File No: STD/1637

August 2018

NATIONAL INDUSTRIAL CHEMICALS NOTIFICATION AND ASSESSMENT SCHEME (NICNAS)

PUBLIC REPORT

1,4-Benzenediamine, 2-(methoxymethyl)-

This Assessment has been compiled in accordance with the provisions of the *Industrial Chemicals (Notification and Assessment) Act 1989* (the Act) and Regulations. This legislation is an Act of the Commonwealth of Australia. The National Industrial Chemicals Notification and Assessment Scheme (NICNAS) is administered by the Department of Health, and conducts the risk assessment for public health and occupational health and safety. The assessment of environmental risk is conducted by the Department of the Environment and Energy.

This Public Report is available for viewing and downloading from the NICNAS website or available on request, free of charge, by contacting NICNAS. For requests and enquiries please contact the NICNAS Administration Coordinator at:

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Director
NICNAS

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SUMMARY

The following details will be published in the NICNAS *Chemical Gazette:*

ASSESSMENT REFERENCE	APPLICANT(S)	CHEMICAL OR TRADE NAME	HAZARDOUS CHEMICAL	INTRODUCTION VOLUME	USE
STD/1637	Cosmetic Suppliers Pty Ltd Coty Australia Pty Ltd HFC Prestige International Australia Pty Ltd	1,4-Benzenediamine, 2-(methoxymethyl)-	Yes	≤ 5 tonnes per annum	Oxidative hair dye

CONCLUSIONS AND REGULATORY OBLIGATIONS

Hazard classification
Based on the available information, the notified chemical is recommended for hazard classification according to the *Globally Harmonised System of Classification and Labelling of Chemicals (GHS),* as adopted for industrial chemicals in Australia. The recommended hazard classification is presented in the following table.

Hazard classification	*Hazard statement*
Acute toxicity, oral (Category 3)	H301 – Toxic if swallowed
Acute toxicity, dermal (Category 3)	H311 – Toxic in contact with skin
Acute toxicity, inhalation (Category 4)	H332 – Harmful if inhaled
Serious eye damage/eye irritation (Category 2A)	H319 – Causes serious eye irritation
Sensitisation, skin (Category 1)	H317 – May cause an allergic skin reaction

The environmental hazard classification according to the *Globally Harmonised System of Classification and Labelling of Chemicals* (GHS) is presented below. Environmental classification under the GHS is not mandated in Australia and carries no legal status but is presented for information purposes.

Hazard classification	*Hazard statement*
Acute aquatic toxicity (Category 2)	H401 – Toxic to aquatic life
Chronic aquatic toxicity (Category 2)	H411 – Toxic to aquatic life with long lasting effects

Human health risk assessment
Under the conditions of the occupational settings described, the notified chemical is not considered to pose an unreasonable risk to the health of workers.

When used in the proposed manner, the notified chemical is not considered to pose an unreasonable risk to public health.

Environmental risk assessment
On the basis of the PEC/PNEC ratio, the notified chemical is not considered to pose an unreasonable risk to the environment.

Recommendations

REGULATORY CONTROLS

Hazard Classification and Labelling

- The notified chemical should be classified as follows:
  - Acute toxicity, oral (Category 3): H301 – Toxic if swallowed
  - Acute toxicity, dermal (Category 3): H311 – Toxic in contact with skin
  - Acute toxicity, inhalation (Category 4): H332 – Harmful if inhaled
  - Serious eye damage/eye irritation (Category 2A): H319 – Causes serious eye irritation
  - Sensitisation, skin (Category 1): H317 – May cause an allergic skin reaction

  The above should be used for products/mixtures containing the notified chemical, if applicable, based on the concentration of the notified chemical present and the intended use/exposure scenario.

- The notified chemical falls within the Schedule 6 general group entry for phenylenediamines in the *Standard for the Uniform Scheduling of Medicines and Poisons* (SUSMP) (2018). The Delegate (and/or the Advisory Committee on Chemicals Scheduling) should consider creating a single new entry for the notified chemical in Schedule 6 of the SUSMP to limit the maximum on head concentration of the notified chemical from oxidative hair dye products to 1.8%. Matters to be taken into consideration include:
  - the SCCS Opinion (2013) concluded that the chemical is safe for use as an oxidative hair dye at a maximum on head concentration of 1.8%, apart from its sensitising potential.

Health Surveillance

- As the notified chemical is a skin sensitiser, employers should carry out health surveillance for any worker who has been identified in the workplace risk assessment as having a significant risk of allergic skin reactions.

CONTROL MEASURES

Occupational Health and Safety

- A person conducting a business or undertaking at a workplace should implement the following safe work practices to minimise occupational exposure during handling of the notified chemical as introduced in hair dye products:
  - Avoid contact with skin and eyes

- A person conducting a business or undertaking at a workplace should ensure that the following personal protective equipment is used by workers to minimise occupational exposure to the notified chemical as introduced in hair dye products:
  - Impervious gloves

  Guidance in selection of personal protective equipment can be obtained from Australian, Australian/New Zealand or other approved standards.

- A copy of the SDS should be easily accessible to employees.

- If products and mixtures containing the notified chemical are classified as hazardous to health in accordance with the *Globally Harmonised System of Classification and Labelling of Chemicals (GHS)* as adopted for industrial chemicals in Australia, workplace practices and control procedures consistent with provisions of State and Territory hazardous substances legislation should be in operation.

Public Health

- Follow the recommendations in Schedule 6 of the *Standard for the Uniform Scheduling of Medicines and Poisons* (SUSMP) (2018) group entry for phenylenediamines when using the notified chemical

introduced in oxidative hair dye formulations, until a specific entry for the notified chemical is included in the SUSMP.

Disposal

- Where reuse or recycling are not appropriate, dispose of the notified chemical in an environmentally sound manner in accordance with relevant Commonwealth, state, territory and local government legislation.

Emergency procedures

- Spills or accidental release of the notified chemical should be handled by physical containment, collection and subsequent safe disposal.

Regulatory Obligations

Secondary Notification
This risk assessment is based on the information available at the time of notification. The Director may call for the reassessment of the chemical under secondary notification provisions based on changes in certain circumstances. Under Section 64 of the *Industrial Chemicals (Notification and Assessment) Act (1989)* the notifier, as well as any other importer or manufacturer of the notified chemical, have post-assessment regulatory obligations to notify NICNAS when any of these circumstances change. These obligations apply even when the notified chemical is listed on the Australian Inventory of Chemical Substances (AICS).

Therefore, the Director of NICNAS must be notified in writing within 28 days by the notifier, other importer or manufacturer:

(1) Under Section 64(1) of the Act; if
- the concentration of the notified chemical in imported oxidative hair dye products exceeds or is intended to exceed 3.6%;
- the on head concentration of the notified chemical exceeds or is intended to exceed 1.8%.

or

(2) Under Section 64(2) of the Act; if
- the function or use of the chemical has changed from an oxidative hair dye, or is likely to change significantly;
- the amount of chemical being introduced has increased, or is likely to increase, significantly;
- the chemical has begun to be manufactured in Australia;
- additional information has become available to the person as to an adverse effect of the chemical on occupational health and safety, public health, or the environment.

The Director will then decide whether a reassessment (i.e. a secondary notification and assessment) is required.

Safety Data Sheet
The SDS of the notified chemical provided by the notifier was reviewed by NICNAS. The accuracy of the information on the SDS remains the responsibility of the applicant.

ASSESSMENT DETAILS

1. APPLICANT AND NOTIFICATION DETAILS

APPLICANTS
Cosmetic Suppliers Pty Ltd (ABN: 91 008 396 245)
Level 31, 1 Market Street
SYDNEY NSW 2113

HFC Prestige International Australia Pty Ltd (ABN: 58 608 686 773)
Level 31, 1 Market Street
SYDNEY NSW 2113

Coty Australia Pty Ltd (ABN: 96 058 696 549)
Level 31, 1 Market Street
SYDNEY NSW 2113

NOTIFICATION CATEGORY
Standard: Chemical other than polymer (more than 1 tonne per year)

EXEMPT INFORMATION (SECTION 75 OF THE ACT)
No details are claimed exempt from publication.

VARIATION OF DATA REQUIREMENTS (SECTION 24 OF THE ACT)
Variation to the schedule of data requirements is claimed for all physico-chemical properties (except for hydrolysis as a function of pH and oxidising properties), acute toxicity, eye irritation, skin sensitisation, repeated dose toxicity, mutagenicity, genotoxicity and reproductive/developmental toxicity.

PREVIOUS NOTIFICATION IN AUSTRALIA BY APPLICANT(S)
NICNAS permits

NOTIFICATION IN OTHER COUNTRIES
EU

2. IDENTITY OF CHEMICAL

MARKETING NAMES
MBB
COLIPA A160
Me^+

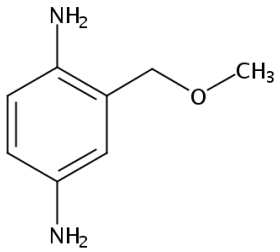
CAS NUMBER
337906-36-2

CHEMICAL NAME
1,4-Benzenediamine, 2-(methoxymethyl)-

OTHER NAME(S)
2-Methoxymethyl-p-phenylenediamine (INCI name)

MOLECULAR FORMULA
$C_8H_{12}N_2O$

STRUCTURAL FORMULA



MOLECULAR WEIGHT
152.19 g/mol

ANALYTICAL DATA
Reference NMR spectra were provided.

3. COMPOSITION

DEGREE OF PURITY
≥ 98.4

IDENTIFIED IMPURITIES

Chemical Name	Benzenemethanol, 2,5-diamino-		
CAS No.	73793-80-3	*Weight %*	130 ppm
Chemical Name	Phenol, 4-amino-2-(methoxymethyl)-		
CAS No.	29785-47-5	*Weight %*	< 34 ppm
Chemical Name	1,4-benzenediamine, 2-methyl-		
CAS No.	95-70-5	*Weight %*	50 ppm
*Hazardous Properties**	H301 (Toxic if swallowed) H312 (Harmful in contact with skin) H332 (Harmful if inhaled) H319 (Causes serious eye irritation) H373 (May cause damage to organs through prolonged or repeated exposure) H317 (May cause an allergic skin reaction) H411 (Toxic to aquatic life with long-lasting effects)		
Chemical Name	Benzene, methyl-		
CAS No.	108-88-3	*Weight %*	≤ 2100 ppm
*Hazardous Properties**	H225 (Highly flammable liquid and vapour) H315 (Causes skin irritation) H373 (May cause damage to organs through prolonged or repeated exposure) H360 (May damage fertility or the unborn child)		

* Information from HCIS, SWA

ADDITIVES/ADJUVANTS
None

4. PHYSICAL AND CHEMICAL PROPERTIES

APPEARANCE AT 20 °C AND 101.3 kPa: White to beige off-white/tan to light pink powder (SCCS, 2013)

Property	Value	Data Source/Justification
Melting Point	80.7 - 81.1 °C	SDS
Boiling Point	Decomposes	SDS
Density	1.2028 g/cm^3	SDS
Vapour Pressure	5.0 × 10^{-9} kPa at 20 °C	SCCS (2013)

Property	Value	Data Source/Justification
Water Solubility	84.9 g/L at 20 °C	SCCS (2013)
Hydrolysis as a Function of pH	The notified chemical undergoes rapid transformation under the test conditions	Measured
Partition Coefficient (n-octanol/water)	log Kow = -0.647 (pH 7)	SCCS (2013)
Adsorption/Desorption	Not determined	The notified chemical is expected to sorb to soil sediment and sludge based on its cationic functionality.
Dissociation Constant	pKa = 8.14	SCCS (2013)
Particle Size	166.6 μm (> 32 μm and ≤ 250 μm)	SCCS (2013)
Flammability	Not highly flammable	SDS
Autoignition Temperature	> 404 °C	SDS
Explosive Properties	Not explosive	SDS
Oxidising Properties	Not oxidising	Measured

DISCUSSION OF PROPERTIES
For full details of tests on physical and chemical properties, refer to Appendix A.

