below 10%, which was regarded as for natural mortality. LC_{50} (14 days) determined for the mixture of dinitrotoluene was 668 mg/kg dry weight. Regarding to weight alterations and symptoms the no-observed-effect-concentration (NOEC) was 316 mg/kg dry weight substrate, the lowest-observed-effect-concentration (LOEC) 562 mg/kg. The lowest tested concentration with mortality was 562 mg/kg dry weight substrate. Data included in table has been corrected for the 80% of 2,4-dinitrotoluene composition.

Results are summarized in Table 3.30:

Table 3.30: Toxicity of 2,4-dinitrotoluene to earthworms

SPECIES	TEST TYPE	Measured/ Nominal	COMMENTS	DURATION (Days)	TOXICITY END POINT (mg/kg dw)	COMMENTS	REFERENCE
Earthworm *(E. foetida)*	Artificial soil	Nominal	OECD 207	14	LC_{50} 497-584 LLC 449 LOEC 449 NOEC 253	V	Heimbach (1990)

LLC: Lowest Letal Concentration; V: Valid

Long-term toxicity

No data on long-term toxicity on earthworms has been provided.

3.2.2.1.3 Microorganisms

No information has been provided on nitrification or carbon mineralization regarding soil microorganisms.

In the OECD (1997) report, downloaded from Internet, it is indicated that significant inhibition of the dehydrogenase activity of natural soil microflora were determined with concentrations of 5 mg/kg dw and 50 mg/kg dw, even after 28 days incubation. This non-

validable information has been considered as not assignable for the assessment and included as additional information.

Table 3.31: Toxicity of 2,4-dinitrotoluene to microorganisms

SPECIES	TEST TYPE	Measured/ Nominal	COMMENTS	DURATION (Days)	TOXICITY END POINT (mg/kg dw)	COMMENTS	REFERENCE
Natural soil microflora	-	-	-	28	Significant inhibition of dehydrogenase activity 5-50	NA Additional information	OECD, 1997

NA: Not Assignable.

3.2.2.1.4 Other terrestrial organisms

In the OECD (1997) there are included some acute and long term tests on the collembola *Folsomia candida*. Data are summarized in Table 3.32. This non-validable information has been included in the assessment as additional information but has not been considered for estimations.

Acute toxicity

An LC_{50} of 42.8 mg/kg dw has been cited in the OECD report (1997) on the collembola (*Folsomia candida*) after an exposure period of 24 hours.

Long-term toxicity

In the same report, OECD (1997), two long-term data on the collembola (*Folsomia candida*) were validated. The first of them indicated an EC_{10} of 3.2 mg/kg dw on the reproduction rate, after 34 days exposure period. In the second one, an EC_{10}, regarding mortality of parental organisms, was cited as 2.8 mg/kg dw, after an exposure period of 34 days. Furthermore, in the same report, with carabidae (*Poecilius cupreus*) significant effects on the feeding rate were determined at a soil concentration of 50 mg/kg dw and with lycosidae (*Pardosa sp.*) significant effects on the feeding rate were determined after 14 days at a soil concentration of 5 mg/kg dw. These data have been included as additional information, but have not been considered for the assessment since no validation was possible.

Table 3.32: Toxicity of 2,4-dinitrotoluene to other terrestrial organisms

SPECIES	TEST TYPE	Measured/ Nominal	COMMENTS	DURATION (Days)	TOXICITY END POINT (mg/kg dw)	COMMENTS	REFERENCE
Acute results							
Collembola (*Folsomia candida*)	-	-	-	1	LC_{50} 42.8	NA, additional information	OECD, 1997
Carabidae (*Poecilius cupreus*)	-	-	-	14	Feeding rate, significant inhibition 50	NA, additional information	OECD, 1997
Lycosidae (*Pardosa sp*.)	-	-	-	14	Feeding rate, significant inhibition 5	NA, additional information	OECD , 1997
Collembola (*Folsomia candida*)	-	-	-	34	LC_{10} 2.8	NA, additional information	OECD, 1997
Long-term results							
Collembola (*Folsomia candida*)	-	-	-	34	Reproduction rate EC_{10} 3.2	NA, additional information	OECD, 1997

LLC: Lowest Lethal Concentration; V: Valid; NA: Not Assignable.

3.2.2.2 Calculation of Predicted No Effect Concentration (PNEC)

Taking into account the existing information, it has been selected a 14d-EC_{50} of 4.9 mg/kg on the tomato *Lipersicum esculentum*. It has also been provided information on acute toxicity on earthworms.

Therefore, accounting the information provided, and according to the TGD an assessment factor of 1000 is applied:

$$PNEC_{soil\ organisms} = \text{lowest end acute toxicity range} / 1000 = 4.9 / 1000 = 4.9\ \mu g/kg$$

$$\mathbf{PNEC_{soil\ organisms} = 4.9\ \mu g/kg}$$

It has also been presented some information regarding hydroponic toxicity tests on plants, following the same approach, it has been selected a lowest EC_{50} of 2.1 mg/l, resulting a PNEC of 2.1µg/l that has been compared with the $PEC_{pore\ water}$. Following the same approach:

$$PNEC_{terrestrial\ plants\ pore\ water} = \text{lowest end acute toxicity range} / 1000 = 2.1 / 1000 = 2.1\ \mu g/l$$

$$\mathbf{PNEC_{terrestrial\ plants\ pore\ water} = 2.1\ \mu g/l}$$

Taken into account the lack of data, and according to the Technical Guidance Document, the equilibrium partitioning method can be applied as a conservative calculation method to identify a potential risk to the soil compartment. Thus, the PNEC has been calculated using the equilibrium partitioning method with the PNEC for aquatic organisms.

$$\mathbf{PNEC_{soil}} = \frac{K_{soil\text{-}water}}{RHO_{soil}} \times PNEC_{water} \times 1000$$

where:
$K_{soil\text{-}water}$ = soil-water partition coefficient = 3.76 m^3/m^3 for 2,4-dinitrotoluene
RHO_{soil} = bulk density of wet soil = 1700 kg/m^3

$$PNEC_{soil} = 3.76\ m^3/m^3 \times 2.5\ \mu g/l \times 1000 / 1700\ kg/m^3 = 5.52\ \mu g/kg$$

$$\mathbf{PNEC_{soil} = 5.52\ \mu g/kg}$$

3.2.3 Atmosphere

No information is available for 2,4-dinitrotoluene to plants and other organisms exposed via air. The very low vapour pressure of the substance means that volatilisation to the atmosphere is likely to be limited and the resulting concentrations are likely to be very low. This means that the possibility of 2,4-dinitrotoluene contributing to atmospheric effects such as global warming, ozone depletion and acid rain are likely to be very small.

3.2.4 Secondary poisoning

According to the low bioaccumulation potential and the rapid elimination of this compound in fish and mammals, no biomagnification potential in top predators is expected from this substance. The possibility of high exposure concentrations in invertebrates feeding on contaminated algae has been considered. Nevertheless, the elimination rate in invertebrates is still enough for assuming low risk of secondary poisoning.

3.3 RISK CHARACTERISATION [5]

3.3.1 Aquatic compartment (incl. STP and sediment)

Local, regional and continental PECs for aquatic and sediment compartments have been compared with the $PNEC_{aquatic}$ = 2 µg/l, derived from available information, and the $PNEC_{sediment}$ = 6.73 µg/kg ww, calculated using the equilibrium partitioning method according to the TGD. For the assessment of the Sewage Treatment Plants, a $PNEC_{microorganisms}$ of 550 µg/l, will be used. The $PNEC_{sea\_water}$ has been estimated as 0. 2 µg/l and the $PNEC_{marine\_seds}$ = 0.673 µg/kg ww. PEC/PNEC assessments are summarized in Table 3.33:

Table 3.33: Risk characterisation for aquatic compartment. Site-specific (sites A, B andD) local assessments for production and processing; assessment for formulation (site E); regional and continental assessments.

LOCAL COMPARTMENT	SITE	PEC	PEC/PNEC
Surface water (µg/l)	A	0.363	0.181
	B	5.28	**2.64**
	D	0.022	0.011
	E	0.142	0.071
Sea water (µg/l)	A	0.06	0.3
Sewage treatment plant (µg/l)	A	3.46	$6.29 \cdot 10^{-3}$
	B	-	**-**
	D	5.85	0.01
	E	28	0.05
Sediment (µg/kg ww)	A	1.22	0.18
	B	17.8	**2.64**
	D	0.08	0.011
	E	0.479	0.07
Marine sediment (µg/kg ww)	A	0.201	0.3

REGIONAL COMPARTMENT	PEC	PEC/PNEC
Surface water (total) (µg/l)	0.0178	0.0089
Surface water (dissolved) (µg/l)	0.0178	0.0089
Sediment (total) (µg/kg wet wt)	0.0542	0.008

[5] Conclusion (i) There is a need for further information and/or testing.
Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.
Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

CONTINENTAL COMPARTMENT	PEC	PEC/PNEC
Surface water (total) (µg/l)	$2.22 \cdot 10^{-4}$	$1.11 \cdot 10^{-4}$
Surface water (dissolved) (µg/l)	$2.22 \cdot 10^{-4}$	$1.11 \cdot 10^{-4}$
Sediment (total) (µg/kg wet wt)	$7 \cdot 10^{-4}$	$1.04 \cdot 10^{-4}$

Conclusions to the risk assessment for the aquatic compartment (including STP and sediment):

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Conclusion (ii) applies to the aquatic and sediment compartment at continental and regional level and for sites A, D and E.

Conclusion (ii) applies to the marine compartment.

Conclusion (ii) applies to the STP.

Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

Conclusion (iii) applies to the need of risk reduction measures for the aquatic compartment and for sediment-dwelling organisms at local level site B. It is expected that any risk reduction measure for surface water would also reduce the risks for sediments.

3.3.2 Terrestrial compartment

Local, regional and continental PECs for the terrestrial compartment have been compared with a $PNEC_{soil}$ of 4.9 µg/kg. The pore water concentration will be compared with the PNEC for terrestrial plants exposed from pore water obtained from the hydroponic tests (2.1 µg/l).

Table 3.34: Predicted levels in terrestrial compartment

LOCAL COMPARTMENT	SITE	PEC	PEC/PNEC
Agricultural soil (total) averaged over 30 days (µg/kg wet wt)	A	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$
	B	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$
	D	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$
	E	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$
Agricultural soil (total) averaged over 180 days (µg/kg wet wt)	A	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$
	B	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$
	D	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$
	E	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$

Grassland (total) averaged over 180 days (μg/kg wet wt)	A	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$
	B	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$
	D	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$
	E	$3.61 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$

Table 3.35: Risk characterisation for terrestrial plants exposed from pore water

LOCAL COMPARTMENT	SITE	PEC	PEC/PNEC
Pore water of agricultural soil	A	$1.63 \cdot 10^{-3}$	$7.76 \cdot 10^{-3}$
	B	$1.63 \cdot 10^{-3}$	$7.76 \cdot 10^{-3}$
	D	$1.63 \cdot 10^{-3}$	$7.76 \cdot 10^{-3}$
	E	$1.63 \cdot 10^{-3}$	$7.76 \cdot 10^{-3}$
Pore water of grassland	A	$1.63 \cdot 10^{-3}$	$7.76 \cdot 10^{-3}$
	B	$1.63 \cdot 10^{-3}$	$7.76 \cdot 10^{-3}$
	D	$1.63 \cdot 10^{-3}$	$7.76 \cdot 10^{-3}$
	E	$1.63 \cdot 10^{-3}$	$7.76 \cdot 10^{-3}$

REGIONAL COMPARTMENT	PEC	PEC/PNEC
Agricultural soil (total) (μg/kg wet wt)	$2.4 \cdot 10^{-3}$	$4.89 \cdot 10^{-4}$
Natural soil (total) (μg/kg wet wt)	$3.6 \cdot 10^{-3}$	$7.34 \cdot 10^{-4}$
Industrial soil (total) (μg/kg wet wt)	3.21	0.65
Pore water of agricultural soil	$1.09 \cdot 10^{-3}$	$5.2 \cdot 10^{-4}$

CONTINENTAL COMPARTMENT	PEC	PEC/PNEC
Agricultural soil (total) (μg/kg wet wt)	$6.65 \cdot 10^{-5}$	$1.36 \cdot 10^{-4}$
Natural soil (total) (μg/kg wet wt)	$1.2 \cdot 10^{-4}$	$2.45 \cdot 10^{-5}$
Industrial soil (total) (μg/kg wet wt)	0.024	0.005
Pore water of agricultural soil	$3.01 \cdot 10^{-5}$	$1.43 \cdot 10^{-5}$

Conclusions to the risk assessment for the terrestrial compartment:

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Conclusion (ii) applies to the terrestrial compartment.

3.3.3 Atmosphere

No effects on the atmosphere are likely in the regional and continental scenarios, because of the low predicted environmental concentrations of 2,4-dinitrotoluene.

Table 3.36: Predicted levels in air compartment

Scale		**PEC** (µg/m³)
Local	Site A	$6.16 \cdot 10^{-6}$
	Site B	$2.75 \cdot 10^{-6}$
	Site D	$1.26 \cdot 10^{-5}$
	Site E	$3.07 \cdot 10^{-6}$
Regional		$2.75 \cdot 10^{-6}$
Continental		$9.14 \cdot 10^{-8}$

Conclusions to the risk assessment for the atmosphere:

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

3.3.4 Secondary poisoning

According to the low bioaccumulation potential and the rapid elimination of this compound in fish and mammals, a low risk for secondary poisoning on birds and mammals is expected from this substance.

Conclusions to the risk assessment for secondary poisoning:

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already

4 HUMAN HEALTH

4.1 HUMAN HEALTH (TOXICITY)

4.1.1 Exposure assessment

4.1.1.1 General discussion

2,4-dinitrotoluene is an orange-yellow crystalline solid with a slight odour. It is slightly soluble in water, soluble in ether, benzene and acetone, and very soluble in chloroform and toluene. This substance has a very low vapour pressure ($7.9 \cdot 10^{-5}$ hPa at 20ºC). No quantitative information concerning the odour threshold is available.

2,4-dinitrotoluene is available commercially as a purified isomer or as a component of DNT mixtures. The commercial or technical grade is a mixture of approximately 76-78% 2,4-dinitrotoluene, 19% of 2,6-dinitrotoluene and 5% other isomers. The isomeric mixture is described as an oily liquid in the literature (Santodonato *et al.*, 1985; DHHS (NIOSH), 1985).

2,4-dinitrotoluene can affect the body if it is inhaled, swallowed or if it is readily absorbed through the skin.

Human exposure to this substance occurs primarily through occupational sources.

4.1.1.2 Occupational exposure

Occupational exposure to 2,4-dinitrotoluene may occur through inhalation and dermal contact with this compound at workplaces where it is produced or used. Oral exposure is assumed to be prevented by good hygiene practices.

The substance is produced and nearly exclusively used in closed systems as an intermediate for further synthesis.

Several sources (OECD-SIDS, 2004; DHHS (NIOSH), 1985; ATSDR, 1998) state that about 99% of 2,4-dinitrotoluene is used as a captive intermediate for obtaining TDA (toluene-2,4-diamine) and TDI (toluene diisocyanate) which, in turn is consumed in the production of flexible polyurethane foams, whereas 1% is used in other applications. For instance, 2,4-dinitrotoluene can be a captive intermediate in the manufacture of TNT or an additive in the production of explosives. It can be used as gelatinizing-plasticizing agent in explosive compositions or as a modifier for smokeless powders.

Uses as intermediates in the synthesis of azodyes have been described as former uses in the literature (HSDB, 2001).

All 2,4-dinitrotoluene manufactured in Europe is processed to TDA in closed installations. The chemical industry utilises technical grade dinitrotoluene, an isomeric mixture composed of approximately 76-78% 2,4-dinitrotoluene, 19% of 2,6-dinitrotoluene and 5% of other isomers. A certain amount of the pure product, consisting mainly of the 2,4-isomer, is imported by the explosives industry in order to use it as an additive in the production of explosives.

Occupational exposure to 2,4-dinitrotoluene is possible during its use as a chemical intermediate essentially in TDI production in closed installations. Most TDI producers use toluene as the starting material, generating 2,4-dinitrotoluene as a captive intermediate in the process. The only end-use of 2,4-dinitrotoluene not involving chemical conversion is its use as a minor component of gunpowder. Therefore, the following scenarios are considered:

Scenario 1: Production and further processing of 2,4–DNT.

Scenario 2: Explosives Manufacture.

Scenario 3: Use of explosives.

External exposure by inhalation and dermal routes is assessed in all scenarios. Some authors (Woollen *et al.*, 1985; Smith, Smith and Kuchar, 1995) have suggested that the skin may be the major route of absorption of dinitrotoluene due to its low vapour pressure.

Occupational exposure information including monitoring data has been obtained from industry. Relevant information from SIDS Initial Assessment on Dinitrotoluene (OECD-SIDS, 2004) has also been used in the assessment. Additional monitoring data has also been obtained from the literature and it has been included on the report.

Quality and recent measured data will be preferred to derive 90^{th} percentile values representing 8h TWA RWC or short term RWC. When the data set is small, values near the highest end of the concentration range will be used. Typical values are also derived.

EASE model (EASE for Windows Version 2.0) has been used to estimate both inhalation and dermal exposure. RISKOFDERM potential dermal exposure model (EXCEL version 2.0, October 2005) has also been used to assess dermal exposure in scenario 2 (Explosives Manufacture).

The assessment has initially been carried out without taking into account the exposure reducing effect of PPE. When PPE is used in accordance with Directive 89/656/EEC (this is, in fact, obligatory around the EU) exposure will be reduced considerably. If information were provided to know that PPE is used by a vast majority of workers and that the PPE is suitable for that situation, a second assessment considering the effect of PPE on reducing exposure will be done, following TGD.

The occupational exposure limits in different countries are summarised in the table 4.1.1.2. It should be noted that these limits refer mostly to the technical grade (mixture of isomers).

Table 4.1.1.2: Occupational Exposure Limits for 2,4-dinitrotoluene

Country	8h-TWA exposure limit		STEL-short term exposure limit		Skin notation	References
	ppm	mg/m^3	ppm	mg/m^3		
USA (ACGIH)	0.03	0.2 A3			YES	ACGIH (2001)
USA (NIOSH)		1.5* Carcinogen			YES	RTECS (2004)
Germany		Carcinogen cat: 2			YES	DFG (2004)
Spain		0.15 Carcinogen C2			YES	INSHT (2005)
Sweden		0.15 C		0.3	YES	Swedish Work Environment Authority, AFS 2000:3

*TWA limit for up to a 10-hour work shift in a 40-hour workweek.
Conversion factor: 1 ppm = 7.57 mg/m^3
1 mg/m^3= 0.13 ppm

4.1.1.2.1 Scenario 1: Production and further processing of 2,4 –dinitrotoluene.

2,4-dinitrotoluene can be produced by a two-step nitration of toluene in a closed system process producing a mixture of approximately 80% ratios of 2,4-dinitrotoluene and 20% of 2,6-dinitrotoluene.

A typical sulphuric acid/nitric acid nitrating mixture reacting with toluene yields 78 wt% 2,4-dinitrotoluene, 18 wt% 2,6-dinitrotoluene, 2.5 wt% 3,4-dinitrotoluene, 1 wt% 2,3-dinitrotoluene and 0.5 wt% 2,5-dinitrotoluene. If the single 2,4-isomer is required, the nitration can be stopped at the mono-stage and pure p-nitrotoluene is obtained by crystallization. Subsequent nitration of the p-nitrotoluene yields only 2,4-dinitrotoluene. 2,4-Dinitrotoluene is hydrogenated to yield TDA and this diamine is reacted with phosgene to yield TDI, which is used to make flexible polyurethane foams (Santodonato *et al.*, 1985).

All European manufacturers have reported production of 2,4-dinitrotoluene as an intermediate for further synthesis. They have also reported that the substance is totally used on site. Manufacturing and processing is similar in all production facilities. The production and further processing of 2,4-dinitrotoluene takes place in closed system. Exposure is therefore only expected in those activities where the system is breached.

2,4-dinitrotoluene is manufactured as a mixture of isomers, predominantly 2,4-dinitrotoluene and 2,6-dinitrotoluene, by nitration of toluene. The process is described as continuous at least by two companies. The product serves as a precursor material for the production of TDA (mixture of isomers) by subsequent catalytic hydrogenation. For the hydrogenation, the produced dinitrotoluene isomer mixture is directly fed into this step of the process, without provisions for outlets or major intermediate storage.

Potential exposure may only occur during activities such as collection of samples for quality control and maintenance. Process temperature has been reported by a company as 70-80ºC.

Sampling is performed with containers that are tightly coupled to the closed reaction system with the sampling arrangement being placed in an encapsulated housing. Sampler type of "dead end" ball valve has been reported by a manufacturer. By this the product stream cannot be opened. Another manufacturer has reported that the sampling device is located in a small box equipped with LEV and with a plexiglass sliding door on the front side.

Decontamination procedures before maintenance are established to prevent exposures.

In cases of repair, the parts of the plant containing product are drained and cleaned in several steps (acid, water, nitrogen) before repair is started.

PPE is reported to be used in these tasks. One company details PPE consisting of safety shoes, helmet, an approved overall, a full mask for eye and respiratory protection (filter also for nitrous gases), and specially selected suitable gloves (temperature resistant to the processing temperature of about 70 to 80ºC; baypren, viton or nitrile rubber gloves reported in the SDS). In cases with longer duration of the repair work, respiratory protection with a supply of fresh air is applied.

Another company has reported that the use of a full face mask with a special filter for organic vapours and suitable rubber gloves are required when the operator enters the sampling point. This is considered by this company as the only task where exposure could be possible. The third company has notified that workers are protected from dermal contact with dinitrotoluene with protecting clothes, glasses, facial screens and gloves. Gloves used in one of its plants (Flexiprof 40) are made with cotton coated by nitrile. In the other plant, gloves used (Mapa Neotex 340-9-91/2) are made with neoprene.

One company states that all persons involved in those tasks are well trained in the use of their personal protective equipment, including cleaning or disposing of possibly contaminated equipment.

Information about the suitability of different glove materials for 2,4-dinitrotoluene is also available from the literature (Fricker and Hardy, 1992; 1994). First nitrile and then neoprene are reported the best protection from materials tested exhibiting the longest breakthrough time values.

Information about the number of exposed workers has been obtained from Industry. European producers have reported a total of 118 workers in theirs plants. This number can be increased in cases of repair. Therefore, it can be estimated that the number of workers potentially exposed to technical grade DNT during its manufacture and use in the synthesis of TDA within Europe is about 150 workers.

Measured data

The analysis of dinitrotoluene is normally performed by gas chromatography with a variety of detectors. Sampling can be performed as described by OSHA (method 44) using a glass fiber filter contained within a Tenax-GC tube. Analysis is conducted by gas chromatography; GC/(TEA/EAP) (OSHA, 1983).

One company has reported the most recent measured data (2005) accompanied by important qualitative information. 2,4-dinitrotoluene is manufactured as a mixture of isomers, predominantly 2,4-dinitrotoluene and 2,6-dinitrotoluene, in a continuous process by nitration of

toluene. The plant is set up as a closed system, where only small amounts of dinitrotoluene can be taken out at certain sampling stations for quality control. The containers used to collect the samples are tightly coupled to the closed reaction system with the sampling arrangement being placed in an encapsulated housing. The amount of the sample is determined by the sampler (type of "dead end" ball valve). By this, the product stream cannot be opened. Most of the samples consist of dinitrotoluene isomer mixture, dissolved at low concentration in a mixture of nitric and sulphuric acid. There is one worker who collects the samples from the different sampling stations and takes them to the quality control laboratory. The preparation of the samples for the different analytical procedures like titrations or GC is done under laboratory conditions.

Measurements were done in the plant during the round tours of the persons checking control points and taking samples for quality control as well as in the laboratory, where the quality control analysis are performed. It is reported that each measurement covers one whole shift. Data are presented in the table below:

Table 4.1.1.2.1-1: Occupational exposure to 2,4-dinitrotoluene (technical grade) during production and further processing

Activity	Type of sampling	No. of samples	Data distribution (mg/m^3)
Round tours checking control points	Personal	8	(< 0.0008; < 0.0008; < 0.0009; < 0.0012; 0.0009; 0.0015; 0.0069; 0.01112)
Taking samples for quality control	Personal	2	< 0.0012-0.0012
Quality control analyses	Personal	2	< 0.0008-0.0018

Sampling and analysis were performed according to the method BGI 505-40 of the German Berufsgenossenschaft. A defined volume of air is drawn with a pump through a glass tube filled with silicagel. The DNT is then desorbed from the silicagel with methanol and measured by gas chromatography with FID using an external calibration standard.

Regarding all the provided information these data are considered of quality. Following TGD, non-detectable data points should be assessed, in a pragmatic approach, using a value of half of the detection limit. A 95th and a 50th percentiles of 0.009 mg/m^3 and 0.00075 mg/m^3 can then be derived from this dataset.

Data from 1999 provided by this company regarding former production sites currently closed were in the range of <0.005 – 0.01 mg/m^3 (18 samples were collected in different sites, the 95th percentile was lower than the detection limit). This range coincides with the measurement data range from its new (and only) production facility.

Long term occupational exposure to 2,4-dinitrotoluene during production and further processing has also been provided by another manufacturer (Industry, 2001) and are shown in the table 4.1.1.2.1-2.

Table 4.1.1.2.1-2: Long-term occupational exposure to 2,4-dinitrotoluene (Technical grade) during production and processing

Task	Type of samples	No. of samples	Data distribution (mg/m^3)
Analytical control laboratory	Stationary	6	(0.01; 0.01; 0.01; 0.01; 0.07; 0.08)
Nitration reactor building	Stationary	3	(0.01; 0.01; 0.09)

Samples have been analysed by gas chromatography. The detection limit is 0.001 mg/m^3. Data points have been obtained from static sampling. Therefore, this dataset is considered of lower quality than the precedent one.

The 95th percentile of 0.009 mg/m^3 derived from a quality dataset is considered as the RWC inhalation exposure value. Instead of 90th, the 95th percentile has been chosen due to the limited extension of the dataset. Typical exposure values can be in the order of 0.0007 mg/m^3 (the 50th percentile).

Modelled data

Production and further processing of 2,4-dinitrotoluene takes place in closed system. The substance is produced in Europe as a precursor material in the production on site of Toluene-2,4-diamine (TDA, isomer mixture). The produced 2,4-dinitrotoluene is fed as a chemical intermediate directly into the next step of the process. Exposure is only expected during activities such as sampling or maintenance that involves breaching the closed systems.

The handling procedures for dinitrotoluene are reported to be the same as for Toluene-2,4-diamine (TDA, isomer mixture), except for filling and shipping that is not done with dinitrotoluene. Therefore, the EU RAR for TDA will be specially considered in the assessment.

Specific information from these tasks has been obtained from industry:

- Sampling is reported by a company to be performed at different sampling stations. The containers used to collect the samples are tightly coupled to the closed reaction system with the sampling arrangement being placed in an encapsulated housing. The amount of the sample is determined by the sampler (type of "dead end" ball valve). By this the product stream cannot be opened. Most of the samples consist of dinitrotoluene isomer mixture, dissolved at low concentration in a mixture of nitric and sulphuric acid. The preparation of the samples for the different analytical procedures like titrations or GC is performed under laboratory conditions. Another manufacturer has reported that the sampling device is located in a small box equipped with LEV and with a plexiglass sliding door on the front side. Quality control is contracted out to an external laboratory.

For maintenance activities the parts of the plan containing product are drained and cleaned in several steps (acid, water, nitrogen) before repair work is started. Maintenance activities do not occur on a daily basis, the equipment or the lines are only occasionally breached if maintenance is necessary. A company has reported that all maintenance activities require prior approval by supervising plant personnel and an approved working instruction checklist

The EASE model (EASE for Windows Version 2.0) has been used to estimate both inhalation and dermal exposure.

This substance has a very low vapour pressure ($7.9 \cdot 10^{-6}$ kPa). For a substance with such a low vapour pressure EASE predicts that exposure will be in the range of 0-0.1 ppm regardless the pattern of use or pattern of control. Process temperature has reported to be 70º-80ºC. Accordingly, the estimated inhalation exposure (Process temperature: 80ºC; non dispersive use with LEV) will be 0-0.1 ppm (0–0.7 mg/m^3). However, these kinds of activities are not carried out along the whole shift. Assuming that they are performed for about one hour per day, an eight-hour TWA exposure range of (0–0.087 mg/m^3) can be calculated.

Because this compound has a very low vapour pressure, dermal exposure may contribute significantly to overall exposure. In order to evaluate dermal exposure to this substance it should be taken into account the high process temperature (70-80ºC). In addition, this substance cause persistent stains (brilliant yellow-orange) as it is stated in Hamill *et al.* (1982) and in Levine *et al.* (1985). Therefore, it is considered than dermal contacts occur only occasionally. Dermal exposure for theses activities is estimated by EASE considering non dispersive use and direct contact:

- Sampling: Taking into account the process temperature (70ºC) immediate dermal contacts should be avoided. Low levels of daily dermal exposure are expected. Potential contact with contaminated surfaces with small amount of cooled dinitrotoluene could be possible. Incidental contact is assumed. The estimated exposure range is 0-21 mg/day, considering an exposed surface of 210 cm^2.

  According to the information provided at the beginning of this section, it could be assumed that the use of suitable gloves is highly accepted by the involved companies. Following TGD a default value of 10% penetration for properly selected gloves is assigned. This leads to a dermal exposure range of 0-2.1 mg/day.

- Maintenance: In cases of repair, the parts of the plant containing product are drained and cleaned by steps (acid, water, nitrogen) before repair work is started. Workers exposure largely depends on the efficacy of decontamination procedures and on the proper use of PPE. According to TGD, the level of contaminant after purging and flushing is highly variable and unpredictable at present. Therefore, dermal exposure can only be qualitatively estimated. Industry has reported that all persons involved are well trained in the use of their personal protective equipment; including cleaning or disposing of possibly contaminated equipment. These activities do not occur on a daily basis. Following EU RAR for TDA they are typically performed not more than once per month leading to a lower dermal exposure level than what assessed for sampling, although perhaps for a larger surface area.

  Therefore, a RWC dermal exposure level of 2.1 mg/day can be assumed for workers exposed to 2,4-dinitrotoluene in this scenario.

Literature data

Levine *et al.* (1985) evaluated in 1983 exposure of workers to technical grade DNT and measured urinary metabolites of dinitrotoluene at a dinitrotoluene manufacturing plant constructed in 1973. They also tried to evaluate the importance of routes of exposure to dinitrotoluene other than the respiratory tract. Seven-hour personal air samples were taken from the breathing zone of several workers; urine was collected over 72 hours; and skin and environmental surfaces were wiped. Chemical analysis was performed using gas chromatography. The concentrations of dinitrotoluene in air ranged from 0.01 to 0.44 mg/m^3 (eleven samples taken). The highest personal air monitoring concentrations and levels of urinary metabolites were found to be for loaders, followed by process operators. Loaders are reported to oversee dinitrotoluene storage tanks, transfers dinitrotoluene product onto tank trucks and tank cars, obtains samples for laboratory analysis and cleans. Operators adjust valves, check gauges, collect samples, and perform simple analysis in the control room laboratory.

Wipe specimens were obtained from the skin of dinitrotoluene workers and from surfaces of objects in the work area that might contact the skin after conclusion of 72-hour monitoring

period. Areas suspected of dinitrotoluene contamination were wiped with a cotton gauze pad soaked in 97% isopropyl alcohol. Samples were analysed by gas chromatography using a flame ionization detector. The limits of reliable measurement extended from 2 μg to 2 mg per sample.

Eight samples were obtained by wiping of the skin (mainly hands and forehead) of different workers. Four samples are related with operators, one is related to a loader and the rest with a control room employee, a stripper operator and a lab analyst. Results showed levels of "not detected" (< 2 μg) to 179.5 μg. Values in the higher part of the range were found in one operator after collecting and analyzing a sample (179.5 μg) and a loader (122.3 μg). 50th percentile was in the order of 48 μg.

The value of 179.5 μg was measured in hands and face of an operator after collecting and analyzing a sample. Assuming a surface area of approximately 2000 cm^2, this value can be converted into 0.089 μg /cm^2/day.

25 samples were collected from different surfaces with values ranged from "not detected" to 433.2 μg (area unspecified). The higher value detected on an environmental surface (433.2 μg) was found on a sample valve handle.

The authors concluded that there is an excess of excreted urinary dinitrotoluene metabolites in operators and loaders with respect to dinitrotoluene in inspired air suggesting that routes other than respiratory may be important.

NIOSH (Ahrenholz, 1980) reported some measurements of dinitrotoluene that were collected during a Health Hazard Evaluation (HHE) conducted in a chemical plant which used dinitrotoluene (technical grade) in the production of TDA. It is reported that dinitrotoluene unloading procedures appear to be the only sources of exposure in the unit besides the fugitive emissions from pumps and clean-up operations. After an initial survey in 1979 where TWA breathing zone concentrations to dinitrotoluene were 0.013 and 0.023 mg/m^3 for a production operator and a tank unloader respectively, a follow-up survey was conducted in 1980 with five additional samples resulting on exposures ranged from not detected to 0.014 mg/m^3 (LOD: 2.8 μg per sample, NIOSH method No. S215). Reported environmental values in the follow-up survey reflect improved spill containment and increased maintenance of the system.

Worker exposure to DNT was also evaluated as part of another NIOSH Health Hazard Evaluation (Fannick, 1980). Full shift breathing zone concentrations of dinitrotoluene for a yardman (who receives the bulk chemical) and an unspecified production area worker were reported to be 0.042 mg/m^3 and 0.032 mg/m^3 respectively. Analysis was performed by gas chromatography. Dinitrotoluene was not detected in an area sample from the control room.

SIDS Initial Assessment on Dinitrotoluene (OECD-SIDS, 2004) mentions that blood levels of all dinitrotoluene isomers were measured in workers of former manufacturing and processing plants of a company as part of the health surveillance program. As result the concentration of dinitrotoluene isomers in all workers blood samples were below the corresponding detection limits.

Conclusions

For inhalation exposure, quality exposure data are available from European producers. These data are considered representative of the European industry.

EASE has been run to model inhalation exposure. However, it must be noted that this model has limitations estimating exposure to very low vapour pressure substances.

There are also some literature data involving inhalation exposure. These data should be regarded with caution since they are more than 25 years old and activities involved are not exactly the same as the activities carried out in current European manufacturing plants, where loading and shipping is not performed with dinitrotoluene.

Therefore, the value of 0.009 mg/m^3, derived from a quality dataset, is considered as the RWC inhalation exposure value. Typical values can be in the order of 0.0007 mg/m^3. Short-term measured data are not available. A RWC short-term value of twice as high as the full shift RWC value is assumed: up to 0.018 mg/m^3.

Dermal exposure has been estimated by EASE as 2.1 mg/day for workers that use suitable gloves as it is the case for this scenario. Scarce literature data are available. One single paper reports some dermal measurement data. In this paper, monitored tasks mostly refer to sampling activities. The obtained exposure range was much lower than the resulting modelled exposures. The higher exposure value obtained was 179.5µg/day. There are some gaps regarding the reported sampling methodology. Therefore, these data should be interpreted with caution.

Comparing both data, the value of 2.1 mg/day obtained by EASE seems an overestimation. However, due to the limited reliability of the literature data it is chosen as RWC for purposes of risk characterisation.

4.1.1.2.2 Scenario 2: Explosives Manufacture

2,4-dinitrotoluene can be used as an additive in the production of explosives due to its gelling, waterproofing, and plasticizing properties.

Industry has reported that explosives manufacturers import the whole amount of used substance.

Information has only been obtained from one European explosives manufacturer. According to this company, a certain amount of the pure product, mainly consisting of the 2,4-isomer, is imported in order to use it as a component of gunpowder. 2,4-dinitrotoluene is incorporated into powdered formulations. Its use as a minor component of gunpowder does not involve chemical conversion. Occupational exposure can occur during the preparations and packing of these explosives mixtures.

It is difficult to estimate the number of workers potentially exposed to this substance in an explosives manufacture scenario. It can be used for the production of explosives for military use which can be classified information. NIOSH had estimated that approximately 500 workers were potentially exposed to 2,4-dinitrotoluene in the production of ammunition and explosives in the USA (DHHS, NIOSH, 1985). One European company has reported that 6-10 workers can be exposed to this substance in the manufacture of gunpowder from a total of 60 workers in its plant. There is no information from other European companies. Therefore, no estimation about the number of exposed workers in Europe can be done.

Biological monitoring studies among workers in an explosives factory indicate that dermal contact may be the major route of 2,4-dinitrotoluene uptake (Woollen *et al*., 1985).

Explosives manufacturing process has been described by one European company.

The manufacturer imports the substance as a powdered crystalline solid which it is described as yellow needle shaped crystals. The substance is imported in quantities up to 30,200 kg in

2004, packed in 15 kg polyethylene bags, all inside a cardboard box. It is used as a minor component (less than 1%) in the production of single-base and double-base gunpowder formulation. The manufacturer has reported that these formulations are solid products in the form of granules without emission of vapours or dust whose function is acting as propellants. The final product is packed in polyethylene bags inside cardboard drums of 25 kg.

2,4-dinitrotoluene is mixed with the rest of the components of the explosive formulation in solid state at room temperature. During production of the formulation only small amounts of the substance are handled (8 kg are weighing with a frequency of six times per shift and this task is shared by two workers per shift). Workers exposure would mainly occur during tasks such as weighing and charging of the system in which the substance is used.

It is reported that these activities are carried out along six minutes per worker and day, five days per week, during 218 days a year. This company has also reported that an analyst can be exposed three times per year during sampling for quality control of the substance received. Similar processes are cited or described in the literature (Stayner *et al.*, 1993; Woollen *et al.*, 1985).

According to this company, PPE are used to prevent exposure. People handling the substance are required to wear respiratory protection, goggles and suitable gloves. Used gloves are made of nitrile rubber (SOL-VEX-Nitrile). It is reported that workers are regularly trained in the use of their personal protective equipment.

Measured data

Only one explosives manufacturer has reported undetectable levels of 2,4-dinitrotoluene. These measurements were done in the place where 2,4-dinitrotoluene is used (weighing and charging tasks). Limit of detection is reported to be <2µg/m^3. Analysis was conducted by gas chromatography GC/ECD. Information about sampling methods was not provided.

Modelled data

The potential for exposure is higher in activities such as weighing and charging of the system in which the substance is used. Information from one manufacturer reports that once the substance is incorporated to the formulation, less than 1% is present in the final product.

The EASE model has been used to estimate both inhalation and dermal exposure to dust. As stated before, the substance is described as yellow needle shaped crystals. Some authors have described DNT as not dusty material but oily crystalline solids at room temperatures (Hunt, Neubauer and Picone, 1980).

To estimate inhalation exposure, non dispersive use and low dust technique is selected. There is no information about the use of LEV. Therefore, dilution ventilation is chosen as the pattern of control. Accordingly, the predicted dust exposure range is 0-5 mg/m^3. This exposure would only occur during a part of the shift. The company has reported an exposure time of six minutes per worker and day. Assuming 15 minutes as a worst case situation, this range is converted into 0-0.15 mg/m^3. Therefore, a value of 0.15 mg/ m^3 is chosen as the RWC.

Dermal exposure is estimated by EASE to be in the range of 0.1-1 mg/cm^2/day (considering non dispersive use and intermittent level of contact) or 42-420 mg/day assuming a skin surface exposed area of 420 cm^2. EASE results are independent of the amount of substance used. Taking into account that this substance has been described as no dusty material and that the quantity of substance handled is rather small, the exposure level is pragmatically assessed as being towards the bottom of this range.

RISKOFDERM potential dermal exposure model (EXCEL version 2.0, October 2005) has also been used to assess dermal exposure in this scenario. This is a set of generic task-based models capable of predicting dermal exposure to both solid and liquids in a wide range of situations. These models are built based on both quantitative and qualitative data gathered through EU RISKOFDERM project. The model predicts median potential exposure rate for the hands and the rest of the body. Using this value and the GSD the model calculates percentiles of the log-normal distribution. Estimated dermal exposure rates can be combined with duration of exposure resulting in potential dermal exposure loadings (RISKOFDERM, 2003; Warren *et al.*, 2006 (*in press*).

The process assessed fits with the exposure model created for mixing, loading and filling tasks which estimates potential dermal exposure rate only to hands (820 cm^2).

Small amounts of 2,4- DNT are dedicated to the use as an additive in the production of explosives. A company has reported 8 kg are weighed with a frequency of six times per shift and this task is shared by two workers per shift. It can be assume that the quantity used in other facilities should not be much higher. The company has reported an exposure time of six minutes per worker and day. Assuming, as a worst case, an expoure duration of 18 minutes (6 minutes per batch; 3 batches per worker), the model provides a median exposure loading of 3 mg and a 95^{th} percentile of 50 mg for hands loading.

Dermal exposure may be lower if suitable gloves are worn. PPE has not been taken into account in the assessment. A company has reported that the use of gloves is highly accepted. Gloves used are regarded as suitable. However, information has only been obtained for one company and it can not be extrapolated to the rest of European facilities. When PPE is used in accordance with Directive 89/656/EEC (this is in fact, obligatory around the EU) dermal exposure will be considerably reduced and therefore, it will be lower than the quoted range.

Exposure during drumming of the gunpowder could be possible. This final product contains up to 1% of the substance. Therefore, lower levels of inhalation and dermal exposure are expected than in the previous assessed tasks.

Conclusions

Limited measured data are available for manufacture of explosives. These data can be considered neither reliable nor representative of this scenario. For inhalation exposure, a RWC of 0.15 mg/m^3 has been estimated by EASE. This value will be used for the purpose of risk characterisation. A RWC short-term value of twice as high as the full shift RWC value is assumed: up to 0.3 mg/m^3. Typical exposure can be pragmatically assessed as half the RWC or 0.075 mg/m^3.

Dermal exposure has been estimated by EASE as 42-420 mg/day. Exposure level is pragmatically assessed as being towards the bottom of this range.

According to RISKOFDERM model, dermal exposure to hands would be up to 50 mg (95^{th} percentile). The input values for the model are chosen to represent the reasonable worst case situation. Therefore, the value representing the median of the resulting exposure distribution should be taken as the reasonable worst case estimate of potential dermal exposure to hands. Then, the reasonable worst case potential dermal exposure model to the hands is estimated to be 3 mg/day.

In contrast to EASE, the RISKOFDERM model is built based on both quantitative and qualitative dermal exposure data. Therefore, its results are preferred and the value of 3 mg/day is chosen as RWC.

The assessment has been carried out without taking into account the use of PPE. When PPE is used in accordance with Directive 89/656/EEC (this is, in fact, obligatory around the EU) dermal exposure will be reduced considerably and therefore, it will be lower than the quoted range.

4.1.1.2.3 Scenario 3: Use of explosives

The recipients of the explosive formulation are companies dedicated to the manufacture of cartridges and munitions. Automatic filling devices are used.

The formulation is emptied into a silo from which it is discharged into cartridges using an automatic filling device. According to industry information, handling is particularly avoided since it could modify the ballistic properties of the formulation. Therefore, potential for exposure could only be possible during charging of the system.

The formulation is a solid product described as granules or flakes without emission of vapours or dust. Industry has reported that the size of these granules is around 1 mm. To estimate inhalation exposure, non dispersive use is selected. Low dust techniques have been chosen, trying to reflect the low dustiness of the product. There is no information about the use of LEV. Therefore, dilution ventilation is selected as the pattern of control. Accordingly the predicted dust exposure range is 0-5 mg/m^3. Assuming that this activity is carried out along one hour per day we can calculate an eight hour TWA exposure range of 0-0.6 mg/m^3. Since the preparation contains up to 1% of 2,4 DNT, this range can be converted into 0-0.006 mg/m^3.

Dermal exposure is estimated to be 0.42-4.2 mg/day, considering non-dispersive use, intermittent level of contact, an exposed surface area of 420 cm^2, and a content of the substance in the formulation up to 1%.

Conclusions

Due to the absence of measured data, results from the EASE model will be used to estimate the reasonable worst case exposure level. The resulting inhalation exposure range is 0-0.006 mg/m^3. The upper value of this range is considered as the RWC exposure level. A RWC short-term value of twice as high as the full shift RWC value is assumed: up to 0.012 mg/m^3. Typical exposure can be pragmatically assessed as half the RWC or 0.003 mg/m^3.

Dermal exposure has been estimated by EASE as 0.42-4.2 mg/day. The upper value of this range is considered as the RWC exposure level. The assessment has been carried out without taking into account the use of PPE. When PPE is used in accordance with Directive 89/656/EEC (this is, in fact, obligatory around the EU), dermal exposure will be considerably reduced and therefore, it will be lower than the quoted range.

Table 4.1.1.2.1-3: Conclusions of the occupational exposure assessment

Exposure Scenario	Relevant Activities	Inhalation exposure						Dermal exposure			
		RWC		Typical		Short- term		Exposure level ($mg/cm^2/day$)	Exposed area (cm^2)	RWC (mg/day)	Method
		mg/m^3	Method	mg/m^3	Method	mg/m^3	Method				
Manufacture	Sampling and maintenance	0.009	Measured	0.0007	Measured	0.018	Calculated	0-0.1	210	0-2.1	EASE (90% protection by suitable gloves)
Explosives Manufacture	Weighing and charging	0.15	EASE	0.075	Calculated	0.3	Calculated		820	3	RISKOFDERM model (unprotected workers)
Use of explosives	Charging	0.006	EASE	0.003	Calculated	0.012	Calculated	0.001-0.01	420	0.42-4.2	EASE (unprotected workers)

4.1.1.3 Consumer exposure

2,4-Dinitrotoluene is primarily used as a chemical intermediate in the production of toluene diisocyanate. This application uses about 99% of 2,4-dinitrotoluene production. Other minor uses are as gelatinizing-plasticizing agent in both commercial and military explosive compositions.

There is no information about 2,4-dinitrotoluene in consumer products. Nevertheless, according to Directive 2003/34/EC, since 15 January 2005, the use of this substance and preparations containing it should not be placed on the market for use by the general public.

4.1.1.4 Humans exposed via the environment

Indirect exposure via the environment is calculated using data for oral intake via food, drinking water and air for local (A, B, D and E sites) and regionalscales. The resultant daily doses for the uptake of 2,4-dinitrotoluene are in the table below. These values have been obtained with EUSES, using the environmental concentrations calculated before for the local and regional scenarios and a standard consumption pattern of food.

Table 4.1.1.4: Daily Human Doses (mg/kg/d)

Intake route	Local site A	Local site B	Local site D	Local site E	Regional
Drinking water	$8.62 \cdot 10^{-6}$	$1.24 \cdot 10^{-4}$	$6.46 \cdot 10^{-7}$	$3.43 \cdot 10^{-6}$	$5.1 \cdot 10^{-7}$
Fish	$4.77 \cdot 10^{-6}$	$6.87 \cdot 10^{-5}$	$3.57 \cdot 10^{-7}$	$2 \cdot 10^{-6}$	$2.8 \cdot 10^{-7}$
Leaf crops	$1.24 \cdot 10^{-7}$	$7.71 \cdot 10^{-8}$	$2.12 \cdot 10^{-7}$	$8.15 \cdot 10^{-8}$	$6.4 \cdot 10^{-8}$
Root crops	$1.8 \cdot 10^{-8}$	$1.8 \cdot 10^{-8}$	$1.81 \cdot 10^{-8}$	$1.8 \cdot 10^{-8}$	$1.2 \cdot 10^{-8}$
Meat	$1.76 \cdot 10^{-10}$	$2.47 \cdot 10^{-9}$	$2.15 \cdot 10^{-11}$	$7.15 \cdot 10^{-11}$	$1.3 \cdot 10^{-11}$
Milk	$1.09 \cdot 10^{-9}$	$1.52 \cdot 10^{-8}$	$1.33 \cdot 10^{-10}$	$4.41 \cdot 10^{-10}$	$7.9 \cdot 10^{-11}$
Air	$1.76 \cdot 10^{-9}$	$5.89 \cdot 10^{-10}$	$3.61 \cdot 10^{-9}$	$8.76 \cdot 10^{-10}$	$5.9 \cdot 10^{-10}$
Total	$1.35 \cdot 10^{-5}$	$1.93 \cdot 10^{-4}$	$1.24 \cdot 10^{-6}$	$5.53 \cdot 10^{-6}$	$8.7 \cdot 10^{-7}$

4.1.1.5 Combined exposure

Exposure to 2,4-dinitrotoluene may reasonably be predicted to arise as a result of combined exposure from workplace and environmental sources.

4.1.2 Effects assessment: Hazard identification and dose (concentration)-response (effect) assessment

4.1.2.1 Toxicokinetics, metabolism and distribution

4.1.2.1.1 Studies in animals

In vivo studies

Inhalation

No available data.

Dermal

No available data.

Oral

Several studies on the toxicokinetics following oral administration of 2,4-DNT have been performed in experimental animals, especially in rats. Most of them were mainly research orientated. Consequently, the protocols were not specifically designed according to OECD guideline 417 and addressed only some of the parameters described. The available data are either published in journals with reviewers or obtained from acceptable studies. Similar results were found with slight concrete discrepancies among studies. Thus, data showed below are considered acceptable for risk assessment.

Rats

Mori, Naruse and Kozuka (1977) investigated the excretion and distribution of 2,4-DNT pure. Three male Wistar rats were administered p.o. with a salad oil solution of ^{3}H-2,4-DNT (50 mg/kg). Urine and faeces were collected separately every day, for seven days. For tissue distribution studies, rats were sacrificed seven days after 2,4-DNT administration and the whole organs were removed excluding: fat, taken from perirenal depots; muscle tissue, taken from thigh bone region of hind legs; and skin, taken from the back. About 21% of the radioactivity administered was excreted in the 1st day-faeces. The amounts of radioactivity in the 2nd day- and 3rd day-faeces were to be about 4 and 1%, respectively. About 13.5% of the radioactivity administered was excreted in the 1st day-urine, but after the 2nd day the excretion of radioactivity was to be trace. In all, about 46% of the radioactivity administered was excreted in the faeces and urine during the seven days. The radioactivity remaining in adipose tissue, skin and liver was 1.5%, 0.6% and 0.4%, respectively, seven days after administration. The amounts of radioactivity in other organs were negligible. The percentage recovery of radioactivity was not provided but from data it appears to be only half of the radioactivity.

Lee *et al* (1975, 1978) carried out a study on the toxicokinetics of 2,4-DNT (98% purity). Groups of three female CD rats were administered, by intragastric intubation, ^{14}C-2,4-DNT suspended in peanut oil as a single dose of 65 mg/kg b.w. Faeces and urine were collected separately as well as expired CO_2. At the termination of each experiment, rats were

anesthetized with ether and aortic blood was collected. Liver, kidneys, brain, lungs, and thigh muscle were removed, weighed and representative samples taken for analysis of radioactivity. The gastrointestinal tract (GI) plus contents was removed and weighed. The GI and the faeces were homogenized and filtered; samples of the filtrate and the filtered residues were assayed for radioactivity. 2,4-DNT was well absorbed. The absorption was essentially completed in 24 hours. Radioactivity recovered at the end of 24 h (88.5% of the dose) averaged 75.9% in the urine; 9.1% in the faeces; 2.8% in the GI, 0.3% in the skeletal muscle (based on 40% of the b.w.) and the liver, 0.1% in whole blood (based on 7% of b.w.) and expired air, and < 0.1% in kidneys, brain and lungs. The majority of the absorbed radioactivity was excreted in the urine. The tissue to plasma radioactivity ratios indicated retention of radioactivity in various tissues. 24 hours after dosing, radioactivity was highly concentrated in liver (ratio of 18.1) and moderately concentrated in kidneys (ratio of 7.4) and lungs (ratio of 6.1); skeletal muscle and brain had concentration ratios greater than one. Another group of three rats were treated with ^{14}C-2,4-DNT for 5 consecutive days to compare the amount of radioactivity in each tissue 24 h after the last dose with that in the tissue 24 h after a single dose. All the tissues receiving 5 daily doses contained 2 to 4.8 times as much radioactivity as those receiving a single dose. 2,4-DNT was metabolised extensively and the parent compound was not detected in urine. Analysis of the radioactivity in the 4- and 24-hour urine samples showed one major peak of radioactivity in both urine samples and one minor peak in only the 24-hour urine sample. Metabolites were only identified in the 24-hour urine sample. They are shown in table 4.1.2.1.1-1.

Table 4.1.2.1.1-1: Metabolites of 2,4-DNT in rat urine collected for 24 hours after oral administration of a single dose of 2,4-DNT (Ring-UL-^{14}C) (Lee *et al* 1975, 1978)

		Conjugates	
Metabolites	**Free**	**Glucuronide**	**Sulphate**
2,4-DNT (I)	0	0	0
4-amino-2-nitrotoluene (II)	0.4	1.2	0.9
2-amino-4-nitrotoluene (III)	0	0.1	0.6
2,4-diaminotoluene (IV)	1.3	5.9	4.3
2,4-dinitrobenzyl alcohol (V)	3.2	27.1	2.9
2,4-/4,2-aminonitrobenzyl alcohols (VI/VII)	2.2	22.5	0.7
2,4-diaminobenzyl alcohol (VIII)	0.9	1.7	3.4
2,4-dinitrobenzoic acid (XIII)	8.0	0.2	0
All identified	16	58.7	12.9
Unidentified	12.5		

Percent of ^{14}C- radioactivity in urine (n = 3 females)

2,4-DNT was metabolised extensively and the parent compound was not detected in urine. The main metabolites found in urine of 24 hours were glucuronide conjugates of 2,4-dinitrobenzyl and 2,4-/4,2-aminonitrobenzyl alcohols (Table 4.1.2.1.1-1). Thus, 2,4-DNT is metabolized in two phases. The first phase consists of reduction of the nitro groups and/or oxidation of the methyl group. One or both nitros may be reduced to amines by the nitro reductase systems found in liver microsomes and other tissues. The methyl group may be oxidized to a benzyl alcohol by the liver microsomal oxidation system. The alcohol can then be further oxidized to a benzaldehyde by alcohol dehydrogenase and to a benzoic acid by aldehyde dehydrogenase. These oxidative and/or reductive products may then undergo the

second phase of metabolism, conjugation, to form glucuronides, sulphates and perhaps other compounds, which are then excreted.

Since 2,4-DNT was concentrated in the liver, which is the site of metabolic biotransformation and biliary excretion, the following experiment was constructed by Lee *et al.* (1978) in order to study the biliary excretion of 2,4-DNT. Three female CD rats were fasted overnight before use. Under ether anesthesia, the common bile duct was cannulated with PE-10 plastic tubing through a midline abdominal incision. After the incision had been closed, rats were administered, by intragastric intubation, ^{14}C-2,4-DNT suspended in peanut oil as a single dose of 65 mg/kg b.w. Bile was collected for the predetermined intervals (0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 23 and 24 hours) and the amount of bile was measured by weighing. Small volumes of blood samples (200 μL) were obtained periodically from the rats by cutting off the tips of their tails and were heparinized. At the end of 24 hours, the rats were anesthetized with ether. Blood was collected from the abdominal aorta. Entire length of the GI tract including their contents was removed and combined with the faeces which were collected without urinary contamination. Radioactivities in the bile, blood, plasma and the GI tract were measured by a liquid scintillation spectrophotometer. The radioactivity appeared in the bile within 15 min after oral administration, increased with time and reached a peak within 2 hours. Thereafter, the rate of biliary excretion decreased. Blood concentration of radioactivity correlated with the rate of biliary excretion. After 24 hours, the total biliary excretion of radioactivity averaged 10.9% of the dose. The radioactivity recovered from the GI tract plus contents and faeces averaged 7.6% of the dose.

Mori, Naruse and Kozuka (1977) investigated the detailed absorption and excretion of 2,4-DNT pure. For absorption and distribution studies, 24 male Wistar rats were given p.o. a salad oil solution of ^{3}H-2,4-DNT (22.2 mg/kg). Blood samples were taken from the jugular vein at certain time intervals; after collecting blood samples, rats were sacrificed by anesthetization and then livers, stomachs, small and large intestine contents were removed. For urinary, and faecal excretion studies, three male Wistar rats were administered p.o. with a salad oil solution of ^{3}H-2,4-DNT (22.2 mg/kg). Urine and faeces were collected for 0-3 h, 3-6 h, 6-9 h and 9-24 h. For the biliary excretion study, after three male rats were anesthetized with ether and the bile duct was cannulated, 2,4-DNT was administered p.o. as a salad oil solution of ^{3}H-2,4-DNT (21.6 mg/kg). Bile samples were collected hourly for 24 h. The time-course of radioactivity level in blood, liver and digestive organ contents, and of biliary, faecal and urinary excretion were observed. Radioactivity was detected in blood, bile and urine from 0.5 h after dosing. Radioactivity level in blood reached a peak at 6 h, gradually declined over the first 9 h, and at 136 h it was about 33% compared with the level at 6 h; its half-life was about 22 h. In the liver, radioactivity levels reached a peak at 6 h after dosing and its peak time paralleled to that of blood. Radioactivity levels in stomach and small intestine contents were rapidly declined at 6 h after dosing. The biliary excretion rate was rising steadily from 6 h after dosing and its peak time was 9-10 h; about 10% of the radioactivity administered was excreted in bile within 24 h. A considerable amount of radioactivity was excreted in the 6-9 h faeces. Based on the results of biliary excretion, it appears that a peak time of faecal excretion depends on the biliary excretion pattern. The excretion rate of urinary radioactivity was more rapid than that of faecal excretion and about 60% of the radioactivity administered was excreted within the first 6 h. These data suggest that the absorption rate from the digestive organs of ^{3}H-2,4-DNT is relatively slow and the faecal excretion radioactivity is originated in the biliary excretion of the radioactivities.

Ellis *et al*. (1979) studied the metabolism of ^{14}C-labelled 2,4-DNT in rats fed 2,4-DNT for 3, 9 and 20 months. Groups of 6 rats/sex/dose were fed 0 or 34/45 mg/kg b.w./day 2,4-DNT

(males/females) for 3 months (3 rats/sex/dose), and 9 months (3 rats/sex/dose). Groups of 3 rats/sex/dose were fed 0 or 3.9/5.1 mg/kg bw/day 2,4-DNT (males/females) for 20 months. Then, ^{14}C-2,4-DNT suspended in peanut oil was administered to each animal by intragastric intubation. Males received a single dose of 57 mg/kg b.w. and females a dose of 65 mg/kg b.w. Immediately after dosing, each rat was placed in metabolic cage for the separate collection of urine and faeces. At the end of 24 hours, blood from the abdominal aorta was collected. Various tissues were removed, weighed and processed for analysis of radioactivity. Metabolism results from rats fed 2,4-DNT for 3, 9 and 20 months were similar to those seen in rats not fed 2,4-DNT before being given the test dose of 2,4-DNT. The oral dose was well absorbed, with a large majority of radioactivity appearing in the urine within 24 hours (42.2-87.8% of the dose). Some radioactivity was found in the gastrointestinal tract (4.2-15.9% of the dose) and faeces (2.8-20.7% of the dose). Very little remained in the tissues, with the liver (organ of metabolism and biliary excretion) and kidney (organ of urinary excretion) having the highest levels of 2,4-DNT-derived radioactivity. Radioactivity recovered at the end of 24 h was 66% of the dose in one female control group and > 90% in the remaining treatment groups. Thin-layer chromatography was used to identify metabolites from extracts of either raw urine or hydrolysed urine samples. Metabolism was extensive (Table 4.1.2.1.1-2).

Table 4.1.2.1.1-2: Metabolites collected in urine of 24 hours after a single oral dose of ^{14}C-2,4-DNT (Ellis *et al.*, 1979)

	Males[b]				Females[b]			
	Fresh		Hydrolysed[c]		Fresh		Hydrolysed[c]	
Metabolites	**Control**	**Test**	**Control**	**Test**	**Control**	**Test**	**Control**	**Test**
Three months[a]								
2,4-DNT	0.02	0.01	1.3	0.8	0	0	0.2	0.4
2,4-/4,2-aminonitrotoluenes	0.5	0.3	3.2	3.0	0.1	0.1	5.9	6.4
2,4-dinitrobenzyl alcohol	1.1	4.8	19.3	20.0	3.1	4.9	18.1	16.7
Aminonitro/diamino benzyl alcohols	1.4	2.1	25.1	26.8	1.6	0.6	23.8	26.0
2,4-diaminotoluene	4.9	4.5	7.8	7.1	5.3	2.8	7.6	6.4
Conjugate and others	92.1	88.3	39.1	40.1	89.9	91.5	42.6	42
Nine months[a]								
2,4-DNT	< 0.1	0.1	0.7	0.6	0.1	< 0.1	0.7	0.4
2,4-/4,2-aminonitrotoluenes	0.3	1.2	7.4	11.6	0.3	0.1	5.2	2.5
2,4-dinitrobenzyl alcohol	13.0	16.7	28.7	38.3	7.6	2.6	35.5	13.5
Aminonitro/diamino benzyl alcohols	0.9	0.9	8.2	8.4	0.2	0.8	10.2	11.4
2,4-diaminotoluene	0.1	0.4	2.2	2.2	0.5	1.2	3.4	4.5
2,4-dinitrobenzoic acid	25.2	22.6	3.1	4.0	31.1	28.0	3.2	4.0
Conjugate and others	60.5	58.1	49.6	34.8	60.3	67.2	41.9	63.7
Twenty months[a]								
2,4-DNT	< 0.1	0	0.4	0.3	< 0.1	< 0.1	0.6	1.0
2,4-/4,2-aminonitrotoluenes	0.3	0.2	6.5	5.6	0.1	0.2	7.2	4.0
2,4-dinitrobenzyl alcohol	9.3	2.0	15.2	13.2	8.3	2.6	14.8	15.0
Aminonitro/diamino benzyl alcohols	0.6	1.7	3.3	3.7	0.3	1.4	4.1	2.8
2,4-diaminotoluene	0.1	0.1	1.6	0.9	0.1	0.1	1.2	1.4
2,4-dinitrobenzoic acid	17.7	12.5	9.7	9.9	18.4	21.1	16.2	16.0
Conjugate and others	72	83.5	63.3	66.4	71.8	74.6	55.9	59.8

[a] 3 and 9 months (34/45 mg/kg b.w./day for ♂/♀); 20 months (3.9/5.1 mg/kg b.w./day ♂/♀). [b] Results expressed as percent of total radioactivity in urine (n = 3 rats). [c] Hydrolyzed by equal volume of 5 N HCl for 1 h in 100°C water bath.

Very small amounts of 2,4-DNT itself was excreted. The major metabolites were dinitro-, aminonitro- and diaminobenzyl alcohols, reflecting oxidation of the side chain and reduction

of the nitro groups. Most of these primary metabolic products were then conjugated before excretion. Data indicated that there were no major differences between dose groups, between sexes or between feeding periods.

Rickert and Long (1980) studied the tissue distribution of 2,4-DNT and its metabolites in male and female Fischer-344 rats. ^{14}C-2,4-DNT was administered dissolved in corn oil as a single dose. Males received 10, 35 or 100 mg/kg and females received 100 mg/kg. Rats were sacrificed at 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 36, 48, 60 or 72 h after dosing and blood was collected. Plasma was separated by centrifugation, and liver, kidney, lung, spleen, fat and brain were removed and frozen for later analyses. In males, peak concentrations of radioactivity in plasma, red blood cells, liver and kidneys were proportional to dose. Levels in liver and kidneys were 5-10 times higher than those in plasma or red blood cells. Levels of radioactivity in other tissues were lower than those in plasma. Terminal half-lives of total radioactivity in various tissues were similar after doses of 10 or 35 mg/kg, but they were shorter after a dose of 100 mg/kg in plasma, red blood cells, liver and kidney. The only clear differences between males and females were the higher retention of radioactivity in red blood cells of females and the concentration of radioactivity in livers of females, which was only half of what was found in males. In addition, concentrations of 2,4-DNT in male kidneys peaked at 4-8 h and were 3-10 times higher than concentrations in female kidneys which peaked at 1 h after dosing. The tissues of both sexes contained parent compound, 2,4-dinitrobenzoic acid, and 2-amino-4-nitrobenzoic acid, but no evidence for the presence of 2,4-diaminotoluene was found. The difference between nanomole equivalents of 2,4-DNT and its metabolites found by specific assay and nanomole equivalents found by radioactivity measurements was two to five times larger in females than in males. The data suggest that dose-dependent changes occur in the elimination and distribution of 2,4-DNT; that females metabolize 2,4-DNT to unidentified metabolites to a greater extent than males; and that hepatocarcinogenicity of 2,4-DNT is not due to its conversion to 2,4-diaminotoluene.

Mori, Naruse and Kozuka (1980) studied the excretion and distribution of ^{3}H-2,4-DNT administered orally at a single dose of 50 mg/kg bw to male Wistar rats fed with standard diet (10 rats, control group) or standard diet containing 5% 2,4-DNT (10 rats, treated group) for four months. The urine and faeces were collected separately every day during the following seven days. Then, rats were sacrificed and whole organs obtained. The radioactivity excreted in faeces of control rats was higher than that excreted in urine. This situation was reversed in 2,4-DNT treated rats. The treatment of rats with 2,4-DNT in feed does not alter the pattern of distribution of the ^{3}H-2,4-DNT, with radioactivity concentrated in liver, skin and adipose tissue of both groups. However, the radioactivity in treated rats was lower than in control rats, suggesting that organs of 2,4-DNT treated rats are saturated by ingesting 5% 2,4-DNT diet for four months.

Mori, Naruse and Kozuka (1981) identified some urinary metabolites of 2,4-DNT in rats after repeated administration. A salad oil solution of 2,4-DNT pure was administered to each of 30 male Wistar rats orally to provide daily doses of 25 mg/kg b.w. for 6 days. The urines were collected daily for 7 days after giving the first dose. Nine metabolites of 2,4-DNT were detected by thin-layer chromatography in the urine. These metabolites, 2-amino-4-nitrotoluene (2A4NT, M-I), 4-amino-2-nitrotoluene (4A2NT, M-II), 2,4-diaminotoluene (2,4-DAT, M-III), 2,4-dinitrobenzyl alcohol (2,4-DNB, M-IV), 2-amino-4-nitrobenzyl alcohol (2A4NB, M-V), 4-amino-2-nitrobenzyl alcohol (4A2NB, M-VI), 2-nitro-4-acetylaminotoluene (2N4AAT, M-VII), 2-amino-4-acetylaminotoluene (2A4AAT, M-VIII) and 2-amino-4-acetylaminobenzoic acid (2A4AAB, M-IX) were identified by comparison of UV and mass spectral data with those of authentic samples. These results suggest that the

primary metabolic reactions of 2,4-DNT in rat are the reduction of a single nitro group to an amino group and the oxidation of the methyl group to CH_2OH, and that N-acetylation of the amino group of the 4-position and subsequent oxidations of CH_2OH to COOH occur as secondary reactions. In addition, it was found that 2-amino-4-acetylaminobenzoic acid was a final metabolic product.

