File No: STD/1596

February 2019

NATIONAL INDUSTRIAL CHEMICALS NOTIFICATION AND ASSESSMENT SCHEME (NICNAS)

PUBLIC REPORT

Manganate(2-), [[*N*,*N*′-1,2-ethanediylbis[*N*-[(carboxy-κ*O*)methyl]glycinato-κ*N*,κ*O*]](4-)]-, potassium (1:2), (*OC*-6-21)-

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	APPLICANTS	CHEMICAL OR TRADE NAME	HAZARDOUS CHEMICAL	INTRODUCTION VOLUME	USE
STD/1596	Yara Australia Pty Ltd and Akzo Nobel Pty Ltd	Manganate(2-), [[*N,N'*-1,2-ethanediylbis[*N*-[(carboxy-κ*O*)methyl]glycinato-κ*N*,κ*O*]](4-)]-, potassium (1:2), (*OC*-6-21)-	ND*	≤ 10 tonnes per annum	Component of micronutrient fertiliser

*Not determined

CONCLUSIONS AND REGULATORY OBLIGATIONS

Hazard classification
Based on the available information, the notified chemical cannot be classified as hazardous according to the *Globally Harmonised System of Classification and Labelling of Chemicals* (GHS) as adopted for industrial chemicals in Australia.

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 according to the label instructions, the notified chemical is not considered to pose an unreasonable risk to public health.

The public report of this assessment will be forwarded to Food Standards Australia New Zealand (FSANZ) for their information and consideration in future dietary surveys when estimating consumer exposure levels to nutrients such as manganese.

Environmental risk assessment
On the basis of the comparison with Australian water quality guideline values and the assessed use pattern, the notified chemical and its transformation products are not considered to pose an unreasonable risk to the environment.

Recommendations

REGULATORY CONTROLS

- The Delegate (and/or the Advisory Committee on Chemicals Scheduling) should consider the notified chemical for listing on the SUSMP with specific label statements for foliar application on crops. This is to control misuse of the chemical to avoid consumer risks through potential dietary exposure.

  Until a scheduling decision becomes available, fertilisers containing the notified chemical should carry the following statements on the product label:

  - This product (or product name) contains Manganese in chelated form and is recommended for the correction of Manganese deficiency in crops. The preferred use is as a soil application although a foliar spray may be used when a soil application is impractical. Ask your agronomist for application advice.

  - CAUTION: This fertiliser contains Manganese and should be used only as recommended. It may prove harmful when misused.

CONTROL MEASURES

Occupational Health and Safety

- A person conducting a business or undertaking at a workplace should implement the following engineering controls to minimise occupational exposure to the notified chemical in powdered form:
  - Adequate ventilation

- 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 in powdered form:
  - Avoid inhalation of dust
  - Minimise dust generation where possible

- 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 in powdered form:
  - Respiratory protection if inhalation of dust is expected

  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.

Disposal

- Where reuse or recycling is 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 containment, physical 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
  - foliar spray application on fruits and vegetables is intended to exceed 2.4 kg/ha (equates to 0.0936 kg manganese/ha) notified chemical/cropping season;

or

(2) Under Section 64(2) of the Act; if

- the function or use of the chemical has changed from component of micronutrient fertiliser, 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 and products containing the notified chemical provided by the notifier were 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
Yara Australia Pty Ltd (ABN: 77 076 301 221)
Level 1, 6 Holt Street
McMAHONS POINT NSW 2060

Akzo Nobel Pty Ltd (ABN: 59 000 119 424)
8 Kellaway Place
WETHERILL PARK NSW 2164

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

EXEMPT INFORMATION (SECTION 75 OF THE ACT)
Data items and details claimed exempt from publication: analytical data, degree of purity, use details and import volume.

VARIATION OF DATA REQUIREMENTS (SECTION 24 OF THE ACT)
Variation to the schedule of data requirements is claimed for all physico-chemical endpoints.

PREVIOUS NOTIFICATION IN AUSTRALIA BY APPLICANTS
None

NOTIFICATION IN OTHER COUNTRIES
Canada, China, EU, Japan, New Zealand and USA

2. IDENTITY OF CHEMICAL

MARKETING NAMES
Dissolvine CXK (product containing the notified chemical at < 30% concentration)
Rexolin CXK (product containing the notified chemical at < 30% concentration)

CAS NUMBER
68015-77-0

CHEMICAL NAME
Manganate(2-), [[*N,N*′-1,2-ethanediylbis[*N*-[(carboxy-κ*O*)methyl]glycinato-κ*N*,κ*O*]](4-)]-, potassium (1:2), (*OC*-6-21)-

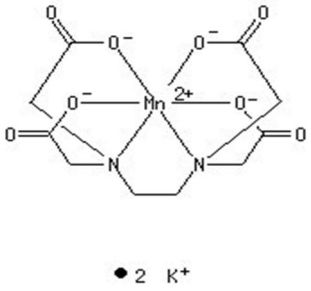
OTHER NAMES
Manganate(2-), [[*N,N*′-1,2-ethanediylbis[*N*-(carboxymethyl)glycinato]](4-)-*N,N*′,*O,O*′,*ON*,*ON*′]-, dipotassium, (*OC*-6-21)-

Manganate(2-), [[*N,N*′-1,2-ethanediylbis[*N*-[(carboxy-κ*O*)methyl]glycinato-κ*N*,κ*O*]](4-)]-, dipotassium, (*OC*-6-21)-

Potassium [(ethylenedinitrilo)tetraacetato]manganate(II)

MOLECULAR FORMULA
$C_{10}H_{12}MnN_2O_8.2K$

STRUCTURAL FORMULA



MOLECULAR WEIGHT
421.3 g/mol

ANALYTICAL DATA
Reference MS and UV spectra were provided.

3. COMPOSITION

DEGREE OF PURITY
> 95%

4. PHYSICAL AND CHEMICAL PROPERTIES

APPEARANCE AT 20 °C AND 101.3 kPa: Green microgranules

Property	Value	Data Source/Justification
Melting Point/Freezing Point	Not determined	Expected to decompose without melting at > 200 °C based on analogue chemical
Boiling Point	Not determined	Expected to decompose without boiling at > 200 °C based on analogue chemical
Density	1,725 kg/m^3 at 20 °C	Measured*
Vapour Pressure	Not determined	Expected to be low based on structure
Water Solubility	> 480 g/L at 20 °C	Estimated
Hydrolysis as a Function of pH	Not determined	Expected to be hydrolytically stable based on the structure
Partition Coefficient (n-octanol/water)	Not determined	Expected to partition to the water phase based on high water solubility of organic moiety
Adsorption/Desorption	Not determined	Expected to be mobile in soil systems due to the high water solubility
Dissociation Constant	Not determined	pKa_1 =11.24; pKa_2= 6.04; pKa_3 =3.74; pKa_4 =1.78 estimated by using ACD/Labs
Particle Size	Inhalable fraction (< 100 μm): 36.7% Respirable fraction (< 10 μm): 1.7%	Measured*
Flash Point	Not determined	-
Flammability	Not determined	Not expected to be highly flammable based on analogue chemical
Autoignition Temperature	Not determined	Not expected to autoignite
Explosive Properties	Not determined	Contain no functional groups that would imply explosive properties
Oxidising Properties	Not determined	Contain no functional groups that would imply oxidising properties

* For the product Dissolvine CXK containing the notified chemical at < 30% concentration

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

Reactivity
The notified chemical is expected to be stable under normal conditions of use.

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. The notified chemical will be imported as a component of micronutrient fertiliser mixture in powder form at < 30% concentration. There will be no reformulation or repackaging in Australia prior to sale to farmers.

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

Year	*1*	*2*	*3*	*4*	*5*
Tonnes	2-10	2-10	2-10	2-10	2-10

PORT OF ENTRY
Melbourne, Sydney, Brisbane, Adelaide, Perth, Hobart and Darwin

IDENTITY OF RECIPIENTS
Yara Australia Pty Ltd
Akzo Nobel Pty Ltd

TRANSPORTATION AND PACKAGING
Products containing the notified chemical at < 30% concentration will be imported as a component of finished soil fertiliser in powder form in 5 kg or 40 kg lined paper bags and transported by road within Australia to regional centres for sale to farmers.

USE
The notified chemical will be used as a component of a micronutrient fertiliser that will be applied only in Mn deficient conditions as determined by foliar and soil testing. The product containing the notified chemical will be applied to vegetables (including leafy vegetables), cut-flowers, potted flowers and pot plants grown in glasshouses and arable crops, including soy-bean, cereals, cotton, maize, oilseed and lucerne, and horticultural crops, including citrus, apple, grapes, peach and plums grown in open fields either as a foliar application or a soil application.

Foliar application
For crops grown in glasshouses, the product containing the notified chemical will be applied at a rate of ≤ 1 g of product/L of water (equivalent to ≤ 0.3 g/L notified chemical). For crops grown in open fields the product containing the notified chemical will be applied at a rate of ≤ 2 kg (equivalent to ≤ 600 g notified chemical) of the product/hectare.

Soil application
Products containing the notified chemical will be applied at a rate of ≤ 15 kg (equivalent to ≤ 4.5 kg notified chemical) of the product/hectare.

In general, the number of applications depends on the type of crop grown and up to four applications in a year may be required with a two-week interval in between.

OPERATION DESCRIPTION
Farmers or farmworkers will move the bags containing the fertiliser mixture (containing the notified chemical at < 30% concentration) to the loading area, weigh-out the required amount of the product and manually add to the make-up tank. The tank will be connected to the spray equipment (boom spray only) or to the on-site drip fertigation system. When boom spray is used for method of application, the farmer will drive the tractor while

the fertigation water containing the notified chemical at ≤ 0.03% concentration is sprayed onto the soil or plant foliage to be treated.

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 and storage	2 - 6	12 - 24
Farmers and farmworkers	1 - 3	1 - 4

EXPOSURE DETAILS

Transport and storage

Transport and storage workers may come into contact with the notified chemical at < 30% concentration only in the event of an unlikely accidental rupture of bags containing the notified chemical.

End-use

Farmers or farmworkers will move the bags containing the fertiliser mixture (containing the notified chemical at < 30% concentration) to the loading area, weigh-out the required amount of the product and manually add to the make-up tank. The tank will be connected to the spray equipment (boom spray only) or to the on-site drip fertigation system. When boom spray is used for application, the farmer will drive the tractor while the fertigation water containing the notified chemical at ≤ 0.03% concentration is sprayed onto the soil or plant foliage to be treated.

The principal route of exposure to the notified chemical will be dermal. However, as the fertiliser mixture is in powder form, during weighing and loading into make-up tank, exposure to the notified chemical via dust through inhalation route and to a lesser extent through ocular route is also possible.