Reactivity
The notified chemical was considered to be stable for more than 5 years if stored dry and protected from light (SCCS, 2013).

Physical hazard classification
Based on the submitted physico-chemical data depicted in the above table, the notified chemical is not recommended for hazard classification according to the *Globally Harmonised System of Classification and Labelling of Chemicals (GHS),* as adopted for industrial chemicals in Australia.

5. INTRODUCTION AND USE INFORMATION

MODE OF INTRODUCTION OF NOTIFIED CHEMICAL (100%) OVER NEXT 5 YEARS
The notified chemical will not be manufactured in Australia. It will be imported into Australia as a component of oxidative hair dye products at ≤ 3.6% concentration.

MAXIMUM INTRODUCTION VOLUME OF NOTIFIED CHEMICAL (100%) OVER NEXT 5 YEARS

Year	*1*	*2*	*3*	*4*	*5*
Tonnes	5	5	5	5	5

PORT OF ENTRY
Sydney or Melbourne

IDENTITY OF MANUFACTURER
Vivimed Labs Europe Ltd (UK)

TRANSPORTATION AND PACKAGING
The notified chemical will be imported as a component of finished hair dying products in containers up to 1 L in capacity that are suitable for retail sale including bottles and tubes. The finished products will be distributed throughout Australia by road to beauty salons and retail shops.

USE
The notified chemical will be used as an oxidative hair colouring agent precursor. The intended maximum concentration of the notified chemical will be 3.6% in oxidative hair dye formulations and 1.8% after mixing with the developer. The notified chemical will be used primarily by professionals and will also be available for home applications.

OPERATION DESCRIPTION
Finished hair dye products containing the notified chemical at ≤ 3.6 concentration will be used primarily by professionals (such as hairdressers and beauty salon workers) and will be available for home use by consumers. The finished products are expected to be applied to the hair by hand.

6. HUMAN HEALTH IMPLICATIONS

6.1. Exposure Assessment

6.1.1. Occupational Exposure

CATEGORY OF WORKERS

Category of Worker	*Exposure Duration (hours/day)*	*Exposure Frequency (days/year)*
Transport/Logistics workers	2	2
Professional salon workers	4	220

EXPOSURE DETAILS

Transport and storage
Transport and storage workers are not expected to be exposed to the notified chemical except in the unlikely event of an accident.

End-use
Dermal exposure to the notified chemical at ≤ 3.6% concentration in finished hair dying products may occur in professionals (e.g. hair dressers or hair salon workers) where the services provided involve the application of the products to clients. Such professionals may use limited personal protective equipment (PPE), such as impervious gloves, to minimise repeated exposure, and good hygiene practices are expected to be in place. If PPE is used, exposure of such workers to the notified chemical is expected to be of a similar or lesser extent than the exposure experienced by consumers using the finished products.

6.1.2. Public Exposure
There will be widespread and repeated exposure of the public to the notified chemical at ≤ 3.6% concentration through the use of hair dye products which will be mixed with the developer at 1:1 ratio before being applied to the hair. The main route of exposure will be dermal, with some potential for accidental ocular or oral exposure.

6.2. Human Health Effects Assessment
The results from toxicological investigations conducted on the notified chemical and its sulfate salt (CAS No. 337906-37-3, referred as analogue chemical) are summarised in the following table. For full details of the studies that were submitted to NICNAS, refer to Appendix B.

Endpoint	*Result and Assessment Conclusion*
Rat, acute oral toxicity – gavage^#	LD50 = 150 - 200 mg/kg bw; toxic
Rat, acute dermal toxicity*	LD50 = 400 mg/kg bw; toxic (SDS)
Rat, acute inhalation toxicity*	LC50 = 1.3 mg/L/4 hour; harmful (SDS)
Skin corrosion – *in vitro* transcutaneous electrical resistance test (100%)*	non-corrosive
Skin corrosion – *in vitro* human skin model test (100%)^	non-corrosive
Skin irritation – *in vitro* reconstructed human epidermis test (1.83%, 6.1% and 100%)*	non-irritating
Eye irritation – *in vitro* isolated chicken eye test (100%)*#	irritating
Eye irritation – *in vitro* isolated chicken eye test (1.8% and 6.1%)*#	non-irritating
Eye irritation – *in vitro* isolated chicken eye test (3% and 10%)^#	non-irritating
Mouse, skin sensitisation – local lymph node assay^	evidence of sensitisation; EC3 = 7.11%
Rat, repeat dose oral toxicity – 91/92 days^	NOAEL = 30 mg/kg bw/day
Genotoxicity – bacterial reverse mutation^	mutagenic
Genotoxicity – *in vitro* mammalian cell gene mutation test^	mutagenic and genotoxic
Genotoxicity – *in vitro* mammalian cell micronucleus test^	equivocal
Genotoxicity – *in vivo* mammalian erythrocyte micronucleus test^	non genotoxic
Genotoxicity – *in vivo* unscheduled DNA synthesis test^	non genotoxic

Endpoint	*Result and Assessment Conclusion*
Genotoxicity – *in vivo* alkaline single cell gel electrophoresis assay^#	non genotoxic (stomach and urinary bladder cells); equivocal (liver cells)
Rat, developmental toxicity^	maternal NOAEL = 30 mg/kg bw/day embryo NOAEL = 90 mg/kg bw/day
Cutaneous absorption – *in vitro* method using pig skin*	1.56 ± 0.36 μg/cm^2
Absorption, distribution, metabolism and excretion^	oral administration: extensively absorbed, readily distributed, extensively metabolised and excreted via urine dermal administration: higher absorption following higher administration dose for longer exposure period

* Test substance was 1,4-benzenediamine, 2-(methoxymethyl)- (notified chemical)
^ Test substance was 1,4-benzenediamine, 2-(methoxymethyl)-, sulfate (1:1) (analogue chemical)
# Data from SCCS (2013)

Toxicokinetics, metabolism and distribution
In an *in vitro* cutaneous absorption study using pig skin for a typical hair dye formulation containing 1.824% notified chemical (with hydrogen peroxide and a reaction partner), the study authors concluded that, under the assumption that a depot effect was absent, a maximum amount of 1.56 ± 0.36 μg/cm^2 (SD) of notified chemical was considered biologically available (n = 11, three donors; receptor fluid + lower skin = 1.341 μg/cm^2 ± 0.219 μg/cm^2). This is equivalent to 0.0896 ± 0.023% of the applied dose (SCCS, 2013). SCCS commented that the dose volume used was too high, three donors instead of four were used for skin samples and recovery was over 72 hours (SCCS, 2013). Therefore 2.33 μg/cm^2 (mean absorption + 2 SD) (0.14% of the applied dose) was used in the calculation for the margin of safety (MOS) of the notified chemical (SCCS, 2013).

In an *in vivo* absorption, distribution, metabolism and excretion (ADME) study in mice, the analogue chemical administered orally was extensively absorbed (84 – 128%), readily distributed into all organs, extensively metabolised and excreted via urine (66 – 77%). Dermal absorption of the test substance was high (21% – mass balance data, 47% – plasma data) after a 24-hour exposure period at a higher administration dose (~100 mg/kg bw, 1.25 mg/cm^2) and low (2% – mass balance data, 2.5% – plasma data) after a 30-minute exposure period at a lower administration dose (~24 mg/kg bw, 0.3 mg/cm^2). When absorbed, excretion occurred primarily via the urine. Three major metabolites (N-[4-amino-2-(methoxymethyl)-phenyl]acetamide, N-[4-amino-3-(methoxymethyl)-phenyl]acetamide and 1,4-di((1-oxo-ethyl)amino)-2-(methoxymethyl)-benzene) were detected in the urine and plasma following all routes of administration. N-acetylation of the parent compound was the major metabolic reaction. No major qualitative differences in the metabolite profile were noted between the oral and dermal routes of administration.

The notified chemical is expected to have a similar ADME profile, considering the analogue chemical is a sulfate salt of the notified chemical.

Acute toxicity
No acute toxicity data were provided for the notified chemical. The LD50 values were established as 100 – 250 mg/kg bw in mice after intraperitoneal administration and 150 – 200 mg/kg bw in rats after oral gavage, based on the results from three *in vivo* genotoxicity studies (micronucleus study in mice, alkaline single cell gel electrophoresis assay in rats and unscheduled DNA synthesis assay in rats) for the analogue chemical (SCCS, 2013). The LD50 values for the notified chemical are adjusted to be 61 – 152 mg/kg bw in mice after intraperitoneal administration and 91 – 122 mg/kg bw in rats after oral gavage, using the conversion factor (0.61) to account for the different molecular weight (SCCS, 2013). Based on the adjusted LD50 values, the notified chemical is recommended to be classified as acute toxicity, oral (Category 3), consistent with the classification in the submitted SDS for the notified chemical.

No acute dermal and inhalation toxicity study reports were provided. However, LD50 of 400 mg/kg bw for acute dermal toxicity and LD50 of 1.3 mg/L/4 hour for acute inhalation toxicity were stated in the submitted SDS for the notified chemical, in which the notified chemical was classified as acute toxicity, dermal (Category 3) and acute toxicity, inhalation (Category 4) by the notifier. No acute dermal or inhalation toxicity data are reported in the SCCS Opinion of the notified chemical (SCCS, 2013).

Irritation
In three *in vitro* studies (transcutaneous electrical resistance test, EpiDerm human skin model test and Episkin reconstructed human epidermis test), the notified chemical and its sulfate salt were found to be non-corrosive or non-irritating to the skin.

The notified chemical was identified as irritating to eyes based on the results from an *in vitro* isolated chicken eye (ICE) study while its aqueous solutions at 1.83% and 6.1% were identified as non-irritating to eyes based on the results from another ICE study (SCCS, 2013). Aqueous solutions of the analogue chemical at 3% and 10% were also identified as non-irritating to eyes based on the results in a separate ICE test (SCCS, 2013). Based on the available data, the notified chemical is not expected to be a strong irritant to eyes at the anticipated use exposure (SCCS, 2013).

Sensitisation
The analogue chemical was a skin sensitiser in mice (local lymph node assay: stimulation indices were 1.1, 1.2, 2.2 and 6.0 at 0.5%, 1.5%, 5% and 15%, respectively. The EC_3 value was calculated to be 7.11%. The EC3 value for the notified chemical is calculated to be 4.3%, using the conversion factor (0.61) to account for the difference in molecular weight (SCCS, 2013). Therefore, the notified chemical is also a skin sensitiser.

Repeated dose toxicity
A repeated dose oral (gavage) toxicity study on the analogue chemical was conducted in rats, in which the test substance was administered at 10, 30 and 90 mg/kg bw/day for 91/92 consecutive days, with a 27/28-day recovery period for high dose and control animals.