Rickert and Long (1981) examined the metabolism and excretion of 2,4-DNT in male and female Fischer 344 rats after different doses. Rats were given a single dose of 10, 35 o 100 mg/kg of ^{14}C-2,4-DNT by gavage in corn oil. Radioactivity was determined in urine and faeces. In both males and females, urine was the major route of elimination of 2,4-DNT at all doses. Urinary excretion was nearly complete at 24 h after the dose and faecal elimination was nearly complete at 48 h. Females excreted significantly more radioactivity in the urine and less in the faeces than did males after the dose of 35 mg/kg, and there were no significant differences between males and females in excretion after doses of 10 or 100 mg/kg. The HPLC profile of urinary metabolites had four early-eluting peaks, present in both sexes and identified as 4-(N-acetyl)amino-2-nitrobenzoic acid, 2,4-dinitrobenzoic acid, 2-amino-4-nitrobenzoic acid and 2,4-dinitrobenzyl alcohol glucuronide. These four compounds accounted for > 85% of the radioactivity excreted in urine. Females excreted a significantly greater percentage of the dose in the urine as 2,4-dinitrobenzyl alcohol glucuronide at doses of 10 or 35 mg/kg bw compared to males. Both sexes showed dose-dependent changes in urinary excretion of 2,4-DNT metabolites. Males excreted a smaller percentage of the dose as 2,4-dinitrobenzoic acid at 100 mg/kg than at 10 or 35 mg/kg. Females excreted less of the dose as 2,4-dinitrobenzoic acid and 2,4-dinitrobenzyl alcohol glucuronide at 100 mg/kg than at 10 or 35 mg/kg. The only significant sex-dependent difference was the greater percentage of 2,4-DNT excreted as 2,4-dinitrobenzyl alcohol glucuronide by females after the dose of 10 or 35 mg/kg bw.

Rickert *et al.* (1981) examined the metabolism, excretion and hepatic covalent binding of ^{14}C-2,4-DNT in conventional, DNT fed (75.8% 2,4-DNT, 19.5% 2,6-DNT and 4% other isomers) for 30 days, and axenic Fischer-344 rats in order to define the role of DNT pre-treatment and intestinal microflora in the disposition and toxicity of 2,4-DNT. Males and females of each group were administered a single oral dose of 35 mg/kg of ^{14}C-2,4-DNT in corn oil. Urine and faeces were collected for 48 hours. Immediately after the last urine collection, animals were sacrificed and the liver removed for analysis of total and covalent bound radioactivity. Axenic rats excreted a smaller percentage of the dose in the urine and faeces than did conventional rats in 48 hours. No differences in 2,4-DNT excretion were produced by 30 days of feeding DNT (35 mg/kg/day) in the diet of males or females. 2,4-DNT was not detected in the urine. The four major metabolites identified in the urine of conventional rats, 4-(N-acetyl)amino-2-nitrobenzoic acid (13-14% of total radiolabel administered), 2,4-dinitrobenzoic acid (10-16% of total radiolabel administered), 2-amino-4-nitrobenzoic acid (2-3% of total radiolabel administered) and 2,4-dinitrobenzyl alcohol glucuronide (16-24% of total radiolabel administered) were also present in the urine of axenic rats, although axenic rats excreted much less 4-(N-acetyl)amino-2-nitrobenzoic acid (1-2% of total radiolabel administered) and 2-amino-4-nitrobenzoic acid (0.3-0.4% of total radiolabel administered) than did conventional rats. The half-times for excretion of 2,4-DNT as 4-(N-acetyl)amino-2-nitrobenzoic acid and 2-amino-4-nitrobenzoic acid were slightly longer than those for 2,4-dinitrobenzoic acid and 2,4-dinitrobenzyl alcohol glucuronide in conventional rats (6-7 hours vs. 5 hours); there were no significant differences ascribable to sex or pre-treatment. In axenic females, only half-times for excretion of 2,4-DNT as 4-(N-acetyl) amino-2-nitrobenzoic acid and 2-amino-4-nitrobenzoic acid were significantly longer than in conventional females (13 and 14 hours vs 6.5 and 6 hours). In axenic males, the half-times for excretion of 2,4-DNT as 4-(N-

acetyl)amino-2-nitrobenzoic acid, 2,4 dinitrobenzoic acid and 2,4-dinitrobenzyl alcohol glucuronide were longer than those in conventional males (18.5, 14 and 16 hours vs. 6.5, 5 and 5 hours). Excretion of 2-amino-4-nitrobenzoic acid in axenic males was extremely slow and variable, and a half-time could not be determined. The ^{14}C hepatic concentration in conventional rats was significantly higher than in axenic rats. Hepatic covalent binding was decreased to 50% in axenic animals. ^{14}C hepatic concentrations were higher in conventional and axenic males than in females of either type. With respect to hepatic covalent binding, it was greater in conventional males than in conventional females, but no sex differences were observed in axenic rats. These data suggest that intestinal microflora plays a major role in the appearance of reduced urinary metabolites and of covalently bound material after 2,4-DNT administration.

Rickert, Schnell and Long (1983) studied the intestinal disposition of 2,4-DNT as well as the time course and degree of covalent binding to hepatic DNA, RNA and protein after administration of ^{14}C-2,4-DNT to rats. Male Fischer-344 rats were administered by gavage 10 or 35 mg/kg of ^{14}C-2,4-DNT dissolved in corn oil. Rats from each group were killed at 1, 2, 4, 8, 12, 24, 48, 96, 192 and 384 h after dosing. Livers were removed, weighed and homogenized. The homogenate was separated into DNA, RNA and protein enriched fractions which were then counted to determine ^{14}C covalently bound. Sections of small intestine and cecal contents were analyzed for total radioactivity and 2,4-DNT metabolites. Radioactivity disappeared from the first quarter of the small intestine rapidily. At the earliest time point studied (1 h), the larger amount of radioactivity was found in the second quarter of the small intestine. As radioactivy disappeared from the first and second quarters, it began to appear in the last half of the small intestine and the cecal contents, peaking at 4-8 h and 8 h, respectively. The segments of intestine examined contained only very small amounts of radioactivity at times longer than 24 h after dosing. An initial peak (1-2 h) of radioactivity appeared in the liver but not significant covalent binding occurred at that time. Appearance of 2,4-dinitrobenzyl alcohol glucuronide in the intestine was followed by a second peak of total hepatic radioactivity (8-12 h). The second peak was followed by a gradual decline up to 16 days. However, the concentration of 2,4-dinitrobenzyl alcohol glucuronide in the intestine declined prior to peak covalent binding in the liver. At 12 h, covalent binding to DNA, RNA and protein became significant and persisted at least until 96 h after dose. Covalent binding to each macromolecular species was proportional to dose. There was no selectivity for any macromolecule. The terminal half-lifes of covalently bound material ranged from 2.9 to 5 days for RNA and protein and from 5.1 to 7.9 days for DNA. These data suggest that activation of 2,4-DNT requires oxidation to 2,4-dinitrobenzyl alcohol, glucuronidation, excretion in the bile, deconjugation, and further metabolism by intestinal microorganisms, followed by reabsorption.

The biliary excretion and enterohepatic circulation of 2,4-DNT were investigated by Medisnky and Dent (1983). The common bile ducts of male and female Fischer-344 rats were cannulated with an uninterrupted cannule at the hepatic and ileal ends. After 24 h, ^{14}C-2,4-DNT was given orally to males at doses of 35, 63 or 100 mg/kg, while females received orally 35 mg/kg bw. Immediately prior to dosing the cannula was snipped and bile collected in a reservoir surgically implanted in the peritoneal cavity. Urine was collected at 6, 12, 18, 24 and 36 h. Bile was also removed at suitable intervals (ranging from every hour to every 6 h). After the last collection of excreta, animals were killed and livers removed and homogenized to determine the amount of ^{14}C covalently bound to hepatic macromolecules. In males, excretion of ^{14}C in bile was linearly related to the dose, and approximately 25% of the dose appeared in bile within 24 h. Females dosed with 35 mg/kg excreted only 18% of the dose in the bile. Around 90% of the radioactivity in the bile was identified as the glucuronide

conjugate of 2,4-dinitrobenzyl alcohol. Biliary excretion of ^{14}C was essentially complete by 24 h for males and 12 h for females. There were no significant differences in the rates of biliary excretion between sexes at 35 mg/kg or as a function of dose in males; mean half times of excretion ranged from 3.3 to 5.3 h. Greater than 90% of urinary excretion of labelled metabolites appeared in urine collected the first 24 hours. From 60 to 90% of the dose was excreted in urine of control rats compared to 20-60% in the urine of rats from which bile was collected; females excreted more ^{14}C in urine than did males, independent of bile flow into the small intestine. In both males and females most of the 2,4-DNT dose excreted in the urine was in form of the oxidized metabolites, 2,4-dinitrobenzyl alcohol glucuronide and 2,4-dinitrobenzoic acid. At the end of 36 hours, only 0.02-0.05% of the radioactivity was detectable in the livers. From 20 to 60% of this radioactivity was covalently bound to hepatic macromolecules. The covalent binding of ^{14}C was less in rats from which bile was collected compared to controls. In addition, in both cannulated and control rats, the covalent binding of ^{14}C was greater in livers from males compared to females. These results indicate that bile is an important route of excretion for 2,4-DNT metabolites and that metabolites excreted in bile can be reabsorbed from the gut.

Shoji *et al.* (1985) quantified the urinary metabolites of 2,4-DNT by HPLC after oral administration. Five male Wistar rats were orally given a salad oil solution of ^{3}H-2,4-DNT (75 mg/kg). Urine was collected over a period of 24 h. 2,4-dinitrobenzoic acid was excreted most abundantly (5.91% of the dose), followed by 2,4-dinitrobenzyl alcohol glucuronide (3.15% of the dose), 2-amino-4-acetylaminobenzoic acid (1.85% of the dose), 2,4-dinitrobenzyl alcohol (0.83% of the dose), 4-amino-2-nitro(2-amino-4-nitro)benzyl alcohol glucuronides (0.45% of the dose), 4-amino-2-nitro(2-amino-4-nitro)benzyl alcohols (0.16% of the dose), and 4-amino-2-nitrotoluene (0.03% of the dose). From these results, it may be concluded that 2,4-DNT is mainly metabolized by oxidation of the methyl group, followed by the formation of glucuronide conjugates in the case of alcoholic metabolites. The potent mutagen 2,4-dinitrobenzaldehyde was not detected in this study.

For a better understanding of the metabolism of 2,4-DNT, Sayama *et al.* (1989) examined the biliary metabolites of 2,4-DNT, 2,4-dinitrobenzyl alcohol and 2,4-dinitrobenzaldehyde in rats using high-performance liquid chromatography. Male Wistar rats were anaesthetized with ether. A polyethylene tube was inserted into the common bile duct, sewn in place and the abdominal wall and skin closed. 2,4-DNT (40 mg/kg), 2,4-dinitrobenzyl alcohol (40 mg/kg) and 2,4-dinitrobenzaldehyde (40 mg/kg) in salad oil were given to the rats orally. The bile was collected over a period of 24 hours. The major biliary metabolite of 2,4-DNT was 2,4-dinitrobenzyl alcohol glucuronide (11.8% of the dose) and the minor metabolites were 2,4-dinitrobenzyl alcohol (0.13%), 2,4-dinitrobenzaldehyde (0.27%), 2-acetylamino-4-nitrotoluene (0.05%), 4-amino-2-nitro(2-amino-4-nitro)benzyl alcohol sulphate (1.5%), 2,4-dinitrobenzoic acid (4.4%), 2-4-diacetylaminobenzoic acid (0.08%) and 2-amino-4-nitrobenzoic acid (0.7%). 2,4-dinitrobenzyl alcohol (0.2%), 2,4-dinitrobenzaldehyde (1.1%), 2,4-dinitrobenzyl alcohol glucuronide (21.4%) and 4-amino-2-nitro(2-amino-4-nitro)benzyl alcohol sulphate (6.2%) were excreted in the bile of rats dosed with 2,4-dinitrobenzyl alcohol. 2,4-dinitrobenzaldehyde (1.6%), 2,4-dinitrobenzyl alcohol (0.04%), 2,4-dinitrobenzyl alcohol glucuronide (21%), 4-amino-2-nitro(2-amino-4-nitro)benzyl alcohol sulphate (4.1%), 2,4-dinitrobenzoic acid (7.7%) and 2,4-diacetylaminobenzoic acid (0.2%) were excreted in the bile of rats dosed with 2,4-dinitrobenzaldehyde. These results indicate that the common biliary metabolites of 2,4-DNT, 2,4-dinitrobenzyl alcohol and 2,4-dinitrobenzaldehyde are 2,4-dinitrobenzyl alcohol and its glucuronide, and 2,4-dinitrobenzaldehyde, and suggest the enterohepatic circulation of 2,4-dinitrobenzaldehyde in the metabolism of 2,4-DNT.

Mori *et al.* (1996) attempted the direct determination of urinary conjugates of 2,4-dinitrobenzyl alcohol in male Wistar rats dosed with 2,4-DNT by HPLC using potassium 2,4-dinitrobenzyl alcohol glucuronide and pyridinium 2,4-dinitrobenzyl alcohol sulphate as authentic compounds. Other metabolites including the unidentified metabolites were also examined by HPLC using authentic compounds. Six rats were dosed orally with a solution of 2,4-DNT in salad oil (75 mg/kg). Urine samples after administration were collected over 24 h. The conjugate detected was 2,4-dinitrobenzyl alcohol glucuronide, which accounted for about 10.7% of the administered dose. No peak corresponding to pyridinium 2,4-dinitrobenzyl alcohol sulphate was detected in urine. 2-amino-4-nitrobenzoic acid (0.71%), 4-amino-2-nitrobenzoic acid (0.52%) and 4-acetylamino-2-nitrobenzoic acid (3.9%), in addition to known metabolites 4-amino-2-nitrotoluene (0.04%), 2-4-dinitrobenzyl alcohol (0.25%), 2,4-dinitrobenzoic acid (6.9%) and 4-acetylamino-2-aminobenzoic acid (3.4%) were detected.

Mice

Lee *et al.,* (1978) performed a study on the toxicokinetics of 2,4-DNT (98% purity) in female albino CD-1 and B6C3F1 mice. ^{14}C-2,4-DNT (34 mg/kg b.w.), suspended in peanut oil, was administered to groups of 4 or 5 mice by intragastric tube. Faeces and urine were collected separately as well as expired CO_2. At termination, each animal was anesthetized with ether or pentobarbital sodium. Aortic blood was collected and various tissues and faeces removed, weighed, and digested in 10 volumes of 2 N NaOH. Blood samples were decolorized by the addition of H_2O_2. Aliquots of tissue and faecal digests, blood, plasma and urine were neutralized, solubilized and counted in the scintillation solution using a liquid scintillation spectrophotometer. Radioactivity recovered at the end of 24 hours in CD-1 and B6C3F1 strains was 94.8% and 92% of the dose, respectively, being the majority recovered in the faeces, 81% and 84% (Table 4.1.2.1.1-3). This could be the result of poor absorption after ingestion or a rapid absorption and metabolism, excretion in the bile, and non absorption of the metabolite(s). No radioactivity was found in the expired air. The tissue to plasma radioactivity ratios indicated retention of radioactivity in liver (ratio of 6.3), kidneys (ratio of 3.4), lungs (ratio of 1.9) and spleen (ratio of 1.7), 24 h after dosing.

Table 4.1.2.1.1-3: Distribution and excretion of radioactivity in two mice strains 24 h after oral administration of a single dose of 2,4-DNT (Ring-UL-^{14}C) (Lee *et al.,* 1978)

	% of administered dose	
	CD-1 (n = 4)	**B6C3F1 (n = 5)**
Gastrointestinal tract plus contents	2.1	0.6
Faeces	81.0	84.0
Whole blood (based on 7% of the b.w.)	0.1	< 0.1
Urine	11.3	7.2
Spleen	< 0.1	< 0.1
Liver	0.2	0.1
Kidney	< 0.1	< 0.1
Brain	< 0.1	< 0.1
Lung	< 0.1	< 0.1
Skeletal muscle (based on 40% of the b.w)	0.1	< 0.1
Recovery	94.8	92.0

The absorbed 2,4-DNT was extensively metabolized with only a small amount of the parent compound excreted in the urine. The main urinary metabolites were glucuronide conjugates of 2,4-dinitrobenzyl and 2,4/4,2-aminonitrobenzyl alcohols (Table 4.1.2.1.1-4). Thus, the main metabolism pathway was oxidation to a benzyl alcohol, with reduction to an aminonitrobenzyl

alcohol, followed by glucuronide conjugation and excretion. Other pathways included reduction of one or both nitro groups to amines, oxidation to a benzoic acid, and excretion as sulphates or as the free compounds.

Table 4.1.2.1.1-4: Metabolites of 2,4-DNT in mouse urine collected for 24 hours after oral administration of a single dose of 2,4-DNT (Ring-UL-^{14}C) (Lee *et al.*, 1978)

		Conjugates	
Metabolites	**Free**	**Glucuronide**	**Sulphate**
2,4-DNT (I)	0.3	0	0
4-amino-2-nitrotoluene (II)	1.0	1.2	10.3
2-amino-4-nitrotoluene (III)	0.1	7.3	2.1
2,4-diaminotoluene (IV)	0.1	3.1	2.0
2,4-dinitrobenzyl alcohol (V)	0.3	19.6	2.7
2,4-/4,2-aminonitrobenzyl alcohols (VI/VII)	0.1	24.5	0.5
2,4-diaminobenzyl alcohol (VIII)	0.2	1.4	0
2,4-dinitrobenzoic acid (XIII)	3.8	0.1	0
All identified	5.8	57.0	17.5
Unidentified	19.6		

Percent of ^{14}C- radioactivity in urine (n = 4 CD-1 females)

Schut *et al.* (1985) studied the elimination and metabolism of a single dose (100 mg/kg) of 2,4-DNT, administered as a tricaprylin solution containing ^{3}H-2,4-DNT to A/J mice by gavage. Mice were killed after 30 min-8 h (4 mice/time points) and their bladder including contents, and large intestine including contents, were removed. For time periods up to 1 h after dosing, mice were placed individually in glass containers and any voided urine was collected by several rinses with distilled water and the rinses were then combined with the bladder contents. For longer periods (> 1 h), mice were placed individually in metabolic cages as before, and urine and faeces were collected separately. An aliquot of each urine sample was removed for quantitation of radioactivity. Faeces and contents of the large intestine of each mouse were combined and, after homogenization, an aliquot was removed for quantitation of radioactivity. For analysis of metabolites the combined urines of each time point were extracted with ethyl acetate-acetone. Four hours after oral administration, only 28.5% of the dose was excreted in urine, which increased to 66% after 8 h. Elimination via the faeces was minimal (< 2.1% of the dose). From 0.5 to 4 h after oral administration, 5.5 to 6.8% of the urinary metabolites were unconjugates while 20.5 to 28.2% were present in the glucuronide fraction. No unchanged 2,4-DNT could be detected in the urine, and 2,4-dinitrobenzyl alcohol represented the most abundant identifiable neutral metabolite. Small amounts of 2,4-diaminotoluene, 2-amino-4-nitrobenzyl alcohol, 2-(N-acetyl)amino-4-nitrotoluene, 4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene were also present. In almost all cases the largest proportion of metabolites represented were unknown, some of which exhibited the chromatographic properties of carboxylic acid metabolites.

Rabbits

Lee *et al.,* 1978 performed a study on the toxicokinetics of 2,4-DNT (98% purity) using two female New Zealand rabbits. A single oral dose of ^{14}C-2,4-DNT, approximately 10% of the acute LD_{50}, suspended in peanut oil was given by intragastric tube to each animal. Faeces and

urine were collected separately as well as expired CO_2. At termination, each rabbit was anesthetized with ether or pentobarbital sodium. Aortic blood was collected and various tissues and faeces were removed, weighed, and digested in 10 volumes of 2 N NaOH. Blood samples were decolorized by the addition of H_2O_2. Aliquots of tissue and faecal digests, blood, plasma and urine were neutralized, solubilized and counted in the scintillation solution using a liquid scintillation spectrophotometer. 2,4-DNT was well absorbed. Radioactivity recovered at the end of 24 hours (89.8% of the dose) averaged 75.2% in the urine, 10.9% in the gastrointestinal tract plus contents, 3.1% in the faeces, 0.2% in the skeletal muscle (based on 40% of the b.w.), 0.1% in whole blood (based on 7% of b.w.), < 0.3% in liver; and < 0.1% in spleen, kidneys, brain and lungs. The majority of the absorbed radioactivity was excreted in the urine. No radioactivity was found in the expired air. The tissue to plasma radioactivity ratios indicated retention of radioactivity in various tissues. 24 hours after dosing, radioactivity was concentrated in the liver (ratio of 8.7) followed by kidneys (ratio of 7), lungs (ratio of 2.2) and spleen (ratio of 1.3); skeletal muscle and brain had concentration ratios lower than one. The absorbed 2,4-DNT was extensively metabolized with only a small amount of the parent compound excreted in the urine. The major metabolite found in urine of 24 hours was the glucuronide conjugate of 2,4-dinitrobenzyl alcohol (Table 4.1.2.1.1-5). Thus, the main metabolism pathway was oxidation to a benzyl alcohol followed by glucuronide conjugation, and excretion. Other pathways were parent compound reduction to aminonitrotoluenes followed by either glucuronide or sulphate conjugation, and benzyl alcohol oxidation to a benzoic acid.

Table 4.1.2.1.1-5: Metabolites of 2,4-DNT in rabbit urine collected for 24 hours after oral administration of a single dose of 2,4-DNT (Ring-UL-^{14}C) (Lee *et al.,* 1978)

		Conjugates	
Metabolites	**Free**	**Glucuronide**	**Sulphate**
2,4-DNT (I)	0.3	0	0
4-amino-2-nitrotoluene (II)	0.4	0.5	7.9
2-amino-4-nitrotoluene (III)	0.6	4.2	1.8
2,4-diaminotoluene (IV)	0.9	4.0	0.7
2,4-dinitrobenzyl alcohol (V)	0.5	40.3	3.0
2,4-/4,2-aminonitrobenzyl alcohols (VI/VII)	0.3	9.4	0.5
2,4-diaminobenzyl alcohol (VIII)	1.0	5.9	0.6
2,4-dinitrobenzoic acid (XIII)	7.5	2.0	0
All identified	11.4	66.4	14.5
Unidentified	7.9		

Percent of ^{14}C- radioactivity in urine (n = 2 females)

Dogs

Lee *et al.,* 1978 performed a study on the toxicokinetics of 2,4-DNT (96% purity) using two female beagle dogs. A single oral dose of ^{14}C-2,4-DNT, approximately 10% of the acute LD_{50}, suspended in peanut oil was given by intragastric tube to each animal. Faeces and urine

were collected separately as well as expired CO_2. At termination, each dog was anesthetized with ether or pentobarbital sodium. Aortic blood was collected and various tissues and faeces were removed, weighed, and digested in 10 volumes of 2 M NaOH. Blood samples were decolorized by the addition of H_2O_2. Aliquots of tissue and fecal digests, blood, plasma and urine were neutralized, solubilized and counted in the scintillation solution using a liquid scintillation spectrophotometer. 2,4-DNT was well absorbed. Radioactivity recovered at the end of 24 hours (97.3% of the dose) averaged 76.4% in the urine, 8.6% in the gastrointestinal tract plus contents, 8.7% in the faeces, 1.6% in skeletal muscle (based on 40% of the b.w.); 1.1% in liver; 0.6% in whole blood (based on 7% of the b.w.); 0.2% in kidneys, < 0.1% in spleen, brain and lungs. The majority of the absorbed radioactivity was excreted in the urine. No radioactivity was found in the expired air. The tissue to plasma radioactivity ratios indicated retention of radioactivity in various tissues. 24 hours after dosing, radioactivity was concentrated in the liver (ratio of 6.9) followed by kidneys (ratio of 4.9), lungs (ratio of 1.7) and spleen (ratio of 1.1); skeletal muscle and brain had concentration ratios lower than one. The absorbed 2,4-DNT was extensively metabolized with only a small amount of the parent compound excreted in the urine. The main metabolites found in urine of 24 hours were glucuronide conjugates of 2,4-dinitrobenzyl and 2,4/4,2-aminonitrobenzyl alcohols (Table 4.1.2.1.1-6). The main metabolism pathway was oxidation to a benzyl alcohol, with reduction to an aminonitrobenzyl alcohol, followed by glucuronide conjugation and excretion. Other pathways were benzyl alcohol oxidation to a benzoic acid and, in less extent, parent compound reduction to aminonitrotoluenes followed by either glucuronide or sulphate conjugation.

Table 4.1.2.1.1-6: Metabolites of 2,4-DNT in dog urine collected for 24 hours after oral administration of a single dose of 2,4-DNT (Ring-UL-^{14}C) (Lee *et al.,* 1978)

		Conjugates	
Metabolites	**Free**	**Glucuronide**	**Sulphate**
2,4-DNT (I)	0.2	0.5	0
4-amino-2-nitrotoluene (II)	0.1	4.6	3.7
2-amino-4-nitrotoluene (III)	0.2	2.9	0.4
2,4-diaminotoluene (IV)	0.2	4.8	0.4
2,4-dinitrobenzyl alcohol (V)	0.1	33.1	1.6
2,4-/4,2-aminonitrobenzyl alcohols (VI/VII)	0.4	17.9	0.2
2,4-diaminobenzyl alcohol (VIII)	0.5	1.4	1.5
2,4-dinitrobenzoic acid (XIII)	5.7	1.0	0
All identified	7.4	66.2	7.8
Unidentified	18.6		

Percent of ^{14}C- radioactivity in urine (n = 2 females)

Monkeys

Lee *et al.,* 1978 performed a study on the toxicokinetics of 2,4-DNT (purity 98%) using two female rhesus monkeys. A single oral dose of ^{14}C-2,4-DNT, approximately 10% of the acute LD_{50}, suspended in peanut oil was given by intragastric tube to each animal. Faeces and urine

were collected separately as well as expired CO_2. At termination, each monkey was anesthetized with ether or pentobarbital sodium. Aortic blood was collected and various tissues and faeces were removed, weighed, and digested in 10 volumes of 2 N NaOH. Blood samples were decolorized by the addition of H_2O_2. Aliquots of tissue and faecal digests, blood, plasma and urine were neutralized, solubilized and counted in the scintillation solution using a liquid scintillation spectrophotometer. 2,4-DNT was well absorbed. Radioactivity recovered at the end of 24 hours (93% of the dose) averaged 81.3% in the urine, 4.7% in the gastrointestinal tract plus contents, 4.8% in the faeces, 1.2% in skeletal muscle (based on 40% of the b.w.); 0.7% in liver; 0.3% in whole blood (based on 7% of the b.w.); < 0.1% in kidneys, spleen, brain and lungs. The majority of the absorbed radioactivity was excreted in the urine. No radioactivity was found in the expired air. The tissue to plasma radioactivity ratios indicated retention of radioactivity in various tissues. 24 hours after dosing, radioactivity was concentrated in the liver (ratio of 17.8) followed by kidneys (ratio of 6.4), spleen (ratio of 2.6), lungs (ratio of 1.9), skeletal muscle (ratio of 1.5) and brain (ratio of 1.3). The absorbed 2,4-DNT was extensively metabolized. The main metabolites found in urine were the glucuronide conjugates of both 2,4-dinitrobenzyl and 2,4-aminonitrobenzyl alcohols (Table 4.1.2.1.1-7). The main metabolism pathway was oxidation to a benzyl alcohol, with reduction to an aminonitrobenzyl alcohol, followed by glucuronide conjugation, and excretion. Other pathways were benzyl alcohol oxidation to a benzoic acid and, in less extent, parent compound reduction to aminonitrotoluenes followed by either glucuronide or sulphate conjugation.

Table 4.1.2.1.1-7: Metabolites of 2,4-DNT in monkey urine collected for 24 hours after oral administration of a single dose of 2,4-DNT (Ring-UL-^{14}C) (Lee *et al.*, 1978)

		Conjugates	
Metabolites	**Free**	**Glucuronide**	**Sulphate**
2,4-DNT (I)	0.4	2.2	0
4-amino-2-nitrotoluene (II)	1.0	0.7	0.4
2-amino-4-nitrotoluene (III)	0.3	6.8	1.6
2,4-diaminotoluene (IV)	0.4	3.3	1.1
2,4-dinitrobenzyl alcohol (V)	1.5	21.5	2.3
2,4-/4,2-aminonitrobenzyl alcohols (VI/VII)	0	17.9	0.2
2,4-diaminobenzyl alcohol (VIII)	0.5	1.3	1.4
2,4-dinitrobenzoic acid (XIII)	4.5	0.4	0
All identified	8.2	54.1	6.9
Unidentified	30.7		

Percent of ^{14}C- radioactivity in urine (n = 2 females)

Intra-peritoneal

Mice

Schut *et al.* (1982) determined the tissue distribution and elimination of 2,4-DNT (1, 10 and 100 mg/kg) administered i.p. as a tricaprylin solution containing ^{3}H-2,4-DNT to A/J mice. After 0.25, 0.5, 0.75, 1, 2, 3 or 4 h, the animals (4 mice/dose/time points) were anesthetized

with diethyl ether and blood was collected from the jugular veins. When the animal urinated during this procedure, the urine was collected. For the longer time periods (0.5 to 4 h), animals were placed individually in containers and the urine was collected when the animal was killed. Immediately after collecting the blood, the peritoneum was opened and carefully blotted to remove any unabsorbed material. The bladder (including contents) was excised and added to the urine, after which the liver, kidneys, adipose tissue, and small and large intestines were removed, weighed and digested. For studies in which 2,4-DNT was re-isolated, mice were injected and bled as described above. The liver, lungs and small intestine were removed, weighed and homogenized. Aliquots of homogenates and blood were extracted with diethyl ether. Immediately before extraction, 2,4-DNT (200 µg/sample) and ^{14}C-2,4-DNT (10,000 dpm/sample) were added to serve as recovery markers. The extracts were evaporated and the residues were chromatographed on thin-layer sheets. After 4 hours, a total of 69.4, 74.8 and 81.7% of the injected dose (1, 10 and 100 mg/kg, respectively) could be accounted for in body fluids and tissues analyzed. The urine was the major route of elimination; at all doses tested, elimination was rapid, especially during the first hour, with 52.5, 60.1 and 70% of the injected dose (1, 10 and 100 mg/kg, respectively) appearing in the urine after 4 h. Amounts eliminated in the faeces over this time period represent only a small fraction of those excreted in the urine. Concentrations of radioactivity in blood were proportional to the dose administered and decreased with time, especially during the first hour after administration. At time points earlier than 2 h, the levels of radioactivity in liver were two- to four-fold higher than in blood although beyond 2 h the levels in liver were comparable to those in blood. A similar situation was observed in kidneys with the exception of the persistence of higher levels than in blood for up to 3 h after administration. Terminal half-lives of radioactive material in liver (1.1 to 1.7 h) and kidney (0.9 to 1.4 h) were not related to dose. After 4 h administration, the distribution of 2,4-DNT in tissues showed no evidence for preferential uptake or retention at any of the doses tested. At all doses, rapid and extensive metabolism of 2,4-DNT by liver and small intestine (virtually complete at 1 h after administration) was observed, as judged by the low amounts of 2,4-DNT (less than 13% of the total ^{3}H/tissue) that could be re-isolated from these tissues. Blood and lungs contained much higher levels of unchanged 2,4-DNT and, in most cases, the extent of 2,4-DNT metabolism was similar in these two tissues, suggesting that the lung is not an active site of 2,4-DNT metabolism.

Schut *et al.* (1985) studied the elimination and metabolism of a single dose (100 mg/kg) of 2,4-DNT, administered as a tricaprylin solution containing ^{3}H-2,4-DNT to A/J mice i.p. Mice were killed after 30 min-8 h (4 mice/time points) and their bladder including contents, and large intestine including contents, were removed. For time periods up to 1 h after dosing, mice were placed individually in glass containers and any voided urine was collected by several rinses with distilled water and the rinses were then combined with the bladder contents. For longer periods (> 1 h), mice were placed individually in metabolic cages as before, and urine and faeces were collected separately. An aliquot of each urine sample was removed for quantitation of radioactivity. Faeces and contents of the large intestine of each mouse were combined and, after homogenization, an aliquot was removed for quantitation of radioactivity. For analysis of metabolites the combined urines of each time point were extracted with ethyl acetate-acetone. After i.p. administration, elimination was rapid with 70% of the dose appearing in the urine within 4 h. Elimination via the faeces was minimal (< 2.1% of the dose). From 0.5 to 4 h after i.p. administration, 3.6 to 8.8% of the urinary metabolites were unconjugates while 2.4 to 8.8% were present in the glucuronide fraction. No unchanged 2,4-DNT could be detected in the urine, and 2,4-dinitrobenzyl alcohol represented the most abundant identifiable neutral metabolite. Small amounts of 2,4-diaminotoluene, 2-amino-4-nitrobenzyl alcohol, 2-(N-acetyl)amino-4-nitrotoluene, 4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene were also present. In almost all cases the largest proportion of metabolites

represented were unknown, some of which exhibited the chromatographic properties of carboxylic acid metabolites.

In vitro studies

There is a substantial information on the *in vitro* metabolisn of 2,4-DNT. Most available data derived from experiments carried out with subcelular hepatic fractions, mainly from rats. In addition, there are data from experiments with other test systems such as hepatocytes and livers from rats, or intestinal microflora from rodents. Finally, data from metabolism by yeasts have been also included at this section.

Rats

a) Subcelular hepatic fractions

Kozuka *et al.* (1978) showed that ^{3}H-2,4-DNT (2.5 µmol), dissolved in ethanol, is metabolised by liver homogenate from male Wistar rats to 2-amino-4-nitrotoluene (23% of the concentration) and 4-amino-2-nitrotoluene (50% of the concentration), under anaerobic incubation conditions.

Lee *et al.* (1978) investigated the *in vitro* metabolism of 2,4-DNT by liver homogenate from male and female CD rats under either aerobic or anaerobic incubation conditions. Four CD rats/sex were sacrificed and the livers removed, weighed, homogenized and centrifuged at 10000 g for 30 min. The incubation medium contained 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.8 mM nicotinamide adenine dinucleotide phosphate, 1 mM ^{14}C-2,4-DNT (0.1 µg/mL), 1.0 mL of 0.2 M tris-HCl pH 7.4, and 0.5 mL of the 10,000 g liver supernatant for a final volume of 2.5 mL. Reactions were conducted for 1 hour. Aerobic and anaerobic reactions as well as quantification of analytes were performed according to acceptable methods. Under aerobic conditions, 2,4-dinitrobenzyl alcohol was the major metabolite in both sexes; males produced more 2,4-dinitrobenzyl alcohol than did females (Table 4.1.2.1.1.-8.). Under anaerobic conditions, the amount of 2,4-dinitrobenzyl alcohol was reduced while the amount of aminonitrotoluenes was increased; males produced more 2,4-/4,2-aminonitrotoluenes than did females (Table 4.1.2.1.1-8.). Despite the anaerobic conditions, the amount of 2,4-dinitrobenzyl alcohol produced by females was higher than that of aminonitrotoluenes, which did not occur in those of males. The pattern of metabolites produced is altered by pretreating male rats with phenobarbital or SKF 525-A. Phenobarbital increased the formation of dinitrobenzyl alcohol. In contrast, pretreatment with SKF 525-A reduced the amount of this metabolite. These observations suggest that 2,4-DNT is metabolized to dinitrobenzyl alcohol by the hepatic microsomal mixed function oxidase system. This study was published in Experientia (Short, Dacre and Lee, 1979).

Table 4.1.2.1.1-8: Aerobic and anaerobic metabolism of 2,4-DNT (ring-UL-^{14}C) by liver homogenate of rats (Lee *et al.,* 1978)

	Metabolic activity (nmol/mg protein)			
	Aerobic		Anaerobic	
Compounds	**Male**	**Female**	**Male**	**Female**
2,4-dinitrobenzyl alcohol	11.1	8.0	4.6	6.1
Aminonitrotoluenes	1.8	1.3	30.4	3.1
2,4-DNT	129	123	105	123
Others	3.8	1.8	4.2	1.8

Dent *et al.* (1981) determined the metabolism of 2,4-DNT by hepatic microsomes from Fischer 344 rats. ^{14}C-2,4-DNT at 100 µM was incubated with microsomes in 66 mM tris HCl pH 7.4 for 1 h. A NADPH generating system comprising NADP, glucose-6-phosphate, and 1 unit/mL glucose-6-phosphate dehydrogenase was included. In a medium supplemented with O_2, the major metabolite formed was 2,4-dinitrobenzyl alcohol, although 4-amino-2-nitrotoluene was also detected. When incubation was performed without supplemented O_2, the amounts of 2,4-dinitrobenzyl alcohol and 4-amino-2-nitrotoluene formed were comparable. Under anaerobic conditions, 2-amino-4-nitrotoluene was the major metabolite formed, 4-amino-2-nitrotoluene and other unidentified metabolites were also present, and no 2,4-dinitrobenzyl alcohol was detected.

Decad *et al.* (1982) examined the metabolism of 2,4-DNT by subcelular hepatic fractions obtained from male Fischer 344 rats. Microsomes or cytosol were incubated with 200 nmol of ^{14}C-2,4-DNT dissolved in DMSO; the final protein concentration was 2 mg/mL. In a medium supplemented with O_2, the major microsomal metabolite formed was 2,4-dinitrobenzyl alcohol, although 4-amino-2-nitrotoluene was also detected. When incubation was performed without supplemented O_2, the amounts of 2,4-dinitrobenzyl alcohol and 4-amino-2-nitrotoluene formed were comparable. Under anaerobic conditions, 2-amino-4-nitrotoluene was the major metabolite formed, 4-amino-2-nitrotoluene and other unidentified metabolites were also present, and no 2,4-dinitrobenzyl alcohol was detected. The major cytosolic metabolite was 4-amino-2-nitrotoluene followed by 2-amino-4-nitrotoluene; 2-(N-acetyl) amino-4-nitrotoluene was also detected and no 2,4-dinitrobenzyl alcohol was present. Pre-treatment of rats with either phenobarbital or Aroclor 1254 increased the metabolism of 2,4-DNT to 2,4-dinitrobenzyl alcohol by 6 to 7 fold, suggesting that oxidative metabolism of 2,4-DNT to 2,4-dinitrobenzyl alcohol was mediated by cytochrome P-450- dependent mixed-function oxidases.

Mori *et al.* (1984b) examined the metabolism of 2,4-DNT by rat liver microsomal and cytosol fractions, under a nitrogen atmosphere, in order to determine whether 2,4-diaminotoluene is produced as a metabolite of 2,4-DNT in rat liver and to characterize the enzymes responsible for the reduction. Data obtained from thin-layer and high pressure chromatography indicated that metabolites produced by microsomal preparations were 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene. This microsomal activity is probably mediated by cytochrome P450 because the reduction is blocked by carbon monoxide and primary amines (aniline, n-octylamine and 2,4-dichoro-6-phenylphenoxyethylamine). In contrast, 2,4-DNT was metabolized via 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene to 2,4-diaminotoluene by citosolic preparations. The greatest part of the reduction activity was due to cytosolic xanthine

oxidase because the reduction was blocked by allopurinol. These results suggest that reduction of 2,4-DNT to 2,4-DAT by cytosolic xanthine oxidase may play a role in 2,4-DNT hepatocarcinogenicity.

Shoji *et al*. (1987) investigated the metabolism of 2,4-DNT, 2,4-dinitrobenzyl alcohol and 2,4-dinitrobenzaldehyde in liver microsomal and cytosolic fractions from male Sprague-Dawley rats. The objectives were to determine whether 2,4-dinitrobenzaldehyde, a potent mutagen, is produced in the oxidation of 2,4-dinitrobenzoic acid and to clarify the nature of the enzymes responsible for the oxidation of 2,4-DNT to 2,4-dinitrobenzyl alcohol, 2,4-dinitrobenzaldehyde and 2,4-dinitrobenzoic acid. Data obtained from HPLC indicated that the major products of 2,4-DNT, 2,4-dinitrobenzyl alcohol and 2,4-dinitrobenzaldehyde in the microsomal and cytosol preparations were 2,4-dinitrobenzyl alcohol, 2,4-dinitrobenzaldehyde and 2,4-dinitrobenzoic acid, and 2,4-dinitrobenzyl alcohol, respectively. Results indicate that 2,4-dinitrobenzaldehyde is an intermediate in the oxidation of 2,4-dinitrobenzyl alcohol to 2,4-dinitrobenzoic acid. In addition, data obtained by incubating 2,4-DNT, 2,4-dinitrobenzyl alcohol or 2,4-dinitrobenzaldehyde with microsomal or cytosol fractions under air, nitrogen and various concentrations of CO in oxygen, using cofactors [NAD (P) and NAD(P)H] and inhibitors (SKF-525A, DMSO, choral hydrate, allopurinol, pyrazole and *o*-phenanthroline) suggest that: a) oxidation of 2,4-DNT to 2,4-dinitrobenzyl alcohol is mediated by microsomal cytochrome P450; b) oxidation of 2,4-dinitrobenzyl alcohol to 2,4-dinitrobenzaldehyde is mediated mainly by cytochrome P450 and NAD-dependent alcohol dehydrogenase; and c) oxidation of 2,4-dinitrobenzaldehyde to 2,4-dinitrobenzoic acid and reduction of 2,4-dinitrobenzaldehyde to 2,4-dinitrobenzyl alcohol may be mediated by NAD(P)-dependent aldehyde dehydrogenases and NAD(P)H-dependent aldehyde reductases, respectively. These results indicate that 2,4-DNT is metabolized stepwise to 2,4-dinitrobenzyl alcohol, 2,4-dinitrobenzaldehyde and 2,4-dinitrobenzoic acid in the rat liver and suggest that the oxidation of 2,4-dinitrobenzyl alcohol to 2,4-dinitrobenzaldehyde is a metabolic activation of 2,4-DNT and that the microsomal cytochrome P-450 and alcohol dehydrogenase may play an important role in the metabolic activation of 2,4-DNT.

Mori *et al.* (1989) investigated the metabolism of 2,4-DNT, 2,4-dinitrobenzyl alcohol and 2,4-dinitrobenzaldehyde by liver microsomal and cytosolic fractions prepared from male Wistar and male Sprague-Dawley rats, in the presence of either NAD(P) or NAD(P)H. Products from 2,4-DNT, 2,4-dinitrobenzyl alcohol and 2,4-dinitrobenzaldehyde metabolism were 2,4-dinitrobenzyl alcohol, 2,4-dinitrobenzaldehyde and 2,4-dinitrobenzoic acid, and 2,4-dinitrobenzyl alcohol, respectively. Therefore, it is concluded that 2,4-dinitrobenzaldehyde is an intermediate in the oxidation of 2,4-dinitrobenzyl alcohol to 2,4-dinitrobenzoic acid, and that the oxidation of 2,4-dinitrobenzyl alcohol to 2,4-dinitrobenzaldehyde and the reduction of 2,4-dinitrobenzaldehyde to 2,4-dinitrobenzyl alcohol is reversible. The activity for the reduction of 2,4-dinitrobenzaldehyde to 2,4-dinitrobenzyl alcohol was the highest among all the reactions examined in both strains, and this activity in Wistar rats was higher than that in Sprague-Dawley rats.

b) Isolated hepatocytes and whole livers

Bond and Rickert (1981) investigated the metabolism of 2,4-DNT by freshly isolated primary hepatocytes from male Fischer 344 rats (untreated or pretreated with sodium phenobarbital, 3-methylcholantrene or Arochlor 1254). Incubations vials contained ^{14}C-2,4-DNT (100 µM), 2 mL of hepatocyte suspension and sufficient Hanks balanced salt solution pH 7.4 to bring the total reaction volume to 5 mL. Kinetic studies with respect to 2,4-DNT were performed over the concentration range of 25-200 µM. When the effects of modifiers on oxidative

metabolism were studied, 7,8-benzoflavone or SKF 525-A dissolved in DMSO were added. Sample blanks consisted of heat-inactivated cells. The major metabolite detected by HPLC was 2,4-dinitrobenzyl alcohol, which accounted for 75-80% of the total metabolites formed. The apparent K_m and V_{max} for the formation of 2,4-dinitrobenzyl alcohol was 58 µmol and 25.5 nmoles/10^6 cells/30 min, respectively. Formation of 2,4-dinitrobenzyl alcohol was enhanced by treatment of rats with Arochlor 1254 (6-fold), sodium phenobarbital (3.5-fold) and 3-methylcholantrene (3.5-fold). *In vitro* additions of either SFK 525-A or 7,8-benzoflavone inhibited the formation of 2,4-dinitrobenzyl alcohol. Hepatocytes incubated under decreased oxygen concentrations (15, 10 or 5% O_2 in N_2) displayed higher levels of reductive metabolism of 2.4-DNT (up to 5-fold) than when incubated in air. Concomitant decreases in oxidative metabolism of 2,4-DNT were observed in these experiments. Hepatocyte metabolism of 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene, two major products of rat cecal metabolism of 2,4-DNT, to 2-(N-acetyl)amino-4-nitrotoluene and 4-(N-acetyl)amino-2-nitrotoluene, respectively, was observed. Further metabolism of these compounds to other metabolites such as 2,4-diaminotoluene was not detected. Incubation of hepatocytes with 2,4-DAT produced no detectable metabolites. These results suggest that hepatic reductive metabolism of 2,4-DNT probably plays a minor role in the overall metabolic scheme of 2,4-DNT.

The sex-dependent metabolism and biliary excretion of 2,4-DNT was evaluated by Bond *et al.* (1981) in isolated perfused livers from male and female Fischer-344 rat. Rats were anesthetized with methoxyfluorane and an abdominal midline incision was made. The bile duct was cannulated with PE-10 tubing. The hepatic portal vein was cannulated, the thoracic aorta was severed and the liver was perfused with an oxygenated fortified Hanks-Henseleit salt solution. The liver was removed, placed in a liver perfusion chamber and perfused (recirculating) with the Hanks-Henseleit salt solution. Bilirubin was added to the perfusate to stimulate initial bile flow. All livers were perfused for 30 min before the addition of ^{14}C-2,4-DNT (20 or 70 µmol) and only livers in which the bile flow rate was at least 0.4 µL/g of liver per min were used. Aliquots were removed from the perfusate reservoir at 5, 20, 15, 30, 45, 60, 90 and 120 min after addition of ^{14}C-2,4-DNT. Bile was collected over 15-min time intervals for the 1st h and over 30-min intervals from the 2nd h after ^{14}C-2,4-DNT addition to the perfusate. Livers from both sexes displayed a capacity for oxidation, reduction, acetylation and conjugation of 2,4-DNT (or its metabolites). Oxidation of 2,4-DNT to 2,4-dinitrobenzyl alcohol followed by glucuronidation to 2,4-dinitrobenzyl alcohol glucuronide was the major route of 2,4-DNT metabolism in both sexes. Formation of 2,4-dinitrobenzyl alcohol glucuronide accounted for 10 to 30% of the total initial 2,4-DNT concentration in perfusates. After perfusion with 20 µM 2,4-DNT, male livers excreted larger quantities of 2,4-dinitrobenzyl alcohol glucuronide in the bile (392 nmol) than female livers (172 nmol); at the same 2,4-DNT concentration, perfusates from female livers contained over 3 times as much 2,4-dinitrobenzyl alcohol glucuronide as male perfusates. These data suggested that female livers transported 2,4-dinitrobenzyl alcohol glucuronide into the bile at slower rates than male livers. At 70 µM 2,4-DNT, the amounts of 2,4-dinitrobenzyl alcohol glucuronide in the bile and perfusate were similar in both sexes. The transport of 2,4-dinitrobenzyl alcohol glucuronide into the bile of male, but not female, livers appeared to be saturated after perfusion with 70 µmol 2,4-DNT. In conclusion, the major difference in the *in vitro* metabolism of 2,4-DNT between males and females is the larger quantities of dinitrobenzyl alcohol glucuronide excreted in the bile of males than females.

c) Intestinal microflora

Guest *et al.* (1982) investigated the metabolism of 2,4-DNT by intestinal microflora from male and female Fischer 344 rats. ^{14}C-2,4-DNT was incubated with cecal contents from rats, under either anaerobic or aerobic conditions; the incubation times ranged from 1 to 240 min. 2,4-DNT was not metabolized in the presence of oxygen. Under anaerobic conditions, 2,4-DNT was rapidly metabolized. In preliminary experiments, utilizing cecal microflora from males and three concentrations of 2,4-DNT (25, 100 or 500 μM), the pattern of metabolites produced was independent of substrate concentration. Furthermore, at the lowest concentration, all 2,4-DNT was consumed within 10 min; at the two higher concentrations, the rate of metabolism was independent of the substrate concentration at 10 min. Thus, all subsequent experiments were performed at 100 μM. When 2,4-DNT (100 μM) was incubated with cecal flora from males, 2,4-DNT was completely consumed by 20 min. The rate of disappearance of 2,4-DNT after 20 min was about 9.9 μmol/min/g. The first metabolites to appear were 2-nitro-4-nitrosotoluene and 4-nitro-2-nitrosotoluene which reached maximal concentrations between 20 and 30 min. As the concentrations of nitroso compounds declined, the concentration of 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene increased, reaching maxima by 60 min. As the concentrations of aminonitro compounds declined, the concentration of diaminotoluene increased. When cecal microflora was incubated with either 2A4NT or 4A2NT, the only product which could be detected was 2,4-DAT. Of the two aminonitro compounds, 2A4NT was more rapidly metabolized to 2,4-DAT. The amount of 2,4-DAT formed did not totally account for the disappearance of either substrate. When 2,4-DAT was added to cecal microflora, a 60% loss of 2,4-DAT was observed over a 2-h incubation. There were no sex-dependent differences in the pathway of metabolism. The reduction of the nitroso compounds to amino compounds and of the amino compounds to the diamino compound is presumed to involve hydroxylamino intermediates which could not be isolated. It is concluded that the intestinal microflora represent the major site of reductive metabolism of 2,4-DNT and may play an important role in the carcinogenic action of 2,4-DNT.

The metabolism of 2,4-DNT by cecal microflora from male Fischer 344 rats was investigated by Dent *et al*. (1981). ^{14}C-2,4-DNT at 100 μM was incubated with 50 mg of cecal content under either aerobic or anaerobic conditions. Cecal microflora did not metabolize 2,4-DNT in an air atmosphere. However, under anaerobic conditions this microflora rapidly reduced 2,4-DNT. Both 2- and 4-nitroso-nitrotoluenes were detected. The cecal microflora reduced the nitro group at the 4 position more rapidly than the nitro group at the 2 position. The amount of 4-nitroso compound exceeded the amount of 2-nitroso compound by a factor of 2 after 10 min of incubation. 4-amino-2-nitrotoluene was present in a 4-fold excess over 2-amino-4-nitrotoluene after 60 min incubation and significant amounts of 2,4-diaminotoluene were detected at this time. The time course of metabolism in cecal microflora showed that 2,4-diaminotoluene was produced by further reduction of the aminonitrotoluenes. When unlabelled 2A4NT or 4A2NT was added to anaerobic incubations of cecal microflora, DAT was produced with either aminonitrotoluene as substrate. These results indicate that the cecal microflora metabolize 2,4-DNT via an ordered sequence of reductive steps. It was proposed that both reductions from nitroso-nitrotoluenes to amino-nitrotoluenes and from amino-nitrotoluenes to diaminotoluene involve the formation of hydroxylamine intermediates. However, none of the proposed intermediates could be isolated.

Mori *et al.* (1985) also examined the intestinal microflora metabolism of 2,4-DNT in male Wistar rats. The study was carried out, under either aerobic or anaerobic conditions, using 2,4-DNT concentrations of 25, 50 or 100 μM and preparations of microflora from the contents of the rat caecum. 2,4-DNT was not metabolised by cecal microflora of rats in the presence of oxygen. However, under anaerobic conditions, 2,4-DNT was reduced to 2-amino-4-

nitrotoluene and 4-amino-2-nitrotoluene via 2-hydroxylamino-4-nitrotoluene and 4-hydroxylamino-2-nitrotoluene. These two aminonitrotoluenes were further reduced to 2,4-diaminotoluene. These data induced to postulate that rat intestinal microflora catalyses the reductive metabolism of 2,4-DNT, suggesting that the reduction of 2,4-DNT to 2,4-diaminotoluene may play an important role in the carcinogenicity of 2,4-DNT.

Mice

a) Subcelular hepatic or other fractions

Lee *et al.* (1978) investigated the *in vitro* metabolism of 2,4-DNT by liver homogenate from male and female CD-1 mice under either aerobic or anaerobic incubation conditions. Four CD mice/sex were sacrificed and the livers removed, weighed, homogenized and centrifuged at 10000 g for 30 min. The incubation medium contained 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.8 mM nicotinamide adenine dinucleotide phosphate, 1 mM ^{14}C-2,4-DNT (0.1 µg/mL), 1.0 mL of 0.2 M tris-HCl pH 7.4, and 0.5 mL of the 10,000 g liver supernatant for a final volume of 2.5 mL. Reactions were conducted for 1 hour. Aerobic and anaerobic reactions as well as quantification of analytes were performed according to acceptable methods. Under aerobic conditions, 2,4-dinitrobenzyl alcohol was the major metabolite in both sexes; females produced more 2,4-dinitrobenzyl alcohol than did males (Table 4.1.2.1.1.-9.). Under anaerobic conditions, the amount of 2,4-dinitrobenzyl alcohol was reduced while the amount of aminonitrotoluenes was increased; males produced more 2,4-/4,2-aminonitrotoluenes than did females (Table 4.1.2.1.1-9.). Despite the anaerobic conditions, the amount of 2,4-dinitrobenzyl alcohol produced by females was higher than that of aminonitrotoluenes, which did not occur in those of males. This study was published in Experientia by Short, Dacre and Lee (1979).

Table 4.1.2.1.1-9: Aerobic and anaerobic metabolism of 2,4-DNT (ring-UL-^{14}C) by livers of mice (Lee *et al.*, 1978)

	Metabolic activity (nmol/mg protein)			
	Aerobic		Anaerobic	
Compounds	**Male**	**Female**	**Male**	**Female**
2,4-dinitrobenzyl alcohol	11.0	16.6	8.4	13
Aminonitrotoluenes	4.0	1.3	10	5
2,4-DNT	107	120	103	120
Others	3.9	3.4	3.8	3.1

Schut *et al.* (1985) investigated the metabolism of 2,4-DNT by liver and lung microsomes of A/J mice. Metabolism of 2,4-DNT by both liver and lung microsomes yielded mainly 2,4-dinitrobenzyl alcohol with lower amounts of 4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene, and their formation was dependent on the presence of oxygen and NAD(P)H. Pre-treatment of mice with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin resulted in increased yields of all three metabolites.

b) Intestinal microflora

Guest *et al.* (1982) investigated the metabolism of 2,4-DNT by intestinal microflora from male Swiss-Webster mice. ^{14}C-2,4-DNT (100 µM) was incubated with cecal contents from

mice, under either anaerobic or aerobic conditions; the incubation times ranged from 1 to 240 min. 2,4-DNT was not metabolized in the presence of oxygen. Under anaerobic conditions, cecal microflora metabolize 2,4-DNT to reduced products. The rate of disappearance of 2,4-DNT after 20 min was > 25 µmol/min/g. The metabolites identified in 20-min incubations were 4-nitro-2-nitrosotoluene, 2-nitro-4-nitrosotoluene, 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene; diaminotoluene was detected in 60 min. In summary, an ordered sequence of reductive metabolism was observed. The 2- and 4-nitro groups were reduced to amino groups via nitroso intermediates which were identified by GC-MS. The reduction of the nitroso intermediate to the amino compound is presumed to involve a hydroxylamino intermediates which could not be isolated. The aminonitrocompounds were then reduced to diaminotoluene; no intermediates in this sequence could be isolated. It is concluded that the intestinal microflora represent an important site of reductive metabolism of 2,4-DNT and may play an important role in the carcinogenic action of 2,4-DNT.

Schut *et al.* (1985) investigated the metabolism of 2,4-DNT by small and large intestine explants or by cecal contents of A/J mice. Aerobic metabolism of 2,4-DNT by small and large intestine explants or by cecal contents yielded 2,4-dinitrobenzyl alcohol, 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene and 4-(N-acetyl)amino-2-nitrotoluene. The proportion of the reduced metabolites, 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene and 4-(N-acetyl) amino-2-nitrotoluene, was much higher in these systems than with liver or lung microsomes and their formation by small intestine and cecal contents was enhanced several-fold under anaerobic conditions, while that of 2,4-dinitrobenzyl alcohol was abolished.

Rabbits

a) Subcelular hepatic fractions

Lee *et al.* (1978) investigated the *in vitro* metabolism of 2,4-DNT by liver homogenate from male and female New Zealand albino rabbits under either aerobic or anaerobic incubation conditions. 4 rabbits/sex were sacrificed and the livers removed, weighed, homogenized and centrifuged at 10000 g for 30 min. The incubation medium contained 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.8 mM nicotinamide adenine dinucleotide phosphate, 1 mM ^{14}C-2,4-DNT (0.1 µg/mL), 1.0 mL of 0.2 M tris-HCl pH 7.4, and 0.5 mL of the 10,000 g liver supernatant for a final volume of 2.5 mL. Reactions were conducted for 1 hour. Aerobic and anaerobic reactions as well as quantification of analytes were performed according to acceptable methods. No sex differences, under aerobic conditions, were found in 2,4-DNT metabolism; 2,4-dinitrobenzyl alcohol was the major metabolite in both sexes (Table 4.1.2.1.1.-10.). Under anaerobic conditions, the amount of 2,4-dinitrobenzyl alcohol was reduced while the amount of aminonitrotoluenes was increased; males produced more 2,4-/4,2-aminonitrotoluenes than did females (Table 4.1.2.1.1-10.). Despite the anaerobic conditions, the amount of 2,4-dinitrobenzyl alcohol produced by females was almost two times higher than that of 2,4-/4,2-aminonitrotoluenes. This study was published in Experientia by Short, Dacre and Lee (1979).

Table 4.1.2.1.1-10: Aerobic and anaerobic metabolism of 2,4-DNT (ring-UL-^{14}C) by livers of rabbits (Lee *et al.*, 1978)

	Metabolic activity (nmol/mg protein)			
	Aerobic		Anaerobic	
Compounds	**Male**	**Female**	**Male**	**Female**
2,4-dinitrobenzyl alcohol	21.5	28.8	16.8	24.4
Aminonitrotoluenes	7.6	8.3	20.3	13.7
2,4-DNT	123	124	116	124
Others	5.4	6.4	5.1	5.2

Dogs

a) Subcelular hepatic fractions

Lee *et al.* (1978) investigated the *in vitro* metabolism of 2,4-DNT by liver homogenate from male and female Beagle dogs under either aerobic or anaerobic incubation conditions. Four males and three females were sacrificed and the livers removed, weighed, homogenized and centrifuged at 10000 g for 30 min. The incubation medium contained 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.8 mM nicotinamide adenine dinucleotide phosphate, 1 mM ^{14}C-2,4-DNT (0.1 μg/mL), 1.0 mL of 0.2 M tris-HCl pH 7.4, and 0.5 mL of the 10,000 g liver supernatant for a final volume of 2.5 mL. Reactions were conducted for 1 hour. Aerobic and anaerobic reactions as well as quantification of analytes were performed according to acceptable methods. No sex differences, under aerobic conditions, were found in 2,4-DNT metabolism; 2,4-dinitrobenzyl alcohol was the major metabolite in both sexes (Table 4.1.2.1.1-11). Under anaerobic conditions, the amount of 2,4-dinitrobenzyl alcohol was reduced while the amount of aminonitrotoluenes was increased; males produced more 2,4-/4,2-aminonitrotoluenes than did females (Table 4.1.2.1.1-11). Under anaerobic conditions, males produced similar amounts of 2,4-dinitrobenzyl alcohol and 2,4-/4,2-aminonitrotoluenes; nevertheless, in females the amount of 2,4-dinitrobenzyl alcohol was 10 times higher than that of 2,4-/4,2-aminonitrotoluenes. This study was published in Experientia by Short, Dacre and Lee (1979).

Table 4.1.2.1.1-11: Aerobic and anaerobic metabolism of 2,4-DNT (ring-UL-^{14}C) by livers of dogs (Lee *et al.*, 1978)

	Metabolic activity (nmol/mg protein)			
	Aerobic		Anaerobic	
Compounds	**Male**	**Female**	**Male**	**Female**
2,4-dinitrobenzyl alcohol	17.1	14	8.6	11.4
Aminonitrotoluenes	0.7	0.6	8	1.1
2,4-DNT	116	126	117	128
Others	1.5	1.6	1.1	1.2

Monkeys

a) Subcelular hepatic fractions

Lee *et al.* (1978) investigated the *in vitro* metabolism of 2,4-DNT by liver homogenate from male and female Rhesus monkeys under either aerobic or anaerobic incubation conditions. Three males and one female were sacrificed and the livers removed, weighed, homogenized and centrifuged at 10000 g for 30 min. The incubation medium contained 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.8 mM nicotinamide adenine dinucleotide phosphate, 1 mM ^{14}C-2,4-DNT (0.1 μg/mL), 1.0 mL of 0.2 M tris-HCl pH 7.4, and 0.5 mL of the 10,000 g liver supernatant for a final volume of 2.5 mL. Reactions were conducted for 1 hour. Aerobic and anaerobic reactions as well as quantification of analytes were performed according to acceptable methods. No sex differences, under aerobic conditions, were found in 2,4-DNT metabolism; the amount of 2,4-dinitrobenzyl alcohol was slightly higher than 2,4-/4,2-aminonitrotoluenes in both sexes (Table 4.1.2.1.1.-12.). Under anaerobic conditions, the same result was found in females. Nevertheless, the amount of 2,4-/4,2-aminonitrotoluenes was markedly increased in males as compared with that of 2,4-dinitrobenzyl alcohol (Table 4.1.2.1.1-12.). Under anaerobic conditions, the metabolism pathway in males was reduction of 2,4-DNT to 2,4-/4,2-aminonitrotoluenes rather than oxidation to 2,4-dinitrobenzyl alcohol. This study was published in Experientia by Short, Dacre and Lee (1979).

Table 4.1.2.1.1-12: Aerobic and anaerobic metabolism of 2,4-DNT (ring-UL-14C) by livers of monkeys (Lee et al., 1978)

	Metabolic activity (nmol/mg protein)			
	Aerobic		Anaerobic	
Compounds	**Male**	**Female**	**Male**	**Female**
2,4-dinitrobenzyl alcohol	20.1	25	13.4	26.8
Aminonitrotoluenes	15.8	17.3	55.8	22.1
2,4-DNT	128	84	89.3	78
Others	7.2	10.6	13.9	9.8

Yeast

Kozuka *et al*. (1978) demonstrated the ability of *Rhodotorula glutinis* for metabolising 2,4-DNT (added to the cell suspension as 2 mL of 1% ethanol solution) to 2-amino-4-nitrotoluene (2% of the concentration) and 4-amino-2-nitrotoluene (14% of the concentration) plus two unidentified hydroxylamine compounds.

Other information

a) 2,4-DNT no-specific toxicokinetic studies

The following studies are not specific toxicokinetic studies but provide information related to metabolism. Therefore, they have been included at this section.

Lee *et al.* (1978) carried out a study in order to assess the effect of 2,4-DNT on drug metabolizing enzymes. Activities of these enzymes were assayed both *in vivo* by the zoxazolamine paralysis time and *in vitro* by the hepatic nitroanisole O-demethylase activity.

Male CD rats were fed diets that contained 0.7% of 2,4-DNT for 2 weeks (145 mg/kg b.w./day). Rats in the positive control group received 50 mg/kg b.w. of phenobarbital sodium twice daily for 3 days. Rats in the negative control were no pre-treated. Zoxazolamine was administered i.p. to rats at a dose of 45 mg/kg bw. The duration of paralysis was measured in terms of the loss of the righting reflex. When compared with no pre-treated rats, phenobarbital pre-treatment decreased the duration of zoxazolamine paralysis while 2,4-DNT pre-treatment did not significantly affect the paralysis duration. The metabolism of nitroanisole by livers was measured in an *in vitro* system. Rats were sacrificed and the livers removed, weighed, homogenized and centrifuged at 9000 g for 30 min. The incubation medium contained 15 μmoles $MgCl_2$, 15 μmoles glucose-6-phosphate, 3 μmoles p-nitroanisole, 0.5 mL of the 9000 g liver supernatant, and 0.5 mL of 0.5 M sodium phosphate buffer pH 7.8. Reactions were conducted for 20 min. The product formed was measured spectrophotometrically at 420 nm. Quantification of both p-nitrophenol and protein content was performed according to acceptable methods. Two weeks of 0.7% of 2,4-DNT in the diet did not change the *in vitro* ability of the liver to convert nitroanisole to nitrophenol. In summary, feeding 0.7% of 2,4-DNT to male rats for two weeks neither affected the liver enzymes involved in the metabolism *in vivo* of zoxazolamine nor affected the *in vitro* liver nitroanisole O-demethylase activity. This study was published in Experientia as a short communication (Short and Lee, 1980).

The influence of dietary 2,4-DNT on the reductase activity was investigated by Mori, Naruse and Kozuka (1980) using incubations of ^{3}H-2,4-DNT with liver homogenate from control or 20 min treated rats, under anaerobic conditions. 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene were used as reduction metabolites of 2,4-DNT. The amounts of ^{3}H-2A4NT, ^{3}H-4A2NT and ^{3}H-2,4-DNT were 22, 50 and 27% in control rats and 12.5, 30 and 56% in treated rats. From these results is evident that the reductase activity in the liver of treated rats was much lower than that in the liver of control rats.

The effects of 2,4-DNT on xenobiotic metabolizing enzymes and the hepatic metabolism and covalent binding of this compound to microsomal proteins *in vitro* were studied by Decad *et al.* (1982). Four groups of male Fischer 344 rats were used: a) rats administered by gavage 2,4-DNT dissolved in corn oil for 5 consecutive days at doses of 14, 35 and 70 mg/kg b.w.; b) rats administered sodium phenobarbital i.p. at a dose of 80 mg/kg/day for 3 days; c) rats given a single i.p. injection of Arochlor 1254 in corn oil 5 days prior to death; d) control rats received i.p. doses of saline or corn oil. Assays for biphenyl 4-hydroxylase, NADPH cytocrome *c* reductase, aryl hydrocarbon hydroxylase and *p*-nitrobenzoic acid reductase activities were performed on freshly prepared microsomes; and methyl red azoreductase activity was measured in freshly prepared cytosol. Cytochrome P450, cytochrome b_5 and total microsomal heme concentrations were determined within 48 h. For metabolism of 2,4-DNT, microsomes or cytosol from control or pretreated rats were incubated with 200 nmol of ^{14}C-2,4-DNT dissolved in DMSO; the final protein concentration was 2 mg/mL. All incubations were carried out under either aerobic or anaerobic conditions. Radioactivity bound to microsomal protein was assessed by exhaustive extraction. Pre-treatment with 2,4-DNT increased liver oxygen-insensitive cytosolic azoreductase activity and decreased microsomal nitroreductase activity. A small but significant increase in liver/b.w. ratio and in hepatic cytochromes P450 and b_5 occurred in the absence of changes in microsomal biphenyl 4-hydroxylase or aryl hydrocarbon hydroxylase activities. The pattern of *in vitro* microsomal metabolism of 2,4-DNT was dependent on oxygen tension; under aerobic conditions, 2,4-dinitrobenzyl alcohol was the major metabolite formed, whereas under anaerobic conditions no 2,4-dinitrobenzyl alcohol was detected; 2-amino-4-nitrotoluene and 2-nitro-4-aminotoluene were the major metabolites. Pre-treatment of rats with either phenobarbital or

Aroclor 1254 increased the metabolism of 2,4-DNT to 2,4-dinitrobenzyl alcohol by 6 to 7 fold, suggesting that oxidative metabolism of 2,4-DNT to 2,4-dinitrobenzyl alcohol was mediated by cytochrome P-450-dependent mixed-function oxidases. Covalent binding studies showed that a maximum of only 7 pmol of 2,4-DNT-derived radioactivity was bound per mg of microsomal protein per hour; this binding was increased to 1 nmol bound/mg protein/h in microsomes from Phenobarbital or Arochlor 1254-pretreated rats. It is concluded that 2,4-DNT treatment had little effect on the activity of some hepatic xenobiotic metabolizing enzymes and was readily metabolized by liver preparations *in vitro*; the pathways of *in vitro* metabolism were dependent on oxygen tension; and this *in vitro* metabolism produced mostly polar metabolites which did no bind appreciably to microsomal macromolecules.

