Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at Community level of

Tetracopper hexahydroxide sulphate [1]
Tetracopper hexahydroxide sulphate hydrate [2]

EC number: 215-582-3
CAS number: 1333-22-8 [1]
12527-76-3 [2]

CLH-O-0000001412-86-42/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

04 December 2014

CLH report

Proposal for Harmonised Classification and Labelling

**Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2**

Substance Name: Tetracopper hexahydroxide sulphate [1]

Tetracopper hexahydroxide sulphate hydrate [2]

EC Number: 215-582-3

CAS Number: 1333-22-8[1] or 12527-76-3[2]

Index Number: 029-004-00-0 (Copper sulphate)

Contact details for dossier submitter: ANSES (on behalf of the French MSCA)

253 avenue du General Leclerc

F-94701 Maisons-Alfort Cedex

+33 1 56 29 19 30

reach@anses.fr

Version number: 3 **Date: 12/12/2013**

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Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity:

Substance name:	Tetracopper hexahydroxide sulphate [1] Tetracopper hexahydroxide sulphate hydrate [2]
EC number:	215-582-3
CAS number:	1333-22-8 [1] or 12527-76-3 [2]
Annex VI Index number:	029-004-00-0 (copper sulphate)
Degree of purity:	> 49 % Cu/% tribasic copper sulphate (dry basis) (equivalent to ≥ 88.9 % /% tribasic copper sulphate*)
Impurities:	See annex I (confidential)

*Calculation on the basis of 55.1 % copper in tribasic copper sulphate

The substance is also generally refered as tribasic copper sulphate in this dossier.

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification:

	CLP Regulation
Current entry in Annex VI, CLP Regulation	Acute Tox. 4 * - H302 Eye Irrit. 2 – H319 Skin Irrit. 2 – H315 Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410
Current proposal for consideration by RAC	Deletion of Eye Irrit. 2 – H319 and Skin Irrit. 2 – H315 Addition of a M-factor of 10 for acute environmental classification Revision of chronic

	environmental classification from category 1 to category 2.
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Acute Tox. 4 - H302 Aquatic Acute 1 – H400, M=10 Aquatic Chronic 2 – H411

Copper and some copper compounds are under review as Biocides (BPD) and/or Plant Protection Product (PPP) Directives and CLH dossier to set or revise their harmonised classification are submitted in parallel for these compounds (see summary in annex II).

In particular, two different types of copper sulphates are reviewed, i.e. copper sulphate pentahydrate and tribasic copper sulphate, and require different classification. They are both currently covered in the general entry “copper sulphate”. The present proposals will therefore lead to the following entries:

Substance name	CAS number	EC number	Classification
Copper sulphate	7758-98-7	231-847-6	Acute Tox. 4 - H302 Eye Irrit. 2 – H319 Skin Irrit. 2 – H315 Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410 M-factor: 10
Copper sulphate pentahydrate	7758-99-8	231-847-6	Acute Tox. 4 - H302 Eye Dam.. 1 – H318 Aquatic Acute 1 – H400, M=10 Aquatic Chronic 2 – H411
Tetracopper hexahydroxide sulphate [1] Tetracopper hexahydroxide sulphate hydrate [2]	1333-22-8 [1] or 12527-76-3 [2]	215-582-3	Acute Tox. 4 - H302 Aquatic Acute 1 – H400, M=10 Aquatic Chronic 2 – H411

Proposed harmonised classification and labelling based on CLP Regulation

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives	None			Conclusive but not sufficient for classification
2.2.	Flammable gases	None			Not relevant
2.3.	Flammable aerosols	None			Not relevant
2.4.	Oxidising gases	None			Not relevant
2.5.	Gases under pressure	None			Not relevant
2.6.	Flammable liquids	None			Not relevant
2.7.	Flammable solids	None			Conclusive but not sufficient for classification
2.8.	Self-reactive substances and mixtures	None			Conclusive but not sufficient for classification
2.9.	Pyrophoric liquids	None			Not relevant
2.10.	Pyrophoric solids	None			Conclusive but not sufficient for classification
2.11.	Self-heating substances and mixtures	None			Conclusive but not sufficient for classification
2.12.	Substances and mixtures which in contact with water emit flammable gases	None			Conclusive but not sufficient for classification
2.13.	Oxidising liquids	None			Not relevant
2.14.	Oxidising solids	None			Conclusive but not sufficient for classification
2.15.	Organic peroxides	None			Conclusive but not sufficient for classification
2.16.	Substance and mixtures corrosive to metals	None			Conclusive but not sufficient for classification
3.1.	Acute toxicity - oral	Acute Tox 4 – H302	None	Acute Tox 4* – H302	
	Acute toxicity - dermal	None			Conclusive but not sufficient for classification
	Acute toxicity - inhalation	None			Conclusive but not sufficient for classification
3.2.	Skin corrosion / irritation	None	None	Skin Irrit 2 – H315	Conclusive but not sufficient for classification
3.3.	Serious eye damage / eye irritation	None	None	Eye Irrit. 2 – H319	Conclusive but not sufficient for

					classification
3.4.	Respiratory sensitisation	None			Data lacking
3.4.	Skin sensitisation	None			Conclusive but not sufficient for classification
3.5.	Germ cell mutagenicity	None			Conclusive but not sufficient for classification
3.6.	Carcinogenicity	None			Conclusive but not sufficient for classification
3.7.	Reproductive toxicity	None			Conclusive but not sufficient for classification
3.8.	Specific target organ toxicity –single exposure	None			Conclusive but not sufficient for classification
3.9.	Specific target organ toxicity – repeated exposure	None			Conclusive but not sufficient for classification
3.10.	Aspiration hazard	None			Conclusive but not sufficient for classification
4.1.	Hazardous to the aquatic environment	Aquatic Acute 1 – H400 Aquatic Chronic 2 – H411	M = 10	Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410	
5.1.	Hazardous to the ozone layer	None			Conclusive but not sufficient for classification

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Signal word: Warning
Pictograms: GHS 07, GHS 09
Hazard statements: H302, H400, H411
Precautionary statements: not harmonised

Proposed notes assigned to an entry: none

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

Tribasic copper sulphate is currently harmonised under the entry "copper sulphate" (index 029-004-00-0).

The harmonised classification for copper sulphate was already present in the 19th ATP.

The current classification was included in the 25th ATP (Directive 98/98/EC). No further discussion on the harmonised classification of copper sulphate occurred since to our knowledge.

Copper sulphate is registered under REACH and relevant information in REACH registration dossier was considered in the preparation of this report.

2.2 Short summary of the scientific justification for the CLH proposal

Tribasic copper sulphate has a moderate acute toxicity by inhalation and a classification as Acute Tox 4 – H302 is proposed.

Taking into account the recommendations of the Annex IV of the Guidance to Regulation (EC) No 1272/2008 Classification, Labelling and Packaging of substances and mixtures, a metal compound is considered as readily soluble if the water solubility is greater or equal to the acute ERV of the dissolved metal ion concentration. The water solubility of Tribasic copper sulfate is equal to 3.42 mg/L and 0.255 mg/L at pH 5.6 and 9.8 respectively. Therefore, this compound is considered as **ready soluble metal compound**.

For acute toxicity classification, the lowest ERV- Tribasic copper sulfate (0.05 mg/l) is below the trigger value of 1 mg/L which leads to the aquatic environmental hazard acute category 1, H400. An M-factor of 10 should also be applied.

For chronic toxicity classification, there is evidence of rapid removal from water column. The lowest chronic ERV- Tribasic copper sulfate (0.013 mg/L) is between the trigger values of 0.01 and 0.1 mg/L which leads to the aquatic environmental hazard chronic category 2, H411.

2.3 Current harmonised classification and labelling

2.3.1 Current classification and labelling in Annex VI, Table 3.1 in the CLP Regulation

The classification of copper sulphate is harmonised in Annex VI of CLP under the index number 029-004-00-0 as follows:

Table 3.1 (CLP)
Acute Tox. 4 * - H302 Eye Irrit. 2 – H319 Skin Irrit. 2 – H315 Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410

2.3.2 Current classification and labelling in Annex VI, Table 3.2 in the CLP Regulation

The classification of copper sulphate is harmonised in Annex VI of CLP under the index number 029-004-00-0 as follows:

Table 3.2 (67/548/EEC)

Xn;	R22
Xi;	R36/38
N; R50-53	

2.4 Current self-classification and labelling

Not relevant.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Tribasic copper sulphate is currently classified under the general entry copper sulphate according to Annex VI of CLP.
Tribasic copper sulphate is an active substance in the meaning of Directive 91/414/EEC. In accordance with Article 36(2) of the CLP Regulation, tribasic copper sulphate shall be subjected to a full harmonised classification and labelling. Therefore, this proposal considers all human health and environmental end points. In particular, modifications of the current harmonised classification are proposed for eye and skin irritation and for the environmental classification (addition of a M-factor), which justify action at community level.

RAC general comment

In addition to tetracopper hexahydroxide sulphate ECHA received CLH proposals for nine other copper compounds or forms of copper from the same dossier submitter (France). The dossier submitter stated that where systemic toxicity is concerned, the toxicologically relevant moiety is the Cu^{2+} ion, which is released to a different degree from all the copper compounds. A comparison of the bioavailability (and hence toxicity) of various copper compounds showed that bioavailability is highest for the most soluble compound copper sulphate. Consequently, the use of copper sulphate data would represent a worst-case scenario for the determination of the systemic toxicity of relatively insoluble copper compounds. For the systemic endpoints the dossier submitter therefore proposed to read-across between the different copper compounds, and introduced identical sections on specific target organ toxicity, mutagenicity, carcinogenicity and reproductive toxicity in the CLH reports for all compounds. The studies reported in these common sections mostly concern copper sulphate pentahydrate, sometimes also other copper compounds. The sections on acute toxicity, skin irritation/corrosion, eye damage/irritation and sensitisation in the CLH reports are specific for each substance/form.

RAC considered the dossier submitter's proposal to group the substances together for consideration of STOT RE and the CMR endpoints. RAC noted that differences in solubility and other physico-chemical properties may potentially impact the toxicity of the various copper compounds, in particular locally after inhalation exposure. RAC noted further that the anions, in particular thiocyanate, might also be a contributing factor to the toxicity. However, these aspects were not addressed in the CLH reports, whereas RAC concluded that these would need a more detailed analysis. As none of the studies with copper sulphate pentahydrate or the other tested copper substances yielded positive evidence for the classification for these endpoints, RAC did not pursue the aspect of grouping the nine substances any further.

Tetracopper hexahydroxide sulphate can exist in several hydrated forms. The EC number in the proposed Annex VI entry covers all hydrated forms of tetracopper hexahydroxide sulphate and all hydrated forms are to be covered by the entry. For clarity, the name and CAS number for a common hydrated form, tetracopper hexahydroxide sulphate hydrate, is also specified in the proposed entry. Tetracopper hexahydroxide sulphate is referred to as tribasic copper sulphate throughout the CLH report.

Although the CLH report makes references to copper sulphate and the dossier submitter considered tetracopper hexahydroxide sulphate to be a form of copper sulphate (and thus currently covered by the Annex VI entry for copper sulphate, Index No. 029-004-00-0), RAC considers this not to be the case and therefore viewed the CLH report as a proposal for a new Annex VI entry.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 5: Substance identity

EC number:	215-582-3
EC name:	Tetracopper hexahydroxide sulphate (CAS 1333-22-8) Tetracopper hexahydroxide sulphate hydrate (CAS 12527-76-3)
CAS number (EC inventory):	1333-22-8 or 12527-76-3
CAS number:	1333-22-8 or 12527-76-3
CAS name:	Copper hydroxide sulfate (Cu4(OH)6(SO4))
IUPAC name:	Tricopper dihydroxide disulfate
CLP Annex VI Index number:	029-004-00-0 (copper sulphate)
Molecular formula:	$Cu_4H_7O_{10.5}S$
Molecular weight range:	461.30 g/mol

Structural formula:

$$Cu_4(OH)_6SO_4 \cdot \frac{1}{2}H_2O$$

1.2 Composition of the substance

Table 6: Constituents (non-confidential information)

Constituent	Concentration range	Remarks
Tetracopper hexahydroxide sulphate [1] *Tetracopper hexahydroxide sulphate hydrate [2]Cas : 1333-22-8 [1] or 12527-76-3[2]*	*> 49 % Cu/% tribasic copper sulphate (dry basis)* *(equivalent to ≥ 88.9 % /% tribasic copper sulphate*)*	*calculation on the basis of 55.1 % copper in tribasic copper sulphate

Current Annex VI entry: see Part A (section 2.3)

Impurities (non-confidential information)

Impurities are confidential. See confidential annex.

Additives (non-confidential information)

Confidential information, see confidential annex.

1.2.1 Composition of test material

Some information in the literature shows that nanomaterials containing copper compounds may exist. However, the information available in the biocidal and plant protection products dossiers and their production process do not seem to indicate that the substance exist under this shape for these applications.

In this context, it was decided not to take into consideration the potential nanoform of copper compounds in this report and the present CLH dossier is proposed for the bulk form of tribasic copper sulphate. A specific dossier and hazard evaluation may be necessary for nanoforms of this substance

The purity of the tested material is specified when available and/or relevant in the different parts of the CLH report.

1.3 Physico-chemical properties

Table 9: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Lumpy powder bluish-green odourless	O'Connor and Mullee, 2000	-
Melting/freezing point	Decomposition before melting point	O'Connor and Mullee, 2000	Measured (EEC A1)
Boiling point	Decomposition before Boiling point	O'Connor and Mullee, 2000	Measured (EEC A2)
Relative density	3.90 (20.5°C)	O'Connor and Mullee, 2000	Measured (EEC A3)
Vapour pressure	Not applicable (from the structure and the melting temperature result, it was anticipated that no definitive value could be obtained due to the negligible volatility of the test material	O'Connor and Mullee, 2000	estimated
Surface tension	72.2 mN/m at 20°C at $<2.9 \times 10^{-3}$ g/L	O'Connor and Mullee, 2000	Measured (EEC A5)
Water solubility	At 20.0 ± 0.5°C (Purity 55.10%) pH 5.6: salt 0.5 g/L; as Cu 0.28 g/L pH 6.2: salt < 3.42×10^{-3} g/L; as Cu 1.88×10^{-3} g/L pH 9.8: salt ≤2.55×10^{-4} g/L; as Cu ≤1.41×10^{-4} g/L	O'Connor and Mullee, 2000	Measured (EEC A6) Flask method
Partition coefficient n-octanol/water	Not relevant (due to the negligible solubility in water and n-octanol)	O'Connor and Mullee, 2000	Estimated
Flash point	Not required (solid)	-	-
Flammability	Not flammable	-	Estimated
Explosive properties	No explosive properties	-	Estimated
Self-ignition temperature	Not auto-flammable	-	Estimated
Oxidising properties	Not oxidizing	-	Estimated
Granulometry	No data		
Stability in organic solvents and identity of relevant degradation products	No data but can be considered as stable in organic solvents as solubility is given below		
Dissociation constant	Due to the negligible water solubility no determination of dissociation constant was performed	-	-
Viscosity	Not required (solid)	-	-
Henry's law constant	No data		

Solubility in organic solvent	heptane	<1000 µg/L	Anon., 1999	Measured (CIPAC MT 181)
	p xylene	<1000 µg/L		
	1,2 dichloroethane	<1000 µg/L		
	acetone	<1000 µg/L		
	ethyl acetate	<1000 µg/L		
	n-octanol	<1000 µg/L		
Reactivity towards container material	No data			

2 MANUFACTURE AND USES

2.1 Manufacture

Not relevant

2.2 Identified uses

Tribasic copper sulphate was notified as a PPP under Directive 91/414/EC. It is fungistatic and bacteristatic in action and is used in the treatment and prevention of fungal and bacterial diseases.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Table 10: Summary table for relevant physico-chemical studies

Physic-chemical properties/Method	**Results**	**Remarks**	**Reference**
Flash point	Not required (solid)	-	
Flammability	Not flammable	Estimated	European Commission. Draft Assessment Report Copper compounds (2007)
Explosive properties	No explosive properties	Estimated	
Self-ignition temperature	Not auto-flammable	Estimated	
Oxidising properties	Not oxidizing	Estimated	

3.1 *Explosive properties*

Copper sulphate tribasic is a stable inorganic substance. None of these components or grouping are associated with explosive hazards. All are stable groupings in high oxidation states. Copper sulphate tribasic therefore will not have explosive properties and experience in use over many years confirms this conclusion.

3.2 *Flammability*

Copper sulphate tribasic is an inorganic salt with copper in a high oxidation state. As such this material is not likely to undergo self heating under bulk storage conditions and is unlikely to auto-ignite. Self heating or auto-ignition has not been observed with copper sulphate tribasic following use for many years.

The determination of flash point is not required because the active substance is a solid.

So we can conclude that copper sulphate tribasic is not flammable.

3.3 *Oxidising potential*

The oxygen is bound up in very stable structural groupings with strong oxygen bonds. The decomposition temperature is also indicating a high energy of activation. Tribasic copper sulphate is considered inert under the conditions of oxidation.

RAC evaluation of physical hazards
Summary of the Dossier submitter's proposal Tetracopper hexahydroxide sulphate is a stable inorganic salt with copper in a high oxidation state. Its physicochemical properties indicate that it is neither explosive, flammable nor oxidising. The dossier submitter proposed no classification for physical hazards. **Comments received during public consultation** No comments were received during the public consultation. **Assessment and comparison with the classification criteria** Since tetracopper hexahydroxide sulphate does not have explosive or oxidising properties and is not (auto-)flammable, RAC supports the non-classification for physical hazards, as proposed by the dossier submitter.

4 HUMAN HEALTH HAZARD ASSESSMENT

Considering that in mammalian the toxic form of any copper salt is the Cu^{2+} ion, a read across between the different salts (copper sulphate, dicopper oxide, copper hydroxide, copper oxide, copper carbonate, copper thiocyanate, copper powder, copper oxychloride and Bordeaux mixture) will be used for assessment of repeated toxicity, mutagenicity, carcinogenicity and reprotoxicity of copper compounds. Therefore, the report of these endpoints will be common in the different CLH report of each compound. However, the acute toxicity and local toxicity as irritation and sensitization will be specific for each substance.

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

The following summary of toxicokinetics of the copper ion Cu^{2+} is derived from the pesticide and biocide assessment reports made for the review of copper compounds under directive 91/414/EEC and 98/8/EEC.

Absorption
Absorption in both rats and humans varies according to diet. For humans: on a copper-adequate diet, absorption is 36 %, on a low copper diet 56 %, and on a high copper diet 12%. Similar figures have been obtained for rats.

Distribution
After oral absorption, when entering interstitial fluid and blood plasma, absorbed copper initially becomes bound to two proteins; albumin and transcuprein. Although the affinity of transcuprein for copper is higher than that of albumin, copper ions are freely exchangeable between them. Most of the copper bound to albumin and transcuprein is rapidly transported via portal blood to the liver (main organ of regulation), although some also goes directly to other tissues, especially to the kidney. The liver controls the distribution of copper to the rest of the body via the bloodstream, bound to ceruloplasmin.

By other routes of exposure (mainly inhalation), absorbed copper does not pass first by the liver, therefore, a wider distribution through the body is possible.

Metabolism
Metabolism does not occur. Copper is a monatomic ion and cannot be metabolised. It is however used in every cell in the body, and every cell can regulate its copper content. Many enzymes and other proteins containing copper have been described.

Interspecies differences
Albumin, one of the major copper transport proteins of the blood, contains histidine in position 3 which is essential for tight binding of copper. In dogs and pigs, this histidine is replaced by a tyrosine, and consequently the albumin does not have the same affinity for copper. Dog and pig albumins have several low-affinity sites for copper, but albumin is still an effective transport protein in those species. Dogs show unusually high levels of copper in the liver, ten times the levels in other species. While dog liver rapidly took up copper injected intravenously, dogs do not appear to be able to excrete copper via the bile as readily as other species. It is possible that dogs express the WND protein less than other species resulting in accumulation of copper in the liver. Based on these differences in albumin structure and the liver of the dog, it was concluded that the dog is not a good animal model for human risk assessment of copper and that is why no dog study is outlined in this report.

Accumulation
Accumulation does not occur except in cases of genetic disease or chronic administration of exceptionally high doses (60 mg/person/day), where copper accumulates in the liver.

Excretion

Excretion in most species is *via* the bile, in a trypsin-independent protein fragment such that entero-hepatic circulation does not occur. A significant amount of copper is excreted bound to metallothioneins contained in intestinal brush border cells sloughed off and lost in faeces. Minor amounts are also excreted in urine and from skin and hair.
Excretion is rapid. An oral dose of 20 mg Cu/kg to rats was completely eliminated from the liver by 48 h. Blood plasma levels did not increase during this period.

Bioequivalence
In mammalian toxicity, it is considered that the toxic form of any copper salt is the Cu^{2+} ion.
This is shown through the comparison of bioavailability and hence toxicity of the most soluble (copper sulphate) and relatively insoluble copper salts. In effect, the use of copper sulphate data would represent a worst-case scenario for the determination of the systemic effect of relatively insoluble copper compounds in mammalian toxicity. This has also been confirmed in a series of bioavailability studies conducted by several authors who have compared the bioavailability of copper sulphate to other copper salts including copper oxide, copper powder, copper thiocyanate and copper carbonate. Moreover, in an other study copper was administered orally to bile-canulated rats, as copper sulphate, copper hydroxide, copper oxychloride, Bordeaux mixture, tribasic copper sulphate and copper (I) oxide. There were no differences in absorption, copper levels in plasma, liver or bile, or in excretion rates between the five forms and copper sulphate. This study demonstrates bioequivalence between the five forms and copper sulphate, such that repeated dose toxicity studies on copper sulphate, or on only one of the five forms, may be considered representative of the other forms for systemic effects.

In 2010, Rodriguez et al, assessed the relative/dissolution of copper ions from copper materials and copper compounds in gastric mimetic fluid, simulated oral exposure.
The copper compounds tested, include: copper wires massive copper materials), copper powder (130 µm median diameter), coated copper flakes (8.5 µm), cupric oxide and cuprous chloride. Loading rates between 100 mg/L and 2 g/L were assessed. The results are expressed as % mass recovered at the end of the bio-elution test and compred with the results obtained from soluble copper sulphate.

The results are summarised in the table below.

Relative bio-solubility of copper and copper compounds, assessed from the recovery of copper after a bio-elution tests in gastric fluids.

1. Material tested	2. Bio-elution recovery 3. (as% of Cu content)
4. Cu massive	5. 0.096-0.105
6. Cu powder	7. 1.1
8. Cu flake	9. 42-71
10. CuO	11. 68-84
12. CuCl	13. 67-94
14. CuSO4	15. 100

The results show a highest solubility of CuSO4 and CuCl.

In conclusion, this study demonstrated large variability in the gastric bio-accessibility of copper bearing materials.

Therefore in order to reduce the number of animal testing, as CuSO4 release more ion Cu^{2+} than the other copper compounds and it is considered that the toxic form is the Cu^{2+} ion, all long term studies by oral routes could be conducted on CuSO4, as the worst case.

4.1.2 Human information

Literature review on ADME

Copper is a micronutrient. It is essential for life and is employed in all living cells. It is used in many enzyme systems, particularly in energy transfer where the property of electron transfer is exploited in photosynthesis and catabolism. It has been the subject of intense research.

Copper is present in almost all foods, with some foods (nuts, shellfish, chocolate) naturally containing more than 20 ppm copper.
Most human diets naturally include between 1 and 2 mg/person/day of copper, with some containing up to 4 mg/person/day. Copper levels in blood and tissues are generally stable. The body is able to maintain a balance of dietary copper intake and excretion that allows normal physiological processes to take place.

As with all micronutrients (minerals), copper is absorbed, used, stored and excreted. This applies at the level of the individual cell, at the organ and at the level of the whole organism. The cell membrane transport mechanisms for copper have been studied extensively, and the genetic codes for the individual transporter proteins are very similar in many different organisms: bacteria, fungi and fish, indicating that the process is ancient.