The No Observed Adverse Effect Level (NOAEL) was established as 30 mg/kg bw/day by the study authors. The SCCS report stated that a NOAEL of 90 mg/kg bw/day (the highest dose tested) and a No Observed Effect Level (NOEL) of 30 mg/kg bw/day may be derived from this study, based on that at the dose level of 90 mg/kg bw/day, minor modifications in biochemistry noted were mostly in the range of historical control values, a small increase in absolute and relative liver weight noted in male animals was considered as an adaptive response, and no significant differences in these parameters were noted after recovery (SCCS, 2013). The equivalent NOAEL for the notified chemical is calculated to be 55 mg/kg bw/day, using the conversion factor (0.61) to account for the difference in molecular weight (SCCS, 2013).

Mutagenicity/Genotoxicity
The analogue chemical was positive in a bacterial reverse mutation assay with metabolic activation and in an *in vitro* mammalian cell gene mutation test using mouse lymphoma L5178Y cells with or without metabolic activation. Equivocal results were reported for the analogue chemical in an *in vitro* mammalian cell micronucleus test using human peripheral lymphocytes. The analogue chemical was negative in an *in vivo* mammalian erythrocyte micronucleus test and in an *in vivo* unscheduled DNA synthesis test. In an *in vivo* alkaline single cell gel electrophoresis assay, the analogue chemical did not induce a biologically relevant increase in DNA damage in cells of stomach and urinary bladder of rats and consequently is not genotoxic in these tissues of rats, while the results were considered equivocal for liver cells treated with the analogue chemical (SCCS, 2013).

Based on the weight of evidence the analogue chemical is not expected to be genotoxic. Read-cross of this result for the notified chemical is considered suitable, considering the analogue chemical is a sulfate salt of the notified chemical.

Developmental toxicity
A prenatal developmental study was conducted using the analogue chemical at 10, 30 and 90 mg/kg bw/day dose levels. The test substance was administered by oral gavage from day 6 to day 19 of gestation. A NOAEL of 30 mg/kg bw/day was established for maternal toxicity, based on the reduction of the body weight gain and food consumption in the 90 mg/kg bw/day dose group, and a NOAEL of 90 mg/kg bw/day was established for developmental toxicity (the highest dose tested).

The equivalent NOAELs for the notified chemical are calculated as 18 mg/kg bw/day for maternal toxicity and 55 mg/kg bw/day for embryo toxicity, using the conversion factor (0.61) to account for the difference in molecular weight (SCCS, 2013).

Health hazard classification
Based on the available information, the notified chemical is recommended for hazard classification according to the *Globally Harmonised System of Classification and Labelling of Chemicals (GHS),* as adopted for industrial chemicals in Australia. The recommended hazard classification is presented in the following table.

Hazard classification	*Hazard statement*
Acute toxicity, oral (Category 3)	H301 – Toxic if swallowed
Acute toxicity, dermal (Category 3)*	H311 – Toxic in contact with skin
Acute toxicity, inhalation (Category 4)*	H332 – Harmful if inhaled
Serious eye damage/eye irritation (Category 2A)	H319 – Causes serious eye irritation
Sensitisation, skin (Category 1)	H317 – May cause an allergic skin reaction

* Based on LD50/LC50 values on the submitted SDS for the notified chemical (no study data submitted to confirm these values)

6.3. Human Health Risk Characterisation

6.3.1. Occupational Health and Safety
Workers involved in professions where the services provided involve the application of hair dye products containing the notified chemical to clients (*e.g.*, hairdressers and hair salon workers) may be exposed to the notified chemical at concentrations up to 3.6%. The greatest potential for exposure is during hair dyeing processes, mainly via skin contact, although ocular exposure may also occur.

Given that the product is a dye, skin contact is expected to be avoided by workers. Workers may use PPE (such as disposable gloves) to minimise repeated exposure, and good hygiene practices are expected to be in place.

Overall, based on the low concentration of the notified chemical in hair dye products and PPE (gloves) are worn, the risk to workers from exposure to the notified chemical is not considered to be unreasonable.

6.3.2. Public Health
Hair dye products containing the notified chemical will be supplied to hairdressing salons and may also be available for public via retail outlets. Therefore, members of the public may potentially be exposed to the notified chemical when mixing/applying the product (at ≤ 3.6% concentration) and when having the product applied to their scalp (at ≤ 1.8% concentration). The degree and type of exposure may vary depending on the frequency of application, the care taken when applying the dye and amount of dye applied.

Eye irritation and skin sensitisation
The notified chemical is covered by the following group entry for phenylenediamines in Schedule 6 of the *Standard for the Uniform Scheduling of Medicines and Poisons* (SUSMP) (2018):

PHENYLENEDIAMINES including alkylated, arylated and nitro derivatives not elsewhere specified in these Schedules:

c) in hair dye preparations **except** when the immediate container and primary pack are labelled with the following statements:
KEEP OUT OF REACH OF CHILDREN, and
WARNING – This product contains ingredients which may cause skin irritation to certain individuals. A preliminary test according to the accompanying directions should be made before use. This product must not be used for dyeing eyelashes or eyebrows; to do so may be injurious to the eye.
written in letters not less than 1.5 mm in height;

Provided that the label directions are followed and disposable gloves are used by the consumers when using hair dye products containing the notified chemical, the risks of skin sensitisation and eye irritation are prevented.

Systemic effects from repeated use
The notified chemical was the subject of a SCCS Opinion (SCCS, 2013) which calculated the margin of safety (MOS) for the notified chemical as follows:

Absorption through the skin	A	2.33 μg/cm^2
Skin area surface	SAS	580 cm^2
Dermal absorption per treatment	SAS × A × 0.001	1.351 mg
Typical body weight of human		60 kg
Systemic exposure dosage (SED)	SAS × A × 0.001/60	0.0225 mg/kg bw/day
No Observed Adverse Effect Level	NOAEL	18 mg/kg bw (derived for maternal toxicity from a developmental toxicity study)
MOS	NOAEL/SED	800

The SCCS (2013) concluded that 'the use of the notified chemical and its sulfate salt as oxidative hair dye with a concentration on head of maximum 1.8% does not pose a risk to the health of the consumer, apart from its sensitising potential'.

The proposed application is for use of the notified chemical in oxidative hair dye products at a maximum on-head concentration of 1.8% that is identical to that assessed by the SCCS. Therefore, systemic repeated dose risks from use of the notified chemical by members of the general public at ≤ 1.8% on-head concentration in oxidative hair dyes is not considered to be unreasonable.

7. ENVIRONMENTAL IMPLICATIONS

7.1. Environmental Exposure & Fate Assessment

7.1.1. Environmental Exposure

RELEASE OF CHEMICAL AT SITE

The notified chemical will be imported into Australia as a component of finished hair dye products. No significant release of the notified chemical to the environment is expected. Accidental spills or releases where packaging is breached are expected to be collected and subsequently disposed of, most likely to landfill.

RELEASE OF CHEMICAL FROM USE

The notified chemical is a component of hair dye products, which are expected to be rinsed off and enter the sewer after use by consumers and professional hair salons. Therefore, under a worst case assumption, the total import volume of the notified chemical is assumed to be discharged to sewer.

RELEASE OF CHEMICAL FROM DISPOSAL

Residues of the notified chemical in end-use containers for cosmetics (3%) are expected to share the fate of the container and be disposed of to landfill, or to be washed to sewer when containers are rinsed before recycling.

7.1.2. Environmental Fate

The notified chemical is not readily biodegradable based on the biodegradation test submitted. Two hydrolysis studies conducted on the notified chemical indicated that the notified chemical rapidly transforms under the conditions of those tests but it is unknown if the notified chemical will be persistent under environmental conditions. Based on the available information, the notified chemical may persist in the environment. For the details of the environmental fate studies please refer to Appendix A and C.

The majority of the notified chemical is expected to be released to the sewage system after use. In waste water treatment processes at sewage treatment plants (STPs). Sludge from STPs containing the notified chemical and its hydrolysis products are expected to be disposed of to landfill or applied to agricultural soils. The notified chemical released into surface waters is expected to degrade or disperse in the aqueous environment and is not expected to bioaccumulate based on its very low log Kow. In sludge, soil and water, the notified chemical is expected to be degraded by abiotic and biotic processes to form water and oxides of carbon and nitrogen.

7.1.3. Predicted Environmental Concentration (PEC)

The notified chemical will be released to sewers following its use in hair dye products. Therefore, under a worst case scenario, it is assumed that 100% of the total import volume of the notified chemical will be discharged into sewers nationwide over 365 days per year. Assuming no removal of the notified chemical in the sewage treatment processes for the worst case scenario, the resultant predicted environmental concentration (PEC) in sewage effluent on a nationwide basis is estimated as follows:

Predicted Environmental Concentration (PEC) for the Aquatic Compartment		
Total Annual Import/Manufactured Volume	5,000	kg/year
Proportion expected to be released to sewer	100%	
Annual quantity of chemical released to sewer	5,000	kg/year
Days per year where release occurs	365	days/year
Daily chemical release:	13.70	kg/day
Water use	200.0	L/person/day
Population of Australia (Millions)	22.613	million
Removal within STP	0%	
Daily effluent production:	4,523	ML
Dilution Factor - River	1.0	
Dilution Factor - Ocean	10.0	
PEC - River:	3.03	µg/L
PEC - Ocean:	0.30	µg/L

STP effluent re-use for irrigation occurs throughout Australia. The agricultural irrigation application rate is assumed to be 1000 L/m^2/year (10 ML/ha/year). The notified chemical in this volume is assumed to infiltrate and accumulate in the top 10 cm of soil (density 1500 kg/m^3). Using these assumptions, irrigation with a concentration of 3.029 µg/L may potentially result in a soil concentration of approximately 2.019×10^{-2} mg/kg. Assuming accumulation of the notified chemical in soil for 5 and 10 years under repeated irrigation, the concentration of notified chemical in the applied soil in 5 and 10 years may be approximately 0.101 mg/kg and 0.202 mg/kg, respectively.

7.2. Environmental Effects Assessment

The results from ecotoxicological investigations conducted on the notified chemical are summarised in the table below. The notifier provided a study on the toxicity to fish embryo instead of a normal fish toxicity study. However, this study was considered relevant for the assessment factor calculation. Details of these studies can be found in Appendix C.

Endpoint	*Result*	*Assessment Conclusion*
Fish Embryo Toxicity	LC50 > 5.69 mg/L	The notified chemical is, at worst, toxic to fish embryos
Daphnia Toxicity	EC50 = 1.68 mg/L	The notified chemical is toxic to aquatic invertebrates
Algal Toxicity	E_rC50 = 2.08 mg/L	The notified chemical is toxic to algae
Inhibition of Bacterial Respiration	EC50 = 22.4 mg/L	The notified chemical is expected to inhibit microbial respiration

Under the Globally Harmonised System of Classification and Labelling of Chemicals (GHS; United Nations, 2009) the notified chemical is considered to be toxic to fish embryo, aquatic invertebrates and algae and is formally classified as 'Acute Category 2: Toxic to aquatic life'. On the basis of lack of ready biodegradability, the notified chemical is classified 'Chronic Category 2: Toxic to aquatic life with long lasting effects'.

7.2.1. Predicted No-Effect Concentration

The predicted no-effect concentration (PNEC) was calculated using the daphnia endpoint (EC50 = 1.68 mg/L), and an assessment factor of 100, as acute ecotoxicity endpoints for three trophic levels are available.