Rowland and Mallet (1983) investigated the effects of dietary carrageeenan and pectin on the metabolic activity of the gut microflora towards 2,4-DNT. Male conventional microflora Sprague-Dawley rats were fed either a control purified diet, or the same diet supplemented with 50 g/kg of pectin or iota carrageenan ad libitum for 50 days. Rats were killed and the cecal contents were suspended in anaerobic phosphate buffer. Cecal microbial nitroreductase activity was determined using 2,4-DNT as substrate. Both pectin and carrageenan increased the weight of cecal contents, and pectin also increased the number of bacteria per caecum. In contrast, carrageenan decreased the cecal bacterial population. Carrageenan significantly decreased the rate of reduction of 2,4-DNT. The results demonstrate that microbial reduction of 2,4-DNT may be altered by diet.

Kedderis, Dyroff and Rickert (1984) used the sulfotransferase inhibitors, 2,6-dichloro-4-nitrophenol and pentachlorophenol, to investigate the role of sulphate ester formation during the *in vivo* bioactivation of 2,4-DNT. Male Fischer 344 rats were administered one of the sulfotransferase inhibitors (40 μmol/kg, i.p.) 45 min prior to oral administration of 28 mg/kg of either ^{14}C-2,4-DNT or ^{3}H-2,4-DNT dissolved in corn oil. Rats were killed 12 h later. Livers were excised, minced and stored until analysis. A portion of each liver was homogenized and covalently bound radiolabel was determined using an exhaustive extraction procedure. The sulfotransferase inhibitors decreased the total hepatic macromolecular covalent binding of 2,4-DNT by 33%. These results suggest that sulfation is important in the biotransformation of 2,4-DNT to reactive metabolites which covalently bind to DNA, RNA or proteins.

Mitchell and Burrows (1995) evaluated the HI4IIE hepatoma cell metabolic responses to 2,4-DNT exposure with and without prior P450 monooxigenase induction by a polychlorinated hydrocarbon. 2,3,4,7,8-pentachlorodibenzfuran (PCDBF) induced cytochrome P-450 (1A1) aryl hydrocarbon hydroxylase (AHH) activity over a wide range of concentrations without significant cytotoxic effects in HI4IIE hepatoma cell cultures. 2,4-DNT did not induce activity itself, but its metabolism was considerably altered when applied to PCDBF induced cultures. The production of 4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene was greatly enhanced in induced cultures as compared to uninduced controls, as was the conversion of radiolabeled 2,4-DNT to relatively more polar metabolites.

b) 2,6-DNT toxicokinetic studies

The toxicokinetics of orally, dermally and inhalative administered 2,6-dinitrotoluene on male F 344 rats was investigated (Hawkins *et al*., 1991). Nine animals were given 1, 7 or 25 mg ^{14}C-2,6-dinitrotoluene (dissolved in polyethylene glycol)/ kg bw orally or dermally (on 10 cm^2 of shaven dorsal skin, application time 6 h, occlusive). A further 9 animals were exposed to a ^{14}C-2,6- dinitrotoluene vapour concentration of 5 or 10.5 mg/m^3 for 6 hours, equivalent to a substance uptake of 1.6 or 7 mg/kg bw. In each group 3 animals were killed 6, 30 and 54 hours after beginning of application and distribution of radioactivity in the bodies was

investigated. Additionally, the radioactivity in urine and faeces was measured. After oral administration, 70-75% was excreted in urine and approximately 20% with the faeces, irrespective of dosage; approximately 5% of the dose remained in the body tissues (excluding the gastro-intestinal tract). Thus, absorption rate was 70-80% of the dose. After dermal application, 3-5% was excreted in urine and approximately 1% with faeces, irrespective of dosage; 0.3-1.1% was found in the body tissues. Thus, only 5-7% of the dose was taken up via the skin. After the uptake through inhalation, 51% of the recovered radioactivity was eliminated with urine and 25% with faeces; 24-33% remained in the body (including skin and fur). However, the authors presume that the high retention of radioactivity in skin and fur is a result of a contamination during dosage. The relative distribution of radioactivity in tissues was independent of application route, dose and absorption rate. The highest concentration was measured in kidneys (killing after 6 h) and liver (killing after 54 h); lower concentrations wore measured in lung, spleen, plasma and whole blood. The testes had the lowest content of radioactivity. The radioactivity in the lung after inhalation was not higher than after oral or dermal application of comparable doses. This is an indication of rapid absorption via the respiratory system. Irrespective of the means of application, approximately 60-70% of radioactivity found in liver proved to be non extractable and therefore, were obviously bound covalently to macromolecules. This study has thus shown that the dominant route of 2,6-dinitrotoluene uptake was via the gastro-intestinal tract and the respiratory system and that dermal absorption plays a less significant role.

4.1.2.1.2 Human data

In vivo studies

Human data derived from three studies carried out in workers exposed to technical-grade DNT.

Turner *et al.* (1985) identified metabolites of 2,4-DNT in the urine of workers exposed to technical-grade DNT (about 80% 2,4-DNT and 20% 2,6-DNT) in a DNT production plant. Metabolites of 2,4-DNT were 2,4-dinitrobenzoic acid, 2-amino-4-nitrobenzoic acid, 2,4-dinitrobenzyl alcohol glucuronide and 2-(N-acetyl)amino-4-nitrobenzoic acid. In addition, the urine contained unchanged 2,4-DNT. The most abundant metabolites were 2,4-dinitrobenzoic acid and 2-amino-4-nitrobenzoic acid, accounted collectively for 74-86% of the DNT metabolites detected. The excretion peak of these metabolites was reached near the end of the work shift, but declined to either very low or non detectable concentrations by the beginning of the following labour day. The elimination half-live of these metabolites ranged between 0.8 and 4.5 hours. The half-lives for 2,4-dinitrobenzoic acid and 2,4-dinitrobenzyl alcohol glucuronide tended to be shorter than those for the metabolites that resulted from both oxidative and reductive metabolism All data collected indicated that urinary metabolites of DNT in humans are qualitatively similar to those found in rats, although quantitative differences exist in the relative amounts of each excreted metabolite.

Levine *et al.* (1985) studied a group of 17 workers exposed to technical-grade DNT (a mixture of 76.4% 2,4-DNT, 18.8% 2,6-DNT and 4.8% other isomers) in a DNT production plant, where personal air sampling revealed levels ranging from 0.6 to 5.9 mg/10 m^3 (0.06-0.59 mg/m^3). 2,4-DNT metabolites were 2,4-dinitrobenzoic acid, 2,4-dinitrobenzyl alcohol glucuronide, 2-amino-4-nitrobenzoic acid, 2-(N-acetyl)amino-4-nitrobenzoic acid and trace amounts of 4-amino-2-nitrobenzoic acid and 4-(N-acetyl)amino-2-nitrobenzoic. They were qualitatively similar to those found in rats, although quantitative differences exist in the

relative amounts of each excreted metabolite. Possibly, there was also a sex difference as regards the pattern of metabolites; three women appeared to excrete relatively more 2,4-dinitrobenzyl alcohol glucuronide than 14 men (33.3% versus 9.5% of all metabolites). The urine contained more metabolites than would have resulted from DNT present in the inhaled air, which strongly indicated dermal absorption. The peak rate of excretion was likely to occur toward the end of the work day or shortly afterwards. A rough estimate of the maximal absorbed daily dose encountered indicated exposure of 0.24-1.00 mg/kg bw in one worker.

Woollen *et al.* (1985) carried out two biomonitoring studies in workers exposed to technical grade DNT in an explosive factory, using urinary metabolites as exposure markers. 2,4-dinitrobenzoic acid was the major metabolite of 2,4-DNT found in urine, and appeared within hours of the onset of exposure. Lesser amounts of 2-amino-4-nitrobenzoic acid, 4-amino-2-nitrobenzoic acid and 4-(N-acetyl)amino-2-nitrobenzoic acid could be also recorded. Nevertheless, the authors speculate with the possibility that 4-amino-2-nitrobenzoic acid may be an artefact produced by the reduction of 2,4-dinitrobenzoic acid during storage and transportation of the samples. Trace levels of 2,4-DNT were also detected but not 2,4-dinitrobenzyl alcohol. In the first study, urine samples of 28 workers (20 males and 8 females) were examined for the presence of 2,4-dinitrobenzoic acid. Metabolite concentrations in urine were extremely low (< 1 mg/L) or non-detectable, prior to starting the work at the beginning of the working week, but the weekly mean concentration for post-shift samples was 17±9.8 mg/L. The levels varied considerably between workers and in individual workers from day to day; however there was no difference in the excretion of this metabolite between males and females. The elimination half-life of 2,4-dinitrobenzoic acid was 2-5 h. However, traces could still be detected after several days, which indicated the presence of a slow compartment. The atmospheric levels of DNT were always lower than the maximum allowed, suggesting that the skin may be a major route of absorption. In the second study, an intensive urine sampling was carried out on five workers over a period of 2 days, with additional samples over the subsequent 2 non working days. Analysis of these urine samples demonstrated that the excretion rate of 2,4-dinitrobenzoic acid rises quite quickly during the working period, indicating that DNT is both absorbed and metabolised. The highest excretion rate was usually recorded near to the end of the working shift.

In vitro studies

Available data derived from two studies which investigated the metabolism of 2,4-DNT by the human intestinal microflora.

Guest *et al.* (1982) investigated the metabolism of 2,4-DNT by human intestinal microflora. Both ileal contents and faeces from male volunteers were collected, processed and incubated with ^{14}C-2,4-DNT (100 µM); the incubation times ranged from 1 to 240 min. 2,4-DNT was not metabolized in the presence of oxygen. Under anaerobic conditions, cecal microflora metabolize 2,4-DNT to reduced products. The rate of disappearance of 2,4-DNT after 20 min was 1.5 and 9 µmol/min/g in the two faecal samples and 1.5 µmol/min/g in the only ileal sample. The metabolites identified in 20-min incubations were 4-nitro-2-nitrosotoluene, 2-nitro-4-nitrosotoluene, 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene; diaminotoluene was not detected in human samples until 240 min in faeces. In summary, an ordered sequence of reductive metabolism was observed. The 2- and 4-nitro groups were reduced to amino groups via nitroso intermediates which were identified by GC-MS. The reduction of the nitroso intermediate to the amino compound is presumed to involve a hydroxylamino intermediates which could not be isolated. The aminonitrocompounds were then reduced to diaminotoluene; no intermediates in this sequence could be isolated. It is concluded that the

intestinal microflora represent an important site of reductive metabolism of 2,4-DNT and may play an important role in the carcinogenic action of 2,4-DNT.

Mori *et al.* (1984a) identified the metabolites produced from ^{3}H-2,4-DNT by *E. coli* strain W3110, isolated from human intestine, using aerobic incubation conditions. Data obtained from thin-layer and gas chromatographies or liquid scintillation counting indicated that the metabolites produced by *E. coli* were two major, 4-amino-2-nitrotoluene (4A2NT) and 2-amino-4-nitrotoluene (2A4NT), and two minor, 4-hidroxylamino-2-nitrotoluene (4HA2NT) and 2-hydroxylamino-4-nitrotoluene (2HA4NT). Monoaminonitrotoluenes metabolites accounting for 18.8% (4A2NT) and 10.2% (2A4NT) of the substrate added. The first metabolites to appear in the reaction mixture were 4HA2NT and 2HA4NT, which reached maximal concentrations at about 8 h. As the concentrations of hydroxylaminonitrotoluenes declined, there was a corresponding increase in the concentrations of 2A4NT and 4A2NT, reaching maxima by about 24 h. This finding indicates that 2,4-DNT is reduced via hydroxylaminonitrotoluene to monoaminonitrotoluenes in *E. coli*.

Other information

The following study has been included in this section although it is not a specific toxicokinetic study but it provides important information on bioactivation of 2,4-DNT.

In order to determine if quantification of DNT-Hb adducts could used to estimate the risk associated with a particular exposure to DNT, Jones *et al.* (2004) developed a method to measure Hb adducts in biological samples.

Hb adducts were measured in blood samples of Chinese working in a TNT factory. The production of TNT involves the exposure to high levels of the intermediate products NT and DNT, which are far more volatile than TNT. In the Chinese factory, the synthesis of TNT was performed by continual batch nitration of NT, then DNT, with sulphuric acid and nitric acid. The workers were grouped according to their job description and work location as follows: group leader, NT-tank, DNT-tank, TNT-tank, laboratory of chemical analyses, transportation of TNT to packaging, packaging, control room, disposal of waste acid, disposal of waste H_2O and non-exposed control workers. In total, 160 blood samples were collected, 99 from exposed workers and 61 from non-matched, non-exposed controls working in the same factory. All participants filled out a questionnaire and their health status was checked.

In order to corroborate adducts identified in the workers resulting from exposure to 2,4-DNT, Hb adducts were also measured in blood samples of three female Wistar rats dosed 0.5 mmol/kg of 2,4-DNT dissolved in tricaprilin, by gavage. Rats were killed after 24 hours.

The isolated Hb (100 mg human Hb or 20 mg rat Hb) was hydrolyzed in 0.1 M NaOH and extracted in the presence of 2,4-DNT specific deuterated standards: 2-amino-4-nitrotoluene (2A4NT), 2-(N-acetyl)amino-4-aminotoluene (2AA4AT), 4-amino-2-nitrotoluene (4A2NT), 4-(N-acetyl)amino-2-aminotoluene (4AA2AT), 2,4-diaminotoluene (24DAT) and 2,4-DNT. Once extracted, the released arylamines were derivatized with PFPA. In rats, for determining the percentage of non-adducted arylamines present in Hb samples, Hb was also extracted at neutral pH. Analytes were quantified against calibration lines of authentic standards using GC-MS-NCI-SIM. All regression lines fitted with $R^2 > 0.98$.

Hb adducts were found in the exposed workers and in the controls working in the same factory, although the mean of all adducts was significantly higher in the exposed group than in the factory control group using both ANOVA and the Mann–Whitney test ($p < 0.0001$). It appears that the factory environment is contaminated since adducts derived from DNT were

not found in the laboratory controls. In general, the adduct levels decrease in the following order among the worker groups: analysis > TNT-tank > NT-tank > DNT-tank > group leaders > control room workers > packing > factory controls. For workers grouped into waste acid, waste H_2O or transportation of TNT, statistical analysis was not performed because the sample number was too low ($n < 5$). The relative levels of each DNT Hb adduct are closely paralleled between the differently grouped workers. The combined level of Hb adducts, in exposed workers, resulting from exposure to 2,4-DNT was 71 ± 68.9 pmol/g Hb. 4A2NT was the major adduct and present in 99% of workers. Low levels of 24DAT, and in some cases similar amounts of 4AA2AT were also detected. The aminonitrotoluene, reduced at the *ortho* position (2A4NT), and the acetylated amine, 2AA4AT, were not identified in the single ion chromatograms of exposed workers.

In rats, the levels of amines found under neutral hydrolysis conditions ranged between 0.7 and 7% of the levels determined under base hydrolysis conditions. This confirmed that >93% of the adduct levels detected in extracts from base hydrolyzed Hb were a direct result of the cleavage of the sulphinic acid amide bond between the cysteine residues on Hb and the arylamine. Basal hydrolysis of Hb from rats dosed with 2,4-DNT yields three amines, 4A2NT (16.3 nmol/g Hb), 24DAT (4.3 nmol/g Hb) and 4AA2AT (0.51 nmol/g Hb). 2A4NT and 2AA4AT adducts were not present.

Generally, less oxidizable arylamines bind, to a larger extent, to Hb. Nitro groups, which are co-planar with the aromatic ring, like the nitro group in the 4 position of 2,4-DNT, are more easily reduced than nitro groups in the *ortho* position to a methyl group forced out of the plane. This corresponds with the highest adduct level found for 4A2NT in both humans and rats.

A similar Hb adduct pattern resulting from 2,4-DNT exposure was found in rats and Chinese workers. However, the ratio for 4A2NT and 24DAT was 24 in humans and 4 in rats. Quantitatively, 4A2NT was not as prevalent in rats as in humans. This difference indicates that the concomitant reduction of both nitro groups in man was less prevalent than in rat.

In previous studies where the health status of workers exposed to DNT was assessed, the most common complaints recorded were mainly due to the ability of DNT to induce MetHb, the secondary effects of which were non-specific health effects. In this study, each worker was examined for adverse health effects linked to exposure to DNT such as inertia, somnolence, insomnia, headache, dizziness and nausea. The health effects were compared with the Hb adduct levels using logistic regression analysis. The odds of suffering from inertia were 3.2 times higher [95% confidence interval (CI) = 1.8–5.8] when the level of 4A2NT Hb adducts increased by one log-unit. Similar odds ratios (OR) were observed with somnolence (3.1, CI = 1.4–6.9), nausea (2.4, CI = 1.3–4.3) and dizziness (5.5, CI = 1.3–24.2). These results inferred that quantification of DNT–Hb adducts provided an effective biomarker of toxicity and could be used to estimate the risk associated with a particular exposure to DNT.

4.1.2.1.3 Summary of toxicokinetics, metabolism and distribution

Absorption

The available information, based on analyses of the urinary metabolites of workers in DNT production plants (Turner *et al.*, 1985; Levine *et al.,* 1985; Woollen *et al.,* 1985), indicate that 2,4-dinitrotoluene is absorbed in humans. Two studies of occupational exposure to technical-grade DNT have suggested that dermal absorption can be a significant route of entry for 2,4-

DNT in humans since the levels of urinary metabolites of 2,4-DNT in loaders and operators at a DNT production plant exceeded those that would have resulted from the inhaled concentrations (Levine *et al.,* 1985; Woollen *et al.,* 1985).

Several studies on toxicokinetics following oral administration of 2,4-DNT were performed in experimental animals. The oral absorption was determined based on these data and taking into account systemic effects observed in toxicity studies (to see sections corresponding to repeated dose toxicity studies).

A single oral dose of radiolabeled 2,4-DNT was well absorbed by rats, rabbits, dogs and monkeys and poorly absorbed by two strains of mice as indicated by excretion data (Lee *et al.,* 1975, 1978). Radioactivity recovered at the end of 24 hours ranged from 88.5% in rats to 97% in dogs. Most of the radioactivity was excreted in the faeces of mice (about 80% of the dose) while only 11% and 7% was recovered in the urine of CD-1 and B6C3F1 strains, respectively. The remaining species excreted most of the radioactivity in the urine (75-81% of the dose) and only 3-9% in the faeces. No radioactivity was found in the expired air. Increased faecal excretion of radioactivity in mice could the result of poor absorption after ingestion or a rapid absorption and metabolism, excretion in the bile, and non absorption of the metabolites. Several metabolites were found in the urine of all species but no parent compound or only small amounts were detected, indicating that once absorbed, 2,4-DNT was extensively metabolised. In view of the similar pattern of urinary metabolites in the five species studied, a sufficiently different metabolism and excretion of 2,4-DNT through the biliary system of the mice is unlikely. Furthermore, 2,4-DNT is significantly less toxic to mice than to rats or dogs. This difference in toxicity is consistent with a poor absorption through the gastrointestinal tract in mice. However, Schut *et al.* (1985) reported that in A/J mice, most of the radioactivity from a single oral dose of radiolabeled 2,4-DNT was excreted in the urine (66% of the dose in 8 h) while elimination via the faeces was minimal (<2.1%) indicating a greater absorption than that of previous cited strains. Thus, mice data suggest that the rate of absorption in mice can be strain-dependent.

The most important information for determining the oral absorption derives from five studies carried out in rats, which showed that 2,4-DNT is rapidly absorbed after a single administration, the absorption is complete within 24 hours; radioactivity level in blood reaches a peak at 6 h and gradually declines over the first 9 h; its half-life in plasma is about 22 h; the urine is the major route of excretion for 2,4-DNT but the bile is relevant; metabolites excreted in bile can be absorbed from the intestine (enterohepatic cycling); and there are no major differences between sexes, dose groups or feeding periods (Lee *et al,.* 1978; Mori, Naruse and Kozuka, 1977; Medisnky and Dent,1983; Rickert, Schnell and Long, 1983; Ellis *et al.,* 1979).

On the basis of this information, the oral absorption was considered to be 100% within 24 hours in rats and by extrapolation, regarding studies done on other species, 100% in rabbits, dogs and monkeys. For humans the worst case was assumed and oral absorption of 2,4-DNT was considered as 100% too.

No data are available for inhalation exposure route. Therefore, based on oral absorption data, the worst case inhalation absorption (i.e. 100%) should be assumed for both animals and humans

No data are available for dermal exposure route. Thus, based on both the physico-chemical properties of the substance (MW= 182.15, log P_{ow}= 1.98) and oral absorption data, a default value for dermal absorption of 100% should be applicable to both animals and humans.

However, the absorption of 2,6-DNT was considered to be nearly 100% by both oral and inhalation routes of administration, and 5-7% following dermal application (Hawkins *et al.*, 1991). The isomers 2,4- and 2,6-DNT have identical molecular weights and show nearly identical physical chemical properties, ie water solubility (2,4-DNT: 166 mg/l vs 2,6-DNT: 145 mg/l), vapour pressure (2,4-DNT: 0.00016 hPa vs 2,6-DNT: 0.00032 hPa) and log Kow (2,4-DNT: 1.98 vs 2.6-DNT: 2.1) among others. Therefore, it seems appropriate to extrapolate 2,4-DNT dermal absorption from that of 2,6-DNT.

Moreover, the US-EPA computer model Dermwin v1.42, computed nearly identical results for the dermal penetration rates of the two isomers. Introducing both solubility and log Kow for 2,4- and 2,6-DNT into the model, 15-min dermal absorbed doses were 0.00016 mg/cm²-event for 2,4-DNT and 0.00017 mg/cm²-event for 2,6- isomer.

Thus, a dermal penetration rate of 10% of 2,4-DNT is considered acceptable. This is also supported by findings obtained in the dermal acute toxicity study, ie all animals treated dermally with 2500 mg/kg bw survived over the 14−day post−exposure observation period without any toxic symptoms (Löser *et al.,* 1982).

Distribution and accumulation

No studies were located regarding distribution in experimental animals following inhalation or dermal exposure to 2,4-DNT. However, the distribution of 2,4-DNT and its metabolites was determined based on data from tissue distribution and toxicity studies carried out in experimental animals following oral administration.

24 hours after a single oral radiolabeled dose of 2,4-DNT, the radioactivity recovered in tissues, including blood, was only 3.6% of the dose in dogs, 2.3% in monkeys and less than 1% in rodents and rabbits. Radioactivity was highly concentrated in the liver (organ of metabolism and biliary excretion) of all species. The liver/plasma radioactivity ratios were 18.1 in rats, 17.8 in monkeys, 8.7 in rabbits, 6.9 in dogs, and. 6.3 in mice. The concentration ratio was also high in the kidney (organ of urinary excretion), and the lung and spleen showed concentration ratios greater than one. In addition, the skeletal muscle and brain of rats and monkeys contained more radioactivity than that in the plasma (Lee *et al.,* 1975, 1978).

Further studies carried out in rats showed that when 2,4-DNT was administered at three dose levels, peak concentrations of radioactivity in plasma, red blood cells, liver and kidneys of rats were proportional to dose (Rickert and Long, 1980); all the rat tissues receiving 5 daily oral radiolabeled doses contained 2 to 4.8 times as much radioactivity as those receiving a single one (Lee *et al.,* 1975, 1978); the qualitative tissue distribution pattern of 2,4-DNT administered as a single oral radiolabeled dose was similar in pre-treated with 2,4-DNT in the diet for 3, 9, 20 (Ellis *et al.,* 1979) or 4 months (Mori, Naruse and Kozuka, 1980) and no pre-treated rats; and radioactivity recovered in tissues was lower in pre-treated rats than in no pre-treated rats, suggesting that organs of 2,4-DNT pre-treated rats were saturated (Mori, Naruse and Kozuka, 1980). The only clear differences between male and female rats administered 2,4-DNT as a single oral radiolabeled dose were the higher retention of radioactivity in red blood cells of females and the concentration of radioactivity in livers of females, which was only half of that found in males; in addition, concentrations of 2,4-DNT in male kidneys peaked at 4-8 h and were 3-10 times higher than concentrations in female kidneys which peaked at 1 h after dosing (Rickert and Long, 1980). Hepatic concentrations of radioactivity in male rats increased in two stages, with the first peak occurring 1-2 hours and the second peak occurring 8-12 hours after a single oral dose; the second peak was followed by a gradual

decline up to 16 days and was thought to be the result of enterohepatic cycling (Rickert, Schnell and Long, 1983).

In experimental animals, 2,4-DNT caused adverse effects in a variety of organs and tissues including the blood, nervous system, liver, kidney and testes; subcutaneous and mammary gland carcinomas were also observed.

Therefore, based on available distribution and toxicity data, it can be said that once absorbed 2,4-DNT and its metabolites are well distributed in all animal species showing a similar pattern of distribution with radioactivity concentrated in liver and kidneys. However, since 2,4-DNT is extensively metabolised and excreted, there is not sufficient evidence to warrant accumulation in these organs.

Considering information from the toxicodynamic studies, it appears to be some suggestive evidence of common target organs following exposure of animals and humans by different routes (oral in animals; inhalatory and dermal in humans). Correlation of toxic effects between humans and animals with regard to hematologic and neurological effects has been noted but insufficient data are available to state definitively whether the other toxic effects observed in animals would also occur in humans. Furthermore, qualitative similarities in metabolism of humans and animals have been noted.

In conclusion, since for systemic effects it is assumed that the tissue distribution pattern will be the same irrespective of the administration route, the distribution pattern of 2,4-DNT for animals orally exposed is considered applicable for both humans and animals exposed by dermal and inhalatory routes.

Metabolism and excretion

The metabolism and excretion of 2,4-DNT has been studied in experimental animals following oral administration.

The routes of excretion were similar in rats, rabbits, dogs and monkeys, with the predominant route being via urine. By 24 hours after a single oral radiolabel dose, the radioactivity recovered in the urine was 75-81% of the dose, and in less extension in faeces (3-9%); no radioactivity was found in the expired air (Lee *et al.,* 1978). With respect to mice, differences between strains were observed. After a single oral radiolabeled dose, most of the radioactivity (80% of the dose) was excreted in the faeces and only about 10% in the urine of CD-1 and B6C3F1 strains within the first 24 hours (Lee *et al.,* 1978); however, in A/J strain, after a single oral dose, urine was the main route of elimination of 2,4-DNT, with 66% excreted after 8 hours; the amount excreted in faeces was lower than 2% of the dose (Schut *et al.,* 1983). Differences in the excretion pattern of mice appear to be justified by the strain-dependent absorption.

Once absorbed, 2,4-DNT was extensively metabolised; no parent compound or only small amounts were detected in the urine. Most information on 2,4-DNT metabolism and excretion derives from studies carried out in rats. However, since a similar 2,4-DNT metabolism pattern has been proposed for all species studied (Lee *et al.,* 1975, 1978), data from rats are considered as representative of all animal species.

Several metabolites have been identified in the urine of rats (Lee *et al.,* 1975, 1978; Mori, Naruse and Kozuka, 1981). The major urinary metabolite was 2,4-dinitrobenzyl alcohol glucuronide; after a single dose of 35 mg/kg, females excreted a greater percentage of the dose as this metabolite than did males (Rickert and Long, 1981). 2,4-dinitrobenzoic acid, 4-

(N-acetyl)amino-2-nitrobenzoic acid, 2-amino-4-nitrobenzoic acid, 4-(N-acetyl)amino-2-aminobenzoic acid, 4-amino-2-nitrobenzoic acid, 2-4-dinitrobenzyl alcohol, and 4-amino-2-nitrotoluene were also detected among others (Shoji *et al.,* 1985; Mori *et al.,* 1996). The metabolite profile following a single oral radiolabel dose was not altered by feeding 2,4-DNT in the diet and there were no major differences between dose groups, between sexes or between feeding periods (Ellis *et al.,* 1979; Rickert *et al.,* 1981).

The bile is also an important route of excretion for 2,4-DNT and its metabolites. In rats, after a single oral radiolabeled dose the excretion rate of urinary radioactivity was more rapid than that of faecal excretion and about 60% of the radioactivity administered was excreted in the urine within the first 6 hours. The biliary excretion rate was rising steadily from 6 h after dosing and its peak time was 9-10 h; about 10% of the radioactivity administered was excreted in bile within 24 h. A considerable amount of radioactivity was excreted in the 6-9 h faeces. Therefore, it can be said that most of the faecal excretion is originated in the biliary excretion of radioactivities, and the absorption rate from the digestive organs is relatively slow (Mori, Naruse and Kozuka, 1977).

The major 2,4-DNT biliary metabolite found in rats was 2,4-dinitrobenzyl alcohol glucuronide; males excreted about 3 times as much of this metabolite as did females (Bond *et al.,* 1981). In addition, 2,4-dinitrobenzoic acid, 4-amino-2-nitro(2-amino-4-nitro)benzyl alcohol sulphate, 2-amino-4-nitrobenzoic acid, 2,4-dinitrobenzaldehyde, 2,4-dinitrobenzyl alcohol, 2-4-diacetylaminobenzoic acid and 2-acetylamino-4-nitrotoluene were detected (Sayama *et al.,* 1989).

Two routes for biotransformation of 2,4-DNT in liver have been proposed. The major route consists on its oxidation via cytochrome P450 to form 2,4-dinitrobenzyl alcohol. This compound is further extensively conjugated by UDP-glucuronosyl transferase to form 2,4-dinitrobenzyl alcohol glucuronide, which can be easily eliminated either via urine or via bile. A minor proportion of 2,4-dinitrobenzyl alcohol can be oxidated again by alcohol dehydrogenase to form 2,4-dinitrobenzaldehyde, which is reoxidated by aldehyde dehydrogenase to 2,4-dinitrobenzoic acid. At this point, both nitro groups of 2,4-dinitrobenzoic acid can be reduced by nitroreductases to form 2-amino-4-nitrobenzoic acid and 4-amino-2-nitrobenzoic acid, both of which can undergo further biotransformations via N-acetyltransferase to yield 2-(N-acetyl)amino-4-nitrobenzoic acid and 4-(N-acetyl)amino-2-nitrobenzoic acid. The minor route consists on its reduction by nitroreductases. The small proportion of 2,4-DNT that does not undergo oxidation of the methyl group can be reduced at positions 2 and 4 by nitroreductases to yield 2-amino-4-nitrotoluene or 4-amino-2-nitrotoluene, which can be finally acetylated by N-acetyltransferases to yield 2-(N-acetyl)amino-4-nitrotoluene and 4-(N-acetyl)amino-2-nitrotoluene.

Both nitro groups of 2,4-DNT were reduced *in vitro* by intestinal microflora to form the corresponding amino derivate. Both reductions are developed in a 2-step process. Initially, the nitro group is reduced to the nitroso form, which is further reduced to the amino group through an intermediate hydroxyl derivate. The product of the complete reduction of 2,4-DNT is the potent mutagenic compound 2,4-diaminotoluene. However, this compound has never been found either in the body of animals dosed with 2,4-DNT or in experiments *in vitro* with perfused livers, even when both 2-amino-4-nitrotoluene and 2-nitro-4-aminotoluene are common metabolites of 2,4-DNT biotransformation by liver and intestinal microflora. It suggests that 2,4-DNT induces hepatocarcinogenicity through a compound different form 2,4-diaminotoluene.

The proposed pathway for the bioactivation of 2,4-DNT in the whole animal is shown in Figure 1.

After an oral administration, 2,4-DNT is oxidated in the liver to 2,4-dinitrobenzyl alcohol, which undergoes a Phase II reaction being conjugated with glucuronide acid. The glucuronic conjugate can be either eliminated via urine or excreted to bile. The conjugate excreted in bile is absorbed in intestine where the glucuronic acid is hydrolysed by glucuronidase, yielding again 2,4-dinitrobenzyl alcohol. This benzyl alcohol is further reduced in intestine at position 4, being the generated 4-amino-2-nitrobenzyl alcohol carried out again to the liver. Once in the liver, 4-amino-2-nitrobenzyl alcohol can be conjugated with sulphate at the hydroxyl group. The sulfoconjugated is unstable and quickly decomposes to electrophilic species with high capability to form covalent binding with DNA. Another possible route for bioactivation of 4-amino-2-nitrobenzyl alcohol re-entered in the liver is its oxidation of the amino group to form 4-hydroxylamino-2-nitrobenzyl alcohol. This compound can be target of reaction of Phase II, especially conjugation with sulphate. The generated molecule is also very unstable and spontaneously changes to other electrophilic species, which can be covalently bound to DNA.

According to the Hb arylamine adducts found in both humans and rats (4A2NT the major one, and low levels of 24DAT and 4AA2AT), the reduction of the nitro group in the *para* position appears to be a determinant factor for the 2,4-DNT bioactivation (Jones *et a.l,* 2005).

The involvement of the conjugation with sulphate in the bioactivation of 2,4-DNT is clearly demonstrated since the previous treatment of rats with inhibitors of sulfotransferases clearly inhibits the binding of 2,4-DNT or its metabolites to hepatic DNA.

2,4-dinitrobenzaldehyde, a potent mutagen, was not found in the urine of rats dosed with 2,4-DNT but was present in the bile, indicating that it is an intermediate in the metabolism of 2,4-DNT. That the oxidation of 2,4-dinitrobenzyl alcohol to 2,4-dinitrobenzaldehyde is reversible is seen from the observation that 2,4-dinitrobenzyl alcohol and its glucuronide are metabolites of 2,4-dinitrobenzaldehyde. These observations support that the enterohepatic circulation of 2,4-dinitrobenzaldehyde is possible and suggest that the oxidation of 2,4-dinitrobenzyl alcohol to 2,4-dinitrobenzaldehyde is a metabolic activation of 2,4-DNT (Sayama et al. 1989).

The gut bacteria are important in the metabolism of 2,4-DNT *in vivo.* The four major metabolites identified in the urine of conventional rats (2,4-dinitrobenzyl alcohol glucuronide, 2,4-dinitrobenzoic acid, 4-(N-acetyl)amino-2-nitrobenzoic acid and 2-amino-4-nitrobenzoic acid) were also present in the urine of axenic rats. However, in axenic rats the amounts of 4-(N-acetyl)amino-2-nitrobenzoic and 2-amino-2-nitrobenzoic excreted in the urine are markedly reduced by comparison with conventional animals. In addition, less radioactivity is covalently bound to hepatic macromolecules in axenic rats compared with conventional rats (Rickert *et al.* 1981). In rats with cannulated bile ducts the collection of bile also markedly reduces the amounts of these metabolites excreted in the urine (Medinsky and Dent, 1983).

These observations indicate that the relationship between liver and intestinal microflora in the metabolism of 2,4-DNT is a complex one. The intestinal microflora is apparently an important site for the metabolism of biliary metabolites and metabolism by intestinal microflora appears to be essential for the production of metabolites that bind covalently to liver macromolecules.

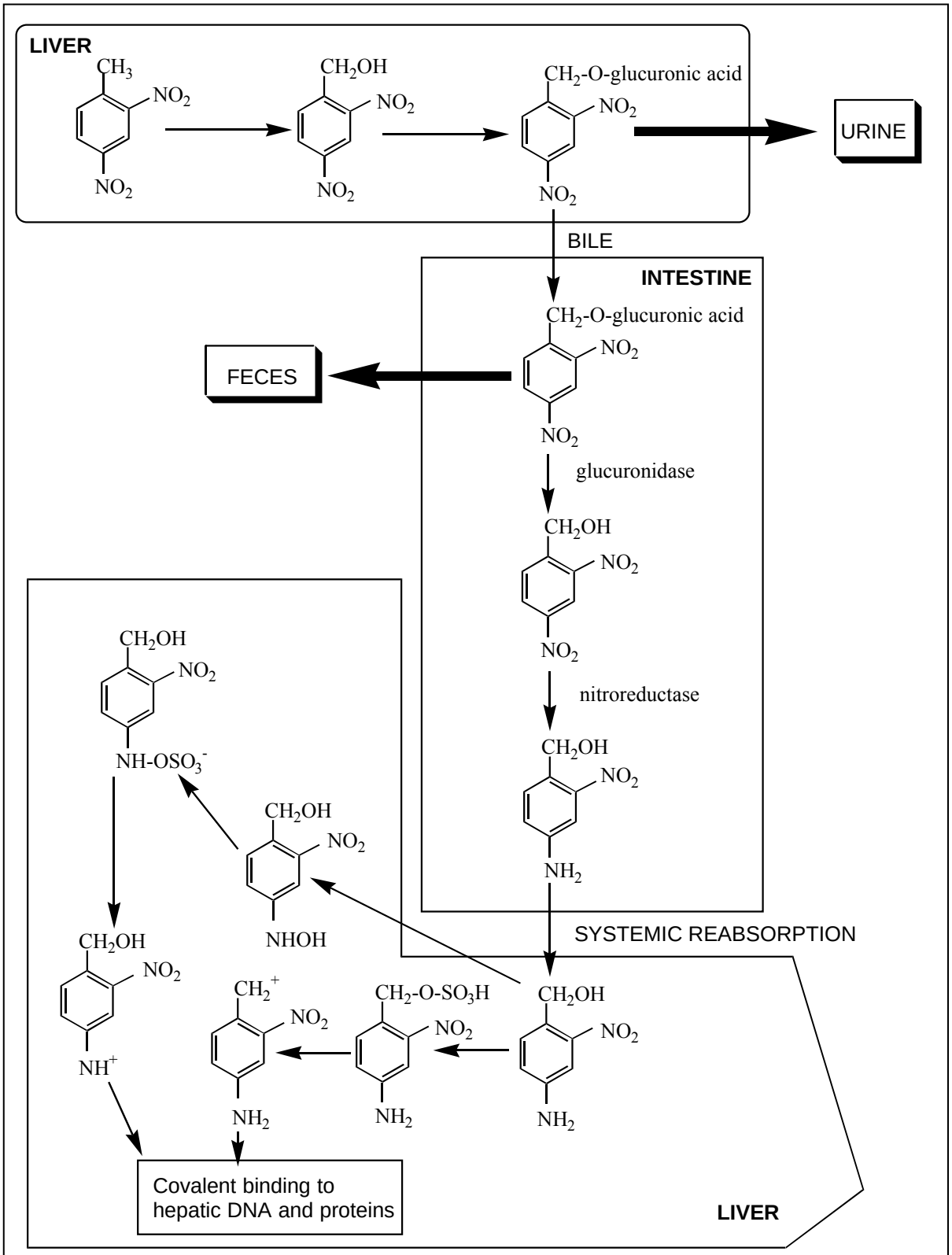


Figure 1: Proposed pathway for the activation of 2,4-dinitrotoluene

The lack of significant sex- or species-dependent differences in the qualitative pattern of cecal microfloral metabolites, combined with the lack of any major differences in rates of reductive metabolism of 2,4-DNT, indicates that the cecal microflora do not play a major role in the

sex- or species-dependent differences in susceptibility to 2,4-DNT carcinogenicity. Indeed, the mouse cecal microflora reduced 2,4-DNT most rapidly and yet mice are apparently less susceptible to the hepatocarcinogenic action of 2,4-DNT than rats.

In rats, sex differences in the metabolism of 2,4-DNT have been observed. After oral doses of 10 and 35 mg/kg, the urine excretion predominates in female rats, while in males biliary excretion is the most important route (Rickert and Long, 1981). This preferential excretion can explain why male urine contains less 2,4-dinitrobenzyl alcohol glucuronide than female urine. The quantitative differences in urinary versus biliary excretion of the glucuronide conjugate by females may account for the sex differences in the susceptibility of the rat to the hepatocarcinogenic effects of 2,4-DNT. Greater urinary excretion may decrease the amount of the glucuronide available to the intestinal microflora for metabolism to a carcinogenic metabolite. Sex differences in the urinary excretion of 2,4-dinitrobenzyl alcohol glucuronide disappears after administration of high doses of 2,4-DNT, suggesting that transport into the bile of 2,4-dinitrobenzyl alcohol glucuronide is saturable in males, but not in females.

The metabolism and excretion of 2,4-DNT in workers exposed to technical-grade DNT by inhalation and dermal routes has been studied by the analysis of urinary metabolites.

The major 2,4-DNT metabolite detected in the urine of workers was 2,4-dinitrobenzoic acid, although lesser amounts of 2-amino-4-nitrobenzoic acid, 2,4-dinitrobenzyl alcohol glucuronide, 2-(N-acetyl)amino-4-nitrobenzoic acid and traces of 4-amino-2-nitrobenzoic acid and 4-(N-acetyl)amino-2-nitrobenzoic were also found. In addition, the urine contained unchanged 2,4-DNT.

As seen in rats, female subjects excreted a higher proportion of urinary metabolites as dinitrobenzyl alcohol glucuronides than did males (Levine *et al.,* 1985).

The appearance of reduced metabolites suggest either that human hepatic enzymes are capable of reduction of the nitro group of 2,4-DNT or that 2,4-DNT (or its metabolites) gain access to the intestinal microflora which is capable of reduction, after which the metabolites are reabsorbed and excreted into urine. In support of the last suggestion, it was observed that the metabolites produced by incubation of 2,4-DNT with human gut were the same as those produced by analogous samples from rats and mice (Guest *et al.,* 1982).

The half-life for excretion of 2,4-DNT metabolites in urine of workers ranged from 0.8 to 4.5 hours. The half-lives for 2,4-dinitrobenzoic acid and 2,4-dinitrobenzyl alcohol glucuronide tended to be shorter than those for the metabolites that resulted from both oxidative and reductive metabolism (Turner *et al.*, 1985). The highest rates of excretion of 2,4-dinitrobenzoic acid occurred near the end of the work shift. The half-life for urinary excretion of 2,4-dinitrobenzoic acid was calculated to be 2-5 hours. This estimate appears to be the initial phase of a biphasic elimination profile since even 3 days after the exposure, detectable levels of 2,4-dinitrobenzoic acid were present in urine (Woollen *et al.*, 1985). These data support that enterohepatic recycling occurred in humans.

In summary, 2,4-DNT metabolism and excretion seem to be qualitatively similar in both humans and rats, but the proportions of nitro-reduced metabolites were lower relative to oxidized metabolites in the urine from humans. These differences may be due more to the particular routes of exposure (inhalation and dermal for humans, oral for rats) than differences in species.

There are no studies on whether 2,4-DNT or its metabolites can cross the placenta or be excreted in breast milk, so it cannot be determined if fetuses may be exposed in utero of if

infants may be exposed via breast milk ingestion. There are also no data to show if 2,4-DNT and its metabolites are stored in maternal tissues and thus might be later mobilized during gestation or lactation; however, 2,4-DNT and its metabolites are not likely to be stored because of their low octanol-water partition coefficient.

4.1.2.2 Acute toxicity

4.1.2.2.1 Studies in animals

In vivo studies

Acute toxicity studies have been carried out in rats, mice and cats. They are summarized in Tables 4.1.2.2.1-1 and 4.1.2.2.1-2.

Table 4.1.2.2.1-1: Summary of acute toxicity of 2,4-DNT in rodents

Route	Species	Dose	LD_{50} (mg/kg b.w.)	Comments	Ref.
Dermal	5 Wistar rats per sex	2500 mg/kg b.w.	> 2500	purity not reported Limit test	Löser 1982
Oral (gavage)	♂ CD rats	Not given	568 (434 – 705)[a]	purity = 98%; 2,6-DNT impurity = 2% Vehicle: peanut oil	Lee *et al.,* 1975
	♀ CD rats	Not given	650 (520 – 743)[a]	purity = 98%; 2,6-DNT impurity = 2% Vehicle: peanut oil	
	♂ rats	Not given	270 (180 - 400)[a]	purity not reported	Vernot *et al.,* 1977
	10 ♂ Wistar rats/dose	125, 200, 315, 500, 800 and 1000 mg/kg b.w.	400 (305 – 520)	purity not reported Vehicle: sesame oil	Hoechst 1977a
	10 ♀ Wistar rats/dose	200, 315, 500, 800 and 1000 mg/kg b.w.	474 (376 – 586)	purity not reported Vehicle: sesame oil	Hoechst 1977b
	5 rats per sex and group	100, 500, 800, 1500 and 2000 mg/kg b.w.	893 (620 – 1190)	purity not reported Vehicle: lutrol	Löser 1981
	♂ albino Swiss mice	Not given	1,954 (1,848 – 2,178)[a]	purity = 98%; 2,6-DNT impurity = 2% Vehicle: peanut oil	Lee *et al.,* 1975
	♀ albino Swiss mice	Not given	1,340 (1,205 – 1,500)[a]	purity = 98%; 2,6-DNT impurity = 2% Vehicle: peanut oil	
	Mice	Not given	1,630 (1,180 – 2,240)[a]	Method not reported.	Vernot *et al.,* 1977

[a] 95% confidence limits

Table 4.1.2.2.1-2: Summary of acute toxicity of 2,4-DNT in cats

Route	Species	Dose	Comments	Ref.
Oral (gavage)	Cats	10 and 50 mg/kg bw in lutrol	At 50 mg/kg bw: 1/1 died (renal insufficiency) ↑ Methaemoglobin (42%) within 7 hours after treatment, but decreased to basal levels after 48 hours. ↑ Heinz-bodies within 48 hours after treatment (55%).	Löser and Schmidt., 1984
Intraperitoneal	Cats	10, 20, 30, 40 mg/kg bw in poppy oil	1/2 cats at 40 mg/kg bw died dose-related increased methaemoglobinaemia level (7-82%) within 5 h after treatment, but decreased within 24h after treatment	Bredow *et al.,* 1942

Inhalation

There are no acute inhalation studies available.

Dermal

Rats

Löser, 1982

Five Wistar rats/sex were exposed dermally to 2500 mg/kg of 2,4-DNT (pure 2,4−DNT, quantitative purity not reported). All animals survived (10/10) over the 14−day post−exposure observation period without any toxic symptoms. This study did not comply with GLP.

Oral

No guideline studies are available. The study carried out by Lee *et al.* (1975) complied, in essence, with modern test guidelines.

Rats

Lee *et al.,* 1975

The solutions of 2,4-DNT for toxicity testing were prepared by saturating peanut oil with 2,4-DNT (2,4-DNT purity = 98%; 2,6-DNT impurity = 2%) and then chemically assaying for the final concentration. Doses of 2,4-DNT were administered by gavage. There is no information available on which doses were tested and which specific effects occurred at the different tested doses. Number of animals per dose and sex was not indicated. The animals were observed for 14 days for delayed mortality or toxic signs. The acute oral LD_{50} (95%

confidence limits) in CD rats was 568 (434 – 705) mg / kg for males, and 650 (520 – 743) mg/kg for females, respectively. The only toxic signs, in most cases, were central nervous system depression which, within a few hours, produced ataxia, respiratory depression and death. Death occurred usually within the first 24 hours or not at all. The surviving animals completely recovered within 48 hours. No gross pathology attributable to the treatment was noted in the animals that died.

Vernot *et al*., 1977

Single oral LD_{50} (95% confidence limits) in male rats was 270 (180 - 400) mg/kg bw of 2,4-DNT. No further details were available.

Hoechst, 1977a

Ten male Wistar rats/dose were administered 125, 200, 315, 500, 800 and 1000 mg/kg b.w. of 2,4-DNT (purity not reported) dissolved in sesame oil. The post exposure observation period was 14 days. The LD_{50} (95% confidence limits) in male Wistar rats was 400 (305 – 520) mg/kg bw.

Table 4.1.2.2.1-3: Mortality of male rats treated with 2,4-DNT (Hoechst, 1977a)

Dose (mg/kg)	Deaths [a]
125	0/10
200	2/10
315	2/10
500	8/9
800	7/10
1000	10/10

[a] Number of deaths / number of animals tested

Hoechst, 1977b

Ten female Wistar rats/dose were administered 200, 315, 500, 800 and 1000 mg/kg b.w. of 2,4-DNT (purity not reported) dissolved in sesame oil. The post exposure observation period was 14 days. The LD_{50} (95% confidence limits) in female Wistar rats was 474 (376 – 586) mg/kg bw.

Table 4.1.2.2.1-4: Mortality of female rats treated with 2,4-DNT (Hoechst, 1977b)

Dose (mg/kg)	Deaths [a]
200	2/10
315	2/10
500	6/10
800	8/10
1000	10/10

[a] Number of deaths / number of animals tested

Löser, 1981

Five rats/sex/dose were treated with 100, 500, 800, 1500 and 2000 mg/kg b.w. of 2,4-DNT (purity not reported) dissolved in lutrol. The post exposure observation period was 14 days. The LD_{50} (95% confidence limits) in rats was 893 (620 – 1190) mg/kg bw.

Table 4.1.2.2.1-5: Mortality of rats treated with 2,4-DNT (Löser, 1981)

Dose (mg/kg)	Deaths [a]
100	0/10
500	2/10
800	4/10
1500	7/10
2000	10/10

[a] Number of deaths / number of animals tested

Mice

Lee *et al.,* 1975

The solutions of 2,4-DNT for toxicity testing were prepared by saturating peanut oil with 2,4-DNT (2,4-DNT purity = 98%; 2,6-DNT impurity = 2%) and then chemically assaying for the final concentration. Doses of 2,4-DNT were administered by gavage. There is no information available on which doses were tested and which specific effects occurred at the different tested doses. Number of animals per dose and sex was not indicated. The animals were observed for 14 days for delayed mortality or toxic signs. The acute oral LD_{50} (95% confidence limits) in albino Swiss mice was 1,954 (1,848 – 2,178) mg / kg for male, and 1,340 (1,205 – 1,500) mg / kg for female, respectively. The only toxic signs, in most cases, were central nervous system depression which, within a few hours, produced ataxia, respiratory depression and death. Death occurred usually within the first 24 hours or not at all. The surviving animals completely recovered within 48 hours. No gross pathology attributable to the treatment was noted in the animals that died.

Vernot *et al.,* 1977

Single oral LD_{50} (95% confidence limits) in mice was 1,630 (1,180 – 2,240). No further details were available.

Cats

Löser *et al.,* 1984

Two male cats were administered either 10 or 50 mg/kg bw of 2,4-DNT (purity = 99%) by gavage in lutrol. Toxic effects were found at the high dose. Methaemoglobin levels increased up to 42% within 7 hours after treatment, and then those decreased to basal levels after 48 hours. Nevertheless, Heinz-bodies were noted within 48 hours after treatment (55%). Furthermore, the cause of death of the high-dose cat 1 week after treatment was renal insufficiency.

Table 4.1.2.2.1-6: Haematological data of male cats after single oral administration of 2,4-DNT (Löser *et al.*, 1984)

		Time after treatment (hours)				
	Dose					
Parameter	**mg/kg b.w.**	**0**	**3**	**7**	**24**	**48**
Met-Hb	10	3	3	3	2	No data
(%)	50 †	2	39	42	10	3
Heinz bodies	10	9	7	9	7	No data
(%)	50	10	12	28	51	55

†: High-dose cat died 1 week after treatment.

Intraperitoneal

Cats

Bredow and Jung, 1942

Groups of 2 cats were administered i.p. 20, 30, 40 mg/kg bw of 2,4-DNT (purity not reported) in poppy oil. In addition, one cat was given i.p. 10 mg/kg bw of 2,4-DNT in poppy oil. The groups treated with 10, 20, 30, 40 mg/kg bw of 2,4-DNT showed dose-response increases in methaemoglobin levels as follow: 7% (n = 1), 36% (n = 2), 53% (n = 2) and 77% (n = 2), respectively, within a few hours after treatment (Table 4.1.2.2.1-7), but decreased within 24h after treatment. Heinz bodies were not noted in the treated animals. One of the two cats treated with 40 mg/kg bw of 2,4-DNT died.

Table 4.1.2.2.1-7: Haematological data of cats after single administration of 2,4-DNT by intraperitoneal injection (Bredow and Jung, 1942)

Cat identity	**Dose of 2,4-DNT**		**Hb**	**MetHb**	**Methaemoglobin**	**Time of maximum toxicity**
	(mg/kg bw)	**(mol·10^{-5} / kg bw)**	**(eq·10^{-4} /kg bw)**	**(eq·10^{-4} /kg bw)**	**(% with respect to initial Hb value)**	**(hours)**
40 K m	10	5.5	5.6	0.4	7	3
41 K F	20	11	2.6	1.0	40	6
42 K n	20	11	4.5	1.4	32	22
43 K m	30	16.5	4.6	2.3	50	2
44 K l	30	16.5	3.5	1.9	55	6
45 K E	40	22	7.5	5.3	71	6
46 K D	40	22	5.3	4.4	82	10 †

Hb: haemoglobin, eq: equivalent weight, †: dead animal

4.1.2.2.2 Human data

No data available

4.1.2.2.3 Summary of acute toxicity

Acute toxicity studies have been carried out in rats, mice and cats.

The only numerical value of any reliability for risk characterization obtained from the oral rodent studies is the LD_{50}. Accordingly, the rodent LD_{50} reported in the literature ranged from180 to 893 mg/kg b.w. in rats, and from 1,340 to 1,954 mg/kg b.w. in mice. Thus, 2,4-DNT would be considered as borderline toxic (T) in rats, and harmful (Xn) in mice. However, most studies on rodent acute oral toxicity have limited quality and do not mention which was the purity of the test substance. The study carried out by Lee *et al.* (1975) complied, in essence, with modern test guidelines and was the only where purity (98%) was reported. In this study, the acute oral LD_{50} (95% confidence limits) was 568 (434–705) mg/kg b.w. and 650 (520–743) mg/kg b.w. in male and female CD rats, respectively, and 1,954 (1,848–2,178) mg/kg b.w. and 1,340 (1,205–1,500) mg/kg b.w. in male and female albino Swiss mice, respectively. Accordingly, 2,4-DNT should be classified as harmful (Xn) in both rats and mice. Results obtained by La and Froines (1992b) support this classification. These authors observed that none of the rats administered 375 mg/kg 2,4-DNT (97-99% purity) by gavage died (to see mutagenicity section).

With respect to the dermal route of exposure, the only available data are from a rat acute toxicity study. All rats treated with 2500 mg 2,4-DNT/kg b.w. survived over the 14-day post-exposure observation period without any toxic symptoms. On the basis of these data, 2,4-DNT should not be classified for dermal acute toxicity. In addition, data from an available rat toxicokinetic study with 2,6-DNT indicated that only 5-7% of the dose was taken up via the skin. Although there are no data available on dermal absorption of 2,4-DNT, it seems appropriate to extrapolate 2,4-DNT dermal absorption from that of 2,6-DNT since the isomers 2,4- and 2,6-DNT have identical molecular weights and show nearly identical physicochemical properties. Consequently, the 2,4-DNT dermal absorption is considered to be 10%. The differences on rat acute toxicity observed between oral and dermal routes of exposure may be justified by the different rates of absorption.

Regarding inhalation there are no available rodent data on acute toxicity. However, it seems reasonable to extrapolate acute toxicity by inhalation from data obtained in oral studies since both oral and inhalation absorption values were estimated to be 100%.

Therefore, based on rodent data 2,4-DNT would be classified for acute toxicity as harmful (Xn; R20/22) by inhalation and oral routes of exposure.

Nevertheless, in the cat acute toxicity study carried out with 2,4-DNT (99.9% purity) increased levels on both methaemoglobin and Heinz bodies were observed at 50 mg/kg b.w. but not at 10 mg/kg b.w. following a single oral administration. The increased levels on methaemoglobin were also observed from 20 to 40 mg/kg b.w. when 2,4-DNT wasadministered i.p.. In addition, one of the two cats administered the highest dose either oral or i.p. died.

In rats, mice and dogs, methaemoglobinemia and other haemolytic anaemia related effects were induced by 2,4-DNT following oral repeated dosing. However, these effects were not reported in the acute toxicity studies performed in rats and mice, and there are noacute toxicity studies in dogs available.

It is known that, in general, the rat, mouse, rabbit, guinea pig and monkey seem to be significantly less sensitive to the formation of MetHb than humans and dogs. On the other hand, the cat is known to be particularly sensitive to the formation of MetHb. In consequence,

2,4-DNT poses a hazard to humans which may be either underestimated if based on rodent data or overestimated if based on cat data.

We consider from a precautionary start point that the cat data are appropriate to be used for hazard identification, C&L proposal and risk characterization.

Therefore, based on cat data (death following oral administration of 50 mg/kg b.w.) 2,4-DNT is considered to be classified for acute toxicity as toxic by oral exposure. Since oral and inhalation absorption values were estimated to be 100% in experimental animals and humans, it seems reasonable to extrapolate toxicity by inhalation from oral toxicity data and to classify 2,4-DNT as toxic by inhalation. Finally, taking into account that the dermal absorption value was estimated to be 10% in rodent and by extrapolation in experimental animals and humans, 2,4-DNT should be considered as borderline toxic by dermal exposure.

Overall, it seems justified to classify 2,4-DNT for acute toxicity as toxic (T; R23/24/25) by inhalation, dermal and oral routes of exposure, and to use the NOAEL of 10 mg/kg b.w. derived from the oral toxicity study in cats as the starting point for risk characterization for acute toxicity.

4.1.2.3 Irritation

Acute dermal and ocular irritation studies performed are summarized in Table 4.1.2.3-1.

Table 4.1.2.3-1: Summary of acute toxicity (irritation) of 2,4-DNT in experimental animals

Exposure	Species	Protocol	Effect	References
Dermal	New Zealand rabbits (n = 6)	Application of the 2,4-DNT (2,4-DNT purity = 98) on intact and abraded skin as a 50% pasta with peanut oil. Responses scored at 24 and 72 hours	Not irritating	Lee *et al.*, 1975
Ocular	New Zealand rabbits (n = 6)	Application of the 2,4-DNT (2,4-DNT purity = 98) as a 50% pasta with peanut oil. Responses scored at 24 and 72 hours	Not irritating	Lee *et al.*, 1975

4.1.2.3.1 Skin

Studies in animals

Lee *et al.*, 1975

Skin irritation tests were carried out according to the modified Draize procedure. New Zealand rabbits with intact and abraded skin in each test group and control group were used (n = 6/group). After application of the 2,4-DNT as a 50% pasta with peanut oil, all animals were examined for signs of erythema and oedema, and the responses scored at 24 and 72 hours. The purity of 2,4-DNT was 98% with 2% of 2,6-DNT as impurity. Some deficiencies existed in both study design and reporting. At room temperature 2,4-DNT is a liquid, and preferably it should have been tested in its pure form. Regarding reporting, neither scores of each rabbit nor when the effects resolved was reported. Nevertheless, due to the concern for reducing

animal testing, this study was considered acceptable for risk assessment. The primary irritation score of 2,4-DNT was 0.25 over control.

Human data

No data available.

4.1.2.3.2 Eye

Studies in animals

Lee *et al.*, 1975

After application of 2,4-DNT New Zealand rabbits were examined for ocular irritation, and the responses scored at 24 and 72 hours. The purity of 2,4-DNT was 98% with 2% of 2,6-DNT as impurity. Some deficiencies existed in both study design and reporting. At room temperature 2,4-DNT is a liquid, and preferably it should have been tested in its pure form. Regarding reporting, the scores of each rabbit were not available. Nevertheless, due to the concern for reducing animal testing, this study was considered acceptable for risk assessment. Primary eye irritation was not seen in the New Zealand rabbits.

Human data

No data available.

4.1.2.3.3 Respiratory tract

No data available.

4.1.2.3.4 Summary of irritation

The only information on the skin and eye irritation potential of 2,4-DNT comes from studies in animals, conducted with a 50% 2,4-DNT solution in peanut oil. In these studies, 2,4-DNT was found to be a mild skin irritant, and did not cause eye irritation. The results obtained by Lee et al. (1975) do not fulfil the EU criteria for skin and eye irritation. However the results were found acceptable to conclude that 2,4 DNT should not be regarded as irritant to the skin and the eye.

4.1.2.4 Corrosivity

According to both eye and skin irritation data, 2,4-DNT is not classified as corrosive substance.

4.1.2.5 Sensitisation

4.1.2.5.1 Studies in animals

Skin

In vivo studies

Lee *et al.,* 1975

Dermal sensitivity was noted in none of ten guinea pigs following 2,4-DNT treatment (2,4-DNT purity = 98%; 2,6-DNT impurity = 2%) by means of Guinea-Pig Maximisation Test (No further details on method and test results are available). This study was the only available study and considered acceptable for risk assessment. Despite deficiencies in reporting, this study was considered to be well conducted. In addition not to ask for further animal testing, this study was considered acceptable for the risk assessment

In vitro studies

No data available

Respiratory tract

No data available.

4.1.2.5.2 Human data

No data available.

4.1.2.5.3 Summary of sensitisation

Dermal sensitivity was not observed in guinea pigs following 2,4-DNT treatment by means of Guinea-Pig Maximisation Test. Thus, 2,4-DNT is not classified as sensitising substance in accordance with the EU criteria.

4.1.2.6 Repeated dose toxicity

4.1.2.6.1 Studies in animals

In vivo studies

Sub-acute toxicity studies

Inhalation

No data available

Dermal

No data available

Oral

The sub-acute oral toxicity of 2,4-DNT was investigated in rats, mice and dogs.

Rats

McGown *et al.*, 1983

Sprague-Dawley rats of one month old (5 animals/sex/group except in control group, which had only 4 females; one removed because of structural defect) were administered 2,4-DNT (purity = 97%; contaminants 2% of 2,6-DNT and unspecified 1%) in the diet for 14 days at 0, 900, 1200, 1900 and 3000 ppm (equivalent to 0, 96, 125, 183 or 260 mg/kg b.w./day for males and 0, 99, 124, 191 or 254 mg/kg b.w./day for females). The purpose of this study was to obtain preliminary data in rats concerning 2,4-DNT effects on tissue histology, blood chemistry, haematology, and urinary parameters. The study was GLP compliant and performed in essence according to OECD guideline 407 with the exception of the exposure (14 days instead of 28 days) and the no inclusion of a recovery period. Gross evidence of toxicity was not observed, but dietary 2,4-DNT caused a dose-dependent decrease in rate of weight gain; this was presumably due to the dose-dependent depression in food consumption. No 2,4-DNT-related effects were found on blood haematology or urinary parameters (because of contamination in urine specimens, only pH and specific gravity measurements were considered valid). Regarding blood chemistry, 2,4-DNT caused dose-related increases in serum cholesterol (significant in all male and female treatment groups) and glucose levels (only significant at the high-dose level in females); increases (not dose-related) were also observed in alanine aminotransferase levels (significant at all dose levels in males) and in the albumin/globulin ratio (significant at 124 and 254 mg/kg b.w./day in females). With respect to histopathological findings, from the low dose hyaline droplet formation was observed in renal tubular epithelial cells of both males and females; a dose-response relationship was not found, although males were more severely affected. 2,4-DNT induced also oligospermia with degenerative changes in the testes of males from 96 mg/kg b.w./day; the degree of testicular changes was dose related. A NOAEL cannot be determined since several effects, among which oligospermia were observed at the lowest dose tested.

Lee *et al.*, 1978

Groups of 8 healthy weanling CD rats/sex/dose were fed diets containing 0, 700, 2000, or 7000 ppm of 2,4-DNT (purity >98%), for 4 weeks equivalent to 0, 38/38, 102/118 or 191/145 mg/kg b.w./day of 2,4-DNT for male/female, respectively. At the end of 4 weeks, 4 rats/sex/dose were sacrificed for necropsy. Treatment was discontinued for 4 rats/sex/dose at the end of 4 weeks. These rats were observed for 4 more weeks and then euthanized for necropsy to study the reversibility of the adverse effects. The study was performed in essence according to OECD guideline 407, with the exception of number of animals (4 instead of 5) and recovery period (28 days instead of 14 days).

Mortality was found at high dose only. Moreover, the mortality of males and females fed the high dose was the same (2/8 and 2/8, respectively). A few rats had episodes of wide spread

stiff-legged gait in the hind legs (further details not available on this topic). Controls increased their body weight along the follow-up period. On the contrary, both feed consumption and body weight of treatment groups decreased as increased the dose of 2,4-DNT. After the 4-week recovery period, the body weight of males and females administered the high dose increased significantly.

Regarding the main haematology findings, males and females receiving the high dose showed decreased erythrocyte count (6.5 vs. $7.1 \cdot 10^6/mm^3$ in controls, $p < 0.05$ for males; and 5.6 vs. $7.8 \cdot 10^6/mm^3$ in controls, $p < 0.05$ for females; respectively) with a compensatory reticulocytosis (4.2% vs. 1.2% in controls, $p < 0.05$ for males; and 6.1% vs. 1.6% in controls, $p < 0.05$ for females; respectively). After the 4-week recovery period males given the high dose showed anaemia with reticulocytosis (erythrocyte count, 6.2 vs. $8.1 \cdot 10^6/mm^3$ in controls, $p < 0.05$; reticulocytes, 5.5% vs. 1.2% in controls, $p < 0.05$). With respect to haematology, data was not available for females at the 8-week sacrifice. Regarding microscopic examination of tissues, authors stated that because of the similarities between the middle and low dose groups, slides from the latter were scanned, but not reported.

Moderate testes atrophy and aspermatogenesis was found in high-dose males (4/4 vs. 0/4 in controls). Those effects were irreversible. Mild to severe haemosiderosis in the spleen was found in all male and female treatment groups, without demonstrable tissue damage. This effect was irreversible (Tables 4.1.2.6.1-1,2).

Table 4.1.2.6.1-1: Splenic haemosiderosis frequency and severity of male rats treated with 2,4-DNT for 4 weeks (Lee *et al.*, 1978)

		Dose (mg/kg bw/day)		
Group	**Haemosiderosis**	**0**	**102**	**191**
4-week	Frequency	0%	75%	75%
	Mild to moderate	0/4	3/4	3/4
	Severe to very severe	0/4	0/4	0/4
4-week stop dose	Frequency	0%	25%	100%
	Mild to moderate	0/4	1/4	2/4
	Severe to very severe	0/4	0/4	2/4

Table 4.1.2.6.1-2: Haemosiderosis frequency and severity in the spleen of female rats treated with 2,4-DNT for 4 weeks (Lee *et al.*, 1978)

		Dose (mg/kg bw/day)		
Group	**Haemosiderosis**	**0**	**118**	**145**
4-week	Frequency	0%	50%	50%
	Mild to moderate	0/4	1/4	1/2
	Severe to very severe	0/4	1/4	0/2
4-week stop dose	Frequency	25%	100%	100%
	Mild to moderate	1/4	2/4	2/2
	Severe to very severe	0/4	2/4	0/2

It was not possible to identify a NOAEL as splenic haemosiderosis was observed at all dose levels. This effect was irreversible. Thus, the LOAEL was considered to be 38 mg/kg bw/day.

Mice

Lee *et al.*, 1978

Groups of 8 young healthy albino Swiss mice/sex/dose were fed 0, 700, 2000 and 7000 ppm, equivalent to 0, 46/53, 132/142, and 332/434 (males/females) mg/kg b.w./day in the diet for 4 weeks over an 8-week observation period. At the end of 4 weeks, 4 mice/sex/dose were sacrificed for necropsy. Treatment was discontinued for 4 mice/sex/dose at the end of 4 weeks. These mice were observed for 4 more weeks and then euthanized for necropsy to study the reversibility of the adverse effects. The study was performed in essence according to OECD guideline 407 with the exception of the number of animals (4 instead of 5) and the recovery period (28 days instead of 14 days).

All of the 32 mice survived along the study period, with the exception of one high-dose male which died in the recovery period. The mean value of the body weights of male and female mice given the high dose was lower than that of the controls, since male and female high-dose mice ingested less feed than controls. When 2,4-DNT in feed was discontinued for 4 weeks, the male and female high-dose mice recovered a healthy body weight. The only histopathologic effect attributable to 2,4-DNT was a mild testis aspermatogenesis found in two of the four high-dose mice (0/4 in controls). Those mice recovered after the 4-week recovery period.

The NOAEL was considered to be 132 mg/kg b.w./day in males on the basis of mild aspermatogenesis observed at the highest dose.