The copper transport mechanisms at the level of the organism form part of the system of homeostasis, the process by which the levels of copper in the body (and ultimately the cell) are regulated. Copper can be considered to show a flattened "U"-shaped dose-response curve.

*The left side of the "U" curve represents defic*iency, where intake is less than the requirement. This can be lethal, especially in children, where copper is needed for growth. Copper deficiency is associated with growth retardation, anaemia, skin lesions, impaired immunity, intestinal atrophy, impaired cardiac function, reproductive disturbance, neurological defects and skeletal lesions. Copper is essential for normal physiological function such as cellular respiration, free radical defence, synthesis of melanin, connective tissue, iron metabolism, regulation of gene expression, and normal function of the heart, brain and immune system.

The central near-horizontal part of the "U" curve represents homeostasis, where intake and excretion are balanced, and copper levels are said to be normal.

The right-hand part of the "U" represents toxicity or excess copper disease.

The natural homeostatic regulation of copper means that an individual on a low copper diet will retain more of an artificial dose of copper than an individual on a high copper diet.

4.1.3 Summary and discussion on toxicokinetics

Copper is widely distributed in biological tissues, where it occurs largely in the form of organic complexes, many of which are metalloproteins and function as enzymes. Copper enzymes are

involved in a variety of metabolic reactions, such as the utilisation of oxygen during cell respiration and energy utilisation. They are also involved in the synthesis of essential compounds, such as the complex protein of connective tissues of the skeleton and blood vessels, and in a range of neuroactive compounds concerned in nervous tissue function.
Copper is present in almost all foods, most human diets naturally include between 1 to 2 mg/person/day of copper, with some containing up to 4 mg/person/day. Copper levels in blood and tissues are generally stable; the body is able to maintain a balance of dietary copper intake and excretion that allows normal physiological processes to take place. Up to 93 % of the copper in the blood is bound to the enzyme caeruloplasmin, with the majority of the rest bound to albumin and amino acids; there is strong evidence that absorbed copper is never released free in the blood or in the cells.

A bioequivalence study was performed to compare copper hydroxide, copper oxychloride, Bordeaux mixture, tribasic copper sulphate and copper (I) oxide with copper sulphate pentahydrate on bile cannulated rats. Absorption, distribution and excretion rates were similar between the six variants of copper following oral ingestion of 20 mg Cu/kg bw; liver was the principal organ of regulation of copper and main excretion was via the bile. Liver copper levels increased significantly following dosing with T_{max} at 12 hours; depuration was rapid, with levels returning to control by 48 hours after dosing. Plasma concentrations in both control and dose rats remained unchanged.
Oral absorption of copper varies according to the diet, for humans a copper-adequate diet results in 36 % absorption, while a low copper diet results in 56 % absorption and a high copper diet in 12 % absorption. Similar figures were found in rat, 50 % oral absorption was considered for this specie. Distribution was directly from the intestine to the liver, which controls the distribution of copper to the rest of the body via the bloodstream, bound to ceruloplasmin. Metabolism does not occur. Copper do not accumulate except in cases of genetic disease or chronic administration of high doses, where copper accumulates in the liver. Excretion is rapid, via the bile, in a trypsin-independent protein fragment such that entero-hepatic circulation does not occur. Significant amounts of copper are excreted bound to metallothioneins contained in intestinal brush border cells sloughed off and lost in faeces; minor amounts are also excreted in urine and from skin and hair.

The natural homeostatic regulation of copper means that an individual on a low copper diet will retain more of an artificial dose of copper than an individual on a high copper diet.

4.2 Acute toxicity

Table 11: Summary table of relevant acute toxicity studies

Method	Results	Remarks	Reference
Oral			
Rat Sprague Dawley 3/sex/dose Tribasic copper sulphate 200-2000 mg/kg bw. Acute exposure 14days post exposure	300 mg/kg bw < LD50 < 2000 mg/kg bw	OECD 423 GLP No deviation Purity: 98.4% (w/w tribasic copper sulphate); 54.2% (w/w copper) Vehicle: 0.5% carboxymethyl cellulose	Sanders, A. (2002a)
Dermal			
Rat Sprague Dawley 5/sex/dose Tribasic copper sulphate 2000 mg/kg bw (limit test) Acute exposure 14 days post exposure	LD50 >2000 mg/kg bw.	OECD 402 GLP No deviation Purity: 98.4% (w/w tribasic copper sulphate); 54.2% (w/w copper) Vehicle: 0.5% carboxymethyl cellulose	Sanders, A. (2002b)

4.2.1 Non-human information

4.2.1.1 Acute toxicity: oral

Reference: Sanders, A. (2002a)
Guideline: OECD 423
GLP: Yes
Deviations: None

Tribasic copper sulphate (batch number L2206, containing 54.2 % w/w copper, content of tribasic copper sulphate 98.4 %) was administered as a dispersion in 0.5 % carboxymethyl cellulose. Groups of three male and three female Sprague-Dawley CD rats weighing 200 g or above were used. The rats were housed in groups of three by sex, acclimatised, and fasted overnight prior to dosing. Food was returned approximately three to four hours after dosing. Dose levels of 200 mg/kg bw (males and females) and 2000 mg/kg bw (females only) were administered by single oral administration by gavage using a metal cannula in 10 mL/kg on Day 1. Animals were observed frequently on the day of dosing and then once daily for the 14-day post-dosing period. Animals were weighed prior to administration and after 7 days (on Day 8) and after 14 days (on Day 15) or at death. Decedents and animals surviving to 14 days were subject to gross necropsy.

There were no mortalities at 200 mg/kg bw. At 2000 mg/kg bw, all females died and the deaths occurred on Day 1 or Day 2. There was a variety of clinical signs recorded in females including piloerection, hunched posture and diarrhoea (200 and 2000 mg/kg bw), and lethargy, decreased respiration rate, laboured respiration and ataxia (2000 mg/kg bw only). Symptoms occurred on Day 1 and all females dosed at 200 mg/kg had recovered by Day 2. No symptoms occurred in males dosed at 200 mg/kg. A summary of mortalities is presented in Table below.

Surviving animals showed weight gain during the study.

No gross findings were recorded in surviving animals. Necropsy finding in animals which died during the study were a blue coloured liquid in the stomach, haemorrhagic or abnormally red lungs, dark liver, dark kidneys, epithelial sloughing of the gastric mucosa and non-glandular region of the stomach and haemorrhagic small intestine.

Table 12: Mortalities following oral administration of tribasic copper sulphate to rats

Dose (mg/kg bw)	**Males**		**Females**	
	Mortality	***Time of death***	***Mortality***	***Time of death***
200	0/3	-	0/3	-
2000	-	-	3/3	Day 1 (2); Day 2

Figures in parenthesis are the number which died on the day specified if more than one.

The acute oral LD_{50} of tribasic copper sulphate to the rat was estimated to be between 300 and 500 mg/kg bw.

4.2.1.2 Acute toxicity: inhalation

No data.

4.2.1.3 Acute toxicity: dermal

Reference: Sanders, A. (2002b)
Guideline: OECD 402
GLP: Yes
Deviations: None

Tribasic copper sulphate (batch number L2206, containing 54.2 % w/w copper, content of tribasic copper sulphate 98.4 %) was moistened with 0.5 % carboxymethyl cellulose prior to application. Five male and five female Sprague-Dawley CD rats weighing 200 g or above were housed individually and acclimatised prior to dosing. A dose level of 2000 mg/kg bw was applied to an area of intact shaven skin, equivalent to approximately 10 % of the total body surface area, on each rat on Day 1 using a syringe. The treated area was covered with surgical gauze and a self-adhesive bandage. After 24 hours, the bandage was removed and the skin wiped with moistened cotton wool to remove residual test substance. Animals were observed for treatment-related clinical signs frequently on the day of administration and once daily for the 14-day post-dosing period. Skin reactions were recorded daily from Day 2. Animals were weighed prior to treatment and after 7 days (Day 8) and 14 days (Day 15). Decedents and animals surviving to 14 days were subject to gross necropsy.

There were no mortalities, no clinical signs of toxicity and no signs of skin irritation.

All animals showed acceptable weight gain during the study.

No gross findings were recorded at necropsy.

The acute dermal LD_{50} of tribasic copper sulphate to the rat was greater than 2000 mg/kg bw for males and females.

4.2.1.4 Acute toxicity: other routes

No data available.

4.2.2 Human information

Inhalation

Little information is available on acute effects in humans and inhalation of copper-containing materials.

Published studies on acute effects in humans appear to have focussed on metal fume fever (MFF)[1] and possible association with copper exposure. This subject has been reviewed extensively by Borak *et al* (2000) with the aim of establishing whether there is an association between exposure to

1 Metal fume fever (MFF) is a transient illness which appears to develop 4-12 hours after occupational exposure to metal fume. MFF presents as an influenza-like illness with cough and dyspnoea followed by fever, sweating and shivering. Other accompanying clinical signs and symptoms are nausea, headache, weakness, a sweet metallic taste, and muscle and joint pain.

copper and MFF. The review was based on seven reports, identified in a literature search as the only reports that contained original descriptions of copper-exposed workers who developed symptoms consistent with MFF. These seven reports are summarised below.

The earliest publication by Hansen (1911) provided a brief report of MFF-like symptoms in 10 males working in a research foundry where scrap copper was melted. The symptoms occurred as an isolated incident. No qualitative or quantitative data concerning exposure were provided. The isolated nature of this incident was considered by Borak *et al* to indicate an association with exposure to contaminants other than copper.

Koelsch *et al* (1923) reported the occurrence of symptoms that included chest discomfort, shivering, nausea and fever in 10 men performing hot rolling of copper bars in a rolling mill. The symptoms, which had not previously been associated with the process, resolved in 24 hours. No qualitative or quantitative exposure data were presented. As with the previous study, the isolated nature of this incident suggested to Borak *et al* that contaminants other than copper were involved.

Friberg and Thrysin (1947) reported MFF-like syndrome in approximately 50 workers involved in cleaning reactor ovens where pulverised copper was used as a catalyst. During the cleaning task, heads and faces of the workers were reported to be covered in dust consisting mainly of cuprous and cupric oxides. Initial symptoms included throat discomfort, burning eyes, nausea and headache, followed by flu-like symptoms, nausea, vomiting, diarrhoea and chest discomfort. In many workers, symptoms persisted for more than 72 hours. Quantitative exposure data was not provided. Dust particles were reported to range from 1-15 µm diameter, with more than 70% >5 µm. Given that MFF is typically associated with fine particles (< 1 µm diameter), Borak *et al* considered that the study did not support association between copper and MFF. Further, the heavy exposure indicated in this study is not generally associated with occurrence of MFF.

Schiotz (1949) reported the occurrence of initial symptoms such as metallic taste, throat dryness and slight chest oppression, followed by shivering, sweating and fever among seven workers involved in pulverising cuprous oxide during the production of marine paint. Symptoms subsided after 20-30 hours. Quantitative exposure data were not provided, although the described working conditions indicated very high levels of exposure.

Gleason (1968) reported symptoms in workers exposed to dust generated during polishing of copper plates with aluminium oxide abrasives. Symptoms were reportedly similar to “the onset of a common cold with chills or warmth, stuffiness of the head, etc”. Lower respiratory symptoms were not reported, nor were other symptoms characteristic of MFF. Quantitative exposure data were limited to a single breathing zone sample, indicating 0.12 mg/m^3, although the study’s author suggested exposure levels may have been “two or three times” higher. In this report, symptoms persisted for several weeks until ventilation was introduced, a feature which is not usually associated with MFF. In view of the absence of many symptoms characteristic of MFF and the persistence of the reported symptoms, Borak *et al* considered that the condition was unlikely to be MFF. Further, co-exposure to aluminium oxide was also likely, a metal also implicated in MFF aetiology.

Hopper (1978) described the single case of a foundry worker who developed an isolated episode of symptoms which included headache, cough, chest pain, chills and shortness of breath. Symptoms occurred shortly after exposure to a molten alloy of copper, beryllium and aluminium, which was poured into vessel containing alcohol and adhesive glue. Exposure data were not presented. Borak *et al* noted the co-exposure to other metals which have been implicated in MFF aetiology and the likely exposure to other potentially harmful substances. Consequently this case-report was not considered as providing evidence of an association between copper and MFF.

Armstrong *et al* (1983) reported symptoms of MFF in a group of 26 workers after cutting brass pipes (containing 90% copper, 10% nickel, and smaller amounts of zinc) with torches in a confined space. Symptoms included fever, chills, headache, dyspnoea and nausea. Exposure data for the different metals were not provided, although a description of the process indicated that high exposure levels were likely. As with the previous two studies, Borak *et al* considered that co-exposure to other metals implicated in MFF prevented identification of copper as the causative agent.

None of the seven studies covered by the review provided adequate exposure data, qualitative or quantitative, to enable identification of the causative agent(s) associated with the reported symptoms. Further, as noted by Borak *et al*, there was a lack of any occupational pattern associated with the MFF symptoms, as indicated by the range of industrial processes covered (foundry work, rolling mill, paint production, metal polishing and pipe cutting). The conclusion of Borak *et al* was that, based on the seven studies identified in the literature search, there is insufficient evidence to conclude that exposure to copper dust or fume causes MFF. Based on data which are currently available, this conclusion would appear to be justified.

Dermal

There are no published data on acute dermal effects of copper or copper compounds.

Oral

Self-poisoning

Self-poisoning with copper sulphate is rare in western countries but has been a common method of suicide among low income groups in some areas of India. The most extensive study concerns 48 cases, including 7 fatalities (15%), admitted to one hospital in Delhi and 5 fatalities reported to other Dehli hospitals (Chuttani *et al*, 1965). The most frequent symptoms observed in subjects were nausea, epigastrial burning and vomiting. In addition, diarrhoea was reported in 14 patients (29%). Biopsy examination of fatalities indicated deep erosions in gastric mucosa, haemorrhage in the stomach and small intestine and oedema in the sub mucosa. Jaundice of variable severity occurred in 11/48 cases (23%). In the more severe cases, palpable liver enlargement, significantly elevated serum glutamic oxaloacetic transaminase (SGOT, 252.4 ±142 IU) and elevated bilirubin (112 ±8.9 mg/litre) were observed. Biopsy examination of liver tissue from fatalities showed centrilobular necrosis and biliary stasis. Post-mortem examination also indicated swollen and congested kidneys with glomerular swelling and necrosis of tubular cells. Anuria was reported in 13/48 patients (27%) and oliguria in 5/48 (10%). Red discolouration of urine was observed, with haemoglobinuria confirmed in some patients. These findings suggest haemolysis and are consistent with other reports. Haematocrit and serum/plasma appearances were not reported. Serum or blood levels of copper in the cases were elevated 2- or 3-fold compared to normal values. Estimated quantities of copper ingested were based on patients' accounts and therefore are unreliable. Consequently, this study provides no reliable data which can be used for human hazard assessment.

Subsequent case reports describe massive overdoses of copper sulphate (175 g) by a 22 year-old Indian male (Mittal, 1972) and 250 g by a 42 year old US male (Jantsch *et al*, 1985). Both patients survived following rapid chelation therapy with single or multiple injections of dimercaprol. The amounts ingested were considerably greater than the highest estimated dose reported by Chuttani and co-workers (1965). It therefore seems probable that survival of these patients was attributable to immediate chelation therapy.

Accidental ingestion

The ingestion of a relatively small amount of copper sulphate (3 g), together with an equal amount of zinc sulphate, by an 86 year-old female patient has also been reported (Hantson *et al*, 1996). The patient was admitted to hospital vomiting blue/green material and she had diarrhoea. Gastric lavage, dehydration and chelation therapy with dimercaprol were performed. The patient then suffered hypotension, bronchial inflammation and ulceration and a decline in respiratory function. These symptoms were interpreted as corrosive pneumonitis. The patient was placed on a mechanical ventilator for three days and subsequently made a complete recovery. In this case, the symptoms may have been exacerbated by the patient's age and health status, but may also have been mitigated to some extent by the co-ingestion of zinc sulphate which may have served to limit copper uptake and the severity of the systemic effects.

Therapeutic treatment

Systemic effects, including renal damage and thrombocytopaenic purpura, were reported in a 17-year old boy who was given 1% copper sulphate (2 mg/day) orally for treating vitiligo (Pande and Gupta, 1969).

4.2.3 Summary and discussion of acute toxicity

Acute oral studies

Tribasic copper sulphate was of moderate toxicity by the oral route (LD_{50} greater than 200 but less than 2000 mg/kg/bw).

The range of clinical signs following exposure was: diarrhoea, piloerection, ataxia, lethargy, hunched posture and impaired respiration. Surviving animals generally showed no gross findings at necropsy, but animals which died during the studies showed discoloration or haemorrhage in the digestive tract, effects on liver and/or kidney and the presence of substances similar to the test material in the stomach or intestines.

Copper has been used in suicide attempts. Most of these involved copper sulphate pentahydrate. Intoxication is associated with emesis, superficial or deep ulcerations of the gastric and intestinal mucosa. Liver histopathology revealed dilatation of central veins, varying degrees of liver cell necrosis and bile thrombi. In kidneys there was congestion of glomeruli swelling or necrosis of tubular cells and haemoglobin casts. These findings are similar to those seen in animal studies. Elevated serum copper levels are only seen in moderate to severe cases of intoxication. Unfortunately, the amount of copper taken in these suicide attempts is never quantified accurately.

Acute dermal studies

Tribasic copper sulphate was of low toxicity by the dermal route (LD_{50} greater than 2000 mg/kg/bw). Clinical signs were absent or very minor.

Acute inhalation studies

No experimental data is available and no case of human fatalities is reported by inhalation exposure.

4.2.4 Comparison with criteria

For tribasic copper sulphate, the oral LD_{50} in rats can be identified between 300 and 500 mg/kg. The oral LD_{50} lies within the range (300-2000 mg/kg) for classification as Acute Tox.4 (H302: Harmful if swallowed) under regulation (EC) 1272/2008.

The dermal LD_{50} lies above the classification cut-off of 2000 mg/kg under regulation (EC) 1272/2008. Therefore no classification is proposed.

No inhalation hazard has been indicated for tribasic copper sulphate. Tribasic copper sulphate is not classified under regulation (EC) 1272/2008.

4.2.5 Conclusions on classification and labelling

Based on the results of the acute oral toxicity studies, a classification Acute Oral Tox.4-H302 is proposed.

No classification is proposed by the inhalation route.

No classification is proposed by dermal route.

RAC evaluation of acute toxicity

Summary of the Dossier submitter's proposal

Two acute toxicity studies (one via the oral route, one via the dermal route) are included in the CLH report, both conducted with tetracopper hexahydroxide sulphate in rats. The oral study (Sanders, 2002a), conducted according to OECD TG 423, determined the LD_{50} to be between 300 and 500 mg/kg bw. In this study two doses were tested, with no animals (3/sex) dying at 200 mg/kg bw while all animals (3 females) died at 2000 mg/kg bw. In the dermal study (Sanders, 2002b), conducted according to OECD TG 402, no animals (5/sex) died at the dose level of 2000 mg/kg bw tested. The LD_{50} was therefore determined to be above 2000 mg/kg bw. The dossier submitter concluded that as the oral LD_{50} value was between 300 and 2000 mg/kg bw, classification as Acute Tox. 4 – H302 is warranted. No classification was proposed for the dermal route, nor for the inhalation route for which no study was available.

The CLH report also contains a review of seven studies reporting on a possible association between copper exposure and Metal Fume Fever (MFF) in humans (Borak *et al.,* 2000). MFF presents as an influenza-like illness with cough and dyspnoea followed by fever, sweating and shivering, accompanied by nausea, headache, weakness, a sweet metallic taste and muscle and joint pain. The dossier submitter concluded (in agreement with the authors of the review) that none of the reports contain enough conclusive evidence to associate copper fumes or particles with MFF. Another review (Chuttani *et al.*, 1965) reports on several cases of self-poisoning by oral ingestion of copper sulphate. Intoxication is associated with nausea, epigastric burning, vomiting, diarrhoea, ulcerations of the gastric and intestinal mucosa, and liver and kidney histopathology. Rapid chelation therapy increases survival.

Comments received during public consultation

One MSCA expressed a general support for the classification proposal, but also commented that a conclusion regarding inhalation toxicity could not be reached due to lack of data and that an acute inhalation study should be requested for tetracopper

hexahydroxide sulphate as an active substance under Directive 91/414/EEC. The dossier submitter considered such a request not necessary, because it is very difficult to generate a proper inhalable atmosphere from the dense aqueous paste of tetracopper hexahydroxide sulphate, the vapour pressure of tetracopper hexahydroxide sulphate is low and, moreover, the authorised plant protection products containing tetracopper hexahydroxide sulphate are not classified for acute inhalation toxicity based on experimental data.

Assessment and comparison with the classification criteria

Following a comparison of the LD_{50} values in the key studies with the criteria, RAC agrees with the conclusion of the dossier submitter that for the oral route tetracopper hexahydroxide sulphate should be classified as **Acute Tox. 4 – H302** and that for the dermal route classification is not warranted.

For the inhalation route, no animal data are available and the available human data are insufficient for classification. No conclusion can be drawn for classification for acute inhalation toxicity.

4.3 Specific target organ toxicity – single exposure (STOT SE)

The human has well recorded homeostatic mechanisms to control excess copper levels in the body by a combination of decreased absorption and increased excretion. Human epidemiological data is available however information is limited regarding doses consumed and exposure. Acute toxicity in humans is infrequent and generally results from ingestion of contaminated foodstuffs/beverages, for suicide purposes.

A paper by Chuttani (Chuttani *et al*, 1965) reviewed 53 cases of copper sulphate poisoning with ingestion varying between 1 and 100g. Jaundice was recorded as a symptom with post mortem examinations showing that the liver had signs of severe histological changes. A kidney biopsy showed swelling and necrosis in two patients, and following an autopsy of patients who had died, a congested kidney was observed. Emesis and irritation of the gastric mucosa was observed in all patients.

A case was reported where a male ingested an estimated 175g of copper sulphate, renal damage was observed (*Mittal*, 1972).

An acute oral was conducted in rats with tribasic copper sulphate (Sanders, A. 2002a). There were no mortalities at 200 mg/kg bw. At 2000 mg/kg bw, all females died. No gross findings were recorded in surviving animals. Necropsy finding in animals which died during the study were a blue coloured liquid in the stomach, haemorrhagic or abnormally red lungs, dark liver, dark kidneys, epithelial sloughing of the gastric mucosa and non-glandular region of the stomach and haemorrhagic small intestine.

Acute dermal toxicity study in rats conducted with tribasic copper sulphate (Sanders, A., 2002b) showed there were no mortalities and no clinical signs of toxicity or skin reactions throughout the observation period.

No acute inhalation studies have been conducted with tribasic copper sulphate.

4.3.1 Summary and discussion of Specific target organ toxicity – single exposure

There was no clear evidence of any specific toxic effects on a target organ or tissue in experimental studies. Clinical signs of toxicity were observed after single exposures to tribasic copper sulphate but were transient in nature and are considered to be unspecific signs of general acute toxicity.
In humans, cases of liver and kidney damage further to a single exposure to copper sulphate were reported but were secondary to either massive or poorly reported doses.

4.3.2 Comparison with criteria

No classification as STOT-SE under regulation (EC) 1272/2008 is proposed. No classification or SCLs are considered necessary.