Predicted No-Effect Concentration (PNEC) for the Aquatic Compartment		
EC50 (Aquatic Invertebrates)	1.68	mg/L
Assessment Factor	100	
Mitigation Factor	1.00	
PNEC	16.8	µg/L

7.3. Environmental Risk Assessment

Risk Assessment	PEC µg/L	PNEC µg/L	Q
Q - River	3.03	16.8	0.18
Q - Ocean	0.30	16.8	0.018

The concentration of the notified chemical in surface waters is expected to be very low based on the reported use pattern and the maximum import volume. The notified chemical is potentially persistent in the environment although it is likely to transform through abiotic processes. The notified chemical is not expected to bioaccumulate, based on its high water solubility and very low partition coefficient. Therefore, on the basis of the PEC (calculated from the maximum annual importation volume and assessed use pattern in hair dye products) to PNEC ratio, the notified chemical is not expected to pose an unreasonable risk to the environment.

APPENDIX A: PHYSICAL AND CHEMICAL PROPERTIES

Hydrolysis as a Function of pH The notified chemical undergoes rapid transformation under the test conditions.

Method OECD TG 111 Hydrolysis as a Function of pH

pH	*T (°C)*	*Degradation after 5 days (%; oxygen reduced)*
4	50	99.8
7	50	98.4
9	50	99.4

Remarks The study was conducted with the notified chemical concentrations of 40 mg/L and test temperature of 50 °C (preliminary test). Test medium buffer solutions at pH 4, 7 and 9 each at atmospheric equilibrium and oxygen reduced conditions were employed. The oxygen concentration at atmospheric equilibrium was recorded to be ~9% whereas at reduced oxygen situation it was ~2%.

The notified chemical does not contain functional groups readily hydrolysable in the environmentally relevant pH range (4-9). However the relatively rapid transformation of the notified chemical at all tested conditions indicate that it undergoes transformation via other routes (e.g. oxidation, di-polymerisation etc.) which are related to the test conditions.

Test Facility Dr U Noack-Laboratorien (2010a)

Hydrolysis as a Function of pH The notified chemical undergoes rapid transformation under the test conditions.

Method OECD TG 111 Hydrolysis as a Function of pH

pH	*T (°C)*	*Concentration after 5 days (% of nominal; oxygen depleted)*
4	50	64
7	50	80
9	50	90

Remarks Samples of the notified chemical were incubated at 50 °C in an oxygen free glove box for 120 hours (5 days). The addition of an antioxidant (3-carboxy-1H-pyrazol-5-one) to the samples did not have a noticeable effect on the long-term stability of the notified chemical.

Test Facility Procter & Gamble (2009)

Oxidizing Properties Not oxidising

Method Commission Directive 92/69/EEC A.17 Oxidizing Properties (Solids)

Remarks Based on that the fastest test item/cellulose mixture burned slower than the fastest reference item/cellulose mixture

Test Facility Dr U Noack-Laboratorien (2008a)

APPENDIX B: TOXICOLOGICAL INVESTIGATIONS

B.1. Corrosion – skin (*in vitro*)

TEST SUBSTANCE: Notified chemical

METHOD: OECD TG 430 *In vitro* Skin Corrosion – Transcutaneous Electrical Resistance (TER) Test

Remarks - Method: No significant protocol deviations. The test substance was applied to the epidermal surface of 3 skin discs for a contact period of 24 hours.

Positive control (hydrochloric acid at approx. 36%) and negative control (sterile distilled water) were run concurrently.

RESULTS

Test Material	*Test Substance Contact Period*	*Mean TER ± SD*
Negative control	24 hrs	15.3 ± 2.3 kΩ
Test substance	24 hrs	14.0 ± 5.0 kΩ
Positive control	24 hrs	0.9063 ± 0.1028 kΩ

Remarks - Results: The skin impedance is measured as TER by using a low-voltage, alternating current Wheatstone bridge and for the purposes of the TER corrosivity assay measurements are recorded in resistance, at a frequency of 100 Hz and using series values.

Corrosive chemicals are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the TER below a threshold level (5 kΩ for rat skin TER).

The mean TER value for the test substance (14.0 ± 5.0 kΩ) was above the cut-off value for non-corrosive (5 kΩ).

The positive and negative controls produced results as expected.

CONCLUSION: The notified chemical was considered unlikely to have the potential to cause skin corrosion under the conditions of the test.

TEST FACILITY: Harlan (2009a)

B.2. Corrosion – skin (*in vitro*)

TEST SUBSTANCE: Analogue chemical

METHOD: OECD TG 431 *In vitro* Skin Corrosion - Human Skin Model Test
EpiDerm™ Human Skin Model

VEHICLE: None

Remarks - Method: The test substance (25 mg moistened with 25 µL water) was applied to the tissues in triplicate. Following exposure periods 3 minutes (room temperature; test 1) and 60 minutes (37 ºC; test 2), the tissues were rinsed, treated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide] and then incubated at 37 °C for 3 hours.

In a preliminary test the test substance was shown not to directly reduce MTT.

Positive control (8.0 N potassium hydroxide) and negative control (sterile distilled water) were run concurrently.

RESULTS

Test material	*Test 1 (3 minute exposure period)*		*Test 2 (60 minutes exposure period)*	
	Mean OD_{540} of duplicate tissues	*Relative mean viability (%)*	*Mean OD_{540} of duplicate tissues*	*Relative mean viability (%)*
Negative control	1.691	100	1.655	100
Test substance	1.793	106.0	1.716	103.7
Positive control	0.238	14.1	0.155	9.4

OD = optical density

Remarks - Results

The relative mean viability of the test substance treated tissues was 106.0 and 103.7% after 3-minute and 60-minute exposure periods respectively.

The positive and negative controls gave satisfactory results, confirming the validity of the test system.

CONCLUSION

The test substance was considered to be non-corrosive to the skin under the conditions of the test.

TEST FACILITY

Harlan (2009b)

B.3. Irritation – skin (*in vitro*)

TEST SUBSTANCE

Notified chemical

METHOD

OECD TG 439 In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method
EpiSkin™ Reconstituted Human Epidermis Model

Vehicle

Sterile water

Remarks - Method

In a pre-test the notified chemical was shown to have the ability to directly reduce MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide). Therefore, the study was performed in parallel on viable and water-killed tissues.

The test substance was applied neat and at two concentrations (1.83% and 6.1% in sterile water).

The test substance (10 µL liquids or 10 ± 2 mg solids moistened with 5 µl sterile water) was applied to the tissues in triplicate. Following exposure periods of 15 minutes (room temperature), the tissues were rinsed, treated with MTT and then incubated at 37 °C for 42 ± 1 hours.

Negative and positive controls were run in parallel with the test substance:

- Negative control: sterile eater
- Positive control: 5% sodium dodecyl sulphate

Cytotoxicity in the colourimetric MTT reduction assay (expressed as percentage viability of treated cultures in comparison to negative controls) and morphological changes identified by histological examination, were evaluated.

- Notified chemical is considered to be non-irritating to the skin if the tissue viability is > 50%.
- Notified chemical is considered to be irritating to the skin if the tissue viability is ≤ 50%.

RESULTS

Test material	*Mean OD_{540} of triplicate tissues*	*Relative mean Viability (%)*	*SD of relative mean viability*
Negative control	0.803 ± 0.052	100	± 6.5

Test material	*Mean OD_{540} of triplicate tissues*	*Relative mean Viability (%)*	*SD of relative mean viability*
Test substance (100%)	0.786 ± 0.026	97.9	± 3.2
Test substance (1.83%)	0.881 ± 0.058	109.7	± 7.2
Test substance (6.1%)	0.866 ± 0.007	107.8	± 0.9
Positive control	0.041 ± 0.008	5.1	± 1.0

OD = optical density; SD = standard deviation

Remarks - Results

The test substance at 1.83%, 6.1% and 100% concentrations did not induce cytotoxicity in the MTT assay indicative of skin irritation. The colour of the test substance did not interfere with the MTT test. The test substance was considered to have the ability to directly reduce MTT but not to have caused interference with the MTT test.

The relative mean viability of the tissues treated with the test substance at 1.83%, 6.1% and 100% concentrations was 109.7%, 107.8% and 97.9% respectively (predicted as non-irritating according to the criteria as > 50%).

Histopathological evaluation showed that the test substance induced no significant epidermal effects at 1.83%, 6.1% and 100% concentrations when compared to the negative control.

The positive and negative controls gave satisfactory results, confirming the validities of the test systems.

CONCLUSION

The notified chemical was considered to be non-irritating under the conditions of the test.

TEST FACILITY

Harlan (2010a)

B.4. Skin sensitisation – mouse local lymph node assay (LLNA)

TEST SUBSTANCE

Analogue chemical

METHOD

OECD TG 429 Skin Sensitisation: Local Lymph Node Assay

Species/Strain: Mouse/CBA/J

Vehicle: Dimethyl sulfoxide

Preliminary study: No

Positive control: Conducted in parallel with the test substance using α-hexylcinnamic aldehyde (HCA) in DMSO.

Remarks - Method: No significant protocol deviations. The concentrations of the test substance were chosen based on the maximum solubility in the vehicle.

RESULTS

Concentration (% w/w)	*Number and sex of animals*	*Proliferative response (Mean DPM)*	*Stimulation Index (Test/Control Ratio)*
Test Substance			
0 (vehicle control)	5F	482 ± 135	-
0.5	5F	529 ± 483	1.1 ± 1.0
1.5	5F	595 ± 163	1.2 ± 0.3
5	5F	1077 ± 748	2.2 ± 1.6
15	5F	2875 ± 1170	6.0 ± 2.4
Positive Control			
25	5F	3476 ± 1609	7.2 ± 3.3

EC3: 7.11%

Remarks - Results: No treatment-related mortalities occurred during the study. Clinical signs included oily fur around the ears of animals treated at 15% concentration on day 0 and orange traces in the bedding of animals treated at 5% and

15% concentrations from days 1-3. Body weights and body weight gains were unaffected by treatment.

The mean lymph node weights of the animals in treatment groups were similar to those in the vehicle control and lower than those in the positive control.

When DMSO was used as the vehicle, the mean SI values for the test substance at concentrations 0.5%, 1.5%, 5% and 15% were 1.1, 1.2, 2.2 and 6.0, respectively. The EC3 value was calculated to be 7.11%. The test substance elicited a SI ≥ 3 and was therefore considered a skin sensitiser.

Mean stimulation index (SI) for the positive control (HCA at 25% in DMSO) was 7.2.

CONCLUSION There was evidence of induction of a lymphocyte proliferative response indicative of skin sensitisation to the test substance under the conditions of the test.

TEST FACILITY MDS (2005)

B.5. Repeat dose toxicity

TEST SUBSTANCE Analogue chemical

METHOD OECD TG 408 Repeated Dose 90-Day Oral Toxicity Study in Rodents

Species/Strain	Rat/HanRcc:WIST (SPF)
Route of Administration	Oral – gavage
Exposure Information	Total exposure days: 91/92 days Dose regimen: 7 days per week Recovery period: 27/28 days
Vehicle	0.4% aqueous solution of ascorbic acid, adjusted with NaOH (1 N) to pH 5.7 – 6.1
Remarks - Method	No significant protocol deviations. Test item concentration were determined by HPLC coupled to an UV detector and quantified with the area under the peak and were acceptable. Under Prescribed storage conditions, the formulations used for dosing will remain stable for more than 5 years.