The notifier has confirmed that, if the product is applied via spray, only boom sprayers will be used. No other methods, such as air-blast or handheld or backpack applicators or aerial application, will be used. As low energy/low pressure equipment will be used in the boom spray, a very low amount of fine spray particles will be generated during spraying. The notifier stated that the end-users are expected to adhere to Australian Pesticides and Veterinary Medicines Authority's (APVMA) operating principals to prevent spray drifts (APVMA, 2008).

The notifier stated that workers will use appropriate PPE including impervious gloves, coveralls, safety glasses and dust masks to minimise repeated exposure. Moreover, good hygiene practices are expected to be in place.

6.1.2. Public Exposure

The products containing the notified chemical will not be made available to the public. Application of products containing the notified chemical by ground-boom application may lead to unintended bystander exposure via chemical spray drift. This may be in the form of single random exposure or repeat exposure of residents who reside adjacent to areas being treated with the product.

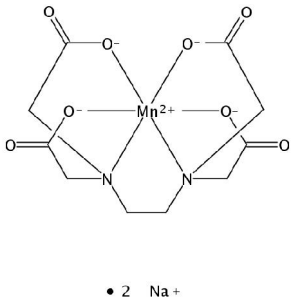
As low energy/low pressure equipment will be used in the boom spray, a very low amount of fine spray particles will be generated during spraying. The notifier stated that the end-users are expected to adhere to Australian Pesticides and Veterinary Medicines Authority's (APVMA) operating principals to prevent spray drifts (APVMA, 2008).

The products containing the notified chemical will be applied via foliar spray and soil application to various food producing crops grown in fields (including soybeans, apples, plums, peaches and grapes) and glasshouses (including various leafy vegetables), up to four times in a growing season. It will be applied during various stages, including fruit and vegetable formation and maturation phases, of crop growth. As the notified chemical will be applied via foliar spray during fruit and vegetable formation and maturation phases, public exposure to the notified chemical may occur via ingestion of fresh produce.

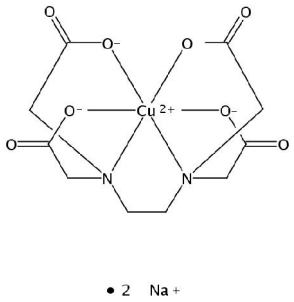
6.2. Human Health Effects Assessment

The results from toxicological investigations conducted on the notified chemical and analogue chemicals are summarised in the following table. For full details of the studies, refer to Appendix B.

Endpoint	*Test Substance*	*Result and Assessment Conclusion*
Rat, acute oral toxicity	Notified chemical	LD50 > 2,000 mg/kg bw; low toxicity
Rat, acute inhalation toxicity	Analogue 1	LC50 > 5.16 mg/L/4 h; low toxicity
Skin irritation (*in vitro*)	Notified chemical	non-irritating
Eye irritation (*in vitro*)	Notified chemical	non-irritating
Mouse, skin sensitisation – Local lymph node assay	Analogue 2	no evidence of sensitisation
Rat, repeated dose oral toxicity with reproductive/developmental toxicity screening – 90 days	Analogue 1	NOAEL (reprod/develop) = 500 mg/kg bw/day
Rabbit, developmental toxicity	Analogue 1	NOAEL (maternal) not established (discolouration of urine and increase creatine kinase levels at all treated groups) NOAEL (develop) = 30 mg/kg bw/day (maternal toxicity observed at higher doses)
Mutagenicity – bacterial reverse mutation	Analogue 2	non mutagenic
Genotoxicity – *in vitro* mammalian cell gene mutation test	Analogue 1	non genotoxic
Genotoxicity – *in vitro* mammalian micronucleus test	Analogue 1	non genotoxic
Genotoxicity – *in vitro* mammalian micronucleus test	Analogue 2	genotoxic



Analogue chemical 1 (EDTA-$MnNa_2$)
(CAS No. 15375-84-5)



Analogue chemical 2 (EDTA-$CuNa_2$)
(CAS No. 14025-15-1)

The analogue chemicals 1 (EDTA-$MnNa_2$) and 2 (EDTA-$CuNa_2$) are metal complexes of ethylenediaminetetraacetic acid (EDTA) similar to the notified chemical. Analogue chemical 1 containing manganese is considered the most suitable analogue as any hazard arising from the notified chemical is expected to be related to the bioavailability of manganese (II) ions. Analogue chemical 2 (containing copper) is used where data gaps exists.

Toxicokinetics, metabolism and distribution

No toxicokinetic data on the notified chemical were submitted.

EDTA salts are poorly absorbed by the oral and dermal route (ECCC/HC, 2017). Following administration of 50 mg radiolabeled EDTA-$CaNa_2$ to rats via oral gavage, oral absorption after 24 hours was 10 and 6% in males and females, respectively, based on urinary excretion. In male humans exposed to 1.5 or 2 mg radiolabeled EDTA-$CaNa_2$ by the oral or dermal route, recovery in urine was only 5% through the oral route after 24 hours and 0.001% through the dermal route. In addition, it has been reported that EDTA and 23 of its salts did not absorb through the skin (ECCC/HC, 2017).

The notified chemical is a highly water soluble (> 480 g/L) manganese compound. Upon dissolution, a negatively charged Manganese-EDTA complex and 2 positively charged potassium (I) ions will exist. The

EDTA moiety surrounds and protects the Mn (II) ion and modifies its reactivity. The Manganese-EDTA complex can be absorbed following oral exposure, but it does not readily penetrate the skin following dermal exposure. However, based on the low oral absorption of EDTA from the gut (<5%), the oral absorption of the Mn-EDTA complex from the gut is expected to be limited (<5%). The notifier stated that the amount of Mn (II) ions absorbed from the gut will depend on the physiological need of the individual. Following inhalation exposure, in view of the relative large particle size, mucociliary clearance from the respiratory tract will generate oral exposure following inhalation, as particles can be returned from the lungs to the back of the throat and swallowed.

Acute toxicity
The notified chemical was found to be of low acute oral toxicity in rats.

No studies were submitted for acute dermal toxicity. No signs of systemic toxicity were observed in dermal irritation studies conducted with the notified chemical. Moreover, based on the physiochemical properties of the notified chemical, it is expected that the absorption of the notified chemical via the dermal route will be limited.

No studies were submitted for acute inhalation toxicity for the notified chemical. Analogue chemical 1 was found to be of low acute inhalation toxicity in rats. Therefore, by inference, the notified chemical is expected to be of low acute toxicity via the inhalation route.

Irritation and sensitisation
The notified chemical was found to be non-irritating to the skin and eyes based on the results from *in vitro* studies.

No studies were submitted for skin sensitisation for the notified chemical. Analogue chemical 2 was determined not to be a skin sensitiser in a mouse local lymph node assay. Therefore, by inference, the notified chemical is not expected to be a skin sensitiser.

Repeated dose toxicity and toxicity for reproduction
No data were submitted for the notified chemical.

Although manganese is an essential micronutrient necessary for normal cellular functioning of humans, excessive accumulation of manganese in the human central nervous system can lead to neurotoxicity that resembles Parkinson's disease (Erikson et al., 2007; ATSDR, 2012). Excessive manganese accumulation in human brain occurs following high dose inhalation or oral exposure (Aschner et al., 2005).

Soluble manganese compounds are classified as hazardous according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) for repeat dose toxicity (Category 1): "causes damage to organs through prolonged or repeated exposure through inhalation and oral routes" based on neurological effects reported in humans with repeated manganese exposure (NICNAS). Clinical signs associated with manganese neurotoxicity in humans include gait abnormalities, postural instability, micrographia, dystonia, rigidity and bradykinesia (Dorman et al., 2006).

The notified chemical is estimated to be highly water soluble (> 480 g/L at 20 °C); however, following dissolution, a negatively charged Manganese-EDTA complex and 2 positively charged potassium (I) ions will exist. The notifier claimed that the EDTA moiety surrounds and protects the Mn (II) ion and modifies its reactivity.

Two repeated dose toxicity studies conducted in rats and rabbits were provided using the analogue chemical 1. In a combined repeated dose oral toxicity study with the reproduction/developmental screening test, rats received analogue chemical 1 (containing manganese) daily by oral gavage at concentrations of 150, 500 and 1,500 mg/kg bw/day during a premating period of 10 weeks and during mating, gestation and lactation (until postnatal day 4) for females and 90 days for males. Compounds with chelating properties such as the notified chemical have been shown to induce repro-toxic effects due to binding of zinc and a related loss of zinc from the body. These effects could be prevented by providing a zinc-supplemented diet to the animals to compensate for the loss of zinc through chelation. To investigate this possibility with the analogue chemical an additional high-dose group was included whereby the animals were fed a standard rodent diet supplemented with zinc carbonate (500 ppm zinc). All other groups received the standard diet for rodents (containing 100 ppm zinc).

The following adverse effects (statistically significant) were observed in animals treated at 1,500 mg/kg bw/day (with or without zinc supplement):

- reduction in live born pups and increase in stillborn pups
- increase in postimplantation loss
- reduction in epididymal sperm motility in males (males treated with mid-dose also showed statistically significant reduction in epididymal sperm motility, however, the reduction in this group was reported to be within the historical control data range and was not considered to be toxicologically significant)
- increase in both the absolute and relative weights of the kidneys in both sexes
- reduction in absolute and relative weights of the spleen in females
- increase in the incidence of very slight diffuse renal subcortical tubular dilation in females

Based on the histopathological findings in kidneys in both sexes, the No Observed Adverse Effect Level (NOAEL) for parental toxicity was established at 500 mg/kg bw/day by the study authors. However, females in all treatment groups had statistically significant increased kidney weight, and pelvic mineralisation in mid and high doses compared to control females. The NOAEL for reproductive and developmental toxicity was established by the study authors as 500 mg/kg bw/day based on reduced sperm motility and increased number of stillborn pups delivered at the high dose. No considerable differences in reproductive effects were observed in the high dose groups with or without zinc supplement.

In a developmental toxicity study, rabbits received analogue chemical 1 (with manganese) daily by oral gavage at concentrations of 10, 30 and 100 mg/kg bw/day from Days 6 to 28 post-coitum. Similar to the combined repeated dose toxicity with reproduction/developmental screening test, an additional high-dose group was included whereby the animals were fed a zinc supplemented diet (i.e. a standard rabbit diet supplemented with zinc carbonate (200 ppm zinc)). All other groups received the standard diet for rabbits (containing 73 ppm zinc).