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)
Summary of the Dossier submitter's proposal No clear evidence of specific toxic effects on organs was reported in the acute toxicity studies. Clinical signs of toxicity were transient in nature and considered to be unspecific signs of general acute toxicity. Liver and kidney damage in human case studies with copper sulphate were seen as secondary to massive or poorly reported doses. The dossier submitter concluded that no classification is warranted for STOT SE. **Comments received during public consultation** No comments were received during the public consultation. **Assessment and comparison with the classification criteria** In the available acute toxicity studies, no clinical signs of toxicity or signs of skin irritation were observed following dermal exposure. Following oral exposure, no clinical signs were observed in male rats whereas in female rats piloerection, hunched posture and diarrhoea (at 200 and 2000 mg/kg bw) and lethargy, decreased respiration rate, laboured respiration and ataxia (at 2000 mg/kg bw) were observed. Symptoms occurred at day 1 for both dose levels, and at 200 mg/kg bw all animals had recovered by day 2. The transient signs at the non-lethal dose level are indicative of non-specific, general acute toxicity, just like the most frequently observed symptoms in human self-poisoning cases (nausea, epigastric burning, vomiting, diarrhoea). RAC agrees with the conclusion of the dossier submitter that tetracopper hexahydroxide sulphate should not be classified for specific target organ toxicity – single exposure (STOT SE).

4.4 Irritation

4.4.1 Skin irritation

Table 13: Summary table of relevant skin irritation studies

Method	Results	Remarks	Reference
Rabbit New Zealand white 3 animals (intact skin) Tribasic copper sulphate 0.5g 4 hours of exposure 72 hours post exposure	Average score 24, 48, 72h Erythema: 0.0 Oedema: 0.0 **Not a skin irritant.**	OECD 404 GLP No deviation Purity: 98.4% (w/w tribasic copper sulphate); 54.2% (w/w copper)	Sanders, A. (2002c)

4.4.1.1 Non-human information

Reference: Sanders, A. (2002c)
Guideline: OECD 404
GLP: Yes.
Deviation No

Tribasic copper sulphate (batch number L2206, containing 54.2% w/w copper, content of tribasic copper sulphate 98.4%) was used for the study. Three male New Zealand white rabbits weighing 2.0 to 3.5 kg were housed singly and acclimatised prior to dosing. On the day prior to application, an area on the dorsal area of the trunk of each animal was clipped free of fur. The following day, 0.5 mL of test material was applied to the intact skin of each rabbit under a cotton gauze patch 2.5 x 2.5 cm in size. The patch was secured with adhesive tape and the trunk of the animal was wrapped with an elastic corset. After 4 hours, the dressings were removed and any residual test substance was removed by swabbing the skin with cotton wool soaked in distilled water. Animals were examined for signs of irritation after 1, 24, 48 and 72 hours and effects scored according to Draize.

No erythema or oedema was recorded in any animal at any time. Faint blue coloured staining of the test site was observed at the 1-hour (all animals) and 24-hour (one animal) assessments.

Tribasic copper sulphate did not cause any irritation to rabbit skin.

4.4.1.2 Human information

No data available

4.4.1.3 Summary and discussion of skin irritation

The available study was performed with 3 rabbits New Zealand. Tribasic copper sulphate was not irritating to rabbit skin. No erythema or oedema was recorded at any observation time (24, 48 and 72 hours).

4.4.1.4 Comparison with criteria

1) Criteria in the CLP classification:

A substance shall be classified as irritant in category 2 if in at least 2 of 3 tested animals mean value for erythema/eschar or for oedema is between 2.3 and 4.0 from gradings at 24, 48 and 72 hours after patch removal or, if reactions are delayed, from grades on 3 consecutive days after the onset of skin reactions. If inflammation persists to the end of the observation period normally 14 days in at least 2 animals, particularly taking into account alopecia (limited area), hyperkeratosis, hyperplasia, and scaling, substance shall be also considered as irritant.

2) Comparison with criteria:

Here, means scores 24 to 72 hours for erythema and oedema were 0.0

4.4.1.5 Conclusions on classification and labelling

In this context and in contrast to the current harmonised classification for the general entry copper sulphate that is classified Skin Irrit 2, available data shows that tribasic copper sulphate does not support classification for skin irritation under CLP regulation criteria. It is therefore proposed not to apply to tribasic copper sulphate the current classification for skin irritation that applies to the general harmonised entry copper sulphate.

RAC evaluation of skin corrosion/irritation
Summary of the Dossier submitter's proposal One skin irritation study with rabbits, conducted with tetracopper hexahydroxide sulphate according to OECD TG 404, is reported in the CLH report (Sanders, 2002c). As no erythema or oedema was observed in any animal at any time point, the dossier submitter concluded that tetracopper hexahydroxide sulphate should not be classified for skin irritation. **Comments received during public consultation** One MSCA supported the proposal for non-classification for skin irritation. One other MSCA expressed general support for the classification proposal. **Assessment and comparison with the classification criteria** Given that all three test-animals scored zero for both erythema and oedema over 24/48/72 h in the available skin irritation study, RAC agrees with the conclusion of the dossier submitter that tetracopper hexahydroxide sulphate should not be classified for skin irritation.

4.4.2 Eye irritation

Table 14: Summary table of relevant eye irritation studies

Method	Results	Remarks	Reference
Rabbit New Zealand white 3 animals (unwashed) Tribasic copper sulphate 0.1 ml ((right eyes) 72 hours post exposure	Average scores (24, 48, 72 hrs): Cornea: 0.0 Iris: 0.0 Conjunctival redness: 0.3 Conjunctival chemosis: 0.0 Effects did not persist after 24 hours **Not an eye irritant**	OECD 405 GLP No deviation Purity: 98.4% (w/w tribasic copper sulphate); 54.2% (w/w copper)	Sanders, A. (2002d)

4.4.2.1 Non-human information

Reference: Sanders, A. (2002d),
Guideline: OECD 405.
GLP: Yes.
Deviations: No

Materials and methods: Tribasic copper sulphate (batch number L2206, containing 54.2% w/w copper, content of tribasic copper sulphate 98.4%) was used for the study. Three male New Zealand white rabbits weighing 2.0 to 3.5 kg were housed individually and acclimatised prior to dosing. 0.1 mL of the test substance was administered into the conjunctival sac of the right eye of each rabbit and the eyelids held together for one second before release. Animals were examined for signs of eye irritation after 1, 24, 48 and 72 hours after administration, and irritation scored according to Draize. Records of non relevant endpoints for classification such as the area of the cornea affected and conjunctival discharge were also made in the study but these are not presented in this summary as they do not affect the outcome.

Finding: Tribasic copper sulphate caused slight conjunctival redness of the eyes in three animals (up to score 2) and conjunctival chemosis in two animals (up to score 1) at one or more assessment times. No cornea opacity or iris lesion was recorded; Effects did not persist after 24 hours. The results are summarised in Table below.

Table 15: Tribasic copper sulphate: summary of individual and mean eye irritation scores according to Draize

Assessment time	Scores according to Draize for animal number											
	Cornea opacity			***Iris lesion***			***Conjunctival redness***			***Conjunctival chemosis***		
	120	121	122	120	121	122	120	121	122	120	121	122
1 hour	0	0	0	0	0	0	1	2	1	0	1	1
24 hours	0	0	0	0	0	0	0	1	0	0	0	0
48 hours	0	0	0	0	0	0	0	0	0	0	0	0
72 hours	0	0	0	0	0	0	0	0	0	0	0	0
Mean score [a]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0
Mean score [a] for three animals	0.0			0.0			0.1			0.0		

[a] Mean scores after 24, 48 and 72 hours (shaded).

The mean eye irritation scores recorded at 24, 48 and 72 hours in two or more animals were 0 (cornea opacity, iris lesion and conjunctival chemosis) and less than 2.5 (conjunctival redness).

4.4.2.2 Human information

No data available

4.4.2.3 Summary and discussion of eye irritation

In an eye irritation study performed according to guideline OECD 405 on 3 rabbits **New Zealand**, the mean eye irritation scores recorded at 24, 48 and 72 hours in two or more animals were 0 (cornea opacity, iris lesion and conjunctival chemosis) and less than 2.5 (conjunctival redness).

4.4.2.4 Comparison with criteria

1) Criteria in the CLP classification :

A substance shall be classified as a substance which could induce reversible eye irritation, classified in Category 2 (irritating to eyes), if when applied to the eye of an animal, a substance produces:

a. At least in 2 of 3 tested animals, a positive response of:
   Corneal opacity ≥ 1 and/or

   Iritis ≥ 1 and/or

   Conjunctival redness ≥ 2 and/or

   Conjunctival oedema ≥ 2

Calculated as the mean scores following grading at 24, 48, and 72 hours after instillation of the test material, and which fully reverse within an observation period of 21 days.

2) Comparison with criteria:

The available study was performed with 3 rabbits New Zealand. The mean eye irritation scores recorded at 24, 48 and 72 hours in two or more animals were 0 (cornea opacity, iris lesion and conjunctival chemosis) and less than 2.5 (conjunctival redness).

In this context and in contrast to the current harmonised classification for the general entry copper sulphate that is classified Eye Irrit 2, available data shows that no classification for eye irritation under CLP regulation criteria is required.

4.4.2.5 Conclusions on classification and labelling

Tribasic copper sulphate is not an eye irritant. No classification under CLP regulation criteria, is required. It is therefore proposed not to apply to tribasic copper sulphate the current classification for eye irritation that applies to the general harmonised entry copper sulphate.

RAC evaluation of eye damage/irritation
Summary of the Dossier submitter's proposal One eye irritation study with rabbits, conducted with tetracopper hexahydroxide sulphate according to OECD TG 405, is reported in the CLH report (Sanders, 2002d). Slight conjuctival redness was seen in one animal at 24 h (Draize score of 1). All other scores were 0 at 24, 48 and 72 h. The dossier submitter concluded that tetracopper hexahydroxide sulphate is not an eye irritant according to the criteria and that therefore no classification for eye irritation is warranted. **Comments received during public consultation** One MSCA supported the proposal for non-classification for eye irritation. One other MSCA expressed a general support for the classification proposal. **Assessment and comparison with the classification criteria** Tetracopper hexahydroxide sulphate caused slight eye irritation in the available eye irritation study, consisting of conjunctival redness (in 3 animals, two with score 1, one with score 2) and chemosis (in 2 animals, both score 1) at the 1 h time point, and conjunctival redness (in 1 animal, score 1) at the 24 h timepoint. No effects on the conjunctivae were seen at 48 and 72 h. Corneal opacity and iris lesions were not observed in any animal at any time point. The mean scores over 24-72 h for corneal opacity (0), iris lesions (0), conjunctival redness (0.33) and chemosis (0) are all below the threshold values for classification (≥1, ≥1, ≥2 and ≥2, respectively, in at least 2 of 3 tested animals). Hence, RAC agrees with the conclusion of the dossier submitter that tetracopper hexahydroxide sulphate should not be classified for eye irritation.

4.4.3 Respiratory tract irritation

No data available.

4.5 Corrosivity

No data available. However, no corrosivity properties are expected for tribasic copper sulphate.

4.6 Sensitisation

4.6.1 Skin sensitisation

Table 16: Summary table of relevant skin sensitisation studies

Method	Results	Remarks	Reference
Guinea pigs Hartley Guinea-pig maximization test 20 test animals 10 control animals Tribasic copper sulphate	No mortalities were observed. 0/20 test animals reveals positive reactions and 0/10control animals **Not sensitising.**	OECD 406 GLP Deviation Purity: 54.2% w/w copper; 98.4% w/w tribasic copper sulphate.	Sanders, A. (2002e)

4.6.1.1 Non-human information

Reference: Sanders, A. (2002e)
Guideline: OECD 406
GLP: Yes.
Deviation No

Tribasic copper sulphate (batch number L2206 containing 54.2% w/w copper, content of tribasic copper sulphate 98.4%) was used for the study. An initial irritation screening test was performed to determine the highest non-irritant concentration for the challenge phase of the study and an irritant concentration for the induction phase. A concentration of 0.1% w/w in the vehicle (distilled water) produced mild to moderate irritation by injection and was selected for the intradermal induction phase. By the topical route, 75% w/w in the vehicle produced only mild to moderate dermal irritation and was selected for the topical induction phase. A concentration of 25% w/w in the vehicle was identified as the highest non-irritant concentration and, together with the lower concentration of 10% w/w in the vehicle, was selected for topical challenge. Adult Dunkin-Hartley guinea pigs, approximately 8 to 12 weeks old, weighing 300 to 450 g at initiation were used for the main test. Intradermal injections of test substance (0.1% w/w) in distilled water, FCA with distilled water (1:1) and test substance (0.1% w/w) in FCA/distilled water (1:1) were administered to the interscapular region of 20 animals. Ten control animals received FCA/distilled water (1:1), distilled water and distilled water at 50% w/w in FCA/distilled water (1:1). Approximately 24 and 48 hours after injection, the sites were scored for erythema. After six days, the interscapular region was clipped. The next day, the topical induction phase was performed on the test animals: a filter paper patch (8 cm^2 in area) was loaded with a 75% concentration of the test substance and applied to the shaved interscapular area previously injected and held in place for 48 hours with an occlusive dressing. Control animals received a filter paper pad loaded with distilled water for the same period. 21 days after initiation, a filter paper patch was loaded with the challenge dose of 25% w/w test substance in vehicle and applied to a naïve shaved site on the right flank of the test and control animals. A second patch loaded with the challenge dose of 10% w/w test substance in vehicle was applied to a naïve shaved site on the left flank of each animal. The patches were covered with an occlusive dressing and held in place for 24 hours. The challenge patches were removed, the sites swabbed to remove residual material, and the animals were examined for erythema as indication of a sensitisation response 24 and 48 hours after removal of the dressings.

Tribasic copper sulphate caused blue staining of the skin after topical induction. No skin reaction was recorded in the control or test animals 24 hours or 48 hours after challenge with the 25% or 10% w/w concentrations in distilled water.

Tribasic copper sulphate did not induce skin sensitisation in the maximisation test.

4.6.1.2 Human information

The few cases of skin sensitisation from exposure to copper or its compounds reported in the literature are restricted to clinical case reports involving small numbers of patients, and in evaluation of a case-series of patients from dermatology clinics.

Allergic dermatitis with positive patch tests

Barranco (1972) reviewed the literature and noted that only six cases of allergic contact dermatitis to copper have been reported by then – 3 cases occurred as a result of contact with brass (copper and zinc alloy). The other cases were due in each case to $CuSO_4$, copper metal, and copper in jewellery respectively. To evaluate the prevalence of skin sensitisation to a range of metals

encountered in the ceramics industry, Motolese and co-workers (1993) assessed 190 enamellers and decorators by patch tests. While the patch tests showed several cases positive to other metals, there was only a single case of a positive patch test to red copper oxide in the group.

Sterry and Schmoll (1985) described contact urticaria with a positive patch test in a patient exposed to copper (II)-acetyl acetonate used in self-adhesive disinfection pads applied to the skin.

Cross-reactivity

Metal objects such as spectacle frames have caused dermatitis (Gaul, 1958), but the role of copper in these cases is uncertain, as there is often concomitant exposure to other known sensitisers such as nickel compounds. Cross-reactivity between copper and other metal sensitisers have been documented. Hackel and co-workers (1991) described a patient with palladium sensitisation who also reacted positively to patch tests using nickel sulphate and $CuSO_4$ (1% petrolatum preparation). Nordlind (1992) showed cross reactivity between $CuSO_4$ and mercuric chloride in patients with oral lesions associated with mercury amalgam restorations.

Skin reactions following use of copper IUD

Barkoff (1976) reported a case of a woman who developed urticaria a month after insertion of a copper-based intra-uterine contraceptive device (IUD). Skin patch tests using 1% $CuSO_4$ solution were negative, but scratch tests using the same test material resulted in an erythematous flare reaction.

Romaguera and Grimalt (1981) described four women who developed papulo-erythematous skin lesions between 1 and 4 months after insertion of a copper-containing IUD. Patch tests were positive for 2% $CuSO_4$ in all four cases, although one of the patients also tested positive to nickel sulphate. All four patients improved after removal of the IUDs and provision of topical treatment.

The first report of IUD-induced copper sensitisation was by Barranco (1972) who obtained a positive patch test with 5% $CuSO_4$ solution. Other subsequent similar reports include those by Frenz and Teilum (1980) and Rongioletti *et al* (1985) who demonstrated a positive patch test reaction to 1% $CuSO_4$ in water in a housewife with a 2-month history of dermatitis, and a copper-containing IUD inserted a few weeks before the onset of symptoms. Removal of the IUD resulted in abatement of the symptoms. Pujol *et al* (1998) reported a case of a woman with a 2-year history of recurrent non-pruritic skin eruption and abdominal pain. It was reported that the woman had had a copper-containing IUD “placed 12 years earlier”. Whilst not clearly stated, this suggests that the same IUD remained in place for the whole period and therefore represents misuse. Patch tests were positive for $CuSO_4$ (2%) and for nickel and cobalt salts. Symptoms resolved after removal of the IUD. The authors suggest the copper-containing IUD as a cause for the dermatitis. However, it is possible that the other substances to which the patient reacted with a positive patch test may be the causative factor.

In an assessment of 37 female patients with side-effects following usage of a copper impregnated IUD, Joupilla *et al* (1979) showed that skin tests to copper were negative despite a history of skin rashes experienced by ten of the patients after insertion of the IUD. Allergy to copper was therefore not thought responsible for the skin and other side effects.

Prevalence of allergic dermatitis from copper salts

To establish the prevalence of irritant and allergic contact dermatitis from pesticides, Lisi *et al* (1987) patch tested 652 outpatients with pre-existing skin disorders. 564 subjects were tested with 1% $CuSO_4$, of which 4 cases (<1%) demonstrated an allergic reaction, with none of the cases deemed to have an irritant reaction to $CuSO_4$. The inclusion of 2% $CuSO_4$ in a routine patch test

series assessing 1190 eczema patients over a three-year period showed a positive reaction to $CuSO_4$ in only 13 patients. Copper salts are not common as skin sensitiers (Karlberg, 1983).

Studies with patch testing of copper as sulphate and as metal revealed only one case in 2660 of an independent allergy to copper (in a subject who worked with copper metal), although a small number of subjects that were sensitised to nickel also showed sensitivity to copper. Copper is not regarded as an allergen (Wöhrl *et al.*, 2001)

These findings indicate the relative rarity of copper compounds in comparison to other metals as a cause of allergic contact dermatitis.

4.6.1.3 Summary and discussion of skin sensitisation

A maximisation test has been performed with guinea-pigs on tribasic copper sulphate. No positive response was observed in tested and control animals in the test (0/10 and 0/20 animals). Moreover, cases of allergy to copper are extremely rare in humans, and copper is not considered a sensitizer.

4.6.1.4 Comparison with criteria

In accordance with the CLP regulation (2^{nd} ATP criteria have been considered), positive results were observed in less than 30% of the test animals, as no positive response was observed in tested and control animals (0/10 and 0/20 animals). Therefore, tribasic copper sulphate will not be classified in category 1 “skin sensitiser”.

4.6.1.5 Conclusions on classification and labelling

Tribasic copper sulphate is not a skin sensitizer to guinea-pig in the maximisation test and therefore no classification is warranted.

RAC evaluation of skin sensitisation

Summary of the Dossier submitter’s proposal

One guinea pig maximisation test (GPMT), conducted with tetracopper hexahydroxide sulphate according to OECD TG 406, is included in the CLH report (Sanders, 2002e). Intradermal and topical induction doses were 0.1% (w/w) and 75% (w/w) at days 1 and 7, respectively. Animals were challenged with 25% (w/w) and 10% (w/w) at day 21 after initiation. No reactions were seen in any of the tested (n=20) or control (n=10) animals.
A few clinical cases of allergic dermatitis upon copper exposure and skin reactions following use of copper-based intrauterine contraceptive devices have been reported, but overall the findings indicate that in comparison with other metals, copper was relatively rarely a cause of allergic contact dermatitis. The dossier submitter concluded, based on the negative GPMT and the rare cases of allergic reactions to copper compounds in humans, that no classification for skin sensitisation for tetracopper hexahydroxide sulphate is warranted.

Comments received during public consultation

No comments were received during the public consultation.

Assessment and comparison with the classification criteria

Given the absence of skin reactions in the available skin sensitisation study, and the few individual cases of allergic reactions in humans, RAC agrees with the conclusion of the dossier submitter that tetracopper hexahydroxide sulphate should not be classified for

skin sensitisation.

4.6.2 Respiratory sensitisation

No data available

Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)A metabolism/bioequivalence study has been performed to demonstrate that the ion, as present in the form of copper sulphate, is similarly or more bioavailable to the other forms of copper following oral administration. Data from studies with the sulphate, and other forms that liberate the copper ion, may be used in the assessment process.

Note: the terms copper sulphate, cupric sulphate, copper sulphate pentahydrate and cupric sulphate pentahydrate have been used by various authors in studies quoted. These terms all refer to the same substance, $CuSO_4.5H_2O$, properly known as cupric sulphate pentahydrate, but more typically called copper sulphate.