Dogs

Lee *et al.*, 1978

Groups of two beagle dogs/sex/dose were treated with 0, 1, 5 or 25 mg/kg b.w./day of 2,4-DNT (purity 98.5 - 99%) in hard gelatine capsules for 4 weeks over an 8-week observation period. The design of the experiment was as follows: At the end of 4 weeks of continuous treatment, 1 dog/sex/dose was sacrificed for necropsy. The treatment for 1 dog/sex/dose was discontinued at the end of 4 weeks and then sacrificed at the end of 8 weeks to study the reversibility of adverse effects.

The two high-dose male dogs died on days 22 and 24. Nevertheless, the two high-dose female dogs survived the 4-week exposure period. Then, it was decided to increase the exposure period of the two high-dose female dogs, in such a way that they died on days 36 and 48 of continuous treatment. Thus, males were more sensitive to 2,4-DNT toxicity than females.

This sub-acute toxicity study and a sub-chronic toxicity study were carried out simultaneously. Since the 13-week study dogs survived the treatment for 4 weeks, demonstrating high interindividual susceptibility to 2,4-DNT toxicity, they were used for the sub-acute toxicity study. Thus, one male and one female dog were treated with the high dose for 4 weeks and allowed to recover for 8 months.

The study was performed according to OECD guideline 407 with the exception of the remarks in the previous paragraphs.

The body weight of the beagle dogs treated with the high dose (25 mg/kg b.w./day) at either fourth week or death was reduced when compared with their initial weights, since feed consumption decreased progressively along the exposure period.

Yellow stain on and near hind legs, pale gums (sometimes blue-tinged), and a neuromuscular incoordination and paralysis were found in all affected dogs treated with the high dose. Regarding neuromuscular effects, the first manifestation was a stiffness and incoordination of the hind legs. As this progressed, the animals were unable to stand. The stiffness progressed upward from hind legs to trunk, forelegs, neck and then head. Furthermore, one high-dose female had transient blindness. After recovery period, dogs appear normal except for occasional poor balance.

Dogs treated with 25 mg/kg b.w./day of 2,4-DNT showed decreased both erythrocyte count (5.07 vs. $6.48 \cdot 10^6/mm^3$ in controls, $p < 0.05$) and haemoglobin (12.7% vs. 15.8% in controls, $p < 0.05$). Furthermore, reticulocytosis increased in a dose-response manner (significantly higher in all treatment groups when compared with that of controls; 0.86%, 1.26%, 1.40%, 1.53% for 0, 1, 5 or 25 mg/kg b.w./day, respectively). Moreover, Heinz bodies were observed in dogs administered the high dose of 2,4-DNT (4.1% vs. 0% in controls). After the 4-week recovery period, the high-dose female had 0.4% Heinz bodies.
Although, mild aspermatogenesis was observed in the dog administered the high dose of 2,4-DNT, this effect was reversible.

The LOAEL was considered to be 1 mg/kg b.w./day on the basis of increased reticulocytosis.

Sub-Chronic toxicity studies

Inhalation

No data available

Dermal

No data available

Oral

The studies described below have been carried out in order to know the effects of 2,4-DNT after oral administration for sub-chronic exposure on different mammalian species. Although, the effects of 2,4-DNT after oral administration for up to 13 weeks in dogs, rats and mice were jointly reported by Lee et al 1978, the effects of 2,4-DNT on each species are indicated separately in the present report.

Rats

Lee *et al.*, 1978, 1985

Groups of 8 healthy weanling CD rats/sex/dose were fed diets containing 0, 700, 2000 or 7000 ppm of 2,4-DNT in the diet (purity > 98%), equivalent to 0, 34/38, 93/108 or 266/145 mg/kg b.w./day of 2,4-DNT for male/female, respectively. At the end of 13 weeks, 4 rats/sex/dose were sacrificed for necropsy. Treatment was discontinued for 4 rats/sex/dose at the end of 13 weeks. These rats were observed for 4 more weeks and then euthanized for necropsy to study the reversibility of the adverse effects. This study was performed in essence according to OECD 408 guideline with the exception of number of animals (4 at each dose level instead of 10, and 4 in the satellite groups instead of 5) and recovery period (28 days instead of 14 days). Nevertheless, it was considered adequate to determine a NOAEL.

Six of the 8 males treated with 266 mg/kg b.w./day of 2,4-DNT died before their scheduled necropsy (after treatment for 6 to 13 weeks). One male administered 93 mg/kg b.w./day died the first week of the 4-week recovery period. All of the 8 females given 145 mg/kg b.w./day of 2,4DNT died in the first 3 weeks of study (blood and tissues samples for examination were not available in most of those females). It was noted a high interindividual susceptibility to 2,4-DNT toxicity, since mortality of female rats from the 4-week study was lower than that of females from the 13-week study at the same time point. With respect to body weight, both males and females showed decreased body weight with reduced food consumption in a dose-response manner. During the recovery period body weight differences between groups remained. Regarding neuromuscular effects, male and female rats fed 266 and 145 mg/kg b.w./day of 2,4-DNT in the diet, respectively, showed wide spread and stiff hind legs.

A dose-response related reticulocytosis was found in males from the middle dose (2.1% vs. 0.7% in controls, $p < 0.05$). Males administered the high dose showed reduced erythrocytes (3.9 vs $7.8 \cdot 10^6/mm^3$ in controls, $p < 0.05$) and haemoglobin levels (12.3% vs. 17.6% in controls, $p < 0.05$), and increased levels of reticulocytes (3.0% vs. 0.7% in controls, $p < 0.05$), although these effects were reversible. Females were not affected.
Regarding microscopic examination of tissues, authors stated that because of the similarities between the middle and low dose groups, slides from the latter were scanned, but not reported. Mild to very severe haemosiderosis in the spleen was found in treatment animals, without demonstrable tissue damage. This effect was irreversible. Control females also showed haemosiderosis.

Table 4.1.2.6.1-3: Haemosiderosis frequency and severity in the spleen of male rats treated with 2,4-DNT for 13 weeks (Lee et al., 1978, 1985)

		Dose (mg/kg bw/day)		
Group	**Haemosiderosis**	**0**	**93**	**266**
13-week	Frequency	50%	100%	100%
	Mild to moderate	2/4	1/4	1/2
	Severe to very severe	0/4	3/4	1/2
13-week stop dose	Frequency	0%	100%	100%
	Mild to moderate	0/4	1/3	1/1
	Severe to very severe	0/4	2/3	0/1

Table 4.1.2.6.1-4: Haemosiderosis frequency and severity in the spleen of female rats treated with 2,4-DNT for 13 weeks (Lee et al., 1978, 1985)

		Dose (mg/kg bw/day)		
Group	**Haemosiderosis**	**0**	**108**	**145**
13-week	Frequency	100%	100%	–
	Mild to moderate	3/4	2/4	–
	Severe to very severe	1/4	2/4	–
13-week stop dose	Frequency	100%	50%	
	Mild to moderate	4/4	1/4	–
	Severe to very severe	0/4	1/4	–

Regarding neurotoxicity, mild gliosis in the cerebrum was found in the 13-week high-dose male (1/1). Furthermore, mild gliosis and demyelination were observed in the cerebellum of the high-stop-dose male (1/1) and one middle-stop-dose male (1/3), respectively. Severe decrease in spermatogenesis were found in males treated with 93 mg/kg b.w./day of 2,4-DNT, whereas those administered 266 mg/kg b.w./day showed aspermatogenesis. The tissue lesions of rats fed 2,4-DNT remained after the recovery period; furthermore, the testicular degeneration apparently progressed.

The LOAEL was considered to be 34 mg/kg b.w./day on the basis of haemosiderosis in the spleen of males.

Kozuka *et al.*, 1979

Twenty male Wistar STD rats were fed 5000 ppm of 2,4-DNT in the diet for six months. 2,4-DNT of the first grade was further purified by column chromatography. Nevertheless, the final purity was not reported. The average 2,4-DNT consumption per day calculated from diet consumptions was about 66 mg in the three months of the first half, and was about 75 mg in the months of the latter half of experimental period. The body-weight corrected dose of 2,4-DNT was calculated, ie 207 mg/kg b.w./day. A control group of 23 rats was fed standard diet. This study was published in a peer-reviewed journal and it was considered adequate for risk assessment.

Mortality of the treatment group was higher (60%, n = 12) than that of the control group (4%, n = 1). The body weight of treated rats decreased progressively (41% reduction at 6 months), and thus the dose of 2,4-DNT increased along the 2,4-DNT-exposure period. Increased relative organ weights of treated rats were noted in liver (7.7 vs. 4.0 g/100 g b.w., $p < 0.01$), spleen (0.234 vs. 0.123 g/100g b.w., $p < 0.01$) and kidney (1.18 vs. 0.68 g/100g b.w., $p < 0.01$). Treated rats showed humpback and jerky incoordination; increased levels of methaemoglobin, triglyceride, blood glucose, aspartate aminotransferase (GOT), lactate dehydrogenase (LDH), alkaline P-ase and acid P-ase were also observed. On the contrary, albumin, A/G ratio and *p*-nitrobenzoic acid reductase decreased. Testicular atrophy and oligospermia were observed at 66 mg/kg/day, the only dose tested (0.383 vs. 0.668 g testes / 100 g body weight, $p< 0.01$).

Mice

Lee *et al.*, 1978; Hong *et al.*, 1985

Groups of 8 young healthy albino Swiss mice/sex/dose were fed 0, 700, 2000 and 7000 ppm, equivalent to 0, 47/52, 137/147, and 413/468 (males/females) mg/kg b.w./day of 2,4-DNT in the diet for 13 weeks over a 17-week observation period. At the end of 13 weeks, 4 mice/sex/dose were sacrificed for necropsy. Treatment was discontinued for 4 mice/sex/dose at the end of 13 weeks. These mice were observed for 4 more weeks and then euthanized for necropsy to study the reversibility of the adverse effects. This study is performed in essence according to OECD 408 guideline with the exception of number of animals (4 at each dose level instead of 10 and 4 in the satellite groups instead of 5) and recovery period (28 days instead of 14 days). Nevertheless, it was considered adequate to determine a NOAEL.

Two of the 8 male mice treated with the high dose died after treatment for 5 to 10 weeks vs. 0/8 in controls. One of the 8 females fed the high dose died after 7 weeks of treatment vs. 0/8 in controls. None of the mice had any obvious signs of morbidity.

Males and females given the high dose showed decreased food consumption and decreased body weight. Mice treated with the high dose showed decreased levels of haemoglobin (10.6% vs. 14% in controls, $p<0.05$ for males, and 14.2% vs. 15.5% in controls, $p < 0.05$ for females) and increased levels of reticulocytes (5.32% vs. 1.25% in controls for males, and 2.19% vs. 1.39% in controls for females). However, after the 4-week recovery period, surviving mice recovered completely.

One low-dose male died at week 11. However, that death was not considered to be treatment related since the low dose was not associated with altered parameters derived from blood analysis, increased/decreased organ weights, and treatment-related lesions.

Therefore, the NOAEL was considered to be 137 mg/kg b.w./day for males/females.

Dogs

Lee *et al.*, 1978; Ellis *et al.*, 1985

Groups of two beagle dogs/sex/dose were treated with 0, 1, 5 or 25 mg/kg b.w./day of 2,4-DNT (purity 98.5 - 99%) in hard gelatine capsules for 13 weeks over a 17-week observation period. The design of the experiment was as follows: At the end of 13 weeks of continuous treatment, 1 dog/sex/dose was euthanized for necropsy. The treatment for 1 dog/sex/dose was discontinued at the end of 13 weeks and they were then euthanized at the end of 17 weeks to study the reversibility of adverse effects.

The study was performed according to OECD guideline 409 with the exception of number of animals (2 instead of 4) and the remarks in the following paragraph.

Due to mortality observed in the 4-week study, the high-dose treatment was discontinued after 4 weeks in one dog for each sex, which were allowed to recover for 8 months. The other high-dose male survived until week 13, whereas the other high-dose female completed the 13-week treatment and was allowed to recover for 4 weeks to study the reversibility of toxic effects.

Dogs treated with 25 mg/kg b.w./day showed a significantly reduced body weight attributed to anorexia, yellow stain on and near hind legs and pale gums (sometimes blue-tinged), as well as incoordination, stiffness, and rigid paralysis. It was found methaemoglobin from 5 mg/kg b.w./day (1.9% vs. 0% in controls, and there were decreased haemoglobin levels (7.4% vs. 16.7% in controls) with Heinz bodies (4.9% vs. 0% in controls) and severe aspermatogenesis at 25 mg/kg b.w./day. In the central nervous system of the high-dose male

were found cerebellar demeylination, gliosis and oedema. The high-dose female showed mild demyelination in both the cerebrum and optic nerve after the 4-week recovery period.

Based on increased methaemoglobin, the NOAEL was considered to be 1 mg/kg b.w./day.

Chronic toxicity studies

Inhalation

No data available

Dermal

No data available

Oral

The studies described below have been carried out in order to know the effects of long-term exposure to 2,4-DNT via oral administration on different mammalian species. Although, the effects of 2,4-DNT after oral administration for 24 months in dogs, rats and mice were jointly reported by Ellis et al 1979, the effects of 2,4-DNT on each species are indicated separately in the present report.

Rats

Ellis *et al.*, 1979; Lee *et al.*, 1985

Groups of 38 healthy weanling CD rats/sex/dose were fed diets containing 0, 15, 100 or 700 ppm of 2,4-DNT (purity 98% 2,4-DNT and 2% 2,6- DNT), equivalent to 0, 0.57/0.71, 3.9/5.1 or 34/45 mg/kg b.w./day (males/females), for either 12 months (4 rats/sex/dose) over a 13-month observation period (4 rats/sex/dose), or 24 months (26 rats/sex/dose) over a 25-month observation period (4 rats/sex/dose). A few extra rodents were added to replace early losses (no further details available).

The objective of this study was to determine the chronic effects of 2,4-DNT following prolonged exposure. It was performed in essence according to OECD guideline 452, with the exception of number of animals per dose level; thus considered acceptable to determine a NOAEL. Findings of this study related to carcinogenicity are also included in section 4.1.2.8.4 summary of carcinogenicity under 4.1.2.8 carcinogenicity.

At 12 months the survival was similar between groups, being almost 100%. Mortality in the high-dose group was higher than in the other groups from month 15. About 50% of the high-dose rats had died by the end of month 20 and all high-dose rats except one female died before the end of month 23. Mortality of control, low- or middle-dose groups reached 50% sometime between months 22nd and 23rd of follow-up period. The three major causes of deaths were pituitary tumours, ulcerated subcutaneous tumours and inanition. The affected rats were lethargic and had rough fur coats. The hindquarters were stained orange to yellow. Some rats exhibited hyperexcitability; a few others had one-sided ataxia or paralysis of the hindquarters or the entire body, crusts of tear pigments around the eyes and snout, and persistent weight loss.

With respect to haematological data, erythrocytes, reticulocytes, hematocrit and haemoglobin of males and females administered either high- or middle-dose for 12 or 18 months were significantly different than those of controls (Tables 4.1.2.6.1-5,6).

Table 4.1.2.6.1-5: Haematological data [a] of male CD rats during feeding of 2,4-DNT (Ellis *et al.*, 1979; Lee *et al.*, 1985)

2,4-DNT (mg/kg/day)	Erythrocytes (10^6 / mm^3)	Reticulocytes (%)	Hematocrit (vol %)	Haemoglobin (g %)
12 months				
Control	7.5 ± 0.2	0.9 ± 0.2	51.8 ± 0.9	16.0 ± 0.2
0.57	7.10 ± 0.07	1.0 ± 0.2	50.4 ± 0.7	15.0 ± 0.2
3.9	6.7 ± 0.2[c]	1.4 ± 0.3	48.2 ± 0.9	15.1 ± 0.3
34	6.2 ± 0.3[c]	1.6 ± 0.3	46.4±1.3[c]	14.3 ± 0.5[c]
18 months				
Control	7.5 ± 0.2	0.4 ± 0.1	49.4 ± 0.6	15.2 ± 0.2
0.57	7.4 ± 0.1	0.8 ± 0.2	48.8 ± 0.7	15.4 ± 0.2
3.9	6.8 ± 0.3	0.7 ± 0.2	47.0 ± 0.8	14.5 ± 0.3
34	6.1 ± 0.3[c]	1.3 ± 0.3[c]	45.2 ± 1.6[c]	12.9 ± 0.5[c]
24 months				
Control	6.3 ± 0.4	0.72 ± 0.04	44 ± 2	13.6 ± 0.8
0.57	6.0 ± 0.4	1.4 ± 0.2	43 ± 2	13.8 ± 0.9
3.9	5.7 ± 0.5	5 ± 2	44 ± 3	13.3 ± 0.8
34[b]	–	–	–	–

Data represent mean value ± SD. [a] Other clinical variables were not altered and are omitted from the table. [b] No survival at 24 months. [c] Significantly different from the respective controls (Dunnett's multiple comparison procedure, $p < 0.05$).

Table 4.1.2.6.1-6: Haematological data [a] of female CD rats during feeding of 2,4-DNT (Ellis *et al.*, 1979; Lee *et al.*, 1985)

2,4-DNT (mg/kg/day)	Erythrocytes (10^6 / mm^3)	Reticulocytes (%)	Hematocrit (vol %)	Haemoglobin (g %)
12 months				
Control	6.5 ± 0.3	1.1 ±0 .2	47 ± 1	14.2 ± 0.5
0.71	6.2 ± 0.2	1.1 ± 0.1	47 ± 1	13.8 ± 0.4
5.1	6.7 ± 0.1	0.8 ± 0.1	47.8 ± 0.6	14.8 ± 0.2
45	5.6 ± 0.1[c]	1.5 ± 0.2	43.0 ± 0.9[c]	13.1 ± 0.3
18 months				
Control	6.9 ± 0.3	0.8 ± 0.1	45 ± 2	13.9 ± 0.4
0.71	6.4 ± 0.2	1.0 ± 0.2	45 ± 1	12.8 ± 0.1
5.1	6.8 ± 0.2	0.8 ± 0.2	46 ± 2	13.8 ± 0.4
45	5.6± 0.3[c]	2.2 ± 0.4[c]	40 ± 2	12.0 ± 0.5[c]
24 months				
Control	6.0 ± 0.5	1.3 ± 0.3	43 ± 2	14.2 ± 0.7
0.71	6.1 ± 0.2	1.4 ± 0.2	43 ± 1	14.1 ± 0.5
5.1	5.4 ± 0.6	3 ± 1	41 ± 3	13 ± 1
45[b]	0.9	9.5	33	9.4

Data represent mean value ± SD. [a] Other clinical variables were not altered and are omitted from the table. [b] Only one female. [c] Significantly different from the respective controls (Dunnett's multiple comparison procedure, $p < 0.05$).

After the recovery periods, the above mentioned effects of anaemia were not observed, with the exception of the decreased erythrocyte levels in high-dose females treated for 12 months (6.3 vs. $7.0 \cdot 10^6$ / mm^3 in controls, $p < 0.05$).

The main treatment-related histopathological findings observed at the highest dose after 12 months (Tables 4.1.2.6.1-7,8) were hyperplastic foci, neoplastic nodules and one carcinoma in one stop-exposure female in liver; severe atrophy of seminiferous tubules in testes (two males with aspermatogenesis vs absence of lesions in controls); and excessive pigmentation (spleen) which could be due to accumulation of iron, as secondary effect to methaemoglobinaemia.

With respect to liver lesions, mild hyperplastic foci were observed in both 12-month and 12-month stop-dose males treated with the low dose (4/4 and 3/4, respectively). Hence 7 of the 8 low-dose males (88%) showed mild hyperplastic foci vs 2 (questionable) of the 7 controls (≤29%). In addition, the mentioned liver lesions developed to neoplastic nodules and hepatocellular carcinoma as increased 2,4-DNT exposure time and dosage. At 24 months, the number and severity of hyperplastic foci did not differ between controls and low-dose males (24-month and 24-month stop-dose groups). According to both 12-month and 24-month results, the lesions were apparent in the low-dose males before than in controls, which could be related to the 2,4-DNT treatment. Hence, the presence of mild hyperplastic foci is considered a critical effect for risk assessment.

Table 4.1.2.6.1-7: Incidence of lesions in rats fed 2,4-DNT for 12 months (Ellis *et al.*, 1979; Lee *et al.*, 1985).

		Dose (mg/kg b.w./day)							
		Males				Females			
		0	**0.57**	**3.9**	**34**	**0**	**0.71**	**5.1**	**45**
Liver									
Hyperplastic foci	Mild	2[?]/4[a]	4/4	3/4	0/4	1/4	0/4	0/4	0/4
	Marked to severe	0/4	0/4	0/4	2/4	0/4	0/4	0/4	¾
Neoplastic nodule		0/4	0/4	0/4	4/4	0/4	0/4	0/4	4/4
Hepatocellular carcinoma		0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Testes									
Atrophy of seminiferous tubules		0/4	0/4	0/4	4/4[b]				
Spleen									
Excessive pigmentation		0/4	0/4	0/4	4/4	0/4	0/4	0/4	¾

[?] Questionable. [a] Number of rats affected / number of rats examined [b] Atrophy of seminiferous tubules was severe.

Table 4.1.2.6.1-8: Incidence of lesions in rats fed 2,4-DNT for 12 months and allowed to recover for 1 month (Ellis *et al.*, 1979; Lee *et al.*, 1985).

		Dose (mg/kg b.w./day)							
		Males				Females			
		0	**0.57**	**3.9**	**34**	**0**	**0.71**	**5.1**	**45**
Liver									
Hyperplastic foci	Mild	0/3[a]	3/4	3/4	1/3	0/4	1/4	1/4	2/4
	Marked to severe	0/3	0/4	0/4	2/3	0/4	0/4	0/4	¾
Neoplastic nodule		0/3	1/4	0/4	2/3	0/4	0/4	0/4	¾
Hepatocellular carcinoma		0/3	0/4	0/4	0/4	0/4	0/4	0/4	¼
Testes									
Atrophy of seminiferous tubules		0/3	0/4	0/4	2/3[b]				
Spleen									
Excessive pigmentation		0/3	0/4	0/4	2/3	0/4	2/4	0/4	4/4

[a] Number of rats affected / number of rats examined [b] Atrophy of seminiferous tubules was severe.

Concerning atrophy of seminiferous tubules, at the high dose the lesion was severe in both 12-month and 12-month stop-dose males whereas controls showed absence of 2,4-DNT-related lesions in testes. From the low dose, both incidence and severity of atrophy of seminiferous

tubules in males dying at unscheduled times were higher than those of controls (Table 4.1.2.6.1-9). Thus, it was found a dose-response relationship between seminiferous tubules atrophy and 2,4-DNT dose. Moreover, low-dose males treated for more than 12 months showed an increased incidence when compared with that of controls (Table 4.1.2.6.1-10). Therefore, atrophy of seminiferous tubules is considered a critical effect for risk assessment.

Table 4.1.2.6.1-9: Severity and incidence of the atrophy of seminiferous tubules in male rats dying at unscheduled times (Ellis *et al.*, 1979; Lee *et al.*, 1985).

		Dose (mg/kg b.w./day)			
		0	**0.57**	**3.9**	**34**
Severity	Mild	1[a]	1	–	–
	Moderate	1	–	–	1
	Marked	–	–	–	1
	Severe	–	2	4	23
N		13	16	13	30
Incidence of severe atrophy of seminiferous tubules		0	0.13	0.31	0.77

[a] Number of rats with degeneration of seminiferous tubules. N, total number of rats dying at unscheduled times.

Regarding high-dose treatment for more than 12 months (Table 4.1.2.6.1-10) there were an increased incidence of hepatocellular carcinoma (males $p > 0.05$ and females $p < 0.05$) with increased hyperplastic foci severity in liver; mammary gland tumours in females; and skin tumours in males.

Table 4.1.2.6.1-10: Incidence of lesions in rats fed 2,4-DNT for more than 12 months (Ellis *et al.*, 1979; Lee *et al.*, 1985).

	Dose (mg/kg b.w./day)							
	Males				Females			
	0	**0.57**	**3.9**	**34**	**0**	**0.71**	**5.1**	**45**
Hyperplastic foci	9/25[a]	10/28	9/19	16/29	7/23	18/35	19/27	13/34
(Percentage)	(36%)	(36%)	(47%)	(55%)	(32%)	(51%)	(70%)	(38%)
Hepatocellular neoplastic nodule [b]	1/25	2/28	1/19	2/29	0/23	3/35	2/27	6/34
(Percentage)	(4%)	(7%)	(5%)	(7%)	(0%)	(9%)	(7%)	(18%)
Hepatocellular carcinoma[b,c]	1/25	0/28	1/19	6/29	0/23	0/35	1/27	18/34
(Percentage)	(4%)	(0%)	(5%)	(21%)	(0%)	(0%)	(4%)	(53%)
Testicular atrophy	4/25	8/28	6/18	25/29				
(Percentage)	(16%)	(29%)	(33%)	(86%)				
Mammary gland tumours[c,d]	0/25	0/28	0/19	2/30	11/23	12/35	17/27	33/35
(Percentage)	(0%)	(0%)	(0%)	(7%)	(48%)	(34%)	(63%)	(94%)
Skin tumours [c,e]	2/25	4/28	3/19	17/30	1/22	3/35	0/27	6/35
(Percentage)	(8%)	(14%)	(16%)	(57%)	(5%)	(9%)	(0%)	(17%)
Pituitary adenoma [c]	9/22	14/23	7/14	2/20	18/23	24/30	20/24	7/23
(Percentage)	(41%)	(61%)	(50%)	(10%)	(78%)	(80%)	(83%)	(30)

[a] Number of rats affected / number of rats examined. The results of rats fed 2,4-DNT for more than 12 months did not include all animals due to some rats that died at night and autolysis hindered examination. To increase the numbers available for calculating incidence, the authors included all rats fed the same dosage which were intended for a metabolism study and a few females intended for a three-generation study but not mated and continued on feed (further details not available).
[b] Classification of liver tumour by Squire was followed.
[c] Number of animals examined denotes the number of living when the first tumour was observed
[d] Including benign and malignant tumours from epithelial or mesenchymal cells.
[e] Including epidermal epithelial and subcutaneous mesenchymal tumours.

Based on these findings, the high dose was considered markedly toxic at 12 months. Although, some treatment-related changes were observed in the middle-dose rats, they have limited quality to consider them as adverse due to the low numbers of animals used at this time. Nevertheless, since liver and testes lesions, between others, increased their incidence/severity after treatment for more than 12 months, even at the low-dose level, a conservative LOAEL of 0.57/0.71 mg/kg b.w./day (males/females) could be considered for chronic toxicity.

Mice

Ellis *et al.*, 1979; Hong *et al.*, 1985

Groups of 58 healthy weanling CD-1 mice/sex/dose were fed diets containing 0, 100, 700 or 5000 ppm of 2,4-DNT (purity 98% 2,4-DNT and 2% 2,6- DNT), equivalent to 0, 13.3/13.7, 96.9/93.8, and 885/911 mg/kg b.w./day (males/females), for either 12 months (4 mice/sex/dose) over a 13-month observation period (4 mice/sex/dose), or 24 months (46 mice/sex/dose) over a 25-month observation period (4 mice/sex/dose). A few extra rodents were added to replace early losses (no further details available).

The objective of this study was to determine the chronic effects of 2,4-DNT following prolonged exposure. It was performed in essence according to OECD guideline 452; hence considered adequate to determine a NOAEL. Findings of this study related to carcinogenicity are also included in section 4.1.2.8.4 summary of carcinogenicity under 4.1.2.8 carcinogenicity.

About 50% of the high-dose mice had died by the end of month 10 and all high-dose mice died before the end of month either 18 (males) or 21 (females). Mortality of control, low- or middle-dose groups reached 50% sometime between months 19th and 21st of follow-up period. The high-dose mice had low body weights when compared with the other groups. There was a pronounced dose effect in the first month of the study with the high-dose mice eating less than the other groups ($p > 0.05$ for males and $p < 0.05$ for females). However, the average feed consumption of mice along the study did not differ significantly between groups, since, the relatively high consumptions of the few surviving high-dose mice during the later months contributed to raise the overall averages.

All high-dose mice exposed for 12 months were hunchbacked and relatively inactive, often resting with their feet tucked in and hair erect as if chilled. When stimulated, the mice became hyperactive, running around the cage with a peculiar, stiff-legged gait. Often their eyes appeared dark and sunken. Red-orange stains, apparently from urine, were noted in the hindquarters.

Decreased erythrocytes and haemoglobin with increased methaemoglobin and Heinz bodies were observed in high-dose mice dying at unscheduled times when compared with controls. Moreover, as shown in Tables 4.1.2.6.1-11,12. Heinz bodies, reticulocytes and methaemoglobin of both males and females treated with the high dose for 12 months were significantly higher than those of controls ($p < 0.05$). These effects were reversible.

Table 4.1.2.6.1-11: Haematological data of male CD-1 mice during feeding of 2,4-DNT (Ellis *et al.*, 1979; Hong *et al.*, 1985)

2,4-DNT (mg/kg/day)	Heinz bodies (%)	Reticulocytes (%)	Haemoglobin (g %)	Methaemoglobin (%)
12 months				
Control	0.0 ± 0.0	1.5 ± 0.2	13.5 ± 0.6	0.0 ± 0.0
13.3	0.0 ± 0.0	1.06 ± 0.08	13.0 ± 0.6	0.0 ± 0.0
96.9	0.0 ± 0.0	1.9 ± 0.1	13.6 ± 0.7	0.0 ± 0.0
885	8 ± 3	4.1 ± 0.8[b]	11.5 ± 0.5	4 ± 2[b]
24 months				
Control	0.0 ± 0.0	1.3 ± 0.4	12 ± 1	0.0 ± 0.0
13.3	0.0 ± 0.0	1.3 ± 0.3	12.8 ± 0.4	1 ± 1
96.9	0.0 ± 0.0	2.0 ± 0.7	11.7 ± 0.9	0.0 ± 0.0
885[a]	–	–	–	–

Data represent mean value ± SD. [a] No survival of the high-dose male at 24 months. [b] Significantly different from the respective controls (Dunnett's multiple comparison procedure, $p < 0.05$).

Table 4.1.2.6.1-12: Haematological data of female CD-1 mice during feeding of 2,4-DNT (Ellis *et al.*, 1979; Hong *et al.*, 1985)

2,4-DNT (mg/kg/day)	Heinz bodies (%)	Reticulocytes (%)	Haemoglobin (g %)	Methaemoglobin (%)
12 months				
Control	0.0 ± 0.0	1.1 ± 0.2	12.5 ± 0.3	0.0 ± 0.0
13.3	0.0 ± 0.0	1.2 ± 0.2	13.1 ± 0.7	0.0 ± 0.0
93.8	0.0 ± 0.0	1.1 ± 0.1	13.3 ± 0.2	0.0 ± 0.0
911	6 ± 3	3.5 ± 0.7[b]	11.8 ± 0.3	3 ± 2[b]
24 months				
Control	0.0 ± 0.0	1.1 ± 0.1	13.9 ± 0.4	0.0 ± 0.0
13.3	0.0 ± 0.0	1.4 ± 0.3	13.4 ± 0.5	0.0 ± 0.0
93.8	0.0 ± 0.0	1.0 ± 0.1	14.5 ± 0.5	0.0 ± 0.0
911[a]	–	–	–	–

Data represent mean value ± SD. [a] No survival of the high-dose female at 24 months. [b] Significantly different from the respective controls (Dunnett's multiple comparison procedure, $p < 0.05$).

After 12-month treatment, the high dose mice showed increased liver weights relative to body weight. The males showed decreased testes weights. The trend in the changes of both liver and testes weight remained after the 1-month recovery period.

Regarding histopathology, treatment-related lesions were found in liver, kidney and gonads (Tables 4.1.2.6.1-,13,14,15). With respect to 12-month treatment, the high-dose mice showed nephropathy, and renal tumours in males; hepatocellular dysplasia; and atrophy in testes and ovaries. Regarding treatment for more than 12 months, livers with hepatocellular dysplasia were observed from the low dose in males and at the high dose in females. From the low dose, kidney showed an increased incidence of toxic nephropathy in both sexes and renal tumours in males. It was noted testicular atrophy from the middle dose, and ovarian atrophy with non functioning follicles at the high dose. The low incidence of tumours in the high dose males

occurred presumably because they died before the lesions developed. Therefore, males were more sensitive than females.

Generalized abnormal pigmentation was found in mice of both sexes treated with the high dose for 12 months and remained after the recovery period. In addition, it was noted from the low dose in males and females treated for more than 12 months. This widespread accumulation of pigment could be due to accumulation of iron, as secondary effect to methaemoglobinaemia.

Table 4.1.2.6.1-13: Incidence of lesions in mice fed 2,4-DNT for 12 months (Ellis et al., 1979; Hong et al., 1985).

	Dose (mg/kg/day)							
	Males				Females			
	0	13.3	96.9	885	0	13.7	93.8	911
Hepatocellular dysplasia	0/4	0/4	0/4	4/4	0/4	0/4	0/4	4/4
Hepatocellular carcinoma	0/4	0/4	0/4	1/4	0/4	0/4	0/4	¼
Toxic nephropathy	1/4	0/4	2/4	3/4	1/4	2/4	1/4	2/4
Kidney carcinoma	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4
Testicular or ovarian atrophy	0/4	0/4	1/4	4/4	1/4	0/4	0/4	2–3/4
Generalized abnormal pigmentation	0/4	0/4	0/4	4/4	0/4	0/4	0/4	4/4

[a] Number of mice affected / number of mice examined

Table 4.1.2.6.1-14: Incidence of lesions in mice fed 2,4-DNT for 12 months and allowed to recover for 1 month (Ellis et al., 1979; Hong et al., 1985).

	Dose (mg/kg/day)							
	Males				Females			
	0	13.3	96.9	885	0	13.7	93.8	911
Hepatocellular dysplasia	0/4	0/4	0/4	4/4	0/4	0/4	0/4	3/4
Hepatocellular carcinoma	0/4	0/4	0/4	2/4	0/4	0/4	0/4	0/4
Toxic nephropathy	1/4	1/4	0/4	3/4	0/4	0/4	0/4	0/4
Kidney adenoma	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4
Testicular or ovarian atrophy	0/4	1/4	0/4	3/4	0/4	0/4	0/4	3/4
Generalized abnormal pigmentation	0/4	0/4	0/4	4/4	0/4	0/4	0/4	4/4

[a] Number of mice affected / number of mice examined

Table 4.1.2.6.1-15: Incidence of lesions in mice fed 2,4-DNT for more than 12 months (Ellis *et al.*, 1979; Hong *et al.*, 1985)

	Dose (mg/kg/day)							
	Males				Females			
	0	**13.3**	**96.9**	**885**	**0**	**13.7**	**93.8**	**911**
Hepatocellular dysplasia	2/33	14/33	12/28	40/40	5/31	3/29	5/31	29/33
(Percentage)	(6%)	(42%)	(43%)	(100%)	(16%	(10%)	(16%)	(88%)
Hepatocellular carcinoma	7/33	9/33	8/28	5/40	2/31	1/29	3/31	1/33
(Percentage)	(21%)	(27%)	(29%)	(13%)	(6%)	(3%)	(10%)	(3%)
Toxic nephropathy	0/33	3/33	3/28	32/40	0/31	3/29	2/31	10/32
(Percentage)	(0%)	(9%)	(11%)	(80%)	(0%)	(10%)	(6%)	(31%)
Kidney tumours	0/33	5/33	16/28	3/40	0/31	0/29	0/31	1/32
(Percentage)	(0%)	(15%)	(57%)	(8%)	(0%)	(0%)	(0%)	(3%)
Testicular or ovarian atrophy	7/32	4/33	11/28	34/39	1/28	2/23	0/27	15/24
(Percentage)	(22%)	(12%)	(39%)	(87%	(4%)	(9%)	(0%)	(63%)
Generalized abnormal pigmentation	0/33	2/33	4/29	38/40	0/31	4/29	8/31	27/33
(Percentage)	(0%)	(6%)	(14%)	(95%)	(0%)	(14%)	(26%)	(82%)

[a] Number of mice affected / number of mice examined

The LOAEL for chronic toxicity was considered to be 13.3/13.7 mg/kg b.w./day (males/females) on the basis of lesions in both kidney and liver.

Dogs

Ellis *et al.*, 1979; Ellis *et al.*, 1985

Groups of six beagle dogs/sex/dose were treated with 0, 0.2, 1.5 or 10 mg/Kg b.w./day of 2,4-DNT (purity 98% 2,4-DNT and 2% 2,6- DNT) in hard gelatine capsules for either 12 months (1 dog/sex/dose) over a 13-month observation period (1 dog/sex/dose), or 24 months (2 dogs/sex/dose) over a 25-month observation period (2 dogs/sex/dose).

This study is performed in essence according to OECD 452 guideline and considered adequate to determine a NOAEL.

The death of three high-dose males was due to neurotoxicity (incoordination and paralysis) during the 8th, 18th and 19th weeks of treatment. The cause of death of one dog was unrelated to treatment. Thus, only two high-dose males survived, both of them were continued to the end of 24 months, where one was necropsied and the other placed on the recovery study. No high-dose male was used for the 12-month necropsy and recovery study. During month 22 of treatment a middle dose female died. It was found a high interindividual susceptibility to 2,4-DNT. Moreover, males were more sensitive than females.

Body weight of dogs treated with the low dose of 2,4-DNT was higher than that of controls, which was higher than that of middle- and high-dose dogs given the high dose. Dogs administered the middle dose of 2,4-DNT showed the lowest body weight. The average feed consumption did not differ significantly among groups.

Neurotoxic symptoms involving the hind legs and finer control of lips and tongue were observed in all high-dose dogs (after weeks 8 to 20) and in one middle-dose male (after week 66). Those neurotoxic symptoms disappeared within a week in the middle-dose male, and were intermittent in the surviving high-dose dogs and accompanied by weight losses. Therefore, soft diets were used when dogs showed severe incoordination in order to increase their body weight; since affected dogs could neither eat nor drink. Then, authors commented that if parenteral nutrition ("hyperalimentation") had been used, the recovery of the severely paralyzed dogs would have been possible. The neurotoxic effects appeared to be cumulative, since the accumulated dose was fairly constant when neurotoxicity was first noted (Table 4.1.2.6.1-16).

Table 4.1.2.6.1-16: Time to first neurotoxic symptoms and accumulated dose of 2,4/DNT in dogs (Ellis *et al.*, 1979, 1985)

Daily dose(mg/kg)	*Days to cause symptoms*	*Accumulated dose (mg/kg)*
25	12	300
10	52	520
5	> 91	> 455
1.5	465	697.5

The daily doses of 5 and 25 mg/kg b.w./day were tested in the 13-week dog study.

Regarding haematological effects of 2,4-DNT at 12 months (Tables 4.1.2.6.1-17,18), reticulocytes and Heinz bodies levels of high-dose males and females were significantly higher than those of controls ($p < 0.05$). Furthermore, erythrocytes and haemoglobin levels of female dogs treated from the middle dose were significantly lower than those of controls ($p < 0.05$). It is likely that due to an adaptive response to 2,4-DNT exposure, the presence of Heinz bodies was not observed in treated dogs after the 24-month exposure period (Tables 4.1.2.6.1-17,18). Both 12-month and 24-month stop-dose dogs were recovered from the anaemia, with no Heinz bodies, and both erythrocyte and reticulocyte levels in the high-dose dogs did not differ from those of controls.

Table 4.1.2.6.1-17: Haematological data of male beagle dogs during feeding of 2,4-DNT (Ellis *et al.*, 1979, 1985).

2,4-DNT (mg/kg/day)	Heinz bodies (%)	Reticulocytes (%)	Erythrocytes ($\cdot 10^6/mm^3$)	Haemoglobin (g %)
12 months				
Control	0 ± 0	¿?	6.0 ± 0.2	15.1 ± 0.4
0.2	0 ± 0	0.7 ± 0.1	5.3 ± 0.2	14.1 ± 0.2
1.5	0 ± 0	0.7 ± 0.1	5.7 ± 0.2	14.4 ± 0.4
10	0.5± 0.4	1.2 ± 0.2[a]	5.2 ± 0.2	14 ± 1
24 months				
Control	0 ± 0	0.7 ± 0.1	5.9 ± 0.2	15.5 ± 0.6
0.2	0 ± 0	0.8 ± 0.2	6.1 ± 0.3	14.9 ± 0.6
1.5	0 ± 0	0.6 ± 0.2	5.9 ± 0.2	14.7 ± 0.6
10	0 ± 0	0.49 ± 0.05	6.7 ± 0.5	16.3 ± 0.8

[a] Significantly different from control dogs (Dunnett's multiple comparison procedure). Entries are ± mean standard error.

Table 4.1.2.6.1-18: Haematological data of female beagle dogs during feeding of 2,4-DNT (Ellis *et al.*, 1979, 1985).

2,4-DNT (mg/kg/day)	Heinz bodies (%)	Reticulocytes (%)	Erythrocytes ($\cdot 10^6/mm^3$)	Haemoglobin (g %)
12 months				
Control	0 ± 0	0.4 ± 0.4	5.9 ± 0.3	15.1 ± 0.4
0.2	0 ± 0	0.84 ± 0.07[a]	5.5 ± 0.1	15.1 ± 0.4
1.5	0 ± 0	0.45 ± 0.06	4.7 ± 0.3[a]	13.7 ± 0.5[a]
10	0.5 ± 0.2	1.6 ± 0.2[a]	4.5 ± 0.3[a]	12.9 ± 0.2[a]
24 months				
Control	0 ± 0	0.4 ± 0.1	6.32 ± 0.06	16.4 ± 0.3
0.2	0 ± 0	0.25 ± 0.02	5.8 ± 0.2	14.2 ± 0.6
1.5	0 ± 0	0.34 ± 0.03	5.7 ± 0.2	14.9 ± 0.2
10	0 ± 0	0.6 ± 0.1	6.6 ± 0.4	16 ± 1

[a] Significantly different from control dogs (Dunnett's multiple comparison procedure).
Entries are ± mean standard error.

The three high-dose male dogs with paralysis, killed for necropsy (at 8, 18 and 19 weeks after treatment) had generalized vacuolization, hypertrophy, endothelial mitosis, and focal gliosis in the cerebellum, as well as some perivascular haemorrhage in the cerebellum and brain stem. These lesions were probably responsible for the incoordination, ataxia and paralysis produced in all the high-dose dogs. The paralysis ceased when the barbiturate overdose used for euthanasia began.

The tissue lesions after the 12-month exposure period were mild bile duct hyperplasia and pigment deposits in the liver of the high-dose female. After the 1-month recovery period, mild

bile duct hyperplasia was noted in the low-dose female, and pigment deposits were found in liver (mild) and kidney (mild to moderate) of both the middle- and high-dose females.

Histopathologic examination at 24 months revealed lesions which were treatment related. The high-dose group at 24th month comprised 2 females and one male. Mild bile duct hyperplasia and clusters of brown pigment-laden Kupffer cells were found in the liver of all 3 high-dose dogs and in one low-dose female. Cystic hyperplasia of the epithelium occurred in the gallbladder of all high-dose dogs (mild in females and moderate in the male), one middle-dose male (mild) and one control female (mild). Brown epithelial pigmentation was noted in the gallbladder of the two high-dose females and in three middle-dose dogs (2 females and 1 male), and in the kidney of the two high-dose females. Excessive pigment was also seen in the spleen of two high-dose dogs (one male and one female). The pigmentation could be due to accumulation of iron as secondary effect to methaemoglobinemia. Treatment-related lesions of dogs given 2,4-DNT for 2 years and allowed to recover for one month were similar to those terminated at the end of two years. Development of tumours was not observed in dogs, presumably due to the relatively limited dosing period.

The NOAEL for chronic toxicity was 0.2 mg/kg b.w./day for males and females on the basis of neurotoxicity (incoordination and paralysis).

4.1.2.6.2 Human data

Levine *et al.*, 1986

Cohorts of 156 and 301 men who had worked for a month or more at jobs where they were exposed to DNT at two ammunition plants were followed up from 1940s-1950s to 1980. The purified 2,4-DNT contained at least 98% 2,4-DNT and about 1% 2,6-DNT. The ratio of observed to expected death using mortality rates for US white males was expressed as the standardized mortality ratio (SMR). Statistical significance was evaluated by computing X^2 with one degree of freedom. Statistical power was estimated by the method of Beaumont and Breslow.

In all, 164 men from the two plants died, compared with 127 expected, using mortality rates for US white males as the standard. The combined SMR of 1.29 for all causes of death was significantly high (p = 0.001), and it increased after 15 years elapsed since entry into study (SMR 1.4; p = 0.00007). Elevated all-cause mortality appeared to be due largely to increased mortality from diseases of the circulatory system (87 cases; SMR 1.40; p = 0.02) and from accidents, poisonings and violence (28 cases; SMR 1.91; p = 0.0007). Deaths due to accidents, poisonings, and violence were elevated only at Radford. All of these deaths occurred following termination of employment. Mortality from all causes (SMRs 1.25 and 133; 95% CI 0.85 to 1.77 and 1.09 to 1.54 at Joiet and Radford, respectively) and mortality from circulatory system diseases (SMRs 1.26 and 1.43; 95% CI 0.65 to 2.34 and 1.12 to 1.79, respectively) were in excess at both plants. The preponderance of excess circulatory system deaths resulted from ischemic heart disease (n = 64 cases) and residual diseases of the circulatory system (n = 13 cases), which included congestive heart failure, cardiac arrest, and arteriosclerosis. Nevertheless, people living in areas surrounding the Joliet and Radford plants at most ages experienced greater mortality from ischemic heart disease than the US population. When observed and expected deaths were tallied using local county experience as standard, mortality from ischemic heart disease remained high at both plants (SMRs 1.33 and 1.38 at Joliet and Radford; 95%CI 0.53 to 2.75 and 0.96 to 1.93, respectively). The combined

SMR was 1.37 (p = 0.05). Moreover, suggestive, but not significant, is the evidence of a relationship between heart disease and duration and intensity of exposure. It should be noted that the level of exposure in workers was not measured and thus there is no information available on the systemic dose of 2,4-DNT absorbed.

This study obtained an excess of heart disease mortality in workers involved in the manufacture and processing of DNT. Nevertheless, this study is of low quality.

Stayner *et al.*, 1992

A retrospective cohort mortality study on 4989 workers exposed to DNT and 5136 unexposed workers was conducted to examine the relationship between DNT exposure and the both cardiovascular disease and ischemic heart disease (IHD) mortality.

The study population was identified from current and former white male workers who had been employed for at least five months at the US Army munitions facility in Radford (Virginia) from 1 January 1949 to 21 January 1980. Information sources were personnel records for all workers employed at the facility, Social Security Administration, Internal Revenue Service, post offices, motor vehicle department and state vital statistics bureaus. Each operation which occurred more than once was rated concerning the probability of exposure to DNT forming 2 groups: workers probably exposed to DNT (n = 4989) and workers not probably exposed to DNT (n = 5136). A modified life-table program was used to compute the expected numbers of deaths by multiplying mortality rates specific for cause, five-year age groups, and five-year calendar groups from data on white males in the US population by the corresponding person-years distribution of the study population. Standardized mortality ratios (SMRs) were calculated by dividing the observed deaths by the number expected. Exact statistical tests (two-tailed) and associated 95% confidence intervals (95% CI) were estimated on the basis of the Poisson distribution. Directly standardized rate ratios (SRRs) were computed with the use of the age and calendar-time distribution of the person-years of the entire study population as the weighting factor.

In the exposed cohort, there were recorded 36 and 253 cases of cerebrovascular and ischemic heart disease mortality, respectively; and 70 and 423 cases in the unexposed cohort, respectively. Thus observed and expected mortality from both cerebrovascular and ischemic heart diseases did not differ significantly (SMR = 0.95, SRR = 0.89; and SMR = 0.98, SRR = 0.99, respectively; 95% CIs were not available) when either the US population or the unexposed group was used as the reference group. In addition, no consistent trends with either age stratum or calendar year were evident for mortality from IHD or cerebrovascular disease. Both exposed and unexposed cohorts experienced an excess in mortality from mental and personality disorders, which was primarily due to alcoholism and violence, when compared with the US population.

DNT-exposure measurements were not performed. Thus, the proportion of exposed workers who absorbed a significant amount of 2,4-DNT was not known. Furthermore, clinical data of the participants different from mortality were not available, and thus it is not known whether participants showed adverse effects related to 2,4-DNT exposure. Hence, this study is of low relevance for risk assessment.

Jones *et al.*, 2005

In previous studies where the health status of workers exposed to DNT was assessed, the most common complaints recorded were due mainly to the ability of DNT to induce MetHb, the

secondary effects of which were non-specific health effects such as headache, dizziness, nausea and drowsiness. In this study, each worker was examined for adverse health effects linked to exposure to DNT such as inertia, somnolence, insomnia, headache, dizziness and nausea. The health effects were compared with the Hb adduct levels using logistic regression analysis. The odds of suffering from inertia were 3.2 times higher [95% confidence interval (CI) = 1.8–5.8] when the level of 4A2NT Hb adducts increased by one log-unit. Similar odds ratios (OR) were observed with somnolence (3.1, CI = 1.4–6.9), nausea (2.4, CI = 1.3–4.3) and dizziness (5.5, CI = 1.3–24.2). These results inferred that quantification of DNT–Hb adducts provided an effective biomarker of toxicity and could be used to estimate the risk associated with a particular exposure to DNT.

4.1.2.6.3 Summary of repeated dose toxicity

Human data

The most common adverse health effect in workers exposed to DNT is related to the ability of DNT to induce MetHb, the secondary effects of which were non-specific health effects such as headache, dizziness, nausea and drowsiness (Jones *et al.,* 2005)

The report of two human studies indicated that in DNT exposed workers there was an excess of mortality due to ischemic heart disease and residual diseases of the circulatory system. However, DNT-exposure measurements were not performed. Thus, the proportion of exposed workers who absorbed a significant amount of 2,4-DNT was not known, and consequently those studies are of low relevance for risk assessment.

Studies in animals

According to the findings mentioned in *Sub-Acute*, *Sub-Chronic* and *Chronic Toxicity Studies in animals* under section *4.1.2.6.1. Repeated Dose Toxicity*, the relevant values for risk assessment are summarized below for rat, mouse and dog studies.

Sub-acute toxicity studies

Rats

Based on splenic haemosiderosis, the LOAEL was considered to be 38 mg/kg b.w./day derived from the 28-day study (Table 4.1.2.6.3-1).

Mice

The NOAEL was considered to be 132 mg/kg b.w./day in males on the basis of mild aspermatogenesis derived from the 4-week study (Table 4.1.2.6.3-1).

Dogs

Males were more sensitive to 2,4-DNT toxicity than females. Moreover, it was found a high interindividual susceptibility to 2,4-DNT effects. The LOAEL was considered to be 1 mg/kg b.w./day on the basis of increased reticulocytosis derived from the 4-week study (Table 4.1.2.6.3-1).

Sub-chronic toxicity studies

Rats

The LOAEL was considered to be 34 mg/kg b.w./day on the basis of haemosiderosis in the spleen of males derived from the 13-week study (Table 4.1.2.6.3-2).

Mice

The NOAEL was considered to be 137 mg/kg b.w./day in males on the basis of mortality derived from the 13-week study (Table 4.1.2.6.3-2).

Dogs

The NOAEL was considered to be 1 mg/kg b.w./day on the basis of increased methaemoglobin derived from the dog 13-week study. The males were more sensitive to 2,4-DNT toxicity than females. Moreover, it was found a high interindividual susceptibility to 2,4-DNT effects (Table 4.1.2.6.3-2).

The critical effect for sub-acute and sub-chronic toxicity studies was substance-induced haemolytic anaemia. The blood was considered as a target organ of 2,4-DNT toxicity, since effects such as clinical signs of hypoxia; decreased counts of haemoglobin and erythrocytes; increased counts of methaemoglobin, Heinz bodies and reticulocytes; and splenic haemosiderosis were found in experimental animals. Haemolytic-anaemia related effects were also observed in long-term studies.

Therefore, the observed changes indicated that the adaptive capacity was exceeded and thus, the secondary effects of haemolytic anaemia were considered relevant for risk assessment.

According to the L/NOAEL values derived from sub-acute and sub-chronic toxicity studies, beagle dogs appear to be the most sensitive species to 2,4-DNT toxicity. Therefore, the relevant LOAEL value for both sub-chronic and sub-acute toxicity studies was 1 mg/kg bw/day on the basis of increased reticulocytosis derived from the 4-week dog study, since a high interindividual variability was found in both 4-week and 13-week dog studies.

Chronic toxicity studies

Rats

The critical effects for risk characterisation derived from the 24-month rat study were presence of hyperplastic foci in the liver and atrophy of seminiferous tubules. With respect to liver lesions, mild hyperplastic foci were observed in both 12-month and 12-month stop-dose males treated with the low dose (4/4 and 3/4, respectively). Hence 7 of 8 the low-dose males (88%) showed mild hyperplastic foci vs 2 (questionable) of the 7 controls (≤29%). In addition, the mentioned liver lesions developed to neoplastic nodules and hepatocellular carcinoma as increased 2,4-DNT exposure time and dosage. At 24 months, the number and severity of hyperplastic foci did not differ between controls and low-dose males. According to both 12-month and 24-month results, the lesions were apparent in the low-dose males before than in controls, which could be related to the 2,4-DNT treatment. Concerning atrophy of seminiferous tubules, at the high dose the lesion was severe in both 12-month and 12-month stop-dose males whereas controls showed absence of 2,4-DNT-related lesions in testes. From the low dose, both incidence and severity of atrophy of seminiferous tubules in males dying at unscheduled times were higher than those of controls (Table 4.1.2.6.1-5). Thus, it was found a dose-response relationship between seminiferous tubules atrophy and 2,4-DNT dose.

Moreover, low-dose males treated for more than 12 moths showed an increased incidence when compared with that of controls (Table 4.1.2.6.1-6).

Thus, the LOAEL was considered to be 0.57 and 0.71 mg/kg b.w./day for male and female CD rats, respectively, on the basis of the hyperplastic foci incidence in the liver and atrophy of seminiferous tubules derived from the 24-month study (Table 4.1.2.6.3-3).

Mice

The LOAEL was considered to be 13 mg/kg b.w./day for male and female CD-1 mice on the basis of lesions in both kidney (males and females) and liver (males) derived from the 24-month study (Table 4.1.2.6.3-3).

Dogs

The NOAEL for male and female beagle dogs was 0.2 mg/kg b.w./day of 2,4-DNT on the basis of neurotoxicity (incoordination and paralysis) derived from the 24-month study (Table 4.1.2.6.3-3).

According to the NOAEL/LOAEL values derived from chronic toxicity studies, both dogs and rats appear to be sensitive species to 2,4-DNT toxicity. Nevertheless, the rat study with exposure duration up to 24 months is considered more appropriate for the risk characterisation of repeated dose toxicity than the 24-month dog study. Thus the critical effects for risk characterisation derived from the 24-month rat study were presence of hyperplastic foci in the liver and atrophy of seminiferous tubules.

Therefore, the relevant LOAEL for risk characterisation is considered to be 0.57 mg/kg b.w./day derived from the 24-month rat study.

Table 4.1.2.6.3-1: Sub-acute toxicity studies

Species and sex	Protocol	Results and comments	References
♂, ♀ Sprague-Dawley rats	Groups of 5 rats/sex/dose were administered 0, 96, 125, 183 or 260 (♂) and 99, 124, 191 or 254 mg/kg b.w./day (♀) in the diet for 14 days. purity = 97%; contaminants 2% of 2,6-DNT and unspecified 1%	From 96(♂) and 99(♀) mg/kg b.w./day it was observed: ↓ b.w. with ↓ food consumption (♂,♀) ↑ Blood cholesterol (♂,♀) ↑ Blood alanine aminotransferase (♂) Hyaline droplet formation (♂, ♀) Oligospermia with degenerative changes in the testes (♂)	McGown *et al.*, 1983
♂, ♀ CD rats	Groups of 8 rats/sex/dose were fed diets containing 0, 38, 102 or 191 (♂) and 0, 38, 118 or 145 mg/kg b.w./day (♀) for 4 weeks over an 8-week observation period. Purity > 98%.	Mortality at 191(♂, 2/8) and 145(♀, 2/8) mg/kg b.w./day Neuromuscular toxicity at 191(♂) and 145(♀) mg/kg b.w./day ↓ b.w. with ↓ food consumption from 38 mg/kg b.w./day (♂,♀) ↓ erythrocyte with ↑ reticulocytes at 191(♂) mg/kg b.w./day at 8 weeks Haemosiderosis from 38 mg/kg b.w./day (♂,♀) Aspermatogenesis at 191 mg/kg b.w./day (♂) Tissue lesions were irreversible LOAEL = 38 mg/kg b.w/day	Lee *et al.*, 1978
♂, ♀ albino Swiss mice	Groups of 8 mice/sex/dose were fed 0, 46, 132 or 332 (♂) and 0, 53, 142 or 434 mg/kg b.w./day (♀) for 4 weeks over an 8-week observation period. Purity 98.5 – 99%	Mortality at 332(♂,1/8) mg/kg b.w./day ↓ b.w. with ↓ food consumption at 332 (♂) and 434 (♀) mg/kg b.w./day Mild aspermatogenesis (reversible) at 332 (♂) mg/kg b.w./day NOAEL = 132 mg/kg b.w./day	Lee *et al.*, 1978
♂, ♀ beagle dogs	Groups of 2 dogs/sex/dose were treated with 0, 1, 5 or 25 mg/kg b.w./day (purity 98.5 - 99%) for 4 weeks over a 8-week observation period	At 25 mg/kg b.w./day it was observed: ↑ Mortality and ♂ more sensitive than ♀ Neuromuscular toxicity (♂, ♀) ↓ b.w. with ↓ food consumption at 25 mg/kg b.w./day (♂, ♀) ↓ erythrocyte and ↓ haemoglobin with ↑ reticulocytes and ↑Heinz bodies (♂, ♀) Haematological effects were reversible Aspermatogenesis ↑ reticulocytes from 1 mg/kg b.w./day LOAEL = 1 mg/kg b.w./day	Lee *et al.*, 1978

↑, ↓ Either increase or decrease statistically significant when compared with controls ($p < 0.05$).

Table 4.1.2.6.3-2: Sub-chronic studies

Species and sex	Protocol	Results and comments	References
♂, ♀ CD rats	Groups of 8 rats/sex/dose were fed diets containing to 0, 34, 93 or 266 (♂) and 0, 38, 108 or 145 mg/kg b.w./day (♀) for 13 weeks over a 17-week observation period. Purity > 98%	↑ Mortality with ↑ interindividual susceptibility and ♀ more sensitive than ♂ at 266(♂, 6/8) and 145(♀, 8/8) mg/kg b.w./day. One ♂ died at 93 mg/kg b.w./day (week 15) Neuromuscular toxicity at 266(♂) and 145(♀) mg/kg b.w./day ↓ b.w. with ↓ food consumption from 34(♂) and 38(♀) mg/kg b.w./day ↓ erythrocyte and ↓ haemoglobin with ↑ reticulocytes at 266(♂) mg/kg b.w./day and ↑ reticulocytes at 93 (♂) mg/kg b.w./day Haematological effects were reversible Haemosiderosis from 34 (♂)mg/kg b.w./day Gliosis and demyelination from 93 (♂)mg/kg b.w./day ↓ spermatogenesis from 93 (♂) mg/kg b.w./day Aspermatogenesis at 266 (♂) mg/kg b.w./day The tissue lesions were irreversible LOAEL = 34 mg/kg b.w./day	Lee *et al.*, 1978, 1985
♂ Wistar rats	One group of 20 rats was fed 207 mg/kg b.w./day in the diet for 6 months and another group of 23 rats was fed standard diet Pure 2,4-DNT	↑ Mortality (12/20 vs. 1/23 in controls) Neuromuscular toxicity ↓ b.w. ↑ methaemoglobin Altered biochemical parameters: triglyceride, blood glucose, albumin, A/G ratio, aspartate aminotransferase, lactate dehydrogenase, alkaline P-ase, acid P-ase and *p*-nitrobenzoic acid reductase Aspermatogenesis	Kozuka *et al.*, 1979
♂, ♀ albino Swiss mice	Groups of 8 mice/sex/dose were fed 0, 47, 137 or 413 (♂) and 0, 52, 147 or 468 mg/kg b.w./day (♀) for 13 weeks over a 17-week observation period. Purity 98.5 - 99%	Treatment-related mortality at 413 (♂, 2/8) and 468 (♀, 1/8) mg/kg b.w./day ↓ b.w. with ↓ food consumption at 413 (♂) and 468 (♀) mg/kg b.w./day ↓ haemoglobin (♂, ♀) with ↑ reticulocytes(♂) at 413 (♂) and 468 (♀) mg/kg b.w./day Haematological effects were reversible NOAEL = 137 mg/kg b.w./day	Lee *et al.*, 1978, Hong *et al.*, 1985

Species and sex	Protocol	Results and comments	References
♂, ♀ beagle dogs	Groups of 2 dogs/sex/dose were treated with 0, 1, 5 or 25 mg/kg b.w./day for 13 weeks over a 17-week observation period Purity 98.5% - 99%	At 25 mg/kg b.w./day it was observed: ↑ Mortality with ↑ interindividual susceptibility at 25 mg/kg b.w./day and ♂ more sensitive than ♀ ↓ b.w. with ↓ food consumption at 25 mg/kg b.w./day (♂, ♀) Neuromuscular toxicity at 25 mg/kg b.w./day (♂, ♀) ↑ Methaemoglobin from 5 mg/kg b.w./day and ↓ haemoglobin with Heinz bodies at 25 mg/kg b.w./day (♂, ♀) Haematological effects were reversible Aspermatogenesis at 25 mg/kg b.w./day (♂) Mild cerebellar demeylination, gliosis and oedema (♂) and mild demyelination in both the cerebrum and optic nerve (♀) at 25 mg/kg b.w./day Tissue lesions were irreversible NOAEL = 1 mg/kg b.w./day	Lee *et al.*, 1978 Ellis *et al.*, 1985

↑, ↓ Either increase or decrease statistically significant when compared with controls ($p < 0.05$).

Table 4.1.2.6.3-3: Chronic toxicity studies

Species and sex	Protocol	Results and comments	References
♂, ♀ CD rats	38 rats/sex/dose were fed diets containing to 0, 0.57, 3.9 or 34 (♂) and 0, 0.71, 5.1 or 45 mg/kg b.w./day (♀) for either 12 months (4 rats/sex/dose) over a 13-month observation period (4 rats/sex/dose), or 24 months (26 rats/sex/dose) over a 25-month observation period (4 rats/sex/dose). Purity > 98%. A few extra rodents were added to replace early losses (no further details available)	↓ survival vs. controls at 34 (♂) and 45(♀) mg/kg b.w./day ↓ terminal body weight at 34 (♂) and 45(♀) mg/kg b.w./day Neuromuscular toxicity (straddling gait, ♂, ♀) at 34 (♂) and 45(♀) mg/kg b.w./day ↓ erythrocytes from 3.9(♂) mg/kg b.w./day for 12 months ↓ erythrocytes (♂, ♀) and ↓ haemoglobin (♂) at 34 (♂) and 45(♀) mg/kg b.w./day for 12 months ↓ erythrocytes and ↓ haemoglobin with ↑ reticulocytes at 34 (♂) and 45(♀) mg/kg b.w./day for 18 months ~↑ reticulocytes (♂, 4.85% vs. 0.72% in controls and ♀, 3.02% vs. 1.34% in controls), at 3.9(♂) and 5.1(♀) mg/kg b.w./day for 24 months Haematological effects were reversible. Hyperplastic foci in the liver from 0.57(♂)mg/kg b.w./day for 12 months Severe hyperplastic foci in the liver at 34 (♂) and 45(♀) mg/kg b.w./day from 12 months Severe atrophy of seminiferous tubules in testes at 34(♂) mg/kg b.w./day for 12 months. Dose-response relationship for atrophy of testes (♂) and 2,4-DNT for more than 12 months (16%, 29%, 33% and 86% for 0, 0.57, 3.9 or 34 mg/kg b.w./day, respectively) Excessive pigmentation in the spleen at 34(♂) and 45(♀) mg/kg b.w./day for 12 months Tissue lesions were irreversible LOAEL = 0.57(♂) and 0.71(♀) mg/kg b.w./day	Lee *et al.*, 1985, Ellis *et al.*, 1979

Species and sex	Protocol	Results and comments	References
♂, ♀ CD-1 mice	Groups of 58 mice/sex/dose were treated with 0, 13.3, 96.9 and 885(♂), and 0, 13.7, 93.8, and 911(♀) mg/kg b.w./day for either 12 months (4 mice/sex/dose) over a 13-month observation period (4 mice/sex/dose), or 24 months (46 mice/sex/dose) over a 25-month observation period (4 mice/sex/dose). Purity > 98%. A few extra rodents were added to replace early losses (no further details available)	↓ survival when compared with controls ($p < 0.05$) at 885(♂) and 911(♀)mg/kg b.w./day for 24 months ↓ b.w. when compared with controls ($p < 0.05$) at 885(♂) and 911(♀) mg/kg b.w./day for 24 months Neuromuscular toxicity at 885(♂) and 911(♀) mg/kg b.w./day ↑ Heinz bodies, reticulocytes and met haemoglobin ($p < 0.05$) at 885(♂) and 911(♀) mg/kg b.w./day for 12 months ↑ Heinz bodies at 96.9 (♂) and 93.8 (♀) mg/kg b.w./day for 12 months and allowed to recover 1 month After the 1-month recovery period, mice partially recovered from the anaemia ♂ were more affected than ♀ Hepatocellular dysplasia from 13.3(♂) and 911(♀) mg/kg b.w./day for more than 12 months Toxic nephropathy from 13.3(♂) and 13.7(♀) mg/kg b.w./day for more than 12 months Testicular atrophy from 96.9(♂) mg/kg b.w./day Non-functional follicles at 911(♀) mg/kg b.w./day Generalized abnormal pigmentation from 13.3(♂) and 13.7(♀) mg/kg b.w./day for more than 12 months Tissue lesions were irreversible LOAEL = 13.3(♂) and 13.7(♀) mg/kg b.w./day	Ellis *et al.*, 1979; Hong *et al.*, 1985

Species and sex	Protocol	Results and comments	References
♂, ♀ Beagle dogs	Groups of 6 dogs/sex/dose were treated with 0, 0.2, 1.5 or 10 mg/Kg b.w./day in hard capsules for either 12 months (1dog/sex/dose) over a 13-month observation period (1dog/sex/dose), or 24 months 2dogs/sex/dose) over a 25-month observation period (2dogs/sex/dose) Purity 98% 2,4-DNT and 2% 2,6-DNT	↓ survival at 10(♂) mg/kg b.w./day ↑ interindividual susceptibility ♂ were more affected than ♀ Neurotoxic symptoms (incoordination and paralysis) from 1.5(♂, week 66) and at 10(♂,♀ after weeks 8 to 22) mg/kg b.w./day ↑ reticulocytes and ↑ Heinz bodies at 10 mg/kg b.w./day (♂,♀) for 12 months ↓ erythrocytes and ↓ haemoglobin from 1.5(♀)mg/kg b.w./day for 12 months Haematological toxic effects were reversible Vacuolization, hypertrophy, endothelial mitosis, and focal gliosis in the cerebellum at 10(♂) mg/kg b.w./day Perivascular haemorrhage in the cerebellum and brain stem at 10(♂) mg/kg b.w./day Bile duct hyperplasia and pigment deposits in the liver at 10(♀) mg/kg b.w./day for 12 months After 24-month exposure period: The high-dose group at 24th month comprised 2 ♀ and 1 ♂ Bile duct hyperplasia and clusters of brown pigment-laden Kupffer cells in the liver at 10 (♂,♀) mg/kg b.w./day (3/3) NOAEL = 0.2 mg/kg b.w./day	Hong *et al.*, 1985 Ellis *et al.*, 1979

↑, ↓ Either increase or decrease statistically significant when compared with controls ($p < 0.05$); ~↑, no significant increase

4.1.2.7 Mutagenicity

There are several data available on the genotoxicity of 2,4-DNT derived from the *in vitro* and *in vivo* studies, which were mostly performed conform to modern guidelines. In some cases, studies were research orientated; consequently, the protocols were not specifically designed according to OECD guidelines. Anyway, the available data are either published in journals with reviewers or obtained from acceptable studies. Therefore, they are considered acceptable for risk assessment.