Based on the discolouration of urine and increased creatine kinase levels in all treated animals, a NOAEL for maternal toxicity was not established by the study authors. At the high dose without zinc supplement in the diet, the foetuses showed signs of retarded skeletal ossification (i.e. higher incidences of unossified metacarpals and reduced ossification of the skull). However no delay in skeletal ossification was observed in high dose animals fed a zinc supplemented diet suggesting that zinc supplementation amended the slight developmental delay. The NOAEL for developmental toxicity was established by the study authors as 30 mg/kg bw/day. The developmental effects were observed in the presence of maternal toxicity.

Minor kidney effects were observed in some animals treated at 100 mg/kg bw/day (with and without zinc supplement). However, a histopathological study conducted (in 2017) on the stored tissues of the treated animals showed that only minimal to slight kidney effects (urothel vacuolation, tubular basophilia, pelvic mineralisation, mineralisation, tubular dilation and tubular vacuolation) were observed in some of the treated animals.

Species differences in neurological responses were reported following high dose manganese exposure. Monkeys have shown similar distribution patterns for manganese ‘within the brain that mimic those seen in heavily exposed people’ (Dorman et al., 2006). It was also reported that ‘rodents do not develop behavioural syndromes comparable to those seen in manganese-poisoned humans or monkeys (Boyes and Miller, 1998, Dorman et al., 2006).

Although the soluble manganese compounds are classified as hazardous (NICNAS), in the absence of clear evidence of neurotoxicity effects as reported by the study authors in the two studies (based on observations of clinical signs in rats and rabbits, and arena testing, neurobehavioral observations, motor activity assessment and brain histopathology in rats), the notified chemical is not classified as hazardous.

Mutagenicity/Genotoxicity
There were no data available on mutagenicity or genotoxicity for the notified chemical.

Analogue chemical 1 tested negative in an *in vitro* micronucleus test in human lymphocytes and in an *in vitro* cell gene mutation test in mouse lymphoma cells. Analogue chemical 2 tested negative in a bacterial reverse mutation assay but a positive response was obtained in an *in vitro* micronucleus test with human lymphocytes. However, it was reported that the percentage of binucleated cells containing micronuclei were only slightly higher than the historical control range of the test facility.

Based on the weight of evidence from these studies, the notified chemical is not expected to be genotoxic.

Health hazard classification
Based on the available information, the notified chemical cannot be classified as hazardous according to the *Globally Harmonised System of Classification and Labelling of Chemicals* (GHS) as adopted for industrial chemicals in Australia.

6.3. Human Health Risk Characterisation

6.3.1. Occupational Health and Safety
Based on the available information, the notified chemical is not recommended for hazard classification according to the GHS. However, soluble manganese compounds pose a concern for repeated dose oral and inhalation toxicity based on neurological effects reported in humans with manganese exposure (NICNAS). The notified chemical is estimated to be highly water soluble (> 480 g/L at 20 °C); however, following dissolution, a negatively charged Mn-EDTA complex and 2 positively charged potassium (I) ions will exist. The EDTA moiety surrounds and protects the Mn (II) ion and modifies its reactivity. Given the strong chelating effect of EDTA for manganese ions, the notifier claims that free manganese ions are not expected to be available for exposure during application of the fertiliser solution and from sprayed crop foliage. However, no data have been provided to justify this claim.

During weighing and loading into the make-up tank, exposure to dust of the product containing the notified chemical at < 30% concentration is possible. The product contains a significant fraction of inhalable particles (36.7% < 100 μm) but only a very small fraction of respirable particles (1.7% < 10 μm). If inhaled, the notified chemical will therefore likely to be deposited in the mucus lining of the upper respiratory tract where it will dissolve and be removed upwards by the mucociliary escalator and subsequently swallowed. Given the risk of systemic toxicity via inhalation control measures should be implemented to reduce inhalation exposure to dusts of the notified chemical, including respiratory protection and adequate ventilation. The notifier has stated that workers will use dust masks when handling the product containing the notified chemical in powdered form.

It is expected that the spray operations will be low energy/low pressure, therefore inhalation exposure to vapours, mists or aerosols during spray application is not likely to occur. Furthermore, the concentration of the notified chemical in spray solution is low (≤ 0.03%), therefore inhalation exposure to the notified chemical will not be significant.

Overall, under the conditions of the occupational settings described, the risk to workers from use of the notified chemical is not considered to be unreasonable.

6.3.2. Public Health
The products containing the notified chemical will not be made available to the public. Bystander risk is possible, but is expected to be limited based on the proposed use pattern. Potential routes of exposure for bystanders are dermal, inhalation and ocular during or immediately after a spraying event, while dermal exposure is the most likely route of exposure during re-entry situations. Workers adherence to good agricultural practice will minimise potential risks for the public during spray application.

Products containing the notified chemical will be applied via foliar spray and soil application to various food producing crops grown in fields (including soybeans, apples, plums, peaches and grapes) and glasshouses (including leafy vegetables), up to four times in a growing season. It will be applied during various stages, including fruit and vegetable formation and maturation phases, of crop growth.

As the notified chemical will be applied via foliar spray during fruit and vegetable formation and maturation phases, manganese residues of the notified chemical could be present on fresh fruits and vegetables, including leafy vegetables. Neurological effects have been reported in humans with excessive manganese exposure through inhalation or oral routes (NICNAS; Dorman et al., 2006). World Health Organisation (WHO) recommends a tolerable daily intake (TDI) of 0.06 mg/kg bw/day for manganese (WHO, 2003). For health reasons, the Australian Drinking Water Guidelines provide a guideline value of 0.5 mg/L for manganese in drinking water, and indicates < 0.5 mg/L as desirable in drinking water (NHMRC, 2011). Based on this guideline value, a 60 kg person drinking 2 L of water per day can be exposed up to 1 mg of manganese/day from drinking water (equivalent to 0.017 mg manganese/kg bw/day).

In case of soybean, cereals and oilseeds, the presence of pod or husk will act as a protective barrier against the notified chemical reaching consumable parts, such as seeds. In case of fruits, foliar application will target leaves

and therefore, only a portion of the applied product is expected to be present on the fruits and vegetables. Moreover, the notifier has stated that the products containing the notified chemical will only be applied as a foliar spray if the concentration of manganese in the leaves is < 30 ppm.

The notifier has provided a worst case consumer exposure scenario through ingestion of manganese residues of the notified chemical from unwashed butter lettuce (lettuce) treated by four foliar applications of the notified chemical:

- Product containing the notified chemical could be applied as a foliar spray on lettuce for 4 times per season at a rate of 2 kg/ha per spray application (8 kg/ha in total per crop season).
- A hectare of lettuce plantation will yield ~ 10,000 to 12,000 kg of lettuce heads. One kg of lettuce will contain 0.8 g of the product (i.e. 8 kg of the product ÷ 10,000 kg of lettuce).
- As the product contains approximately 3.9% manganese, 1 kg of lettuce will contain 0.0312 g of manganese (i.e. 0.039 × 0.8 g).
- An edible part of lettuce head weighs ~ 360 g and a person would consume ~100 g of unwashed lettuce/day.
- Therefore, 3.12 mg of manganese will be available in 100 g of unwashed lettuce.
- This equates to 0.052-0.045 mg of manganese/kg bw/day for a 60-70 kg person.
- This value is below the WHO recommended TDI level of 0.06 mg/kg bw/day for manganese (WHO, 2003).

Members of the public may consume various fruits and vegetables containing manganese residues of the notified chemical. However, exposure to the manganese residues at significant levels (as calculated above with four spray applications and unwashed) is not expected due to:

- low concentration (< 0.03%) of the notified chemical in one spray application;
- watering between spray applications will wash off residues on crops; and
- watering prior to harvest, or rain following application, will wash off residues on crops.

Given the strong chelating effect of EDTA for manganese ions, the notifier claims that free manganese ions are not expected to be available for exposure following application of fertilisers. However, misuse of the notified chemical (e.g. use it when there is no manganese deficiency in crops) on crop foliage may cause repeated exposure to the notified chemical (and manganese) in consumers through their diets that could lead to daily systemic doses higher than the WHO TDI level for manganese.

To prevent misuse of the notified chemical, the notifier has agreed to add the following label statements on fertiliser products containing the notified chemical:

- *This product (or product name) contains Manganese in chelated form and is recommended for the correction of Manganese deficiency in crops. The preferred use is as a soil application although a foliar spray may be used when a soil application is impractical. Ask your agronomist for application advice.*

- *CAUTION: This fertiliser contains Manganese and should be used only as recommended. It may prove harmful when misused.*

The public report of this assessment will be forwarded to Food Standards Australia New Zealand (FSANZ) for their information and consideration in future dietary surveys when estimating consumer exposure levels to nutrients such as manganese when applied on fresh fruits and vegetables as a foliar spray.

Overall, when used in the proposed manner according to the label instructions, the notified chemical is not considered to pose an unreasonable risk to public health.

7. ENVIRONMENTAL IMPLICATIONS

7.1. Environmental Exposure & Fate Assessment

7.1.1. Environmental Exposure

RELEASE OF CHEMICAL AT SITE

The notified chemical will not be manufactured in Australia. It will be imported as a component of micronutrient fertiliser mixture in powder form. There will be no reformulation or repackaging in Australia prior to sale to farmers. Any accidental spills during transport are expected to be collected and recycled or disposed of to landfill.

RELEASE OF CHEMICAL FROM USE

The notified chemical will be used as a component of a micronutrient fertiliser that will be used in micronutrient deficient soils. The product containing the notified chemical will be applied either as a foliar application or a soil application. For foliar application, it will be applied at a rate of ≤ 1 g of product/L of water (equates to ≤ 0.3 g/L notified chemical) or at a rate of ≤ 2 kg (equivalent to ≤ 600 g notified chemical) of product/hectare. For soil application, it will be applied at a rate of ≤ 15 kg (equivalent to ≤ 4.5 kg notified chemical) of product/hectare. Up to four applications may be required with a two-week interval in between. The fertiliser mixture containing the notified chemical will be mixed with water and connected to the spray equipment (boom spray only) or to on-site drip fertigation system. When boom spray is used for method of application, the farmer will drive the tractor while the fertigation water containing the notified chemical at ≤ 0.03% concentration is sprayed onto the soil or plant foliage to be treated. Notified chemical residues remaining in application equipment are expected to be delivered to soil during subsequent use of the equipment.

RELEASE OF CHEMICAL FROM DISPOSAL

During use, the total amount of notified chemical is expected to be applied to the soil or plant foliage as the fertiliser. However, unwanted, unused fertiliser is likely to be disposed of by an authorised waste disposal company.