RESULTS

Group	*Number and Sex of Animals*	*Dose mg/kg bw/day*	*Mortality*
control	10 per sex	0	0/10
low dose (LD)	10 per sex	10	0/10
mid dose (MD)	10 per sex	30	0/10
high dose (HD)	10 per sex	90	0/10
control recovery	5 per sex	0	0/10
high dose recovery	5 per sex	0	0/10

Mortality and Time to Death

There were no treatment-related mortalities.

Clinical Observations

Orange discolouration of urine was observed in all HD animals from day 5 and in all MD animals from day 35 of treatment, which was considered related to the test item colour and not of any toxicological significance. Salivation was frequently observed in HD animals and in MD females and was frequently observed also before administration. Burrowing (taking bedding material in the mouth) was observed infrequently once in one HD and one MD male and several times in one HD female. The salivation and burrowing were considered due to the taste of the formulation and not of any toxicological significance.

Slight and localised alopecia was observed in one HD, one MD and three LD females during the treatment period. Damage to the tail (kinked) was observed in one male and one female in the HD group, three MD males and two LD males on several occasions throughout the study. Hunched posture was reported in one HD male and one LD male during the last week of treatment. Vocalisation when touched was recorded in one HD and one LD female.

Mean grip strength in HD males was significantly lower than those of controls after the treatment period, whereas the mean forelimb grip strength in males and the mean fore- and hind limb grip strength values of the females were similar to the values in control animals.

A significant reduction in locomotor activity was noted from 30-40 minutes post administration in HD males when compared to the control males at the end of the treatment. Females in this group had increased locomotor activity during 40-50 minutes post administration.

Ophthalmoscopy examinations showed uni- or bilateral corneal opacities in 8/15 males (53.3%) and 3/15 females (20.0%) in HD group, 4/10 in males (40%) and 4/10 females (40%) in the MD group, and 4/10 males (40%) and 1/10 females (10%) in the LD group. Persistent pupillary membrane in the vitreous body was observed in 1/15 females (0.07%) in the HD group, 4/10 males (40%) and 1/10 females (10%) in the MD group, 1/10 males (10%) and 1/10 females (10%) in the LD group, and 1/15 males (0.07) and 2/15 females (1.3%) in the control group. Persistent hyaloid vessel in the vitreous body was observed in 3/15 males (20%) and 1/15 females (0.07%) in the HD group; 4/10 males (40%) and 1/10 females (10%) in the MD group, and 1/10 male (10%) and 1/10 female (10%) in the LD group. These findings were comparable with the historical control data of Wistar rats. The study authors stated that the increased incidences of the corneal opacities were not test substance-related but a secondary effect of the animals' reaction to the taste of the dose formulation.

No treatment-related effects were noted on mean daily food consumption or mean body weights.

Laboratory Findings – Clinical Chemistry, Haematology, Urinalysis

HD animals: Elevated mean reticulocyte counts and shifted reticulocyte maturity indices were observed in both sexes from older (low fluorescent) cells to younger (high fluorescent) cells. A reduction in the haematocrit level was observed in female recovery group and was higher than the control values measured at week 13. Mean relative thromboplastin time was only slightly increased in females and marginally increased in males, although the value was statistically significant.

The aspartate aminotransferase activity was elevated in males and the levels of lactate dehydrogenase and creatinine kinases were elevated in both sexes.

Animals of both sexes showed statistically significantly reduced glucose levels, elevated mean cholesterol levels and triglyceride levels (only statistically significant in females). Mean phospholipid levels were statistically significantly elevated in both sexes.

Elevated levels of lactate dehydrogenase in both sexes coincided with elevated creatine kinase levels. Significantly elevated sodium levels were observed in both sexes and significantly reduced potassium levels were noted in females only. Calcium levels and protein levels were elevated in males but unaffected in females.

Urinalysis examination showed marginally elevated ketone values and number of erythrocytes and leukocytes in the urine in males, whereas females only had relatively elevated leukocytes when compared to controls.

MD and LD animals: No test-item related adverse effects were noted on reticulocytes, haematocrit values and urinalysis parameters. Sodium levels were significantly elevated in MD males and females and LD males, when compared with controls. Elevated chloride levels were noted in MD males and significantly lower globulin levels were noted in MD females. LD males had significantly elevated mean glucose levels and elevate sodium levels when compared with controls.

Effects in Organs

HD animals: Males showed elevated mean absolute liver weights, mean liver-to-body weight ratios and mean liver-to-brain weight ratio values when compared to the controls. One male had minimal hepatocellular hypertrophy after 13 weeks. All findings were reversible after the recovery period. Females showed elevated mean absolute kidney weights and statistically significantly increased mean kidney-to-body weight ratios.

Focal and/or general discoloration was noted in the stomach of one female, ovaries of one female, thymus of one male, and seminal vesicles of one male. One male was reported to have a renal cyst.

MD animals: Females showed elevated mean absolute kidney weights and elevated mean kidney-to-body weight ratios, with no statistical significance.

Focal and/or general discoloration of the thymus was observed in two males and one female and in the seminal vesicles in two males. Renal pelvis dilation was noted in two males.

LD animals: Increases in the mean absolute heart weight and mean heart-to-brain weight ratio were noted in females, compared to the controls. However, the mean heart-to-body weight ratio was nearly identical to that of the control females and no supportive histopathological changes were noted.

Focal and/or general discoloration in the seminal vesicles was noted in one male. Minor dermal injuries were reported in one female.

Remarks – Results
Dose-dependent trend in the increased absolute liver weights in HD males was of statistical significance and correlated with the statistically significant increase of aspartate aminotransferase actively.

CONCLUSION
The No Observed Adverse Effect Level (NOAEL) was established as 30 mg/kg bw/day by the study authors.

TEST FACILITY Harlan (2010b)

B.6. Genotoxicity – bacteria

TEST SUBSTANCE	Analogue chemical
METHOD	OECD TG 471 Bacterial Reverse Mutation Test Plate incorporation procedure
Species/Strain	*Salmonella typhimurium*: TA1535, TA1537, TA98, TA100, TA102
Metabolic Activation System	S9 mix from phenobarbital/β-naphthoflavone induced rat liver
Concentration Range in Main Test	a) With metabolic activation: 3 – 5000 µg/plate b) Without metabolic activation: 3 – 5000 µg/plate
Vehicle	Deionised water
Remarks - Method	A preliminary test was performed at 3 – 5000 µg/plate and reported as main Test 1 as no toxic effects were noted at up to 5000 µg/plate. Vehicle and positive controls were used in parallel with the test material. Positive controls: i) without metabolic activation: sodium azide (TA100, TA1535), methyl methane sulfonate (TA102) and 4-nitro-o-phenylene-diamine (TA1535, TA98); ii) with metabolic activation: 2-aminoanthracene

RESULTS

Metabolic Activation	*Test Substance Concentration (µg/plate) Resulting in:*			
	Cytotoxicity in Preliminary Test	*Cytotoxicity in Main Test*	*Precipitation*	*Genotoxic Effect*
Absent				
Test 1	> 5000	> 5000	> 5000	negative
Test 2		> 5000	> 5000	negative
Present				
Test 1	> 5000	> 5000	> 5000	positive
Test 2		> 5000	> 5000	positive

Remarks - Results In the absence of metabolic activation, no significant increases in the

frequency of revertant colonies were noted for any of the bacterial strains, with any dose of the test substance.

In the presence of metabolic activation, substantial and dose dependent increases in revertant colonies were noted in strains TA1535, TA1537, TA98 and TA100.

The negative and positive controls gave satisfactory responses, confirming the validity of the test system.

CONCLUSION The test substance was mutagenic to bacteria in the presence of metabolic activation, under the conditions of the test.

TEST FACILITY RCC (2007a)

B.7. Genotoxicity – *in vitro*

TEST SUBSTANCE Analogue chemical

METHOD OECD TG 476 *In vitro* Mammalian Cell Gene Mutation Test
Species/Strain Mouse
Cell Type/Cell Line Lymphoma L5178Y cells
Metabolic Activation System S9 mix from phenobarbital/β-naphthoflavone induced rat liver
Vehicle Cell culture medium (RPMI + 3% horse serum)
Positive controls Without metabolic activation: ethyl methane sulfonate
With metabolic activation: Benzo[a]pyrene
Remarks - Method Dose selection for main tests was based on the results in a range-finding test carried out at 10.2 - 2636 µg/mL, in the absence of metabolic activation.

Metabolic Activation	*Test Substance Concentration (µg/mL)*	*Exposure Period*	*Expressi on Time*	*Selection Time*
Absent				
Test 1	10, 50, 100, 150, 200, 250, 300*, 350*, 400*, 450*, 500	4 h	72 h	11-14 days
Present				
Test 1	400, 600, 800, 1000*, 1250*, 1500*, 1750*, 2000*, 2250	4 h	72 h	11-14 days

*Cultures selected for metaphase analysis.

RESULTS

Metabolic Activation	*Test Substance Concentration (µg/mL) Resulting in:* *Cytotoxicity in Preliminary Test*	*Cytotoxicity in Main Test*	*Precipitation*	*Genotoxic Effect*
Absent				
Test 1	> 264	> 250	> 500	positive
Present				
Test 1		> 1000	> 2250	positive

Remarks - Results In the absence of metabolic activation, a dose dependent increase in mutants was noted and in the presence of metabolic activation, this increase held on a plateau level before decreasing slightly.

Colony sizing showed test substance induced clastogenic effects.

The negative and positive controls gave satisfactory responses, confirming the validity of the test system.

CONCLUSION The test substance was considered to be mutagenic and clastogenic to mouse lymphoma L5178Y cells.

TEST FACILITY BSL (2002)

B.8. Genotoxicity – *in vitro*

TEST SUBSTANCE	Analogue chemical
METHOD	Similar to OECD TG 487 In Vitro Mammalian Cell Micronucleus Test
Species/Strain	Human
Cell Type/Cell Line	Peripheral lymphocytes
Metabolic Activation System	S9 mix from Aroclor 1254 induced rat liver
Vehicle	Dimethyl sulfoxide
Remarks - Method	Dose selection for the main tests was based on toxicity noted in a range-finding study carried out at 5.805 – 1600 µg/mL. Negative (vehicle and untreated) and positive controls (4-nitroquinoline 1-oxide and cyclophosphamide) were run concurrently with the test substance.

Metabolic Activation	*Test Substance Concentration (µg/mL)*	*Exposure Period*	*Harvest Time*
Absent			
Test 1	200*, 300*, 400, 450*, 500, 550, 575*, 600, 625, 650, 675, 700, 750, 800, 850	20 h	72 h
Test 2	200*, 300*, 350*, 400, 420, 440, 460, 480, 500, 525, 550, 575, 600, 650, 700	20 h	96 h
Present			
Test 1	500, 750, 1000*, 1250, 1500, 1750*, 2000, 2250, 2560*	3 h	72 h
Test 2	473.1, 750, 1000, 1250, 1500, 1750, 2000*, 2250*, 2560*	3 h	96 h

*Cultures selected for metaphase analysis

RESULTS

Metabolic Activation	*Test Substance Concentration (µg/mL) Resulting in:*			
	Cytotoxicity in Preliminary Test	*Cytotoxicity in Main Test*	*Precipitation*	*Genotoxic Effect*
Absent				
Test 1	> 345.6	> 550	>200	positive
Test 2	> 345.6	> 200	>200	positive
Present				
Test 1	> 1600	> 2560	500	positive
Test 2	> 1600	> 2560	>2000	positive

Remarks - Results

Without metabolic activation
In Tests 1 and 2, statistically significant increases in the frequencies of micronucleated binucleate (MNBN) cells were noted at the two highest concentrations analysed. However, the increases were considered to be of questionable biological importance by the study authors as the increases were generally small and with exception of the highest concentration analysed in Test 2, only single cultures exhibited MNBN cell frequencies that exceeded historical vehicle control values. Furthermore, dose levels inducing these increased MNBN cell frequencies also showed high cytotoxicity.