The different papers are commented and summarised in tables, and a global evaluation is given at the end. Moreover, genotoxicity studies carried out with 2,4-DNT metabolites have been also included and commented.

4.1.2.7.1 Studies in vitro

Bacterial studies with 2,4-DNT

They are summarised in table 4.1.2.7.1-1.

Gene mutation

The potential mutagenicity of 2,4-DNT, before and after ozonation in water, was investigated by Cotruvo *et al.* (1977) in *Salmonella typhimurium* TA98, TA100, TA1535, TA1536, TA1537 and TA1538 strains, in the absence and presence of a metabolic activation system (rat liver homogenate) using suspension and plate incorporation methods. Reaction of 2,4-DNT with ozone was rapid (20 minutes), but no new products were detected in the gas chromatography profile. At the highest concentration tested (0.08 µg/plate) the compound was not mutagenic before and after ozonation.

2,4-DNT mutagenicity was determined by Ellis *et al.* (1978) in the Ames' *Salmonella*/microsome plate incorporation test using TA1535, TA1537, TA1538, TA98 and TA100 strains. 2,4-DNT (purity ≥ 98%) was tested at concentrations of 10, 30, 100, 300 and 1000 µg/plate in the absence and presence of a metabolic activation system. 2,4 DNT was mutagenic in TA1538 both in absence (from 10 µg/plate) or presence (at 1000 µg/plate) of a metabolic activation system, and in TA100 with and without metabolic activation from 300 µg/plate. Toxicity was observed at 1000 µg/plate in TA1537 and TA1538 in the absence of a metabolic activation system.

The mutagenicity of 2,4-DNT (purity ≥99.98%) dissolved in DMSO was determined by Couch *et al.* (1981) in 3 *Salmonella typhimuriun* assays: the Ames' *Salmonella*/microsome test in TA1535, TA1537, TA1538, TA98 and TA100 strains (plate incorporation method as described by Ames *et al.*, 1975), a quantitative reverse mutation assay in TA1538 and TA98 strains (suspension method as described by Skopek *et al.,* 1978), and a forward mutation assay using resistance to 8-azaguanine as a marker in TM677 strain (suspension method as described by Skopek *et al.,* 1978). S9 derived from livers of Arochlor-treated male F-344 rats was used in all assays. In the Ames' *Salmonella*/microsome test 2,4-DNT increased the number of revertants per plate in TA98 and TA1538 strains in a concentration dependent manner although the only data reported were those corresponding to the highest non-toxic concentration tested (200 µg/plate); mutagenicity was not observed in the presence of S9. In suspension assays, treatments which caused greater than 90% cytotoxicity relative to solvent

controls were not reported. In the quantitative reversion assay, 2,4-DNT, when tested at 50, 100, 250 and 500 µg/mL, caused concentration related increases in mutant fraction in both strains with or without S9; however, only results obtained with TA98 were shown since this strain was the most sensitive to the mutagenicity of 2,4-DNT in this suspension assay. 2,4-DNT also produced concentration related increases in 8-azaguanine-resistant fraction of TM677 strain in the presence and absence of S9 although the only data reported were those corresponding to the concentration of 500 µg/mL.

The mutagenic activity of 2,4-DNT was tested by Tokiwa *et al* (1981) in the Ames' *Salmonella*/microsome test (plate incorporation method as described by Ames *et al.*, 1975) using *Salmonella typhimuriun* TA1535, TA1536, TA1537, TA1538, TA98 and TA100 strains. S9 was derived from livers of Arochlor 1254-treated rats. 2,4-DNT was non mutagenic in TA1535 and mutagenic in the remaining strains. Nevertheless, only data corresponding to TA100 and TA98 were shown. The mutagenic activity was presented as revertants/µg of 2,4-DNT incorporated in the plate. 2,4-DNT was considered a weak mutagen based on the mutagenicity observed in both TA100 (at 1000 µg or 500 µg in the absence or presence of S9, respectively) and TA98 (at 500 µg in the absence of S9).

2,4-DNT (purity greater than 99%), at concentrations of 100, 200 and 300 µg/plate, was tested for mutagenicity by Mori *et al.* (1982) in the Ames test (plate incorporation method as described by Ames, Lee and Durston, 1973) using *Salmonella typhimurium* strains TA98 and TA100 in the absence of metabolic activation system. The solvent control was DMSO. Positive controls were 2NF and 4NQO. 2,4-DNT induced concentration related increases in the number of revertants per plate in both strains but these increases were less than 3-fold that induced spontaneously. Therefore, 2,4-DNT was considered a weak mutagen.

Mutagenicity of 2,4-DNT was tested by Spanggord *et al* (1982) in the Ames' *Salmonella*/microsome test (plate incorporation method as described by Ames *et al.*, 1975) using *Salmonella typhimuriun* TA1535, TA1537, TA1538, TA98, TA100 and TA100NR3 (nitroreductase-deficient) strains. S9 was derived from livers of Arochlor 1254-treated Sprague-Dawley rats. Concurrent negative and positive controls were included. 2,4-DNT was tested at a minimum of five concentrations in a range of 10-5000 µg/plate. It was mutagenic in TA100 without S9 and TA100NR3 with S9. The mutagenic potency (calculated as revertants/µg 2,4-DNT tested in TA100) was 0.28.

2,4-DNT (purity 99%) was tested for mutagenicity using the preincubation procedure of the Ames test as described by Yahagi *et al*, 1975. *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 were used. Liver S9 was prepared from male Sprague-Dawley rats and Syrian hamsters induced with Arochlor 1254. 2,4-DNT concentrations of 33, 100, 333, 1000 and 3333 µg/plate, in addition to the concurrent solvent (DMSO) and positive controls, were tested on each strain in the presence or absence of S9. Three plates were used, and the experiment was repeated no less than 1 week after completion of the initial test. Results were reported by Haworth *et al.* (1983). 2,4-DNT was considered mutagenic because it increased the number of revertants per plate in TA100 strain in a concentration dependent manner (with and without rat or hamster S9). The highest concentration tested (3333 µg/plate) was toxic in all strains.

In a four laboratory study reported by Dunkel *et al*. (1985), 2,4-DNT (purity >99%) was tested for mutagenicity using the plate incorporation procedure described by Ames *et al.*, 1975. *Salmonella typhimurium* strains TA98, TA100, TA1537 and TA1538, and *Escherichia coli* WP2 *uvrA* were used. Liver S9 was prepared from male Fischer 344 rats, B6C3F1 mice and Syrian hamsters uninduced or induced with Arochlor 1254. The concentration range

tested was 0.3-10000 µg/plate and at least six concentrations were tested for each strain/activation combination in each laboratory. All plates were prepared in triplicate and concurrent positive and negative controls were run. 2,4-DNT was judged mutagenic in *Salmonella typhimurium* (TA100, TA98 and TA1538 strains) but not in *Escherichia coli.* 2,4-DNT was mutagenic for TA100 in all laboratories and mutagenicity was observed in all activation combinations. 2,4-DNT was mutagenic for TA98 in two laboratories and mutagenicity was observed in nearly all activation combinations (except in the presence of hamster S9). In addition, positive results were obtained in TA1538 in the absence of S9 in one laboratory and in the presence of mouse S9 in another one. Cytotoxicity was observed at one or more than one high concentrations tested in each laboratory.

2,4-DNT (purity greater than 99%) was tested for mutagenicity by Mori *et al*. (1985b) in the Ames' *Salmonella*/microsome test (plate incorporation method as described by Ames, McCann and Yamasaki, 1975) using *Salmonella typhimurium* strains TA98 and TA100. Liver S9 was prepared from male Sprague-Dawley rats pre-treated with phenobarbital-Na (PB) and 5,6-benzoflavone (BF). The solvent control was DMSO. Positive controls were 2NF, 4NQO and BP. 2,4-DNT was tested at concentrations of 250, 500, 750, 1000, 1250 and 1500 µg/plate. 2,4-DNT was mutagenic in both strains with and without S9. The addition of S9 increased the mutagenic response in both strains. 2,4-DNT, however, appeared to be only a weak mutagen because the number of revertants/plate induced was less than 3-fold that induced spontaneously in strains TA98 and TA100. Toxicity was observed from 1250 µg/plate in both strains.

The mutagenic activity of 2,4-DNT was determined by Furukawa, Kawai and Kawai (1985) using the Yahagi's preincubation method on *Salmonella typhimurium* TA98 strain in the absence of metabolic activation system. 2,4-DNT when tested at 0.02, 0.2, 2, 20 and 200 µg/plate was found non mutagenic.

Ashby, 1986 reported mutagenicity results obtained in two independent experiments carried out with 2,4-DNT pure in *Salmonella typhimurium* TA100 strain. In the first experiment, 2,4-DNT, dissolved in acetone, was tested at concentrations of 8, 40, 200, 1000 and 5000 µg/plate in the presence and absence of an unspecified metabolic activation system (S9). In the second experiment, 2,4-DNT, dissolved in DMSO, was tested at concentrations of 100, 200, 500, 1000, 2000 and 4000 µg/plate without S9. 2,4-DNT was mutagenic in both experiments and either in presence or absence of S9. Cytotoxicity was observed at the highest concentrations tested (at 5000 µg/plate in the 1st experiment, and from 1000 µg/plate in the 2nd experiment).

2,4-DNT mutagenicity was tested by Kawai *et al.* (1987) using the pre-incubation method in *Salmonella typhimurium* TA100 and TA98 strains with and without S9. 2,4-DNT was a weak mutagen in TA100 either in presence or absence of S9. Cytotoxicity was observed at 2500 µg/plate.

The mutagenicity of 2,4-DNT (97% purity) in *Salmonella typhimurium* (TA98 and TA100 strains) was evaluated by Dellarco and Prival (1989) using three different methods: the standard plate incorporation test with rat S9, the modified preincubation method with flavin mononucleotide (FMN) in the presence of hamster S9 or rat S9, and the preincubation protocol without FMN in the presence of hamster S9. Concurrent negative and positive controls were run in all assays. 2,4-DNT, dissolved in *p*-dioxane was tested at concentrations of 0.1, 0.3, 1, and 3 µmoles/plate. S9 was derived from Arochlor 1254-induced hamster or rat liver. 2,4-DNT was found to have a clear FMN-dependent mutagenic activity only in the modified preincubation assay with hamster S9 in TA98 but not in TA100. This fact suggests

that extracellular nitro reduction is necessary for optimal detection of the mutagenic activity of this compound.

Einistö *et al.* (1991) tested 4-5 concentrations of 2,4-DNT in *Salmonella typhimurium* TA98, TA98NR (nitroreductase deficient), TA98/1.8-DNP_6 (*O*-acetyltransferase deficient), YG1021 (nitroreductase overproducing) and YG1024 (*O*-acetyltransferase overproducing), using the preincubation method, without metabolic activation. The nitroreductase-overproducing strain was considered the most sensitive for detecting 2,4-DNT mutagenicity, followed by the *O*-acetyltransferase-overproducing strain, and both nitroreductase- and *O*-acetyltransferase deficient strains showed a decreased sensitivity when compared with TA98.

2,4-DNT (purity greater than 99%) was tested for mutagenicity by Sayama *et al.* (1998) in the Ames test (plate incorporation method as described by Ames, McCann and Yamasaki, 1975) using *Salmonella typhimurium* TA98, TA100, YG1021, YG1024, YG1026, YG1029, YG1041 and YG1042 strains. 2,4-DNT, dissolved in DMSO, was tested at 5-7 concentrations (0-5 μmoles) in the absence of S9. Strains YG1021, YG1024 and YG1041 have been constructed from TA98; strains YG1026, YG1029 and YG1042, from TA100. These strains have elevated levels of either nitroreductase (NR) (YG1021 and YG1026), or *O*-acetyltransferase activity (OAT) (YG1024 and YG1029), or both NR and OAT activities (YG1041 and YG1042). 2,4-DNT was not mutagenic for TA98 but mutagenic for the remaining strains, YG1021 (125 revertants/μmol), YG1024 (759 revertants/μmol), YG1041 (3657 revertants/μmol), TA100 (234 revertants/μmol), YG1026 (230 revertants/μmol), YG1029 (920 revertants/μmol) and YG1042 (6439 revertants per μmol). The highest mutagenic activity was observed in strains YG1041 and YG1042, suggesting that 2,4-DNT requires high levels of NR and OAT activities to exert this mutagenicity.

DNA damage

Nakamura *et al.* (1987) examined the ability of 2,4-DNT to induce *umu* gen expression (defined on a basis of increased β-galactosidase activity by 2-fold over the background level) in *Salmonella typhimurium* TA1535/pSK1002, in the presence or absence of S9 from liver of rats pretreated with phenobarbital and 5,6-benzoflavone. The assay was carried out according to the procedure described by Oda *et al.* (1985). 2,4-DNT, dissolved in DMSO, at the highest concentration tested (100 μg/ml) was not genotoxic in the *umu* test.

Oda *et al.* (1992) developed a new tester strain (*Salmonella typhimurium* NM1011, which has a high nitroreductase activity) for the *umu* assay to detect the genotoxic activities of various kinds of mutagenic nitroarene compounds (among them, 2,4-DNT). The assay was carried out according to the procedure described by Oda *et al.* (1985). The results were compared with those obtained with *Salmonella typhymurium* TA1535/pSK1002 or *Salmonella typhimurium* NM1000 (a nitroreductase deficient strain). 2,4-DNT was tested at 6.25, 12.5, 25 and 50 μg/ml, in the absence of a metabolic activation system. NM1011 showed about 2.63 times higher induction of *umu*C gene expression by 2,4-DNT than TA1535/pSK1002. 2,4-DNT did not induce *umu*C gen expression in NM1000 strain.

Oda *et al.* (1993) developed a new tester strain, *Salmonella typhimurium* NM3009, which has high *O*-acetyltransferase (OAT) and nitroreductase (NR) activities, for the *umu* assay to detect the genotoxic activities of various kinds of mutagenic nitroarene compounds (among them, 2,4-DNT). The assay was carried out according to the procedure described by Oda *et al.* (1985, 1992). The sensitivity of the strain NM3009 was compared with those of the parent TA1535/pSK1002 strain, the NR-overexpressing strain NM1011, the NR-deficient strain NM1000, the OAT-overexpressing strain NM2009, and the OAT-defective strain NM2000.

The order of sensitivities to induce *umu*C gene expression toward 2,4-DNT was NM3009 > NM2009 > NM1011 > TA1535/pSK1002 > NM2000. 2,4-DNT did not induce *umu*C gen expression in NM1000.

Öztürk and Durusoy (1999) investigated the genotoxic potency of 2,4-DNT using the *umu* test and the standard SOS chromotest. The *umu* test was performed as described by Oda *et al*. (1993) using *Salmonella typhimurium* strains NM2009 and NM3009. The SOS chromotest was carried out as recommended by Quillardet and Hofnung (1985) using *Escherichia coli* strain PQ37 both in the presence and absence of S9 from liver of male Sprague-Dawley rats pretreated with 3-methylcholantrene (MC) and Phenobarbital (PB). 2,4-DNT (purity>95%) was dissolved in DMSO and tested at concentrations of 0.1, 1, 10 and 100 μg/assay in the SOS chromotest, and 0.1, 1, 2.5, 10, 12.5, 25 and 100 μg/assay in the *umu* test. 2,4-DNT was weakly positive in strain NM3009, but negative in strain NM2009 and in the SOS chromotest. The highest concentration tested was cytotoxic in both tests.

Table 4.1.2.7.1-1: Bacterial genotoxicity studies with 2,4-DNT

Test	System	Dosage	Results	Comments	Reference
Reverse mutation (suspension and plate incorporation method, ±S9)	*S. typhimurium* TA98, TA100, TA1535, TA1536, TA1537, TA1538 S9 from rat liver.	Up to 0.08 μg/plate	Negative	Purity not given. 2,4-DNT tested before and after ozonation. No detailed information.	Cotruvo, 1977
Reverse mutation (plate incorporation method, ±S9)	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100. S9 from rat liver	10, 30, 100, 300, 1000 μg/plate	Positive in both TA100 (±S9) and TA1538 (±S9). ↓ mutagenicity to TA1538 (+S9)	≥ 98% purity Cytotoxicity at 1000 μg/plate in both TA1537and TA1538 (-S9).	Ellis *et al*, 1978
Reverse mutation (plate incorporation method, ±S9)	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100. S9 from liver of Arochlor-induced male F-344 rats.	Up to 200 μg/plate	Positive in both TA98 (-S9) and TA1538 (-S9)	≥ 99.98% purity. No cytotoxicity	Couch *et al.,* 1981
Quantitative reverse mutation (suspension method, ±S9)	*S. typhimurium* TA1538, TA98. S9 from liver of Arochlor-induced male F-344 rats.	50, 100, 250, 500 μg/mL	Positive in both TA98 (±S9) and TA1538 (±S9). TA98 (the most sensitive strain)	≥ 99.98% purity. Survival ≤ 10% at higher than 500 μg/mL	
Forward mutation (suspension method, ±S9)	*S. typhimurium* TM677. S9 from liver of Arochlor-induced male F-344 rats.	Up to 500 μg/mL	Positive in TM677 (±S9)	≥ 99.98% purity Survival ≤ 10% at higher than 500 μg/mL	
Reverse mutation (plate incorporation method, ±S9)	*S. typhimurium* TA1535, TA1536, TA1537, TA1538, TA98, TA100. S9 from liver of Arochlor-induced rats.	Unspecified	Weak positive in TA100 (±S9) and TA98 (-S9). Positive also in TA1536, TA1537 and TA1538 (data not shown).	Purity not given. Mutagenicity at 500 μg/plate in TA98; at 1000 μg/plate (-S9) or at 500 μg/plate (+S9) in TA100.	Tokiwa *et al.,* 1981
Reverse mutation (plate incorporation method, -S9)	*S. typhimurium* TA98, TA100	100, 200, 300 μg/plate	Weak positive in both TA98 and TA100	> 99% purity No data on cytotoxicity	Mori *et al.,* 1982

Test	System	Dosage	Results	Comments	Reference
Reverse mutation (plate incorporation method, ±S9)	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100, TA100NR3 S9 from liver of Arochlor-induced Sprague-Dawley rats	10-5000 µg/plate	Positive in TA100 (-S9) and TA100NR3 (+S9)	Purity not given TA100NR3 is NR-deficient	Spanggord *et al.*, 1982
Reverse mutation (preincubation method, ±S9)	*S. typhimurium* TA1535, TA1537, TA98, TA100. S9 from liver of Arochlor-induced male Sprague-Dawley rats and Syrian hamsters	33, 100, 333, 1000, 3333 µg/plate	Positive in TA100 (±S9 from rat or hamster)	99% purity Cytotoxicity at 3333 µg/plate in all strains.	Haworth *et al.*, 1983
Reverse mutation (plate incorporation method, ±S9)	*S. typhimurium* TA1537, TA1538, TA98, TA100. *E. coli* WP2 *uvrA* S9 from liver of either untreated or Arochlor-treated male Fischer 344 rats, B6C3F1 mice and Syrian hamster	Four laboratories tested the following concentration range 62.5-2000 µg/plate 0.3-10000 µg/plate 10-3333.3 µg/plate 10-6666 µg/plate	Positive in TA100 (±S9 from rat, mouse and hamster), TA98 (±S9 from rat and mouse) and TA1538 (±S9 from mouse)	> 99% purity Cytotoxicity at the high dose levels tested in each laboratory (one or more than one concentration)	Dunkel *et al.*, 1985
Reverse mutation (plate incorporation method, ±S9)	*S. typhimurium* TA98, TA100. S9 from liver of PB and BF pre-treated male Sprague-Dawley rats	250, 500, 750, 1000, 1250, 1500 µg/plate	Weak positive in both TA98 and TA100 (±S9). Mutagenicity increased with S9.	> 99% purity Cytotoxicity from 1250 µg/plate in both strains.	Mori *et al.*, 1985b
Reverse mutation (preincubation method, -S9)	*S. typhimurium* TA98	0.02, 0.2, 2 , 20, 200 µg/plate	Negative	Purity not given.	Furukawa *et al.*, 1985
Reverse mutation (plate incorporation method, ±S9)	*S. typhimurium* TA100	8, 40, 200, 1000, 5000 µg/plate (1st exp., ±S9); 100, 200, 500, 1000, 2000, 4000 µg/plate (2nd exp, -S9)	Positive (±S9), in both experiments	High purity. Cytotoxicity at 5000 or from 1000 µg/plate in the 1st and 2nd exp., respectively.	Ashby, 1986.
Reverse mutation (preincubation method, ±S9)	*S. typhimurium* TA98, TA100	Up to 2500 µg/plate	Positive (±S9) in TA100	Purity not given Cytotoxicity at 2500 µg/plate	Kawai *et al.*, 1987
Reverse mutation with S9: 1) plate incorporation, 2) modified preincubation (+FMN), or 3) preincubation (-FMN) tests	*S. typhimurium* TA98, TA100 S9 from liver of Arochlor-induced rats (1 and 2) or hamsters (2 and 3)	0.1, 0.3, 1, 3 µmoles/plate	Positive in TA98 in the modified preincubation test (+S9 of hamster)	97% purity Cytotoxicity at 3 µmoles/plate in the modified preincubation test (+S9 of hamster)	Dellarco and Prival, 1989
Reverse mutation (preincubation method, -S9)	*S. typhimurium* TA98, TA98NR TA98/1.8-DNP_6, YG1021, YG1024	4 or 5 unspecified concentrations	Positive. Highest activity in both YG1021 and YG1024	Purity not given YG1021 (↑ NR) YG1024 (↑ OAT)	Einistö *et al.*, 1991

Test	System	Dosage	Results	Comments	Reference
Reverse mutation (plate incorporation method, -S9)	*Salmonella typhimurium* TA98, TA100, YG1021, YG1024, YG1026, YG1029, YG1041 and YG1042 strains	0-5 µmoles	Positive except in TA98, being the highest mutagenic activity observed in YG1041 and YG1042.	> 99% purity YG1041 and YG1042 strains have high levels of NR and OAT activities	Sayama *et al.,* 1998
DNA damage (*umu* test, ±S9)	*S. typhimurium* TA1535/pSK1002 S9 from liver of PB and BF pre-treated rats	Up to 100 µg/mL	Negative	Purity not given.	Nakamura *et al.,* 1987
DNA damage (*umu* test, -S9)	*S. typhimurium* NM1011, NM1000, TA1535/pSK1002	6.25, 12.5, 25, 50 µg/mL	Positive in both NM1011and TA1535/pSK1002 Highest activity in NM1011	Purity not given NM1011 has a high level of NR	Oda *et al.,* 1992
DNA damage (*umu* test, -S9)	*S. typhimurium* TA1535/pSK1002, NM1000, NM1011, NM2000, NM2009, NM3009	Unspecified	Positive except in NM1000, being the highest mutagenic activity observed in NM3009	Purity not given NM3009 has high levels of NR and OAT activities	Oda *et al.,* 1993
DNA damage (*umu* test, -S9)	*S. typhimurium* NM2009, NM3009	0.1, 1, 2.5, 10, 12.5, 25, 100 µg/assay	Weak positive in NM3009	> 95% purity Cytotoxicity at 100 µg/assay	Öztürk and Durusoy, 1999
DNA damage (SOS chromotest, ±S9)	*E. coli* PQ37 S9 from liver of MC and PB pre-treated Sprague-Dawley rats	0.1, 1, 10, 100 µg/assay	Negative	> 95% purity Cytotoxicity at 100 µg/assay	

Bacterial studies with 2,4-DNT metabolites

Gene mutation

2,4-DNT urinary metabolites, 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), 2,4-dinitrobenzyl alcohol (2,4-DNB), 2-amino-4-nitrobenzyl alcohol (2A4NB), 4-amino-2-nitrobenzyl alcohol (4A2NB), 2-nitro-4-acetylaminotoluene (2N4AAT), 2-amino-4-acetylaminotoluene (2A4AAT), 2-amino-4-acetylaminobenzoic acid (2A4AABA), 2,4-dinitrobenzoic acid (2,4-DNBA) and 2,4-diaminotoluene (2,4-DAT), with purity greater than 99%, and a 2,4-DNT putative metabolite, 2,4-dinitrobenzaldehyde (2,4-DNA1), with purity of 97%, were tested for mutagenicity by Mori *et al.* (1982). Mutagenicity assay was carried out according to Ames, Lee and Durston, (plate incorporation method) using *Salmonella typhimurium* strains TA98 and TA100 in the absence of metabolic activation system. The solvent control was DMSO. Positive controls were 2NF and 4NQO. Each compound was tested at concentrations ranging between 5 and 1000 µg/plate. 2,4-DAT, 2N4AAT, 2A4AAT, 2A4AABA and 2,4-DNBA were non-mutagens. 2 aminonitrotoluenes (2A4NT, 4A2NT), 2 aminonitrobenzyl alcohols (2A4NB and 4A2NB) and 1 dinitrobenzyl alcohol (2,4-DNB) were increasingly mutagenic in that order, in both strains, however they were only weak mutagens at mM concentrations. In contrast, 2,4-dinitrobenzaldehyde (2,4-DNAl) was mutagenic even at µM concentrations in both strains. The observation that 2,4-DNB and 2,4-DNBA are major metabolites of 2,4-DNT strongly suggest that 2,4-DNAI could be an intermediary metabolite

in the oxidation of 2,4-DNB to 2,4-DNBA. Thus, it appears that a metabolic conversion of 2,4-DNT to 2,4-DNAI may be correlated to the 2,4-DNT hepatocarcinogenicity.

2,4-DNT urinary metabolites, 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), 2,4-dinitrobenzyl alcohol (2,4-DNB), 2-amino-4-nitrobenzyl alcohol (2A4NB), 4-amino-2-nitrobenzyl alcohol (4A2NB), 2-nitro-4-acetylaminotoluene (2N4AAT), 2-amino-4-acetylaminotoluene (2A4AAT), 2-amino-4-acetylaminobenzoic acid (2A4AABA), 2,4-dinitrobenzoic acid (2,4-DNBA) and 2,4-diaminotoluene (2,4-DAT), with purity greater than 99%, and a possible intermediate in the oxidation of 2,4-DNB to 2,4-DNBA, 2,4-dinitrobenzaldehyde (2,4-DNA1), with purity of 98%, were tested for mutagenicity by Mori *et al*. (1985b) in the Ames' *Salmonella*/microsome test (plate incorporation method as described by Ames, McCann and Yamasaki, 1975) using *Salmonella typhimurium* strains TA98 and TA100. Liver S9 was prepared from male Sprague-Dawley rats pre-treated with phenobarbital-Na (PB) and 5,6-benzoflavone (BF). The solvent control was DMSO. Positive controls were 2NF, 4NQO and BP. Each compound was tested at concentrations ranging between 0 and 2000 µg/plate. 2A4NT, 4A2NT, 2N4AAT, 2A4AABA and 2,4-DNBA were non-mutagens. In contrast, 2A4AAT, 2A4NB, 4A2NB, 2,4-DAT, 2,4-DNB and 2,4-DNAl were more mutagenic than 2,4-DNT, being increasingly mutagenic in that order. 2A4AAT, 2A4NB and 4A2NB were weak mutagens at mM concentrations, and 2,4-DAT, 2,4-DNB and 2,4-DNAl were mutagenic at µM concentrations. Most of mutagens were mutagenic in both strains, except 2A4AAT that was only mutagenic in TA98, and in both presence and absence of S9, except 2A4AAT and 2,4-DAT that were only mutagenic in the presence of S9. These results suggest that the metabolic conversion of 2,4-DNT to 2,4-DNB, 2,4-DNAl and 2,4-DAT, a known carcinogen, may contribute to the carcinogenicity of 2,4-DNT.

2,4-DNT bacterial metabolites, 2-hydroxylamino-4-nitrotoluene (2HA4NT), 4-hydroxylamino-2-nitrotoluene (4HA2NT), 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), 2,2'-dimethyl-5,5'-dinitroazoxybenzene (2,2'-DM-5,5'-DNAOB) and 4,4'-dimethyl-3,3'-dinitroazoxybenzene (4,4'-DM-3,3'-DNAOB), with purity greater than 99%, were tested for mutagenicity by Sayama *et al.* (1998) in the Ames test (plate incorporation method as described by Ames, McCann and Yamasaki, 1975) using *Salmonella typhimurium* TA98, TA100, YG1021, YG1024, YG1026, YG1029, YG1041 and YG1042 strains. The compounds were dissolved in DMSO and tested at 5-7 concentrations (0-5 µmoles) in the absence of S9. No mutagenic activity was detected in TA98 and TA100. 2,2'-DM-5,5'-DNAOB was mutagenic in one strain (YG1041); 4,4'-DM-3,3'-DNAOB in three strains (YG1041, YG1026, YG1042); both 2HA4NT and 4HA2NT in five strains (YG1024, YG1026, YG1029, YG1041 and YG1042); and both 2A4NT and 4A2NT in six strains (YG1021, YG1024, YG1026, YG1029, YG1041 and YG1042). The greatest mutagenic activities of these compounds were found in both YG1041 and YG1042 strains. The relative mutagenic activities were 4A2NT < 2A4NT < 2,4-DNT < 4HA2NT < 2,2'-DM-5,5'-DNAOB = 2HA4NT << 4,4'-DM-3,3'-DNAOB toward YG1041, and 4A2NT < 2A4NT < 2,4-DNT < 4HA2NT = 4,4'-DM-3,3'-DNAOB < 2HA4NT toward YG1042. Thus, it can be said that aminohydroxylamino dimethylazoxybenzenes or aminohydroxylamino dimethylazobenzenes produced either by reduction of hydroxylaminonitrotoluenes or by reduction of dimethyl dinitroazoxybenzenes could be considered the active metabolites responsible for the mutagenic activity of 2,4-DNT in bacteria.

Yeast studies with 2,4-DNT

DNA damage

The potential genotoxicity of 2,4-DNT (purity not given), before and after ozonation in water, was investigated by Cotruvo *et al.* (1977) in the mitotic recombination assay with *Saccharomyces cerevisiae* D3 strain, in the absence and presence of a metabolic activation system (rat liver homogenate), using a suspension method. Reaction of 2,4-DNT with ozone was rapid (20 minutes), but no new products were detected in the gas chromatography profile. At the highest concentration tested (0.004%) 2,4-DNT was neither genotoxic nor toxic before ozonation; there was an increased number of mitotic recombinants after ozonation but the response was not concentration related.

Mammalian cell studies with 2,4-DNT

They are summarised in table 4.1.2.7.1-2.

Gene mutation

Lee et al. (1978) tested the ability of 2,4-DNT (purity 98.5-99%) to induce single gene mutations in Chinese hamster ovary (CHO-K1) cells. The concentrations used were selected from a single cell survival curve obtained according to the method of Puck and Kao (1967). The only information given was that the 1% and 5% cell survival concentrations (193 and 155 µg/ml, respectively) did not cause mutations.

The CHO/HGPRT mutagenicity assay was performed by Couch *et al.* (1979) as described by Hsie *et al.* (1975) and later modified by O'Neill *et al.* (1977). 2,4-DNT (purity 99.98%), dissolved in DMSO, was tested in the presence of S9 from liver of Arochlor-induced male Fischer-344 rats, under aerobic or anaerobic incubation conditions. Anaerobic conditions (reduced oxygen tensions) were achieved by the method of Mallet and coworkers (1977). In addition, primary hepatocytes from Arochlor-induced male Fischer-344 rats were also used as activation system under aerobic incubation conditions. When Arochlor-induced S9 was used as an activating system under aerobic conditions, concentrations of 2,4-DNT up to 2mM produced neither a cytotoxic nor a mutagenic response; higher concentrations (up to 6 mM) reduced viability but were not mutagenic. Under anaerobic conditions, at 0.6 mM 2,4-DNT survival was reduced (approximately 30%) and the 6-thioguanine-resistant fraction of surviving cells increased (approximately $40x10^{-6}$); at 0.8 mM survival was < 10% and no mutagenicity was observed. When both anaerobic (2.5 hr) and aerobic (2.5 hr) conditions were used, the mutagenicity results were not different from those obtained with anaerobic treatment alone. When Arochlor-induced hepatocytes were used as an activating system, at the highest concentration tested (100 µM 2,4-DNT) survival was reduced (< 25% and >12.5%) and the 6-thioguanine-resistant fraction of surviving cells increased (approximately $30x10^{-6}$); however, the mutant frequencies produced by 2,4-DNT, like those obtained in the presence of S9 under anaerobic conditions, were relatively low.

Abernethy and Couch (1982) evaluated the mutagenicity of 2,4-DNT (purity ≥ 99.98%), dissolved in DMSO, in the CHO/HGPRT system with the CHO K1 line. The method used for determining induced mutation frequency has been described by Hsie *et al.* (1975) and O'Neill *et al.* (1977). Cells were treated with and without S9 mix from livers of Arochlor-induced male F-344 rats. 2,4-DNT, tested up to 3 mM, did not induce mutations at the *hgprt* locus of CHO cells either in the presence or absence of S9-mix. The concentration required to reduced cell survival by 50% was 1.8 mM (without S9) and 2.5 mM (with S9)

Styles and Cross (1983) tested 2,4-DNT (pure) at five concentrations ranging from 1.6 to 1000 µg/ml in the P388 mouse lymphoma gene mutation assay (TK +/-). Cells in exponential growth were exposed to 2,4-DNT both in the presence and absence of unspecified S9 mix for

30 min and then incubated for optimum expression time before exposure to the selective agent and the subsequent mutation and survival assays. 2,4-DNT without S9 produced a concentration-related decrease in survival and an increase in mutation frequency that was statistically significant. When S9 was included there was concentration-related cytotoxicity but although the mutation frequency increased with concentration, this effect was not statistically significant.

Chromosomal aberration

Loveday *et al.* (1989) tested the ability of 2,4-DNT (purity 99%) to induce chromosomal aberrations in cultured Chinese hamster ovary (CHO) cells. Growth and treatment conditions were based on procedures described by Galloway *et al* (1985). 2,4-DNT, dissolved in DMSO, was tested at concentrations of 100, 300 and 1000 µg/ml in the presence (2 hour-treatment) or absence (8 hour-treatment) of S9 mix from liver of Arochlor 1254-induced male Sprague-Dawley rats. The total duration of the non-activated and activated experiments were 10 and 12 hours, respectively, to give 10 hours of growth in medium with serum for each experiment. 2,4-DNT did not induce chromosomal aberrations either in presence or absence of S9.

Huang *et al.* (1996a, 1996b) evaluated the genotoxicity of 2,4-DNT by the chromosomal aberration test in human peripheral lymphocytes. 2,4-DNT (purity not given) dissolved in DMSO was added to cultures of whole blood from a healthy male donor 48 hours after initiation. Cultures were incubated for an additional 24 hours after treatment. The number of cells with chromosome aberrations among 100 well-spread metaphase cells in one culture was recorded. The percentage of aberrant cells (number of aberrant cells/ number of metaphase cells scored) was 16.5%, 24.6%, 51.0% and 73.2% at 2,4-DNT concentrations of 0.0005, 0.002, 0.010 and 0.050 mmol/L, respectively, in all cases higher than that of solvent control (1.8%).

Mutagenicity data on 2,4-DNT were reported by JETOC (1996). 2,4-DNT (purity not given) dissolved in DMSO was tested for chromosomal aberrations in CHL cells at concentrations of 0.05, 0.1, 0.2, 0.3 and 0.4 mg/mL (24 and 48 hour long-term treatments without S9) or 0.5, 1, 2, 3 and 4 mg/mL (unspecified short-term treatment with and without S9). S9 used was not specified. 2,4-DNT induced structural chromosome aberrations after the short-term treatment, with and without S9, when tested at 2 and 3 mg/mL; metaphases were not observed at 4 mg/mL. No chromosomal aberrations were induced by 2,4-DNT after long-term treatments; toxicity was observed at 0.4 and 0.3 mg/mL after 24 and 48 hours of treatment, respectively.

DNA damage

Loveday *et al.* (1989) tested the ability of 2,4-DNT (purity 99%) to induce sister chromatid exchanges (SCEs) in cultured Chinese hamster ovary (CHO) cells. Growth and treatment conditions were based on procedures described by Galloway *et al* (1985). 2,4-DNT, dissolved in DMSO, was tested in the presence (2 hour-treatment before BrdUrd addition) or absence (2 hour-treatment before BrdUrd addition and 24 hour-treatment after BrdUrd addition) of S9 mix from liver of Arochlor 1254-induced male Sprague-Dawley rats. The total duration of both non-activated and activated experiments was approximately 26 hours. In the absence of S9 mix, 2,4-DNT was tested at concentrations of 10, 30 and 100 µg/ml. In the presence of S9 mix, 2,4-DNT was tested at concentrations of 100, 300 and 1000 µg/ml (1st experiment) or 840, 1010 and 1260 µg/ml (2nd experiment). 2,4-DNT produced a small but reproducible increase in SCEs with S9 mix. In the first experiment, there was a positive trend and a 19% increase in SCEs was seen at 1000 µg/ml; precipitation was observed at this concentration.

Increases of 20% and 23% were observed at 1010 and 1260 μg/ml, respectively, in the second experiment; precipitation was seen at both concentrations. The experiment without S9 mix showed no increase in SCEs, although delayed harvest times (30.50 hours) were used at the two highest concentrations. The next highest doses had insufficient M2 cells for analysis.

Bermudez, Tillery and Butterworth (1979) examined the ability of pure 2,4-DNT to induce DNA damage using the Unscheduled DNA Synthesis (UDS) assay in primary hepatocytes from male Fischer 344-rats, as described by Williams (1977). 2,4-DNT, dissolved in DMSO, was tested for toxicity in an initial experiment using a wide range of concentrations ($1x10^{-2}$ to $1x10^{-7}$ M). Only those concentrations where acute toxicity was not evident were scored for UDS. At the non-toxic concentrations of $1x10^{-3}$ and $1x10^{-4}$ M (as judged by cell morphology) 2,4-DNT did not induce a significant response (i.e. an average greater than 5 net grains/nucleus).

Working and Butterworth (1984) determined the ability of 2,4-DNT to induce DNA damage using the UDS assay in spermatogenic cells, which were isolated by trypsin digestion of male Fischer-344 rat testes and cultured for 18 hours in the presence of ^{3}H-thimidine. 2,4-DNT (purity >99%) dissolved in DMSO was tested at concentrations of 10, 100 and 1000 μM. Spermatogenic cell stages were identified according to standard morphological criteria (LeBlond and Clermont, 1952) and nuclear grains were counted in mid- to late-pachytene stage primary spermatocytes and postmeiotic round spermatids. 2,4-DNT did not induce UDS in either pachytene spermatocytes or round spermatids. Toxicity, as measured by trypan blue exclusion, was observed at 1000 μM with >95% of nonviable cells.

The genotoxic potential of 2,4-DNT was assessed by Butterworth *et al.*, (1989) using the UDS assay in primary human hepatocytes from discarded surgical material. Small portions of apparently healthy liver not need for pathological examination were obtained from two patients (a 30-year-old female admitted for surgical resection of two suspected benign liver tumours, and a 6-year-old female who underwent surgery to remove a liver sarcoma), placed in ice-cold saline and transported to the laboratory. Human hepatocytes of sufficient viability to measure DNA repair were successfully prepared by collagenase perfusion techniques. The cells were allowed to attach to collagen substrate for periods of 2 hours (1st patient) or 1.5 hours (2nd patient) and subsequently incubated with ^{3}H-thimidine and 2,4-DNT for 18 hours. 2,4-DNT (purity 99.98%), dissolved in DMSO, was tested at concentrations of 0.01, 0.1 and 1 mM. UDS was quantified autoradiographically. 2,4-DNT did not induce UDS in human hepatocytes. Toxicity was observed at 1 mM (2nd patient). For purposes of comparison 2,4-DNT was also evaluated in the UDS assay using primary rat hepatocytes and negative results were obtained (data from Bermudez, Tillery and Butterworth, 1979). The lack of genotoxic activity of 2,4-DNT is accord with the concept that reduction by intestinal flora is required in addition to hepatic metabolism for activation.

Sina *et al.* (1983) developed an alkaline elution/rat hepatocyte assay to concomitantly measure DNA damage (single-strand breaks) and cytotoxicity induced by xenobiotics. Hepatocytes were isolated from uninduced rats by collagenase perfusion, exposed to 2,4-DNT at 0.03, 0.3, 1 and 3 mM for 3 hours, harvested and analyzed. Citotoxicity was estimated by glutamate-oxalacetate transaminase release or trypan blue dye exclusion. DNA determinations were done fluorimetrically. A greater than 30% reduction in cell viability below control levels was considered as biologically significant cytotoxicity, and a 3-fold increase in elution rate compared with the concurrent control, as a DNA damage response. 2,4-DNT caused DNA damage (an increase in elution rate of 5.1-7.0-fold over the control) at the concentration of 3 mM, which was cytotoxic (viability was 58% of control). DNA single-strand breaks seen at cytotoxic concentrations may be primary, due to direct chemical attack on some portion of the

macromolecule, or secondary, resulting from extensive cell repair, cell lysis, or release of intracellular degradative enzymes within the cell.

Table 4.1.2.7.1-2: *In vitro* mammalian cell genotoxicity studies with 2,4-DNT

Test	System	Dosage	Results	Comments	Reference
Gene mutation (HGPRT +/-, - metabolic activation)	Chinese hamster ovary (CHO-K1) cells	Unspecified	Negative at 155 and 193 μg/ml	98.5-99% purity. 5% and 1% survival at 155 and 193 μg/ml, respectively	Lee *et al.* (1978)
Gene mutation (HGPRT +/-, + metabolic activation)	Chinese hamster ovary (CHO) cells. S9 from liver of Arochlor-induced male F-344 rats (aerobic, anaerobic, or anaerobic + aerobic incubation)	Up to 6 mM (aerobic incubation) Up to 0.8 mM (anaerobic incubation)	Negative (aerobic incubation) Positive at 0.6 mM (anaerobic, anaerobic + aerobic incubation)	99.98% purity ↓ Survival at 6 mM (aerobic incubation) 30% and < 10% survival at 0.6 and 0.8 mM, respectively (anaerobic incubation)	Couch *et al* (1979)
	Chinese hamster ovary (CHO) cells. Arochlor-induced male F-344 rat primary hepatocytes (aerobic incubation)	Up to 100 μM	Positive at 100 μM	99.98% purity <25% but >12.5% survival at 100 μM.	
Gene mutation (HGPRT +/-, ±S9)	Chinese hamster ovary (CHO-K1) cells. S9 from liver of Arochlor-induced male F-344 rats	Up to 3 mM	Negative (±S9)	≥ 99.98% purity 50% survival at 1.8 mM (-S9) or 2.5 mM (+S9)	Abernethy and Couch (1982)
Gene mutation (TK +/-, ±S9)	P388 mouse lymphoma cells	1.6-1000 μg/ml	Positive (-S9) in a concentration dependent manner	High purity. Concentration-related toxicity (±S9)	Styles and Cross (1983)
Chromosome aberration (±S9)	Chinese hamster ovary (CHO) cells. S9 from Arochlor 1254-induced male Sprague-Dawley rats.	100, 300, 1000 μg/ml Treatments were 8 h (-S9) and 2 h (+S9)	Negative	99% purity	Loveday *et al.* (1989)
Chromosome aberration (-S9)	Human peripheral lymphocytes	0.0005, 0.002, 0.0010, 0.050 mmol/L 24 h treatment.	Positive from the low concentration tested	Purity not given	Huang *et al.* (1996a, 1996b)
Chromosome aberration (±S9)	CHL cells	0.05, 0.1, 0.2, 0.3, 0.4 mg/mL (24 and 48 h treatment, -S9) 0.5, 1, 2, 3, 4 mg/mL (short-term treatment, ±S9)	Positive in short-term treatment from 2 mg/mL (±S9)	Purity not given Toxicity at 4 mg/mL in short-term treatment (±S9), at 0.4 or 0.3 mg/mL after treatment for 24 or 48 h (-S9)	JETOC (1996)

Test	System	Dosage	Results	Comments	Reference
DNA damage (SCEs, ±S9)	Chinese hamster ovary (CHO) cells	10, 30 and 100 µg/ml (-S9) 100, 300, 1000 µg/ml (1st exp) and 840, 1010, 1260 µg/ml (2nd exp) (+S9)	Positive (+S9)	99% purity Precipitation at ≥1000 µg/ml. Cytotoxicity at concentrations higher than 100 µg/ml (-S9)	Loveday *et al.* (1989)
DNA damage (UDS, -S9)	Male Fischer -344 rat hepatocytes	$1x10^{-3}$ and $1x10^{-4}$ M	Negative	High purity. Cytotoxicity at concentrations $>1x10^{-3}$ M	Bermudez, Tillery and Butterworth (1979)
DNA damage (UDS, -S9)	Male Fischer -344 rat spermatocytes and spermatids	10, 100, 1000 µM	Negative in both spermatocytes and spermatids	> 99% purity Cytotoxicity at 1000 µM	Working and Butterworth (1984)
DNA damage (UDS, -S9)	Human hepatocytes from discarded surgical material of two patients	0.01, 0.1 and 1 mM	Negative	99.98% purity Cytotoxicity at 1mM in one patient	Butterworth *et al.* (1989)
DNA damage (alkaline elution, -S9	Rat hepatocytes	0.03, 0.3, 1 and 3 mM	Positive (DNA single strand breaks) at 3 mM	Purity not given. Cytotoxicity at 3 mM	Sina *et al.* (1983)

4.1.2.7.2 Studies in vivo

Studies in somatic cells

Mammalian studies with 2,4-DNT

They are summarised in table 4.1.2.7.2-1.

Gene mutation

No data available

Chromosomal aberration

The cytogenetic effect of 2,4-DNT was determined by Lee *et al.,* 1978 in lymphocytes and kidney cell cultures obtained from CD male rats fed diets containing 93 mg/kg/day 2,4-DNT (98.5-99% purity). At the end of 5 and 19 weeks consumption, blood samples were drawn from the tail vein and lymphocytes cultured according to the method of Moorhead et al. (1960). At the end of 5 and 13 weeks consumption, kidney tissues were removed at necropsy and cultured by the trypsinization method of Fernandes (1958). In both cultures, polyploidy was estimated by examination of 200 cells and morphological aberrations were examined from photographic negatives of up to 50 metaphase cells. Four animals per control group and at least three animals per each treatment group were used. In lymphocyte cultures, treatment with 2,4-DNT did not cause any significant changes in the chromosome frequency distribution or number of tetraploids; however, the number of chromatid breaks and gaps was increased after treatment for 5 weeks, but this effect was not statistically significant until after 19 weeks. In kidney cultures, although non-significant, an increase in the number of

tetraploids was observed after treatment for 13 weeks; nevertheless, significant increases in the number of chromatid breaks and gaps were observed after treatment for 5 or 13 weeks, being the number of breaks and gaps increased with the duration of treatment.

To assess the mutagenic potential of 2,4-DNT (98% purity), Ellis *et al.* (1979) performed cytogenetic analysis of tissue cultures from chronically treated dogs and rats. At the end of 24 months, femur bone marrow was removed at necropsy and processed by the method of Eggen (1969); kidney tissue samples were also removed at necropsy and cultured by the trypsinization method of Fernandes (1958). Chromosome analysis was carried out according to the method of Moorhead and Nowell (1964). Polyploidy was estimated by examination of 200 cells and morphological aberrations were examined from photographic negatives of up to 50 metaphase cells. Beagle dogs were administered 2,4-DNT (10 mg/kg b.w./day) in hard gelatine capsules, and in both kidney and bone marrow cultures, at least three animals were used per treatment or control group. Kidney cultures from the treated dogs had slightly increased tetraploids but the increase was not significant and bone marrow cultures were normal; in addition 2,4-DNT did not cause any morphological chromosome aberrations. CD rats were fed diets containing 2,4-DNT as a middle dose level of 100 ppm (3.9 and 5.1 mg/kg b.w./day in males and females, respectively) or a high dose level of 700 ppm (34 and 45 mg/kg b.w./day in males and females, respectively). There were used five animals per middle dose level for bone marrow cultures, six animals per middle dose level for kidney cultures, one animal per high dose level for both cultures and four animals per control group for both cultures. The kidney cultures from middle dose rats had a significant increase in tetraploid frequency, but the increase was relatively small (1.50) when compared to that of control group (0.50); furthermore, the bone marrow cultures and those from the high dose rat showed no such effect. There were not any apparent morphological aberrations of chromosomes in bone marrow or kidney cultures of rats. Cytotoxicity for these tissue cultures was not reported but based on 2,4-DNT toxicokinetics and toxicity data it is presumably that the substance reach these target tissues.

DNA damage

Mirsalis and Butterworth (1982) examined the induction of unscheduled DNA synthesis (UDS) in rat hepatocytes following *in vivo* treatment with 2,4-DNT. Three male Fischer-344 rats were administered 100 mg/kg of 2,4-DNT (98% purity) in corn oil by gavage. Hepatocytes were isolated 12 hours after treatment by liver perfusion and cultured with ^{3}H-thymidine. UDS was measured by quantitative autoradiography as net grains/nucleus (NG); cells in repair were defined as those with ≥ 5 NG. 2,4-DNT produced only a weak response (NG = 1.5 ± 0.3; % of cells in repair = 33 ± 1) when compared with control (NG = -3.4 ± 0.2; % of cells in repair = 2 ± 1).

Mirsalis *et al.* (1982) tested the genotoxic potential of 2,4-DNT using the *in vivo* unscheduled DNA synthesis (UDS) assay in rat hepatocytes. Male Fischer-344 rats (3 animals per treatment) were administered 50 and 200 mg/kg of 2,4-DNT (>99.9% pure) in corn oil by gavage. Hepatocytes were isolated 12 hours after treatment by liver perfusion and cultured with ^{3}H-thymidine. UDS was measured by quantitative autoradiography as net grains/nucleus (NG); cells in repair were defined as those with ≥ 5 NG. 2,4-DNT was genotoxic in the UDS assay with NG values of 0.8 ± 1.3 (at 50 mg/kg) and 21.1 ± 3.9 (at 200 mg/kg) when compared with control (NG = -4.4 ± 0.5), and % of cells in repair of 25 ± 6 (at 50 mg/kg) and 83 ± 3 (at 200 mg/kg) when compared with control (3 ± 1).

DNA covalent binding

Rickert *et al.* (1983) studied the time course and degree of covalent binding to hepatic DNA, RNA and protein after administration of ^{14}C-2,4-DNT. Male Fischer-344 rats were administered by gavage 10 or 35 mg/kg of ^{14}C-2,4-DNT dissolved in corn oil. Rats from each group were killed at 1, 2, 4, 8, 12, 24, 48, 96, 192 and 384 h after dosing. Livers were removed, weighed and homogenized. A 3 mL portion of liver homogenate (equivalent to 1 g tissue) was fractionated into DNA, RNA and protein fractions. Covalent binding to each macromolecular species was proportional to dose. There was no selectivity for any macromolecule. The time course of appearance and disappearance of covalently bound material was similar regardless the dose administered. Little covalent bound material was present until 8 h after dosing. Covalent binding peaked between 12 and 24 h and then slowly declined. The terminal half-lifes of covalently bound material ranged from 2.9 to 5 days for RNA and protein and from 5.1 to 7.9 days for DNA.

Kedderis, Dyroff and Rickert (1984) used the sulfotransferase inhibitors, 2,6-dichloro-4-nitrophenol and pentachlorophenol, to investigate the role of sulphate ester formation during the *in vivo* bioactivation of 2,4-DNT. Male Fischer 3444 rats were administered one of the sulfotransferase inhibitors (40 µmol/kg, i.p). 45 min prior to oral administration of 28 mg/kg of either ^{14}C-2,4-DNT or ^{3}H-2,4-DNT dissolved in corn oil. Rats were killed 12 h later. Livers were excised, minced and stored until analysis. A portion of each liver was homogenized and covalently bound radiolabel was determined using an exhaustive extraction procedure. The sulfotransferase inhibitors decreased the total hepatic macromolecular covalent binding of 2,4-DNT by 33%. Purification of hepatic DNA by hydroxylapatite chromatography indicated covalent binding at levels of 45 pmol equivalents/mg DNA. 2,6-dichloro-4-nitrophenol decreased the binding of 2,4-DNT to DNA by > 84%, while the decrease due to pentachlorophenol was 33%. These results suggest that sulfation is important in the biotransformation of 2,4-DNT to reactive metabolites which covalently bind to DNA.

La and Froines (1992a) examined the ability of 2,4-DNT to induce *in vivo* DNA adduct formation. Three male Fischer-344 rats were administered single doses of 4.1, 41, 123, 410, 1230 and 2046 µmol/kg 2,4-DNT (97-99% purity), dissolved in DMSO, by i.p. injection. Rats were killed 18 hours later and DNA from liver measured for adduct formation using ^{32}P-postlabelling and thin layer chromatography. DNA adduct formation was observed from 4.1 µmol/kg 2,4-DNT. It was found a dose-response relationship. 2,4-DNT produced three adduct spots. Adduct levels were reported as ratios of adducted nucleotides to total nucleotides (or relative adduct labelling, RAL). At the dose of 410 µmol/kg 2,4-DNT, the RAL (x10^7 total nucleotides) was 7.6 ± 1.7 (adduct 1) 1.0 ± 0.2 (adduct 2) and 1.0 ± 0.4 (adduct 3)

La and Froines (1992b) examined the ability of 2,4-DNT to induce *in vivo* DNA adduct formation. Fischer-344 rats were administered a single dose of 150 mg/kg 2,4-DNT (97-99% purity), dissolved in DMSO, by i.p. injection. Rats were killed 18 hours later. Liver, kidney and lung were isolated from males, and mammary glands from females DNA from these organs was measured for adduct formation using ^{32}P-postlabelling and thin layer chromatography. Data were expressed as means ± standard deviations for three animals. 2,4-DNT was found to bind hepatic DNA and produced three different adduct spots on autoradiograms. Adduct 1 produced the highest intensity of radioactivity, comprising approximately 85% of the total; adducts 2 and 3 accounted for 10% and 5%, respectively. 2,4-DNT also induced DNA binding in kidney, lung and mammary gland. DNA adducts from extrahepatic sites were chromatographically identical to those from liver, but their yields were significantly lower. In order to examine the relationship between the administered dose of 2,4-DNT and DNA adduct levels in liver, rats were treated i.p. with different doses ranged from

0.75 to 375 mg/kg 2,4-DNT. There was an increase in adduct yield as a function of concentration. Adducts from 2,4-DNT increased 200-fold between 0.75 and 225 mg/kg. In addition, treatment by gavage and intraperitoneal injection were compared for qualitative and quantitative differences in adduct formation. When three male Fischer-344 rats were administered 150 mg/kg of 2,4-DNT by either gavage or intraperitoneal injection, no differences were noted. The two treatments method produced identical adduct maps on autoradiograms, and adduct yields from the two routes of administration were not significantly different. To study the kinetics of hepatic DNA adduct formation and removal, male Fischer-344 rats administered i.p. 50 mg/kg 2,4-DNT were killed at different time points (6 hours to 14 days) and examined. Maximum hepatic adduct levels were obtained 20-24 hours following treatment. Adducts were then removed slowly over the time. DNA adduct removal appeared to follow biphasic kinetics, with a rapid loss over the first 72 hours, followed by a slower rate of elimination. Significant amounts of adducts were found to persist over the time. After 2 weeks, 42% of maximum levels were detected for 2,4-DNT induced adducts. With respect to toxicity, none of the rats administered 2,4-DNT (to 375 mg/kg) died as a result of exposure to the chemical irrespective of the route of administration; liver sections taken from rats treated with 50 mg/kg 2,4-DNT showed no observable signs of necrosis.

Table 4.1.2.7.2-1: *In vivo* somatic mammalian cell genotoxicity studies with 2,4-DNT

Test	System	Dosage	Results	Comments	Reference
Chromosome aberration (CA)	Lymphocytes of CD male rats	93 mg/kg/day in feed for 5 and 19 weeks	Positive after 19 weeks for structural CA (↑chromatid breaks)	98.5-99% purity	Lee *et al,* (1978)
	Kidney cultures of CD male rats	93 mg/kg/day in feed for 5 and 13 weeks	Positive for structural CA (↑ chromatid breaks) after both 5 and 13 weeks.	98.5-99% purity The chromatid breaks increased with the duration of treatment	
Chromosome aberration (CA)	Kidney and bone marrow cultures from chronically treated Beagle dogs	10 mg/kg/day in hard gelatine capsules	Negative in both cultures	98% purity	Ellis *et al.* (1979)
Chromosome aberration (CA)	Kidney and bone marrow cultures from chronically treated CD rats	3.9 or 34 mg/kg/day in feed (♂); 5.1 or 45 mg/kg/day in feed (♀)	Negative in both cultures	98% purity A significant ↑ in tetraploid was observed only at the low dose in kidney cultures	Ellis *et al.* (1979)
DNA damage (UDS)	Hepatocytes of Fischer-344 rats	100 mg/kg by gavage (rats killed 12 h after dosing)	Weak positive	98% purity	Mirsalis and Butterworth (1982)
DNA damage (UDS)	Hepatocytes of male Fischer-344 rats	50 and 200 mg/kg by gavage (rats killed 12 h after dosing)	Positive in a dose-dependent manner	> 99.9% purity	Mirsalis *et al.* (1982)
DNA covalent binding	Liver of male Fischer-344 rats	10 and 35 mg/kg by gavage (rats killed 1, 2, 4, 8, 12, 24, 48, 96, 192 and 384 h after dosing)	Positive	High purity Covalent binding peaked between 12 and 24 h	Rickert *et al.* (1983)

Test	System	Dosage	Results	Comments	Reference
DNA covalent binding	Liver of male Fischer-344 rats	28 mg/kg, oral (rats killed 12 h after dosing)	Positive	> 98%purity Inhibitors of sulfotransferases decreased the covalent binding	Kedderis, Dyroff and Rickert (1984)
DNA covalent binding	Liver of male Fischer-344 rats	4.1, 41, 123, 410, 1230 and 2046 µmol/kg i.p. (rats killed 18 h after dosing)	Positive from 4.1 µmol/kg in a dose-dependent manner	97-99% purity 3 adducts	La & Froines (1992a)
DNA covalent binding	Liver, kidney, lung (♂) and mammary glands (♀) of Fischer-344 rats	150 mg/kg i.p. (rats killed 18 h after dosing)	Positive	97-99% purity 3 adducts in all cases (in liver higher yield than in extrahepatic sites)	La & Froines (1992b)
	Liver of male Fischer-344 rats	From 0.75 to 375 mg/kg i.p. (rats killed 18 h after dosing)	Positive in a dose-dependent manner	97-99% purity	
	Liver of male Fischer-344 rats	150 mg/kg i.p. or by gavage (rats killed 18 h after dosing)	Positive	97-99% purity No differences between the two administration routes	
	Liver of male Fischer-344 rats	50 mg/kg i.p. (rats killed from 6 hours to 14 days after dosing)	Positive	97-99% purity Maximum hepatic adduct levels at 20-24 hours; 42% of maximum levels after 2 weeks.	

Mammalian studies with 2,4-DNT metabolites

DNA covalent binding

La and Froines (1992a) examined the ability of three urinary metabolites of 2,4-DNT to induce *in vivo* DNA adduct formation. Three male Fischer-344 rats were administered 2,4-diaminotoluene, 2-amino-4-nitrotoluene or 4-amino-2-nitrotoluene, reported to be 97-99% purity, dissolved in DMSO, by i.p. injection at a single dose of 410 µmol/kg. Rats were killed 18 hours later and DNA from liver measured for adduct formation using ^{32}P-postlabelling and thin layer chromatography. The three compounds produced three adduct spots identical to those produced by 2,4-DNT. Adduct levels were reported as ratios of adducted nucleotides to total nucleotides (or relative adduct labelling, RAL). The 2,4-diaminotoluene RAL (x10^7 total nucleotides) was 17.5 ± 4.2 (adduct 1), 1.5 ± 0.4 (adduct 2) and 2.1 ± 0.4 (adduct 3). The 2-amino-4-nitrotoluene RAL (x10^7 total nucleotides) was 7.7 ± 1.3 (adduct 1), 0.9 ± 0.1 (adduct 2) and 1.1 ± 0.2 (adduct 3). The 4-amino-2-nitrotoluene RAL (x10^7 total nucleotides) was 8.7 ± 2.3 (adduct 1), 0.8 ± 0.1 (adduct 2) and 1.3 ± 0.3 (adduct 3). Adducts yields from 2,4-diaminotoluene were significantly different than yields from the two nitro-substituted compounds. Yields from 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene were comparable with those from 2,4-DNT.

Studies in germ cells

Insect studies with 2,4-DNT

Gene mutation

Woodruff *et al.* (1985) tested the ability of 2,4-DNT to induce sex-linked recessive lethal (SLRL) mutations in *Drosophila melanogaster*. 2,4-DNT (99% purity) was administered to adult Canton-S males by feeding or injection. In feeding, males were treated for 3 days in glass shell vials containing a glass fiber disk soaked with 0.2-0.5 mL of solution (2,4-DNT dissolved in ethanol 10%); concentrations tested were 1000 and 10000 ppm. In injection, 0.2-0.3 µL of freshly prepared solution (2,4-DNT dissolved in ethanol 10% and then diluted in 0.7% NaCl) was injected into the males; concentrations tested were 200 and 10000 ppm; males were allowed to recover for 24-48 hours before mating. The SLRL test was performed as described by Wurgler *et al.* (1977) using the standard *Basc* mating scheme. Individual wild-type males were mated with 3 new *Basc* females for each of 3 broods (2, 2, and 2 days). Lethal mutation was declared if no wild-type males were recovered among 20 or more *Basc males or Basc/*± females. Retests were performed to confirm lethality. Where a few F_2 or F_3 wild-type males survived among a large number of progeny, a lethal was declared if wild-type males were less than 5% of the total *Basc* males or *Basc* ± females. 2,4-DNT induced SLRL mutations after injection at 10000 ppm but failed to induce lethal mutations after feeding.

Chromosomal aberration

Woodruff *et al.* (1985) tested 2,4-DNT (99% purity) for the induction of reciprocal translocations in *Drosophila melanogaster*. 0.2-0.3 µL of freshly prepared solution (2,4-DNT dissolved in ethanol 10% and then diluted in 0.7% NaCl) was injected into the Canton-S males; concentration tested was 10000 ppm; males were allowed to recover for 24-48 hours before mating. The reciprocal translocation test was performed as described by Valencia *et al.* using the standard mating scheme for T (2; 3) and T (Y; A). Wild-type males were mated with *bw; e* or *bw; st* females. Females were subcultured at 3-4-day intervals for at least 17-18 days. A translocation was judged to have occurred only in crosses with total counts of 20 or more progeny. Retests were performed to confirm translocations. T (Y; 2; 3) events were counted as two translocations. 2,4-DNT was not found to induce reciprocal translocations.

Mammalian studies with 2,4-DNT

They are summarised in table 4.1.2.7.2-2.

Chromosomal aberration

Rats

The mutagenic effect of 2,4-DNT was studied by Lee *et al.* (1978) using the dominant lethal test in Charles River CD rats. Three groups of four or five males were fed standard rodent feed or feed containing either 0.02 % or 0.2% of 2,4-DNT (purity > 98%). The intake by the high dosage males was 93 mg/kg b.w./day. After 13 weeks of feeding, each male was mated with three females. On day 13 of gestation each female was killed and the uterus examined for implantation sites, dead and viable foetuses, and resorptions. Data were expressed as fertility index (confirmed pregnancies/sperm-positive females x 100) and implant viability index (viable foetuses/total implants x 100). When compared to controls, rats fed 0.02% of 2,4-DNT

had reduced fertility (67±14 vs. 92±8) and implant viability indexes (62±24 vs. 92±1), but the results were not statistically significant; rats fed 0.2% (93 mg/kg b.w./day) of 2,4-DNT had significantly reduced fertility (20±13 vs. 92±8) and implant viability indexes (0 vs. 92±1).

Ellis *et al.* (1979) performed four dominant lethal mutation studies in Charles River CD for testing the mutagenicity of 2,4-DNT (98% 2,4-DNT, 2% 2,6-DNT). For the first study, groups of four, four and five males were fed 0, 0.02 or 0.2% 2,4-DNT for 10 weeks, respectively. For the second study, groups of eight, nine, seven and ten males were fed 0, 0.0015, 0.01 and 0.07% 2,4-DNT for 13 weeks, respectively. For the third study, groups of ten rats were fed 0, 0.15 or 0.2% 2,4-DNT and a group of 15 rats was fed 0.5 % 2,4-DNT for 13 weeks. For the fourth study, groups of 24 males were fed 0, 0.07, 0.10 or 0.15% 2,4-DNT for 13 weeks; to get maximum information during the last study, it was also measured body weight and feed consumption. In all studies, at the end of the treatment, each male was mated to two virgin females, and at mid-term of pregnancy the females were killed and the following data collected: number of fertile males per number of males treated, number of pregnant females per number of mated females, number of corpora lutea per pregnant female, and the number of total implants, dead implants and live implants per pregnant female. In addition, after mating, 10 of the males in each group of the fourth study were killed and necropsied for examination of any morphological changes in their genital organs and the remaining rats were fed plain feed without 2,4-DNT for a 13 week recovery period and they were then similarly necropsied. In the first study, when compared to controls, rats fed 0.02% 2,4-DNT for 10 weeks had reduced fertility (67±14 vs. 92±8) and implant viability indexes (62±24 vs. 92±1), but the results were not statistically significant; rats fed with 0.2% 2,4-DNT had significantly reduced fertility (20±13 vs. 92±8) and implant viability indexes (0 vs. 92±1). In the second study, live implants per female, dead and viable foetus and resorptions in treatment groups and controls did not differ significantly. In the third study, only three of the 15 high dose (0.5 % 2,4-DNT) males survived the 13 weeks' feeding and none mated, so they were functionally sterile; males from the middle (0.2 % 2,4-DNT) and low (0.15% 2,4-DNT) dose groups did mate but there were no viable foetuses; about two-thirds of the plugs from middle dose males and one-third of those from low dose males had no apparent sperm, indicating sterility. In the fourth study, 2,4-DNT produced a dose-related decrease in weight gain. There were some decreases in feed consumption. There was a dose-related increase in spermless vaginal plugs and a dose-related decrease in fertility (pregnancies). There was only one, non-viable foetus in only one of the females mated to males fed 0.15% 2,4-DNT. The variations in corpora lutea/dam were within normal limits. The lack of an effect on implant viability index, despite the drastic effect on implantation index, showed that there was no apparent dominant lethal mutation effect. Nearly all males fed 0.07, 0.1 or 0.15% 2,4-DNT for 13 weeks had marked to severe atrophy or degeneration of seminiferous tubules of the testes and too few or no spermatozoa in ducts of the epididymis. After the treatment was discontinued for 13 weeks, there was no evidence of any recovery for those lesions.