7.1.2. Environmental Fate

The notified chemical is expected to be applied to plant foliage and topsoil as fertiliser. The submitted study conducted on an analogue (analogue chemical 3; EDTA-$CaNa_2$) indicates that the organic moiety of the notified chemical is not readily biodegradable within 28 days but shows high biodegradation rate in natural waters at pH=8. Generally, EDTA-metal complexes are considered to be inherently biodegradable. For the details of the environmental fate studies please refer to the Appendix C. Supplementary environmental fate characteristics of the notified chemical were sourced from published documents including the report provided by the notifier which is based on European Union Risk Assessment Report 2004. In addition, metal ion exchange reactions may occur in soils. The notified chemical (EDTA-MnK_2) will be converted to Fe or Ca-EDTA complexes depending on the pH in the environment (European Union Risk Assessment, 2004). The Ca-EDTA complex is susceptible to biodegradation at pH > 8, whereas the Fe EDTA complex is susceptible to photodegradation (European Union Risk Assessment 2004). The half-life of the notified chemical was reported to be < 1-7 days in soil systems at different pH (Aboulroos, 1981). Released manganese may form soluble and insoluble complexes with organic and inorganic ligands and undergo biochemical oxidation and reduction in aqueous and soil environments (Cerrato et al., 2010). The distribution of manganese between solution and solid phases is related to the soil characteristics, biocycling and seasonal fluctuations of water tables and redox gradients (Reisenauer, 1988). Bioavailable manganese may be rapidly converted to the insoluble manganese compounds or adsorbed or precipitated as manganese (II) with another soil constituent (Reisenauer, 1988). A small and highly variable fraction may remain as manganese (II) ions in the soil solution (Reisenauer, 1988).

The environmental chemistry of manganese is governed by pH and redox conditions in the aquatic, soil and sediment environments. The acidic pH and reducing conditions may favour manganese mobilisation (Heal, 2001). A significant proportion of manganese may be also associated with suspended solids depending on the cation exchange capacity and the organic composition of the soil (WHO, 2004).

The microelements contained in liquid fertilisers such as manganese is necessary for plant metabolism as they are components of the active centres of many indispensable enzymes, and take part in photosynthesis and water photolysis and other processes (Borowiec et al., 2007). Therefore, the notified chemical in the form of bioavailable manganese species is expected to be taken up by plants and crops in nutrient deficient soils.

Manganese bioavailability is controlled by soil properties, plant characteristics, and the interactions of plant roots and the surrounding soil.

The notified chemical (EDTA-MnK_2) is expected to be highly soluble and may reach aquatic environment from overspray, spray-drift or run off. The notified chemical will transform under environmental conditions and rapidly form different manganese species which may have variable solubility and bioavailability in complex soil, aquatic and sediment systems. However, efficient and economic use of fertilisers, in addition to good farming practices, is expected to minimise loss of the notified chemical to the aquatic environment. Generally, a bioavailable fraction of manganese has high potential to bioconcentrate in lower trophic organisms (plankton, aquatic plants and some fish) but has low potential for biomagnification from lower trophic levels to higher (Williams et al., 2012).

7.1.3. Predicted Environmental Concentration (PEC)

The notified chemical is intended as part of a nutrient replacement program for agricultural land and actual application rates will depend on specific crop nutrient requirements. The product containing the notified chemical will be applied either as a foliar application or a soil application. For foliar application, it will be applied at a rate ≤ 2 kg of product (equivalent to ≤ 600 g notified chemical)/hectare. For soil application, it will be applied at a rate of ≤ 15 kg of product (equivalent to ≤ 4.5 kg notified chemical)/hectare. Up to four applications in a year may be required with a two-week interval in between.

Since the half-life of the notified chemical is short, the predicted environmental concentrations were calculated based on the application rates of the notified chemical and total manganese.

Soil compartment

The notified chemical will be released into soils as a result of its application to agricultural soils by ground boom sprayer and drip irrigation. The recommended annual maximum application rate of the notified chemical [4.5kg notified chemical /ha×4times]$_{soil}$+[4times×0.6kg notified chemical/ha]$_{foliar}$ = 20.4 kg notified chemical/hectare or 20.4 kg/ha×[54.94/421.3] = 2.66 kg of manganese/hectare, results in a worst case PEC_{soil} [notified chemical] = 13.6 µg/kg and PEC_{soil} [$manganese_{Total}$] = 1.77 µg/kg in the 10 cm of the soil system assuming soil density of 1500 g/cm^3. PEC_{soil} values decrease further with soil depth. The increase in manganese in soil is considered well within natural variability of this element in Australian soil (4 and 13,000 mg/kg; Naidu et al., 1996).

Aquatic compartment

The notified chemical may reach aquatic environments from overspray, spray drift during application by ground boom sprayer, or in run-off. Direct overspray is unlikely based on the reported use pattern. For run-off a worst-case edge-of-field scenario may be considered assuming a 100 mm rainfall event with 20 mm of run-off and 5% of the applied chemical contained in the run-off water (https://apvma.gov.au/node/805). This does not consider the uptake by crops, degradation and mobility of the notified chemical. Given the short half-life of the notified chemical accumulation between applications is not expected, and the resulting concentration from a run-off event after a single application is 1,275 µg/L {[(0.6 + 4.5 kg/ha) × 0.05] ÷ 200 m^3}. Its transformation product (manganese), at the proposed volume and application rate of the notified chemical, will not significantly contribute to the background concentration in water and sediment from the run-off route, given manganese is ubiquitous in the environment and its concentration will be dependent on the natural fate and cycle of manganese species.

Exposure to the aquatic compartment from spray drift as a result of application by ground boom sprayer can be modelled using the AgDRIFT® model (AgDRIFT Spray Drift Task Force Spray Software, Version 2.0.09). The PEC arising from spray drift is calculated for both the notified chemical (EDTA-MnK_2) and total manganese assuming a water body 15 cm deep and 3 m wide. The variables in the model that affect spray drift are the droplet size and height of the boom. Since the notified chemical will be used as a fertiliser, a coarse droplet size is likely to be used, and a high boom height is assumed based on the worst-case scenario. Generally, off-target exposure is increased with fine droplets size and increased boom height. The percent drift at 0 m, 1 m and 5 m is 18%, 6.79% and 2.1% of the nominal application rate, respectively. With the application rate of 20.4 kg of notified chemical/hectare $PEC_{spray\ drift}$ for a water body at 0 m, 1 m and 5 m are 2,480 µg/L, 920 µg/L, and 280 µg/L, respectively. With an application rate of 2.66 kg manganese/ha the $PEC_{spray\ drift}$ for a water body at 0 m, 1 m and 5 m are 320 µg manganese/L, 120 µg manganese/L, and 37 µg manganese/L, respectively.

A significant proportion of the notified chemical will be lost due to uptake in plants or may be associated with the solid phase; therefore, the calculated PEC is an overestimate of the aquatic exposure.

7.2. Environmental Effects Assessment

The results from ecotoxicological investigations conducted on analogue chemical 1 (EDTA-$MnNa_2$) are summarised in the table below. Details of these studies can be found in Appendix C. In some cases, additional ecotoxicological endpoints have been sourced from the published literature to supplement the submitted information. A small proportion of released manganese may be bioavailable to aquatic organisms and the toxicity of the notified chemical may be attributed to the soluble form of manganese (Mn (II)). Both released manganese and uncomplexed EDTA show higher toxicity to aquatic life than Mn-EDTA complex (Sorvari and Sillanpää, 1996). Transformation products such as Fe and Ca-EDTA complexes are not expected to be harmful to the aquatic life similarly to analogue chemical 1 based on the submitted information by the notifier.

Endpoint	*Result*	*Assessment Conclusion*
Analogue chemical 1		
Acute toxicity		
Fish Toxicity	96 h LC50 > 1000 mg/L	Not harmful to fish
Algal Toxicity	72 EC50 > 100 mg/L	Not harmful to algae
Inhibition of Bacterial Respiration	3 h IC50 > 640 mg/L	Not inhibitory to microbial respiration
Chronic toxicity		
Daphnia Toxicity	21 d NOEL = 156 mg/L	Not harmful to aquatic invertebrates
Mn (II)		
Acute toxicity		
Daphnia Toxicity	24 h EC50 = 56* mg/L	Harmful to aquatic invertebrates
Daphnia Toxicity	48 h EC50 = 0.8-76.3[§] mg/L	Very toxic or toxic or harmful to aquatic invertebrates
Algae Toxicity	72 h EC50 = 8.3[§] mg/L	Toxic to algae
Fish Toxicity	96 h LC50=2.4-3350[§] mg/L	Toxic or harmful or not harmful to fish

[*] Sorvari and Sillanpaa (1996)
[§] WHO (2004)

Based on the above ecotoxicological endpoints for analogue chemical 1, the notified chemical is not expected to be harmful. Therefore, the notified chemical is not formally classified under the *Globally Harmonised System of Classification and Labelling of Chemicals (GHS)* (United Nations, 2009) for acute and chronic toxicities.

WHO (2004) report indicates that manganese species in ionic form may be very toxic to aquatic life. However, manganese species are not expected to be present in ionic form in significant amounts in the natural environment therefore, the ANZECC (2000) guideline limits for manganese in surface waters for ecosystem protection will be considered as more representative for risk characterisation purposes.

No ecotoxicity data was provided to describe toxicity of the notified chemical and manganese species to soil- and sediment-dwelling organisms.

7.2.1. Predicted No-Effect Concentration

The notified chemical is not toxic to the limits of testing for species relevant to the aquatic environment. Therefore the predicted no-effect concentrations have not been calculated for the aquatic compartments using the assessment factor methodology for industrial chemicals. Rather for determining the risks to the environment in the risk characterisation, the effects endpoints were directly compared to the predicted environmental concentrations in accordance with the methodology outlined in the Environmental Risk Assessment Guidance Manual for Agricultural and Veterinary Chemicals (EPHC, 2009) or were compared to the ANZECC (2000) guideline limits for manganese in surface waters for ecosystem protection.

7.3. Environmental Risk Assessment

The application of the notified chemical to fields by ground boom sprayer or drip irrigation has the potential to result in exposure to aquatic organisms in the nearby water bodies. The risk quotient for the aquatic compartment for the notified chemical was not calculated as the notified chemical is not considered to be harmful to aquatic life, and therefore, does not pose a risk to the environment based on low hazard and use pattern.