Table 17: Summary table of relevant repeated dose toxicity studies

Method	Results	Remarks	Reference
Oral			
Rat Fisher 344/N 5/sex/dose/species Copper sulphate pentahydrate Drinking water 15 days 0, 300, 1000, 3000, 10000 ppm Correspond to 0, 10, 29, 45, 36 mg Cu/kg bw/d in males rats and 0, 10, 26, 31, 31 mg Cu/kg bw/d in females rats	10000 ppm: all rats died or were killed moribund. Clinical signs included ruffled fur, emaciation, abnormal posturing, hypoactivity, dyspnoea, tremors and prostration. 3000 ppm: Significant ↓ mean bw gains. ↓water consumption (poor palatability of the solution). 300 and 1000 ppm: ↑ size and number of protein droplets in epithelial cells of the proximal convoluted tubules of the kidney of males. LOAEL of 300 ppm (equivalent to 10 mg Cu/kg bw/d)	No guideline GLP Deviation: 15d instead of 28 days Purity: 99-100%	Hébert, C.D., Elwell, M.R., Travlos, G.S., Fitz, C.J. and Bucher, J.R. (1993)
Mice B6C3F1 5/sex/dose/species Copper sulphate pentahydrate Drinking water 15 days 0, 300, 1000, 3000, 10000 ppm Correspond to 10, 24, 58 and 133 mg copper/kg bw/d in males mice and 15, 36, 6 and174 mg copper/kg bw/d in females mice	> 3000 ppm: mortality, significant ↓ mean bw gains. ↓water consumption Microspcopic cellular depletion in several tissues. NOAEL: 1000 ppm (equivalent to 24 or 36 mg Cu/kg bw/d for males and females)	No guideline GLP Deviation: 15d instead of 28 days Purity: 99-100%	Hébert, C.D., Elwell, M.R., Travlos, G.S., Fitz, C.J. and Bucher, J.R. (1993)
Rat Fisher 344/N 5/sex/dose/species	≥ 2000ppm: Chronic inflammation of the liver.Hyperplasia and hyperkeratosis of the squamous mucosa on the limiting ridge separating	No guideline GLP Deviations: 15	Hébert, C.D., Elwell, M.R., Travlos, G.S., Fitz, C.J. and

Copper sulphate pentahydrate Feeding studies 15 days 0, 1000, 2000, 4000, 8000, 16000 ppm Correspond to 23, 44, 162, 196, 285 mg Cu/kg bw/d in males mice and 23,46, 92, 198, 324 mg Cu/kg bw/d in females rats	the forestomach from the glandular stomach. Depletion of haematopoetic cells in bone marrow occurred. A minimal to mild decrease in erythroid haematopoesis was seen in the spleens.There was an increase in the number and size of protein droplets in the cytoplasm and lumen of the renal cortical tubules in the male and female rats similar to that seen in the drinking water studies. NOAEL of 1000 ppm (equivalent to 23 mg Cu/kg bw/d)	days instead of 28 days. Purity: 99-100%	Bucher, J.R. (1993)
Mice B6C3F1 5/sex/dose/species Copper sulphate pentahydrate Feeding studies 15 days 0, 1000, 2000, 4000, 8000, 16000 ppm in diet Correspond to 0, 43, 92, 197, 294, 717 mg Cu/kg bw/d in males mice and 0, 53, 104, 216, 398, 781 mg Cu/kg bw/d in females mice	16000ppm: ↓ significantly bw gains in female. ↓ mean food consumption. ≥ 2000ppm: minimal hyperplasia and hyperkeratosis of the squamous mucosa on the limiting ridge separating the forestomach from the glandular stomachs. NOAEL of 1000 ppm (43 and 53 mg Cu/kg bw/d in male and female, respectively)	No guideline GLP Deviations: 15 days instead of 28 days. Purity: 99-100%	Hébert, C.D., Elwell, M.R., Travlos, G.S., Fitz, C.J. and Bucher, J.R. (1993)
Rat Fisher 344/N 10/ animals sex/dose Copper sulphate pentahydrate Feeding studies 90 days 0, 500, 1000, 2000, 4000, 8000 ppm in diet Corresponds to 8, 16, 32, 66, 140 mg Cu/kg bw/d in male and 9, 17, 34, 68, 134 mg Cu/kg bw/d in female rats	≥4000ppm: ↓bw gain; Haematological changes. Hyperplasia and hyperkeratosis in the forestomac mucosa, probably as a result of irritant effects of the compound. ≥ 2000ppm: histological changes in the liver and kidney were recorded. NOAEL of 1000 ppm in rat (16 or 17 mg Cu/kg bw/d for males and females)	No guideline GLP Purity: 99-100%	Hébert, C.D., Elwell, M.R., Travlos, G.S., Fitz, C.J. and Bucher, J.R. (1993)
Mice B6C3F1 10/ animals sex/dose Copper sulphate pentahydrate Feeding studies 90 days 0, 1000, 2000, 4000, 8000 and 16000 ppm in diet Corresponds to 44, 97.2, 187.3, 397.8, 814.7 mg Cu/kg bw/d in male and 52.2, 125.7, 266.7, 536 and 1058 mg Cu/kg bw/d in female mice	≥4000ppm: ↓bw gain; Hyperplasia and hyperkeratosis in the forestomac mucosa, probably as a result of irritant effects of the compound. NOAEL of 2000 ppm (97.2 mg Cu/kg bw/din male and 125.7 mg cu/kg bw/d in female) in mouse	No guideline GLP Purity: 99-100%	Hébert, C.D., Elwell, M.R., Travlos, G.S., Fitz, C.J. and Bucher, J.R. (1993)
Rats	The treatment was associated with reduced bodyweight gains and toxicity	No guideline	Haywood, S (1980a)

4 males per group (9 groups) Copper sulphate Feeding study 1, 2, 3, 6, 9 or 15 weeks 2000 mg/kg diet Correspond to 165 mg Cu/kg bw/day	to the liver and kidneys. Toxicity (including hyperplasia and cellular damage) was marked at 6 weeks of dietary administration, but by 15 weeks, animals had shown almost total adaptation and recovery at the cellular level. NOAEL < 165 mg Cu/kg/d	No GLP	
Rats Wistar 4 males/group Copper sulphate Feeding study Exposure during 2, 3, 4, 5, 6 or 15 weeks 0, 3000, 4000, 5000 or 6000 ppm Correspond to 150, 200, 250 or 300 mg Cu/kg bw/day)	Toxicity after 6 weeks followed by regeneration of the liver up to 5000 ppm. 6000 ppm resulted in unsustainable liver damage and death by six weeks.	No guideline No GLP	Haywood, S. (1985)
Rats 4 males/group Copper sulphate Feeding study Killed at intervals of 1, 2, 3, 6, 9 and 15 weeks 0 or 2000 ppm Cu Correspond to 200 mg/kg bw/day in the young rat, or 100 mg/kg bw/day in the older rat	Dietary copper, administered at high levels to weanling rats was associated with increased blood and plasma copper concentrations after six weeks (with an initial transient rise in plasma concentration in the first week) to reach a maximum at nine weeks. Similarly, ceruloplasmin activity increased significantly at six weeks. Alanine aminotransferase activity rose gradually from the first week to reach a maximum at nine weeks. Alkaline phosphatase activity and bilirubin concentration showed no change. The changes in enzyme activity and ceruloplasmin levels coincide with liver toxicity seen at higher levels in subsequent studies, and may reflect increased competence to manage high levels of copper following the initial insult.	No guideline stated No GLP	Haywood, S. and Comerford, B. (1980b)
Inhalation			
Guinea pigs 6 male/group exposed daily for 5 minutes aerosols Inhalation 0.4% aqueous solutions of either copper oxychloride (containing 50% copper) or copper oxychloride (containing 37.5% copper) plus zineb (16%). Animals killed after 60, 120, 200, 270 and 420 periods of exposure	After 70 days of exposure animals showed copper inclusions within swollen Kupffer cells and histiocytes in the portal tracts and subcapsular areas. In three animals killed after 270 days of exposure, a close association was noted between the lesion reported and perisinusoidal and portal fibrosis. The exposure of limited numbers of animals to copper formulations indicates that animals show similar lesions to humans.	No guideline No GLP	Pimentel (1969)

Rat Sprague Dawley 10/ animals sex/dose for low, med-low and med-high dose and 20/ animals sex/dose for control and high dose Cuprous oxide Dust aerosol Whole-body inhalation exposure as a 6-hour/day exposure 0, 0.2, 0.4, 0.8 and 2 mg/m^3 for 1, 2, 3, or 4 weeks 13-week recovery period	Following a 13-week recovery period at 2 mg/m^3, there were no test substance related effects on hematology parameters, BALF parameters, or lung, lymph node or nasal histopathology. The effects on lung weights were greatly reduced, but still slightly detectable following the recovery period. But there were no microscopic findings or changes in BALF parameters that correlated with the higher lung weights at the recovery necropsy.	OECD 412 GLP	Kirkpatrick, 2010
Dermal			
Rabbit 5/sex/groups 3 weeks exposure Copper hydroxide 1000 or 2000 mg/kg/day of the formulation Correspond to 500 or 1000 mg Cu/kg bw/day)	2000 mg/kg: 3 deaths not related to treatment. Body weight loss. Increased incidence of dermal necropsy findings. The skin of treated animals was discoloured blue by test material. NOAEL = 1000 mg/kg bw/d (=500mg Cu/kg bw/d)	OECD 410 No GLP Purity not stated	Painter O.E. (1965)

4.6.3 Non-human information

4.6.3.1 Repeated dose toxicity: oral

Reference: Hébert, C.D, (1993)
Guideline: No
GLP: Yes
Several studies were realised:

- Studies on rats and mice by drinking exposure during 15 days,
- Studies on rats and mice by diet exposure during 15 days,
- Studies on rats and mice by diet exposure during 92 days.

Duration of treatment: 15 days
Deviations:

- Study duration is less than recommended,
- no haematology or clinical chemistry investigations,
- adrenals and spleen are not weighted at necropsy.

These deficiencies do not, however, necessarily compromise the validity of the data generated.

Five males and five females Fischer 344/N rats and 5 males and 5 females B6C3F1 mice were exposed to copper sulphate pentahydrate at concentrations of 0, 300, 1000, 3000, 10000 ppm in the drinking water.
Five others males and females Fischer 344/N rats and 5 males and females B6C3F1 mice were exposed to copper sulphate pentahydrate at concentrations of 0, 1000, 2000, 4000, 8000 and 16000 ppm in the diet.

During the studies, clinical observation and mortality were reported. At termination all animals were given a full macroscopic examination and body and organ weights (liver, thymus, right kidney, right testis, heart, lungs, brain) were determined. Histopathological examination was performed on control animals (plant diet or untreated drinking water), any unscheduled kill animals, all animals in the highest dose group with 60% survival rate and all animals in higher dose groups. Target organs (liver, kidney, forestomach) were examined to a no-effect level in lower exposure groups.

Drinking water studies results:

Table 18: Body weight, water and compound consumption in rats

	Dose level (ppm)				
	0	**300**	**1000**	**3000**	**10000**
Male					
Final body weight (g)	169	171	174	88**	-
Water consumed (g/day)	17.9	17.3	14.7	4.8	1.0
Calculated compound consumption (mg/kg/day)	0	41	113	175	140
Female					
Final body weight (g)	139	141	131	75**	-
Water consumed (g/day)	16.3	15.3	11.3	3.2	0.9
Calculated compound consumption (mg/kg/day)	0	39	102	121	120

** P < 0.01.

Table 19: Body weight, water and compound consumption in mice

	Dose level (ppm)				
	0	**300**	**1000**	**3000**	**10000**
Male					
Final body weight (g)	27.2	27.7	26.5	21.1**	-
Water consumed (g/day)	4.8	3.6	2.4	1.6	1.1
Calculated compound consumption (mg/kg/day)	0	41	95	226	524
Female					
Final body weight (g)	22.2	21.5	21.1	14.6**	-
Water consumed (g/day)	5.6	4.1	2.8	1.3	1.1
Calculated compound consumption (mg/kg/day)	0	58	140	245	683

** P < 0.01

Clinical signs of both rats and mice in the highest two groups included ruffled fur, emaciation, abnormal posturing, hypoactivity, dyspnoea, tremors and prostration. Animals from the two highest groups also showed a decreased water consumption, which was attributed to poor palatability of the cupric sulphate solution.Final mean body weight gains for surviving animals of both species from the 3,000 ppm groups were significantly reduced.

All rats and all mice in the 10,000 ppm groups and one female rat, one male mouse and three female mice in the 3,000 ppm groups died or were killed moribund during the study.

Any changes in absolute organ and relative organ weights were attributed to the lower body weights of animals receiving 3,000 ppm, rather than a direct toxic effect of treatment. Microscopic lesions in rats were limited to an increase in the size and number of protein droplets in epithelial cells of the proximal convoluted tubules of the kidney of males in the 300 and 1,000 ppm groups. No kidney lesions were observed in female rats or in mice of either sex. The only microscopic lesion in mice was cellular depletion, present in numerous tissues in mice from the two highest dose groups and which was attributed to the marked decrease in water consumption and body weight gain in these groups.

Concentrations of cupric sulphate above 3,000 ppm were lethal to rats and mice within two weeks. Slight kidney changes were observed in male rats at 300 and 1,000 ppm but female rats and mice of both sexes were not affected.

Feeding studies results:

Table 20: Body weight, food and compound consumption in rats

	Dose level (ppm)					
	0	**1000**	**2000**	**4000**	**8000**	**16000**
Male						
Final body weight (g)	184	186	183	178	151**	122**
Food consumed (g/day)	14.6	15.2	14.7	14.4	13.3	9.2
Calculated compound consumption (mg/kg/day)	0	92	180	363	777	1275
Female						
Final body weight (g)	138	139	138	136	128*	106*
Food consumed (g/day)	11.4	11.6	11.2	11.7	11.7	7.1
Calculated compound consumption (mg/kg/day)	0	89	174	637	769	1121

* $P < 0.05$.
** $P < 0.01$

Table 21: Body weight, food and compound consumption in mice

	Dose level (ppm)					
	0	**1000**	**2000**	**4000**	**8000**	**16000**
Male						
Final body weight (g)	25.1	25.1	25.4	24.6	23.6	23.6
Food consumed (g/day)	4.4	4.1	4.5	4.7	3.3	4.0
Calculated compound consumption (mg/kg/day)	0	168	362	773	1154	2817
Female						
Final body weight (g)	21.2	21.4	20.2	20.8	20.2	20.0*
Food consumed (g/day)	4.1	4.3	4.0	4.3	3.8	3.7
Calculated compound consumption	0	210	408	849	1563	3068

(mg/kg/day)						

* P < 0.05.

No animals died or were killed during the study. Final mean body weights gains of male and female rats of 8,000 and 16,000 ppm groups and of female mice receiving 16,000 ppm were significantly lower than the controls. These decreases were attributed to decreased feed consumption in animals, considered to be due to the poor palatability of the feed mixture rather than to specific cupric sulphate toxicity.

Changes in organ weights and organ to body weight ratios were sporadic and were considered to be related to decreased body weights rather than to toxicity of the cupric sulphate.

Microscopic findings in rats at 2,000 ppm and above included hyperplasia and hyperkeratosis of the squamous mucosa on the limiting ridge separating the forestomach from the glandular stomach. A similar finding was observed in mice but the severity was minimal. This was considered due to the irritant effects of cupric sulphate and the authors noted that there were no adverse effects on the health of the animals. Additionally in rats, chronic active inflammation of the liver characterised as minimal to mild mononuclear inflammatory cell infiltrate was observed in males at 8,000 ppm (4/5) and 16,000 ppm (5/5) and in females at 16,000 ppm (3/5). Depletion of haematopoetic cells in bone marrow occurred in male and female rats in the 8,000 and 16,000 ppm groups, consisting of a decreased cellularity of bone marrow erythroid/myeloid elements and an increase in the prominence of fat cells normally present in the bone shaft. In several high dose animals bone mass (cortex and trabecular density) was reduced when compared to controls. This was considered a consequence of reduced body weight gain rather directly related to treatment. A minimal to mild decrease in erythroid haematopoesis was seen in the spleens of rats in the 16,000 ppm group. There was an increase in the number and size of protein droplets in the cytoplasm and lumen of the renal cortical tubules in the male and female rats of the three highest dose groups, similar to that seen in the drinking water studies.

Microscopic findings were more severe in rats than in mice and at levels of 2,000 ppm and above included hyperplasia and hyperkeratosis of the squamous mucosa of the limiting ridge of the stomach. This finding was minimal in mice, and may have been associated with the sulphate ion, rather than copper. Administration at 4,000 ppm and above was associated with inflammation of the liver, changes in the kidney similar to the drinking water study and changes in bone marrow cells.

Duration of treatment: 92 days:
Deviations:

- No ophthalmoscopy was performed,
- adrenals were not weighted at necropsy.

Copper sulphate pentahydrate was administered in the diet to groups of 10 male and 10 female Fischer 344/N rats at dietary levels of 0, 500, 1,000, 2,000, 4,000 and 8,000 ppm for 92 days. Also groups of 10 male and 10 female B6C3F1 mice received treated diet at levels of 0, 1,000, 2,000, 4,000, 8,000 and 16,000 ppm for 92 days. Chemical analyses of the formulations showed that they were within ±10% of theoretical concentrations.

Clinical signs and mortality were reported but schedule for observations were not indicated.

Bodyweights and organ weights (liver, thymus, right kidney, right testis, heart, lungs and brain) were determined at the termination of the study for all rats and mice.

Haematology and clinical chemistry evaluations (haematocrit, haemoglobin concentration, mean cell volume, platelets, erythrocyte count, total and differential leukocyte count, reticuloocyte count, blood urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, 5'-nucleotidase, bile salts) were performed on Days 5 and 21 on supplemental rats (10 animals/sex/per group) and on the main study rats on Day 92 (termination).

Urinalysis (clarity, colour, volume, specific gravity, creatinine, glucose, total protein, aspartate aminotransferase (AST), N-acetyl-3-glucosaminidase (NAG)) was performed on Day 19 on supplemental rats (10 animals/sex/per group) and on the main study rats on Day 92.

Gross necropsy was performed on all animals.

Histopathology was performed on decedents, all control animals, on animals from the highest dose group with 60% survival rate and on any higher dose group animals. Target organs (liver, kidney and forestomach) were examined to a no-effect level in the lower exposure groups.

No mortality was reported. There were no clinical signs observed that could be directly attributed to treatment among rats and mice.

Food consumption was generally similar to the controls in all groups in both rats and mice except for the highest dose group in the rat (8,000 ppm).

Final mean body weight gains were significantly reduced in male rats in the two highest dose groups (4,000 and 8,000 ppm) and in female rats in the highest dose group (8,000 ppm).

Treated mice showed a dose-related reduction in body weight gain that occurred earlier than in the rat and was more severe at the higher dose levels.

Haematology showed significant changes in rats of both sexes at all time points but generally limited to the 2,000, 4,000 and 8,000 ppm dose groups. Initially (day 5), significant increases in haematocrit (HCT), haemoglobin (HGB), platelet count and erythrocytes (RBC) were seen in the 8,000 ppm group which were consistent with polycythemia related to dehydration. Also on day 5, significant decreases in reticulocyte count, mean cell volume (MCV), and mean cell haemoglobin (MCH) were noted in high-dose animals. By Day 21, HCT and HGB levels were significantly decreased for male rats in the 2 highest dose groups and female rats in the 3 highest dose groups together with MCV and MCH and these persisted until the end of the study. Significant increases in RBC and reticulocytes were noted in high dose males at the end of the study.

Clinical chemistry showed significant elevations of alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) activities throughout the study, indicating hepatic injury. Decrease in alkaline phosphatise (AP) activity were noted on days 5 and 21 in both sexes in the two highest dose groups, but AP activity had returned to control levels by day 92. Total protein and albumin concentrations were significantly decreased and urea nitrogen increased in the two highest dose groups at all time points. Variations occurred in other parameters with reversal of trends at differing time points.

Significant changes in urinalysis parameters included an increase in aspartate aminotransferase (AST) and N-acetyl-β-D-glucosaminidase (NAG) and 5'-nucleotidase (5'NT, males only) activities in the two highest dose groups.

Generally, absolute organ weights of both species were reduced in the two highest dose groups when compared with the controls and the relative organ weights were similar or increased with decreasing body weight. It was considered that the changes could be attributed to the lower final mean body weight in the higher dose groups.

Gross and histopathology observations showed (tables 22 and 23):

- For forestomach:

Gross lesion in rats was characterized by an enlargement of the limiting ridge in all animals in the 4000 and 8000 ppm groups and in 7 females and 9 males in the 2000 ppm group.

In mice, the limiting ridge had focal white discoloration of the squamous mucosa where it forms a junction with the glandular gastric mucosa.

Histopathological findings included dose-related minimal to moderate hyperplasia with hyperkeratosis of the squamous mucosa of the forestomach from 2000 ppm, at the site of the limiting ridge. Severe incidences of this lesion were often accompanied by an increase in the number of inflammatory cells and/or oedema in the lamina propria of the limiting ridge. Rats were more severely affected than mice at similar dose levels. The difference between the species may be associated with the lower stomach pH (less acidic) in the rat. It may be anticipated that the hydrochloric acid in the stomach may react with copper sulphate to produce copper chloride and sulphuric acid. This acid may have caused the irritation, and the rat stomach, being adapted to a less-acidic environment, showed more effects than the mouse.

- For liver:

There was a dose-related increase in the incidence and severity of chronic inflammation in the livers of rats, characterised by multiple foci of a mixture of mononuclear inflammatory cells. Staining of the livers for the presence of copper showed a presence in the 4,000 and 8,000 ppm groups. At 8,000 ppm staining had a clear periportal to midzonal distribution and consisted of a few to numerous red granules of 1 to 2 mm in the cytoplasm of hepatocytes. At 4,000 ppm staining was periportal and there was a marked reduction in the number of cells stained and in the number of granules per cell. Positive minimal staining of livers for copper was evident in the high dose male and female mice and consisted of only a few positive-staining hepatocytes in the entire liver section.

- For kidneys:

Changes in the kidneys included an increase in the size and number of cytoplasmic protein droplets present in the epithelium of proximal convoluted tubules of rats at doses of 2,000 ppm and higher and was less severe in females than in males. Many of the protein droplets in the male rats had large irregular crystalline shapes, which were not present in the females. Minimal nuclear enlargement (karyomegaly) in renal tubule cells was present in the high dose group. Degeneration of renal tubule epithelium was present in three females from the 8,000 ppm group. Positive staining for copper was seen in the kidneys at 4,000 ppm, and to a greater extent, in the 8,000 ppm groups and consisted of red granules in the cytoplasm of the renal tubule epithelium and a diffuse red staining of the protein droplets in the cytoplasm and the tubule lumen. There was no staining for copper in the kidneys of any mice.

In conclusion, administration of copper sulphate pentahydrate to rats and mice for 92 days via the

diet produced hyperplasia and hyperkeratosis in the forestomach mucosa, although this may be associated with the sulphate ion, rather than copper.

The NOAEL for this lesion was 1,000 ppm for rats and 2,000 ppm for mice.

In rats damage to the liver was produced with a NOAEL of 1000 ppm for males and 2000 ppm for females.

In rats damage to the kidney was produced with a NOAEL of 1000 ppm for both sexes.

A NOAEL for mice could not be derived for liver and kidney toxicity as lesions were not seen in these organs even at the highest concentration.

Sperm morphology and vaginal cytology were also realised. There were no changes in testis, epididymis or cauda epididymis weight, or spermatid counts or sperm motility in males of either species at any dose level. Similarly, there were no changes in oestrous cycle length or in the timings in each phase of the cycle in females of either species.

Table 22: Rats - histopathological findings - incidence and severity

	Incidence and mean severity ( ) at dose level (ppm)					
	0	**500**	**1000**	**2000**	**4000**	**8000**
Male						
Forestomach, hyperplasia and hyperkeratosis	0	-	-	10 (1.6)	10 (2.8)	10 (2.8)
Liver, inflammation	0	-	0	1 (1.0)	10 (1.0)	10 (1.9)
Kidney, droplets	0	-	0	3 (1.0)	10 (2.0)	10 (2.5)
Kidney, karyomegaly	0	-	0	0	0	10 (1.0)
Female						
Forestomach, hyperplasia and hyperkeratosis	0	-	-	7 (1.3)	10 (2.5)	10 (2.5)
Liver, inflammation	0	-	0	0	6 (1.2)	10 (1.9)
Kidney, droplets	0	-	1 (1.0)	9 (1.0)	10 (1.0)	10 (1.0)
Kidney, karyomegaly	0	-	0	0	0	10 (1.1)
Kidney, degeneration	0	-	0	0	0	3 (1.3)

Mean severity (in brackets) based on number of animals with lesions 1, minimal; 2, mild; 3, moderate; 4, marked

Table 23: Mice -histopathological findings - incidence and severity

	Incidence and mean severity ( ) at dose level (ppm)					
	0	**1000**	**2000**	**4000**	**8000**	**16000**
Male						
Forestomach, hyperplasia and hyperkeratosis	0	-	0	2 (1.0)	6 (1.0)	10 (1.6)
Female						
Forestomach, hyperplasia and hyperkeratosis	0	-	0	5 (1.0)	8 (1.0)	10 (1.7)

[a] mean severity (in brackets) based on number of animals with lesions 1, minimal; 2, mild; 3, moderate; 4, marked

Reference: Haywood, S. (1980a)
Guideline: No
GLP: No

Male weanling rats of uniform age and weight were allocated to nine groups of four animals. Groups 1 to 6 were fed powdered laboratory diet (Spillers expanded) to which 2000 mg/kg diet as $CuSO_4$ (equivalent to 165 mg Cu/kg/d) had been added, for up to 15 weeks. Groups 7 to 9 received unsupplemented diet and served as controls.
Rats on the copper supplemented diets from groups 1 to 6 were killed in weeks 1, 2, 3, 6, 9 and 15 respectively.
Two control animals were killed at the same time.
Animals were exsanguinated under ether anaesthesia and liver and kidneys were dissected free and weighed. Slices of the liver and kidney were preserved for histological examination; other parts were frozen (-70^{o}C) and triplicate samples analysed for copper content following acid digestion using atomic absorption spectrophotometry.

There were no deaths.

Animals receiving copper showed reduced body weight gain, and reduced liver weight. The liver to body organ weight ratio was similar in all groups.

Macroscopic liver changes were recorded from week 6, when clearly defined peripheral areas of necrosis were recorded in the right and median lobes. By week 9, pale areas were still visible but not so clearly defined, and by week 15 the livers were apparently normal, except for fine scarring on the lobular surface.