With metabolic activation
In Test 1, statistically significant increases in the frequencies of MNBN cells were noted at all concentrations analysed. However, the increases were considered to be of questionable biological importance by the study authors as the increases were generally small and only one single culture (at 2560 µg/mL) exhibited a MNBN cell frequency that exceeded the historical vehicle control range.

In Test 2, no statistically significant increases in the frequencies of MNBN

cells were noted.

The results of the negative and positive controls confirmed the validity of the test system.

CONCLUSION

The test substance induced micronuclei in cultures of human peripheral blood lymphocyte cells treated *in vitro* under the conditions of the test. However, the positive findings were considered by the study authors to be of questionable biological importance.

TEST FACILITY

Covance (2006)

B.9. Genotoxicity – *in vivo*

TEST SUBSTANCE

Analogue chemical

METHOD

OECD TG 474 Mammalian Erythrocyte Micronucleus Test

Species/Strain: Mouse/NMRI

Route of Administration: Intraperitoneal

Vehicle: Aqua dest.

Remarks - Method: Dose selection for the main tests was based on the findings from a pre-experiment for toxicity carried out at 2000, 250 and 100 mg/kg bw stepwise.

Group	*Number and Sex of Animals*	*Dose mg/kg bw*	*Sacrifice Time hours*
Negative control (vehicle)	5 M, 5 F	0	24
low dose	5 M, 5 F	10	24
mid dose	5 M, 5 F	50	24
high dose	5 M, 5 F	100	24
high dose	5 M, 5 F	100	48
positive control (CPA)	5 M, 5 F	40	24

CPA = cyclophosphamide.

RESULTS

Doses Producing Toxicity

In the pre-experiment for toxicity, the total 2 female animals treated at 2000 mg/kg bw showed toxic symptoms including palpebral closure, lethargy and convulsions and died after 10 minutes. Two fresh female animals were then treated at 250 mg/kg bw and both showed palpebral closure and lethargy and died after 6 hours. Three additional male and 3 female additional animals were then treated at 100 mg/kg bw. Palpebral closure and lethargy was noted in all animals at 1 hour after treatment and no symptoms were noted at 6, 24, 48 and 72 hours post-treatment. No unscheduled deaths occurred at this dose level.

In the main experiment, 10 male and 10 female animals treated at 100 mg/kg bw showed palpebral closure and lethargy at 1 hour after treatment and no symptoms were noted at 6, 24 and 48 hours post-treatment. No unscheduled deaths occurred at this dose level.

The mean relative PCE (relative PCE = proportion of polychromatic erythrocytes among total erythrocytes) was slightly decreased in the 100 mg/kg bw group (male animals at 48 h and female animals at 24 and 48 h), when compared to the negative controls. This effect was considered by the study authors to be biologically relevant, indicating the test substance had reached the bone marrow.

Genotoxic Effects: The number of cells with micronuclei was not significantly different to the negative control.

Remarks - Results: The concurrent negative and positive controls gave satisfactory responses

confirming the validity of the test system.

CONCLUSION — The test substance was not clastogenic under the conditions of this *in vivo* mouse bone marrow micronucleus test.

TEST FACILITY — BSL (2003)

B.10. Genotoxicity – *in vivo*

TEST SUBSTANCE — Analogue chemical

METHOD — OECD TG 486 Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*

Species/Strain — Rat/Wistar Hanlbm: WIST (SPF)

Route of Administration — Oral – gavage

Vehicle — Deionised water

Remarks - Method — Doses were adjusted due to the toxic potential of the test substance. The highest dose of test substance applied in the main experiment was estimated in pre-experiments to be closer to the maximum tolerated dose.

The main experiment was performed using male rats only, since the males could tolerate slightly higher dosages than females without inducing mortality.

Group	*Number and Sex of Animals*	*Dose mg/kg bw*	*Sacrifice Time hours*
vehicle control	4 M	0	4 h
vehicle control	4 M	0	16 h
low dose	4 M	75	4 h
low dose	4 M	75	16 h
high dose	4 M	150	4 h
high dose	4 M	150	16 h
positive control (DMH)	4 M	80	4 h
positive control (2-AAF)	4 M	80	16 h

DMH = N,N'-dimethylhydrazinedihydrochloride; 2-AAF = 2-acetylaminofluorene

RESULTS

Doses Producing Toxicity — In the pre-experiments, 3 animals died (1 male and 1 female treated at 500 mg/kg bw and 1 female treated at 200 mg/kg bw). No natalities occurred at 100 and 150 mg/kg bw dose levels.

In the main study, low and high dose animals showed ruffled fur and reduction in spontaneous activity.

Genotoxic Effects — The viability of the hepatocytes was not substantially affected due to the *in vivo* treatment at any dose of the treatment period or dose groups. The inter-individual variations for the numbers and viabilities of the isolated hepatocytes were in the range of the historical laboratory control data.

Remarks - Results — No dose level revealed UDS induction in the hepatocytes of the treated animals when compared to the concurrent vehicle controls. Both the nuclear grains and the resulting net nuclear gains were not significantly enhanced due to the *in vivo* treatment for 4 h or 16 h. Therefore, the net nuclear gain values obtained after treatment were considered negative. No substantial shift to higher values was observed in the percentage of cells in repair.

In vivo treatment with positive controls showed significant increases in the number of nuclear and net nuclear gain counts.

	The change in the urine colour of the treated animals, which was reported to occur in the main study, could be attributed to the systemic distribution of the test substance, showing its bioavailability.
CONCLUSION	The test substance was reported to be not clastogenic under the conditions of this in vivo unscheduled DNA synthesis (UDS) test in mammalian liver cells.
TEST FACILITY	RCC (2007b)

B.11. Developmental toxicity

TEST SUBSTANCE	Analogue chemical
METHOD	OECD TG 414 Prenatal Developmental Toxicity
Species/Strain	Rat/Sprague-Dawley Crl: OFA.SD
Route of Administration	Oral – gavage
Exposure Information	Exposure days: Days 6 through 19 of gestation Dose regimen: Daily
Vehicle	0.4 % ascorbic acid in water
Remarks - Method	No significant protocol deviations

RESULTS

Group	*Number of Animals*	*Dose mg/kg bw/day*	*Mortality*
1	25 F	0	0
2	25 F	10	0
3	25 F	30	0
4	25 F	90	0

Mortality and Time to Death

No mortalities were recorded. All dams survived to scheduled necropsy.

Effects on Dams

No treatment-related effects were observed in dams treated at the lowest dose (10 mg/kg bw).

Orange-coloured urine was observed in dams at the two highest doses (30 and 90 mg/kg bw). Other clinical signs including hair-loss (1/25 at 10 mg/kg bw) and reddish vaginal discharge (2/25 at 10 mg/kg bw; 4/25 at 30 mg/kg bw and 5/25 at 90 mg/kg bw) were considered to be incidental or related to pregnancy by the study authors. Mean food consumption and mean body weight gain were statistically significantly reduced at 90 mg/kg bw, during gestations days 6-9 compared to the control values. No treatment-related macroscopic changes and mean uterus weights were observed in dams in any groups. Incidental symptoms observed included alopecia (1/25 at 0 mg/kg bw and 1/25 at 10 mg/kg bw); renal pelvic dilation 2/25 at 10 mg/kg bw and yellow fluid or a haematoma in the uterus 1/25 at 10 mg/kg bw and 1/25 at 30 mg/kg bw. Slight increase in the incidence of late resorption was observed in one female and one female showed one late resorption and no viable foetus at 90 mg/kg bw as compared with the control group.

Effects on Foetus

No external abnormalities and no visceral or skeletal malformation related to the treatment were observed in foetuses. Slightly higher incidence of foetuses with delayed ossification of the cranium, paws or sternum were observed at 90 mg/kg bw as compared to the control group and the historical control data. Similar but less marked increase was observed in the 10 and 30 mg/kg bw groups but no statistical significance was attained for any of these findings.

All of the above findings were considered by the study authors to be indicative of a minor delay in foetal ossification and too slight to be of physiological consequence. A slightly greater incidence of rudimentary 14th ribs in all treated groups was observed, but was considered by the study authors to be of no toxicological significance.

Remarks - Results
The effects observed in animals at the highest dose were considered to be related to the test item.

CONCLUSION
The No Observed Adverse Effect Level (NOAEL) was established as 30 mg/kg bw/day by the study authors for maternal toxicity, based on the reduction of the body weight and food consumption in the high dose group.

The NOAEL for developmental toxicity/teratogenicity was reported as 90 mg/kg bw/day.

TEST FACILITY Ricerca (2012)

B.12. Toxicokinetic: dermal absorption *in vitro*

TEST SUBSTANCE Notified chemical (radiolabelled)

METHOD OECD TG 428 Skin Absorption: *In Vitro* Method
Test Guidelines of the COLIPA Task Force for "*In Vitro* Assessment of Percutaneous Absorption and Penetration of Cosmetic Ingredients" comprising a Standard Protocol for *in vitro* Cutaneous Absorption/Penetration with Pig Skin

STUDY DESIGN AND OBJECTIVE
Cutaneous absorption of radiolabelled test substance at 1.824% in a typical hair dye formulation in the presence of hydrogen peroxide and a reaction agent (under oxidative conditions) was investigated in vitro in pig skin preparations, which were continuously rinsed with physiological receptor fluid at 32 ± 2 ºC.

Two independent experiments were performed with 6 diffusion cells per experiment. The mean value of all valid skin samples (n = 11 from 3 donors) in contact with the hair dye formulation were used for calculations.

Integrity of skin preparations was determined by examining penetration characteristics with tritiated water. After checking skin integrity 400 mg of the formulation (100 mg/cm^2) containing 1.824% test substance was applied onto the skin samples (1.824 mg test substance/cm^2) and left for 30 mins. The skin samples were then washed off with shampoo and water. The amount of the test substancel in washings was determined by scintillation counting. The amount of the test substance in the receptor fluid was determined at 16, 24, 40, 48, 64 and 72 hrs by the same method. At termination, skin samples were heat-treated and the upper layers (stratum corneum and upper stratum germinativum) were separated from the lower layers (lower stratum germinativum and upper dermis). Both skin compartments were extracted separately and radioactivity was quantified by scintillation counting.

RESULTS
Skin integrity: 0.7% to 2.5% of applied dose was found after 4 hours in the receptor fluids, which were within the limits of acceptance (≤ 2.0% of applied dose) for 11 skin samples used for determination, while remaining 1 sample (> 2.0% of applied dose) was not used for the calculation of the mean value.