In Australia, the ANZECC (2000) guideline limits for manganese are 1,700 µg/L in surface waters for ecosystem protection. At the proposed application rate of the notified chemical it may lead to the maximum predicted environmental concentrations in surface waters of 320 µg manganese/L per hectare from spray drift which is significantly below a freshwater moderate reliability trigger value of 1700 µg manganese/L. Generally,

concentrations of dissolved manganese in natural waters rarely exceed 1,000 µg manganese/L and are usually less than 200 µg manganese/L (WHO, 2004).
It is noted that manganese is ubiquitous in the environment, and that manganese introduced to the soil environment represents just a small fraction of the total manganese concentration in surface soils. Background concentrations of manganese in Australian rural surface soils vary between 4 and 13,000 mg/kg (Naidu et al., 1996). Given significant proportion of manganese species will be taken up by plants and crops, no significant increase in environmental levels on manganese is expected from the use of the notified chemical as a fertiliser in nutrient deficient soils. Similarly the concentration of manganese in sediment will be dependent on its fate and behaviour in the whole aquatic system, including the overlying water, and no significant increase in environmental levels of manganese in sediment is expected.

On the basis of the comparison with Australian water quality guideline values and the assessed use pattern, the notified chemical and its transformation products are not considered to pose an unreasonable risk to the environment. Additionally, good agricultural practices could ensure that the wastage and potential contamination of water bodies from overspray, drift or runoff are minimised.

APPENDIX A: PHYSICAL AND CHEMICAL PROPERTIES

Density 1,725 kg/m^3 at 20 °C

Method OECD TG 109 Density of Liquids and Solids.
Remarks The density was determined using a pycnometer.
Test Facility Akzo Nobel (2013a)

Particle Size Inhalable fraction (< 100 μm): 36.7%
Respirable fraction (< 10 μm): 1.7%

Method OECD TG 110 Particle Size Distribution/Fibre Length and Diameter Distributions.

Range (μm)	*Mass (%)*
< 100	36.7
< 10	1.7
< 5	0.9

Remarks Determined using laser light scattering method.
Test Facility Akzo Nobel (2013b)

APPENDIX B: TOXICOLOGICAL INVESTIGATIONS

B.1. Acute toxicity – oral

TEST SUBSTANCE	Notified chemical (47.9% aqueous solution)
METHOD	OECD TG 423 Acute Oral Toxicity – Acute Toxic Class Method.
Species/Strain	Rat/Wistar Crl:WI (Han)
Vehicle	Nil
Remarks - Method	No protocol deviations. Dose was adjusted for concentration of notified chemical in test substance.

RESULTS

Group	*Number and Sex of Animals*	*Dose mg/kg bw*	*Mortality*
1	3F	2,000	0/3
2	3F	2,000	0/3

LD50	> 2,000 mg/kg bw
Signs of Toxicity	There were no unscheduled mortalities during the study. There were no observed adverse clinical signs.
Effects in Organs	No abnormalities were noted at macroscopic examination.
Remarks - Results	The body weights were within the range commonly recorded for this strain and age of rats.
CONCLUSION	The test substance is of low acute toxicity via the oral route.
TEST FACILITY	WIL Research (2013a)

B.2. Acute toxicity – inhalation

TEST SUBSTANCE	Analogue chemical 1 (92.3% purity)
METHOD	OECD TG 436 Acute Inhalation Toxicity – Acute Toxic Class Method
Species/Strain	Rat/Wistar WU (Crl;[W1]WU)
Vehicle	Water
Method of Exposure	Nose-only exposure.
Exposure Period	4 hours
Physical Form	Liquid aerosol
Particle Size	Mass median aerodynamic diameter (MMAD): 3.3-3.4 µm
Remarks - Method	No significant protocol deviations.

RESULTS

Group	*Number and Sex of Animals*	*Concentration (g/m^3)*		*Mortality*
		Nominal	*Actual*	
1	3 per sex	29.9	5.16	0/6

LC50	> 5.16 mg/L/4 hours
Signs of Toxicity	Slight sniffing was heard shortly after exposure in one male and two females and all three females showed slightly soiled head fur. No treatment related abnormalities were observed after day 2. Reduction in body weight gain was observed in all animals on days 1 and 3.
Effects in Organs	Petechiae (spots caused by bleeding) were observed on both lobes of the thymus in one male and on one side of the lungs of one female.

Remarks - Results	Effects of exposure were limited to decreased body weight gain up to day 3 and breathing abnormalities shortly after exposure. No mortality occurred.
CONCLUSION	The analogue chemical 1 is of low acute toxicity via inhalation.
TEST FACILITY	TNO (2010a)

B.3. Irritation – skin (in vitro)

TEST SUBSTANCE	Notified chemical (47.9% aqueous solution)
METHOD	OECD TG 439 In vitro Skin Irritation: Reconstructed Human *Epidermis* Test Method
Vehicle	Nil
Remarks - Method	No significant protocol deviations. Positive and negative controls were run in parallel with the test substance: - Negative control: phosphate buffered saline - Positive control: 5% sodium dodecyl sulphate The MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, thiazolyl blue] -assay was used to determine cell viability.

RESULTS

Test material	*Mean OD_{570} of triplicate tissues*	*Relative mean cell viability (%)*
Negative control	0.918	100
Test substance	0.856	93
Positive control	0.125	14

Remarks - Results	The test substance was shown not to directly reduce MTT. The relative mean tissue viability for the test substance as compared to the negative control was 93%. As the relative mean tissue viability for the test substance was above 50%, it is considered a non-irritant. The positive and negative controls gave satisfactory results, confirming the validity of the test.
CONCLUSION	The notified chemical was non-irritating to the skin under the conditions of the test.
TEST FACILITY	WIL Research (2013b)

B.4. Irritation – eye (in vitro)

TEST SUBSTANCE	Notified chemical (47.9% aqueous solution)
METHOD	OECD TG 437 (adopted July 2013) Bovine Corneal Opacity and Permeability Test Method for Identifying Ocular Corrosives and Severe Irritants
Vehicle	Nil
Remarks - Method	No significant protocol deviations. Positive and negative controls were run in parallel with the test substance: - Negative control: physiological saline - Positive control: 10% (w/v) benzalkonium chloride in physiological saline

RESULTS

Test material	*Mean opacities of triplicate tissues*	*Mean permeabilities of triplicate tissues*	*IVIS*
Vehicle control	-1	0.00	-1.0
*Test substance**	0	-0.001	0.0
*Positive control**	87	2.782	129

IVIS = in vitro irritancy score
*Corrected for background values

Remarks - Results

A mean in vitro irritancy score of 0.0 was obtained for the test substance. The test substance is therefore not considered to be corrosive or a severe eye irritant.

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

CONCLUSION

The notified chemical was not a severe eye irritant (IVIS ≤ 3) under the conditions of the test.

TEST FACILITY

WIL Research (2012)

B.5. Skin sensitisation – mouse local lymph node assay (LLNA)

TEST SUBSTANCE

Analogue chemical 2 (92.5% purity)

METHOD

OECD TG 429 Skin Sensitisation: Local Lymph Node Assay
Species/Strain: Mouse/CBA/J
Vehicle: Water with 1% pluronic L92
Preliminary study: Yes
Positive control: α-Hexylcinnamaldehyde (not conducted in parallel)
Remarks - Method: A preliminary study was conducted using 25% and 50% of the test substance. Very slight erythema was observed on one animal at a concentration of 50%. Variation in ear thickness during the observation period was less than 25% from day 1. Based on these results, the highest concentration selected for the main study was 50%.

RESULTS

Concentration (% w/w)	*Number and sex of animals*	*Proliferative response (DPM/lymph node)*	*Stimulation Index (Test/Control Ratio)*
Test Substance			
0 (vehicle control)	5 F	513	1.0
10	5 F	466	0.9
25	5 F	571	1.1
50	5 F	612	1.2
Positive Control			
0 (vehicle control)*	5 F	300	1.0
5	5 F	518	1.7
10	5 F	502	1.7
25	5 F	1423	4.7

*Acetone/Olive oil (4:1)

Remarks - Results

White/grey test substance residues were present in the dorsal surface of the ears on both animals on days 1-5 in the preliminary study and in all animals on days 1-3 and one animal on day 4 in the main study. The study authors asserted that this effect did not affect scoring of the skin reactions.

CONCLUSION

There was no evidence of induction of a lymphocyte proliferative response indicative of skin sensitisation to the analogue chemical 2.

TEST FACILITY WIL Research (2013c)

B.6. Repeated dose oral toxicity study with the reproduction/developmental toxicity screening

TEST SUBSTANCE Analogue chemical 1 (92.3% purity)

METHOD OECD TG 422 Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test

Species/Strain Rats/Wistar

Route of Administration Oral – gavage/drinking water

Exposure Information Males: 90 days (10 weeks premating, during mating and up to the day of scheduled sacrifice)
Females: 10 weeks premating, during mating and gestation, and up to day lactation

Vehicle Tap water

Remarks - Method Compounds with chelating properties such as the notified chemical have been shown to induce repro-toxic effects due to binding of zinc and a related loss of zinc from the body. These effects could be prevented by providing a zinc-supplemented diet to the animals to compensate for the loss of zinc through chelation. To investigate this possibility with the analogue chemical an additional high-dose group was included whereby the animals were fed a standard rodent diet supplemented with zinc carbonate (500 ppm Zn). All other groups received the standard diet for rodents (containing 100 ppm Zn).

No significant protocol deviations.

RESULTS

Group	*Number and Sex of Animals*	*Dose (mg/kg bw/day)*	*Mortality*
Control[1]	12/sex	0	0/24
low dose[1]	12/sex	150	0/24
mid dose[1]	12/sex	500	0/24
high dose[1]	12/sex	1,500	0/24
high dose + extra zinc[2]	12/sex	1.500	0/24

1 – fed with standard pellet diet which contains 100 mg/kg of zinc
2 – fed with standard diet supplemented with zinc carbonate (500 ppm of zinc)

Mortality and Time to Death

No unscheduled mortalities occurred during the study.

Clinical Observations

Abnormal contraction of the eyelid muscle (blepharospasm) was observed in a mid-dose group male from week 7 until sacrifice. An abnormal opening of vagina was observed in a mid-dose female during mating period. Vaginal haemorrhagic discharge was observed in a high-dose female.

- Statistically significant body weight gain decreases and increases were observed at various times during the dosing period.

Laboratory Findings – Clinical Chemistry, Haematology, Urinalysis

Following statistically significant findings were observed:

- reduction in alkaline phosphatase activity (ALP) in mid, high and high + zinc dose males and high and high + zinc dose females. The study authors indicated that these differences were mainly due to a relative high concentration of ALP in the control group animals, as compare to historical control data.
- reduction in aspartate aminotransferase activity (ASAT) in mid-dose males.
- reduction in alanine aminotransferase activity (ALAT)in low, high and high + zinc dose males. The study authors indicated this was due to relatively high concentration of ALAT in the control group which is at the upper end of historical control data.