Histological changes were noted from week 2, with hypertrophy of the periportal parenchymal cells. Copper was present in the outer zones of lobules in sections stained with rubeanic acid.
In week 3, inflammatory foci were present, restricted to the periportal zone. Lesions consisted of aggregates of hypertrophied hyperchromatic parenchymal cells, some of which showed signs of necrosis. There was marked deposition of copper in outer zones of the lobules, pericanalular in distribution.
By week 6 there were marked changes in the livers of all animals, although there was considerable individual variation. The changes were always more severe in the right and median lobes. Necrosis was widespread, with marked cellular inflammatory reaction consisting of polymorpho-nuclear neutrophil leukocytes and mononuclear cells. There was extensive copper in the cells, considered to be lysosome-bound copper. There was also bile duct hyperplasia and some attempted regeneration of still-viable cells.
By week 9 there was extensive regeneration of parenchymal tissue, and individual cells were normal in size, with plentiful glycogen. Necrosis was limited to a cuff of cells in the periportal zone

and the cellular response had subsided but was still present. Copper had largely disappeared from the rubeanic acid-stained sections.
By week 15, all livers showed advanced healing, although there was still architectural distortion of the right and median lobes. Bile duct hyperplasia was still present, and necrotic remnants consisting of eosinophilic (hyaline) bodies occasionally with nuclear material, were present in portal areas.

In the kidney, macroscopic changes were limited to greenish discolouration in some animals at week 6.

Histological changes were noted from week 3, when small eosinophilic droplets were present in the cytoplasm of the proximal convoluted tubules. Extrusion of the droplet-containing cells into the lumen of the tubule was common. Copper was not detected in the rubeanic acid stained sections. By week 6 there were marked changes in the proximal convoluted tubule, although there was considerable individual variation in degree. The cytoplasmic droplets were larger, more numerous and assumed the appearance of green globules. Rubeanic acid staining revealed copper in particulate form, and in the droplets visible in the H & E stained sections. In some kidneys there was extensive desquamation of the epithelial cells of the proximal convoluted tubule, with the lumen frequently obliterated by debris. Regeneration was also evident among surviving cells, with mitosis common. The remainder of the nephron was unaffected.
By week 9 the regeneration was mostly completed, with copper still present in rubeanic acid stained sections.
At week 15, regeneration was complete, with little particulate copper in the rubeanic acid stained sections.

Analysis of copper content in both liver and kidney matched the rubeanic acid staining; both rose to maximum values in week 6, after which levels fell.

Dietary administration of copper as sulphate was associated with histological changes in the liver and kidney, reaching a maximum after six weeks of treatment, followed by recovery to week 15. Initially copper accumulated with little effect, but from 2-3 weeks, histological changes were evident in both tissues. Accumulation eventually caused a crisis, associated with severe necrosis, followed by regeneration and recovery.

Reference: Haywood, S. (1985)
Guideline: No
GLP: No

Male weanling Wistar rats were caged in fours and allocated to groups receiving 0, 3000, 4000, 5000 or 6000 ppm copper as copper sulphate for up to 15 weeks. All rats were fed a standard laboratory diet (Labsure Animal Diet, RHM Agriculture South Ltd., with a copper content of 10 ppm). Animals were regularly inspected and weighed. In each dietary group, a cage of four rats was killed after 2, 3, 4, 5, 6 and 15 weeks of dietary administration. At necropsy, the liver and kidneys were removed for histological examination. Kidney and parts of the right median liver lobe were preserved for histological examination (H & E or Gomoris reticulin stain for general histopathology, and rubeanic acid and rhodanine for copper, and orcin for ‘copper-associated protein); other samples were frozen (-70^{o}C) and triplicate samples analysed for copper content following acid digestion using atomic absorption spectrophotometry

Rats at 3000 ppm showed reduced weight gain, with 'staring' coats between weeks 4 and 5, but by week 15, coats were described as sleek and the animals active, although they weighed less than controls (202 g compared to 438 g for controls).
Rats at 4000 and 5000 ppm showed clinical deterioration between 3 and 4 weeks and subsequent recovery.
Rats at 6000 ppm showed no weight gain. Two animals died in week 2, and by week 6 the remaining animals showed weight loss and deteriorating condition and were sacrificed.

Control mean liver copper concentration was 17.8 μg/g dry weights.
At 3000 ppm, liver copper concentration rose rapidly to 4780 μg/g dry weight between 4 and 5 weeks, but fell significantly to 2412 μg/g dry weight at week 6. By week 15, copper content had fallen further to the same level as at week 2 (approximately 1500 μg/g dry weight).
At 6000 ppm, maximum liver concentrations occurred at week 2 (approximately 3800 μg/g dry weight), and fell only to 2000 μg/g dry weight by week 6, when the animals were terminated.

Renal copper concentration in controls was 34 μg/g dry weights.
Renal copper concentration at 3000 ppm rose more slowly than in the liver, with a maximum of 1188 μg/g dry weight between 4 and 5 weeks which was maintained to 15 weeks.
In the kidney, copper concentration at 6000 ppm continued to rise to week 4, when it equalled the liver value (approximately 2500 μg/g dry weight).

Similar patterns occurred in the liver and kidney at 3000 and 4000 ppm, although the maximum occurred earlier at week 3 (values not stated in paper).

Histological findings in the liver at up to 5000 ppm showed an earlier onset, but were essentially similar to those seen in the earlier study, with hepatic hypertrophy and necrosis, followed by regeneration and recovery.
At 6000 ppm necrotic changes were evident in the first week, increased in severity to weeks 2-3, and resulted in chronic hepatitis at 6 weeks.

Renal histopathology at 3000 ppm was similar to that seen at 2000 ppm in the earlier study. However, at 4000 and 5000 ppm, the findings showed earlier onset and correlated with the earlier liver findings. Findings were more marked, with numerous copper-staining granules and droplets in the cells of the proximal convoluted tubule. Extrusion of droplets and exfoliation of whole cells was common in the distal or collecting tubules, with extensive degeneration in many proximal tubules, with the occlusion of the lumen by copper-containing debris and its passage into the distal tubule. By week 15, regeneration was complete. The author concluded that the kidney has the capacity to excrete copper as well as the liver in cases of copper overload, and excrete high doses of copper via the urine.

Dietary doses of 6000 ppm (approximately equivalent to 300 mg/kg bw/day) of copper produced unsustainable liver damage by 6 weeks of administration. Doses of between 3000 and 5000 ppm (approximately equivalent to 150 and 250 mg/kg bw/day) result in liver and kidney damage after between 2 and 5 weeks, with subsequent full recovery by week 15. Regeneration of both organs takes place at 5000 ppm and below, and the kidney appears to develop the capacity to excrete copper when the liver is overloaded.

Reference: Haywood, S. and Comerford, B. (1980b)
Guideline: No
GLP: No

Copper sulphate was administered in the diet to six groups of four male weanling rats at a level of 2,000 mg copper/kg diet. Three similar groups of rats received unsupplemented diet and served as controls. Animals from group 1 to 6 were killed at intervals of 1, 2, 3, 6, 9 and 15 weeks and 2 control animals were killed at each of these time.
During the necropsy of each animal, a blood sample was withdrawn from the vena cava.
The samples (5-10 mL) were taken into lithium heparin anti-coagulant; 1 mL was retained for copper analysis and plasma obtained from the remainder. Copper content was determined in the whole blood and plasma. Alanine aminotransferase (GPT), ceruloplasmin (plasma copper oxidase), alkaline phosphatase and bilirubin were determined on plasma.

Copper content in blood and plasma: the copper concentration in plasma rose significantly after Week 1 but fell to normal at Week 2. Both the plasma copper and the blood copper concentrations increased significantly at Week 6 and thereafter although a slight fall occurred in Week15.

Plasma enzyme activities and bilirubin concentrations: GPT activity was significantly greater than the control value at Week 1 and thereafter rose to a maximum activity around 6 to 9 weeks which was maintained until Week 15. This early rise in activity coincided with the time of pathological changes in the liver seen at higher dose levels but there was not the subsequent decline to parallel the regeneration of the liver.

Alkaline phosphatase activity did not differ greatly from the control value throughout the trial.
Ceruloplasmin activity was similar to the control value for the first three weeks but was high at Week 6 and thereafter.
Bilirubin concentration was similar to the control throughout the trial
Dietary copper, administered at high levels to weanling rats (2,000 ppm, equivalent to 200 mg/kg bw/day in the young rat, or 100 mg/kg bw/day in the older rat) was associated with increased blood and plasma copper concentrations after six weeks (with an initial transient rise in plasma concentration in the first week) to reach a maximum at nine weeks. Similarly, ceruloplasmin activity increased significantly at six weeks. Alanine aminotransferase activity rose gradually from the first week to reach a maximum at nine weeks. Alkaline phosphatase activity and bilirubin concentration showed no change.

The changes in enzyme activity and ceruloplasmin levels coincide with liver toxicity seen at higher levels in subsequent studies, and may reflect increased competence to manage high levels of copper following the initial insult.

4.6.3.2 Repeated dose toxicity: inhalation

Reference: Pimentel, J.C. (1969)
Guidelines: Not standard
GLP: No

Four groups of six guinea pigs housed in poorly ventilated glass cages were treated, by inhalation, as follows: one group was untreated and served as controls; one group was treated with a finely pulverised Bordeaux mixture (solution of copper sulphate neutralised with hydrated lime). This was done three times a day, such that the atmosphere of the cage was completely saturated with the spray. The second group was similarly treated with a solution of wine tartar using a Flit spray gun and the fourth group treated three times a day with sulphur dioxide fumes produced by burning ‘sulphur wicks’ such as are used for the disinfection of wine vats. The animals were treated daily

for at least 6 months. The guinea pigs were radiographed at the start of the study, at the second month and at the end of the 6 months treatment. Radiographic changes were noted in the animals treated with Bordeaux Mixture. Four of these animals were sacrificed at the end of treatment and two were retained untreated for three months when further radiographs were taken before sacrifice. Histopathological examination of the pulmonary lesions was performed, including staining for copper.
The lungs of the animals exposed to sulphur dioxide showed scanty intra-alveolar cells containing yellow/dark brown granules; staining indicated the presence of sulphur-containing amino acids. The wine tartar spray treated animals showed occasional inter-alveolar cells with a brown/yellow granular pigment that stained for copper. The Bordeaux Mixture-treated animals killed at the end of treatment showed micronodular lesions, characterised by foci involving a variable number of alveoli filled with plugs of desquamated macrophages with inclusions of a substance rich in copper. Additionally, in one guinea pig small histocytic granulomas were seen in the septa with the appearance of fibro-hyaline scars similar to those found in human cases. In the two animals killed three months after exposure an apparently total regression of the lesions was noted on the radiograph. Microscopic examination revealed fibrous bands, small groups of alveoli filled with macrophages, hyaline deposits and small areas of condensation of the reticulin fibres of the septa in regions not involved when using the routine stains.

Reference Kirkpatrick (2010)
Guideline: OECD 412
GLP: Yes
Deviations: None

Cuprous oxide was administered via whole-body inhalation exposure as a 6-hour/day exposure duration to male and female Sprague Dawley Crl:CD(SD) rats for 1, 2, 3, or 4 weeks (5 days/week), test substance-related effects observed at exposure levels of 0.2, 0.4, 0.8 and 2.0 mg/m^3 (particle size of 1.725 µm MMAD +- 1.73 µm GSD).
For the core study, 20 males and 20 females per concentration (control and high) and 10 males and 10 females per concentration (low, med-low and med-high) were used. For the satellite study which evaluate whether a plateau was observed when a time course was conducted for effects following 5, 10 and 15 exposures, 10 males and 10 females per exposure (control and high) and time point (1,2 or 3 weeks) were used.

After 4 weeks of exposure, there was an exposure concentration-related increase in microscopic findings in the lung, and increased lung, bronchial lymph node, and mediastinal lymph node weights. Lung histopathology included alveolar histiocytosis, acute inflammation, and perivascular mononuclear cell infiltrates. At 0.2 mg/m^3, alveolar histiocytosis was minimal, progressing to moderate severity at 0.8 and 2.0 mg/m^3.

Higher blood neutrophil counts were observed following 4 weeks of exposure to cuprous oxide. Inhalation exposure resulted in higher LDH, total protein, and total cell counts, and a higher proportion of neutrophils in the bronchoalveolar lavage fluid of rats following 1, 2, and 3 weeks of exposure (2.0 mg/m^3 group on study days 5, 12, and 19) and following 4 weeks of exposure at the end of exposure evaluation (0.2 mg/m^3 or higher, except 0.4 mg/m^3 or higher for total cell count, at study week 3 after a minimum of 20 exposures as the first week of exposure is study week 0). In the nasal cavity after 4 weeks of exposure, findings considered test substance-related were minimal olfactory epithelium degeneration in a small number of males from the 0.8 and 2.0 mg/m^3 groups and mild subacute inflammation in a small number of males from the 2.0 mg/m^3 group.

Most test substance-related effects at 2.0 mg/m^3 appeared to show a peak in the effect prior to completion of 4 weeks of exposure and therefore, the results were consistent with a possible plateau. Only lung weights and the incidence of lymphoid hyperplasia of the bronchial lymph node in males appeared to continue to increase relative to control through 4 weeks of exposure.

At the lowest exposure level of 0.2 mg/m^3, the inflammatory effects in the alveoli were minimal and present in only 2 of 10 animals. There was no microscopic evidence for alveolar epithelial or endothelial cell injury or the presence of edema at any exposure level.

Following a 13-week recovery period at 2 mg/m^3, there were no test substance related effects on hematology parameters, BALF parameters, or lung, lymph node or nasal histopathology. The effects on lung weights were greatly reduced, but still slightly detectable following the recovery period. But there were no microscopic findings or changes in BALF parameters that correlated with the higher lung weights at the recovery necropsy.

4.6.3.3 Repeated dose toxicity: dermal

Reference: Paynter, O.E. (1965)
Guideline: OECD 410
GLP: No
Deviations: Yes

- Animals were treated five days per week for three weeks, instead of continuously for 28 days, as recommended,
- haematology and histological investigations were performed, but the number of parameters investigated was smaller than the modern guideline,
- the test was performed on a formulation, not on the technical material.

Copper hydroxide (wettable powder formulation KOCIDE101) was applied as a 53% w/v aqueous suspension to the shaved backs of adult albino rabbits. Suspensions of test material were applied at 1000 and 2000 mg/kg bw/day of the formulation which represente 500 and 1000 mg/kg bw/day copper as hydroxide. The control group consisted of 5 males and 5 females and the test groups of 10 males and 10 females. Animals were treated for five days per week for three weeks. Half of the animals by sex in each group were subject to mild abrasion of the skin prior to dosing at the beginning of each week. The dose site was covered with a light gauze bandage. Animals were treated for 6 – 8 hours per day. At the end of each exposure, bandage and collar were removed and the treated area washed lightly with water and wiped dry. All animals were sacrificed three or four days after the last application and necropsied. Sections of liver, kidney and skin were preserved.

There were two deaths in the low dose group and three deaths in the high dose group. None of the deaths in dose groups could be conclusively related to the test material; all deaths were considered to be due to apparent gastroenteritis. There were no indications of irritation.

There was an overall mean bodyweight loss in the high dose group. There were no adverse effects on food consumption.

There were no adverse effects of treatment on haematological or urinalysis parameters.

Necropsy and histopathology revealed degenerative changes in the skin. Five control animals showed minimal findings such as focal leukocyte infiltration of the dermis or focal peri-follicular thickening. There were no histological findings in the skin of low dose animals. Ten high dose

animals (five abraded and five intact skin) showed skin abnormalities at histopathology. Findings included epidermal thickening, focal leukocyte infiltration of the dermis, keratin thickened or distorted, atrophied hair follicles. There were single instances of dermal fibrosis, dermal oedema, eschar formation and slight ulceration.

4.6.3.4 Repeated dose toxicity: other routes

No data available.

4.6.3.5 Human information

O'Donohue, J.W. (1993) reports a case of chronic self-administration. A 26-year-old Irishman took 30 mg Cu/day for two years (apparently without ill effect), then increased the dose to 60 mg Cu/day in the third year and suffered liver failure.

Araya, M, (2001) reports that relatively low concentrations of free copper in water induce nausea in humans. In an international trial, 179 individuals were given water containing copper sulphate at 0, 2, 4, 6 or 8 mg Cu/L in a 200 mL bolus of water (equivalent to a dose of 0, 0.4, 0.8, 1.2 and 1.6 mg Cu). Subjects were monitored for nausea and other symptoms. The no-adverse-effect-level for nausea was 4 mg Cu/L. However, this represents a taste effect of a soluble copper salt in water. Copper sulphate is a gastric irritant, and the nausea is probably associated with irritation of the stomach. Natural levels of copper in food include 6 mg/kg (= ppm) for shrimp and liver, 10 mg/kg for mushrooms, and 27 mg/kg for dark (bitter) chocolate. Consumption of 200 g of shrimp or liver in a meal, or 160 g of mushrooms, or 50 g of dark chocolate (which would each provide the same amount of bound copper as was administered in drinking water in the drinking water nausea study) would not be expected to induce nausea.

Araya et *al.* (2003) report another study, in which copper sulphate was administered by the same protocol than the previous investigation but in bottled spring water rather than deionised water and using an entirely female study population (n=269). Consistent with the previous study, nausea was the earliest and most commonly reported gastrointestinal symptom, occurring mostly within 15 min of copper ingestion with a no-adverse-effect-level of 4 mg Cu/L.

Olivares et *al.*(1998) report a study in which the effect of copper supplementation in the drinking water at the level of 2 mg/L was investigated in formula-fed and breast-fed infants from 3 to 12 months old in Chile. This study failed to demonstrate any adverse effects in infants who had consumed water with a copper content during the first 12 months of life. The only observed effect in children with copper relative to control was an increase in ceruloplasmine at 9 months only.

Other human epidemiological data are available and summarised in section 4.10.

4.6.3.6 Other relevant information

No data available.

4.6.3.7 Summary and discussion of repeated dose toxicity

Oral route:
Several studieswere available for the assessment of the toxicity after repeated administration:

- 15-days drinking water studies in rat and mice
- 15-days feedings studies in rat and mice
- 15-weeks feedings studies in rat
- 90-days feedings studies in rat and mice
- Human data

2 week drinking study:
All rats and all mice in the 10000 ppm groups and one female rat died, one male mouse and three female mice in the 3000 ppm groups died or were killed during the study. Clinical signs of both rats and mice in the highest two groups included emaciation, abnormal posturing, hypoactivity, dyspnoea, tremors and prostration. Final mean body weight gains for surviving animals of both species from the 3000 ppm groups were significantly reduced. Animals from the two highest groups also showed a decreased water consumption, which was attributed to poor palatability of the cupric sulphate solution. Microscopic lesions in rats were limited to an increase in the size and number of protein droplets in epithelial cells of the proximal convoluted tubules of the kidney of males in the 300 and 1000 ppm groups. No kidney lesions were observed in female rats or in mice of either sex.

2 week feeding study: No animals died or were killed during the study. Final body weights of male and female rats of 8,000 and 16,000 ppm groups and of female mice receiving 16,000 ppm were significantly lower than the controls. Mean food consumption for rats in the 16,000 ppm group and for mice in the 8,000 and 16,000 ppm groups was lower than controls. These decreases were considered to be due to the poor palatability of the feed mixture rather than to specific cupric sulphate toxicity. Microscopic findings in rats at 2,000 ppm and above included hyperplasia and hyperkeratosis of the squamous mucosa on the limiting ridge separating the forestomach from the glandular stomach. A similar finding was observed in mice but the severity was minimal. This was considered due to the irritant effects of cupric sulphate and the authors noted that there were no adverse effects on the health of the animals. Additionally in rats, chronic active inflammation of the liver characterised as minimal to mild mononuclear inflammatory cell infiltrate was observed in males at 8,000 ppm (4/5) and 16,000 ppm (5/5) and in females at 16,000 ppm (3/5). Depletion of haematopoetic cells in bone marrow occurred in male and female rats in the 8,000 and 16,000 ppm groups, consisting of a decreased cellularity of bone marrow erythroid/myeloid elements and an increase in the prominence of fat cells normally present in the bone shaft. In several high dose animals bone mass (cortex and trabecular density) was reduced when compared to controls. This was considered a consequence of reduced body weight gain rather directly related to treatment. A minimal to mild decrease in erythroid haematopoesis was seen in the spleens of rats in the 16,000 ppm group. There was an increase in the number and size of protein droplets in the cytoplasm and lumen of the renal cortical tubules in the male and female rats of the three highest dose groups, similar to that seen in the drinking water studies.

90-day feeding studies (Hebert, 1993):
Fischer rats (10 males and 10 females per group) were treated with copper sulphate (hydrated salt) administered in the diet at doses of 0, 500, 1,000, 2,000, 4,000 and 8,000 ppm for 92 days. B6C3F1 mice (10 males and 10 females) were treated to concentration of 0, 1,000, 2,000, 4,000, 8,000 and 16,000 ppm for 92 days. All rats and mice, except one female rat in the 1,000 ppm group (accidental death), survived to the end of the study. Final mean body weight gains were significantly reduced in male rats in the two highest dose groups (4,000 and 8,000 ppm) and in female rats in the highest dose group (8,000 ppm). Treated mice showed a dose-related reduction in body weight gain that occurred earlier than in the rat and was more severe at the higher dose levels. Food consumption was generally similar to the controls in all groups in both rats and mice except for the highest dose group in the rat (8,000 ppm).

Significant changes in haematology were noted in rats of both sexes at all time points but generally limited to the 2,000, 4,000 and 8,000 ppm dose groups. The effects were more marked in males. Significant increases in haematocrit (HCT), haemoglobin (HGB), platelet count and erythrocytes (RBC) were seen in the 8,000 ppm group which were consistent with polycythemia related to dehydration. By Day 21, HCT and HGB levels were significantly decreased together with MCV and MCH and these persisted until the end of the study. Significant increases in RBC and reticulocytes were noted in high dose males at the end of the study. There was a dose-related increase in the incidence and severity of chronic inflammation in the livers of rats, characterised by multiple foci of a mixture of mononuclear inflammatory cells.
Staining of the livers for the presence of copper showed a presence in the 4,000 and 8,000 ppm groups. At 8,000 ppm staining had a clear periportal to midzonal distribution and consisted of a few to numerous red granules of 1 to 2 mm in the cytoplasm of hepatocytes. At 4,000 ppm staining was periportal and there was a marked reduction in the number of cells stained and in the number of granules per cell. Positive minimal staining of livers for copper was evident in the high dose male and female mice and consisted of only a few positive-staining hepatocytes in the entire liver section.
Changes in the kidneys included an increase in the size and number of cytoplasmic protein droplets present in the epithelium of proximal convoluted tubules of rats at doses of 2,000 ppm and higher, and was less severe in females than in males. Many of the protein droplets in the male rats had large irregular crystalline shapes, which were not present in the females. Minimal nuclear enlargement (karyomegaly) in renal tubule cells was present in the high dose group. Degeneration of renal tubule epithelium was present in three females from the 8,000 ppm group. Positive staining for copper was seen in the kidneys at 4,000 ppm, and to a greater extent, in the 8,000 ppm groups and consisted of red granules in the cytoplasm of the renal tubule epithelium and a diffuse red staining of the protein droplets in the cytoplasm and the tubule lumen. There was no staining for copper in the kidneys of any mice. There was a reduction in iron-positive granules in the cytoplasm of splenal macrophages in the 8,000 ppm group, which was also evident but less prominent in the 2,000 and 4,000 ppm dose groups.

Human epidemiological data

Data are available however information are limited regarding doses consumed and exposure. The information is based on estimated quantities of copper ingested, which are reliant on patient accounts and are therefore biased, or effects observed are of differing severity which are not consistent with reported copper exposure concentrations. A case study is available detailing an individual who consumed 30mg/day of copper as a dietary supplement (well above the Tolerable upper intake level of 5 mg/day suggested by the Scientific Committee on Food) for 2 years (10 times the RDA) with no apparent ill effects. He then increased the copper intake to 60mg/day and was finally admitted to hospital showing signs of malaise and jaundice. His symptoms included cirrhosis of the liver and six weeks after admission to hospital he was given a liver transplant and made a good postoperative recovery (O'Donohue *et al*, 1993).