Lane *et al.* (1985) tested the potential of 2,4-DNT (purity not indicated, recristalized) to produce reproductive and dominant lethal effects in Sprague Dawley rats. Fifty rats were randomly divided into five groups. The control group received a daily oral dose of Mazola corn oil for five days. The positive control group received TEM dissolved in corn oil as a single oral dose. The other three groups received 2,4-DNT dissolved in corn oil as a daily oral dose of 60, 180 or 240 mg/kg/day for five days. Since there were excessive deaths in the highest 2,4-DNT dose group which would make it difficult the statistical evaluation of the results a new group receiving 240 mg/kg 2,4-DNT was created one week after the other groups. Each male was allowed to mate with two females for five days each week. Females were sacrificed on day 14 of gestation. The uteri and ovaries were examined for living and

dead implantations and corpora lutea. Mating lasted for seven weeks except for the high 2,4-DNT dose which was extended by six weeks to look for the possible reversibility of the effects. 60 mg/kg/day 2,4-DNT produced no adverse reproductive or dominant lethal effects; no signs of toxicity were seen other than slight cyanosis. 180 mg/kg/day 2,4-DNT produced cyanosis but only three statistically significant effects in the pre-implantation loss index (higher than in control at week 2), the mating index (lower than in control at week 5) and the corpora lutea index (lower than in control at week 5). 240 mg/kg/day 2,4-DNT produced weight loss, cyanosis and death during the week after dosing. A number of adverse reproductive effects were observed among the rats receiving this high dose, most noticeably the mating index of weeks 1-6. The low number of pregnant females made difficult to interpret the other data for those weeks, and therefore, it was decided to allow this portion of the study to continue six weeks longer than the other doses. After week 7 of the study, the mating index was not significantly different from control to allow the other data to be more easily interpreted. The implantation index, the pre-implantation loss index, and the foetal index were significantly altered in week 7. Thereafter, only the pre-implantation loss index of weeks 8 and 9 were statistically significant from control. By the thirteenth week, all reproductive and dominant lethal indices had recovered and were comparable to control values. It was therefore concluded that 2,4-DNT did not cause dominant lethal mutations but did adversely affect reproductive performance when tested at 240 mg/kg/day, however reproductive effects were slowly reversible. The positive control produced its classical dominant lethal effects.

Mice

The mutagenic effect of 2,4-DNT (purity > 98%) was studied by Lee *et al.* (1978) using the dominant lethal test in albino Swiss mice. Four males were fed standard rodent feed, five males were fed 0.2% (137 mg/kg b.w./day) of 2,4-DNT for 13 weeks, and three males were fed 0.7% (332 mg/kg b.w/day) of 2,4-DNT for 4 weeks. After treatment, each male was mated with three females. Mating was determined by the presence of a vaginal plug and females were allowed to deliver. Data were expressed as fertility index (confirmed pregnancies/sperm-positive females x 100) and implant viability index (viable foetuses/total implants x 100). The low dose of 2,4-DNT produced no effects. The high dose of 2,4-DNT, greatly reduced the fertility index but had no effect on the implant viability index. The reduced fertility index was due to a majority of the females having no implants. However, females bearing young had normal numbers of implants and pups.

The mutagenic effect of 2,4-DNT was tested by Soares and Lock (1980) in DBA/2J mice using the dominant lethal test. Two groups of 20, 10- to 12-weeks-of-age, mice were treated on two consecutive days with 250 mg/Kg of purified 2,4-DNT either by i.p. injection (IP) or gavage (PO); two positive control groups of 10 mice each received on two consecutive days 125 mg/Kg of EMS (PO) or 0.15 mg/Kg of TEM (IP); and negative controls received on two consecutive days Hanks Balanced Salt Solution (HBBS) IP and PO (five mice each) or corn oil IP and PO (5 mice each). After 48 hours post-treatment, mice were paired with three 12-weeks of age CD-1 females, which were replaced for other three after seven nights. This mating scheme was repeated for a total of seven weeks post-treatment. Females were killed seventeen days after the initial exposure to a male and their uterine contents examined for living foetuses and post-implantation deaths. Statistical analysis of the number of implantations and the percent of post-implantation death showed no indications of induced increases in dominant lethals for groups treated with 250 mg/Kg of 2,4-DNT PO or IP. Positive controls resulted in consistent reductions in the total number of implantations as well as increases in the percent post-implantation death. Mice treated with 250 mg/Kg of 2,4-DNT

PO or IP showed consistent decreases in fertility, which were significant in weeks 2, 3 and 6 (PO group) and weeks 2 and 4 (PI group).

Table 4.1.2.7.2-2: *In vivo* germinal mammalian cell genotoxicity studies with 2,4-DNT

Test	System	Dosage	Results	Comments	Reference
Dominant lethal	Charles River CD rats (males treated by feeding)	0.02 or 0.2% for 13 weeks. 93 mg/kg/day was the intake by the high level dose	A significant decrease of the implant viability index at 0.2% (no viable foetuses).	> 98% purity. The fertility index was significantly reduced at 0.2%, probably due to 2,4-DNT effect on spermatogenesis. Further testing is required to separate toxic effect from mutagenic effect.	Lee *et al,* (1978)
Dominant lethal	Charles River CD rats (males treated by feeding)	0.02 or 0.2% for 10 weeks	A significant decrease of the implant viability index at 0.2% (no viable foetuses).	98% purity. The significant decreased fertility index at 0.2%, probably due to 2,4-DNT effect on spermatogenesis, casts doubt on the dominant lethal mutation effect.	Ellis *et al.* (1979)
		0.0015, 0.01 or 0.07% for 13 weeks	No effects on implant viability	98% purity. No effects on fertility	
		0.15, 0.2 or 0.5% for 13 weeks	No viable foetuses from males fed 0.15 or 0.2%.	98% purity. Survived males fed 0.5% (3/15) did not mate; males fed 0.2 or 0.15% mated. ~2/3 and 1/3 of the plugs from males fed 0.2 and 0.15%, respectively, had no apparent sperm. These results indicate sterility.	
		0.07, 0.1 or 0.15% for 13 weeks with a recovery period of 13 weeks	Only one non-viable foetus in only one of the females mated to males fed 0.15%. Based on the lack of an effect on implant viability index, despite the drastic effect on implantation index, it is considered that 2,4-DNT did not induce dominant lethal mutations.	98% purity. A dose-related in both increased spermless vaginal plugs and decreased fertility. Atrophy or degeneration of testes seminiferous tubules and too few or no spermatozoa in nearly all males fed 2,4-DNT without recovery for those lesions.	

Test	System	Dosage	Results	Comments	Reference
Dominant lethal	Sprague-Dawley rats (males treated orally). Mating lasted for 7 weeks except for the highest dose which was extended by 6 weeks to see the reversibility of effects.	60, 180 or 240 mg/kg/day for 5 days.	Negative for dominant lethal mutations.	Purity not given. Reproductive effects were observed among rats receiving 240 mg/kg/day, most noticeably the mating index of weeks 1-6, but these effects were slowly reversible.	Lane *et al.* (1985)
Dominant lethal	Albino Swiss mice (males treated by feeding)	0.2% (137 mg/kg/day) for 13 weeks or 0.7% (332 mg/kg/day) for 4 weeks.	Negative for dominant lethal mutations	> 98% purity. 0.7% 2,4-DNT greatly reduced the fertility index due to a majority of the females having no implants.	Lee *et al,* (1978)
Dominant lethal	DBA/2J mice (males treated i.p or by gavage). Mating lasted for 7 weeks.	250 mg/kg for 2 days	Negative for dominant lethal mutations	Purified 2,4-DNT. Decreases in fertility were significant in weeks 2, 3 and 6 (mice treated by gavage and weeks 2 and 4 (mice treated i.p.)	Soares and Lock (1980)

4.1.2.7.3 Other information

Soares and Lock (1980) tested the ability of 2,4-DNT to induce sperm abnormalities in DBA/2J mice. From the dominant lethal test described above, the following groups were selected for testing sperm abnormalities: The two groups of 20, 10- to 12-weeks-of-age, mice treated on two consecutive days with 250 mg/Kg of purified 2,4-DNT either by i.p. injection (IP) or gavage (PO); the positive control group of 10 mice which received on two consecutive days 0.15 mg/Kg of TEM (IP); and the negative controls which received on two consecutive days Hanks Balanced Salt Solution (HBBS) IP and PO (five mice each) or corn oil IP and PO (5 mice each) . After 48 hours post-treatment, mice were paired with three 12-weeks of age CD-1 females, which were replaced for other three after seven nights. This mating scheme was repeated for a total of seven weeks post-treatment. Following the conclusion of the last mating period (i.e., eight weeks post-treatment) sperm was collected from the vas deferens of 10 mice each from treatment groups. Slides were coded and scored for the percent of morphologically abnormal sperm. Mice treated with 2,4-DNT showed no significant increase in the percent of morphologically abnormal sperm over the control values. Mice treated with TEM showed an increase in the mean percent of abnormal sperm, but the increase was not significant.

4.1.2.7.4 Summary of mutagenicity

The genotoxicity of 2,4-DNT has been investigated in a comprehensive range of *in vitro* and *in vivo* assays including gene mutation, chromosomal aberration, DNA damage and DNA binding as endpoints.

The information in bacteria indicates that, under standard conditions, 2,4-DNT is clearly mutagenic, albeit weakly, in *Salmonella typhimurium* both in the presence and absence of a rat liver metabolic activation system. Various modifications either of the activation conditions or the inherent metabolic profile of the bacterium have resulted in a more marked mutagenicity. Thus, 2,4-DNT was not mutagenic in TA98 strain using the standard plate incorporation test with or without rat liver S9, but was found to have a clear flavin mononucleotide-dependent mutagenic activity in a modified preincubation assay with hamster S9. Thus, it is suggested that extracellular nitro reduction is necessary for optimal detection of 2,4-DNT mutagenicity. In the absence of a metabolic activation system, the highest mutagenic activity was observed in strains with elevated levels of both nitroreductase and *O*-acetyltransferase activities (YG1041 and YG1042) when compared to that observed in the parent strains (TA98 and TA100) suggesting that 2,4-DNT requires high levels of both activities to exert its mutagenicity. The greatest mutagenic activities of bacterial 2,4-DNT metabolites, in the absence of a metabolic activation system, were also found in both YG1041 and YG1042 strains. The relative mutagenic activities were 4A2NT < 2A4NT < 2,4-DNT < 4HA2NT < 2,2'-DM-5,5'-DNAOB = 2HA4NT << 4,4'-DM-3,3'-DNAOB toward YG1041, or 4A2NT < 2A4NT < 2,4-DNT < 4HA2NT = 4,4'-DM-3,3'-DNAOB < 2HA4NT toward YG1042. According to these findings, aminohydroxylamino dimethylazoxybenzenes or aminohydroxylamino dimethylazobenzenes produced either by reduction of hydroxylaminonitrotoluenes or by reduction of dimethyl dinitroazoxybenzenes could be considered the active metabolites responsible for the mutagenic activity of 2,4-DNT in bacteria. With respect to DNA damage, 2,4-DNT was genotoxic in the *Salmonella typhimurium umu* test. As occurred in mutation tests, the most sensitive strain was NM3009, which has high *O*-acetyltransferase and nitroreductase activities.

2,4-DNT was not mutagenic in the CHO/HGPRT system, when tested in the presence of rat liver S9 under usual (aerobic) assay conditions. However, it was mutagenic either when CHO cells were incubated with rat liver S9 under anaerobic (reduced oxygen tension) conditions or when primary rat hepatocytes were used as metabolic activation system. In addition, 2,4-DNT was a direct mutagen in the P388 mouse lymphoma/TK system. Results on clastogenicity were contradictory. Thus, in one study, 2,4-DNT of 99% purity did not induce chromosomal aberrations in CHO cells (with or without S9), whereas in two other studies, 2,4-DNT of unknown purity was reported to be clastogenic in human lymphocytes (without S9) and in CHL cells (with and without S9). With respect to DNA damage, 2,4-DNT induced sister chromatid exchanges in CHO cells when tested in the presence of rat liver S9. There was no evidence of unscheduled DNA synthesis in rat (hepatocytes, spermatocytes and spermatids) or human (hepatocytes) cells incubated with 2,4-DNT. This lack of 2,4-DNT genotoxic activity is accord with the concept that reduction by intestinal flora is required in addition to hepatic metabolism for activation. Finally, 2,4-DNT of unknown purity was reported to cause DNA damage (single-strand breaks) when tested at cytotoxic concentrations in the alkaline elution/rat hepatocyte assay.

Dogs administered 2,4-DNT (10 mg/kg b.w./day) in hard gelatine capsules for 2 years had no chromosomal aberrations in their bone marrow or kidney. 2,4-DNT when fed to male and female rats at up to 45 mg/kg b.w./day for 2 years did not induce either chromosomal aberrations in the bone marrow or kidney. Nevertheless, 2,4-DNT when fed to male rats at 93 mg/kg b.w./day for 19 weeks induced chromosomal aberrations (chromatid breaks) in lymphocytes; in addition, significant increases in the number of chromatid breaks were also observed after treatment for 5 and 13 weeks in kidney cultures, being the number of breaks increased with the duration of treatment. With respect to DNA damage, unscheduled DNA synthesis was induced by 2,4-DNT when administered at up to 200 mg/kg by gavage to male

rats. DNA covalent binding was observed in several organs (liver, kidney, lung and mammary glands) of rats administered i.p. a single dose of 150 mg/kg of 2,4-DNT, the binding being highest in the liver. Moreover, when rats were administered 2,4-DNT by either gavage or i.p., no differences in liver DNA covalent binding were noted. The sulfotransferase inhibitors decreased the covalent binding to DNA, indicating that sulfation is important in the biotransformation of 2,4-DNT to reactive metabolites.

In *Drosophila melanogaster*, 2,4-DNT induced sex-linked recessive lethal mutation after injection, but failed to induced lethal mutations after feeding and translocations after injection.

The decreased implant viability index of males fed 0.2% for 10 weeks (Lee *et al.,* 1978) or 13 weeks (the first experiment carried out by Ellis *et al.,* 1979) suggested a mutagenic effect. However, the decreased fertility indexes, probably due to the effect of 2,4-DNT on spermatogenesis casted doubt on this interpretation. Therefore, additional experiments were carried out by Ellis *et al.,* 1979 to determine if there was a dose of 2,4-DNT which would reduce implant viability without reducing fertility. Results from these additional experiments showed a close relationship between decreased implant viability index and sterility. Therefore, taking into account all available information it was concluded that 2,4-DNT did not induce dominant lethal mutations when administered to rats for 5 days (by gavage) or up to 13 weeks (by feeding). Negative results were also obtained for dominant lethal mutations in mice administered 2,4-DNT for 2 days (by gavage) or up to 13 weeks (by feeding). In addition, 2,4-DNT did not induce sperm abnormalities in mice.

In conclusion, the weight of evidence indicates that 2,4-DNT is an *in vivo* mutagenic agent for somatic cells. Therefore, 2,4-DNT is classified as mutagenic category 3 (Xn, R68).

The type of results leading to classification of chemicals in category 3 is generally regarded as an alert for possible carcinogenic activity. Taking into account this fact, the information derived from mutagenicity studies carried out with urinary metabolites of 2,4-DNT is considered valuable in order to determine the 2,4-DNT carcinogenic mode of action.

Urinary metabolites of 2,4-DNT were tested for mutagenicity in the *Salmonella/*microsome test. 2A4AAT, 2A4NB, 4A2NB, 2,4-DAT, 2,4-DNB and 2,4-DNAl were more mutagenic than 2,4-DNT, being increasingly mutagenic in that order. Among them, 2,4-DAT (only mutagenic in the presence of S9) has been shown to be a hepatocarcinogen in rats. The observation that 2,4-DNB and 2,4-DNBA are major metabolites of 2,4-DNT strongly suggest that 2,4-DNAI (a putative metabolite of 2,4-DNT) could be an intermediary metabolite in the oxidation of 2,4-DNB to 2,4-DNBA. Thus, the metabolic conversion of 2,4-DNT to 2,4-DNB, 2,4-DNAl and 2,4-DAT, a known carcinogen, may contribute to the carcinogenicity of 2,4-DNT.

Three urinary metabolites (2,4-DAT, 2A4NT and 4A2NT) induced *in vivo* DNA adduct formation. They produced three adducts identical to those produced by 2,4-DNT. Adducts yields from 2,4-DAT were significantly different than yields from the two nitro-substituted compounds. Yields from 2A4NT and 4A2NT were comparable with those from 2,4-DNT. A complex metabolic pathway has been proposed for 2,4-DNT bioactivation involving reduction of the nitro groups by intestinal microflora. 2A4NT and 4A2NT are major intermediates in the reduction of 2,4-DNT. Subsequent reactions in the liver are then though to produce the ultimate DNA binding species. The formation of identical adducts by the four compounds may derive from their conversion to other common metabolic intermediates. The relatively lower adduct yields from 2,4-DNT and the two aminonitrotoluenes may reflect their complex

metabolism in generating reactive species. In conclusion, results suggest that the differences in carcinogenic potency between 2,4-diaminotoluene and 2,4-dinitrotoluene may be explained in part by quantitative differences in the extent of DNA adduct formation.

4.1.2.8 Carcinogenicity

4.1.2.8.1 Studies in animals

In vivo studies

Inhalation

No data available.

Dermal

No data available.

Oral

Regarding carcinogenicity, there are few oral studies available in Fischer 344 rats and B6C3F1 mice carried out under similar methodology to OECD guideline 451.

Rats

NCI study, 1978

Fifty Fischer 344 rats/sex were treated with 80 ppm (low dose) of 2,4-DNT (greater than 95% purity) in the diet for 18 months, whereas other 50 Fischer 344 rats/sex were feed standard diet for 18 months (low-dose control). As rats treated with 80 ppm did not demonstrate the desired weight depression, after 51 weeks, a new group of 50 rats/sex receiving 200 ppm (high dose) of 2,4-DNT in the diet for 18 months was started with a new control (high-dose control, 25 rats/sex). Estimated body-weight-corrected doses of 2,4-DNT were calculated by using the default reference values for biological parameters (Appendix VI, Chapter 2, TGD), since 2,4-DNT doses were reported in the NCI study as dietary concentrations.

$$D_{b.w.} = \frac{D_{diet} \cdot 0.040 \cdot W^{0.479}}{W}$$

$D_{b.w.}$ body-weight-corrected dose (mg/kg b.w./day), D_{diet} dietary concentration (ppm), W body weight (kg).

The low dose was equivalent to 4.7 and 6.3 mg/kg b.w./day for males and females, respectively, whereas the high dose was 11.8 and 15.7 mg/kg b.w./day for males and females, respectively. After the exposure period, observation of the rats continued for 26 weeks. This carcinogenicity study is performed according to the OECD 451 guideline.

Survival of male and female treatment groups did not differ significantly from its corresponding control group. With respect to body weight, high dose male rats weighed

approximately 25 % less than their controls, whereas high-dose females about 10% less than their controls.

The incidence of primary tumours was assessed (Table 4.1.2.8.1-1). No integumentary tumours were seen in controls, whereas in treated males, the incidence of fibromas of skin/subcutaneous tissue was increased significantly in a dose-dependent manner; sporadic occurrence of squamous-cell papillomas (1/49 low dose), basal-cell carcinoma (1/49 low dose), fibrosarcomas (1/49 low dose, 2/49 high dose), and lipomas (3/49 high dose). The incidence of hepatocellular carcinoma in liver of males fed the low- and high-dose was slightly higher than that of high and low-dose controls. Nevertheless, that difference was not statistically significant.

Table 4.1.2.8.1-1: Incidence of primary tumours in male Fischer 344 rats treated with 2,4-DNT for 18 months over a 24-month observation period (NCI , 1978)

	Dose (mg/kg b.w./d)			
	Low dose		High dose	
	0	4.7	0	11.8
Hepatocellular carcinoma	0/45	3/49	0/25	3/48
Fibroma (Subcutaneous tissue or skin)	0/46	7/49[a]	0/25	13/49 [a]
Lipoma (Subcutaneous tissue)	0/46	0/49	0/25	3/49
Islet-cell adenoma or carcinoma Pancreatic islets	1/45	3/45	2/25	3/48
Leukemia (hematopoietic system)	3/46	4/49	4/25	3/49
Adenoma NOS or basophil adenoma (Pituitary)	9/44	5/44	3/21	0/35
Pheochromocytoma (adrenal)	6/45	3/46	2/25	6/45
C-cell adenoma or C-cell carcinoma (Thyroid)	3/42	3/41	0/23	5/47
Interstitial-cell tumour (testes)	44/45	43/46	19/24	46/49

[a] Significantly different from controls ($p < 0.05$)

In females, the incidence of mammary gland fibroadenoma of high-dose group was significantly higher than in controls (Table 4.1.2.8.1-2). The incidence of hepatocellular carcinoma in low- and high-dose groups did not differ significantly when compared with that of its corresponding low- and high-dose controls.

Table 4.1.2.8.1-2: Incidence of primary tumours in female Fischer 344 rats treated with 2,4-DNT for 18 months over a 24-month observation period (NCI , 1978)

	Dose (mg/kg b.w./d)			
	Low dose		High dose	
	0	6.3	0	15.7
Hepatocellular carcinoma	1/47	0/49	0/23	1/50
Fibroma (Subcutaneous tissue or skin)	0/48	0/49	0/23	3/50
Fibroadenoma (mammary gland)	9/48	12/49	4/23	23/50 [a]
Leukemia (hematopoietic system)	2/48	2/49	2/23	4/50
Adenoma NOS or chromophobe adenoma (Pituitary)	19/46	22/45	8/21	14/40
Pheochromocytoma (adrenal)	2/47	2/49	2/23	0/50
C-cell adenoma or C-cell carcinoma (Thyroid)	2/45	2/45	3/21	6/48
Endometrial stromal polyp (uterus)	15/46	14/47	6/23	11/49

[a] Significantly different from controls ($p < 0.05$)

Treatment groups and controls did not differ significantly in terms of non-neoplastic, degenerative, proliferative and inflammatory lesions.

Mice

NCI study (1978)

Groups of 50 B6C3F1 mice/ sex were treated with 80 ppm (low-dose) of 2,4-DNT (greater than 95% purity) in the diet for 18 months, whereas other 50 B6C3F1 mice / sex were feed standard diet for 18 months (low-dose control). After 29 weeks, a new group receiving 400 ppm (high-dose) of 2,4-DNT in the diet for 18 months was started with a new control (high-dose control, 50 B6C3F1 mice/ sex), since the mice treated with 80 ppm did not demonstrate desired weight depression.

Estimated body-weight-corrected doses of 2,4-DNT were calculated by using the Default reference values for biological parameters (Appendix VI, Chapter 2, TGD), since 2,4-DNT doses were reported in the NCI study as dietary concentrations.

$$D_{b.w.} = \frac{D_{diet} \cdot 0.064 \cdot W^{0.7242}}{W}$$

$D_{b.w.}$ body-weight-corrected dose (mg/kg b.w./day), D_{diet} dietary concentration (ppm), W body weight (kg).

The low dose was equivalent to 12.0 and 12.9 mg/kg b.w./day for males and females, respectively, whereas the high dose was 60.2 and 64.5 mg/kg b.w./day for males and females, respectively. After exposure period, observation of the mice continued for 13 weeks. This carcinogenicity study is performed according to OECD 451 guideline.

Both treatment with 2,4-DNT and sex did not influence mice survival along follow-up period. Weigh gain at the end of the study was 91% and 82% for low- and high-dose males, respectively, and 89% and 76% for low- and high-dose females when compared with that of controls (100%). Treatment groups and controls did not differ significantly in the incidence of the primary tumours.

This study was performed in essence according to OECD guideline 451 and considered adequate for risk assessment.

Other information

Under this section are described studies carried out in rats and mice which are non OCDE (451 or 453) guideline, non GLP compliant and considered inadequate to test carcinogenicity.

Rats

Oral

Leonard, Graichen and Popp, 1987

2,4-DNT (99.9% purity) was administered in the diet (27 mg/kg b.w./day) to 28 male Fischer 344 rats for 12 months in order to determine hepatocarcinogenicity. Hepatic microsomes and cytosol were prepared from 4 animals after 6 weeks and 6 months of feeding. At 12 months, blood samples from the remaining 20 rats were collected for determining GGT and ALT, and their livers and lungs evaluated histopathologically. This study was published in a peer-reviewed journal.

Body weight gain of treated animals was about 25% less than that of controls at the end of the study. Moreover, liver weights were significantly elevated in treated rats at 6 and 12 months. No early deaths were indicated. One treated rat had hepatic neoplastic nodule at 12 months and no liver tumour was noted in the controls. With respect to non-neoplastic lesions, hepatocytic degeneration and vacuolation were noted in treated rats. Furthermore, acidophilic and basophilic cell foci were observed in 70% and 10% of the livers, respectively; neither type of focus was apparent in the controls. The hepatic epoxide oxidase (EH) activity was significantly increased only in the treated animals at 6 months (ca. 200% of control activity, $p < 0.05$).

This study has limited quality to evaluate carcinogenicity, due to small number of used animals, short exposure period and incomplete histopathology.

Leonard, Lyght and Popp, 1983

A single administration of 2,4-DNT (75 mg/kg, purity ≥ 99.4%) in combination with partial hepatectomy, did not initiate hepatocytes in male CDF® (F344)/Cr1BR rats when assayed using hepatic initiation-promotion protocols.

Leonard, Adams and Popp, 1986

A rat hepatic initiation-promotion protocol was used to determine the relative hepatocyte foci activity of technical grade DNT, 2,4-DNT and 2,6-DNT. Both GGT^+ foci number (foci/cm^3) and mean volume in liver (right anterior, median and right posterior lobes) of male CDF® (F344)/CrlBR rats fed 2,4-DNT (27 mg/kg/day) for 6 weeks after diethylnitrosamine initiation (150 mg/kg) were significantly higher as compared to those of rats fed either 2,4-DNT or phenobarbital (500 ppm of diet) alone. Following 12 weeks of 2,4-DNT feeding to diethylnitrosamine-initiated rats, both GGT^+ foci number and mean volume in liver (right anterior and right posterior lobes) were significantly higher than those of rats fed either 2,4-DNT or phenobarbital alone. Additionally, foci number in the median lobe of diethylnitrosamine-initiated 2,4-DNT-treated rats was higher than that of diethylnitrosamine-initiated controls, although that difference was not statistically significant (536 ± 216 foci/cm^3 in initiated 2,4-DNT-treated rats vs 201 ± 154 foci/cm^3 in initiated controls). Thus, authors found that the promoting effect observed with 2,4-DNT was similar to that obtained with phenobarbital, a known promoter.

<u>Mice</u>

Dermal

Slaga *et al.*, 1985

Lute purity varied from 98% (impurities 2-NT and 2,6-DNT) to 92 – 95% (impurity 2,6-DNT. No data available regarding GLP compliance. In the tumour initiation study, 2,4-DNT (1, 5 and 10 mg/kg,) was given once to Spencer mice (either topical or i.p. initiation) followed by once weekly promotion with 4 µg of TPA (12-O-tetradecanoylphorbol-13-acetate) for 30 weeks in an initiation-promotional protocol using TPA as the promoter. The test compound was found to be negative as skin tumour initiator. Nevertheless, authors pointed out that the histological studies suggested that 2,4-DNT might have skin tumour promoting activities, since the hyperplastic and dark cell response of 2,4-DNT at 5 and 10 mg/kg was similar to the maximum response obtainable with 1µg of TPA. Furthermore, 2,4-DNT (1, 5 and 10 mg/kg)

administered once a week for 52 weeks did not induce skin tumours in Sencar mice by the complete carcinogenesis protocol. Number of animals per group was not indicated.

Oral

Stoner *et al.*, 1984

2,4-DNT (92- 95% with the major impurity being 2,6-DNT) was inactive by means of the strain A mouse lung tumour bioassay when given 2 times per week for 12 weeks (po) to A/J mice (26/sex/dose) at 50, 125 and 250 mg/kg.

Intraperitoneal

Schut *et al.*, 1982b

In male A/Jax mice (52/dose), 25, 62.5 and 25 mg/kg of 2,4-DNT (purity > 99.4%) were injected ip three times weekly for an exposure period of 8 weeks. All animals were followed up 22 weeks after last injection. In this strain A mouse lung tumour bioassay, the average number of lung tumours per mouse in response to 2,4-DNT was not significantly different from that observed in controls.

Maronpot *et al.*, 1983

Maronpot et al. (1983) reported negative results for 2,4-DNT administered orally or ip in the lung tumour bioassay with A/Jax mice, but positive results were reported using female A/St mice.

Slaga *et al.*, 1985

In the lung tumour bioassay, 2,4-DNT was injected i.p. into groups of 30 Strain A mice three times a week for eight consecutive weeks. The animals were followed up for 16 weeks after the last injection. Three dose levels of 2,4-DNT were tested: 1200, 600 and 240 mg/kg. None of the doses of 2,4-DNT tested in the mouse lung tumour assay gave an unequivocally positive response.

Stoner *et al.*, 1991

Summarized results on selected chemicals, including 2,4-DNT, that were tested for carcinogenicity by means of the strain A mouse lung tumour bioassay. 2,4-DNT was found negative in this review.

<u>In vitro studies</u>

No data available.

4.1.2.8.2 Human data

Stayner *et al.*, 1993

A retrospective cohort study was carried out on 4,989 DNT-exposed workers and 7,436 unexposed workers at a munition facility in order to test the hypothesis that DNT exposure is

associated with an increased risk of both liver and biliary-tract cancers. A purified form of DNT containing approximately 98% 2,4-DNT and 2% 2,6-DNT was used at the study facility. Participants had worked for at least 5 months at the study facility between January 1, 1949 and January 21, 1980. Quantitative DNT-exposure measurements were not available. Instead, jobs were classified into three types: probably exposed if they had worked at least 1 day with probable exposure to DNT, possibly exposed and definitely unexposed to DNT. Workers who had been employed in jobs classified as possibly exposed were not included in this study. Analyses included standardized mortality ratios (SMRs) using rates for the United States, standardized rate ratios (SRRs) computed by direct comparison between exposed and unexposed cohorts, and 95% confidence intervals (CIs).

DNT-exposed workers showed a significant increase in hepatobiliary cancer mortality (6 cases), when compared with that of unexposed workers (4 cases; SRR, 3.88; 95%CI, 1.04 - 14.41; and p = 0.04). Regarding indirect comparisons, the SMR for hepatobiliary cancer was 2.67 (95%CI, 0.98-5.83; and p = 0.052) when compared to the U.S. population. Furthermore, there was no relationship between duration of DNT exposure and hepatobiliary cancer mortality.

The power of the analyses was limited. A small number of hepatobiliary cancer cases in both DNT-exposed and unexposed cohorts was observed. With respect to duration of exposure, only 7% (6,430/98,673) of the cohort person-years were in the 5 or more years of DNT-exposure category. In addition, workers with minimal exposure were classified as probably exposed, which could underestimate the risk associated to DNT exposure. Concerning risk factors for hepatobiliary cancer, relevant differences were not noted either between both DNT-exposed and unexposed cohorts for direct comparisons, or between DNT-exposed workers and reference population for indirect comparisons. Nevertheless, the SRR approach was considered more appropriate than the indirect standardisation approach, because the unexposed cohort is likely to be more similar to DNT-exposed cohort than the US general population. In the medical records of the hepatobiliary cancer cases exposed to DNT, there was no evidence of risk factors which explained the observed cancer. The exception was one of the exposed liver cancer cases who had previously developed posttransfusion hepatitis and cirrhosis, which may have contributed to the development of liver cancer in this case.

This study associated an excess of hepatobiliary cancer with jobs where workers were supposedly exposed to purified 2,4-DNT. However, neither DNT-exposure estimates nor measurements were performed. Thus, the proportion of exposed workers who absorbed a significant amount of 2,4-DNT was neither known nor estimated, and consequently there was not sufficient evidence to establish an association between 2,4-DNT exposure and hepatobiliary cancer. Nonetheless, the excess in hepatobiliary cancer mortality observed among DNT exposed workers in this study is similar to the findings from experimental studies of DNT exposed animals. Thus, this study adds some support for the hypothesis that occupational exposure to DNT may be carcinogenic.

Brüning *et al.*, 1999

Between 1984 and 1997, 6 cases of urothelial cancer and 14 cases of renal cell cancer occurred in a group of 500 underground mining workers in the copper-mining industry of the former German Democratic Republic (GDR). A descriptive study was carried out on the 20 cases of cancer and a representative sample (n = 183), in order to describe the exposure to

explosives containing technical grade DNT and the occurrence of cancer. The cancer cases and the representative sample of 183 miners were examined by using information sources such as cancer registers, health records, standard medical examination and personal interviews. In addition smoking histories, former diseases of the kidneys, and histories of renal diseases and of cancers within the families were recorded. With respect to exposure assessment, rods of Donarit were the exclusive explosives used since the early 1950s. Donarit contained approximately 30% technical DNT, which consisted of approximately 75% 2,4-DNT and 20% 2,6-DNT. Miners had to work and handle DNT-containing explosives under extreme working conditions, since very narrow seams of approximately 70 cm in height were typical for the mines. Furthermore, Donarit was produced in the form of long explosive sticks only, which then had to be broken manually into smaller pieces to be used. This was regularly done with bare hands, without any skin protection. Quantitative DNT-exposure measurements were not available. Instead, the exposures were categorized according to the types and durations of professional contact with DNT. The miners were exposed via two routes: inhalation of the smoke after explosions, and direct skin contact upon handling DNT-containing explosive sticks, placing the sticks in the bore holes, and loading or unloading explosive materials (Table 4.1.2.8.2-1).

Table 4.1.2.8.2-1: Scoring system for semiquantitative levels of exposure (Brüning *et al.*, 1999)

Exposure	Frequency of exposure	Points [a]
Skin Contact		
A	Occasionally	1
B	Regularly, ie, at least three times per week, for less than 4 hours each time	2
C	Regularly, ie, at least three times per week, for more than 4 hours each time	3
Exposure due to inhalation		
D	Occasionally	1
E	Regularly, ie, at least three times per week	2

[a] For every 10-year period started

Accordingly, jobs were classified into four types: low exposure (0-4 points), medium exposure (5-8 points), high exposure (9-12 points) and very high-exposure (≥ 13 points). In addition, all identified tumour patients were genotyped by polymerase chain reaction. Genomic DNA was isolated from lymphocytes. Genotypes of the polymorphic glutathione transferases GSTM1 and GSTT1, and the N-acetyltransferase NAT2 were determined. The statistical analysis was not described in the article. We could not ascertain whether authors computed standardized mortality/morbidity ratios (SMRs).

From the existing archived health records, 14 renal cell cancer cases (12 of the clear-cell type, two of the chromophilic type) and six urothelial cancer cases (five bladder carcinomas, one transitional-cell carcinoma of the renal pelvis) were identified within a total number of the 500 miners with former exposures to DNT. There is no information available regarding DNT-related symptoms recorded in the health records of the 20 cancer cases. In approximately 25% of the DNT-exposed representative group, the health records indicated signs of liver disorders (ie, increased values of the serum enzymes alanine aminotransferase, aspartate aminotransferase and gamma-glutamyltransferase), anaemia or breathing problems. With

respect to the DNT-exposure distribution, the categorization of the 14 DNT-exposed kidney tumour cases revealed a distribution similar to that of the representative group of 183 miners (low exposure, n = 23, 12.6%; medium exposure, n = 78, 42.6%; high exposure, n = 52, 28.4% and very high exposure, n = 30, 16.4%). Thus, a relationship between DNT exposure and renal cell cancer was not achieved. The urothelial tumours were concentrated in the high-exposure group (medium exposure n = 1; high exposure n = 4; and very high exposure n =1). It should be noted that only one urothelial cancer case was in the very-high exposure group and all six cases of urothelial cancer were smokers. Regarding distribution of genotypes of the polymorphic enzymes GSTM1, GSTT1 and NAT2, all six urothelial cancer cases were identified as slow acetylators (NAT2). In the biotransformation of 2,4-DNT, one or both nitros may be reduced to aminotoluenes or diaminotoluene. This is relevant since, in the pathogenesis of human bladder cancer due to occupational exposure to aromatic amines acetylation by NAT2 is regarded as a detoxication step. Regarding GSTM1 and GSTT1, genotype distributions were similar to those of the normal German population. The authors stated in the discussion that "the standard incidence rates for renal cell cancer and urothelial cancer, according to the cancer registry of the former GDR, are 14.5 and 19.1 respectively. The cluster of tumours observed in this study would correspond to an enhanced risk for renal cell cancer and for urothelial cancer by factors of 4.5 and 14.3, respectively". However, they did not mention whether/how they calculated the expected cases based on the former GDR population, the standardised mortality ratio (SMR) with confident intervals and the significance of the statistic test. Furthermore, this study was limited, since a small number of cancer cases were observed.

This study found an excess of both urothelial cancer and renal cell cancer among miners exposed to technical grade DNT. However, the absence of an adequate statistical analysis questions the validity of the presumed association between both renal and urothelial tumours and exposure to 2,4-DNT in humans. In addition, the cases of this study were also exposed to significant amounts of 2,6-DNT. Hence, there was not sufficient evidence to establish an association between 2,4-DNT exposure and 2,4-DNT-related carcinogenicity. Nevertheless, renal and urothelial tumours observed in this study were similar to those found in 2,4-DNT-exposed mice. Thus, this study adds some support for the hypothesis that occupational exposure to 2,4-DNT may be carcinogenic.

4.1.2.8.3 Summary of carcinogenicity

Human data

There is no valid study available. Instead, there are two studies which added some support for the hypothesis that occupational exposure to DNT may be carcinogenic, since the excess in cancer mortality observed among DNT exposed workers is similar to the findings from experimental studies of DNT exposed animals. Those studies associated an excess of hepatobiliary cancer and both urothelial cancer and renal cell cancer with jobs where workers were supposedly exposed to purified 2,4-DNT and miners supposedly exposed to technical grade DNT, respectively.

Rats

Thus, this study adds some support for the hypothesis that occupational exposure to DNT may be carcinogenic.

There are two adequate studies in rats (Table 4.1.2.8.4-1), one of them being a chronic toxicity study. The data of the chronic toxicity study were considered relevant for carcinogenicity. The same signs of carcinogenicity (ie skin/subcutaneous tissue fibromas in males, mammary gland fibroadenomas in females and hepatocarcinomas in both sexes) were found in both chronic (Ellis *et al.*, 1979; Lee *et al.*, 1985) and carcinogenicity (NCI, 1979) studies. Hence, it suggests a positive association. The dose which produced tumours in females in the chronic study was in the dose-level range of the carcinogenicity study. On the other hand, the high-dose of the chronic study (34/45 mg/kg b.w./day for males/females) reduced significantly survival and showed a high toxicity.

With respect to females, the incidence of mammary gland fibroadenoma in Fischer 344 rats treated with 15.7 mg/kg b.w./day (NCI, 1979) and CD rats treated from 5.1 mg/kg b.w./day (Ellis *et al.*, 1979; Lee *et al.*, 1985) was significantly higher than in controls.

In males, significant increased incidences of subcutaneous tissue or skin fibroma were found in CD rats administered 34 mg/kg b.w./day (Ellis *et al.*, 1979; Lee *et al.*, 1985) and in Fischer 344 rats treated from 4.7 mg/kg b.w./day (NCI, 1979); sporadic occurrence of squamous-cell papillomas, basal-cell carcinoma, fibrosarcomas, and lipomas (NCI, 1979).

The incidence of hepatocellular carcinoma in liver of males treated with 4.7 and 11.8 mg/kg b.w./day for 18 months was higher ($p > 0.05$) than in controls (NCI, 1979). Despite of the no significant excess, the same tumour occurred with a significant increased incidence in CD rats given 34/45 mg/kg b.w./day (Ellis *et al.*, 1979; Lee *et al.*, 1985); hence it was included herein. At that dose, most of the treated rats showed neoplastic nodules in liver after 12-month treatment (Ellis *et al.*, 1979; Lee *et al.*, 1985).

Mice

There are two adequate studies in mice, one of them being a chronic toxicity study (Table 4.1.2.8.4-1). In one study no carcinogenic effect was reported (NCI, 1979). Data of the chronic toxicity study were considered relevant for carcinogenicity, since renal tumours were observed in males (Ellis *et al.*, 1979; Hong *et al.*, 1985).

In males the incidence of kidney tumours (both benign and malignant) was significantly elevated in groups treated with 13.3 and 96.9 mg/kg b.w./day for more than 12 months.

In addition, carcinomas in liver of males and females were found at 885 and 911 mg/kg b.w./day respectively for 12 months (1/4 and 1/4, respectively), and in male livers at 885 mg/kg b.w./day for 12 months and allowed to recover for 1 month (2/4) *vs.* none in controls.

In summary, there is a good evidence of an increase in tumour incidence in rats and mice. Furthermore, 2,4-DNT is an in vivo mutagen and thus genotoxicity is likely to be the underlying mechanism or an important contributory factor of carcinogenicity. Therefore, according to EU criteria, 2,4-dinitrotoluene is considered carcinogenic category 2 and then classified as T R45.

Table 4.1.2.8.3-1: Summary of the relevant carcinogenicity data.

Species and sex	Protocol	Results and comments	References
♂, ♀ Fischer 344 rats	50 Fischer 344 rats / sex were treated with 0, 4.7 or 11.8(♂) and 0, 6.3 or 15.7(♀) mg/kg b.w./day for 18 months over a 24-month observation period Purity > 95%	↑ incidence of hepatocellular carcinoma (♂) at both 4.7 and 11.8 mg/kg b.w./day for 18 months (3/49, 6% vs. 0/45, 0%; and 3/48, 6% vs. 0/25, 0%; respectively) ↑ incidence of mammary gland fibroadenoma (♀) at 15.7 mg/kg b.w./day for 18 months ($p = 0.016$) ↑ incidence of tissue or skin fibroma (♂) at both 4.7 ($p = 0.008$) and 15.7 ($p = 0.003$) mg/kg b.w./day for 18 months; sporadic occurrence of squamous-cell papillomas, basal-cell carcinoma, fibrosarcomas, and lipomas	NCI 1978
♂, ♀ CD rats	Chronic study 38 rats/sex/dose were fed diets containing 0, 0.57, 3.9 or 34 (♂) and 0, 0.71, 5.1 or 45 mg/kg b.w./day (♀) for either 12 months (4 rats/sex/dose) over a 13-month observation period (4 rats/sex/dose), or 24 months over a 25-month observation period (4 rats/sex/dose). Purity > 98% A few extra rodents were added to replace early losses (no further details available)	↑ hepatocellular carcinoma incidence at 34(♂) and 45(♀) mg/kg b.w./day for more than 12 months (6/29, 21% vs. 1/25, 4%; and 18/34, 53% vs. 0/23, 0%, $p < 0.05$; respectively) ↑ Mammary gland fibroadenoma incidence at both 5.1 (♀, 17/27, 63%) and 45 (♀, 33/35, 94%) mg/kg b.w./day for more than 12 months vs. controls (♀, 11/23, 48%) ↑ Skin fibroma incidence at 34 (♂, 17/30, 57% vs. 2/25, 8%) mg/kg b.w./day for more than 12 months	Lee *et al.*, 1979, 1985
♂, ♀ CD-1 mice	Chronic study Groups of 58 mice/sex/dose were treated with 0, 13.3, 96.9 and 885(♂), and 0, 13.7, 93.8, and 911(♀) mg/kg b.w./day for either 12 months (4 mice/sex/dose) over a 13-month observation period (4 mice/sex/dose), or 24 months (46 mice/sex/dose) over a 25-month observation period (4 mice/sex/dose). Purity > 98%. A few extra rodents were added to replace early losses (no further details available)	Carcinoma in kidney at 885 (♂, 1/4) mg/kg b.w./day for 12 months vs. none in controls Adenoma in kidney at 885 (♂, 1/4) mg/kg b.w./day for 12 months and allowed to recover for 1 month vs. none in controls Carcinomas in liver at both 885 (♂, 1/4) and 911 (♀, 1/4) mg/kg b.w./day for 12 months, and at 885 (♂, 2/4) mg/kg b.w./day for 12 months and allowed to recover for 1 month vs. none in controls ↑ incidence of renal tumour at 13.3(♂, 5/33, 15%) and 96.9(♂, 16/28, 57%) mg/kg b.w./day vs. controls (0/33, 0%) for more than 12 months Hepatocellular dysplasia from 13.3(♂) and 911(♀) mg/kg b.w./day for more than 12 months ♂ more sensitive than ♀	Ellis *et al.*, 1979; Hong *et al.*, 1985

4.1.2.9 Toxicity for reproduction

4.1.2.9.1 Effects on fertility

Studies in animals

They are summarised in Tables 4.1.2.9.1-3 and 4.1.2.9.1-4.

Rat repeated dose toxicity studies: observations on fertility

In repeated-dose studies in rats (Lee *et al* 1978, Ellis *et al* 1979, Kozuka *et al* 1979 and McGowan *et al* 1983) relevant observations to reproduction-fertility were reported. Details of theses studies are described in the Section of repeated dose toxicity. Here they are indicated only those data relevant for reproduction-fertility.

Lee *et al*. (1978)

The males consumed an average of 0, 34, 93 or 266 mg/kg/day 2,4-DNT (purity >98%) and the females 0, 38, 108 or 145 mg/kg/day. After 4 weeks, male rats fed 266 mg/kg/day had moderate atrophy and aspermatogenesis in the testes. After 13 weeks, males fed 266 mg/kg/day had very severe atrophy and aspermatogenesis in the testes. Males fed 93 mg/kg bw/day had similar lesions ranging in degree from moderate to very severe. Testicular lesions caused by middle or high levels of 2,4-DNT were apparently not reversible after treatment was discontinued for 4 weeks. The NOAEL for male reproductive organ was considered to be 34 mg/kg bw/day on the basis of atrophy and aspermatogenesis in the testes in rats orally exposed for 4 weeks.

Ellis *et al.,* (1979)

In a 24 months feeding study, CD rats (38/38 males/females per groups) consumed an average of 0.57/0.71, 3.9/5.1 and 34/45 mg/kg bw/day. All four high dose males fed 2,4-DNT (34 mg/kg bw/day) for 12 months and two of three high dose males allowed to recover for one month had very severe atrophy of seminiferous tubules, with almost complete lack of spermatogenesis and a decrease of testis weights. Females fed the highest dose (45 mg/kg bw/day) in both groups suffered from a decrease in ovary weights. After 24 months, no rats from the high dose group survived. Eight rats from each dosage group were bled for clinical chemistry and killed for necropsy. The last group, compounded for the surviving rats from each dosage group, was allowed to recover for one month. Both male groups (dosed with 0.57 and 3.9 mg/kg bw/day) suffered from atrophy of seminiferous tubules. The number of animal affected at 0.57 and at 3.9 is not clearly different from control when only the surviving animals are considered. Under this point of view, the observation might be considered not relevant. However, taking into account the accumulative number of animals, surviving and dead animals (See Table in repeated dose toxicity section), the number and severity is markedly higher in animal dosed and so the observation at 0.57 and 3.9 should be considered of concern. For male fertility a LOAEL of 0.57 mg/kg bw/day may be established on the basis of atrophy of seminiferous tubules, and decrease of spermatogenesis and decrease of testis weight at higher doses. However, 2,4-DNT did not have significant influence on the fertility of females at these doses.

Kozuka *et al.* (1979)

Wistar rats fed dietary concentrations of 347-395 mg/kg/day 2,4-DNT for 6 months suffered from a markedly decrease of the relative weight of testis, and also testicular atrophy was seen.

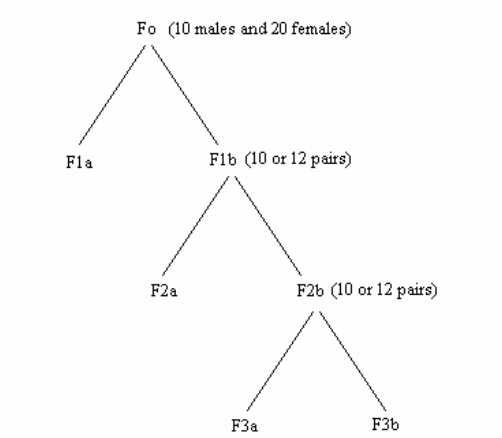
McGown *et al.* (1983)

Sprague-Dawley rats of one month old (5 animals/sex/group except in control group, which had only 4 females; one was removed because of a structural defect) were administered 2,4-DNT (purity = 97%; contaminants 2% of 2,6-DNT and unspecified 1%) in the diet for 14 days at 0, 900, 1200, 1900 and 3000 ppm (equivalent to 0, 96, 125, 183 or 260 mg/kg b.w./day for males and 0, 99, 124, 191 or 254 mg/kg b.w./day for females). From the low dose males exhibited oligospermia with degenerative changes, such as syncytial cell formation and focal spermatic granuloma, in a dose-dependent manner. The lowest dose caused a decrease in the thickness of spermatogenic cell layers. No histopathological changes were found in the reproductive organs of females. Conclusion: A LOAEL of 96 mg/kg bw/day is derived on the basis of decrease in the thickness of spermatogenic cell layers. No changes found in female reproductive organs at any dose.

Rat fertility studies

Ellis *et al.* (1979)
In a multigenerational study, 2,4-DNT (98% pure) was tested in CD rats. This is considered a key study and so is here reported in details as possible. Some limitations were found in the report of the original study as only average results are presented without data of individual animals.

The experimental design is illustrated below:



The initial rats used as parental generation (F_0) started at the same time as rats used in the chronic toxicity study (See Ellis *et al.,* 1979 in chronic toxicity section) and received the same control and substance containing diet as in the chronic study. The concentrations of 2,4-DNT in the diet were 0.0015%, 0.01% and 0.7%. Males/females consumed 0.57/0.71 (low), 3.9/5.1 (middle) and 34/45 (high) mg/kg b.w./day, respectively. For the F_0 generation, 10 male and 20 female CD rats from each dosage group were mated after receiving the test diets for 6 months. Each male was housed with two females from the same dosage group for 14 days. Offspring from the matings (F_{1a}, first litters) were discarded at weaning. The F_0 rats were again mated. Twenty to 24 offspring of each sex from the second litters (F_{1b}) were randomly selected from each dosage group at weaning. The F_0 females and surplus pups were discarded; the F_0 males were retained in the chronic study. Each F_{1b} male was mated with a female within the same dosage group for 14 days at 3 months of age. The F_{2a} generation was discarded at weaning and the F_{1b} rats were terminated at weaning of the F_{2b} pups. The F_{2b} rats were then selected and mated at 3 months of age according to the same

procedure used for F_{1b}. The study was terminated upon weaning of the F_{3b} rats. This study complied, in essence, with OECD guideline 416, and thus it was considered adequate.

At birth, all offsprings were examined for gross physical abnormality and the number of live and dead pups of each litter was recorded. Survival and body weight were recorded at 0, 4 and 21 days. The general health of the parent generation was quantified by the weight at first mating (Table 4.1.2.9.1-1)

Reproductive performance for each parental generation was quantified by: the mating ratio (copulations over male-female pairing) and fertility ratios for each sex (number with offspring to number of that sex mated).

Reproductive performance of each litter was quantified by the following variables comparing treated animals and controls: litter size, weight at birth, live-born index (% of total number of pups liveborn), viability index (% of liveborn surviving to 4 days), lactation index (% of young alive at day 4 surviving to weaning), the weight at weaning and the sex ratio (number of males over total number of offspring).

There were not significant differences between groups, with the exception of a decreased viability index of high-dose F_{1b} generation, and weight at weaning of low-dose and middle-dose F_{3b} generation ($p < 0.05$). (Table 4.1.2.9.1-2)

The mean body weights at the time of the first mating for both males and females given the high dose were decreased (Table 4.1.2.9.1-1). The body weights from F_0 and F_1 generation given the high dose of 2,4-DNT when compared with their respective controls were 77-75% for males and 77-90% for females, for F_0-F_1, respectively.

In Table 4.1.2.9.1-2 the quantitative data reported in the original study is reported.

The following variables were compared between treated animals and controls: litter size, weight at birth and weaning, live-born index, viability index and lactation index. There were not significant differences between groups, with the exception of a decreased viability index of high-dose F_{1b} generation, and weight at weaning of low-dose and middle-dose F_{3b} generation ($p < 0.05$).

No treatment related effect on fertility of both males or females were apparent. However the fertility of both males and females was reduced for all groups of the F_0 generation. This was interpreted as due to their older age.

However, the absence of the F_2 parental generation for the group given the high dose and the few animals mated in the F_1 indicated an adverse effect on reproductive performance. This adverse effect on reproduction was clarified by the quantitative data for individual litters (Table 4.1.2.9.1-2). No treatment related effects were apparent on liveborn index, weight at birth, weight at weaning or the sex ratio. Although not statistically significant, the mean litter size appeared to be reduced for F_{1a} and/or F_{1b}. This effect did not persist with subsequent parental generation.

The viability and lactation indexes were also reduced for one or both litters born to the F_0 generation. With the exception of the viability index for F_0 litters born to dams receiving the high dose, these effects did not appear to be related to treatment. This lowered viability resulted from maternal neglect and death during parturition.

The incidences of deaths during parturition of F_{1a} were 1/10, 4/13, 3/10 and 4/11 for F_0 dams given 0, 0.71, 5.1 and 45 mg/kg bw/day, respectively. The incidence during parturition of F_{1b} were 1/5, 0/6, 0/6 and 4/5 for F_0 dams given 0, 0.71, 5.1 and 45 mg/kg bw/day, respectively. The

deaths were associated with a prolonged parturition, excessive haemorrhage and retention of placental or foetal-placental units. In some cases placentas were still attached to the uterus. The occurrence of deaths during parturition in the control group suggests that these deaths may be related to the age of the dams at first mating. However, 2,4-DNT enhanced the occurrence of these deaths.

All three high-dose females from F_{1b}, which were mated at 3 months of age, produced and weaned offspring as well as control dams even though one female had a mammary tumour at time of mating. However, none of the three females produced second litters: one with the tumour failed to mate; in a second female with a vaginal plug, sperm was absent in vaginal smear and the third female failed to produce offspring.

No anomalies were detected in the offspring from any of the matings. Birth weights, postpartum survival when parturition was normal, weight at weaning and the lack of a teratogenic effect indicate that any 2,4-DNT received via the placenta or milk was of little consequence.

In brief, no quantitative treatment related effect on fertility of both males and females was apparent in the three generation study. The fertility of both males and females was reduced for all groups of the F_0 generation. This was interpreted as due to their older age. The viability and lactation indexes were also reduced for one or both litters born to the F_0 generation. With the exception of the viability index for F_0 litters born to dams receiving the high dose, these effects did not appear to be related to treatment. This lowered viability resulted from maternal neglect and death during parturition. However, the absence of the F_2 parental generation for the group given the high dose and the few animals mated in the F_1 appears to indicate an adverse effect on male fertility. Therefore, the NOAEL of 3.9 mg/kg could be considered for fertility effects.

Table 4.1.2.9.1-1: Age, weight and fertility of three generations of rats given 2,4 DNT (Ellis *et al.*, 1979)

					Males		Females		
2,4 DNT (mg/kg bw/ day) (male/female)	Generation	Age at first mating (months)	Mating Ratio	Pregnancy Ratio	Fertille Mated	Weight (g) at first mating	Fertile Mated	Weight (g) at first mating	Duration of gestation (days)
0/0	F_0	8	30/40[a]	17/30[b]	7/10	612±12[c]	13/22	333±7[c]	23
	F_1	3	38/38	38/38	15/13	475±9	19/19	276±3	22
	F_2	3	39/40	38/39	20/20	438±11	20/20	265±5	22
0.57/0.71	F_0	8	32/38	20/32	8/10	618±12	15/21	347±9	23
	F_1	3	29/32	24/29	14/16	489±9	14/16	272±3	22
	F_2	3	39/40	38/39	19/20	451±9	20/20	260±5	22
3.9/5.1	F_0	8	29/38	17/29	7/10	593±18	12/20	311±5	23
	F_1	3	39/40	36/39	19/20	469±6	20/20	267±4	22
	F_2	3	38/40	38/38	20/20	456±9	20/20	264±5	22
34/45	F_0	8	33/38	16/33	8/10	464±13[d]	12/21	255±6[d]	23
	F_1	3	4/6	¾	3/3	355±10[d]	3/3	249±5	22

a=Number of copulations detected by vaginal smear to the number of male-female pairings.
b=Number of confirmed pregnancies to the number of copulations;
c=Mean±S.E;
d=Significantly different from the mean value of the respective control generation (Dunnett's multiple comparison procedure)

Table 4.1.2.9.1-2: Reproductive performance of female rats given 2,4 DNT (Ellis *et al.* ,1979)

2,4 DNT (mg/kg bw/day) (male/female)	Litter Nº	Litter Size	Live-born Index	Weight at birth	Viability Index	Lactation Index	Weight at weaning	Sex ratio males:total
0	F_{1a}	6.9±1.1 (10)[a]	85±7	7.2±0.3	70±15	61±15	45±8 (7)	23:33
	F_{1b}	12.7±1.8 (5)	91±5	7.0±0.5	100	91±6	55±7 (6)	13:35
	F_{2a}	12.7±0.6 (19)	98±1	7.0±0.2	98±2	96±3	43±2 (19)	111:222
	F_{2b}	14.0±0.3 (19)	96±2	7.2±0.1	98±1	92±6	42±1 (18)	122:253
	F_{3a}	11.3±0.4 (23)	97±2	7.1±0.1	96±2	95±3	39±1 (23)	123:249
	F_{3b}	12.2±0.6 (18)	99±1	6.4±0.1	94±2	94±3	40±1 (18)	93:216
0.57/0.71	F_{1a}	6.8±1.0 (13)	83±9	7.1±0.2	69±13	91±6	53±2 (9)	28:56
	F_{1b}	8.2±1.9 (6)	100	7.2±0.8	99±1	70±6	45±9 (6)	16:32
	F_{2a}	13.9±0.4 (14)	97±1	6.6±0.1	99±1	95±3	41±1 (14)	92:178
	F_{2b}	15.4±0.6 (10)	95±2	6.8±0.3	96±4	83±6	41±2 (10)	58:140
	F_{3a}	12.2±0.6 (23)	95±2	7.1±0.2	96±1	93±2	36±1 (23)	95:266
	F_{3b}	13.2±0.4 (19)	98±1	6.4±0.2	95±3	87±6	35±1 (18)[b]	93:242
3.9/5.1	F_{1a}	4.9±1.1 (10)	89±10	7.4±0.1	60±16	61±18	38±10 (7)	22:35
	F_{1b}	9.7±1.1 (6)	88±6	7.5±0.4	83±17	83±17	44±13 (6)	24:44
	F_{2a}	13.8±0.4 (18)	99±1	6.9±0.2	94±6	95±5	42±1 (18)	119:245
	F_{2b}	14.2±0.8 (18)	97±1	7.1±0.2	94±6	97±1	41±2 (17)	108:242
	F_{3a}	12.6±0.5 (20)	98±2	6.9±0.1	94±2	87±5	35±1 (20)	105:243
	F_{3b}	13.6±0.5 (20)	98±2	6.5±0.1	97±1	91±3	35±2 (20)[b]	123:265
34/45	F_{1a}	4.5±1.1 (11)	94±4	7.5±0.4	64±15	90±10	53±6 (7)	14:29
	F_{1b}	7.4±1.2 (5)	90±5	7.0±0.3	20±20[b]	78	44 (1)	4:7
	F_{2a}	11.0±1.0 (3)	100	6.4±0.2	89±11	100	35±1 (3)	19:29

a=Mean ± S.E. and in parentheses the number of litters included in the mean.
b=Significantly different from the mean value of the respective control litters (Turkey's omega procedure)

Lane *et al.* (1985): Reproduction study after 5 days male exposure.

Fifty male rats were randomly divided into five groups. One group received 10 ml/kg oral dose of Mazola corn oil for five days. Another group received 0.5 mg/kg triethylenemelamine dissolved in corn oil in a single oral dose. The other three groups received 60, 180 or 240 mg/kg/day 2,4-

DNT (purity not indicated, recristalized) dissolved in corn oil for five consecutive days. A new group receiving 240 mg/kg 2,4-DNT was created one week after the other groups, since there were excessive deaths in the high-dose group which would make it difficult to statistically evaluate the results. Each male was allowed to mate with two females for five days every week and the insemination was determined by microscopic examination of vaginal smears for the presence of sperm. Due to the low number of pregnant females in the high-dose group by the sixth week, the mating lasted thirteen weeks while in the other groups it lasted 6 weeks. Thus, there could be a possible reversibility of the effects of 2,4-DNT since the number of pregnant high-dose females increased by the thirteenth week of mating. Females were sacrificed on day 14 of gestation in order to examine their reproductive system. The findings in groups which were administered 2,4-DNT were the followings:

A 60 mg/kg/day 2,4-DNT dose did not produce adverse reproductive effects and it was only seen a slight cyanosis. At 180 mg/kg/day 2,4-DNT dose, the mating index (total number of pregnant females/total number of females allowed to mate x 100) may be slightly decreased, but this effect is temporary. It produced cyanosis. A 240 mg/kg/day 2,4-DNT dose produced weight loss, cyanosis and death one week after administering it. Mating index and DF/LF (total number of dead foetuses/total number of living foetuses) were severely affected. Implantation index (total number of implantations sites/total number of pregnant females), preimplantation loss index (total number of corpora lutea – total number of implantation sites/total number of pregnant females) and foetal index (total number of living foetuses/total number of pregnant females) were significantly altered in week 7. These indices were different from control values before week 7, by week 11 there appeared to be little difference between the data from treated and control animals. It was concluded that 60 mg/kg/day did not produce adverse reproductive effects, 180 mg/kg/day affects in a moderate and reversible way the mating and foetal index, and 240 mg/kg/day 2,4-DNT for five days affects severely the reproductive system in rats, although these effects are slowly reversible. Conclusion: A NOAEL of 60 mg/kg/day is deduced for effects on fertility on the basis of moderate and reversible affectation of the mating and foetal index (LOAEL = 180 mg/kg/day).

Bloch *et al.* (1988): Male fertility-spermatogenesis study

In a 3 weeks study, 30 adult male Sprague-Dawley rats were fed control, 1000 ppm (0.1%) or 2000 ppm (0.2%) 2,4-DNT (97%pure). This feeding was equivalent to 26.2 mg/kg bw/day and 51.7 mg/kg bw/day. This was deduced from default food consumption estimated as recommended in TGD Apendix VI using the formulae $F=0.040 \cdot W^{0.479}$, W being the average body weight of then group along the study). Rats were randomly distributed into groups of 10 rats each (although one rat died of unknown causes during the treatment in the group treated with 0.2% 2,4-DNT). To evaluate the spermatogenic effects and the reproductive endocrine system the following parameters were evaluated: body, testis and epididymal weights, testis morphology using electron microscopy, cauda epididymal sperm reserves and serum LH, FSH and testosterone levels.

Body weights in all rats increased during the study, but at the end of the study the weights were reduced in animals treated with 26.2 or 51.7 mg/kg bw/day. Testis weights were not significantly affected after the treatment, however a decrease in epididymal weight was seen when 51.7 mg/kg bw/day was administered. Examinations of testis morphology with electron microscopy showed focal alterations at the 26.2 mg/kg bw/day level (such as vacuolization and lipid accumulation in Sertoli cells, prominence of multinucleated spermatids and mild irregularity of the basal lamina), although the damages were limited and variable. At the 51.7 mg/kg bw/day level Sertoli cells presented varying sized vesicles, swollen mitochondria and distended endoplasmic reticulum.

Furthermore, extensive degenerative changes were seen in spermatocytes and spermatids. In control animals Sertoli cells had a normal appearance.

The total sperm count was analyzed and it was found that it decreased by 63% in rats fed 51.7 mg/kg bw/day. In this group it was also seen a 100% increase in LH levels and a 25% increase in FSH levels, but the levels of testosterone were not affected. Rats fed 26.2 mg/kg bw/day showed no significant effects on sperm concentrations or serum hormone levels.

These findings indicate that 2,4-DNT is capable to affect the spermatogenesis and the reproductive endocrine system.

Conclusion: For male fertility a LOAEL of 26.2 mg/kg bw/day is deduced on the basis of focal alterations in testis morphology in a three week study in SD rats. At the next higher dose of 51.7 mg/kg bw/day, a decrease in sperm concentration, increase in serum hormonal levels and degenerative changes in spermacytes and spermatids and anomalies in Sertoli cells was observed.

Mouse repeated dose toxicity: observations on fertility

In mouse repeated dose toxicity studies in (Lee *et al.,* 1978; Ellis *et al.,* 1979), relevant observations to reproduction-fertility were reported. Details of theses studies are described in the section of repeated toxicity. Here only data relevant to reproduction-fertility is presented:

Lee *et al.* (1978)

After 4 weeks feeding 47, 137 or 413 mg/kg/day, two of the males fed 413 mg/kg/day 2,4-DNT had mild depression on spermatogenesis. After 13 weeks there were no apparent 2,4-DNT induced lesions in the testes. NOAEL of 137 mg/kg/day may be deduced on the basis of mild depression on spermatogenesis.

Ellis *et al.* (1979)

In a study mice were fedg 0, 13.5, 95 or 900 mg/kg/day for 12 months (58 males, 58 females). In 4 of 4 high dose males and 1 of 4 middle dose male testicular atrophy was seen. In the 900 mg/kg/day female group a mild ovary atrophy was seen in 2 of 4 females. A second group of four male and four female mice allowed to recover during one month without treatment, the same lesions were seen as reported in the group not allowed to recover but they were often less severe. After 24 months, no mice from the high dose group survived. Eight mice from each dosage group were bled for clinical chemistry and killed for necropsy. A mild atrophy of the testes was seen. In female mice there were no treatment related fertility lesions. The last group was compounded for the surviving mice from each dosage group, treated for 24 months and allowed to recover for one month. A mild atrophy of the testes was seen. Non functioning follicles with lacking of corpora lutea were present in some females. Conclusions: A NOAEL of 13.5 mg/kg bw/day was considered on the basis of mild atrophy in testis observed at 95 mg/kg bw/day. In female mice there were no treatment related fertility effects observed.

Dog repeated dose toxicity: observations on fertility.

In dog repeated dose toxicity studies (Lee *et al* 1978), relevant observations to reproduction-fertility were reported:

Lee *et al.* (1978): The administration of 1 or 5 mg/kg/day for 13 weeks did not cause any apparent adverse effect. Dogs treated with 25 mg/kg/day suffered from a moderate decrease in spermatogenesis after 4 weeks, which became severe after 13 weeks. After the recovery period the histological appearance was normal. Conclusion: A NOAEL of 5 mg/kg/day may be deduced

on the basis of a moderate spermatogenesis decrease after 4 weeks and severe after 13 weeks observed at the next higher dose of 25 mg/kg bw/day

Table 4.1.2.9.1-3: Summary target effects and NOAEL-LOAEL of 2,4 DNT on male fertility

Species (Type study)	Exposure period/route (doses)	Target effect and NOAEL-LOAEL	Reference
CD rat. (Repeated)	Up to 13 weeks/ dietary (34, 93, 266 mg/kg/day)	Atrophy of the testis. Depression of spermatogenesis NOAEL=34 mg/kg/day	Lee *et al.* 1978
CD rat. (Repeated)	12-24 months/dietary (0.57, 3.9 or 34mg/kg/day)	Atrophy of seminiferous tubules. Depletion of spermatozos in ductules. Decrease of the testis weight LOAEL=0.57 mg/kg/day (based on atrophy of seminiferous tubules at 12-24 months).	Ellis *et al.* 1979
Wistar-STD rat	6 months/dietary (347-395 mg/kg/day)	Decrease of testis weight and testicular atrophy.	Kozuka *et al.*, 1979
Sprague-Dawley rat (Repeated)	14 days/dietary (0, 96, 125, 183 or 260 mg/kg/day)	Testicular atrophy and oligospermia. Syncytial cell formation. Focal spermatic granuloma. Decreased thickness of spermatogenic cell layers LOAEL=96 mg/kg/day (based on decreased thickness of spermatogenic cell layers)	McGown *et al.*, 1983
S. D. rat (Male fertility spermatogenesis)	3 weeks/dietary (26.2 or 51.7 mg/kg/day)	Focal alterations of the Sertoli cells, multinucleated spermatids in some tubules. Decrease in weight epididymes and epididymal sperm reserves, 25% increase in FSH levels and 50% LH levels LOAEL=26.2 mg/kg/day	Bloch *et al.*, 1988
S. D. rat (Male-Reproduction)	Males exposed 5 days by gavage before mating (60, 180, 240 mg/kg/day)	Decrease mating index on week 5, implantation sites and pregnant females. Increased dead foetuses. NOAEL=60 mg/kg/day	Lane *et al.*, 1985
CD rats (three generation reproduction study)	Since 6 months prior to F_0 mating. (0.57/0.71, 3.9/5.1, 34/45 mg/kg bw/day) (male/female).	No quantitative treatment related effect on fertility of both males and females. However, the absence of the F_2 parental generation for the group given the high dose and the few animals mated in the F_1 appears to indicate an adverse effect on male fertility. NOAEL=3.9 mg/kg/day	Ellis *et al.*, 1979
Alb. Sw. mouse (Repeated)	Up to 13 weeks/dietary (47, 137, 413 mg/kg/day)	Mild depression of spermatogenesis. NOAEL=137 mg/kg/day	Lee *et al.*, 1978
CD-1 mouse (Repeated)	12-24 months/dietary (13.5, 95, 900 mg/kg/day)	Testicular atrophy. Decrease of the testis weight. NOAEL=13.5 mg/kg/day	Ellis *et al.*, 1979
Beagle dog (Repeated)	Up to 13 weeks/capsule (1, 5, 25 mg/kg/day)	Decrease in spermatogenesis after 4 weeks NOAEL=5 mg/kg/day	Lee *et al.*, 1978

LOAEL=0.57 mg/kg/day (atrophy of seminiferous tubules, 12-24 months feeding in CD rats).