- reduction in bilirubin levels in high and high + zinc dose females. The study authors indicated this was due to relatively high concentration of bilirubin in the control group which is higher than the mean of historical control data.
- increase in the concentration of cholesterol in mid and high + zinc dose males. The study authors stated the cholesterol concentration in the control group is at the lower end of the historical data and no statistically significant difference was observed in high dose males therefore this effect was considered to be of minor toxicological relevance.
- shorter prothrombin time in low-dose females.
- reduction in urine volume in high-dose males. As indicated under Clinical Observations section, increased water consumption was observed in high dose groups males and this contradicts with the reduced urine volume measured in these groups. The study authors stated that this may be due to the fact that these measurements were done on different days under different experimental (housing) conditions. As described under Effects in Organs section, the changes in urinary effects were accompanied by increase in absolute and relative kidney weights and renal histopathological changes consisting of very slight diffuse subcortical tubular dilation.
- increase concentration of creatinine levels in high + zinc dose females. The study authors indicated that absolute amount of creatinine excreted was not affected and asserted that the increase in creatinine was due to differences in the urine volume.
- increase in sodium and sodium/creatinine levels in high and high + zinc dose group of both sexes. The study authors indicated this effect was probably due to the high sodium intake of the animals via the test substance.

Reproductive and developmental findings
Statistically significant increase in mean duration of gestation period (22 days for high + zinc group and for all other groups 21 to 22 days) was observed for high + Zn group. The study authors indicated that based on a small number of animals exposed this effect was not considered treatment related. Moreover, a gestation period of 22 days is a usual gestation period for this strain of rats.

High and high + zinc dose males showed treatment related effects on epididymal sperm motility and derived parameters. However, no differences were observed in epididymal sperm count, epididymal sperm morphology and testicular sperm count among the control and treatment groups. The changes did not affect the reproductive performance of these high dose animals.

High dose group males showed statistically significant reduction in testicular parenchyma, however, no effects were observed on the number of spermatozoa per gram of testicular parenchyma or the daily sperm production.

Following statistically significant findings were observed:
- reduction in the mean number of (live) pups delivered in high and high + zing dose groups whereas the number of stillborn pups in these groups was increased.
- increase in the mean number of pup mortality between post-natal days 1-4 was observed in high + zinc dose group.
- number of pups (4 out of 6) were pale and only one pup survived (but dehydrated) on day 4 in the high + zinc dose group.

Effects in Organs
Statistically significant increase in the absolute and the relative kidney weights in high and high + zinc dose group males were observed. Females treated with all doses showed statistically significant (but reported by the study authors as not treatment related) increase in absolute kidney weights. Increased incidence of very slight diffuse renal subcortical tubular dilation was observed in both sexes (statistically significant in females only) in high and high + zinc dose groups.

Females exposed to low and mid-doses showed statistically significant (but reported by the study authors as not treatment related) increase in absolute liver weights.

Both the absolute and relative weights of the spleen of high + zinc dose females were statistically significantly reduced, but there were no histopathological changes in the spleen.

Statistically significant lower incidence of pelvic mineralisation was noted in high and high + zinc dose males. Mid-dose group females showed statistically significant higher incidence of very slight papillary mineralisation.

The study authors indicated this strain of rats shows a large variation in the incidence of renal mineralisation this effect is not considered to be treatment related.

Remarks – Results

The study authors stated that "there were some neuro-behavioural effects reported in rats including slightly tilted head and distance travelled in males (statistically significant), but the study authors considered these to be non-treatment related. Neurobehavioural testing showed a dose-related decrease in arousal in males in week 8-10 of the study. As the decrease was not supported by behavioural changes in measures of the same functional domain, and as it was not consistently observed from first occurrence until the end of the neurobehavioural study period (week 10), it was not considered as clear evidence of neurotoxicity induced by the test substance".

Regarding chelating properties and subsequent reproductive toxicity effects of EDTA-$MnNa_2$, no considerable differences in reproductive effects were observed in the high and high + Zinc dose groups animals. Therefore it can be concluded that additional Zinc is not required to compensate for EDTA-$MnNa_2$ chelating effects. The study authors stated that the reproductive toxicity of EDTA-$MnNa_2$ was probably due to the presence of manganese.

CONCLUSION

The No Observed Adverse Effect Level (NOAEL) for parental toxicity was established as 500 mg/kg bw/day in this study, based on increased urinary sodium concentration and histopathological effects in kidneys at 1,500 mg/kg bw/day.

The NOAEL for reproductive toxicity was established by study authors as 500 mg/kg bw/day in this study, based on reduce sperm motility at 1,500 mg/kg bw/day.

The NOAEL for developmental toxicity was established by study authors as 500 mg/kg bw/day in this study, based on reduced number of live born pups and increased post-implantation loss at 1,500 mg/kg bw/day.

TEST FACILITY TNO (2010b)

B.7. Genotoxicity – bacteria

TEST SUBSTANCE	Analogue chemical 2 (47.2% aqueous solution)
METHOD	OECD TG 471 Bacterial Reverse Mutation Test. Plate incorporation (Test 1) and pre-incubation procedure (Test 2)
Species/Strain	*Salmonella typhimurium*: TA1535, TA1537, TA100 and TA98
Metabolic Activation System	S9 mix from Aroclor1254-induced rat liver
Concentration Range in Main Test	a) With metabolic activation: 100 – 10,000 µg/plate b) Without metabolic activation: 100 – 10,000 µg/plate
Vehicle	Distilled water
Remarks - Method	Negative control: distilled water Positive control: with S9-mix: 2-aminoanthracene without S9-mix: *N*-methyl-N'-nitro-N-nitrosoguanidine (TA100 and TA1535); 4-nitro-phenylendiamine (TA98); and 9-aminoacridine chloride monohydrate (TA1537). Preliminary toxicity test was not conducted.

RESULTS

Metabolic Activation	*Test Substance Concentration (µg/plate) Resulting in:*			
	Cytotoxicity in Preliminary Test	*Cytotoxicity in Main Test*	*Precipitation*	*Genotoxic Effect*
Absent				
Test 1	-	> 10,000	> 10,000	Negative
Test 2	-	> 10,000	> 10,000	Negative
Present				
Test 1	-	> 10,000	> 10,000	Negative
Test 2	-	> 10,000	> 10,000	Negative

Remarks - Results	In Test 1, due to high spontaneous rate of revertants (> 50%), no evaluation was performed for TA98 strain. No biologically relevant increases in revertant colony numbers of any of the tester strains were observed during the test in either the presence or absence of metabolic activation. The positive controls induced a distinct increase of revertant colonies during the study indicating the validity of the test system.
CONCLUSION	The analogue chemical 2 was not mutagenic to bacteria under the conditions of the test.
TEST FACILITY	BASF (1992)

B.8. Genotoxicity – in vitro mammalian cell gene mutation test

TEST SUBSTANCE	Analogue chemical 1 (91% purity)
METHOD	OECD TG 476 In vitro Mammalian Cell Gene Mutation Test.
Species/Strain	Mouse
Cell Type/Cell Line	Lymphoma/L5178Y tk +/- 3.7.2C line
Metabolic Activation System	S9 mix from Aroclor 1254-induced rat liver
Vehicle	Culture medium (RPMI 1640)
Remarks - Method	No significant protocol deviations. Negative control: culture medium (RPMI 1640). Positive control: Without S9: methyl methanesulphonate With S9: 3-methyl-cholantrene Preliminary cytotoxicity test was not conducted.

Metabolic Activation	*Test Substance Concentration (µg/mL)*	*Exposure Period*	*Harvest time*
Absent			
Test 1	14, 28, 56, 112, 224*, 320*, 458*, 654*, 934*, 1335*, 1907*, 2724*, 3891*	24 h	48 h
Test 2	14*, 28, 56*, 112*, 224*, 320*, 458*, 654*, 934*, 1335*, 1907, 2724, 3891	24 h	48 h
Test 3	13, 26, 51*, 102*, 204*, 292, 417*, 595, 851*, 1215, 1736*, 2480*, 3543*	4 h	48 h
Present			
Test 1	14, 28, 56, 112, 224, 320*, 458*, 654*, 934*, 1335*, 1907*, 2724*, 3891*	4 h	48 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	-	≥ 14	> 3891	Negative
Test 2	-	≥ 14	> 3891	Negative
Test 3	-	> 3543	> 3543	Negative
Present				
Test 1	-	≥ 934	> 3891	Negative

Remarks - Results	In Test 1, without metabolic activation, an increase in the mean mutant frequency (MF) by more than 88 but less than 126 mutants/1,000,000

clonable cells was observed for cells treated with the test substance at 934, 1335, 1907, 2724 and 3891 µg/mL. However a clear dose response relationship was not observed.

In Test 2, without metabolic activation, an increase in mean MF (more than 88 but less than 126 mutants/1,000,000 clonable cells) was observed for cells treated with the test substance at 934 and 1335 µg/mL.

An increase in mean MF by more than 88 but less than 126 mutants/1,000,000 clonable cells is considered an equivocal result under the test guideline.

The increases in the MF occurred at concentrations causing high cytotoxicity (relative total growth ≤ 12% in Test 1 and < 10% in Test 2). The study authors therefore considered that these increases were not biologically relevant.

In Test 3 without metabolic activation no increase in MF was observed, further supporting the conclusion from Test 1 and 2. No increase in MF was also observed in Test 1 with metabolic activation.

The study authors considered the high cytotoxicity to be caused by chelation of essential metals.

CONCLUSION The analogue chemical 1 was not clastogenic to mouse lymphoma L5178Y cells treated in vitro under the conditions of the test.

TEST FACILITY TNO (2015)

B.9. Genotoxicity - In vitro mammalian cell micronucleus test

TEST SUBSTANCE Analogue chemical 1 (92.3% purity)

METHOD OECD TG 487 *In Vitro* Mammalian Cell Micronucleus Test.

Species/Strain	Human
Cell Type/Cell Line	Lymphocytes
Metabolic Activation System	S9 mix from Aroclor 1254-induced rat liver
Vehicle	Culture medium
Remarks - Method	A preliminary cytotoxicity test was not conducted.