Inhalation exposure:

Two studies are available and were performed in guinea-pigs and rats. Other data are available in human in the chronic/cancerogenicity section (See 4.10.2).

The study of Pimentel (1969) was not performed according to standard guideline. In this study, Guinea-pigs showed interstitial pulmonary lesions, possibly leading to respiratory insufficiency (without a NOAEL).

In human, similar pulmonary lesion were seen after inhalation of Bordeaux mixture. However, in theses epidemiological data analysis different confusing situation were identified (smoking, wood dust, arsenic, etc…) and therefore no link could be established.

Furthermore, in the 4-weeks inhalation toxicity study, performed with current guideline in rat, there was an exposure concentration-related increase in microscopic findings in the lung, and increased lung, bronchial lymph node, and mediastinal lymph node weights. Lung histopathology included alveolar histiocytosis, acute inflammation, and perivascular mononuclear cell infiltrates. However, following a 13-week recovery period, the effects on lung weights were greatly reduced and the microscopic findings were no more observed. As the effects were reverible, they are not considered as severe or significant;

Dermal exposure:
Three-week dermal exposure in rabbit show slight dermal effects above 500 mg Cu/kg bw/d**.**

4.6.3.8 Summary and discussion of repeated dose toxicity findings relevant for classification as STOT RE according to CLP Regulation

Oral route:
Target organs of copper upon oral administration were the liver (inflammation), kidneys (histopathological changes) and hyperplasia and hyperkeratosis of the forstomach in rats, haematological changes were also observed in this specie, while mice were less sensitive, showing adverse effects only in the stomach. Thus, minimal to moderate effects were observed at > 232 mg tribasic copper sulfate/kg bw/day (32 mg Cu/kg bw/d). More severe effects were observed above 479mg tribasic copper sulfate /kg bw/day;

Inhalation route:
The 4-weeks study in rat, performed with standard guideline is considered the most relevant.
In this study, no irreversible adverse effects were observed up to 2 mg/m^3 Cu.

Dermal route:
No adverse effects were observed at or below 500 mg/kg bw Cu in the available study (3 weeks exposure).

4.6.3.9 Comparison with criteria of repeated dose toxicity findings relevant for classification as STOT RE

Evaluation of non human data
Oral route:
In the 90-day study in rat at the dose level of 232 mg/kg bw tribasic copper sulphate, liver and kidney change were observed but were not considered “significant/severe” effects at this dose level.
In the 90-day study in mice, no adverse effects were observed below 50 mg/kg bw/d.

Overall, in the available studies in mice and rat, no serious adverse effects were observed below the harmful cut-off values for classification Cat 2 (10-100 mg/kg bw) in the CLP regulation.
Therefore, no classification is warranted.

Inhalation route:

In the 4-weeks study by inhalation in rat, no serious adverse effects were observed at the maximum tested concentration (2 mg/m^3). Therefore, no classification is warranted according to the CLP criteria.

Dermal route:
In the 3 weeks study performed in rabbit, no adverse effects were observed at or below 500 mg/kg Cu. No classification is therefore necessary according to the CLP criteria.

Evaluation of human data
Human epidemiological data is available however information is limited regarding doses consumed and exposure. The information is based on estimated quantities of copper ingested, which are reliant on patient accounts and are therefore biased, or effects observed are of differing severity which are not consistent with reported copper exposure concentrations.
A case study is available detailing an individual who consumed 30mg/day of copper as a dietary supplement (well above the Tolerable upper intake level of 5 mg/day suggested by the Scientific Committee on Food) for 2 years (10 times the RDA) with no apparent ill effects. He then increased the copper intake to 60mg/day and was finally admitted to hospital showing signs of malaise and jaundice. His symptoms included cirrhosis of the liver and six weeks after admission to hospital he was given a liver transplant and made a good postoperative recovery (O'Donohue et al, 1993). Other human epidemiological data are available and summarised in section 4.10.

Weight of evidence of all data, including human incidents, epidemiology, and studies conducted in experimental animals, do not to support classification for specific target organ toxicity following repeated exposure.

4.6.3.10 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification according to DSD

No classification is considered necessary for repeated exposure.

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)
Summary of the Dossier submitter's proposal No data on tetracopper hexahydroxide sulphate are available in the CLH report. However, in light of the proposal to read-across between the different copper compounds for systemic endpoints (see section "RAC general comment" above), the dossier submitter included in the CLH report several animal studies with repeated exposure to other copper compounds (predominantly copper sulphate pentahydrate) for various durations and routes, as well as some human data. Hébert *et al.* (1993) reported on oral 15-day drinking water and feeding studies and 90-day feeding studies in both rats and mice, all conducted with copper sulphate pentahydrate but none guideline compliant. In addition, three studies where copper sulphate was administered in the diet at one or several doses for up to 15 weeks and animals sacrificed at several intervals, were also reported (Haywood, 1980, 1985; Haywood & Comerford, 1980). One OECD TG 412 compliant 28-day rat inhalation study conducted with dicopper oxide (Kirkpatrick, 2010) is included as well as an older non-guideline compliant study where guinea pigs were exposed via inhalation to Bordeaux mixture for about 6 months (Pimentel & Marques, 1969). Finally, an OECD TG 410

compliant dermal rabbit study is included (Paynter, 1965), with exposure to copper dihydroxide for 3 weeks (5 days per week). A human case study of chronic oral self-administration of copper causing liver failure (O'Donohue *et al.*, 1993) and human volunteer studies demonstrating nausea associated with copper sulphate in drinking water (Araya *et al.*, 2001, 2003) are also reported, as are human case studies of chronic inhalation exposure to Bordeaux Mixture causing pulmonary lesions (e.g. Pimentel & Marques, 1969; Pimentel & Menezes, 1975, 1977).

Inhalation exposure to dicopper oxide resulted in no irreversible adverse effects up to the highest dose tested in rats (2 mg/m^3). Following dermal exposure to rabbits, degenerative skin abnormalities were only observed at 1000 but not at 500 mg copper/kg bw/day. Human data is poorly reported and doses are difficult to estimate. Following oral exposure in rats, target organs of copper were the liver (inflammation), kidneys (histopathological changes) and forestomach (hyperplasia and hyperkeratosis), with some evidence of haematological changes. Mice were less sensitive, with adverse effects limited to the forestomach. According to the dossier submitter, no serious adverse effects were observed in the available oral studies below the cut-off value for classification (100 mg/kg bw/day for a 90-day study). After considering all available human and animal data, the dossier submitter concluded that they do not support classification for specific target organ toxicity following repeated exposure.

Comments received during public consultation

No comments were received during the public consultation.

Assessment and comparison with the classification criteria

RAC notes that no data are available on tetracopper hexahydroxide sulphate. The CLH report contains data on other copper compounds (predominantly copper sulphate pentahydrate), from which the dossier submitter proposed to read-across to tetracopper hexahydroxide sulphate. In view of the considerations presented in the section "RAC general comment", RAC has not pursued the aspect of grouping any further. RAC concludes that in the absence of relevant data no proposal for classification for specific target organ toxicity following repeated exposure can be made for tetracopper hexahydroxide sulphate.

4.7 Germ cell mutagenicity (Mutagenicity)

Copper has been extensively investigated in a series of mutagenicity studies in various salts. The majority of studies were from the literature, but there are three core guideline compliant GLP studies.

Table 24: Summary table of relevant *in vitro* and *in vivo* mutagenicity studies

Method	Results	Remarks	Reference
In vitro			
Ames *S. typhimurium* TA 98, TA100, TA1535, TA1537, TA102. Copper sulphate pentahydrate Five concentration: 50, 100, 200, 400 and 800 µg/plates	Cytotoxicity at 800 µg/plates and at 200 and 400 µg/plates with S9 +S9: negative -S9: negative	OECD 471 GLP Purity: 99-100.5%	Ballantyne (1994)

+/- metabolic activation system Positive and negative controls Pre-incubation: 1 hour with metabolic activation			
Ames *S. typhimurium* TA 102 Copper sulphate 10, 30, 100, 300, 100 and nM/plate Positive and negative controls Triplicate	+S9: Not investigated -S9: negative	No guideline No GLP Lack of data	Marzin, D.R., Phi, H.V. (1985)
Ames *S. typhimurium* TA 98, 100, 1535, 1537, 1538, and *E. coli.*WP2 Copper sulphate Up to 5000 µg/plate. Oxine copper Up to 50 µg/plate.	**Copper sulphate** Cytotoxicity not stated. +S9: negative -S9: negative **Oxine copper** Cytotoxicity above 5µg/plate +S9: negative -S9: negative	Guidelines followed with lacks. No GLP	Moriya, M., Ohta, T., Watanabe, K., Miyazawa, T., Kato, K. Shirasu, Y. (1983)
In vitro UDS Primary rat hepatocytes Copper sulphate Concentrations : 7.9, 15.7, 41.4, 78.5µM (incubation 20h) +/- hydroxyurea Positive and negative controls Triplicate	Lowest concentration non-cytotoxic Highest concentration moderately cytotoxic +S9: Not investigated -S9: Positive Significant stimulation of 3H-thymidine incorporation into the DNA, both in presence and absence of hydroxyurea and at all concentrations (dose-dependent).	Guideline not stated No GLP Lacks of data	Denizeau, F., Marion, M. (1989)
Ames *S. typhimurium* TA 98, 100, 1535, 1537, 1538. Technical Bordeaux mixture First replicate: 33, 100, 333, 1000, 3333, 10000 µg/plate Second replicate: 312.5, 625, 1250, 2500, 5000, 10.000 µg/plate. Third: 1000,2000, 3000, 4000, 5000, 6000µg/plate (TA 98 and 100)	+S9: negative -S9: negative	OECD 471 GLP Deviation: lack of strain TA 102 or *E. coli.*WP2 Purity: not stated	Dillon, D. M., Riach, G. C. (1994a)
Ames *S. typhimurium* TA 98, 100, 1535, 1537, 1538 Copper oxychloride.	Toxic effects observed above 3333µg/plate +S9: negative -S9: negative	OECD 471 No GLP Deviation: only 2 tested strain Purity: 98.3%	Dillon, D. M., Riach, G. C. (1994b)

First: 33, 100, 333, 1000, 3333, 10000µg/plate Second: 312.5, 625, 1250, 2500, 5000, 10000 µg/plate Third: 1000, 2000, 3000, 4000, 5000, 6000 µg/plate (TA 98 and 100)			
Ames *S. typhimurium* TA 98, TA100 Copper Nordox Technical 0.1, 1.0, 10, 20 µg/plate.	+S9: negative -S9: negative		Bossotto, A., Allegri, R., Chujman, A., Terceño, A., Mannocci, S. (2000)
Ames *S. typhimurium* TA 98,TA102, TA1535, TA1537 Copper chloride 160ppm and 200ppm (no more precision).	+S9: negative -S9: negative	Guideline not stated No GLP Deviations: lack of data Purity: not stated	Wong P.K. (1988)
Rec-assay Cold incubation assay in recombination-repair deficient strains of *Bacillus Subtilis* CuCl and $CuCl_2$	+S9: Not investigated -S9: negative	Non-guideline study No GLP Deviations: Lack of information on concentrations. Purity: not stated	Kanematsu, N., Hara, M., Kada, T. (1980)
UDS and SCE assays CHO V79 cells Copper (II) nitrate	+S9: Not investigated -S9: Assay showed binding to DNA and weak positive SCE	Non-guideline study Deviations: Lack of information on concentrations. No positive control, experimented not duplicated. Purity: not stated	Sideris, E.G., Charalambous, A.T. Katsaros, N. (1988)
In vivo			
Mouse micronucleus CD-1 mice 5/sex/groups Copper sulphate Oral gavage First: LD50 745 mg/kg Main study: 2 days at 447 mg/kg (i.e. 113.76 mg Cu/kg). Twice on consecutive days Sacrifice 24 and 48h, after second treatment. Positive control	Negative Decreased ratio of PCE to NCE after 24h compared to vehicle control indicated that copper sulphate had been absorbed into the bone marrow.	OECD 474 GLP Purity: 99-100.5%	Riley, S.E. (1994)
UDS Rat (hepatocytes) Wistar rats. 6 males/group	Negative No production of a group mean net grain counter greater than	No guideline GLP Purity: 99-100.5%	Ward, P.J. (1994)

Copper sulphate Oral gavage 632.5, 2000 mg/kg (equivalent to 161 and 509 mg copper/kg/day), once sampling times: 12-14h or 2-4h post dosing positive and negative controls	1.0 in primary cultures of hepatocytes treated with 3H thymidine. No more than 1.0 % of cells found in repair at either dose.		
Swiss mice Groups of 3 mice Copper sulphate Bone marrow chromosome aberration study, micronucleus assay and sperm abnormality assay in the mouse ip injection **Bone marrow chromosome aberration study** IP inj, doses 5, 10, 20 mg/kg,. Mice killed after 6h (20 mg/kg), 24h (5, 10, 20mg/kg) and 48h (20mg/kg). Other group with IP inj.of 4 mg/kg/day during 5days. Mice killed 24h after the last injection Oral or SC: dose: 20mg/kg; sacrifice 24 h later. **Micronucleus** 5, 10, 20mg/kg/day; 2 inj. at 24h interval. Mice killed 6h after the 2nd. inj. **Sperm abnormality assay** IP inj. Doses: 1, 2, 4 mg/kg/day 5 consecutive days. Sacrifice 35 days after the first inj. 500 sperm examined for each animal.	**Bone marrow chromosome aberration** Aberrations such as gaps more frequent than, breaks, fragments, exchange of rings. Greatest effect with IP inj. **Micronucleus:** Significant dose-dependent increase in the incidence of micronuclei. **Sperm abnormality assay:** Significant dose-related increase in the mean number of abnormal sperm (head shapes, tail attachments and double tailed sperm).	Guideline not stated No GLP Lack of information	Bhunya, S.P. Pati, P.C. (1987)
Bone marrow chromosome aberration	Positive	Guideline not stated (meet OECD 475)	Agarwal, K., Sharma, A.

Swiss albino Mice 6 mice/groups Copper sulphate IP injection Doses: 1.1, 1.65, 2.0, 3.3, 6.6 mg/kg. Sacrifice of 6 mice at 6, 12, 24h, after treatment for each dose Positive control: Mitomycine C.	The aberrations induced were mainly of the chromatid type and only in the higher dose groups were chromosomal breaks significantly enhanced. There were positive trends with increasing dose for the number of chromosomal aberrations per cell and the % damaged cells at all hours of exposure.	No GLP	Talukder, G. (1990)
Mouse micronucleus Male CBA mice 5 animals/group Copper sulphate i.p. injection Doses: 6.6, 13.2, 19.86 mg/kg. Sacrifice of 6 mice at 24h (all doses) or 48h (6.6 mg/kg), after treatment. Positive controls: Cyclophosphamide and Vincristine sulphate.	Negative Reduced PE/NE ratio indicated that copper sulphate had been absorbed into the bone marrow. Deviation: Statistical analyses not performed	Guideline not stated GLP: not stated Deviation: no statistical analysis Purity: not satated	Tinwell, H. Ashby, J. (1990)

4.7.1 Non-human information

4.7.1.1 In vitro data

The *in vitro* systems, particularly those involving isolated mammalian cells, may not be valid in the risk assessment of copper. Copper absorbed by the body is always bound , and transfer from blood/plasma to cells is regulated such that copper passed through the cell membrane is also bound to metallothioneins within the cell, before being incorporated in various enzymes. The *in vitro* tests bypass these strict control mechanisms and effectively present the cell with a totally artificial situation of excess free copper ion. The free copper ion is highly reactive, and the presence of high quantities of free ion in cell cultures will cause disruption of the cellular processes.

As *in vitro* data are not appropriate to assess genotoxicity of copper (Arce, 1998) and that several data *in vivo* were available, *in vitro* data are only summarized in the table above.

4.7.1.2 In vivo data

Reference: Ward, P. J. (1994)
Guideline: No
GLP: No
Deviations: None

Doses of 623.5 and 2000 mg/kg bw of copper II sulphate pentahydrate were administered to male Wistar rats by gavage at a dose volume of 10 mL/kg bw to groups of 6 rats. Doses were administered on two occasions separated by 2 hours. Negative control animals received water only. Positive control animals received an oral dose of 2-Acetamidofluorene, suspended in corn oil at 75 mg/kg (experiment 1) or dimethylnitrosamine, suspended in water at 10 mg/kg (experiment 2).After 12-14 hours (experiment 1) or 2-4 hours (experiment 2), the rats were killed and the livers perfused with collagenase to provide a primary culture of hepatocytes. Cultures were made from 5 animals per group and were treated with [^{3}H] thymidine. Slides were treated with photographic emulsion to prepare autoradiograms, and examined microscopically. The net grain count, the number of grains present in the nucleus minus the number of grains in 3 equivalent areas of cytoplasm, was determined.

Negative (vehicle) controls and positive controls confirmed the validity of the assay. Treatment with 632.5 or 2000 mg/kg bw copper II sulphate (equivalent to 161 or 509 mg copper/kg bw) did not produce a group mean net grain count greater than -1.0, nor were there any more than 1.0% cells found in repair at either dose (table 25).

Copper II sulphate pentahydrate has no genotoxic activity in the in vivo rat liver UDS assay.

Table 25: Group mean net grain counts for experiment 1 and 2

12-14 hour sacrifice time

Dose (mg/kg)	Net nuclear grain count (NG)		Net grain count of cells in repair		Percent of cells in repair (NG≥5)	
	Mean	SD	Mean	SD	Mean	SD
0 water	-1.3	0.6	0	-	-	-
632.5	-1.3	0.3	10.2	6.4	0.6	0.9
2000	-1.0	0.3	5.5	0.9	1	1
75 2-AAF	12.7	0.9	13.7	0.8	90.0	4.0

2-4 hour sacrifice time

Dose (mg/kg)	Net nuclear grain count (NG)		Net grain count of cells in repair		Percent of cells in repair (NG≥5)	
	Mean	SD	Mean	SD	Mean	SD
0 water	-2.2	0.3	0	-	-	-
632.5	-2.2	0.2	0	-	-	-

2000	-3.2	0.5	0	-	-	-
10 DMN	17.2	2.8	17.3	2.7	99.6	0.9

Reference: Riley, S. E. (1994)
Guideline: OECD 474
GLP: Yes
Deviations: Yes

- Only one dose tested in the main study.

Copper II sulphate pentahydrate was administered orally by gavage to groups of male and female CD-1 mice. In the main study, mice were treated on two consecutive days at 447 mg/kg bw/day to groups of 5 male and female mice, that were killed either 24 or 48 hours after the second dose. Groups of mice were also dosed on two consecutive days with vehicle (distilled water) only and killed either 24 or 48 hours after the second dose, and other groups of 5 male and 5 female mice were dosed with the positive control cyclophosphamide dissolved in purified water at 80 mg/kg bw and killed after 24 hours.
Erythrocytes of bone marrow were observed in all animals, in order to determine polychromatic/normochromatic erythrocytes ratio and frequency of micronucleated PCE/1000 cells determined.

Several animals in the main study died prior to scheduled sacrifice (5 out of 10 males and 3 out of 10 females), indicating that it would not have been possible to administer the test material at a significantly higher dose. Mice treated with copper II sulphate pentahydrate showed decreased ratios of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) when sampled after 24 hours, compared to concurrent vehicle controls, indicating that copper II sulphate pentahydrate had been absorbed into the bone marrow. The PCE/NCE ratios seen in animals sampled at 48 hours were similar to those of control animals. Mice treated with copper II sulphate pentahydrate exhibited frequencies of micronucleated PCE which were similar to vehicle controls at all sampling times. There were no instances of statistically significant increases in micronucleus frequency for any group receiving the test chemical at either sampling time. The positive control animals exhibited increased numbers of micronucleated polychromatic erythrocytes, such that the frequency of micronuclei was significantly greater than in concurrent controls (table 26).

Table 26: Summary of group mean findings

Treatment group (mg/kg) twice	Kill time (hours)	Sex	Mean ratio PCE/NCE	Group mean frequency of micronucleated PCE (per 1000)	
				Per sex	Per treatment group
Vehicle control	24	♂	1.07	0.4	0.35
		♀	1.20	0.3	
	48	♂	1.44	0.38	0.33
		♀	0.83	0.3	
447	24	♂	0.70	0.6	0.5
		♀	0.84	0.4	
	48	♂	1.12	0.5	0.45
		♀	1.32	0.4	
Positive control CPA, 80+	24	♂	0.52	26.87	28.07

		♀	0.48	29.27	

Conclusion: Copper II sulphate pentahydrate did not induce micronuclei in the polychromatic erythrocytes of the bone marrow of mice at 447 mg/kg bw/day (equivalent to 113.76 mg copper/kg bw/day), a dose at which limited mortality was observed.

Reference: Bhunya, S.P. and Pati, P.C. (1987)
Guideline: No
GLP: No
Deviations: Yes

- Only three animals per group were used,
- no positive control group,
- in the micronucleus test animals were killed 6 h after the last injection.

Three parameters were analysed:

- Bone marrow chromosome aberration assay,
- micronucleus assay,
- sperm abnormality assay.

Bone marrow chromosome aberration assay:
Swiss mice, with an average body weight of 25g, were administered hydrated copper sulphate, by a single intraperitoneal injection, at dose levels of 5, 10 and 20 mg/kg and groups of three were killed after 6 h (20 mg/kg), 24 h (5, 10 and 20 mg/kg) and 48 h (20 mg/kg). Another group of animals was administered the test article at a dose level of 20 mg/kg divided into 5 equal parts, each part administered daily by intraperitoneal injection (4 mg/kg/d during 5 days) and the animals were killed 24 h after the last injection. A similar group of animals was administered double distilled water and served as controls. Further groups of animals were given a single administration of the test article orally or by subcutaneous injection at a dose level of 20 mg/kg and were killed after 24 h. Groups of animals were given double distilled water by similar methods to serve as controls. Colchicine was used, shortly before sacrifice, as a spindle inhibitor. Bone marrow smears were prepared and 100 metaphases per animal were scored for aberrations.

Micronucleus assay:
The test article was administered to groups of three Swiss mice by two intraperitoneal injections, separated by 24 h, at dose levels of 5, 10 and 20 mg/kg. A similar group received double distilled water and served as controls. The animals were killed 6 h after the second injection. Bone marrow smears were prepared and 1,000 erythrocytes per animal scored for micronuclei.

Sperm abnormality assay:
The test article was administered to groups of three Swiss mice by intraperitoneal injection at dose levels of 5, 10 and 20 mg/kg, each dose being split into five equal parts and each part being injected daily at 24 h intervals. A similar group received double distilled water and served as controls. The animals were killed 35 days after the first injection. Sperm were collected from the cauda epididymides and slides prepared. Five hundred sperm from each animal were examined and sperm abnormalities categorised. Statistical analyses were performed on each series of tests.

Bone marrow chromosome aberration assay:

Treatment induced aberrations such as gaps, breaks, fragments, double minutes, exchanges and rings, with gaps being more frequent than breaks. Repeated exposure of fractionated doses induced less aberration than that of the equivalent dose as a single dose. The greatest effect was produced when copper sulphate was administered by intraperitoneal injection (table 27).

Table 27: Chromosomal aberrations (%)

Kill (h)	**Dose level (mg/kg)**			
	0	**5**	**10**	**20**
Single intraperitoneal injection				
6	-	-	-	4.00*
24	-	4.00*	4.66*	5.00*
48	0.70	-	-	4.33*
Multiple intraperitoneal injection				
120	-	-	-	4.00*
Oral				
24	0.66	-	-	4.00*
Subcutaneous injection				
24	0.66	-	-	4.66*

* Statistically significant using an equality of proportion test

In all cases, chromosomal aberrations were predominantly chromatid gaps and when gaps are excludes, results were similar to negative controls.