Table 4.1.2.9.1-4: Summary target effects and NOAEL-LOAEL of 2,4 DNT on female fertility

Species (Type study)	Exposure period/route (doses)	Target effect and NOAEL-LOAEL	Reference
CD rat (Repeated)	Up to 13 weeks/dietary (38, 108, 145 mg/kg/day)	Not effects	Lee *et al.* 1978
CD rat (Repeated)	12-24 months/dietary (0.71, 5.1, 45 mg/kg/day)	Decrease of ovary weight at the highest dose tested	Ellis *et al.*, 1979
S. D. rat (Repeated)	14 days/dietary (99, 124, 191 or 254 mg/kg/day)	Not effects.	McGown *et al.,* 1983
CD rats (three generation reproduction study)	Since 6 months prior to F_0 mating. 0.57/0.71, 3.9/5.1, 34/45 mg/kg bw/day (male/female).	No quantitative treatment related effect on fertility of both males and females. However, the absence of the F_2 parental generation for the group given the high dose and the few animals mated in the F_1 appears to indicate an adverse effect on male fertility.	Ellis *et al.*, 1979
Alb Sw mouse (Repeated)	Up to 13 weeks/dieraty (52, 147, 468 mg/kg/day)	Not effects	Lee *et al.*, 1978
CD-1 mouse (Repeated)	12-24 months/dietary (13.5, 95, 900 mg/kg/day)	Decrease of ovary weight. Ovary atrophy. Non-functioning follicles with lacking of corpora lutea- NOAEL= 95 mg/kg/day (based on ovary atrophy)	Ellis *et al.*, 1979
Beagle dog (Repeated)	Up to 13 weeks/capsule (1, 5, 25 mg/kg/day)	Not effects	Lee *et al.*, 1978

NOAEL=95 mg/kg/day (ovary atrophy, 12-24 months feeding in CD mice).

4.1.2.9.2 Developmental toxicity

Studies in animals

They are summarised in Table 4.1.2.9.2-1.

Rats

No specific studies of developmental toxicity are available. However in the 3-generation reproduction study (Ellis *et al.,* 1979), it was reported that at birth, all offprint were examined for gross physical abnormality. No anomalies were detected in the offspring from any of the matings. Birth weights, postpartum survival when parturition was normal, weight at weaning and the lack of a teratogenic effect indicate that any 2,4-DNT received via the placenta or milk was of little consequence. In this study rat were dosed by feeding with an estimated 2,4-DNT intake of 0.57/0.71, 3.9/5.1 and 34/45 mg/kg bw/day for males/females, respectively.

Viability index was also evaluated in this study. As described above (in fertility section; 4.1.2.9.1), the viability and lactation indexes were reduced for one or both litters born to the F_0 generation. With the exception of the viability index for F_0 litters born to dams receiving the high dose, these effects did not appear to be related to treatment. This lowered viability resulted from maternal neglect and death during parturition and the decrease in viability in F_0 generation was interpreted as due to age of rat.

No significant developmental effect was observed at the tested doses, including the high dose of 34/45 mg/kg bw/day for males/females, respectively. However, it is not clear whether visceral and skeletal abnormalities were examined.

Mice

Smith *et al.,* 1983; Harding *et al.*, 1987

Fifty timed-pregnant female CD-1 mice (between the seventh and fourteenth gestation days) were administered 390 mg/kg 2,4-DNT by gavage once a day for eight consecutive days. These mice ranged in age from six to eight weeks old. The concentration of 2,4-DNT was chosen based on the results of the maximum tolerated dose test, ie 0, 310, 525, 1250, 2500 and 3500 mg/kg b.w./day were tested. This study was performed according to OECD guideline 414 with some significant protocol deviations, such as only one dose level was tested; 10mL/kg b.w. dissolved in corn oil as a vehicle were administered, which exceed the maximum recommended volume, ie 0.4 mL/kg b.w.; and the tested dose induced toxicity, since female mortality was higher than 10% (6 of the 34 pregnant females died). A maternal NOAEL could not be established in this study. Developmental endpoints are reported (viability and weight of litter postpartum parameters at 12h and on day 1 and 3), but no visceral or skeletal abnormalities were investigated or at least not reported. This study has limited quality.

The number of non-pregnant mice was the same in both groups (n = 16 *vs.* n = 16 in controls). Therefore it is deduced (no specifically state in the original study) that 34 females were pregnant after the mating period.

All of the controls survived along the study period and 34 females were pregnant, one was unaccounted after day 18 (reason not indicated) and so, for the final evaluation of viability reference 33 pregnant dams had to be accounted. One did not produce living litter and 2 showed resorption and therefore, 30 dams showed viable litters at the final necropsia. However the original report (Smith *et al*., 1983) accounted for 31 viable litter over 34 total pregnant, while in the revised version published (Harding *et al.,* 1987) it was reported a most appropriate data of 30 over 33 (90.9%), which rounded represented the same 91% index.

Severe maternal mortality was observed in treated animals; 10 dosed mated females (of which 4 were pregnant) died during the dosing period (not attributed to gavage error). Other 5 died during post dosing observation (from which 2 were pregnant) and one seems that was not observed for final necropsia. Therefore, 28 females were the actual living animals examined at necropsia for evaluation the viability of litters. From them, 23 showed viable litters and 5 showed resoptions. Again a discrepancy between both reports in 1983 and 1987 are observed. In the former, a ratio of 23/34 (68%) (viable litter/pregnant dams) is reported while in the later a more realistic ratio of 23/28 (82 %) is indicated.

The reproductive index reported in the original study of 1983 (ratio of the number of animals producing viable litters divided by the total number of mice ever pregnant) showed some discrepancies with the later published version (Harding *et al.,* 1987). In the 1983-report it is indicated that there is a significant difference between treated dams and controls index, reported to be 23/34, 68% vs. 31/34, 91% in controls, $p = 0.01$. However, the apparent lower number of viable litter in treated dams was mainly due to maternal toxicity and the value of this index had to be evaluated taken into consideration that 15 animals died during treatment (of which 6 were pregnant dams), and one was not examined at necropsia. Therefore the actual living pregnant treated dams examined were 28. From them, 23 produced viable litters, and thus it is reflected by the same author in the later report in 1987 for the same study. Therefore a ratio of 23/28 (82.1%) is deduced versus 30/33 (90.9%) in control which did not differ significantly ($p>0.05$). This Raporteur agrees with this later evaluation.

All the other parameters pre- and postpartum did not show any significant differences: initial bw (day 7), maternal bw prior to delivering (day 18), maternal bw 3 days postpartum (those with

viable litters), mean body weight of litters, live and dead pups per litter at 12h, pups viability per litter during days 1-3 postpartum, weight litter at day 1 and 3. No observations of abnormalities were reported in this study.

No significant developmental effects are observed at a dose high enough to cause important maternal toxicity (See Table 4.1.2.9.2-1). Nevertheless, this study is not considered acceptable for evaluation of developmental toxicity. As the only tested dose level is relatively high and mortality of dams was observed at this level, it is difficult to evaluate and use this study for the developmental toxicity of 2,4-DNT at lower dose levels. Furthermore, it is not clear whether visceral and skeletal abnormalities were examined.

Table 4.1.2.9.2-1: Effects of 2,4-DNT on developmental toxicity

Protocol	Result	Reference
Female CD-1 mice were administered 390 mg/kg/day (pure 2,4-DNT) by gavage for 8 days (gestation days 7-14)	Maternal mortality (6/34 pregnant dams and 9/16 in non pregnant, vs. 0/34 in controls) (Resorbed+stillborn) litters 5/28 vs. 3/33 in controls Reproduction index 23/28 (82%) in treated pregnant dams versus (no significant p>0.05) No differences in litter weight and viability after 12 h, 1 day and 3 days after partum. Treatment produced maternal mortality but showed no significant adverse effects on the evaluated developmental endpoints. No observations of abnormalities were reported in this study.	Smith *et al.*, 1983; Harding *et al.*, 1987
Abnormalities evaluation in offspring of the 3-generation reproductive study in rat testing to dose levels of 0.57/0.71, 3.9/5.1 and 34/45 mg/kg bw/day for males/females, respectively.	No abnormalities were detected in the offspring from any of the matings. Birth weights, postpartum survival when parturition was normal, weight at weaning and the lack of a teratogenic effect indicate that any 2,4-DNT received via the placenta or milk was of little consequence.	(Ellis *et al.*, 1979)

Other information

Price *et al.,* 1985: Teratologic evaluation of DNT (technical-grade) in rats

Female Fischer 344 rats were administered technical-grade DNT by gavage at 0, 14, 35, 37.5, 75, 100 and 150 mg/kg bw/day since gestation day 7 (i.e., 7 days after sperm detection date) until gestation day 20, when dams were sacrificed (Table 4.1.2.9.2-2). The technical-grade DNT contained the following isomers: 2,4-DNT (76%), 2,6-DNT (19%), 3,4-DNT (2.4%), 2,3-DNT (1.5%), 2,5-DNT (<1%) and 3,5-DNT (<1%). Laboratory-grade corn oil was employed as the vehicle for DNT and was administered alone as the treatment for the vehicle control group. Hydroxyurea suspended in sterile distilled water was used as the positive control agent. Selection of doses for DNT were based upon pilot data which indicated that exposure of timed-pregnant

Fischer 344 rats (75 and 150 mg/kg bw/day, po) on gestation day 9 through 12 was well tolerated. Thus, mated females, from the first of three breeding dates received dosages of 0, 35, 75, or 150 mg/kg bw/day DNT. During the treatment period, mortality of pregnant females given 150 mg /kg bw/day DNT was unexpectedly high (6/13 died between gestation day 11 and 18). Therefore, mated animals from the second and third breedings were treated with 14, 37.5 or 100 mg /kg bw/day DNT (Table 4.1.2.9.2-2).

The following parameters were recorded for each dam on gestation day 20: b.w., liver weight, spleen weight, number of corpora lutea, gravid uterine weight and status of uterine implantation sites (i.e., implantation resorptions, dead foetuses, live foetuses). Uteri with no visible implantation sites were treated with 10% ammonium sulphide solution in order to stain implantation sites which had undergone very early resorption. Live foetuses were dissected from the uterus and the following observations recorded: uterine position, b.w., crown-rump length, placental weight, sex and gross morphological abnormalities. Maternal and fetal (pooled by litter) blood samples were collected and analyzed for methemoglobin content. In addition, blood samples from each pregnant dam and from one male and one female fetus per litter in the 100 mg/kg/day DNT-treated group were collected for a reticulocyte count. Blood samples from dams assigned to the 100 mg/kg/day DNT-treated group on the third breeding date as well as from one male and one female fetus per litter were evaluated for complete blood count. Following blood collection, 50% of the foetuses in each litter were decapitated; foetal heads were fixed and examined for malformations. Decapitated foetuses were examined for visceral malformations, and foetal liver and spleen weights were recorded. Skeletons from all foetuses (50% decapitated and 50% intact) were stained and examined for malformations.

This study was conducted in general according to OECD TG 414 and it is considered relevant for risk characterization.

Table 4.1.2.9.2-2: Distribution of experimental subjects across dose groups and breeding dates in the teratologic evaluation of technical grade dinitrotoluene in Fischer 344 rats (Price *et al.,* 1985)

	Treatment (mg/kg bw/day)							
		Hydroxyurea	Technical grade DNT					
	Vehicle	**200**	**14**	**35**	**37.5**	**75**	**100**	**150**
Total females treated	37	36	22	13	22	13	23	13
Deaths (n)	0	0	1	1	0	0	1	6
Deaths (%)	0	0	4.5	7.7	0	0	4.3	46.2
Assignment of surviving females for teratologic evaluation								
First breeding	9	9	0	7	0	7	0	6
Second breeding	7	6	6	0	6	0	6	0
Third breeding	6	7	7	0	7	0	7	0
Total females assigned	22	22	13	7	13	7	13	6
Pregnant (n)	20	20	10	7	12	6	12	5
Pregnant (%)	91	91	77	100	92	86	92	83

Mortality rates for the DNT (14, 35, 37.5, 75, 100 or 150 mg/kg bw/day) groups were 4.5, 7.7, 0, 0, 4.3 and 46.2% of treated females, respectively. No deaths occurred in the positive control or vehicle control groups. At sacrifice on gestation day 20, dams treated with 100 mg/kg b.w./day DNT exhibited statistically significant increases in methaemoglobin, reticulocyte count, red blood cell size, red blood cell distribution width and platelet count. Statistically significant decreases in red blood cell count and hematocrit were also observed. A significant difference among treatment groups was observed for maternal weight gain during gestation but only the highest dose group showed a statistically significant decrease in weight when pairwise comparisons were made with the vehicle control group (Table 4.1.2.9.2-3). Treatment-related increases in maternal relative

liver and spleen weight, and a dose-related decrease in absolute maternal weight gain during gestation (i.e., minus gravid uterine weight) were observed across all DNT groups (Table 4.1.2.9.2-3). There were no statistically significant differences among groups for the incidence of resorptions, or live or dead foetuses when these measures were expressed as the percentage of affected implants per dam. A notable increase in prenatal mortality occurred at the high dose (16.8% resorptions or late fetal deaths per litter for controls vs 49.6% for DNT) but did not reach statistical significance (Table 4.1.2.9.2-3).

Table 4.1.2.9.2-3: Maternal and uterine status following exposure of Fisher 344 rats to technical grade DNT or vehicle on gestational days 7 through 20[a] (Price *et al.*, 1985)

		Dinitrotoluene (mg / kg bw/ day)					
	Vehicle	**14**	**35**	**37.5**	**75**	**100**	**150**
Pregnant dams Number sacrificed	20	10	7	12	6	12	5
Weight gain, gd 0-20 (g)[b]	61.8±3.6†	64.9±4.6	66.1±6.0	55.8±5.8	64.8±6.8	52.7±4.5	8.1±20.1**
Gravid uterine weight (g)	37.7±3.2	47.8±2.4	39.4±3.3	33.6±5.2	41.7±5.9	37.4±3.8	22.1±9.8
Absolute weight gain (g)[c]	24.1±2.1‡§	17.2±3.9*	26.7±4.3	22.2±2.1	23.1±2.3	15.2±1.9**	-14.0±13.4**
Liver weight (% b.w.)	4.09±0.08‡§	3.9±0.1*	4.12±0.09	3.96±0.09	4.6±0.1**	4.58±0.08**	4.8±0.4
Spleen weight (% b.w.)	0.20±0.003‡	0.185±0.007	0.22±0.011*	0.215±0.006*	0.245±0.010**	0.32±0.027**	0.28±0.059*
% Resorptions[d]	16.8±5.4	2.3±1.5	4.1±4.1	14.6±5.2	11.0±9.3	12.7±5.4	49.6.±22.3
% Dead foetuses[d]	0	2.4±1.6	0	0	1.3±1.3	0	3.6±3.6
% Live foetuses[d]	83.2±5.4	95.4±1.9	95.9±4.1	85.4±5.2	87.7±9.1	87.3±5.4	50.4±20.6

[a] Data are expressed as X± SE using dam or average litter values as the experimental unit. [b] Includes uterine weight.
[c] Weight gain during gestation minus gravid uterine weight. [d] Expressed as the percentage of total implants per dam gd gestation days.
* $p < 0.05$ Mann-Whitney U test (two tailed), ** $p < 0.01$ Mann-Whitney U test (two tailed).
† $p < 0.05$ Kruskal-Wallis one-way ANOVA, ‡ $p < 0.01$ Kruskal-Wallis one-way ANOVA, § $p < 0.01$ Jonckheere's test.

No statistically significant effects on foetal growth or morphological developments as a result of DNT treatment were observed (Tables 4.1.2.9.2-4. and 4.1.2.9.2-5).

Table 4.1.2.9.2-4: Status of live foetuses from Fisher 344 rats following maternal exposure to technical grade DNT or vehicle on gestational days 7 through 20[a] (Price *et al.*, 1985)

		Dinitrotoluene (mg / kg bw/ day)					
	Vehicle	**14**	**35**	**37.5**	**75**	**100**	**150**
Nº litters with live foetuses	20	10	7	12	6	12	3
Live litter size [b]	7.3±0.7	9.2±0.7	9.0±0.9	6.4±1.1	8.3±1.3	7.3±0.9	7.3±1.7
Male/live×100 (%)	48.8±5.0[c]	53.0±6.3	46.4±4.5	44.4±8.0	52.7±4.9	43.0±5.9	57.4±22.8
b.w. (g)	3.21±0.05	3.39±0.07	3.29±0.07	3.34±0.06	3.29±0.13	3.17±0.08	3.14±0.18
Crown-rump length (cm)	3.55±0.03	3.51±0.07	3.57±0.07	3.58±0.07	3.53±0.05	3.46±0.07	3.53±0.15
Liver weight (% bw)	8.09±0.11‡	7.38±0.12	8.35±0.14	7.82±0.10	8.44±0.29	8.12±0.08	8.50±0.30
Spleen weight (% bw)	0.097±0.005‡	0.081±0.008	0.131±0.006**	0.084±0.004	0.119±0.003*	0.085±0.004	0.128±0.012
Placental weight (g)	0.494±0.022	0.539±0.054	0.440±0.009	0.536±0.046	0.453±0.018	0.510±0.028	0.458±0.057

[a] Data are expressed as X± SE using dam or average litter values as the experimental unit
[b] Live litter size was not analyzed statistically; for an evaluation of foetal viability see Table 4.1.2.9.2-3 "% live foetuses"
[c] Sex of one foetus was not recorded
* $p < 0.05$ and ** $p < 0.01$ using Mann-Whitney U test (two tailed); ‡ $p < 0.01$ using Kruskal-Wallis one-way ANOVA

Table 4.1.2.9.2-5: Summary of malformations in Fisher 344 rat foetuses following maternal exposure to technical grade DNT, hydroxyurea or vehicle on gestational days 7 through 20 (Price *et al.*, 1985)

			Dinitrotoluene (mg / kg bw/ day)					
	Vehicle	**hydroxyurea**	**14**	**35**	**37.5**	**75**	**100**	**150**
External malformations								
Foetuses [a]	0/146	30/146	1/92	2/63	0/77	0/50	0/88	0/22
Litters [b]	0/20	9/19***	1/10	2/7	0/12	0/6	0/12	0/3
Visceral malformations								
Foetuses [a]	0/72	18/86	1/47	0/31	0/40	0/27	0/43	0/11
Litters [b]	0/20	5/19*	1/10	0/7	0/12	0/6	0/12	0/3
Skeletal malformations								
Foetuses [a]	4/146	23/144 [d]	3/92	0/63	1/77	0/50	4/88	0/22
Litters [b]	3/20	10/19*	3/10	0/7	1/12	0/6	2/12	0/3
Summary								
Total foetuses [a]	4/146	42/146 [d]	4/92	2/63	1/77	0/50	4/88	0/22
Total litters [b]	3/20	13/19**	4/10	2/7	1/12	0/6	2/12	0/3
Malformed foetuses per litter [c]	3.8±2.6	30.6±8.3**	4.1±1.7	3.2±8.8	0.8±0.8	0	5.8±4.3	0

[a] Malformed foetuses / total foetuses examined

[b] Litters with one or more malformed foetuses / total of litters examined

[c] Nº of malformed foetuses in the litter / total Nº of live foetuses in the litter × 100; Data are expressed as X SE using litter as the experimental unit

[d] Two malformed foetuses were preserved for archival reference and were not evaluated for skeletal malformations

** $p < 0.01$ Mann-Whitney U test (two tailed)

* $p < 0.05$ Fisher exact probability test (two tailed); ** $p < 0.01$ Fisher exact probability test (two tailed)

*** $p < 0.001$ Fisher exact probability test (two tailed)

In conclusion, DNT failed to produce a teratogenic effect in Fischer 344 rats following oral administration throughout the postimplantation phase of gestation. Prenatal viability was reduced only at a dose of DNT which approached the maternal LD_{50}, thus providing no evidence for selective sensitivity of the developing conceptus to the toxic effects of DNT.

4.1.2.9.3 Effects on fertility and developmental toxicity: human data

There are three studies about effects on reproduction in male workers exposed to both technical-grade DNT (80% 2,4-DNT and 20% 2,6-DNT) and diaminotoluene (2,4 and 2,6 isomers). Their quality for the risk assessment of 2,4-DNT is very limited due to the fact that workers were exposed to other chemicals such as 2,6-DNT or diaminotoluene. Although, air concentration of DNT and diaminotoluene was available, exposure measurements on workers were not performed. Thus, the proportion of exposed workers who absorbed a significant amount of DNT and diaminotoluene was not known. It should be noted that diaminotoluene-exposed experimental animals showed both reproductive and teratogenic effects (EHC 74, 1987).

Consequently, those three studies are considered to be inadequate for 2,4-DNT risk assessment. Nevertheless, a brief description of those studies is provided below.

NIOSH, 1980

The US National Institute for Occupational Safety and Health (NIOSH) evaluated the reproductive health of 44 workers in a plant manufacturing diaminotoluenes. Three groups were classified for the study (exposed, n=9; without exposure for the last two years, n=12; and without exposure, n=9). Exposure usually involved both diaminotoluene and DNT. Regarding air sampling, concentrations of DNT in air were lower than the OSHA recommended standard of 1.5 mg/m^3. With respect to reproductive effects, an increased rate of spontaneous abortions (6/18 in exposed group vs 4/23 in unexposed group), decreased sperm count and reduction in the

proportion of the large morphological type of sperm were found in exposed workers compared with the controls. The participants constituted a limited-size sample and there was some risk of selection bias, since volunteers were not randomly selected. This study was of limited quality.

Ahrenholz and Meyer, 1982

The NIOSH evaluated the reproductive health of 50 workers. Employees were classified into three groups for the study (potential exposure, n=15; without exposure for the last two years, n=23; and without exposure, n=12). Exposure usually involved both diaminotoluene and DNT. Regarding air sampling, concentrations of DNT in air were lower than the OSHA recommended standard of 1.5 mg/m^3. Fifty volunteers were examined, 41 of whom provided semen specimens. Results obtained from the medical study determined that there were not significant differences between the exposed and control groups regarding liver function test, renal function tests, sperm counts and sperm morphologies. The ratio miscarriage/live births were higher in the DNT group than that of unexposed group (1/7 in DNT group vs 3/38 in unexposed group). However, the validity of that result is low, since it was based on a small number of cases. Additionally, there was some risk of selection bias, since volunteers were not randomly selected. This study was of limited quality.

Hamill *et al.*, 1982

In order to test the reproduction hazards after exposure to DNT and diaminotoluene, 84 exposed workers and 119 unexposed workers were studied at Olin's chemical complex at Lake Charles. The sources of information were blood and semen analysis, medical examinations, and reproductive and fertility questionnaires. The tested parameters were testicular volume, an assessment of serum follicle-stimulating hormone, and sperm count and morphology. Regarding air sampling, concentrations of DNT in air were lower than the OSHA recommended standard of 1.5 mg/m^3. No differences were found between the exposed and control groups. The authors concluded that exposure to DNT and diaminotoluene was not associated with reproductive effects. This study was of limited quality.

4.1.2.9.4 Summary of toxicity for reproduction

Fertility observations in repeated dose studies

Male fertility

For male fertility, LOAEL of 0.57 mg/kg bw/day is considered on the basis of atrophy of seminiferous tubules, and decrease of spermatogenesis and decrease of testis weight at higher doses in rat orally exposed for 12 and 24 months.

Female fertility

For female fertility, the relevant NOAEL was considered to be 95 mg/kg bw/day (LOAEL of 900 mg/kg bw/day) based on ovary atrophy of mice exposed for 12-24 months (Ellis *et al.,* 1979).

Special comments on multigeneration study

It is concluded that although no quantitative treatment-related effect can be deduced on reproduction in the three generation study, the decreased viability (not statistically significant) of

high dose F_{1b} generation and in particular the absence of F_2 parental generation are considered indications of adverse effects on male fertility. Therefore, the NOAEL for impaired fertility was considered to be 3.9 mg/kg b.w

Developmental toxicity studies

In the mouse teratogenicity study (Smith et al 1983, Harding et al 1987), pregnant treated dams dosed with 390 mg/kg/day for 8 days during organogenesis period (gestation days 7-14), showed increased incidence of mortality when compared with that of controls. Teratogenic effects have not been observed at the tested dose. This study is not considered acceptable for evaluation of developmental toxicity. As the only tested dose level is relatively high and mortality of dams was observed at this level it is difficult to evaluate and use this study for the developmental toxicity of 2,4-dinitrotoluene at lower dose levels. Furthermore, it is not clear whether visceral and skeletal abnormalities were examined.

In the three generation study in rats (Ellis et al 1979) no anomalies were detected in the offspring from any of the matings. Birth weights, postpartum survival when parturition was normal, weight at weaning and the lack of a teratogenic effect indicate that 2,4-DNT received via the placenta or milk was of little consequence. Furthermore, it is not clear whether visceral and skeletal abnormalities were examined.

In the teratogenic study carried out in rats (Price *et al.,*1985), DNT (technical-grade) failed to produce a teratogenic effect in Fischer 344 rats following oral administration throughout the postimplantation phase of gestation. Prenatal viability was reduced only at a dose of DNT which approached the maternal LD_{50}, thus providing no evidence for selective sensitivity of the developing conceptus to the toxic effects of DNT.

Human data

The data obtained from workers exposed to 2,4-DNT, did not allow to get conclusive data useful for risk characterization.

Classification for reproduction-fertility and reproduction-development

Based on effects observed in rats, mice, dogs, and also in humans, it can be concluded that 2,4-DNT may impair fertility. In particular, depression in spermatogenesis, decrease of testis weight, decrease of weight of epidymes and epidymal sperm, morphological alteration of Sertoli cells, increase in FSH and LH levels were reported in several studies. Furthermore, reduced capacity of mating and inducing pregnant females was also observed in males exposed for 5 days and increase in foetal deaths when males were exposed prior to mating. This observation suggests also that the decrease in viability observed in the multigeneration study at the highest dose and the absent of F_2 parental generation may be caused by male impaired fertility. Overall, there is consistent information supporting that 2,4-DNT is causing damage to reproduction-(fertility) in male. Although, other toxic effects coexisted at doses which caused adverse effects on fertility, those effects on fertility are not considered to be a secondary non-specific consequence of the other toxic effects. Therefore, according to EU criteria, 2,4-dinitrotoluene is considered to be classified as harmful for reproduction category 3 (Xn, R62). The LOAEL of 0.57 mg/kg b.w./day based on atrophy of seminiferous tubules observed in the 24-month rat study is considered the starting point for risk characterization for reproduction toxicity (fertility).
With respect to developmental toxicity, according to data from the teratogenicity study carried out with DNT (technical-grade) the treatment-related developmental effects are regarded as a

secondary effect due to parental systemic toxicity. It seems to be justified to extrapolate data from DNT to 2,4-DNT since 2,4-DNT is the major isomer of DNT (technical-grade). Therefore, no need of classification is deduced for reproduction-development according to EU criteria.

4.1.3 Risk characterisation [6]

4.1.3.1 General aspects

Data on toxicokinetics of 2,4-DNT in humans are limited to three *in vivo* studies, based on analyses of the urinary metabolites of workers exposed to technical-grade DNT in DNT production plants, and two *in vitro* studies that investigated the metabolism of 2,4-DNT by the human intestinal microflora. However, a lot of studies following oral administration have been performed in experimental animals, mainly in rats; *in vitro* experiments provided additional information.

The oral absorption was considered to be 100% within 24 hours in rats and by extrapolation, regarding studies done on other species, 100% in rabbits, dogs and monkeys. For humans the worst case was assumed and oral absorption of 2,4-DNT was considered as 100%. Based on oral absorption data, the worst case inhalation absorption (i.e. 100%) should be assumed for both animals and humans. The absorption of 2,6-DNT was considered to be nearly 100% by both oral and inhalation routes of administration, and 5-7% following dermal application (Hawkins *et al.*, 1991). The isomers 2,4- and 2,6-DNT have identical molecular weights and show nearly identical physical chemical properties, ie water solubility, vapour pressure and log Kow. Therefore, it seems appropriate to extrapolate 2,4-DNT dermal absorption from that of 2,6-DNT. Moreover, the US-EPA computer model Dermwin v1.42, computed nearly identical results for the dermal penetration rates of the two isomers. Thus, a dermal penetration rate of 10% of 2,4-DNT is considered acceptable. This is also supported by findings obtained in the dermal acute toxicity study, ie all animals treated dermally with 2500 mg/kg bw survived over the 14−day post−exposure observation period without any toxic symptoms (Löser et al., 1975).

Once absorbed 2,4-DNT and its metabolites are well distributed in all animal species showing a similar pattern of distribution with radioactivity concentrated in liver and kidneys. There is not sufficient evidence to warrant accumulation in these organs. Since for systemic effects it is assumed that the tissue distribution pattern will be the same irrespective of the administration route, the distribution pattern of 2,4-DNT for animals orally exposed is considered applicable for both humans and animals exposed by dermal and inhalatory routes.

The routes of excretion were similar in rats, rabbits, dogs and monkeys, with the predominant route being via urine. By 24 hours after a single oral dose, the radioactivity recovered in the urine was 75-81%, and in less extension in faeces (3-9%); no radioactivity was found in the expired air.

Several metabolites have been identified in the urine of rats. The major urinary metabolite was 2,4-dinitrobenzyl alcohol glucuronide; 2,4-dinitrobenzoic acid, 4-(N-acetyl)amino-2-nitrobenzoic acid, 2-amino-4-nitrobenzoic acid, 4-(N-acetyl)amino-2-aminobenzoic acid, 4-amino-2-nitrobenzoic acid, 2-4-dinitrobenzyl alcohol, and 4-amino-2-nitrotoluene were also detected

[6] Conclusion (i) There is a need for further information and/or testing.
Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.
Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

among others. The metabolite profile following a single oral radiolabel dose was not altered by feeding 2,4-DNT in the diet and there were no major differences between dose groups, between sexes or between feeding periods.

The bile is an important route of excretion for 2,4-DNT and its metabolites in rats. The major biliary metabolite of 2,4-DNT was 2,4-dinitrobenzyl alcohol glucuronide; 2,4-dinitrobenzoic acid, 4-amino-2-nitro(2-amino-4-nitro)benzyl alcohol sulphate, 2-amino-4-nitrobenzoic acid, 2,4-dinitrobenzaldehyde, 2,4-dinitrobenzyl alcohol, 2-4-diacetylaminobenzoic acid and 2-acetylamino-4-nitrotoluene were also detected.

In rats, sex differences in the excretion of 2,4-DNT metabolites have been observed. For male and female rats similarly treated, the urine excretion predominates in females while in males biliary excretion is the most important route; in addition, females excreted a greater percentage of the dose in the urine as 2,4-dinitrobenzyl alcohol glucuronide than did males. Biliary excretion measured after 24 h was greater for males (25%) than for females (18%), essentially complete within 24 hours for males and 12 hours for females, and there were no significant differences in the rates of biliary excretion between sexes; mean half times of excretion ranged from 3.3 to 5.3 h. Regarding urinary excretion, radioactivity excreted in the urine of rats which bile was not collected (60-90%) was higher than in the urine of rats which bile was collected (20-60%), indicating that biliary metabolites were absorbed from the intestine (enterohepatic cycling). Whether or not bile was collected, females excreted more radioactivity in urine than males.

The gut bacteria are important in the metabolism of 2,4-DNT *in vivo*. The four major metabolites identified in the urine of conventional rats (2,4-dinitrobenzyl alcohol glucuronide, 2,4-dinitrobenzoic acid, 4-(N-acetyl)amino-2-nitrobenzoic acid and 2-amino-4-nitrobenzoic acid) were also present in the urine of axenic rats. However, in axenic rats the amounts of 4-(N-acetyl)amino-2-nitrobenzoic and 2-amino-2-nitrobenzoic excreted in the urine are markedly reduced by comparison with conventional animals. In addition, a less radioactivity is covalently bound to hepatic macromolecules in axenic rats compared with conventional rats.

These observations indicate that the relationship between liver and intestinal microflora in the metabolism of 2,4-DNT is a complex one. The intestinal microflora is apparently an important site for the metabolism of biliary metabolites and metabolism by intestinal microflora appears to be essential for the production of metabolites that bind covalently to liver macromolecules.

The following pathway has been proposed for the bioactivation of 2,4-DNT in the whole animal (Figure 1, 4.1.2.1.3 section). After an oral administration, 2,4-DNT is oxidated in the liver to 2,4-dinitrobenzyl alcohol, which undergoes a Phase II reaction being conjugated with glucuronide acid. The glucuronic conjugate can be either eliminated via urine or excreted to bile. The conjugate excreted in bile is absorbed in intestine where the glucuronic acid is hydrolysed by glucuronidase, yielding again 2,4-dinitrobenzyl alcohol. This benzyl alcohol is further reduced in intestine at position 4, being the generated 4-amino-2-nitrobenzyl alcohol carried out again to the liver. Once in the liver, 4-amino-2-nitrobenzyl alcohol can be conjugated with sulphate at the hydroxyl group. The sulfoconjugated is unstable and quickly decomposes to electrophilic species with high capability to form covalent binding with DNA. Another possible route for bioactivation of 4-amino-2-nitrobenzyl alcohol re-entered in the liver is its oxidation of the amino group to form 4-hydroxylamino-2-nitrobenzyl alcohol. This compound can be the target of reaction of Phase II, especially conjugation with sulphate. The generated molecule is also very unstable and spontaneously changes to other electrophilic species, which can be covalently bound to DNA.

The metabolism and excretion of 2,4-DNT in workers exposed to technical-grade DNT by inhalation and dermal routes has been studied by the analysis of urinary metabolites. The major

2,4-DNT metabolite detected in the urine of workers was 2,4-dinitrobenzoic acid, although lesser amounts of 2-amino-4-nitrobenzoic acid, 2,4-dinitrobenzyl glucuronide, 2-(N-acetyl)amino-4-nitrobenzoic acid and traces of 4-amino-2-nitrobenzoic acid and 4-(N-acetyl)amino-2-nitrobenzoic were also found. In addition, the urine contained unchanged 2,4-DNT.

As seen in rats, female subjects excreted a higher proportion of urinary metabolites as dinitrobenzyl alcohol glucuronides than did males.

The appearance of reduced metabolites suggest either that human hepatic enzymes are capable of reduction of the nitro group of 2,4-DNT or that 2,4-DNT (or its metabolites) gain access to the intestinal microflora which is capable of reduction, after which the metabolites are reabsorbed and excreted into urine. In support of the last suggestion, it was observed that the metabolites produced by incubation of 2,4-DNT with human gut were the same as those produced by analogous samples from rats and mice.

The half-life for excretion of 2,4-DNT metabolites in urine of workers ranged from 0.8 to 4.5 hours. The half-lives for 2,4-dinitrobenzoic acid and 2,4-dinitrobenzyl alcohol glucuronide tended to be shorter than those for the metabolites that resulted from both oxidative and reductive. The highest rates of excretion of 2,4-dinitrobenzoic acid occurred near the end of the work shift. The half-life for urinary excretion of 2,4-dinitrobenzoic acid was calculated to be 2-5 hours. This estimate appears to be the initial phase of a biphasic elimination profile since even 3 days after the exposure, detectable levels of 2,4-dinitrobenzoic acid were present in urine.These data support that enterohepatic recycling occurred in humans.

In summary, 2,4-DNT metabolism and excretion seem to be qualitatively similar in both humans and rats, but the proportions of nitro-reduced metabolites were lower relative to oxidized metabolites in the urine from humans. These differences may be due more to the particular routes of exposure (inhalation and dermal for humans, oral for rats) than differences in species.

There are no studies on whether 2,4-DNT or its metabolites can cross the placenta or be excreted in breast milk, so it cannot be determined if fetuses may be exposed in utero of if infants may be exposed via breast milk ingestion. There are also no data to show if 2,4-DNT and its metabolites are stored in maternal tissues and thus might be later mobilized during gestation or lactation; however, 2,4-DNT and its metabolites are not likely to be stored because of their low octanol-water partition coefficient.

Based on cat data (death following oral administration of 50 mg/kg b.w.) 2,4-DNT is considered to be classified for acute toxicity as toxic by oral exposure. Since oral and inhalation absorption values were estimated to be 100% in experimental animals and humans, it seems reasonable to extrapolate toxicity by inhalation from oral toxicity data and to classify 2,4-DNT as toxic by inhalation. Finally, taking into account that the dermal absorption value was estimated to be 10% in rodent and by extrapolation in experimental animals and humans, 2,4-DNT should be considered as borderline toxic by dermal exposure. Overall, it seems justified to classify 2,4-DNT for acute toxicity as toxic (T; R23/24/25) by inhalation, dermal and oral routes of exposure, and to use the NOAEL of 10 mg/kg b.w. derived from the oral toxicity study in cats as the starting point for risk characterization for acute toxicity.

According to available animal studies, 2,4-DNT is considered not irritant for skin and eye, and, consequently not corrosive.

No dermal sensitivity was observed in the Guinea-Pig Maximisation Test. Thus, 2,4-DNT is not classified as sensitising substance in accordance with the EU criteria.

The most common adverse health effect in workers exposed to DNT is related to the ability of DNT to induce MetHb, the secondary effects of which were non-specific health effects such as headache, dizziness, nausea and drowsiness. Although an excess of mortality due to ischemic heart disease and residual diseases of the circulatory system have been reported for exposed workers, the evidence is only limited and then these data are of low relevance for risk assessment.

Several studies have been investigated the toxicity of 2,4-DNT following repeated oral administration to rats, mice and dogs. The LOAEL for chronic toxicity was considered to be 0.57 mg/kg b.w., based on the hyperplastic foci incidence in the liver and atrophy of seminiferous tubules derived from the 24-month study in rats.

2,4-DNT is clearly mutagenic in *Salmonella typhimurium* strains both in the presence and absence of a rat liver metabolic activation system. The highest mutagenic activity was observed in strains with elevated levels of both nitroreductase and *O*-acetyltransferase activities, and extracellular nitro reduction was necessary for optimal detection of 2,4-DNT mutagenicity in standard strains. Aminohydroxylamino dimethylazoxybenzenes or aminohydroxylamino dimethylazobenzenes produced either by reduction of hydroxylaminonitrotoluenes or of dimethyl dinitroazoxybenzenes, could be considered the active metabolites responsible for the 2,4-DNT mutagenic activity in bacteria. With respect to DNA damage, 2,4-DNT was genotoxic in the *Salmonella typhimurium umu* test. As occurred in mutation tests, the most sensitive strain was NM3009, which has high *O*-acetyltransferase and nitroreductase activities.

2,4-DNT was not mutagenic in the CHO/HGPRT system, when tested in the presence of rat liver S9 under usual (aerobic) assay conditions. However, it was mutagenic either when CHO cells were incubated with rat liver S9 under anaerobic (reduced oxygen tension) conditions or when primary rat hepatocytes were used as metabolic activation system. In addition, 2,4-DNT was a direct mutagen in the P388 mouse lymphoma/TK system. Results on clastogenicity were contradictory. Thus, in one study, 2,4-DNT of 99% purity did not induce chromosomal aberrations in CHO cells (with or without S9), whereas in two other studies, 2,4-DNT of unknown purity was reported to be clastogenic in human lymphocytes (without S9) and in CHL cells (with and without S9). With respect to DNA damage, 2,4-DNT induced sister chromatid exchanges in CHO cells when tested in the presence of rat liver S9. There was no evidence of unscheduled DNA synthesis in rat (hepatocytes, spermatocytes and spermatids) or human (hepatocytes) cells incubated with 2,4-DNT. This lack of 2,4-DNT genotoxic activity is accord with the concept that reduction by intestinal flora is required in addition to hepatic metabolism for activation. Finally, 2,4-DNT of unknown purity was reported to cause DNA damage (single-strand breaks) when tested at cytotoxic concentrations in the alkaline elution/rat hepatocyte assay.

Dogs administered 2,4-DNT (10 mg/kg b.w./day) in hard gelatine capsules for 2 years had no chromosomal aberrations in their bone marrow or kidney. 2,4-DNT when fed to male and female rats at up to 45 mg/kg b.w./day for 2 years did not induce either chromosomal aberrations in the bone marrow or kidney. Nevertheless, 2,4-DNT when fed to male rats at 93 mg/kg b.w./day for 19 weeks induced chromosomal aberrations (chromatid breaks) in lymphocytes; in addition, significant increases in the number of chromatid breaks were also observed after treatment for 5 and 13 weeks in kidney cultures, being the number of breaks increased with the duration of treatment. With respect to DNA damage, unscheduled DNA synthesis was induced by 2,4-DNT when administered at up to 200 mg/kg by gavage to male rats. DNA covalent binding was observed in several organs (liver, kidney, lung and mammary glands) of rats administered i.p. a single dose of 150 mg/kg of 2,4-DNT, the binding being highest in the liver. Moreover, when rats were administered 2,4-DNT by either gavage or i.p., no differences in liver DNA covalent

binding were noted. The sulfotransferase inhibitors decreased the covalent binding to DNA, indicating that sulfation is important in the biotransformation of 2,4-DNT to reactive metabolites.

In *Drosophila melanogaster*, 2,4-DNT induced sex-linked recessive lethal mutation after injection, but failed to induced lethal mutations after feeding and translocations after injection.

2,4-DNT did not induce dominant lethal mutations when administered to rats for 5 days (by gavage) or up to 13 weeks (by feeding). Negative results were also obtained for dominant lethal mutations in mice administered 2,4-DNT for 2 days (by gavage) or up to 13 weeks (by feeding). In addition, 2,4-DNT did not induce sperm abnormalities in mice.

In conclusion, the weight of evidence indicates that 2,4-DNT is an *in vivo* mutagenic agent for somatic cells. Therefore, 2,4-DNT is classified as mutagenic category 3 (Xn, R68).

Regarding carcinogenicity in rats, there are two studies considered adequate, one of them being a chronic toxicity study. The same signs of carcinogenicity (ie skin/subcutaneous tissue fibromas in males, mammary gland fibroadenomas in females and hepatocarcinomas in both sexes) were found in both studies. The dose that produced tumours in females in the chronic study was in the dose-level range of the carcinogenicity study. On the other hand, the high-dose of the chronic study (34/45 mg/kg b.w./day for males/females) reduced significantly survival and showed a high toxicity. With respect to females, the incidence of mammary gland fibroadenoma in Fischer 344 rats treated with 15.7 mg/kg b.w./day (NCI, 1979) and CD rats treated from 5.1 mg/kg b.w./day (Ellis et al., 1979; Lee et al., 1985) was significantly higher than in controls. In males, significant increased incidences of subcutaneous tissue or skin fibroma were found in CD rats administered 34 mg/kg b.w./day (Ellis et al., 1979; Lee et al., 1985) and in Fischer 344 rats treated from 4.7 mg/kg b.w./day (NCI, 1979); there was a sporadic occurrence of squamous-cell papillomas, basal-cell carcinoma, fibrosarcomas, and lipomas (NCI, 1979). The incidence of hepatocellular carcinoma in liver of males treated with 4.7 and 11.8 mg/kg b.w./day for 18 months was higher ($p > 0.05$) than in controls (NCI, 1979). Despite of the not significant excess, the same tumour occurred with a significant increased incidence in CD rats given 34/45 mg/kg b.w./day (Ellis et al., 1979; Lee et al., 1985); hence it was included herein. At that dose, most of the treated rats showed neoplastic nodules in liver after 12-month treatment (Ellis et al., 1979; Lee et al., 1985).

Regarding carcinogenicity in mice, there are two studies considered adequate, one of them being a chronic toxicity study. In one study no carcinogenic effect was reported. Data of the chronic toxicity study were considered relevant for carcinogenicity, since renal tumours were observed in males. In males the incidence of kidney tumours (both benign and malignant) was significantly elevated in groups treated with 13.3 and 96.9 mg/kg b.w./day for more than 12 months. In addition, carcinomas in liver of males and females were found at 885 and 911 mg/kg b.w./day respectively for 12 months (1/4 and 1/4, respectively), and in male livers at 885 mg/kg b.w./day for 12 months and allowed to recover for 1 month (2/4) vs. none in controls.

In summary, there is a good evidence of an increase in the tumour incidence in rats and mice. These observations are consistent with genotoxic aetiology, which is consistent with the findings from the genotoxicity studies. In addition, two studies support the hypothesis that occupational exposure to DNT may be carcinogenic. Those studies associated an excess of hepatobiliary cancer and both urothelial cancer and renal cell cancer with jobs where workers were supposedly exposed to purified 2,4-DNT and miners supposedly exposed to technical grade DNT, respectively. Therefore, according to EU criteria, 2,4-DNT is considered carcinogenic category 2 and then classified as T R45.

In sub-acute, sub-chronic and chronic studies, effects on reproduction such as decrease of testis weights, decrease in spermatogenesis or atrophy of the seminiferous tubules have been observed

in males from different species (rats, mice, dogs) and these effects are the most commonly reported. After a short term feeding study (5 days) a decrease of sperm positive females has been reported.

In a three generation study, the absence of the F_2 parental generation for the group given the high dose and the few animals mated in the F_1 indicated an adverse effect on fertility. Furthermore, a decreased viability index of high-dose F_{1b} generation ($p < 0.05$) was found when compared with controls. None of the three high-dose females from F_{1b} produced second litters.

In the teratogenicity study, pregnant treated dams showed increased incidence of mortality when compared with that of controls. Teratogenic effects have not been observed at tested doses.

The data obtained from workers exposed to 2,4-DNT, although not conclusive, showed the same effects on reproduction as those obtained in sub-acute, sub-chronic and chronic studies in experimental animals, ie reduction of sperm counts.

Fertility effects in rats, mice, dogs, and also in humans support the current classification of toxic for reproduction category 3 (Xn,, R 62) according to EU criteria. The LOAEL for reproduction toxicity is considered to be 0.57 mg/kg b.w./day based on atrophy of seminiferous tubules observed in the 24-month rat study.

With respect to developmental toxicity, the effects observed are regarded as a secondary effect due to parental systemic toxicity, therefore, no need of developmental classification according to EU criteria.

4.1.3.2 Workers

The most probable route of human exposure to 2,4-dinitrotoluene is inhalation and dermal contact of workers involved in the production and use of this substance. Three scenarios were used for risk caharacterization: production and further processing (scenario 1), explosives manufacture (scenario 2), and uses of explosives (scenario 3).

The following assumptions have been made:

- The body weight of the average worker is 70 kg and the worker breathes 10 m^3 of air during an 8-hours working day.
- In the absence of quantitative data on the bioavailability of 2,4-dinitrotoluene, 100% absorption by inhalation route has been assumed.
- Extrapolating from 2,6-DNT data, 10% absorption of 2,4-DNT by dermal route has been assumed.

The exposure estimations used in the workers risk characterisation are summarised in section 4.1.1.2 (Table 4.1.1.2.1-3).

Comparison of exposure and effects

When considering the risks to human health arising from occupational exposure to 2,4-dinitrotoluene, the key areas of concerns are for repeated dose toxicity, mutagenicity, carcinogenicity and toxicity for reproduction (fertility).

4.1.3.2.1 Acute toxicity

The NOAEL of 10 mg/kg b.w. derived from an acute cat oral toxicity study carried out by Löser and Schmidt (1984) is used as starting point for the risk characterization.

Inhalation exposure

The minimal MOS is considered to be 25 based on the following assessment factors: 2 x 2.5 for interspecies differences and 5 for intraspecies differences.

Starting with the NOAEL of 10 mg/kg b.w., a worker respiratory volume of 10 m^3/8 h, a human body weight of 70 kg, and oral and inhalation absorption values of both 100%, a corresponding human NAEC of 70 mg/m^3 was derived.

When the human NAEC of 70 mg/m^3 is compared with the short-term exposure of 0.018 mg/m^3 (scenario 1), 0.3 mg/m^3 (scenario 2) and 0.012 mg/m^3 (scenario 3), it results in MOS of 3888.89, 233.33 and 5833.33 for scenarios 1, 2 and 3 respectively. The comparison between minimal MOS and the calculated MOS indicates no concern for inhalation exposure and **conclusion (ii)** is reached.

Dermal exposure

The minimal MOS is considered to be 25 based on the following assessment factors: 2 x 2.5 for interspecies differences and 5 for intraspecies differences

Starting with the NOAEL of 10 mg/kg b.w., corrected for differences in absorption between routes (dermal absorption 10%), and a human body weight of 70 kg, a corresponding human NAEL of 7000 mg/person/day was derived.

When the human NAEL of 7000 mg/person/day is comparesd with the level of exposure, 2.1 mg/p./d. (scenario 1), 3 mg/p./d. (scenario 2) and 4.2 mg/p./d. (scenario 3), it results in MOS of 3333.33 (scenario 1), 2333.33 (scenario 2), and 1666.67 (scenario 3). The comparison between minimal MOS and the calculated MOS indicates no concern for dermal exposure and **conclusion (ii)** is reached.

4.1.3.2.2 Irritation and corrosivity

Based on animal testing, 2,4-dinitrotoluene is not considered either irritant or corrosive for skin and eyes.

Therefore, for the irritation/corrosivity effects, **conclusion (ii)** is reached.

4.1.3.2.3 Sensitisation

Based on animal testing, 2,4-dinitrotoluene is not considered a dermal sensitising.

Therefore, for the dermal sensitisation effects, **conclusion (ii)** is reached.

4.1.3.2.4 Repeated dose toxicity

The LOAEL of 0.57 mg/kg b.w. derived from a 24-month rat oral toxicity study is used as starting point for the risk characterization.

Inhalation Exposure

The minimal MOS is considered to be 150 based on the following assessment factors: 4 x 2.5 for interspecies differences, 5 for intraspecies differences and 3 for extrapolation LOAEL to NOAEL.

Starting with a LOAEL of 0.57 mg/kg b.w., a worker respiratory volume of 10 m^3/8 h, a human body weight of 70 kg, and oral and inhalation absorption values of both 100%, a corresponding human LAEC of 3.99 mg/m^3 was derived.

The risk characterization for the different scenarios is shown in Table 4.1.3.2.4-1.

Table: 4.1.3.2.4-1: Risk characterisation for repeated dose toxicity (inhalation)

Exposure scenario	Inhalation			
	Shift average value (mg/m^3)	LAEC mg/m^3	MOS	**Conclusion**
Production and further processing **(scenario 1)**	0.009(1)	3.99	443	(ii)
	0.0007(2)		5700	(ii)
Explosives manufacture **(scenario 2)**	0.15(1)	3.99	27	**(iii)**
	0.075(2)		53	**(iii)**
Use of explosives **(scenario 3)**	0.006(1)	3.99	665	(ii)
	0.003(2)		1330	(ii)

(1) RWC (2) Typical exposure

These MOS are considered enough for scenarios 1 and 3 and **conclusion (ii)** is reached. However, in scenario 2, MOS value is considered of concern both for RWC value and for the typical exposure value and **conclusion (iii)** is reached.

Dermal Exposure

The minimal MOS is considered to be 150 based on the following assessment factors: 4 x 2.5 for interspecies differences, 5 for intraspecies differences and 3 for extrapolation LOAEL to NOAEL.

Using the LOAEL of 0.57 mg/kg b.w. as starting point, corrected for differences in absorption between routes (dermal absorption 10%), and a human body weight of 70 kg, a corresponding human LAEL of 399 mg/person/d was derived.

The risk characterization for the different scenarios is shown in Table 4.1.3.2.4-2.

Table: 4.1.3.2.4-2: Risk characterisation for repeated dose toxicity (dermal)

Exposure scenario	Dermal contact			
	Exposure range (mg/day)(1)	LAEL mg/person /day	MOS	**Conclusion**
Production and further processing **(scenario 1)**	2.1	399	190	**(ii)**
Explosives manufacture **(scenario 2)**	3	399	133	**(iii)**
Use of explosives **(scenario 3)**	4.2	399	95	**(iii)**

(1)RWC

The MOS value is considered of no concern in scenario1 and conclusion (ii) is reached and of concern in scenarios 2 and 3 and **conclusion (iii)** is reached

Combined exposure

Systemic health effects due to combined exposure have to be assessed in addition to route specific risk assessment.

Combined exposure is calculated by the formula:

$$MOS = \frac{[LOAEL_{oral\text{-}rat} * ABS_{oral\text{-}rat}]}{[Expo_{inh\text{-}human} * (RV_{human}/bw_{human}) * ABS_{inh\text{-}human}] + [Expo_{derm\text{-}human} * ABS_{derm\text{-}human}] + [Expo_{oral\text{-}human} * ABS_{oral\text{-}human}]}$$

The calculated MOS_{comb} are 142.5, 6.33 and 83.09 for scenarios 1, 2 and 3, respectively. Comparing minimal MOS (150) with the calculated MOS_{comb} indicate concern for combined exposure for all scenarios and **conclusion (iii)** is reached.

4.1.3.2.5 Mutagenicity

2,4-dinitrotoluene has shown to be an *in vivo* mutagenic agent for somatic cells of mammals. Therefore, it is of concern for mutagenicity and **conclusion (iii)** is reached.

4.1.3.2.6 Carcinogenicity

A quantitative risk characterization for carcinogenicity according to the T25 approach (Draft TGD 2005) was performed for 2,4-DNT in order to estimate the level of concern for the different scenarios. Briefly, the dose-descriptor T25, the chronic dose that will give 25% of tumours after correction for spontaneous incidence, was calculated with Expression 1. *Dc* was the exposure/observation-period-corrected dose of 2,4-DNT (mg/kg bw/day) and *i* was the cancer incidence after correction for spontaneous incidence.

$T25 = Dc \cdot \frac{0.25}{i}$	Exp. 1

Dc was calculated by using Expression 2 where w_1 and w_2 were the exposure and observation periods (months), respectively.

$Dc = D \cdot \frac{w_1}{24} \cdot \frac{w_2}{24}$	Exp. 2

Incidence after correction for spontaneous incidence was computed with Expression 3.

$\mathrm{i} = \frac{\text{treatment incidence} - \text{control incidence}}{1 - \text{control incidence}}$	Exp. 3

The animal dose descriptor (T25) was converted into a human dose descriptor (HT25) by using the Expression 4.

$HT25 = \frac{T25}{(\frac{bw_{human}}{bw_{animal}})^{0.25}}$	Exp. 4

The body weigh of animals at 12 months was introduced in the Expression 4 (Table 4.1.3.2.6-1), since most of the animals died before 24 months. According to default biological parameters, human b.w. was considered to be 70 kg for both males and females.

The critical dose descriptors are those obtained in the life-time exposure studies, since workers could be exposed to 2,4-DNT for years. The carcinogenicity of 2,4-DNT was investigated in two oral carcinogenicity studies performed in rats and mice. Moreover, there are two oral chronic toxicity studies available which were carried out in rats and mice.

Skin/subcutaneous tissue fibromas in males, mammary gland fibroadenomas in females and hepatocarcinomas in both sexes were found in both chronic and carcinogenicity rat studies. Moreover, male mice showed treatment-related kidney tumours (both benign and malignant). Thus, the carcinogen was effective in multiple tissue sites and across species and genders.

With respect with site/species/strain/gender activity, HT25 calculated values were highly consistent among studies, ie most of the calculated HT25 values were within a few units of mg/kg bw/day (Table 4.1.3.2.6-1).

In rats, the lowest male HT25 for hepatocarcinoma was calculated from the carcinogenicity study, which was similar to that in females calculated from the chronic study (4.13 mg/kg bw/day in males vs 5.22 mg/kg bw/day in females).

Concerning dose-response relationships, the HT25 calculated values increased with the (corrected) dose for all the treatment-related tumours observed in both rats and mice (Table 4.1.3.2.6-1). Thus, a supralinear dose-response relationship could be obtained.

Table 4.1.3.2.6-1: Human dose descriptor (HT25) values calculated from doses of 2,4-DNT and tumour incidence after correction for spontaneous incidence

Species	Exposure/observation period	Tumour	Sex	Incidence			Dose	Corrected dose (1)	Animal body weight (2)	T25	HT25	Reference
	(months)			Controls	Treatment	Corrected	(mg/kg bw/day)	(mg)	(mg)	(mg/kg bw/day)	(mg/kg bw/day)	
CD Rats	24/24	Hepatocellular carcinoma	Male	1/25	6/29	17.4%	34	–	0.438	48.89	13.75	Ellis et al., 1979; Lee et al., 1985
			Female	0/23	18/34	52.9%	45	–	0.255	21.25	5.22	
		Mammary gland fibroadenoma	Female	0/23	17/27	29.0%	5.1	–	0.373	4.39	1.19	
					33/35	89.0%	45	–	0.255	12.63	3.10	
		Skin fibroma	Male	2/25	17/30	52.9%	34	–	0.438	16.07	4.52	
Fischer 344 Rats	18/24	Hepatocellular carcinoma	Male	0/45	3/49	6.1%	4.7	3.53	0.475	14.39	**4.13**(3)	NCI 1978
				0/25	3/48	6.3%	11.8	8.85	0.475	35.40	10.16	
		Mammary gland fibroadenoma	Female	4/23	23/50	34.6%	15.7	11.78	0.275	8.50	2.13	
		Skin fibroma	Male	0/46	7/49	14.3%	4.7	3.53	0.475	6.17	1.77	
				0/25	13/49	26.5%	11.8	8.85	0.475	8.34	2.39	
CD-1 Mice	24/24	Kidney tumours (both benign and malignant)	Male	0/33	5/33	15.2%	13.3	–	0.045	21.95	3.49	Ellis *et al.*, 1979; Hong *et al.*, 1985
					16/28	57.1%	96.9	–	0.045	42.39	6.75	

(1) Dose corrected to a exposure/observation period of 24 months
(2) Body weight at 12 months
(3) HT25 value selected for carcinogenicity risk characterisation

Regarding risk characterisation, the use of the HT25 value derived from malignant tumours, ie hepatocellular carcinomas, was preferred. Therefore, the HT25 value of 4.13 mg/kg bw/day on the basis of hepatocellular carcinomas in low-dose male rats derived from the carcinogenicity study was the critical value used for carcinogenicity risk characterisation.

The life-time cancer risk for workers (wLR) is obtained by Expression 5, where *wE* was worker exposure estimation (mg/kg bw/day). For workers the exposure time is 8 hours per day, 5 days per week, 48 weeks per year for 40 years, ie a correction factor of 2.8 (Exp. 5).

$wLR = \frac{\frac{wE}{2.8}}{\frac{HT25}{0.25}}$	Exp. 5

With regard of modifying elements, the starting assumption was achieved for mechanisms including genotoxicity, mechanistic relevance to humans and toxicokinetics. Regarding supralinear dose-response relationship, the selected HT25 value was calculated from a dose which produced a very low cancer incidence (Table 4.1.3.2.6-1). Therefore, it is unlikely that linear and supralinear extrapolations would differ substantially. With respect to site/species/strain/gender activity of 2,4-DNT, the actual life-time cancer risk could be higher than the calculated risk. Overall, 2,4-DNT activity was into the medium potency range (1 mg/kg bw/day < T25 = 14.39 mg/kg bw/day < 100 mg/kg bw/day).

All wLR calculated values were higher than 10^{-5} (Table 4.1.3.2.6-2).

Table 4.1.3.2.6-2: Life-time cancer risk for workers

Scenario	Route of exposure	Exposure[(1)]	Exposure[(2)]	Total exposure	wLR [(3)]
Production and further processing **(scenario 1)**	Inhalation	$9{,}00 \cdot 10^{-3}$	$1{,}29 \cdot 10^{-3}$	$4.29 \cdot 10^{-3}$	$9.27 \cdot 10^{-5}$
	Dermal	$2{,}10 \cdot 10^{-1}$	$3{,}00 \cdot 10^{-3}$		
Explosives manufacture **(scenario 2)**	Inhalation	$1{,}50 \cdot 10^{-1}$	$2{,}14 \cdot 10^{-2}$	$2.57 \cdot 10^{-2}$	$5.56 \cdot 10^{-4}$
	Dermal	$3 \cdot 10^{-1}$	$4.29 \cdot 10^{-3}$		
Use of explosives **(scenario 3)**	Inhalation	$6{,}00 \cdot 10^{-3}$	$8{,}57 \cdot 10^{-4}$	$6.86 \cdot 10^{-3}$	$1.48 \cdot 10^{-4}$
	Dermal	$4{,}20 \cdot 10^{-1}$	$6{,}00 \cdot 10^{-3}$		

(1) RWC; inhalation, mg/m^3; systemic dose following dermal exposure, mg/day (Dermal absorption 10%)
(2) RWC; inhalation and systemic dose following dermal exposure (Dermal absorption 10%), mg/kg bw/day
(3) wLR, life-time cancer risk for workers calculated by Exp. 5 (wLR = (wE/2.8)/(HT25/0.25)); HT25 value of 4.13 mg/kg bw/day on the basis of hepatocellular carcinomas in low-dose male rats derived from the carcinogenicity study.

2,4-dinitrotoluene is classified as carcinogenic category 2. There is a good evidence of an increase in tumour incidence in rats and mice, consistent with a genotoxic mechanism of carcinogenicity supported by findings from the genotoxicity studies. Overall, conclusion (iii) is reached in worker scenarios 1, 2 and 3.

4.1.3.2.7 Toxicity for reproduction

Effects on fertility

The LOAEL of 0.57 mg/kg b.w. derived from a 24-month rat oral toxicity study is used as starting point for the risk characterization for toxicity for reproduction (fertility).

The minimal MOS is considered to be 150 based on the following assessment factors: 4 x 2.5 for interspecies differences, 5 for intraspecies differences and 3 for extrapolation LOAEL to NOAEL.

Inhalation Exposure

Starting with a LOAEL of 0.57 mg/kg b.w., a worker respiratory volume of 10 m^3/8 h, a human body weight of 70 kg, and oral and inhalation absorption values of both 100%, a corresponding human LAEC of 3.99 mg/m^3 was derived.

This human LAEC is compared with the exposure information (Table 4.1.3.2.7-1).

Table: 4.1.3.2.7-1: Risk characterisation for toxicity for reproduction (inhalation)

Exposure scenario	Inhalation			
	Shift average value (mg/m^3)	LAEC mg/m^3	MOS	**Conclusion**
Production and further processing **(scenario 1)**	0.009(1)	3.99	443	(ii)
	0.0007(2)		5700	(ii)
Explosives manufacture **(scenario 2)**	0.15(1)	3.99	27	**(iii)**
	0.075(2)		53	**(iii)**
Use of explosives **(scenario 3)**	0.006(1)	3.99	665	(ii)
	0.003(2)		1330	(ii)

(1)RWC; (2) Typical exposure

The comparison between these MOS and minimal MOS indicates no concern for scenarios 1 and 3 and **conclusion (ii)** is reached. However, scenario 2 is considered of concern both for RWC value and for the typical exposure value and **conclusion (iii)** is reached.

Dermal Exposure

The minimal MOS is considered to be 150 based on the following assessment factors: 4 x 2.5 for interspecies differences, 5 for intraspecies differences and 3 for extrapolation LOAEL to NOAEL.

Using the LOAEL of 0.57 mg/kg b.w. as starting point, corrected for differences in absorption between routes (dermal absorption 10%), and a human body weight of 70 kg, ,a corresponding human LAEL of 399 mg/person/d was derived.

The risk characterization for the different scenarios is shown in Table 4.1.3.2.7-2.

Table: 4.1.3.2.7-2: Risk characterisation for toxicity for reproduction (dermal contact)

Exposure scenario	Dermal contact			
	Exposure range (mg/day)[(1)]	LAEL mg/person /day	MOS	**Conclusion**
Production and further processing **(scenario 1)**	2.1	399	190	**(ii)**
Explosives manufacture **(scenario 2)**	3	399	133	**(iii)**
Use of explosives **(scenario 3)**	4.2	399	95	**(iii)**

[(1)]RWC

The MOS value is considered of no concern in scenario1 and conclusion (ii) is reached and of concern in scenarios 2 and 3 and **conclusion (iii)** is reached

Combined exposure

Systemic health effects due to combined exposure have to be assessed in addition to route specific risk assessment.

Combined exposure is calculated by the formula:

$$MOS = \frac{[LOAEL_{oral-rat} * ABS_{oral-rat}]}{[Expo_{inh-human} * (RV_{human}/bw_{human}) * ABS_{inh-human}] + [Expo_{derm-human} * ABS_{derm-human}] + [Expo_{oral-human} * ABS_{oral-human}]}$$

The calculated MOS_{comb} are 142.5, 6.33 and 83.09 for scenarios 1, 2 and 3, respectively. Comparing minimal MOS (150) with the calculated MOS_{comb} indicate concern for combined exposure for all scenarios and **conclusion (iii)** is reached.

Developmental toxicity

Based on available data, developmental toxicity is regarded as a secondary effect due to parental systemic. Therefore, for developmental toxicity effects, **conclusion (ii)** is reached.

4.1.3.2.8 Summary of risk characterization for workers

When considering the risks to human health arising from occupational exposure to 2,4-dinitrotoluene, the key areas of concern are for repeated dose toxicity, carcinogenicity, mutagenicity and toxicity for reproduction (fertility).

The mutagenicity and carcinogenicity effects of 2,4-dinitrotoluene do not allow the identification of a threshold level of exposure below which there would be no risk for the development of these effects in humans. In addition, the life-time cancer risk calculated for workers indicate concern for mutagenicity and carcinogenicity as a consequence of

inhalation and dermal exposure for all worker scenarios (1, 2 and 3). Therefore it is considered that risk reduction measures are required and **conclusion (iii)** applies.

Regarding repeated dose toxicity and toxicity for reproduction (fertility), the calculated MOS are judged not to be enough for one worker scenario (2) as a consequence of inhalation, and for two worker scenarios (2 and 3) as a consequence of dermal exposure. Consequently, **conclusion (iii)** can be derived for inhalation for scenario 2, and for dermal exposure for scenarios 2 and 3.

On the other hand, there is no concern for the remaining end-points: acute toxicity by inhalation and dermal route; irritation/corrosivity for skin and eyes; sensitisation; repeated dose toxicity and toxicity for reproduction (fertility) for two worker scenarios (1 and 3) by inhalation and for one worker scenario (1) by dermal route. Therefore, **conclusion (ii)** applies.

4.1.3.3 Consumers

Exposure of the consumers is not assumed to exist. Therefore, the **conclusion (ii)** is reached.