Negative control: culture medium
Positive control (clastogen):
Without S9: mitomycin C
With S9: cyclophosphamide
Aneugenic positive control: vinblastine sulphate

Metabolic Activation	*Test Substance Concentration (µg/mL)*	*Exposure Period*	*Harvest Time*
Absent			
Test 1	125, 250, 500, 1000*, 2000*, 3891*	4 h	24 h
Test 2	1000, 1500, 2000, 2500*, 3000*, 3891*	20 h	48 h
Present			
Test 1	125, 250, 500, 1000*, 2000*, 3891*	4 h	24 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	-	≥ 3891	> 3891	Negative

Metabolic Activation	*Test Substance Concentration (µg/mL) Resulting in:* *Cytotoxicity in Preliminary Test*	*Cytotoxicity in Main Test*	*Precipitation*	*Genotoxic Effect*
Test 2	-	≥ 3000	> 3891	Negative
Present				
Test 1	-	≥ 3891	> 3891	Negative

Remarks - Results

In Test 1 (in the presence of S9-mix) and Test 2 (in the absence of S9-mix), the highest concentration (3,891 µL/mL) tested showed cytotoxicity (20% and 31% respectively). Slight to no cytotoxicity was observed at all other concentrations.

In Test 2 (in the absence of S9-mix), 26% and 35% cytotoxicity was observed at 3,000 and 3,891 µL/mL, respectively. Slight cytotoxicity was noted at all other concentrations.

The test substance did not induce a dose dependent statistically significant increase in the number of binucleated cells containing micronuclei at any concentrations analysed.

The positive controls behaved as expected, confirming the validity of the test system.

CONCLUSION

The analogue chemical 1 was not clastogenic to human lymphocytes treated in vitro under the conditions of the test.

TEST FACILITY

TNO (2010c)

B.10. Genotoxicity - In vitro mammalian cell micronucleus test

TEST SUBSTANCE

Analogue chemical 2 (92.7% purity)

METHOD

OECD TG 487 *In Vitro* Mammalian Cell Micronucleus Test.

Species/Strain: Human

Cell Type/Cell Line: Lymphocytes

Metabolic Activation System: S9 mix from Aroclor 1254-induced rat liver

Vehicle: Culture medium

Remarks - Method:

Negative control: culture medium

Positive control (clastogen):

Without S9: mitomycin C

With S9: cyclophosphamide

Aneugenic positive control: vinblastine sulphate

Preliminary cytotoxicity test was not conducted.

Metabolic Activation	*Test Substance Concentration (µg/mL)*	*Exposure Period*	*Harvest Time*
Absent			
Test 1	7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000*, 2000*, 3977*	4 h	24 h
Test 2	62.5*, 125*, 250*, 500, 750, 1000, 1500, 2000, 2500, 3000, 3977	20 h	48 h
Present			
Test 1	7.8, 15.6, 31.3, 62.5, 125, 250, 500*, 1000, 2000*, 3977*	4 h	24 h

*Cultures selected for micronuclei 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	-	≥ 3977	> 3977	Negative
Test 2	-	≥ 125	> 3977	Positive
Present				
Test 1	-	≥ 2000	> 3977	Negative

Remarks - Results

In Test 1 (without S9), 41% cytotoxicity was observed in cells treated at 3,977 µg/mL.

In Test 2 (without S9), only dead cells were observed at the four high concentrations (1,500, 2,500, 3,000 and 3,977 µg/mL). Some binucleated cells were detected at 750 and 1,000 µg/mL. At 500, 250 and 125 µg/mL cytotoxicity was 77%, 53% and 35%, respectively.

In Test 1 (with S9), 45%, 35% and 18% cytotoxicity was observed at 3,977, 2,000 and 1,000 µg/mL, respectively. At lower concentrations cytotoxicity fluctuated between 6% and 13%.

In Test 1 (with or without S9) the test substance did not show a statistically significant increase in the number of binucleated cells containing micronuclei, at any of the concentrations analysed.

In Test 2 (without S9) a dose dependent statistically significant increase in the number of binucleated cells containing micronuclei was observed at 250, 125 and 62.5 µg/mL. However the percentage of binucleated cells containing micronuclei at these concentrations were only slightly higher than the historical control range of the test facility.

The proportion of the large and small micronuclei induced by the test substance was not statistically different from the response of the aneugen vinblastine sulphate. The observed similar proportions of large and small micronuclei are considered to be an indication for aneugenic effects.

CONCLUSION

The analogue chemical 2 was aneugenic to human lymphocytes treated in vitro under the conditions of the test.

TEST FACILITY

TNO (2013b)

B.11. Developmental toxicity study

TEST SUBSTANCE

Analogue chemical 1 (91% purity)

METHOD

OECD TG 414 Prenatal Developmental Toxicity Study.

Species/Strain: Rabbit (females)/New Zealand White (SPF)

Route of Administration: Oral – gavage

Exposure: Days 6 to 28 post-coitum, inclusive

Vehicle: Water

Remarks - Method: Compounds with chelating properties such as the notified chemical have been shown to induce repro-toxic effects due to binding of zinc and a related loss of zinc from the body. These effects could be prevented by providing a zinc-supplemented diet to the animals to compensate for the loss of zinc through chelation. To investigate this possibility with the analogue chemical an additional high-dose group was included whereby the animals were fed a standard rabbit diet supplemented with zinc carbonate (~200 ppm Zn). All other groups received the standard diet for rabbits (containing 73 ppm Zn).

Group	*Number and Sex of Animals*	*Dose (mg/kg bw/day)*	*Mortality*
Control[1]	22 F	0	0/22
low dose[1]	22 F	10	0/22
mid dose[1]	22 F	30	0/22
high dose[1]	22 F	100	0/22
high dose (extra zinc)[2]	22 F	100	0/22

1 – fed with standard pellet diet which contains 73 mg/kg of zinc
2 – fed with standard diet supplemented with zinc carbonate (200 ppm of zinc)

Maternal findings

Mortality and Time to Death

No unscheduled mortality occurred during the study period. The study authors noted that one female was inadvertently terminated early (on day 25 post-coitum instead of day 29 post-coitum).

Clinical Observations

Grey discolouration of urine was observed in 15/22 animals in low-dose group and all treated animals in the other groups.

Histopathology examination notes that females treated with 100 mg/kg bw/day (without and with zinc supplement) showed:

- minimal urothel vacuolation in urethra in 5 and 4 females treated without and with zinc supplement, respectively. Eight females (4 females/ treatment) showed slight urothel vacuolation in urethra.
- minimal urothel vacuolation in urinary bladder in 6 and 9 females without and with zinc supplement respectively and slight urothel vacuolation in urinary bladder in 16 and 12 females without and with zinc supplement, respectively.

Diarrhoea was noted in 9, 16, 20 and 21 animals in low-, mid-, and high-dose (with and without extra zinc) groups, respectively. Faeces containing mucus was observed in 1, 2, 7 and 5 animals in low-, mid-, and high-dose (with and without extra zinc) groups, respectively. The study authors stated that generally ~ 5% of the chelating agents are absorbed in the gut and rest are excreted via faeces. Further rabbit's gut has a delicate microflora therefore the test substance may have disturbed the functioning of the intestine and this may have resulted in diarrhoea.

In both high-dose groups no change in body weight was observed on average on day 9 post-coitum. This recovered during the remainder of treatment. However this did not reach the levels of control animals in the animals without extra zinc body weights.

Statistically significant reduction in food consumption was observed in mid-dose group on days 9-13, in high-dose group on days 9-16 and in high-dose (with extra zinc) group on days 6-13.

Laboratory Findings – Clinical Chemistry, Urinalysis

Treatment related, (not statistically significant), increase in mean creatine kinase levels were observed in all treated animals. Twenty per cent of animals treated with10 mg/kg bw/day and 50% of animals treated with 30 and 100 mg/kg bw/day and 60% of animals treated with 100 (with zinc supplement) mg/kg bw/day showed higher (> 800 U/L, range 811 to 25,280 U/L) than the normal (200 to 800 U/L) range of mean creatine kinase levels for this age group of rabbits (16 weeks). The histopathology evaluation (submitted by the notifier following completion of the Day 90 reports) conducted on the tissue samples of the treated females showed that minimal (grade 1) to slight (grade 2) kidney effects (urothel vacuolation, tubular basophilia, pelvic mineralisation, mineralisation and tubular vacuolation) were observed in some of the treated animals at 100 mg/kg bw/day without and with zinc supplement.

Following histopathology findings were observed in animals treated at 100 mg/kg bw/day (with and without zinc supplement):

- minimal urothel vacuolation in 8 females (4 females/ treatment group)
- minimal pelvic mineralisation in 1 and 2 females without and with zinc supplement respectively
- slight (in 3 and 5 females without and with zinc supplement respectively) and minimal (in 1 female without zinc supplement) mineralisation
- minimal tubular vacuolation in a female without zinc supplement
- minimal tubular basophilia in 6 and 12 females without and with zinc supplement, respectively
- slight tubular dilation in 1 female without zinc supplement

No such findings were observed in animals treated with 10 or 30 mg/kg bw/day of the test substance.

In addition, individual females in the mid- and high-dose (with and without extra zinc) groups showed increased LDH concentrations. All remaining clinical chemistry parameters were unaffected by treatment in all groups.

Apart from discolouration of the urine in all treated groups, the mean urinalysis parameters were unaffected by treatment in all groups.

Effects in Organs

In the low-, mid-, and high-dose (with and without extra zinc) groups, discoloured contents (grey) of the urinary bladder was observed in 7, 14, 14 and 13 females, respectively.

There were no other necropsy findings reported that were considered toxicologically relevant.

Developmental findings

No treatment-related effects on litter size in any group were observed. Slightly lower mean foetal body weights were noted at 100 mg/kg. This was considerably higher for high-dose (without extra zinc) group than high-dose (with extra zinc) group. These changes, however, were not statistically significant.

Increased incidence (not statistically significant) of caudal shift of pelvic girdle was observed in the high-dose (with and without extra zinc) groups. There was no dose response relationship and the study authors stated that the incidences were within the historical control range.

In the high-dose (without extra zinc) group, the foetuses showed signs of retarded skeletal ossification, i.e. higher (but not statistically significant) incidences of unossified metacarpals and reduced ossification of the skull. These affected foetuses had low weights in line with reduced maternal body weights.

In the high-dose (with extra zinc) group, no delay in skeletal ossification was observed despite a slightly lower foetal body weight and a slightly lower maternal body weight.

All other developmental parameters were unaffected by treatment in all dose groups.

RESULTS

Remarks - Results

Maternal toxicity was observed at all treated doses. Discoloured urine and treatment related increased creatine kinase levels were observed at 10 mg/kg bw/day and higher, reduced food intake was observed at 30 mg/kg bw/day and higher, and reduced body weight gain was observed at 100 mg/kg bw/day which did not fully recover in animals without extra zinc.

No developmental toxicity was observed at 10 and 30 mg/kg bw/day. The developmental effects observed at 100 mg/kg bw/day were seen in the presence of maternal toxicity. The developmental effects were not observed at 100 mg/kg bw/day where the rabbits were fed a zinc-supplemented diet.