Micronucleus assay:
There was a significant dose-dependent increase in the incidence of micronuclei (table 28). However, a statistically significant increase in the frequency of nuclei in lysis compared to controls was also reported for all doses investigated, indicating that all the doses of copper sulfate used in this study were cytotoxic.

Table 28: Bone marrow counts (mean %)

	Dose level (mg/kg)			
	0	**5**	**10**	**20**
Number cells examined	3000	3000	3000	3000
Poly and normochromatic erythrocytes with micronuclei	0.15	0.98	1.41	1.76
Poly/normochromatic ratio	0.88	1.10	1.10	1.10
Immature white cells with micronuclei	0.06	0.40	0.88	1.23
Nuclei in lysis	-	0.20	0.30	0.46
Total	0.21	1.58*	2.59*	3.45*

* Statistically significant using an equality of proportion test

Sperm abnormality assay:

There was a significant dose-related increase in the mean number of abnormal sperm. Varieties of abnormal sperm were induced, including various head shapes, tail attachments, double headed and double tailed sperm (table 29).

Table 29: Incidence of sperm abnormality

	Dose level (mg/kg)			
	0	**5**	**10**	**20**
Number sperm examined	1500	1500	1500	1500
Number abnormal sperm	62	87	166	231
Mean %	2.06	5.80*	11.60*	15.40*

* Statistically significant using an equality of proportion test

Conclusion: Results indicated that copper sulphate solution administered by intra-peritoneal injection (where free copper is injected directly to the abdominal cavity) caused mutagenic activity in bone marrow cells and in sperm. However, this route of administration is inappropriate, as it avoids the normal processes of copper absorption and distribution.
Chromosomal aberration study in vivo where copper is administered orally (the natural route, whereby uptake is controlled by homeostatic mechanisms) is positive (at 20 mg/kg). However, chromosomal aberrations were predominantly chromatid gaps and when gaps are excludes, results were similar to negative controls. Moreover, only three animals are used whereas in the guideline 10 animals are recommended.

Dose, route and time influenced significantly the frequency of chromosomal aberration, incidence of micronucleus and sperm abnormality. The study deviated from the guideline and the findings are consequently considered to be unreliable.

Reference: Agarwal, K., Sharma, A. and Talukder, G. (1990)
Guideline: No. Generally meets requirements of OECD 475
GLP: No.
Deviations: Yes

- Groups of six male mice were used for each dose level at each time point,
- no cytotoxicity was observed and reported in this study,
- only 50 metaphase plates from each 6 animals per dose were scored, whereas OECD guideline 475 require that “At least 100 cells should be analysed for each animal.

The test article, copper sulphate pentahydrate in isotonic saline, was administered by intraperitoneal injection to groups of Swiss albino male mice at dose levels of 1.1, 1.65, 2.0, 3.3 and 6.6 mg/kg. Prior to sacrifice (1.5 h) the mice were injected with 4 mg/kg colchicine, a spindle inhibitor. Groups of six mice were killed at 6, 12 and 24 h after treatment for each dose. A similar group of mice was treated with 1.5 mg/kg mitomycin C (a positive control article) and then animals killed after 6 h. Bone marrow smears were prepared by standard methods and 50 metaphases from each of the six animals from each group were scored for aberrations, excluding gaps.

The aberrations induced were mainly of the chromatid type (isochromatid breaks and chromatid gaps) and only in the higher dose groups were chromosomal breaks significantly enhanced. When gaps were excluded, there were positive trends with increasing dose for the number of chromosomal aberrations per cell and the % of cells with at least one chromosomal aberration at all time points and doses investigated. Further analysis of the data demonstrated that both chromosome aberrations/cell and % of cells with at least one chromosomal aberration (excluding gap) were significantly higher at 6h compared to 12 and 24h at all doses investigated, indicating a relative early onset of clastogenesis. The highest concentration of copper sulphate produced higher values in the chromosomal aberrations per cell and % damaged cells at 6 and 12 h exposure than the positive control, mitomycin C (Table 30).

Table 30: Chromosomal aberrations

Exposure (h)	Mitomycin C 1.5 mg/kg	Dose level (mg/kg)					
		0	1.1	1.65	2	3.3	6.6
Chromosome aberrations (excluding gaps)							
6	0.077	0.017	0.053	0.060	0.073	0.067	0.100
12		0.017	0.023	0.040	0.037	0.050	0.087
24		0.010	0.037	0.047	0.047	0.040	0.050
% damaged cells with at least 1 aberration							
6	7.667	1.670	5.330	6.000	7.330	6.670	10.00
12		1.670	2.330	4.000	3.670	5.000	8.670
24		1.000	3.670	4.670	4.670	4.000	5.000

Conclusion: Results show that copper sulphate is a moderate clastogenic agent in mice causing a significant increase in aberrations at higher dose levels. Intraperitoneal injection bypasses the natural mechanisms for binding copper, and is not an appropriate route to assess oral exposure.

Reference: Tinwell, H. and Ashby, J. (1990)
Guideline: No but very close
GLP: No
Deviations: Yes

- Statistical analyses were not performed.

The study was performed as a direct response to the previous study and published in the same journal. Hydrated copper sulphate dissolved in sterile deionised water was administered by a single intraperitoneal injection to groups of six male CBA mice at dose levels of 6.6, 13.2 and 19.86 mg/kg. Other groups of six mice of the same age, sex and strain were given distilled water and served as controls. Positive control articles, cyclophosphamide and vincristine sulphate dissolved in sterile deionised water, were administered to groups of mice at dose levels of 65 mg/kg (two mice) and 0.1 mg/kg (one mouse), respectively. A dose volume of 10 mL/kg was used. The animals were killed 24 h (all doses) or 48 h (6.6 mg/kg dose only) after treatment. Bone marrow smears were prepared and 2,000 polychromatic erythrocytes (PE) were assessed for micronucleated PE. The ratio of PE to normocytes (NE) was determined from 1,000 erythrocytes. Statistical analyses were not performed as the results were considered to be obvious.

No toxicity was reported at the lowest dose level (6.6 mg/kg) during the course of the experiment. At the other two dose levels, the animals were reported as appearing subdued. In addition, a marked depression in erythropoiesis (reduced PE/NE ratio) was observed at both 13.2 and 19.86 mg/kg, indicating cytotoxicity. The dose of 19.86 mg/kg (60% of LD50) was estimated to be the maximum tolerated dose. Copper sulphate failed to induce micronuclei in the bone marrow at any of doses or time points investigated. These results conflict with those of the preceding study.

Table 31: Mean bone marrow counts

		Dose level (mg/kg)					Cyclophos-phamide (65 mg/kg)	Vincristine sulphate (0.1 mg/kg)
		0 Test 1	0 Test 2	6.6	13.2	19.8		
MPE/1000 PE	at 24 h	2.6	1.5	3.3	2.1	2.0	65.25	10.5
	at 48 h			2.5				
PE/NE ratio	at 24 h	0.9	0.9	1.0	0.5	0.45	0.7	0.7
	at 48h			0.9				

Conclusion: Copper sulphate did not induce micronuclei in the bone marrow of mice. As this conflict with a preceding study and the age and sex of the animals were the same, there is the possibility of a strain specific bone marrow response, although no precedent exists.

4.7.2 Human information

No data available.

4.7.3 Other relevant information

Literature review on copper genotoxicity:

Reference: Arce, G. T. (1998), Griffin, Unpublished report.

This report is a summary of evidence from the literature. The report emphasises the essential nature of copper including its presence in the cell nucleus associated with stabilising genetic materials and with DNA polymerases. Copper appears to be essential for the replication of DNA, and transcription of RNA.
The report notes that *in vitro* systems, particularly those involving isolated mammalian cells, may not be valid in the risk assessment of copper. Copper absorbed by the body is always bound, and transfer from blood/plasma to cells is regulated such that copper passed through the cell membrane is also bound to metallothioneins within the cell, before being incorporated in various enzymes. The *in vitro* tests bypass these strict control mechanisms and effectively present the cell with a totally artificial situation of excess free copper ion. The free copper ion is highly reactive, and the presence of high quantities of free ion in cell cultures will cause disruption of the cellular processes. These effects may be manifest as gene mutations, but their occurrence is not evidence for mutagenic activity of copper, but shows that the proper concentration of copper is vital for the correct functioning of all cells.
Copper has rarely been found to be mutagenic alone. In combination with certain chemicals or UV light, it can cause mutation by allowing the production of hydroxyl radicals, where excess copper is the catalyst producing oxidation through the Cu (II)/Cu(I) redox cycle. The report also notes that copper, like iron, has been shown to be responsible for inducing mutations through the formation of metal-generated free radicals, often in the presence of another chemical.
One such report cited the role of copper in DNA strand breaks when the chemical menadione is added to Chinese hamster fibroblast cultures. No additional copper was added. There is enough natural copper present in the cells: menadione induced the release of sufficient stored copper from the cell to produce hydrogen peroxide through the redox reaction, which produced sufficient oxygen free radicals to cause DNA damage. Similar studies have been performed with UV light, hydroquinone and ascorbic acid.

4.7.4 Summary and discussion of mutagenicity

The potential mutagenicity of copper compounds has been investigated in a number of in vitro assays in bacterial and mammalian cells, and in several in vivo assays.

Ames tests were negative. Two *in vitro* studies were positive. The *in vitro* UDS positive results is considering not relevant as the *in vivo* UDS study was negative. The SCE weak positive result is considered equivocal due to the lack of information in this study.

Two *in vivo* tests performed by the oral route (a micronucleus assay and a UDS test of Riley, S.E., 1994 and Ward, P.J., 1994, respectively) presented no concern about their validity and were negative. Only the chromosomal aberrations study of Bhunya (1987) presented positive results at 20 mg/kg. However, chromosomal aberrations were predominantly chromatid gaps and when gaps are excludes, results were similar to negative controls. Moreover, only three animals are used whereas in the guideline 10 animals are recommended. Consequently, the findings were not considered.
Results of these studies provide no evidence that copper compounds are mutagenic *in vivo* upon oral administration.

Following non-oral exposure, two tests via ip (intra-peritoneal) (Bhunya, S.P., 1987 and Argawal, K., 1990) showed positive results, although they had some short comings: no positive control had been used for one, a low number of animals had been used and a low number of cells examined, and both studies were no GLP. Moreover, this route of administration is inappropriate, as it avoids the normal processes of copper absorption and distribution. Furthermore, these results are in conflict with an additional well-conducted negative *in vivo* micronucleus study *via* intraperitoneal injection (Tinwell, H., 1990).

To conclude, a number of studies have been performed, but several suffer of deficiencies. Consideration of the weight of evidence from *in vitro* and *in vivo* tests, leads to the conclusion that copper compounds are likely not mutagenic.

Overall, data indicates that copper compounds do not meet the criteria for classification as a genotoxic.

4.7.5 Comparison with criteria

1) Criteria in the CLP classification:

A substance shall be classified in category 2 for germ cell mutagenicity endpoint if the substance causes concern for humans owing to the possibility that they may induce heritable mutation in the germ cells of humans. This classification is based on:

- Positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from:
    - Somatic cell mutagenicity tests in vivo, in mammals (mammalian bone marrow chromosome aberration test, mouse spot test or mammalian erythrocyte micronucleus test); or
    - Other in vivo somatic cell genotoxicity test (UDS or SCE assay) which are supported by positive results from in vitro mutagenicity assays (in vitro mammalian chromosome aberration test, in vitro mammalian cell gene mutation test or bacterial reverse mutation test).

2) Comparison with criteria:

For copper compounds, positive results were observed for bone marrow micronucleus assay (Bhunya, 1987) and bone marrow chromosome aberration assays (Bhunya, 1987 and Agarwal, 1990) when the substance was administered by **intra-peritoneal route**. However, this route is considered as inappropriate as it avoids the normal process of copper absorption and distribution. And another bone marrow micronucleus assay (Tinwell, 1990), with less deficiency than the Bhunya's study, was available and gave negative result. Moreover, two in vivo reliable test (bone

marrow micronucleus assay (Riley, 1994), UDS in hepatocyte cells (Ward, 1994) performed by the oral route (natural route, whereby uptake is controlled by homeostatic mechanisms) were negative.

4.7.6 Conclusions on classification and labelling

In this context, the available data do not support a classification for mutagenicity endpoints.

However, there was insufficient evidence to exclude a local genotoxic potential of copper as some studies by I.P route were positive (but with a low reliability) and that UDS and SCE *in vitro* tests without metabolic activation were also positive.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier submitter's proposal

No data on tetracopper hexahydroxide sulphate are available in the CLH report. However, in light of the proposal to read-across between the different copper compounds for systemic endpoints (see section "RAC general comment" above), the dossier submitter included in the CLH report mutagenicity studies with other copper compounds (predominantly copper sulphate pentahydrate).

Ten *in vitro* studies were very briefly summarised in tabular form. Three Ames tests conducted with copper sulphate (pentahydrate) and another four conducted with Bordeaux Mixture, dicopper chloride trihydroxide, copper Nordox Technical and copper chloride were all reported as negative as well as a rec-assay with copper chloride. An unscheduled DNA synthesis (UDS) test conducted with copper sulphate in primary hepatocytes and an UDS and sister chromatid exchange (SCE) assay with copper nitrate in Chinese hamster V79 cells showed positive results in the absence of metabolic activation. The dossier submitter did not discuss these studies further in the report, as *in vitro* data are not considered appropriate to assess the genotoxic potential of copper. This is because absorbed copper is normally always bound to proteins in the body, where the *in vitro* tests present the cells with free copper, which is highly reactive.

Five *in vivo* studies are included in the CLH report, all conducted with copper sulphate pentahydrate. A negative mouse bone marrow micronucleus assay (Riley, 1994) and a negative rat liver USD assay (Ward, 1994) administering copper sulphate pentahydrate by gavage are presented. In addition, three studies administering copper sulphate pentahydrate by intra-peritoneal (IP) injection to mice are included. Two bone marrow chromosome aberration assays were concluded as positive as well as a sperm abnormality assay and one out of two micronucleus assays (Bhunya & Pati, 1987; Agarwal et al., 1990; Tinwell & Ashby, 1990). Mice also scored positive for bone marrow chromosome aberrations following oral and subcutaneous administration of copper sulphate pentahydrate (Bhunya & Pati, 1987). Considering that the IP route bypasses the normal processing of copper in the body, that there were conflicting results for two IP micronucleus assays, and that two reliable studies via the oral route (where uptake is controlled by homeostatic mechanisms) were negative, the dossier submitter concluded that the available data do not support classification for germ cell mutagenicity for copper compounds, including tetracopper hexahydroxide sulphate.

Comments received during public consultation

For five of the ten copper compounds under consideration, one MSCA commented that the available genotoxicity data are insufficient to evaluate, and thus to conclude on, the genotoxic potential of copper compounds. The dossier submitter responded that in their

opinion the data do not meet the criteria for classification, but acknowledged that insufficient evidence exists to exclude a genotoxic potential via the IP route, referring also to the EFSA peer review of copper substances (EFSA, 2008) where it was concluded that genotoxicity is not of concern upon oral administration, but that there is insufficient evidence to exclude a (local) genotoxic potential upon non-oral administration.

Assessment and comparison with the classification criteria

RAC notes that no data on tetracopper hexahydroxide sulphate are available. The CLH report contains data on other copper compounds (predominantly copper sulphate pentahydrate), from which the dossier submitter proposed to read-across to tetracopper hexahydroxide sulphate. In view of the considerations presented in the section "RAC general comment", RAC has not pursued the aspect of grouping any further. RAC concludes that in the absence of relevant data no proposal for classification for germ cell mutagenicity exposure can be made for tetracopper hexahydroxide sulphate.

4.8 Carcinogenicity

Table 32: Summary table of relevant carcinogenicity studies

Method	Results	Remarks	Reference
Rat Sprague-Dawley Daily in diet Sodium copper chlorophyllin 20/sex/group exposed for 104 weeks 2/sex/group exposed for 10 weeks and 3/sex/group exposed for 52 weeks 0.1, 1 or 3% (=2.7, 27 or 80 mg Cu/kg bw/day)	3% 22% Survival *vs* 30% in control. Plasma copper level slightly elevated (303 µg/100ml *vs* 180µg/100mL in control). There were no indication of increased tumour incidence at 104 weeks	Guideline not stated No GLP Deviations: Number of animals too small to concluded on a carcinogenic potential. Numbers of organs were not examined.	Harrisson, J.W.E., Levin, S.E., Trabin, B. (1954)
Rat Sprague-Dawley Daily in diet Copper sulphate 25/sex/group for 44 weeks 530, or 1600 ppm (=27 or 80 mg Cu/kg bw/day)	1600 ppm Marked reduction in bw in comparison to control. ↓food efficiency. Moderate ↑ in blood urea nitrogen. ↑ liver, kidney, stomach weight. Icteric pigmentation and abnormal cytoplasmic staining properties of liver. ≥ 530 ppm Marked accumulation of copper levels in liver and kidneys.	Guideline not stated No GLP Deviations: No report but a published paper. Number of animals too small to concluded on a carcinogenic potential. Numbers of organs were not examined. The study duration is short: 44 weeks.	Harrisson, J.W.E., Levin, S.E., Trabin, B. (1954)
Rat Sprague-Dawley Daily in diet Copper gluconate 25/sex/group for 44 weeks 1600 ppm (=80 mg Cu/kg bw/day)	1600 ppm 90% of the animals died between the fourth and eight month. Marked reduction in bw in comparison to control. ↑ in blood urea nitrogen. Marked accumulation of copper levels in liver and kidneys. ↑ liver, kidney, stomach weight. Icteric pigmentation and abnormal cytoplasmic staining properties of liver.	Guideline not stated No GLP Deviations: No report but a published paper. Number of animals too small to concluded on a carcinogenic potential. Numbers of organs were not examined. The study duration is short 44 weeks.	Harrisson, J.W.E., Levin, S.E., Trabin, B. (1954)

Rats 4 male weanling /groups For 1, 2, 3, 6, 9, and 15 weeks In diet 2000 ppm copper (equivalent to approximately 200 mg/kg bw/day)	No deaths. ↓body weight gain.↓ liver weight. Copper content in both liver and kidney rose to maximum values in week 6, after which levels fell. Dietary administration of copper as sulphate at 2000 ppm was associated with histological changes to the liver and kidney, reaching a maximum after six weeks of treatment, followed by recovery to week 15. Initially copper accumulated with little effect, but from 2-3 weeks, histological changes were evident in both tissues. Accumulation eventually caused a crisis, associated with severe necrosis, followed by regeneration and recovery.	No guideline study No GLP Deviations: too short to be used for carcinogenicity assessment.	Haywood (1980)
Rats Wistar Male weanling Copper sulphate 4/groups for 1, 2, 3, 4, 5, 6 and 15 weeks In diet 0, 3000, 4000, 5000 or 6000 ppm approximately equivalent to 150, 200, 250, 300 mg/kg bw/day	<u>3000 ppm</u> ↓body weight gain. Liver copper concentration rose rapidly between 4 and 5 weeks but fell significantly at week 6. By week 15, copper content had fallen to the same level as at week 2. Renal copper concentration rose more slowly than in the liver, with a maximum between 4 and 5 weeks. This concentration declined very slightly to week 15. Liver and kidney damage between 2 and 5 weeks, subsequent full recovery. Renal histopathology at 3000 ppm was similar to that seen at 2000 ppm in the earlier study. <u>4000 and 5000 ppm</u> Clinical deterioration between 3 and 4 weeks and subsequent recovery. Liver and kidney damage between 2 and 5 weeks, with subsequent full recovery. The findings showed earlier onset and were correlated with the earlier liver findings. Findings were more marked. <u>6000 ppm</u> No weight gain. Two animals died in week 2. At week 6 remaining animals showed weight loss and deteriorating condition and were sacrificed. Maximum liver concentrations at week 2 and fell only by week 6. In the kidney, copper concentration rise until week 4, when it equalled the liver value. Necrotic liver changes evident in the first	No guideline study No GLP Deviations: too short to be used for carcinogenicity assessment.	Haywood, S., (1985)

	week, increased in severity to weeks 2-3, and resulted in chronic hepatitis at 6 weeks.		
Rat Male weanling Sequential kills 15, 20, 29 and 52 we Diet Copper sulphate 3000ppm for 52 weeks (250 mg Cu/kg bw/d) 3000 ppm for 15 weeks followed by 6000 ppm for 3 weeks	Animals treated with copper at 3000 ppm for one year showed no long-term evidence of liver toxicity: an adaptive response was shown similar to the earlier shorter study, and at 52 weeks, copper concentrations were lower than at 15 weeks. Animals previously treated with copper at 3000 ppm for 15 weeks that were then given 6000 ppm (double the dose) for three weeks did not show altered liver copper concentrations, whereas previously untreated rats of the same age and strain given 6000 ppm copper showed moderate to severe hepatocellular necrosis.	No guideline study No GLP Deviations: Yes This study can not be considered as a key study, as it only focus on growth rate and liver copper content. The longest of the 3 experiments(52 weeks) does not allow the assessment of the carcinogenic potential of copper.	Haywood, S., Loughran, M. (1985)
Carcinogen co-administration Rats 5/sex/groups Exposed for 16 or 19 months In diet or in finely ground maize *Liver carcinogen: p*-dimethylaminobenzene (DMAB) at 0.9% w/w Copper acetate and/or ferric citrate were also added at 0.5% and 2.0% respectively to some groups	Copper, when added to rat diets containing the known carcinogen *p*-dimethylaminobenzene significantly reduced the incidence of liver tumours, and delayed the onset of histological changes leading to cirrhosis and hyperplasia.	No guideline study No GLP The design of the study did not permit assessment of tumour incidence of copper administered alone. However, if copper were to have any carcinogenic action either alone, or as a co-carcinogen, this type of study would certainly have shown an increased incidence of tumours, and an earlier onset.	Howell, J.S. (1958)
Investigation of the effects of oral CuSO4 on the incidence of 7,12-dimethylbenz(α)anthracene (DMBA) induced ovarian tumours, tumours of the breast and lymphomas in C57BL/6J mice Mouse C57BL/6J Female 10-12 animals/group Copper sulphate pentahydrate 46 weeks Oral drinking water	The incidences of ovarian tumours after 46 weeks were 0/10, 0/12, 11/11 and 6/11 in the untreated controls, copper treated mice, DMBA-treated mice and DMBA-copper-treated mice respectively. This suggests that copper sulphate may possibly inhibit DMBA-induced tumour development. CuSO4 had no effect on the incidence of DMBA-induced adenomas of the lung, lymphomas and breast tumours.	No guideline study No GLP Purity not stated	Burki, H.R. and Okita, G.T. (1969)

198g/L			
Rat Sprague Dawley 50-58 animals/male/group 9 months Oral diet Copper sulphate The excess Cu diet contained 800 ppm Cu as $CuSO_4$	Liver necrosis (3/32) and transitional nodules in the liver (1/32) was observed at 40 mgCu/kg/bw/day whereas one kidney tumour (1/42) was observed in the low Copper group (not thought significant). Decreased body weight gain and increased mortality were found in the high copper group. Exposure to known carcinogens increased the incidence of liver necrosis and transitional nodules and each induced a similar incidence of liver tumours in rats fed excess copper or copper-deficient diets. In the DMN group, 17/30 rats on the copper-deficient diet and kidney tumours compared to 0/29 given excess copper. The incidence of AAF-induced extrahepatic neoplasms was apparently reduced by the excess copper diet. (5/30 vs 11/27).	No guideline study No GLP Purity not stated	Carlton, W.W. and Price, P.S. (1973)

4.8.1 Non-human information

4.8.1.1 Carcinogenicity: oral

Reference: Harisson (1954)
Guideline: No
GLP: No (Prior to GLP).

Deviations:

- This is not a report but a published paper in *J. American Pharm ASS.*,
- number of animals too small, with several interim sacrifices, all being not due to bad conditions,
- due to the small number of rats per group it is impossible to make any conclusion on a carcinogenic potential,
- number of organs were not examined**,**
- adrenals were not weighed at necropsy, clinical chemistry parameters not performed..