4.1.3.4 Humans exposed via the environment

The human exposure estimations (EUSES) via the environment both for local (four sites) and regional scenarios were used in the risk characterisation. They are summarised in section 4.1.1.4 (Table 4.1.1.4). The values for the total human intake of 2,4-dinitrotoluene are compared with the LOAEL of 0.57 mg/kg b.w. (rat oral chronic).

Comparison of exposure and effects

When considering the risks to human health arising from indirect exposure to 2,4-dinitrotoluene via the environment the key areas of concern are for repeated dose toxicity, mutagenicity, carcinogenicity and toxicity for reproduction (fertility).

4.1.3.4.1 Repeated dose toxicity

The LOAEL of 0.57 mg/kg b.w. derived from the chronic oral toxicity study with rats is used as starting point for the risk characterization for repeated dose toxicity. Comparing this LOAEL with the estimated internal total human daily intake levels, the calculated MOSs are 42222, 2953, 459677 and 103074 for local scenarios A, B, D and E, respectively, and 655172 for the regional scenario.

The minimal MOS is considered to be 300 based on the following assessment factors: 4 x 2.5 for interspecies differences, 10 for intraspecies differences and 3 for extrapolation LOAEL to NOAEL. Accordingly, the comparison between these MOS and minimal MOS indicates no concern for the regional scenario and for all local scenarios and **conclusion (ii)** is reached.

4.1.3.4.2 Mutagenicity

2,4-dinitrotoluene has shown to be an *in vivo* mutagenic agent for somatic cells of mammals. Therefore, it is of concern for mutagenicity and **conclusion (iii)** is reached.

4.1.3.4.3 Carcinogenicity

A quantitative risk characterization for carcinogenicity according to the T25 approach (Draft TGD 2005) was performed for 2,4-DNT in order to estimate the level of concern for the different scenarios, as described in "4.1.3.2.6. Carcinogenicity" under section "4.1.3.2. Workers". The HT25 value of 4.13 mg/kg bw/day on the basis of hepatocellular carcinomas in low-dose male rats derived from the carcinogenicity study was the critical value for carcinogenicity risk characterisation (4.1.3.2.6. Carcinogenicity under section 4.1.3.2. Workers).

The lifetime cancer risks for humans exposed via the environment (eLR) are obtained directly from HT25 and scenario-specific exposures (eE) by the formula eLR = eE/(HT25/0.25).

The life-time cancer risk for human exposed via environment of local site B scenario was higher than 10^{-5} (Table 4.1.3.4.6-1).

Table 4.1.3.4.3-1: Life-time cancer risk for humans exposed via environment

Scenario	Exposure [(1)]	eLR [(2)]
Local site A	$1{,}35 \cdot 10^{-5}$	$8{,}17 \cdot 10^{-7}$
Local site B	$1{,}93 \cdot 10^{-4}$	$1{,}17 \cdot 10^{-5}$
Local site D	$1{,}24 \cdot 10^{-6}$	$7{,}51 \cdot 10^{-8}$
Local site E	$5{,}53 \cdot 10^{-6}$	$3{,}35 \cdot 10^{-7}$
Regional	$8{,}70 \cdot 10^{-7}$	$5{,}27 \cdot 10^{-8}$

(1) Exposure (mg/kg bw/day)
(2) eLR, life-time cancer risk for humans exposed via environment calculated by eLR = eE/(HT25/0.25); HT25 value of 4.13 mg/kg bw/day on the basis of hepatocellular carcinomas in low-dose male rats derived from the carcinogenicity study.

2,4-dinitrotoluene is classified as carcinogenic category 2. There is a good evidence of an increase in tumour incidence in rats and mice, consistent with a genotoxic mechanism of carcinogenicity supported by findings from the genotoxicity studies. Overall, **conclusion (iii)** is reached in local site B scenario.

4.1.3.4.4 Toxicity for reproduction

Fertility

The LOAEL of 0.57 mg/kg b.w. derived from a 24-month rat oral toxicity study is used as starting point for the risk characterization. Comparing this LOAEL with the estimated internal total human daily intake levels, the calculated MOSs are 42222, 2953, 459677 and 103074 for local scenarios A, B, D and E, respectively, and 655172 for the regional scenario. Taking into account that the minimal MOS is considered to be 300, these

calculated MOSs indicate no concern for the regional scenario and for all local scenarios and **conclusion (ii)** is reached.

Development

Based on available data, developmental toxicity is regarded as a secondary effect due to parental systemic. Therefore, for developmental toxicity effects, **conclusion (ii)** is reached.

4.1.3.4.5 Summary of risk characterization for humans exposed via the environment

When considering the risks to human health arising from indirect exposure to 2,4-dinitrotoluene via environment the key areas of concern are for mutagenicity and carcinogenicity.

The mutagenicity and carcinogenicity effects of 2,4-dinitrotoluene do not allow the identification of a threshold level of exposure below which there would be no risk for the development of these effects in humans. However, based on the calculated life time cancer risk, the risk is judged to be tolerable for the regional scale and for local sites (A, D and E). Nevertheless, the calculated life-time cancer risk indicates concern for carcinogenicity for the local site B. Therefore, conclusion **(iii)** is reached for both carcinogenicity and mutagenicity as a consequence of oral exposure arising from the for the local site B.

The calculated MOS for oral exposure of man via the environment in both local and regional scales are judged to be enough regarding repeated dose toxicity and toxicity for reproduction (fertility) and **conclusion (ii)** is reached.

4.1.3.5 Combined exposure

Exposure to 2,4-dinitrotoluene may reasonably be predicted to arise as a result of combined exposure from workplace and environmental sources.

The risk to human health under conditions of combined exposure is dominated by occupational exposure.

4.2 HUMAN HEALTH (PHYSICO-CHEMICAL PROPERTIES)

4.2.1 Exposure assessment

4.2.1.1 Workers

2,4-dinitrotoluene is not volatile and not flammable. Thermal and hazardous decompositions are not expected when handled correctly.

4.2.1.2 Consumers

2,4-dinitrotoluene has not been detected in consumer products.

4.2.1.3 Humans exposed via the environment

Not applicable.

4.2.2 Effects assessment: Hazard identification

4.2.2.1 Explosivity

2,4-dinitrotoluene does not show a transmission of detonation under 150º C.

4.2.2.2 Flammability

2,4-dinitrotoluene is not a flammable solid (autoflamability: 300º C). It does not ignite readily.

4.2.2.3 Oxidizing potential

2,4-dinitrotoluene is not an oxidising agent.

4.2.3 Risk characterisation

4.2.3.1 Workers

There is no risk of concern in the industry setting, regarding its physico-chemical properties. Adequate safety measures are taken and information is provided on the label and safety data sheet. Therefore, since risk reduction measures already being applied are considered sufficient, **conclusion (ii)** is reached.

4.2.3.2 Consumers

Concerning use by consumers, no current exposure has been identified. Therefore, **conclusion (ii)** is reached.

4.2.3.3 Humans exposed via the environment

Not applicable.

5 RESULTS [7]

5.1 INTRODUCTION

5.2 ENVIRONMENT

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Conclusion (ii) applies to the aquatic and sediment compartment at continental and regional level and for sites A, D and E.

Conclusion (ii) applies to the marine compartment.

Conclusion (ii) applies to the STP compartment.

Conclusion (ii) applies to the atmospheric compartment.

Conclusion (ii) applies to the terrestrial compartment and,

Conclusion (ii) also applies to secondary poisoning according to the low bioaccumulation potential and the rapid elimination of this compound in fish and mammals, a low risk for secondary poisoning on birds and mammals is expected from this substance.

Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

Conclusion (iii) applies to the need of risk reduction measures for the aquatic compartment and for sediment-dwelling organisms for one site at local level (site B). Nevertheless, it is expected that any risk reduction measure for surface water would also reduce the risks for sediments.

[7] Conclusion (i) There is a need for further information and/or testing.
Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.
Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

5.3 HUMAN HEALTH

5.3.1 Human health (toxicity)

5.3.1.1 Workers

Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

This conclusion is reached because of:

- concerns for carcinogenicity and mutagenicity as a consequence of inhalation and dermal exposure arising from all worker scenarios.
- concerns for repeated dose toxicity and toxicity for reproduction (fertility) as a consequence of dermal exposure arising from manufacture and use of explosives (worker scenarios 2 and 3).
- concerns for repeated dose toxicity and toxicity for reproduction (fertility) as a consequence of inhalation arising from manufacture of explosives (worker scenario 2).

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

This conclusion applies to acute toxicity by inhalation and dermal route; irritation/corrosivity for skin and eyes; sensitisation; repeated dose toxicity and toxicity for reproduction (fertility) for two worker scenarios (1 and 3) by inhalation and for one worker scenario (1) by dermal route, because these endpoints are of no concern.

5.3.1.2 Consumers

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

This conclusion applies because exposure of consumers is not assumed to exist.

5.3.1.3 Humans exposed via the environment

Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

This conclusion is reached because of:

- concerns for carcinogenicity and mutagenicity as a consequence of oral exposure arising from the local site B**.**

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

This conclusion is reached for repeated dose toxicity and toxicity for reproduction (fertility) because the calculated MOS for oral exposure of man via the environment in both local and regional scales are judged to be enough for these endpoints.

5.3.1.4 Combined exposure

The risk to human health under conditions of combined exposure is dominated by occupational exposure.

5.3.2 Human health (risks from physico-chemical properties)

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

This conclusion is reached because the risk assessment shows that risks are not expected, and risk reduction measures already being applied are considered sufficient.

6 REFERENCES

Abernethy DJ and Couch DB (1982). Cytotoxicity and mutagenicity of dinitrotoluenes in Chinese hamster ovary cells. Mutat. Res., **103**, 53-59.

ACGIH, American Conference of Governmental Industrial Hygienists: (2001) TLVs® and BEIs®.

Adema, D.M.M., J.H. Canton, W. Slooff, and A.O. Hanstveit (1981). Research for a Useful Combination of Test Methods to Determine the Aquatic Toxicity of Environmentally Dangerous Chemicals (Consecutive System of Rep. No. CL81/100, Natl. Inst. Public Health Environ. Hyg.: 107 p. In: Aquire database (2004).

Adema, DMM and Henzen, L. (1989). A comparison of plant toxicities of some industrial chemicals in soil culture and soil-less culture. Ecotoxicology and Environmental Safety, **18**: 219-229.

Ahrenholz SH (1980). Health hazard evaluation determination. Report no HE 79-113-728. Olin Chemical Co., Brandenberg, K.Y. Hazard evaluations and technical assistance branch, NIOSH.

Ahrenholz SH and Meyer CR (1982). Health Hazard Evaluation. NIOSH, Cincinnati, Ohio. Report No. HETA 81-295-1155.

Al-Ghusain *et al*., Huang, C.P. (ed) (1994). 2,4-dinitrotoluene: toxicity evaluation and treatability studies. Hazardous and industrial wastes: proceedings of the 26th mid-atlantic industrial waste conference. University of Delaware, Newark, 61-68.

Andrews CC, Osmon JL. (1977). The effects of light on TNT and other explosives in aqueous solutions. Weapons Quality Engineering Center, Naval Weapons Support Center, Crane, Indiana. WQEC/C 77-32. NTIS AD-A0361 32.

Ashby J (1986). Carcinogen/mutagen screening strategies. Mutagenesis, **1**, 309-317.

ATSDR, Agency for Toxic Substances and Disease Registry (1998). US Department of Health and Human Services. Toxicological profile for 2,4- and 2,6-Dinitrotoluene.

Bailey, HC; Spangord, RJ; Javitz, HS and Liu, DHW. (1984). Toxicity of TNT (Trinitrotoluene) wastewater to aquatic organisms. Volume 4. Chronic toxicity of 2,4-dinitrotoluene and condensate water (AD-A153536). SRI International, Menlo jPark. California, USA, 80pp.

Bausum, HT *et al*., (1992). J Environ Sci. Health, **A27**: 663-695

Bausum HT, Mitchell WR, Major MA. (1992). Biodegradation of 2,4- and 2,6-dinitrotoluene by freshwater microorganisms. J Environ Sci Health **A27**: 663-695.

Bayer, AG. (1985). Acute toxicity of 2,4-dinitrotoluene to *Braquidario rerio* and *Leuciscus idus*. Unpublished report.

Bayer, AG. (1986). Bestimmung der Chronischen Daphnientoxizität. Unpublished report.

Bayer, AG. (1990). Phytotoxicity of DNT, unpublished report (1990).

Bayer, AG (1991). Anaerobic degradation of dinitrotoluene according to EPA guideline 796.3140. Unpublished report.

Bermudez E, Tiller-y D and Butterworth BE (1979). The effect of 2,4-dinitrotoluene and isomers of dinitrotoluene on unscheduled DNA synthesis in primary rat hepatocytes. Environ. Mutagen., **1**, 391-398.

Bloch E, Gondos B, Gatz M, Varma S and Thysen B (1988). Reproductive toxicity of 2,4-dinitrotoluene in the rat. Toxicol. Appl. Pharmacol., **94**, 466-472.

Bond JA, Medinsky MA, Dent JG and Rickert DE (1981). Sex-dependent metabolism and biliary excretion of [2,4-14C] dinitrotoluene in isolated perfused rat livers. J. Pharmacol. Exp. Ther., **219**, 598-603.

Bond JA and Rickert DE (1981). Metabolism of 2,4-dinitro[14C]toluene by freshly isolated Fischer-344 rat primary hepatocytes. Drug. Metab. Dispos., **9**, 10-14.

Bradley PM; Chapelle FH and Landmeyer JE. (1994). Microbial transformation of nitroaromatics in surface soils and aquifer materials. Appl. Environ. Microbiol, **60**: 2170-2175.

Bradley PM, Chapelle FH, Landmeyer JE. (1995). Degradation of 2,4-DNT, 2,6-DNT, and 2,4,6-TNT by indigenous aquifer microorganisms. Bioremediation of Recalcitrant Organics **7**:267-271.

Bradley, PM; Chapelle, FH; Landmayer, JE and Schumacher, JG. (1997). Potential for intrinsic bioremediation of a dinitrotoluene-contaminated aquifer. Ground Water, **35(1):** 12-17.

Bredow M and Jung F (1942). Arch. Exp. Pathol. Pharmakol., **200**, 335 – 353.

Bringmann, G. (1975). Bestimmung der biologischen Schadwirkung wassergefährder Stoffe aus der Hemmung der Zellvermehrung der Blualge Microcistis. Gesundheits-Ingenieur, **96** 11.9: 238-241.

Bringmann, G and Kühn, R. (1971). Biologischer Abbau von Nitrotoluolen und Nitrobenzolen mittels Azotobacter agilis. Gesundheits-Ingenierur, **9(92**): 273-276

Bringmann, G and Kühn, R. (1977). Befunde der Schadwirkung wassergefährdender Stoffe gegen *Daphnia magna*. Z. f. Wasser-und Abwasser-Forchung, **10:** 161-166.

Bringmann, G and Kühn, R. (1977b). Grenzwerte der Schdwikung wassegefährdender Stoffe gegen Bakterien (Pseudomonas putida) und Grünalgen (Scenedesmus quadricauda) im Zellvermehrungshemmtes. Z. f. Wasser-und Abwasser-Forchung, **10**: 87-98.

Bringmann, G and Kühn, R. (1981). Verleich der Wirkun von Schadstoffen auf flagellate sowie ciliate bzw. Auf holozoische bakterienfressende sowie saprozoische Protozoen. Gwf-Wasswe/Abwasser, **122** H7: 308-313.

Broderius, SJ; Kahl, MD and Hoglund, MD. (1995). Use joint toxic response to define the primary mode of toxic action for diverse industrial organic chemicals. Environemtnal Toxicology and chemistry, **14(9):** 1591-1605.

Brodsky, Jan, Brodesser, Josef, Bauer, Christa and Römbke, Jörg (1997). The environmental fate of six existing chemicals in laboratory test. Chemosphere, 3, 515-538.

Brüning T, Chronz C, Thier R, Havelka J, Ko Y and Bolt HM (1999). Occurrence of Urinary Tract Tumors in Miners Exposed to Dinitrotoluene. JOEM, **41**, 144-149.

BUA Report 41 (1989). Nitrotoluenes. GDCh-Advisory Committee on Existing Chemicals of Environmental Relevance. 104pp.

BUA, 1992. Dinitrotoluene. BUA report 12 (July 1987). Supplementary report (December 1992). Edited by the GDHh-Advisory Committee on Existing Chemicals of Environmental Relevance.

Bunch, R.L. and Chambers, C.W. (1967). A biodegradability test for organic compounds. J. Water Poll Control Fed., **39:** 181-187.

Butterworth BE, Smith-Oliver T, Earle L, Loury DJ, White RD, Doolittle DJ, Working PK, Cattley RC, Jirtle R, Michalopoulos G and Strom S (1989). Use of primary cultures of human hepatocytes in toxicology studies. Cancer. Res., **49**, 1075-1084.

Canton, J.H., D.M.M. Adema, and D. De Zwart. (1984). Research After the Usefulness of Three Egg-Laying Fish Species in Routine Toxicity Research Rep. No.668114-002, Natl.Inst.Public Health Environ.Hyg.:18 p. In: Aquire database (2004).

Carbonell, G., Ramos, C., Pablos, M.V., Ortiz, J.A. and Tarazona, J.V. (2000) A system dynamic model for the asssessment of different exposure routes in aquatic ecosystems. Science of Total Environment, 247, 107-118.

Carolina Biological Supply Co. http://www.carolina.com/.

Chism, JP; Turner, MJ and Rickert, DE. (1984). The metabolism and excretion of mononitrotoluenes by Fisher 344 rats. The America Society for Pharmacology and Experimental Therapeutics, **12(5):** 596-602.

Cotruvo JA (1977). Investigation of mutagenic effects of products of ozonation reactions in water. Ann. N.Y. Acad. Sci., **298**, 124-140.

Couch DB, Bermudez E, Decad GM and Dent JG (1979). The influence of activation systems on the metabolism of 2,4-dinitrotoluene and its mutagenicity to CHO cells. Banbury Rep., **2**, 303-309.

Couch DB, Allen PF and Abernethy DJ (1981). The mutagenicity of dinitrotoluenes in Salmonella typhimurium. Mutat. Res., **90**, 373-383.

CSTEE. (2003). Opinion of the Scientific Committee on Toxiciy, Ecotoxicity and the Environment on "The scientific basis of the national provisions on short-chains chlorinated paraffins (SCCPs) being more restricitive than those laid down in Directive 2002/ 45/EC that The Netherlands intends to maintain in accordance with article 95(4) of the EC Treaty. October 2003.

Davidova, IA and Suflita, JM. (1997). Transformation of 2,4-dinitrotoluene by anaerobic bacteria. Abstracts of the General Meeting of the American Society for Microbiology, Miami Beach. Florida, pp 514.

Davies EM; Murria, HE; Liehr, JG and Powers, EL. (1981). Basic microbial degradation rates and chemical by products of selected organic compounds. Water Res., **15:** 1125-1127.

Decad GM, Graichen ME and Dent JG (1982) Hepatic microsomal metabolism and covalent binding of 2,4-dinitrotoluene. Toxicol. Appl. Pharmacol., **62** (2), 325-334.

Deener, J.W., Sinnige, T.L., Seinen, W. and Hermens, J.L.M. (1987). Quantitative structure-activity relationship for the toxicity and bioconcentration factor of nitrobenzene derivates towards the guppy (*Poecilia reticulata*). Aquatic Toxicology, **10,** 115-129.

Dellarco VL and Prival MJ (1989). Mutagenicity of nitro compounds in Salmonella typhimurium in the presence of flavin mononucleotide in a preincubation assay. Environ. Mol. Mutagen., **13**, 116-127.

Dent JG, Schnell SR and Guest D (1981). Metabolism of 2,4-dinitrotoluene by rat hepatic microsomes and cecal flora. Adv. Exp. Med. Biol., **136**, 431-436.

Deutsche Forschungsgemeinschaft (2004), List of MAK and BAT Values.

DHHS (NIOSH) (1985). Current Intelligence Bulletin 44 Dinitrotoluene. US Department of Health and Human Services. DHHS (NIOSH) Publication No 85-109, Cincinnaty, Ohio.

Dillert R, Brandt M, Fomefett I, *et al*. (1995). Photocatalytic degradation of trinitrotoluene and other nitroaromatic compounds. Chemosphere **30:**2333-2341.

Dodard, SG; Renoux, AY; Hawari, J; Amplemen, G; Thiboutot, S and Sunahara, GI. (1999). Ecotoxicity characterisation of dinitrotoluene and some of their reduced metabolites. Chemosphere, **38(9):** 2071-2079.

Drzyzga, O; Gorontzy, T; Smidt, A; and Blotevogel, KH. (1995). Toxicity of explosives and related compounds to the luminescent bacterium Vibrio fischeri NRRL-B-11177. Arch. Environ. Contam. Toxicol, **28(2):** 229-235.

Dunkel VC, Zeiger E, Brusik D, McCoy E, McGregor D, Mortelmans K, Rosenkranz HS and Simmon VF (1985). Reproductibility of microbial mutagenicity assays: Il. Testing of carcinogens and noncarcinogens in Salmonella typhimurium and Escherichia coli. Environ. Mutagen., **7**, 1-248.

Einistö P, Watanabe M, Ishidate M and Nohmi T (1991). Mutagenicity of 30 chemicals in Salmonella typhimurium strains possessing different nitroreductase or O-acetyltransferase activities. Mutat. Res., **259**, 95-102.

Ellis HV III, Hodgson JR, Hwang SW, Halfpap LM, Helton DO, Andersen BS, VanGoethem D and Lee CC (1978). Mammalian toxicity of munition compounds: Phase I. Supplement. Acute oral toxicity, primary skin and eye irritation, dermal sensitization, disposition and metabolism and Ames test. USAMRCD Contract No. DAMD-17-74-C-4073. Report No. 6, 1-39.

Ellis HV, Hagensen JH, Hodgson JR, Minor JL, Hong CB, Ellis ER, Girvin JD, Helton DO, Herndon BL and Lee C.C (1979). Mammalian toxicity of munitions compounds. Phase BI: Effects of lifetime exposure. Part I. 2,4-Dinitrotoluene. Final report no. 7. Midwest Research Institute, Kansas City, MO. Contract no. DAMD 17-74-C-4073, ADA077 692.

Ellis HV, Hong CB, Lee CC, Dacre JC and Glennon JP (1985). Subchronic and chronic toxicity studies of 2,4-dinitrotoluene. Part I. Beagle dog. J. Am. Coll. Toxicol., **4**, 233-242.

Elwell, MR. (1996). NTP technical report on comparative toxicity and carcinogenicity studies of o-Nitrotoluene and o-Toluidine hydrochloride (CAS Nos 88-72-2 and 636-21-5). Administered in feed to male F344/N rats. US Department of Health and Human Services. Public Health Service National Institutes of Health. NIH Publication 96-3936.

EPA. (1971). Algal Assay Procedures: Bottle Test. National Eutrophication Research Program. Corvallis, Oregon. 82 pp.

EPA. (1975). Methods for acute toxicity testing with fish, macroinvertebrates, and amphibians. Ecotoxicological Research Series, EPA-66013-75-009, National Environmental Research

Center, Office of Research and Development, U.S. Environmental Protection Agency, Corvallis, Oregon.

Fannick N (1980). Health hazard evaluation determination report no. HHE-77-99-726, Dupont, Chambers Works Deepwater, N.J. Hazard evaluations and technical assistance branch, NIOSH Cincinnati, OH.

Freedman, DL; Shanley, RS and Scholze RJ. (1996). aerobic biodegradation of 2-4-dinitrotoluene, aminonitrotoluene isomers, and 2,4-diaminotoluene. J. Hazard Mater, **49(1):** 1-14.

Fricker C and Hardy JK (1992). Protective glove material permeation by organic solids. Am.Ind.Hyg.Assoc.J., **53** (12), 745-750.

Fricker C and Hardy JK (1994). The effect of an alternate environment as a collection medium on the permeation characteristics of solid organics through protective glove materials. Am.Ind.Hyg.Assoc.J., **55** (8), 738-742.

Fossi MC; Marsili L; Neri G; Casini S; Bearzi G; Politi E; Zanardelli M and Panigada S. (2000). Skin biopsy of Mediterranean cetaceans for the investigation of interspecies susceptibiloity to xenobiotic contaminants. Marine Environmental research, **50:** 517-521.

Fossi MC; Casini S; Marsili L; Ausili A and Notarbartolo di Sciara G. (2001b) Are Mediterranean top predators exosed to toxicological risks due to endocrine disrupters?. Ann. NY Acad. Sci, **948:** 67-74.

Furukawa H, Kawai N, Kawai K (1985). Frameshift mutagenicity of dinitrobenzene derivatives on Salmonella typhimurium Ta 98 and Tm elevation of calf thymus DNA by dinitrobenzene derivatives. Nucleic. Acids Symp. Ser., **16**, 5-8.

Guest D, Schnell SR, Rickert DE and Dent JG (1982). Metabolism of 2,4-dinitrotoluene by intestinal microorganisms from rat, mouse, and man. Toxicol. Appl. Pharmacol., **64**, 160-168.

Gustafson, D.I. (1989). Groundwater ubiquity score: A simple method for assessing pesticide leachability. Envir. Toxicol. Chem., **8:** 339-357.

Haderlein SB, Weissmahr KW, Schwarzenbach RP. (1996). Specific adsorption of nitroaromatic explosives and pesticides to clay minerals. Environ. Sci. Technol. **30,** 612-622.

Haidour, A and Ramos, JL. (1996). Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene and 2,6-dinitrotoluene by *Pseudomonas sp.*Environ Sci. Technol, **30**: 2365-2370.

Hanstveit, A.O., F.I. Kappers and Canton J.H.. (1985). Research for a Useful Combination of Tests Methods to Determine the Aquatic Toxicity of Environmentally Dangerous Chemicals-Further Research After the Rep. No. R85/083, Natl. Inst. Public Health Environ. Hyg.: 26 p.

Hamill PVV, Steinberger E, Levine RJ, Rodriguez-Rigau LJ, Lemeshow S and Spitz Avrunin J (1982). The epidemiologic assessment of male reproductive hazard from occupational exposure to TDA and DNT. J. Occup. Med., **24**, 985-993.

Harding B, Schuler R, Burg J, Booth G, Hazelden K, Mackenzis K, Piccirillo V and Smith K (1987). Teratogenesis Carcinogenesis and mutagenesis, 7, 29-48.

Hartley, WR. (1981). Evaluation of selected subacute effects of the nitrotoluene group of munitions compounds on fish and potential use in aquatic toxicity evaluations. Evaluation of subacute effects of 2,4-dinitrotoluene on the bluegill sunfish *Lepomis macrochirus*. Dissertation

of a study undertaken in partial fulfilment of the requirements for degree of Doctor Science. Tulane University School of Public Health and Tropical Medicine. New Orleans, LA 70112.

Hawkins, D.R.; Elsom, L.F.; Girkin, R.; Dighton, M.: The absorption and excretion of ^{14}C-2,6-dinitrotoluene after oral, dermal and inhalation administration. Huntingdon Research Center Ltd. (Huntingdon, England), unpublished report No. CMA 3/901373 from the 14.10.1991 to Chemical Manufacturers Association (Washington, USA).

Haworth S, Lawlor T, Mortelmans K, Speck W and Zeiger E (1983). Salmonella mutagenicity test results for 250 chemicals. Environm. Mutagen., **5**, 3-142.

Heimbach, F. (1990). Toxicity of dinitrotoluol to earthworms. Report Nº: HBF / Rg 133; Study Nº: E 310 0454-7. Bayer.

Ho PC. (1986). Photooxidation of 2,4-dinitrotoluene in aqueous solution in the presence of hydrogen peroxide. Environ Sci Technol **20:**260-267.

Hoechst (1977a). Akute orale Toxizität von 2,4-dinitrotoluol an männlichen SPF-Wistar-Ratten. Bericht Nr. 1110/77.

Hoechst (1977b). Akute orale Toxizität von 2,4-dinitrotoluol an weiblichen SPF-Wistar-Ratten. Bericht Nr. 1111/77.

Hong C.B, Ellis JV, Lee CC, Sprinz H, Dacre JC and Glennon JP (1985). Subchronic and chronic toxicity studies of 2,4-dinitrotoluene. Part III. CD-l mice. J. Am. Coll. Toxicol., **4**, 257-269.

Howard, P.H. (1989). Handbook of Environmental Fate and Exposure Data for Organic Chemicals. Lewis Publ., Chelsea, 574 pp.

Huang QG, Liu YB, Wang LS and Han S (1996a). Genotoxicity of substituted nitrobenzenes and the quantitative structure-activity relationship. J. Environm. Sci., **8** (1), 103-109.

Huang QG, Kong LR, Liu YB and Wang LS (1996b). Relationships between molecular structure and chromosomal aberrations in in vitro human lymphocytes induced by substituted nitrobenzenes. Bull. Environm. Contam. Toxicol., **57**, 349-353.

Hughes, JB; Wang, CY and Zhang, C. (1999). Anaerobic biotransformation of 2,4-dinitrotoluene and 2,6-dinitrotoluene by *Clostridium acetobutylicum*: a pathway through dihydroxylamino intermediates. Environmental Science and Technology., **33:** 1065-1070

Hunt RJ, Neubauer NR and Picone RF (1980). An improved procedure for sampling and analysis of dinitrotoluene vapor concentrations. Am. Ind. Hyg. Assoc. J., **41** (8), 592-599.

INSHT (2005), Límites de exposición profesional para agentes químicos en España. 2005.

Jackson, MM; Hou, L; Banerjee, HN; Sridhar, R; Dutta, SK. (1999). Disappearance of 2,4-dinitrotoluene and 2-amino,4,6-dinitrotoluene by *Phanerochaete chrysosporium* under non-lignolytic conditions. Bulleting of Environmental Contamination and Toxicology, **62:** 390-396.

JETOC (1996). Mutagenicity test data of existing chemical substances. Japan Chemical Industry Ecology-Toxicology and Information Center, Japan.

Jones CR, Liu Y-Y, Sepai O, Yan H and Sabbioni G (2005). Hemoglobin adducts in workers exposed to nitrotoluenes. Carcinogenesis, **26**, 133-143.

Kawai A, Goto S, Matsumoto Y and Matsushita H (1987). Mutagenicity of aliphatic and aromatic nitro compounds. Jpn. J. Ind. Health, **29**, 34-54.

Kedderis GL, Dyroff MC and Rickert DE (1984). Hepatic macromolecular covalent binding of the hepatocarcinogen 2,6-dinitrotoluene and its 2,4-isomer in vivo: modulation by the sulfotransferase inhibitors pentachlorophenol and 2,6-dichloro-4-nitrophenol. Carcinogenesis, **5**, 1199-1204.

Kozuka H, Mori M, Katayama K, Matsurashi T, Miyamara T, Mori Y and Magamara S (1978). Studies on Metabolism and Toxicity of dinitrotoluenes - Metabolism of Dinitrotoluenes by Rhodotorula glutinis and Rat Liver Homogenate. Hishi Kagaku., **24**, 252-259.

Kozuka H, Mori M and Nause Y (1979). Studies on the metabolism and toxicity of dinitrotoluenes. Toxicological study of 2,4-dinitrotoluene (2,4-DNT) in rats in long-term feeding. J. Toxicol. Sci., **4**, 221-228.

Künh, R; Pattard, M and Penark, K. (1988). Damaging effects of environmental chemicals in the *Daphnia magna* reproduction test as a basis for evaluation of the environmental hazard in the aquatic system. Report UBA-F8. Institut für Wasser- Berlin. Germany.

Künh, R and Pattard, M. (1990). Results of the harmful effects of water pollutants to green algae (*Scenedesmus subpicatus*) in the cell multiplication inhibition test. Water Research, **24** (1): 31-38.

La DK and Froines JR (1992a). Comparison of DNA adduct formation between 2,4 and 2,6-dinitrotoluene by 32P-postlabelling analysis. Arch. Toxicol., **66**, 633-640.

La DK and Froines JR (1992b). P-postlabelling analysis of DNA adducts from Fisher-344 rats asministered 2,4-diaminotoluene. Chem. Biol. Interactions, **83**, 121-134.

Lane RW, Simon GS, Dougherty RW, Egle JL, Borzelleca JR and Borzelleca JF (1985). Reproductive toxicity and lack of dominant lethal effects of 2,4-dinitrotoluene in the male rat. Drug Chem. Toxicol., **8**, 265-280.

Lang, PZ; Wang, Y.; Chen, DB; Wang, N.; Zhao, XM and Ding, Yz. (1997). Bioconcentration, elimination and metabolism of 2,4-dinitrotoluene in carps (*Cyprinus carpio L.*). Chemosphere, **35**(8): 1799-1815.

Lee CC, Dilley JV, Hodgson JR, Helton DD, Wiegand WJ, Roberts DN, Anderson BS, Halfpap LM, Jurtz LD and West N (1975). Mammalian toxicity of munition compounds: Phase I. Acute oral toxicity, primary skin and eye irritation, dermal sensitization, and disposition and metabolism. Report no. 1. Contract DAMD17-74-c-4073; Midwest Research Institute Project no. 3900-B.

Lee CC, Ellis HV, Kowalski JJ, Hodgson JR, Hwang SW, Short RD, Bhandari JC, Sanyer JL, Reddig TW and Minor JL (1978). Mammalian toxicity of munitions compounds. Phase II: Effects of multiple doses. Part II: 2,4-Dinitrotoluene. Progress report No. 3. Midwest Research Institute, Kansas City, MO. Contract no. DAMD 17-74-C-4073.

Lee CC, Hong CB, Ellis HV, Dacre JC and Glennon JP (1985). Subchronic and chronic toxicity studies of 2,4-dinitrotoluene. Part II. CD rats. J. Am. Coll. Toxicol., **4**, 243-256.

Lee YS, Hunter JV. (1985). Effect of ozonation and chlorination of Environmental Protection Agency priority pollutant. In: Jolley RL, Bull RJ, Davis WP, *et al*., eds. Water chlorination:

Chemistry, environmental impact and health effects. Vo1 **5**. Chelsea, MI: Lewis Publishers, Inc. 1515-1526.

Leonard TB, Adams T and Popp JA (1986). Dinitrotoluene isomer-specific enhancement of the expression of diethylnitrosamine-initiated hepatocyte foci. Carcinogenesis, **7**, 1797-1803.

Leonard TB, Graichen M.E. and Popp JA (1987). Dinitrotoluene isomer-specific hepatocarcinogenesis in F344 rats. JNCI, 79, 1313-1319.

Leonard TB, Lyght O and Popp JA (1983). Dinitrotoluene structure-dependent initiation of hepatocytes in vivo. Carcinogenesis, **4**, 1059-1061.

Levine RJ, Turner MJ, Crume YS, Dale ME, Starr TB and Rickert DE (1985). Assessing exposure to dinitrotoluene using a biological monitor. J. Occup. Med., **27** (9), 627-638.

Levine RJ, Andjelkovich DA, Kersteter SL, Arp EW, Balogh SA, Blunden PB and Stanley JM (1986). Heart disease in workers exposed to dinitrotoluene. J. Occup. Med., **28**, 811-816.

Liu, DH; Spanggord, RJ; Bailey, HC; Javitz, HS and Jones, DCL. (1983). Toxicity of TNT wastewaters to aquatic organisms. Volume II. Acute toxicity of condensate wastewater and 2,4-dinitrotoluene. SRI International. Report LSU-4262. Vol **2.** Menlo Park Califormia.

Liu, DH; Bailey, HC and Pearson, JG. (1983b). "Toxicity of a complex munitions wastewater to aquatic organisms". Aquatic toxicology and Hazard Assessment: Sith Simposium, ASTM STP 802, W.E. Bishop, RD Cardwell, and BB Heidolph, Eds., American society for Testing and Materials, Philadelphia, pp. 135-150.

Liu, DH; Thomson, K and Anderson, AC. (1984). Identification of nitroso compounds from biotransformation of 2,4-dinitrotoluene. Applied Environmental Microbiology, **47**(6): 1295-1298.

Long, RM and Rickert, DE. (1982). Metabolism and excretion of 2,6-dinitro[^{14}C]toluene in vivo and in isolated perfused rat livers. Drug Metab. Dispos., **10:** 455-458.

Löser E (1981). 2,4-dinitrotolulol, Untersuchungen zur akuten oralen Toxizität an männlichen und weiblichen Wistar-Ratten. Bayer AG.

Löser E (1982). 2,4-dinitrotolulol rein, Untersuchungen zur akuten dermalen Toxizität an männlichen und weiblichen Wistar-Ratten. Bayer AG.

Löser E and Schmidt WM (1984). 2,4-dinitrotolulol, Untersuchungen zur akuten oralen Toxizität an der Katze, Einfluss auf Met-Haemoglobingehalt und Zahl der HEINZ-Innenkörper im peripheren Blut. Bayer AG.

Loveday KS, Lugo MH, Resnick MA, Anderson BE and Zeiger E (1989). Chromosome aberration and sister chromatid Exchange tests in Chinese hamster ovary cells in vitro: II. Results with 20 chemicals, Environ. Mol. Mutagen., **13**, 60-94.

Maronpot RR, Witschi HP, Smith LH and McCoy JL (1983). Recent experience with the strain A mouse pulmonary tumor bioassay model. Environmental Science Research Series, **27**, 341- 349.

McGown EL, Knudsen JJ, Makovec GT and Marrs GE (1983). Fourteen-day feeding study of 2,4-dinitrotoluene in male and female rats. U.S. Army Medical Research and Development Command, Division of Research Support, Letterman Army Institute of Research. AD-A126069.

Medinsky MA and Dent JG (1983) Biliary excretion and enterohepatic circulation of 2,4-dinitrotoluene metabolites in Fischer-344 rats. Toxicol. Appl. Pharmacol., **68**, 359-366.

Mirsalis JC, Tysonn CK and Butterworth BE (1982). Detection of genotoxic carcinogens in the in vivo-in vitro hepatocyte DNA repair assay. Env. Mut., **4**, 553-562.

Mirsalis JC and Butterworth BE (1982). Induction of unscheduled DNA synthesis in rat hepatocytes following in vivo treatment with dinitrotoluene. Carcinog., **3**, 241-245.

Mitchell WR and Burrows EP (1995). Nitroreduction of 2,4-dinitrotoluene in vitro by cytochrome P-450 induced H4IIE cells. Chemosphere., **31** (2), 2767-2777.

MITI, (1992). Biodegradation and Bioaccumulation Fata of Existing Chemicals Based on the CSSL Japan. Compiled under the Supervision of Chemical Products Safety Division, Basic Industries Bureau MITI, Ed. By CITI, October 1992. Published by Japan Chemical Industry Ecology-Toxicology & Information Center.

Mori, M; Naruse, Y and Kozuka, H. (1977). Studies on the metabolism and toxicity of dinitrotoluenes –on the absorption and excretion of tritium-labelled 2,4-dinitrotoluene (3H-2,4-dinitrotoluene) in the rat. Radioisotopes, **26:** 780-783.

Mori M, Naruse Y and Kozuka H (1980). Studies on the metabolism and toxicity of dinitrotoluenes changes of excretion, distribution and metabolism of 3H-2,4-dinitrotoluene 3H-2,4-DNT) in rats. Radioisotopes, **29** (7), 338-340.

Mori M, Naruse Y and Kozuka H (1981). Identification of urinary metabolites of 2,4-dinitrotoluene 2,4-DNT) in rats. Chem. Pharm. Bull (Tokyo)., **29**, 1147-1150.

Mori M, Miyahara T, Taniguchi K, Hasegawa K, Kozuka H, Miyagoshi M and Nagayama T (1982). Mutagenicity of 2,4-dinitrotoluene and its metabolites in Salmonella typhimurium. Toxicol. Lett., **13**,1-5.

Mori M, Matsuhashi T, Miyahara T, Shibata S, Izima C and Kozuka H (1984a). Reduction of 2,4-dinitrotoluene by Wistar rat liver microsomal and cytosol fractions. Toxicol. Appl. Pharmacol., **76**, 105-112.

Mori M, Miyahara T, Hasegawa Y, Kudo Y and Kozuka H (1984b). Metabolism of dinitrotoluene isomers by Escherichia coli isolated from human intestine. Chem. Pharm. Bull (Tokyo)., **32**, 4070-4075.

Mori M, Kudo Y, Nunozawa T, Miyahara T and Kozuka H (1985a). Intestinal metabolism of 2,4-dinitrotoluene in rats. Chem. Pharm. Bull (Tokyo)., **33**, 327-332.

Mori M, Miyahara T, Moto-o K, Fukukawa M, Kozuka H, Miyagoshi M and Nagayama T (1985b). Mutagenicity of urinary metabolites of 2,4-dinitrotoluene to Salmonella typhimurium. Chem. Pharm. Bull., **33**, 4556-4563.

Mori M, Kawajiri T, Sayama M, Miyahara T and Kozuka H (1989). Metabolism of 2,4-dinitrotoluene and 2,6-dinitrotoluene, and their dinitrobenzyl alcohols and dinitrobenzaldehydes by Wistar and Sprague-Dawley rat liver microsomal and cytosol fractions. Chem. Pharm. Bull (Tokyo)., **37**, 1904-1908.

Mori MA, Shoji M, Dohrin M, Kawagoshi T, Honda T and Kozuka H (1996). Further studies on the urinary metabolites of 2,4-dinitrotoluene and 2,6-dinitrotoluene in the male Wistar rat. Xenobiotica., **26**, 79-88.

Nakamura S, Oda Y, Shimada T, Oki I and Sugimoto K (1987). SOS-inducing activity of chemical carcinogens and mutagens in Salmonella typhimurium TA1535/pSK 1002: examination with 151 chemicals. Mutat. Res., **192**, 239-246.

NCI. (1978). Bioassay of 2,4-dinitrotoluene for possible carcinogenicity. CAS No. 121-14-2. Washington, DC: National Cancer Institute, U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health. NCI-CG-TR-54.

Neumeier, W, Hass, R & Von Löw, E. (1989). Mikrobieller Abbau von Nitroaromaten aus einer ehemaligen Sprengstoffproduktion. Forum Städte Hyg., **40:** 32-37.

NIOSH (1980). Health Hazard Evaluation Determination Report. National Institute for Occupational Health, Cincinnati, Ohio. BE 79-113-728.

Nishino, SF; Paoli, GC and Spain, JC. (2000). Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. Appl. Environ Microbiol., **66 (5):** 2139-2147.

Noguera DR, Freedman DL. (1996). Reduction and acetylation of 2,4-dinitrotoluene by a Pseudomonas aeruginosa strain. Applied Environ Microbial **62**: 2257-2263.

Noguera, DR and Freeman, DL. (1997). Characterisation of products from the biotransformation of 2,4-dinitrotoluene by denitrifying enrichment cultures. Water Environ Res., **69**(3): 260-268.

OECD, (1997). Screening Information Datasheets (SIDS) for High Production Volume Chemicals. Processed by IRPTC International Register of Potentially Toxic Chemicals. VOLUME **4**, part 1. A Contribution to International Programme on Chemical Safety (IPCS).

Oda Y, Shimada T, Watanabe M, Ishidate M Jr. and Nohmi T (1992). A sensitive umu test system for the detection of mutagenic nitroarenes in Salmonella typhimurium NM 1011 having a high nitroreductase activity. Mutat. Res., **272**, 91-99.

Oda Y, Yamazaki H, Watanabe M, Nohmi T and Shimada T (1993). Highly sensitive umu test system for the detection of mutagenic nitroarenes in Salmonella typhimurium NM3009 having high O-acetyltransferase and nitroreductase activities. Environ. Mol. Mut., **21**, 357-364.

OECD-SIDS (2004). SIDS initial assessment report on the OECD HPV chemical 2,4-dinitrotoluene.

OSHA (1983). Sampling and Analytical Methods. Method 44.

Öztürk K and Durusoy M (1999). The detection and comparison of the genotoxic effects of some nitro aromatic compounds by the umu and SOS chromotest systems. Toxicol. Lett., **108**, 63-68.

Pearson, JG; Glennon, JP; Barkley, JJ and Highfill, JW. (1979). An approach to the toxicological evaluation of a complex industrial wastewater. Aquatic Toxicology. ASTM STP 667, LL Marking and RA Kimirie, Eds., American Society for Testing and Materials. 284-301 pp.

Price CJ, Rochelle WT, Marks TA, Paschke LL, Ledoux TA and Reel JR (1985). Teratologic evaluation of dinitrotoluene in the Fischer 344 rat. Fundamental and Applied Toxicology, **5**, 948-961.

Randall, T.L., and P.V. Knopp. (1980). Detoxification of Specific Organic Substances by Wet Oxidation J.Water Pollut.Control Fed. **52**(8): 2117-2130. In: Aquire database (2004).

Rickert DE and Long RM (1980). Tissue distribution of 2,4-dinitrotoluene and its metabolites in male and female Fischer-344 rats. Toxicol. Appl. Pharmacol., **56**, 286-293.

Rickert DE and Long RM (1981). Metabolism and excretion of 2,4-dinitrotoluene in male and female Fischer 344 rats after different doses. Drug. Metab. Dispos., **9**, 226-232.

Rickert, DE; Long, RM; Krakowa, S and Dent, JG. (1981). Metabolism and excretion of 2,4-[^{14}C]dinitrotoluene in convectional and axenic Fisher-344 rats. Toxicology and Applied Pharmacology, **59:** 574-579.

Rickert DE, Schnell SR and Long RM (1983). Hepatic macromolecular covalent binding and intestinal disposition of [14C]dinitrotoluenes. J. Toxicol. Environ. Health, **11**, 555-567.

Rickert, DE; Chism, JP and Kedderis, GL. (1986). Metabolism and carcinogenicity of nitrotoluenes. Advances in experimental medicine and biology, **197:** 563-571.

Rickert, DE. (1987). Metabolism of nitroaromatic compounds. Drug Metabolism Review, **18**(1): 23-53.

Rippen, G. (1987). Handbuch Umweltchemikalien. Stoffstaen - Prüfverfahren - Vorschriften. ecomed Verlag, Landsberg, Loseblattsammlung.

RISKOFDERM, (2003). Deliverable **43:** Final report of work part 3. Responsible partner: TNO.

Roembke J, Bauer C, Brodesser J, Brodsky J, Danneberg G, Heimann D, Renner I, Schallnass H-J (1995). Grundlagen fuer die Beurteilung des oekotoxikologischen Gefaehrdungspotentials von Altstoffen im Medium Boden - Entwicklung einer Teststrategie (Basis for the Assessment of the Ecotoxicological Potential of "Old Chemicals" in the Terrestrial Environment - Development of a Testing Strategy). Research Report UBA-FB 106 04 103, UBA Text 53/95.

Roth M, Murphy JM. (1979). Evaluation of the ultraviolet-ozone and ultraviolet-oxidant treatment of pink water. Cincinnati, OH: U.S. Environmental Protection Agency, Office of Research and Development, Industrial Environmental Research Laboratory. EPA/600/12. NTIS PB300763.

Rowland IR, Mallett AK, Wise A and Bailey E (1983). Effect of dietary carrageenan and pectin on the reduction of nitro-compounds by the rat caecal microflora. Xenobiotica., **13**, 251-256.

RTECS, NIOSH, (2004). XT1575000

Santodonato J, Bosch S, William N, Becker J and Neal M (1985). Monograph on human exposure to chemicals in the workplace: 2,4-Dinitrotoluene. National Cancer Institute, U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health. NCI-CP-26002-03.

Sayama M, Mori M, Ishida M, Okumura K and Kozuka H (1989). Enterohepatic circulation of 2,4-dinitrobenzaldehyde, a mutagenic metabolite of 2,4-dinitrotoluene, in male Wistar rat. Xenobiotica., **19**, 83-92.

Sayama M, Mori M, Shoji M, Uda S, Kakikawa M, Kondo T and Kodaira K (1998). Mutagenicities of 2,4- and 2,6-dinitrotoluenes and their reduced products in Salmonella typhimurium nitroreductase- and O-acetyltransferase-overproducing Ames test strains. Mutat. Res., **420**, 27-32.

Schut HAJ, Loeb TR and Stoner GD (1982). Distribution, elimination, and test for carcinogenicity of 2,4-dinitrotoluene in strain A mice. Toxicol. Appl. Pharmacol., **64**, 213-220.

Schut HA, Dixit R, Loeb TR and Stoner GD (1985). In vivo and in vitro metabolism of 2,4-dinitrotoluene in strain A/J mice. Biochem. Pharmacol., **34**, 969-976.

Shoji M, Mori M, Moto-o K, Kozuka H and Honda T (1985). High-performance liquid chromatographic determination of urinary metabolites of 2,4-dinitrotoluene in Wistar rats. Chem. Pharm. Bull (Tokyo), **33**, 1687-1693.

Shoji, M; Mori, M-A; Kawajiri, T; Sayama, M; Mori, Y; Miyahara, T; Honda, T and Kozuza, H. (1987). Metabolism of 2,4-Dinitrotoluene, 2,4-Dinitrobenzyl alcohol and 2,4-Dinitrobenzaldehyde by rat liver microsomal and cytosol fractions. Chem. Pharm Bull, **35**(4): 1579-1586.

Short RD, Dacre JC and Lee CC (1979). A species comparison of 2,4-dinitrotoluene metabolism in vitro. Experientia, **35**, 1625-1627.

Simmons, M. S. and Zeep, Richard G. (1986). Influence of humic substances on photolysis of nitroaromatic compounds in aqueous systems. Wat. Res., 20, 899-904.

Sina JF, Bean CL, Dysart GR, Taylor VI and Bradley MO (1983). Evaluation of the alkaline elution/rat hepatocyte assay as a predictor of carcinogenic/mutagenic potential. Mutat. Res., **113**, 357-391.

Slaga TJ, Triplett LL, Smith LH and Witscni HP (1985). Carcinogenesis of nitrated toluenes and benzenes skin and lung tumor assays in mice. Oak Ridge National Laboratory, Biology Division. Oak Ridge. Army Project Order No. 1809 DOE-IAG 40-1016-79.

Smith KN (1983). Determination of the reproductive effects in mice of nine selected chemicals. NIOSH, Cincinnati, Ohio. 210-81-6011.

Smith EF, Smith HJ and Kuchar EJ (1995). Monitoring of dinitrotoluene and its metabolites in urine by spectrophotometry of their coupled aryldiazonium salts. Am. Ind. Hyg. Assoc. J., **56** (12): 1175-1179.

Soares ER and Lock LF (1980). Lack of indication of mutagenic effects of dinitrotoluenes and diaminotoluenes in mice. Environ. Mutagen., **2**, 111-124.

Spanggord RJ, Mortelmans KE, Griffin AF and Simmon VF (1982). Mutagenicity in Salmonella typhimurium and structure-activity relationships of wastewater components emanating from the manufacture of trinitrotoluene. Environ. Mutagen., **4**, 163-179.

Stayner LT, Dannenberg AL, Thun M, Reeve G, Bloom TF, Boiniger M and Halperin W (1992). Cardiovascular mortality among munitions workers exposed to nitroglycerin and dinitrotoluene. Scand. J. Work Environ. Health, **18** (1), 34-43.

Stayner LT, Dannenberg AL, Bloom T and Thun M (1993). Excess hepatobiliary cancer mortality among munitions workers exposed to dinitrotoluene. J. Occup. Med., **35** (3), 291-296.

Steiner,AA. (1968). Soilless culture. In: Proceedings of the 6th colloquium of the Internationsl Potash Institute Florence/Itale. Int. Potash Institute, Berne, Switzerland.

Stoner GD, Greisiger EA, Schut HAJ, Pereira MA, Loeb TR, Klaunig JE and Branstetter DG (1984). A comparison of the lung adenoma response in Strain A/J mice after intraperitoneal and oral administration of carcinogens. Toxicol. Appl. Pharmacol., **72**, 313-323.

Stoner GD (1991). Lung tumors in strain A mice as a bioassay for carcinogenicity of environmental chemicals. Experimental Lung Research, **17**, 405-423.

Styles JA and Cross MF (1983). Activity of 2,4,6-trinitrotoluene in an in vitro mammalian gene mutation assay. Cancer. Lett., **20**, 103-108.

Swedish Work Environment Authority, AFS 2000:3. Occupational exposure limit values and measures against air contaminants.

Tabak, HH; Quave, SA; Mashni, CI and Barth, EF. (1981). Biodegradability studies for predicting the environmental fate of organic priority pollutants. USEPA. Municipal Environmental Protection Agency.

Tanabe, S and Tatsukawa, R. (1992). Chemical modernization and vulnerability of cetaceans: increasing toxic threat of organochlorine contaminants. In: Persistent pollutants in marine ecosystems. Edited by CH Walker and DR Livingstone. Pergamon Press. 161-177.

Tokiwa H, Nakagawa R and Ohnishi Y (1981). Mutagenic assay of aromatic nitro compounds with Salmonella typhimurium. Mutat. Res., **91**, 321-325.

Turner MJJr., Levine RJ, Nystrom DD, Crume YS and Rickert DE (1985). Identification and quantification of urinary metabolites of dinitrotoluenes in occupationally exposed humans. Toxicol. Appl. Pharmacol., **80**, 166-174.

USEPA. (1999). Risk Reduction Engineering Laboratory, Cincinnati. Ohio: Treatability database, version 4.0.

Van den Dikkenberg, RP; Canton, LA; Mathijssen-Spiekman, LAM and Roghair, CJ. (1989). Usefulness of *Gasterosteus aculeatus* – the three-spined sticklebacks - as a test organisms in routine toxicity tests. Report. Order No PB90-244989. Avail. NTIS. Rijksinst. Volksgezond. Milieuhyg. RIVM. Bilthoven. Netherlands., 28 pp.

Valli, K; Brock, BJ; Joshi, DK and Gold, MH. (1992). Degradation of 2,4-dinitrotoluene by the lignin-degrading *Fungus phanerochaete chrysosporium*. Appl. Environ. Microbiol., **58**(1): 221-228

Vernot EH, MacEwen JD, Haun CC and Kinkead ER (1977). Acute toxicity and skin corrosion data for some organic and inorganic compounds and aqueous solutions. Toxicol. Appl. Pharmacol., **42**, 417-423.

Wang, Y; Wang, Z; Wang, C and Wang W. (1999). Uptake of weakly hydrophobic nitroaromatics from water by semipermeable membrane devices (SPMDs) and by Goldfish (*Carassius auratus*). Chemosphere, **38**(1): 51-66.

Warren ND, Marquart J, Christopher Y, Laitinen J and van Hemmen JJ (2006). Task-based dermal exposure models for regulatory risk assessment. Ann. Occup. Hyg., (*in press*).

Woodruff RC, Mason JM, Valencia R and Zimmering S (1985). Chemical mutagenesis testing in Drosophila. V. Results of 53 coded compounds tested for the National Toxicology Program. Environ. Mutagen., **7**, 677-702.

Woollen BH, Hall MG, Craig R and Steel GT (1985). Dinitrotoluene: an assessment of occupational absorption during the manufacture of blasting explosives. Int. Arch. Occup. Environ. Health., **55** (4), 319-330.

Working PK and Butterworth BE (1984). An assay to detect chemically induced DNA repair in rat spermatocytes. Environ. Mutagen., **6**, 273-286.

ABBREVIATIONS

2A4AAB	2-Amino-4-Acetylaminobenzoic acid
2A4AAT	2-Amino-4-Acetylaminotoluene
2AA4AT	2-(N-Acetyl)Amino-4-Aminotoluene
4AA2AT	4-(N-acetyl)amino-2-aminotoluene
4A2NB,	4-Amino-2-Nitrobenzyl alcohol
ADI	Acceptable Daily Intake
AF	Assessment Factor
A/G	Albumin/Globulin
AHH	Aryl Hydrocarbon Hydroxylase
ALT	Alanine Aminotransferase
2A4NB	2-Amino-4-Nitrobenzyl Alcohol,
2A4NT	2-Amino-4-Nitrotoluene
4A2NT	4-Amino-2-Nitrotoluene
ASTM	American Society for Testing and Materials
ATP	Adaptation to Technical Progress
AUC	Area Under The Curve
B	Bioaccumulation
BBA	Biologische Bundesanstalt für Land- und Forstwirtschaft
BCF	Bioconcentration Factor
BF	5,6-Benzoflavone
BMC	Benchmark Concentration
BMD	Benchmark Dose
BMF	Biomagnification Factor
bw	body weight / *Bw*, *b.w.*
C	Corrosive (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)
CA	Chromosome Aberration
CA	Competent Authority
CAS	Chemical Abstract Services
CEC	Commission of the European Communities
CEN	European Standards Organisation / European Committee for Normalisation
CHL	Chinese Hamster Lung
CHO	Chinese Hamster Ovary
CI	Confidence Interval
CMR	Carcinogenic, Mutagenic and toxic to Reproduction
CNS	Central Nervous System

COD	Chemical Oxygen Demand
CSTEE	Scientific Committee for Toxicity, Ecotoxicity and the Environment (DG SANCO)
CT_{50}	Clearance Time, elimination or depuration expressed as half-life
2,4-DAT or 24DAT	2,4-Diaminotoluene
$D_{b.w.}$	body-weight-corrected Dose
D_c	Exposure/observation-period-corrected dose
D_{diet}	Dietary Concentration
d.wt	dry weight / dw
DF	Dead foetuses
Dfi	daily food intake
DG	Directorate General
DIN	Deutsche Industrie Norm (German norm)
2,2'-DM-5,5'-DNAOB	2,2'-Dimethyl-5,5'-Dinitroazoxybenzene
4,4'-DM-3,3'-DNAOB	4,4'-Dimethyl-3,3'-Dinitroazoxybenzene
DMSO	Dimethyl Sulfoxide
DNA	DeoxyriboNucleic Acid
2,4-DNAl	2,4-Dinitrobenzaldehyde
2,4-DNB	2,4-Dinitrobenzyl Alcohol
2,4-DNBA	2,4-dinitrobenzoic acid
2,4-DNT	2,4-Dinitrotoluene
2,6-DNT	2,6-Dinitrotoluene
DNT	Dinitrotoluene
DOC	Dissolved Organic Carbon
DT50	Degradation half-life or period required for 50 percent dissipation / degradation
DT90	Period required for 50 percent dissipation / degradation
E	Explosive (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)
EASE	Estimation and Assessment of Substance Exposure Physico-chemical properties [Model]
EbC50	Effect Concentration measured as 50% reduction in biomass growth in algae tests
EC	European Communities
EC10	Effect Concentration measured as 10% effect
EC50	median Effect Concentration
ECB	European Chemicals Bureau
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECVAM	European Centre for the Validation of Alternative Methods
EDC	Endocrine Disrupting Chemical
eE	Environmental exposure

EEC	European Economic Communities
EH	Hepatic Epoxide Oxidase
EINECS	European Inventory of Existing Commercial Chemical Substances
ELINCS	European List of New Chemical Substances
eLR	Life-time cancer risk for humans exposed via the environment
EMS	Ethylmetthanesulphonate
EN	European Norm
EPA	Environmental Protection Agency (USA)
ErC50	Effect Concentration measured as 50% reduction in growth rate in algae tests
ESD	Emission Scenario Document
EU	European Union
EUSES	European Union System for the Evaluation of Substances [software tool in support of the Technical Guidance Document on risk assessment]
F(+)	(Highly) flammable (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)
FAO	Food and Agriculture Organisation of the United Nations
FELS	Fish Early Life Stage
FID	
FMN	Flavin Mononucleotide
FSH	Follicle-Stimulating Hormone
GC	Gas Chromatography
GDR	German Democratic Republic
GGT	Gamma-Glutamyltranspeptidase)
GI	Gastrointestinal
GLP	Good Laboratory Practice
GSD	Geometric standard Deviation
GOT	Aspartate Aminotransferase
GST	Glutathione S-Transferase
2HA4NT	2-Hydroxylamino-4-Nitrotoluene
4HA2NT	4-hydroxylamino-2-nitrotoluene
Hb	Haemoglobin
HBBS	Hanks Balanced Salt Solution
HEDSET	EC/OECD Harmonised Electronic Data Set (for data collection of existing substances)
HELCOM	Helsinki Commission -Baltic Marine Environment Protection Commission
HPLC	High Pressure Liquid Chromatography
HGPRT	Hypoxanthine-Guanine Phosphoribosyl transferase
HPVC	High Production Volume Chemical (> 1000 t/a)
IARC	International Agency for Research on Cancer

IC	Industrial Category
IC50	median Immobilisation Concentration or median Inhibitory Concentration
2HA4NT	2-Hydroxylamino-4-Nitrotoluene
4HA2NT	4-Hidroxylamino-2-Nitrotoluene
HT25	Human equivalent of T25
IHD	Ischemic Heart Disease
ILO	International Labour Organisation
i.p. or IP	Intraperitoneal
IPCS	International Programme on Chemical Safety
I	Cancer incidence after correction for spontaneous incidence
ISO	International Organisation for Standardisation
IUCLID	International Uniform Chemical Information Database (existing substances)
IUPAC	International Union for Pure and Applied Chemistry
JEFCA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
Koc	organic carbon normalised distribution coefficient
Kow	octanol/water partition coefficient
K_m	Michaelis Constant
Kp	solids-water partition coefficient
L(E)C50	median Lethal (Effect) Concentration
LAEL	Lowest Adverse Effect Level
LC50	median Lethal Concentration
LD50	median Lethal Dose
LDH	Lactate Dehydrogenase
LEV	Local Exhaust Ventilation
LF	Living foetuses
LH	Luteinizing Hormone
LLNA	Local Lymph Node Assay
LAEL	Lowest Adverse Effect Level in humans
LOAEL	Lowest Observed Adverse Effect Level
LOD	Limit of Detection
LOEC	Lowest Observed Effect Concentration
LOED	Lowest Observed Effect Dose
LOEL	Lowest Observed Effect Level
MAC	Maximum Allowable Concentration
MATC	Maximum Acceptable Toxic Concentration
MC	Main Category

MC	3-Methylcholantrene
MetHb	Methaemoglobin
MITI	Ministry of International Trade and Industry, Japan
MOE	Margin of Exposure
MOS	Margin of Safety
MS	Mass Spectrum
MW	Molecular Weight
N	Dangerous for the environment (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC
2N4AAT	2-Nitro-4-Acetylaminotoluene
NADP or NAD (P)	Nicotinamide Adenine Dinucleotide Phosphate
NADPH or NAD (P) H	Dihydronicotinamide Adenine Dinucleotide Phosphate
NAEL	No Adverse Effect Level
NAT	N-acetyltransferase
NCI	National Cancer Institute
2NF	2-Nitrofluorene
NG	Net Nuclear Grain
NIOSH	National Institute for Occupational Safety and Health
NOAEC	No Observed Adverse Effect Concentration
NAEC	No Adverse Effect Concentration in humans
NAEL	No Adverse Effect Level in humans
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
NOEC	No Observed Effect Concentration
NOS	Not Otherwise Specified
4NQO.	4-Nitroquinoline 1-Oxide
NR	Nitroreductase
NTP	National Toxicology Program (USA)
O	Oxidizing (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)
OAT	*Ortho*-Acetyltransferase
OECD	Organisation for Economic Cooperation and Development
OEL	Occupational Exposure Limit
OJ	Official Journal
OR	Odd Ratio
OSHA	Occupational Safety and Health Administration
OSPAR	Oslo and Paris Convention for the protection of the marine environment of the Northeast Atlantic

P	Persistent
PB	Phenobarbital
PBT	Persistent, Bioaccumulative and Toxic
PBPK	Physiologically Based PharmacoKinetic modelling
PBTK	Physiologically Based ToxicoKinetic modelling
PCDBF	2,3,4,7,8-Pentachlorodibenzfuran
PEC	Predicted Environmental Concentration
pH	logarithm (to the base 10) (of the hydrogen ion concentration $\{H^+\}$
pKa	logarithm (to the base 10) of the acid dissociation constant
pKb	logarithm (to the base 10) of the base dissociation constant
PNEC	Predicted No Effect Concentration
PO	Oral AdministrationChinese hamster lung
POP	Persistent Organic Pollutant
PPE	Personal Protective Equipment
ppm	parts per million
QSAR	(Quantitative) Structure-Activity Relationship
R phrases	Risk phrases according to Annex III of Directive 67/548/EEC
RAL	Relative Adduct Labelling
RAR	Risk Assessment Report
RC	Risk Characterisation
RfC	Reference Concentration
RfD	Reference Dose
RNA	RiboNucleic Acid
RPE	Respiratory Protective Equipment
RWC	Reasonable Worst Case
S phrases	Safety phrases according to Annex III of Directive 67/548/EEC
SAR	Structure-Activity Relationships
SBR	Standardised birth ratio
SCE	Sister Chromatic Exchange
SD	Standard Deviation
SDS	Safety Data Sheet
S.E.	Standard Error
SETAC	Society of Environmental Toxicology And Chemistry
SKF 525-A	2-Diethylaminoethyl 2:2-Diphenylvalerate Hydrochloride
SLRL	Sex-Linked Recessive Lethal
SMR	Standardized Mortality Ratio
SNIF	Summary Notification Interchange Format (new substances)

SRR	Standardized Rate Ratio
SSD	Species Sensitivity Distribution
STP	Sewage Treatment Plant
T(+)	(Very) Toxic (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)
T25	The chronic dose rate that will give 25% of tumours after correction for spontaneous incidence
TDI	Tolerable Daily Intake
TEM	Triethylenemelamine
TG	Test Guideline
TGD	Technical Guidance Document
TNsG	Technical Notes for Guidance (for Biocides)
TNO	The Netherlands Organisation for Applied Scientific Research
TNT	Trinitrotoluene
TPA	12-O-Tetradecanoylphorbol-13-Acetate
TWA	
UC	Use Category
UDS	Unscheduled DNA Synthesis
UN	United Nations
UNEP	United Nations Environment Programme
US EPA	Environmental Protection Agency, USA
UV	Ultraviolet Region of Spectrum
UVCB	Unknown or Variable composition, Complex reaction products of Biological material
vB	very Bioaccumulative
V_{max}	Maximum Rate of Reaction
vP	very Persistent
vPvB	very Persistent and very Bioaccumulative
v/v	volume per volume ratio
W	body weight
w/w	weight per weight ratio
W_1	Exposure period
W_2	Observation period
wE	Worker exposure estimation
WHO	World Health Organization
wLR	Life-time cancer risk for workers
WWTP	Waste Water Treatment Plant
Xn	Harmful (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)

Xi	Irritant (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)

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The report provides the comprehensive risk assessment of the substance 2,4-dinitrotoluene.

It has been prepared by Spain in the frame of Council Regulation (EEC) No. 793/93 on the evaluation and control of the risks of existingsubstances, following the principles for assessment of the risks to humans and theenvironment, laid down in Commission Regulation (EC) No. 1488/94.

Part I - Environment
This part of the evaluation considers the emissions and the resulting exposure to the environment in all life cycle steps. Following the exposure assessment, the environmental risk characterisation for each protection goal in the aquatic, terrestrial and atmosphericcompartment has been determined.
The environmental risk assessment concludes that there is concern for the aquatic compartment and sediment dweling organisms at one local site. There is no concern for the atmosphere, the terrestrial ecosystem and mcro-organisms in the sewage treatment plant

Part II – Human Health
This part of the evaluation considers the emissions and the resulting exposure to humanpopulations in all life cycle steps. The scenarios for occupational exposure, consumerexposure and humans exposed via the environment have been examined and the possiblerisks have been identified.
The human health risk assessment concludes that there is concern for workers with regard to mutagenicity and carcinogenicity at all worker scenarios and with regard to repeated dose toxicity and toxicity for reproduction (fertilty) from manufacture and use of explosives. There is also concern for humans exposed via the environment with regard to carcinogenicity and mutagenicity from one local site. For consumers and for human health (physico-chemical properties) there is no concern.

The conclusions of this report lead to risk reduction measures to be proposed by the Commission's committee on risk reduction strategies set up in support of Council Regulation (EEC) N. 793/93.