CONCLUSION

The No Observed Adverse Effect Level (NOAEL) for maternal toxicity was not established due to the discolouration of the urine and increased creatine kinase levels in all treated animals.

The NOAEL for developmental toxicity was established by the study authors as 30 mg/kg bw/day in this study, based on skeletal malformations observed at 100 mg/kg bw/day group.

TEST FACILITY WIL Research (2016) and Charles River (2018)

APPENDIX C: ENVIRONMENTAL FATE AND ECOTOXICOLOGICAL INVESTIGATIONS

C.1. Environmental Fate

C.1.1. Ready biodegradability

TEST SUBSTANCE: Analogue chemical 3

METHOD: OECD TG 301 D Ready Biodegradability: Closed Bottle Test.

Inoculum	Lake, Ditch and River Water
Exposure Period	49 days
Auxiliary Solvent	None
Analytical Monitoring	Theoretical Oxygen Demand ($ThOD_{NH3}$)
Remarks - Method	The Closed Bottle tests are performed according to modified OECD Test Guidelines. To assess the potential of natural ecosystem biodegradation the test substance was added at a concentration of 8 mg/L into biological oxygen demand (BOD) bottles filled with water obtained from 3 different aquatic systems in the Netherlands, the shallow freshwater lake Ketelmeer, the river IJssel near Arnhem and a ditch near Zevenaar. Biodegradation was measured by following the course of the oxygen decrease. Tests were run over 49 days at both pH 6.5 and 8.0, adjusted with 1 N HCl. Inhibition of the endogenous respiration of the inoculum by the test substance was not detected, therefore inhibition of biodegradation due to initial high concentration of the test substance is not expected. River, lake and ditch water biodegradation tests, without test substance added, were run in parallel to the test substance aqueous inoculum biodegradation tests. The actual concentration of the test solution was not determined. Deviations from the closed bottle test procedure were: the complete filling of the bottles with river, lake and ditch water, respectively, instead of dilution of the inoculum into a mineral salts medium with activated sludge; the adjustment of pH to 6.5 and 8.0 instead of 7.0: the extended test time to 49 days.

RESULTS

$CaNa_2EDTA$ in River Water			*$CaNa_2EDTA$ in Lake Water*			*$CaNa_2EDTA$ in Ditch Water*		
Day	*% Degradation*		*Day*	*% Degradation*		*Day*	*% Degradation*	
	pH 6.5	pH 8.0		pH 6.5	pH 8.0		pH 6.5	pH 8.0
0	0	0	0	0	0	0	0	0
7	0	0	7	2	0	7	4	0
14	0	9	14	0	0	14	4	4
21	2	47	21	2	8	21	4	8
28	12	72	28	2	53	28	6	62
35	47	75	35	17	79	35	11	89
42	83	-	42	45	-	42	70	-
49	-	-	49	60	-	49	81	-

Remarks - Results: The validity of the test was demonstrated by high endogenous respiration and oxygen concentrations > 0.2 mg/L in all bottles during the test period. Biodegradation of > 60% was found within 28 days in alkaline (pH 8.0) river and ditch water inoculum tests and at 35 days in the lake water inoculum test. There was a longer lag phase to the onset of biodegradation in the pH 6.5 tests which had a > 60% degradation after 42 days in the river and ditch water inoculum tests and at day 49 for the lake water. On the basis of these the prolonged closed bottle tests, the test substance is considered to be somewhat biodegradable at pH 8.0 and pH 6.5 in the natural waters tested, but does not meet the 10-day window test criteria for ready biodegradability under the OCDE 301 D guideline.

CONCLUSION The analogue chemical 3 is readily biodegradable in natural waters.

TEST FACILITY Akzo Nobel (1999)

C.2. Ecotoxicological Investigations

C.2.1. Acute toxicity to fish

TEST SUBSTANCE Analogue chemical 1

METHOD OECD TG 203 Fish, Acute Toxicity Test - static.

Species	*Danio rerio* (zebra fish)
Exposure Period	96 hours
Auxiliary Solvent	None
Water Hardness	229 mg $CaCO_3$/L
Analytical Monitoring	HPLC
Remarks – Method	The test was conducted in accordance with the guideline recommendations. During the preliminary range finding test, performed as a non-GLP test, 3 fish per concentration were exposed to control, 1, 10 and 100 and 1000 mg/L. No effects were observed in any exposure concentrations. The definitive test was, therefore, performed with a control and the highest concentration.

RESULTS

Concentration mg/L		*Number of Fish*	*Mortality*				
Nominal	*Actual*		*1 h*	*24 h*	*48 h*	*72 h*	*96 h*
Control	< LOD	7	0	0	0	0	0
1000	987.85	7	0	0	0	0	0

LC50	>1000 mg/L at 96 hours.
NOEC	>1000 mg/L at 96 hours.
Remarks – Results	All quality criteria were met, with the exception of temperature which during the test fell below the lower boundary of 21 ºC recommended in the guideline. The variation was < 2 ºC and was not considered to have influenced the results. The test solutions were analysed at the beginning and end of the exposure period. Concentrations were within ± 20% of the nominal concentrations, therefore, responses are calculated based on nominal concentrations. The LC_{50} and NOEC concentrations could not be calculated as no mortality was observed at any concentration of the test substance.

CONCLUSION The analogue chemical 1 is not harmful to fish.

TEST FACILITY Akzo Nobel (2009a)

C.2.2. Acute toxicity to aquatic invertebrates

TEST SUBSTANCE Analogue chemical 1

METHOD OECD TG 211 & 202 Daphnia sp. Reproduction Test – Semi-static.

Species	*Daphnia magna*
Exposure Period	21 days *[chronic study]*
Auxiliary Solvent	None
Water Hardness	5.7 – 6.7 mg $CaCO_3$/L
Analytical Monitoring	HPLC
Remarks - Method	The test was conducted using a combination of OECD 211 and 202 guidelines for the conduct of reproductive daphnid tests. Due to poor

performance of the daphnids in synthetic medium, relating to pH fluctuations, natural surface water with M4 media vitamins added was used in the test. Water hardness was lower than recommended but as the daphnids showed excellent performance in this test medium it is not considered to have impacted the test outcome. All other conditions were in accordance with guideline requirements. Parent animals are fed a diet of 0.1 to 0.2 mg of carbon per daphnid per day, in the form of the algae *Chlorella vulgaris*.

RESULTS

Concentration mg/L		*Number of Parent D. magna*	*Number live of neonates per parent*	*Parent weight µg*
Nominal	*Actual*		*21 d*	*21 d*
Control	Control	10	111	1.25
4.7	4.6	10	104	1.2
15.2	ND	9	110	1.2
48.8	ND	10	119	1.25
156	ND	10	107	1.0
500	519.6	9	28	0.5

EC50 365 mg/L at 21 days
NOEC 156 mg/L at 21 days
Remarks - Results The validity criterion for the coefficient of variation (less than 25% in the control based on the number of living neonates for each parent alive at the end of the test) was achieved. The lowest and highest test solutions were analysed on 6 occasions during the test. The mean measured concentrations were within ± 20% of the nominal concentrations, therefore, the effect data are based on the nominal test concentrations. Two parents died during the test. Parental weight was found to be the most sensitive endpoint based on the EC_{10}-values. EC_{50} for reproduction was 365 mg/L and for parental weight was 397 mg/L. The reproductive NOEC was determined to be 156 mg/L based on Dunnett's test.

CONCLUSION The analogue chemical 1 is not harmful to aquatic invertebrates.

TEST FACILITY Akzo Nobel (2010a)

C.2.3. Algal growth inhibition test

TEST SUBSTANCE Analogue chemical 1

METHOD OECD TG 201 Alga, Growth Inhibition Test.
Species *Pseudokirchneriella subcapitata*
Exposure Period 72 hours
Concentration Range Nominal: 15, 48, 154, 492 and 1573 mg/L
Actual: 13, NM, 129, NM and 1287 mg/L
Auxiliary Solvent None
Water Hardness Not determined
Analytical Monitoring HPLC
Remarks - Method The method used followed the OECD 201 guideline recommendations with the following modification: Selected growth media components were 150% enriched to minimize the influence of nutrient deficiency caused by the chelating properties of the test chemical, allowing the actual toxicity of the test compound to be more accurately determined. An increase of 2.0 pH units was recorded in the control and lowest concentration replicates. This is slightly more than the guideline recommended increase, but is not considered to have negatively influenced the results.

RESULTS

Biomass		*Growth*	
E_bC50 mg/L at 72 h	*NOEC mg/L*	*ErC50 mg/L at 72 h*	*NOEC mg/L*
>100 (95% CI: 59 – 204)	Not reported	>100 (95% CI: 204-18430)	15

Remarks - Results: The study fulfilled the test validity criteria based on the mean coefficient of variation for section-by-section specific growth rates in the control cultures and the coefficient of variation of average specific growth rates during the whole test period in the replicate control cultures. The test solution was not renewed during the 72 h test period. Concentrations of the 0, 15, 154 and 1573 mg/L test solution were measured at the start and the end of the 72 hr test. The mean measured concentrations remained within 20% of the nominal and, therefore, the nominal concentrations were used to calculate the effect levels. The 72 h E_bC50 and E_rC50 for the alga were both determined to be > 100 mg/L. The NOEC for growth rate was 15 mg/L and the LOEC was 48 mg/L.

CONCLUSION: The analogue chemical 1 is not harmful to algae.

TEST FACILITY: Akzo Nobel (2009b)

C.2.4. Inhibition of microbial activity

TEST SUBSTANCE: Analogue chemical 1

METHOD: OECD TG 209 Activated Sludge, Respiration Inhibition Test.
- Inoculum: Activated sludge
- Exposure Period: 3 hours
- Concentration Range: Nominal: 0, 40, 80, 160, 320, 640 mg/L; Actual: Not determined
- Remarks – Method: The method used followed the OECD 209 guideline recommendations with no deviations or amendments.

RESULTS
- IC50: >640 mg/L at 3 hours
- NOEC: >640 mg/L at 3 hours
- Remarks – Results: The test validity was demonstrated by the reference compound, 3,5-dichlorophenol EC_{50} of 7 mg/L which was within the prescribed range of 5 to 30 mg/L, and the control respiration rates which are within 15% of each other. The inhibitory effect of the test substance at each exposure concentration is expressed as a percentage of the two controls. No inhibition of the respiration of the activated sludge was measured at 640 mg/L, the highest concentration tested.

CONCLUSION: The analogue chemical 1 does not inhibit microbial respiration in activated sludge.

TEST FACILITY: Akzo Nobel (2010b)

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