Amount of test substance in:	*μg/cm^2 (Mean ± SD, n = 11)*	*% of applied dose* (Mean ± SD, n = 11)*
Receptor fluid (72 hrs)	1.341 ± 0.328	0.077 ± 0.019
Lower skin (72 hrs)	0.219 ± 0.143	0.013 ± 0.008
Upper skin (72 hrs)	7.177 ± 3.211	0.411 ± 0.179
Rinsing solution (30 mins)	1618.43 ± 43.40	92.88 ± 1.59
Total balance (recovery)**	1712.17 ± 25.10	98.47 ± 1.79

* Corrected for individual applied dose
** Total is corrected for losses on application tips

The majority of the test substance was found in the rinsing solutions (1618.43 ± 43.40 μg/cm^2). Small amounts were found in the upper skin (7.177 ± 3.211 μg/cm^2), the lower skin (0.219 ± 0.143 μg/cm^2) and in the fractions of receptor fluid collected within 72 hours (1.341 ± 0.328 μg/cm^2). The mass balance of the test substance found 95.2 to 101.0% recovery for all 11 skin samples with acceptable integrity. With respect to the receptor fluid the applied fraction was predominantly detectable within the first fraction collected (fractions 0-16 hours).

At the end of the experiment (after 72 hours) the amounts of the test substance in the receptor fluid declined, indicating that the test substance remaining on or in the skin samples after 72 hours did not tend to migrate to deeper layers. This assumption was further supported by the fact that relatively low amounts of the test substance were found in the lower skin compartment compared to upper skin and the total amount in the receptor fluid after 72 hours. The amounts of the test substance found in the upper skin were thus not considered by the study authors to be biologically available (no depot effect).

CONCLUSION
The study authors concluded that, under the assumption that a depot effect was absent, a maximum amount of 1.56 ± 0.36 μg/cm^2 of the test substance was considered biologically available (receptor fluid + lower skin = 1.341 μg/cm^2 ± 0.219 μg/cm^2).

TEST FACILITY Cosmital SA (2009)

B.14. Toxicokinetic: ADME Study

TEST SUBSTANCE	Analogue chemical (radiolabelled)
METHOD	OECD TG 417 Toxicokinetics OECD TG 427 Skin Absorption: In Vivo Method
Species/Strain	Rat/Wistar (female)
Route of Administration	Intravenous (IV) (Groups 1 and 6) Oral – gavage (Groups 2, 3, 7 and 8) Dermal (Groups 4, 5, 9 and 10)
Vehicle	IV administration: 0.9% NaCl Oral administration: Milli-Q water Dermal administration (Groups 4 and 9): Milli-Q water and 81905108B (water based vehicle mimicking use conditions, i.e. including typical ingredients found in hair dye formulations – non-oxidative conditions) Dermal administration (Groups 5 and 10): dimethyl sulfoxide (DMSO)
Dose	IV: 25 mg/kg bw Oral (Groups 2 and 7): 25 mg/kg bw Oral (Groups 3 and 8): 100 mg/kg bw Dermal (Groups 4 and 9): ~24 mg/kg bw (0.3 mg/cm^2) Dermal (Groups 5 and 10): ~100 mg/kg bw (1.25 mg/cm^2)
Duration of exposure (dermal)	30 minutes (Groups 4 and 9) 24 hours (Groups 5 and 10)
Duration of observation	72 hours

STUDY DESIGN AND OBJECTIVE
Groups 1 – 5 were used to define the absorption, distribution, metabolism and excretion (ADME) of the test substance and a total C^{14} radioactivity material balance was determined for these groups. 4 animals were used for each group.

Groups 6 – 10 were used to determine the toxicokinetics (TK) and metabolites of the test substance. 6 animals were used for each group.

In dermal administration groups, animals were collared to prevent ingestion. For the dermal low dose group, a water based vehicle mimicking end use conditions of the test substance under non-oxidative conditions, was used (81905108B). The design of the dermal high dose was to achieve a relatively high bioavailability (ie. 24 hour exposure, occlusive conditions, DMSO as vehicle) compared to standard application conditions for hair dye formulations (30 mins exposure, un-occluded, water based vehicle mimicking use conditions).

Animals in mass-balance groups were housed individually in metabolism cages to obtain a total ^{14}C-radioactivity balance per animal. Urine and faeces were collected in 0 - 8, 8 - 24, 24 - 48, 48 - 72 and 72 - 96 h intervals. Animals were euthanized 72 h after dose administration and tissues and organs collected. Total radioactivity was determined by liquid scintillation counting. For metabolism evaluation, selected faeces and urine samples were pooled per group and the metabolic profile was investigated by LC- PDA -RAD-MS (Liquid Chromatography- Photodiode Array- Radioactivity- Mass Spectrometry).

In the TK groups, blood was sampled from three animals at 0.5, 1, 2, 4, 8, 24, 48 and 72 h after dosing. Total radioactivity and the test substance equivalents were determined.

RESULTS

No mortalities occurred. No clinical signs were noted in animals of the oral exposure groups except all animals in the highest exposure group displayed piloerection on Day 1. No clinical signs were noted in animals of the IV exposure groups. After dermal exposure, all animals displayed red discharge from nose and eye, relating to the collar around the neck (preventing animals from grooming).

Oral absorption was calculated using urine data and also using plasma data. The oral absorption was 99% (low dose group; group 2) and 84% (high dose group; group 3) when calculated from the urine data and 97% (low dose group; group 2) and 128% (high dose group; group 3) when calculated from the plasma data.

Dermal absorption in the low dermal dose group (group 4) was low (2.0% or 0.005 μg/cm^2) and higher in the high dose group (group 5) (21% or 0.29 μg/cm^2). It could not be concluded by the study authors that the amount retained in the application skin site may eventually become systemically available. The skin residues were therefore considered potentially absorbed and potentially systemically available. Therefore, the absorbed fraction and the potentially absorbed fraction were combined to yield the 'total potentially absorbed fraction'. This was 2.7% (0.01 μg/cm^2) and 22% (0.3 μg/cm^2) of the applied dose in the low and high dose dermal groups respectively. Differences in absorption between the dermal dosing groups were due to dosing vehicle, concentration, duration of exposure (24 vs. 0.5 hour) and the use of the penetration enhancer DMSO. When calculated from the plasma data, the absorption was 2.5 and 47% in the low and high dose groups, respectively.

From the plasma data, oral absorption was rapid with T_{max} of 0.5 - 2 hours. Dermal absorption was moderately rapid with a T_{max} of 1 - 2 hours in the low and high dose groups, respectively. This was also noted with the mass-balance data; however, the absorption calculated from the plasma concentrations was approximately twice the absorption calculated from the mass-balance data, partially due to the variability noted in the plasmakinetic data in all animals. It was stated by the study authors that since the mass-balances of the dermal groups were all within the required criteria (100 ± 10%), these absorption percentages were more reliable. Terminal half-lives were 1.22 – 3.94 hours.

Urine was the most important route of excretion of the test substance. Urinary excretion accounted for 78% of the IV dose, 77% of the low oral dose, 66% of the high oral dose, 1% after low dermal dosing and 16% after high dermal dosing. Urinary excretion was highest during the first 8 hours after IV and low oral dosing and thereafter a decreasing excretion rate was noted with increasing time intervals. The excretion was more evenly divided over the 0 – 8 hours and 8 – 24 hours interval in the high oral and dermal groups. This could indicate a slower absorption or elimination of the test substance at high doses.

Faecal excretion accounted for 12% of the IV dose, 13 - 17% after oral dosing, 0.6% after low dermal dosing and 4% after high dermal dosing.

At termination, the average total remaining radioactivity in blood, carcass plus tissues ranged between 0.9% and 1.7% of the administered dose, indicating no major accumulation of radioactivity. Plasma concentrations were below the limit of qualification in dermal groups (Groups 4 and 5) and were in the same order of magnitude for IV and oral groups (Groups 1 – 3). Blood concentrations in Groups 1 – 3 were at least 2-fold higher than plasma concentrations, indicating some distribution of the test substance into the red blood cells.

The average total recovery in Groups 1 - 5 was 89% - 95% of the applied dose.

Results are summarised in the following tables:

Mass balance data

Group No.	*Test item dose level/concentration*	*Dosing route*	*Absorption %*	*Excretion via urine/faeces (%)*
1	25 mg/kg bw	IV	100	78/12
2	25 mg/kg bw	Oral	99	77/13
3	100 mg/kg bw	Oral	84	66/17
4	24 mg/kg bw; 30 mg/mL; 0.3 mg/cm^2	Dermal low	2*	1/0.6
5	100 mg/kg bw; 125 mg/mL; 1.25 mg/cm^2	Dermal high	21*	16/4

* without skin residue

Toxicokinetic data

Group No.	*Test item dose level/concentration*	*Dosing route*	*F_{abs} %*	*C_{max} mg/kg*	*AUC_{last} hr*mg/kg*
6	25 mg/kg bw	IV	100	-	126
7	25 mg/kg bw	Oral	97	25.0	123
8	100 mg/kg bw	Oral	128	66.8	653
9	24 mg/kg bw; 30 mg/mL; 0.3 mg/cm^2	Dermal low	2.5	1.47	3.26
10	100 mg/kg bw; 25 mg/mL; 1.25 mg/cm^2	Dermal high	47	36.8	300

F_{abs}: absolute oral/dermal bioavailability, calculated as (AUC_{last} po or dermal / AUC_{last} IV)*(dose IV/dose po or dermal)*100%

Seven potential metabolite peaks of the test substance were detectable in urine samples and 2 potential metabolite peaks were noted in plasma samples. No radioactivity peaks could be detected in faeces extracts.

Proposed metabolites detected in urine

*Rad Rt**	*m/z***	*MS Rt****	*Metabolic reaction*	*Interdosing comparison (%)*			
				IV (77.8)	Oral (77.4-65.6)	Dermal low (1.1)	Dermal high (15.7)
11.8-12.3	357	11.8	N-acetyl conjugation combined with glucose conjugation (tentative)	+	+	-	+
	195/212	11.9	N-[4-amino-2-(methoxymethyl)-phenyl]acetamide (AS884)	+	+	-	+
				16.7	11.3-9.7	-	23.7
12.3-12.7	388	12.1	N-acetyl conjugation combined with carboxylation followed by glucuronidation (tentative)	+	+	-	+
	151/153	12.2	Parent compound: 1,4-Diamino-2-methyoxymethyl-benzene sulfate	-	-	-	-
				< 5	< 5-5.7	-	< 5
16.6-16.7	193/195	16.3	N-[4-amino-3-(methoxymethyl)-phenyl]acetamide (AS886)	+	+	-	+
				22.8	20.6-41.4	-	28.4
17.5-18.2	354	17.4	N-acetyl conjugation combined with mercapturic acid conjugation	+	+	-	+
				< 5	< 5-6.0	-	< 5
18.8-18.9	254	18.5	1,4-Di((1-oxo-ethyl)amino)-2-(methoxymethyl)-benzene (AS885)	+	+	+	+
				45.3	52.6-26.6	-	35.3

* = radioactivity retention time

** = mass-to-charge ratio

*** = mass spectrometry retention time

Metabolites detected and identified in plasma samples

*Rad Rt**	*m/z***	*MS Rt****	*Metabolic reaction*	*Interdosing comparison (%)*			
				IV	Oral (low-high)	Dermal low	Dermal high
16.4-16.8	195	16.2	N-[4-amino-3-(methoxymethyl)-phenyl]acetamide (AS886)	+	+	-	+
				87.3-100	100-100	-	100
18.9	254	184	1,4-Di((1-oxo-ethyl)amino)-2-(methoxymethyl)-benzene (AS885)	+	+	+	+
				0-12.7	-	-	-

* = radioactivity retention time
** = mass-to-charge ratio
*** = mass spectrometry retention time

N-acetylation was the major route of metabolic conversion of the test substance to form AS886 and then further N-acetylated to form the di-acetylated derivative AS885. To a lesser extent, the test substance was N-acetylated to form AS884. In addition monoacetylation observed in combination with mercapturic acid conjugation or other suggested modifications in the urine that were based on only tentative structure proposals.

CONCLUSION
The test substance administered orally was extensively absorbed, readily distributed into all organs, extensively metabolised and excreted via urine. Dermal absorption of the test substance was high after a 24-hour exposure period and low after a 30-minute exposure period. When absorbed, excretion occurred primarily via the urine. Three major metabolites were detected in the urine and plasma following all routes of administration. N-acetylation of the parent compound was the major metabolic reaction. No major qualitative differences in the metabolite profile were noted between the oral and dermal routes of administration.

TEST FACILITY Notox BV (2009)

APPENDIX C: ENVIRONMENTAL FATE AND ECOTOXICOLOGICAL INVESTIGATIONS

C.1. Environmental Fate

C.1.1. Ready biodegradability

TEST SUBSTANCE	Notified chemical
METHOD	OECD TG 301 B Ready Biodegradability: CO2 Evolution Test
Inoculum	Activated sludge
Exposure Period	28 days
Auxiliary Solvent	None
Analytical Monitoring	Total organic carbon content (TOC)
Remarks - Method	No variations from the guidelines were recorded and good laboratory practice (GLP) principles were followed. A toxicity control was run.

RESULTS

Test substance		*Sodium benzoate*	
Day	*% Degradation*	*Day*	*% Degradation*
6	0	6	61
8	1.5	8	72
14	7.5	14	93
21	14.5	21	100
28	14.5	28	100

Remarks - Results	All validity criteria were satisfied. The toxicity control attained 45% degradation after 14 days indicating the notified chemical is not toxic to the inoculum. The test substance was found to be biodegradable (14.5%) under the conditions of the test. The total CO_2 evolution in the inoculum control at the end of the test was 59.7 mg/L. However, as biodegradation did not reach the pass level of > 60% CO_2 production within the 10 day window, it cannot be classed as readily biodegradable.
CONCLUSION	The notified chemical is not readily biodegradable.
TEST FACILITY	Dr U Noack-Laboratorien (2008b)

C.2. Ecotoxicological Investigations

C.2.1. Acute toxicity to fish embryos

TEST SUBSTANCE	Notified chemical
METHOD	Zebrafish Embryo Toxicity (FET) Test, Semi-static OECD-Guideline draft (2006) with modifications
Species	*Danio rerio* (Zebra fish)
Exposure Period	72 hours
Auxiliary Solvent	None
Water Hardness	30 – 215 mg $CaCO_3$/L
Analytical Monitoring	HPLC
Remarks - Method	A semi-static exposure was performed (24 h renewal into pre-saturated 24 well micro-titre plates). Based on the results of a preliminary static range finding test, concentrations of 0, 0.5, 1, 2, 4 and 8 mg/L were used. Test solutions of the selected test concentrations were prepared by dilution of a stock solution. Sixty replicates per test concentration and a control were tested. Each replicate had 1 egg (20 eggs per plate were set up). A positive control (3,4-dichloroaniline) at 3.7 mg/L was used in each test to confirm the performance of each test.

Results

Table 1. Observations in the Test Vessels after 72 h (3 replicates)

Geometric mean measured test item concentration (mg/L)	*Effects at 72 hours*							
	n	*h*	*L*	*s*	*c*	*p*	*b*	*t*
5.69	46/52	46/52	34/52	5/33	1/19	-	-	-
2.83	53/53	53/53	41/53	-	1/18	-	-	-
1.40	56/56	56/56	53/56	-	-	-	-	-
0.671	58/59	58/59	42/59	-	1/20	-	-	-
0.286	58/58	58/58	53/58	-	-	-	-	-
Reference	3/10	-	1/3	-	-	1/4	1/3	10/14
Control	57/57	57/57	55/57	-	-	-	-	-

[1]n = normal development; h = heartbeat; L = larvae; s = no tail detachment (lethal); c = coagulated egg; p = no pigmentation; b = no blood circulation; t = no heartbeat; - = no observations

Table 2. Cumulative mortality (%) in the Test Vessels (mean of 3 replicates)

Geometric mean measured test item concentration (mg/L)	*Test duration (h)*		
	24	*48*	*72*
5.69	13.3	13.3	23.3
2.83	11.7	11.7	13.3
1.40	6.67	6.67	6.67
0.671	0	1.67	3.33
0.286	3.33	3.33	3.33
Reference	58.3	76.7	95
Control	3.33	5	5

LC50 (72 h)	> 5.69 mg/L (geometric mean measured concentration)
NOEC (72 h)	Not applicable due to low mortalities in all tested concentrations.
Remarks – Results	All draft validity criteria were fulfilled. The dissolved oxygen was 100 % of the air saturation value at the beginning of the study. The overall fertility rate was 98%. The water temperature was maintained at 25.8 ± 0.36 ºC throughout the study. Overall survival of fertilised eggs in the negative control was 95% and the mortality in the positive control was 95%. Recovery rates of the notified chemical were in a range of 37 – 109. All results are based on the geometric mean measured test item concentration. The notified chemical caused a mortality of 23.3% at a nominal concentration of 8 mg/L (geometric mean measured notified chemical concentration 5.69 mg/L). Therefore the LC50 (72 h, nominal notified chemical concentration) was > 8 mg/L.
CONCLUSION	The notified chemical is, at worst, toxic to fish embryos.
TEST FACILITY	Dr U Noack-Laboratorien (2013a)

C.2.2. Acute toxicity to aquatic invertebrates

TEST SUBSTANCE	Notified chemical
METHOD	OECD TG 202 Daphnia sp. Acute Immobilisation Test and Reproduction Test Semi - static
Species	*Daphnia magna*
Exposure Period	48 hours
Auxiliary Solvent	None
Water Hardness	Not reported
Analytical Monitoring	High Pressure Liquid Chromatography (HPLC)
Remarks - Method	No variations from the guidelines were recorded and good laboratory practice (GLP) principles were followed. A stock solution (16 mg/L) was prepared by dissolving 16 mg of the test

substance in 1000 mL dilution water and test solutions were prepared by dilution of this stock solution. Test solutions were renewed daily. A reference item ($K_2Cr_2O_7$) was tested no less than 1 month prior to the current study.

RESULTS

Concentration mg/L		*Number of D. magna*	*% Immobilised*	
Nominal	*Geometrical mean measured concentration*		*24 h*	*48 h*
Control		20	0	0
1.00	0.85	20	0	0
2.00	1.63	20	5	35
4.00	3.08	20	80	100
8.00	6.13	20	90	100
16	12.5	20	90	100

EC50 1.68 mg/L at 48 hours (Confidence limit 1.67 – 1.7)
NOEC 0.85 mg/L at 48 hours

The EC50-values after 24 and 48 hours were calculated by sigmoidal dose-response regression.

Remarks - Results The concentrations of test substance were analysed at all concentration levels after 0 and 24 hours (fresh media) and 24 and 48 hours (old media) via HPLC analysis. The measured concentrations at test start were in the range of 91 - 96 % of the nominal values. At the end of the test the test item gave recoveries in the range of 57 - 81 %. Therefore, all effect values are based on geometric mean exposure concentrations. The reference item had a 24 h EC50 of 1.57 mg/L.

The validity criteria for the test were met. The dissolved oxygen concentration was ≥ 3 mg/L throughout the test.

CONCLUSION The notified chemical is toxic to aquatic invertebrates.

TEST FACILITY Dr U Noack-Laboratorien (2013b)

C.2.3. Algal growth inhibition test

TEST SUBSTANCE Notified chemical

METHOD OECD TG 201 Alga, Growth Inhibition Test
EC Council Regulation No 440/2008 C.3 Algal Inhibition Test

Species *Desmodesmus subspicatus*
Exposure Period 72 hours
Concentration Range Nominal: 1.5, 3.0,6.0, 12 and 24 mg/L
Actual: 0.340, 0.680, 1.61, 3.67 and 8.83 mg/L
Auxiliary Solvent None
Water Hardness 0.24 mmol Ca+Mg/L.
Analytical Monitoring High Pressure Liquid Chromatography (HPLC)
Remarks - Method No variations from the guidelines were recorded and good laboratory practice (GLP) principles were followed.

A stock solution (200 mg/L) was freshly prepared by dissolving 200 mg of the test substance in 1000 mL dilution water and test solutions were prepared by dilution of this stock solution.

RESULTS

Biomass		*Growth*	
E_bC50 *mg/L at 72 h*	NOEC *mg/L*	E_rC50 *mg/L at 72 h*	*NOEC mg/L*
1.78 (confidence limit 1.51 - 2.02)	0.68	2.08 (confidence limit 1.89 - 2.21)	1.61

Remarks - Results

EC50 values of growth rate and yield inhibition after 72 h were calculated by sigmoidal dose-response regression.

The concentrations of test substance were analysed at all concentration levels after 0, 24, 48 and 72 hours via HPLC analysis. The measured concentrations at test start were in the range of 93 - 98 % of the nominal values. At the end of the test the test item gave recoveries in the range of 6 - 14 %. Therefore, all effect values are based on geometric mean exposure concentrations.

The validity criteria for the test were met. The cell growth increased 208-fold after 72 h in the control. The temperature during the test was in the range of 21-24 °C. The pH-value in the control replicates increased from 7.95 to 8.24. The mean coefficients of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3) in the control cultures was 11.1 %. Coefficient of variation of average specific growth rates during the whole test period in replicate control cultures was 1.13 %.

CONCLUSION

The notified chemical is toxic to algae.

TEST FACILITY

Dr U Noack-Laboratorien (2013c)

C.2.4. Inhibition of microbial activity

TEST SUBSTANCE

Notified chemical

METHOD

OECD TG 209 Activated Sludge, Respiration Inhibition Test.

Inoculum

Activated sludge

Exposure Period

3 hours

Concentration Range

Nominal: 3.2, 10, 32, 100, 320 and 1000 mg/L.

Remarks – Method

The test was conducted in accordance with the test guideline without significant deviations. Good Laboratory Practice (GLP) was followed.

Activated sewage sludge was exposed to the test substance at concentrations of 3.2, 10, 32, 100, 320 and 1000 mg/L for a period of 3 hours at a temperature of 18 - 22 °C with the addition of a synthetic sewage as a respiratory substrate.

Copper (II) sulphate pentahydrate was used as a reference substance.

EC50

22.4 mg/L (Confidence limit 16.1 – 29.6)

Remarks – Results

The EC50-value of the reference item was calculated with probit analysis using software SIGMAPLOT (Windows), SPSS CORPORATION

The validity criteria for the test were satisfied. The two control respiration rates differ not more than 15 % in the study. The EC50 (3 hours) of copper (II) sulphate pentahydrate was in the accepted range of 54 - 156 mg/L.

CONCLUSION

The notified chemical is expected to inhibit microbial respiration.

TEST FACILITY

Dr U Noack-Laboratorien (2010b)

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