Potassium sodium copper chlorophyllin **(104 weeks, but interim sacrifices, see below).**

Twenty males and 20 females Sprague-Dawley rats were dosed with 0, 0.1, 1, and 3 % of potassium sodium copper chlorophyllin. in the diet (equivalent of 53, 530 and 1600 ppm copper in the diet or equivalent of 2.7, 27 and 80 mg Cu/kg b.w./day). The animals were observed at least three times a week for mortality and clinical observations. Body weights, food and water consumption were measured weekly.
During the course of the study 5 males and 5 females from each group were paired for mating for a period of one week.
The females were allowed to litter and rear pups to maturity. Numbers of pups born and the number raised to maturity were counted.
Haematology and urinalysis were performed at regular intervals throughout the study.
Necropsies were performed after 10 weeks (2 animals per sex per group), 52 weeks (3 animals per sex per group)and 104 weeks (up to 10 animals per sex per group) and organ weights (heart, lungs, liver, spleen, kidneys, stomach, brain, uterus, ovaries, seminal vesicles testes) were determined. Samples of liver, kidneys and spleen were examined for copper and iron content from animals killed after 10, 52 and 104 weeks.
Histopathology was performed on all animals from the 52-week kill and at terminal sacrifice. Plasma and faecal samples were taken after 62 days and analysed for copper and ‘chlorophyllin’ content.

Mortality: Control group 30 %, group 0,1 % in diet 18 %, group 3 % in diet 22 %. There is no indication, in the published paper, for the 1 % in diet group.

Bodyweight: At 3% (80 mg/kg), there is a slight decrease in comparison to controls but there were no significant differences in body weights and body weight gains in the chlorophyllin treated animals compared with the controls over the 104 weeks of the study.

Food consumption and food efficiency were similar for all groups.

Mating: Not all females were pregnant, although the period allowed for mating was only 1 week. Mean numbers of pups born were 7.2 for controls and 6.5 to 9 for the treated groups. The number of pups raised to maturity was 5.2 for the controls and 4.5 to 6.2 for the treated groups. There were no differences that could be attributed to treatment. The report does not state the duration of pre-mating treatment.

Haematology and urine examinations: There were no differences in any of the parameters measured including the oxygen carrying capacity of haemoglobin.

Plasma chlorophyllin and plasma copper:

Table 33: Plasma chlorophyllin and copper

Diet	Chlorophyllin	Copper
	µg/mL	µg/100 mL
Control	None	189
2.7 mg/kg b.w	None	174
27 mg/kg b.w	58	196
80 mg/kg b.w	116	303

Plasma copper levels were slightly elevated in the high dose group

.

Tissue stored copper: The high dose animals had a slightly higher liver copper concentration (not significant) after two years treatment compared with the controls. Kidney and spleen copper contents of the chlorophyllin treated animals were similar to the controls.

Necropsy, organ weights and histopathology: Organ weight analysis and necropsy findings at the interim and final kills were not adversely affected by treatment.

Findings at terminal kill included ventricular oedema, areas of pulmonary consolidation, occasional liver tumour and occasional cystic areas, retention cysts and minor congestion of the kidneys, pituitary tumours, hyperplasia of the lymphoid tissue of the small intestine and occasional atrophy of the reproductive organs. The study authors reported that these findings were distributed among control and test groups and were consistent with the age and strain of animals. No detail on the incidence of these tumours was available.

There were no significant differences in organ weight ratios of the chlorophyllin treated animals.

At 1600 ppm, the kidneys, liver, stomach, small intestine and spleen of animals sacrificed after 52 weeks, showed only tinctorial changes with no cell injury. All sections of control and test animals showed interstitial scarring, tubular atrophy, and dilatated tubules filled with hyaline material and minor inflammatory changes in kidney, at termination. Apart from minor adrenal cortical changes of a cystic and old hemorrhagic nature in the cortex of 2 high level animals and a small adenoma at the same dose there were no adverse effects at histopathological examination of the chlorophyllin treated animals.

There was no observation of increased tumour incidence in rats at 104 weeks.

Copper administered to rats as potassium sodium copper chlorophyllin showed moderate adverse effects following prolonged (104 weeks) dietary administration at 1600 ppm (*ca* 80 mg Cu/kg bw/day). NOAEL = 27 mg Cu/kg bw/day.

- **Copper sulphate (42 weeks)**

Twenty-five males and 25 females Sprague-Dawley rats were fed diets containing copper sulphate, equivalent in copper content to the copper in the 3 and 1% potassium sodium copper chlorophyllin diets, i.e. 1,600 ppm and 530 ppm (equivalent of 80 and 27 mg Cu/kg b.w./day), respectively for up to 44 weeks A third control group received the basal diet only. Similar data were collected throughout this study as in the study with potassium sodium copper chlorophyllin.

An interim sacrifice was carried out at 33 weeks in which 4 animals from the control group and 4 animals from the group fed 1600 ppm Cu were sacrificed. The balance of the animals was continued in the study, and all surviving animals of all groups were sacrificed at 40 – 44 weeks.

Mortality: A higher proportion of the high dose sulphate treated animals died compared to controls.

Bodyweight: The growth of animals on the high level of $CuSO_4$ was adversely affected by treatment. This was readily discernible at the 26^{th} week, when male control animals and animals receiving 530 ppm Cu weighed at least 50% more than animals on the 1600 ppm Cu intake. Animals of both sexes receiving 530 ppm copper as sulphate showed body weights that were essentially similar to controls.

Food consumption and food efficiency:. Although the intake of food was less during the first twelve weeks, the gain in weight per gram of food consumed was similar for all groups

Blood and urine examinations: Blood nonprotein nitrogen levels were high in the high dose (83 mg% with expected range = 60-70 mg%).

Tissue-stored copper: The liver copper levels were several times higher than the controls or the chlorophyllin treated animals and were produced in relatively shorter time. In the high dose sulphate treated animals showed higher levels in kidney and spleen than the chlorophyllin treated animals.

Necropsy, organ weights and histopathology: Treated animals (killed in Weeks 33 and 42) findings in the high dose groups included bronzed kidneys exhibiting sharp demarcation between the cortex and the medulla; bronzed or yellowish livers; hypertrophied ridges between the cardiac and peptic portions of the stomach, occasional ulcers and some blood; bloody mucous in the intestinal tract.

Some slight differences in the organ weight ratios in the treated animals were probably related to the lower body weights of the treated animals rather than a direct result of treatment. Stomach weight ratios of the high dose female animals were increased compared with controls.
Other organs examined were heart, lungs, liver, spleen, kidneys, uterus, ovaries, seminal vesicles, testes and brain. There were increase of liver, kidneys and stomach weights at 1600 ppm.

Histopathology was performed on the organs of animals in the 1600 ppm group (sacrificed at 30 to 35 weeks), and also on the liver, kidney and testes of animals in the 530 ppm group (sacrificed at 40 to 44 weeks). The following organs were normal in all animals: Spleen; adrenals; small intestine; large intestine; stomach; sciatic nerve. The livers of animals in the 1600 ppm group showed well-

defined abnormalities of a toxic nature in both males and females; icteric pigmentation was increased and cytoplasmic staining properties were abnormal. The kidneys of animals in the 1600 ppm group showed minor changes. Varying degrees of testicular degeneration were noted in both treatment groups; the ovaries of the females were not noticeably affected to any degree. The kidneys, liver and testes of all the control animals were found to be normal. No microscopic evidence of neoplasms was reported.

Copper administered to rats as sulphate showed adverse effects following prolonged (but limited to 44 weeks) dietary administration at 1600 ppm and a far less extent at 530 ppm (equivalent to approximately 27 mg Cu/kg bw/day). The NOAEL is ≤ 27 mg Cu/kg bw/day).

- ***Copper gluconate*** **(42 weeks)**

Guideline: No
GLP: No
Deviation: Yes

- Number of animals too small (25 males and 25 females per group). Only one or two group(s) of treated animals,
- the study duration is short 44 weeks. Due to the short duration it is impossible to make any conclusion on a carcinogenic potential,
- number of organs not convenient.

Twenty-five males and 25 females were fed diet containing copper gluconate with a copper equivalent to 1,600 ppm or 80 mg Cu/kg b.w./day up to 44 weeks.

Mortality: Ninety percent died between the fourth and eight month

Bodyweight: There was a very marked reduction of bodyweight gains, from week 8 in males and week 26 in females.

Food consumption and food efficiency: Slight variations were observed, although the intake of food was less during the first twelve weeks, the gain in weight per gram of food consumed was similar for all groups.

Blood and urine examinations: Blood nonprotein nitrogen levels were high in the high dose (109 mg% with expected range = 60-70 mg%).

Copper content of tissues: The liver copper levels were several times higher than the controls or the chlorophyllin treated animals and were produced in relatively shorter time. The very high levels seen in the gluconate treated animals correlated with the high death rate and the high blood non-protein nitrogen in these animals. In the high dose gluconate treated animals showed higher levels in kidney and spleen than the chlorophyllin treated animals.

Necropsy, organ weights and histopathology: Treated animals findings included bronzed kidneys and livers, hypertrophied limiting ridges in the stomach with occasional ulcers and bloody mucous in the intestinal tract. The stomachs of some animals were sometimes flabby and distended.

Some slight differences in the organ weight ratios in the gluconate treated animals were probably related to the lower body weights of the treated animals rather than a direct result of treatment. Stomach weight ratios of the high dose gluconate animals were increased compared with controls.

The uterus and ovary weight ratios were reduced in the gluconate treated females, and mean testis weight was slightly reduced in the gluconate treated males at 42 weeks.
There were minor histopathological changes, but not consistent, in the kidney sections of the high dose animals. Icteric pigmentation was increased in the liver with abnormal cytoplasmic staining properties.
There were no observations of increased tumour incidence

Copper administered to rats as gluconate showed marked adverse effects following prolonged (but limited to 44 weeks) dietary administration at 1600 ppm (equivalent to approximately 27 mg Cu/kg bw/day).NOAEL is < 1600 ppm or < 80 mg Cu/kg bw/day.

Table 34: Copper content of tissues (mg Cu/100 g tissue)

Week	**Dose level (%) potassium sodium copper chlorophyllin**			
	0	**0.1**	**1**	**3**
Liver – Males				
10	0.41	0.47	0.58	0.56
52	0.78	1.46	0.81	1.06
104	1.82	1.47	1.85	2.18
Liver – Females				
10	0.48	0.57	0.74	0.56
52	1.09	1.14	2.43	2.14
104	1.10	1.85	2.02	3.71
Kidney – Males				
10	1.07	1.47	1.58	1.48
52	2.08	1.52	1.83	2.11
104	3.45	2.03	2.35	2.48
Kidney – Females				
10	1.72	1.52	1.57	1.65
52	4.46	2.44	3.79	2.97
104	2.25	2.55	3.19	3.22
Spleen – Males				
10	0.96	0.52	0.40	0.68
52	1.83	2.92	3.05	2.36
104	3.38	3.34	2.75	3.01
Spleen – Females				
10	1.59	0.46	0.72	0.52
52	4.00	3.26	3.46	3.61
104	6.96	1.92	2.34	2.96
	Dose level (ppm) copper sulphate and copper gluconate			
	0	**530 sulphate**	**1600 sulphate**	**1600 gluconate**
Liver – Males				
Term	1.16	12.47	38.28	75.1
Liver – Females				
Term	1.78	32.36	45.77	56.6
Kidney – Males				
Term	2.48	3.49	15.83	59.57
Kidney – Females				
Term	3.53	6.91	12.11	54.1
Spleen – Males				
Term	3.34	5.63	13.91	12.39
Spleen – Females				
Term	4.83	5.12	6.07	13.77

The two studies of Haywood 1980 and 1985 are summarized in the repeated toxicity part.

Reference: Haywood, S., and Loughran M. (1985)
Guideline: No
GLP: No

Male weanling Wistar rats were given 3000 ppm copper as copper sulphate via the diet for one year (equivalent for 250 mg Cu/kg b.w/day). At 15, 20, 29 and 52 weeks, groups of three or four rats were weighted, killed and the livers examined. In a second experiment, sixteen male weanling Wistar rats were fed diet containing 3000 ppm copper as copper sulphate for 15 weeks. At 15 weeks, four rats were killed and the livers examined as before. The remaining animals were given a diet containing 6000 ppm copper as copper sulphate (equivalent for 500 mg Cu/kg b.w/day) for a further three weeks at which time they were also killed and the livers examined. A further 16 rats were given control diet for 15 weeks, four were killed and the livers examined, and the remaining rats were also given the diet containing 6000 ppm copper as copper sulphate. At 18 weeks the animals were killed and the livers examined.

There were no deaths reported.

In the first experiment, at 52 weeks, the control group mean body weight was 513 g, and the group mean body weight of rats receiving 3000 ppm was 433 g. Mean liver copper concentration in the treated animals was 1303 μg/g at 15 weeks and fell to 440 μg/g at 52 weeks.

In the second experiment, the change of diet at 15 weeks to 6000 ppm did not affect the condition of the 'primed' rats previously fed copper at 3000 ppm, but the unprimed group were lethargic with ruffled coats. Liver copper content of the 'primed' group did not alter significantly (1395 μg/g compared to 1342 μg/g at week 18) at the change of diet, but liver copper content of the unprimed group rose from 18.0 μg/g at week 15 to 1835 μg/g at week 18 – higher than the primed animals.
Histologically, at 15 weeks, animals that had received 3000 ppm showed complete lobular recovery with only some fine residual scarring and some hyalinised cells in the portal areas, in line with recovery seen in earlier studies. There were no further changes in primed animals receiving 6000 ppm for the additional three weeks. In the animals with no previous copper supplementation, there was moderate to severe hepatocellular necrosis with an associated inflammatory response after 3 weeks administration of diet containing 6000 ppm.

Animals treated with copper at 3000 ppm for one year showed no long-term evidence of liver toxicity: an adaptive response was shown similar to the earlier shorter study, and at 52 weeks, copper concentrations were lower than at 15 weeks. Animals previously treated with copper at 3000 ppm for 15 weeks that were then given 6000 ppm (double the dose) for three weeks did not show altered liver copper concentrations, whereas previously untreated rats of the same age and strain given 6000 ppm copper showed moderate to severe hepatocellular necrosis.

No information on tumors development was available in this study.

The following studies did not permit assessment of tumour incidence of copper administered alone but showed a beneficial effect of copper when administered together with known carcinogens. In this context, theses studies must be only be considered illustrative. However, if copper were to have

any carcinogenic action either alone, or as a co-carcinogen, this type of study would certainly have shown an increased incidence of tumours, and an earlier onset. It did neither.

Reference: Howell, J.S. (1958)
Guideline: No
GLP: No

During experiment A, groups of 5 male and 5 female rats received the known carcinogen *p*-dimethylaminoazobenzene in either standard laboratory diets or maize supplemented with ferric acid and copper acetate for their whole lifespan. Liver biopsies were performed regularly. Experiment B was performed to confirm the inhibitory effect of copper acetate. Groups of 5 male and 5 female rats received dimethylaminobenzebe (DMAB) in maize with or without ferric acid at 2% or copper acetate at 0.5%. In addition, groups with alternating feeding were included to reduce the likelihood of copper acetate interfering with DMAB absorption in the gut. The animals were sacrificed when palpable liver tumours were observed. Spleen weights were determined and histopathology of liver and spleen was conducted.

Copper, when added to rat diets containing the known carcinogen p-dimethylaminobenzene significantly reduced the incidence of liver tumours, and delayed the onset of histological changes leading to cirrhosis and hyperplasia.

It was concluded that copper has a beneficial effect in reducing the action of the carcinogen. The study indicates that copper has no carcinogenic potential when administered in the diet.

Reference: Carlton, W.W. and Price, P.S., (1973)
Guideline: No
GLP: No
Deviations: Yes

A study was carried out to determine whether a high level of Cu would have an inhibitory effect on the induction of neoplasia by acetylaminofluorene (AAF) or dimethylnitrosamine (DMN) and to determine whether the incidence of neoplasia would be increased, or whether neoplasms would appear earlier in rats fed a diet low in Cu.

Six experimental groups of Sprague-Dawley rats were included in this study. Three groups were fed a basal diet containing 1 ppm Cu (“Cu-deficient diet”) (equivalent for 0.05 mg Cu/kg bw/d) and a further 3 groups received the basal diet supplemented with $CuSO_4$ to give a Cu concentration of 800 ppm (“excess-Cu diet”) (equivalent for 40 mg Cu/kg bw/d). Within each of these two dietary regimens, one group received DMN in the drinking water and the other received AAF in the diet. Groups without these carcinogens served as controls. The initial number of animals used in each group was as follows: Cu-deficient control, 50 rats; Cu-deficient-DMN, 74 rats; Cu-deficient-AAF, 55 rats; excess-Cu-control, 58 rats; excess-Cu-DMN, 102 rats; excess-Cu-AAF, 65 rats. The numbers in each group varied because preliminary studies showed that higher DMN concentrations were toxic.

DMN was added to the drinking water for 6 months at a concentration of 50 ppm for 4 days out of every 8. Similarly, AAF was added to the diets for 6 months at a concentration of 0.06% for 4 days out of every 8.

After 90 days, 5 rats from each diet group were killed. Each 30 days thereafter, an additional 5 animals from each group were killed. Spleen, kidneys, lungs, heart, thyroid gland, adrenals,

duodenum and pancreas were taken from each animal and fixed in 10% formalin. The liver was divided into 2 portions; one of which was retained for analysis of Cu content; the other was fixed in formalin. Liver and enlarged neoplastic kidneys were weighed prior to fixation. Fixed tissues were processed, sectioned and stained with H&E for histological examination.

Liver and kidney Cu concentrations were determined by atomic absorption spectrophotometry. The analyses were run in triplicate and precautions were taken to prevent Cu-contamination of the tissues.

Rats fed the Cu-deficient control diet consistently had the highest mean bodyweights. Mean weights of other groups decreased in the following sequence: Cu-deficient-DMN; excess-Cu control and excess-Cu DMN had similar mean weights; Cu-deficient AAF; excess-Cu-AAF. AAF was considered to be markedly toxic.

After 3 months, mortality in the 6 groups was as follows:

Table 35: Mortality after 3 months and at termination

	Mortality after 3 months	Study termination
Cu-deficient control	2%	16% (minimum)
Cu-deficient-DMN	38%	57% (maximum)
Cu-deficient-AAF	15%	-
Excess-Cu control	33%	45%
Excess-Cu -DMN	69%	-
Excess-Cu-AAF	39%	54%

Macroscopic investigations showed that:

- Livers from control rats fed both Cu-deficient and excess-Cu diets were grossly normal.

The incidence of hepatic neoplasms in DMN-treated rats was similar for the Cu-deficient and excess-Cu groups. Livers of rats fed the Cu-deficient-DMN diet for 3 or 4 months varied in appearance from those that were grossly normal to those with severe macroscopic changes. Some were tan-coloured and slightly swollen. Features of livers from rats fed this diet for 5-8 months included: swelling, colour variation, presence of clear cysts, haematocysts and/or neoplasms. Livers from excess-Cu-DMN rats were either normal or slightly off-colour after 3 and 4 months. Few further changes were observed after 5 and 6 months, except for prominent capsular vessels. Cysts, swollen lobes and haematocysts occurred in livers of rats fed for 7 months. Livers from 4 rats killed after 8 months were more severely affected; haematocysts were observed in 2 livers and a neoplasm in one other.

Gross hepatic lesions were observed at monthly samplings in Cu-deficient-AAF rats. At 3 months, these included discoloration, enlargement and presence of focal pale areas. After 4 months, a few clear cysts were also present. Later, livers were pale, cystic and enlarged. Neoplasms of varying size were found in all lobes. At 3 months, the surface of the liver of one rat fed the excess-Cu-AAF diet was converted into a mass of nodules. This was also seen in one or more livers at the other autopsy periods, and was more marked on the visceral surface. Clear cysts were also present peripherally after 5 months. Increased hepatic size, cysts and small white foci appeared after 6 months. Neoplasms were larger after 7 months, and all livers at 8 months had clear cysts, neoplasms and capsular nodularity.

The numbers of hepatic neoplasms in AAF-treated rats on the Cu-deficient and excess-Cu diets were similar and it appeared that the concentration of Cu had no effect upon the incidence of

hepatic neoplasms. However, the latency period may have been slightly increased, as hepatocellular carcinomas and metastases occurred 1 month later in the excess-Cu group.

- Kidneys grossly enlarged with neoplasms were seen after 5 months in Cu-deficient-DMN rats.

The kidneys of 4/5 rats had neoplasms of various sizes. After 6 months, neoplasms were present in all 5 rats. Grossly apparent neoplasms were present in 3/5 rats examined after 7 months. Only one renal neoplasm was obvious at autopsy in 5 rats killed after 8 months. 3/13 rats on this treatment which died during the study had grossly apparent renal neoplasms.

- Abnormalities observed at autopsy in Cu-deficient-DMN rats included pale, expanding masses in the lungs of 2 rats.

Grossly detectable neoplasms were observed in the lungs of excess-Cu-DMN rats after 7 and 8 months.

Neoplasms at locations other than the liver were most numerous in Cu-deficient-AAF rats. After 5 months, 3 rats had grossly obvious neoplasms in one or more of the following locations: ventral throat area, middle of side, groin area and base of ear. After 6 months, neoplasms were noted in the lungs of 2 rats and in the spleen of another. At month 7, neoplasms were present in the ventral thorax, spleen, abdomen, perianal region, base of ear, right rear leg and small intestine.

Fewer extrahepatic neoplasms were found in excess-Cu-AAF rats (17% compared with 40% in the excess-Cu-AAF). Those that occurred werelocated at the base of the ear, along the lateral abdomen and in the lungs. It was considered that the Cu supplement acted to reduce the number of extrahepatic neoplasms.

No gross abnormalities were observed in the urinary bladder of animals in any group.

Histopathology showed that:

- Commonly occurring non-neoplastic lesions in the livers of carcinogen-treated rats included biliary-ductule cell hyperplasia, proliferation of biliary ducts and the presence of haematocysts.

Transitional nodules were localized groups of hepatocytes showing only minimal deviation of nuclear morphology and no compression of the surrounding parenchyma. Hepatomas were larger foci of hepatocytes showing changes in nuclear morphology and causing compression of the surrounding parenchyma. Hepatocellular carcinomas were large, highly cellular neoplasms showing marked alterations in nuclear and cytoplasmic morphology, containing areas of necrosis and blood cysts and invading blood and lymph vessels. In addition to hepatomas and hepatocellular carcinomas, a fibrosarcoma and cholangiocarcinoma were observed in Cu-deficient-DMN rats. Hepatomas, hepatocellular carcinomas, cholangiomas and one cholangiocarcinoma were observed in livers of Cu-deficient-AAF rats. The Cu level of the diet appeared to have no effect on the incidence rate of hepatic neoplasms.

- Fibrosarcomas, adenomas and adenocarcinomas were seen in kidneys of Cu-deficient-DMN rats.

One fibrosarcoma was found in a kidney from a rat fed the Cu-deficient control diet. No renal neoplasms were observed either grossly or microscopically in the rats from other groups killed for

autopsy. One renal adenoma was observed in a rat that died after 7 months on the excess-Cu-DMN treatment.

- Neoplasms in locations other than liver and kidneys included those of the lung, spleen, skin and -intestine.

The neoplasms observed included adnexal gland adenocarcinomas, keratoacanthomas, splenic lymphoma, alveolar-cell adenomas and adenocarcinomas, adenocarcinoma arising from the epithelium of the intestinal mucosa, squamous cell carcinomas of the skin and lungs, fibrosarcoma of the dermis and a rhabdomyosarcoma. The incidences of these neoplasms were less in rats receiving excess Cu and a carcinogen.

Table 36: Incidence of hepatic lesions and neoplasms in rats fed copper-deficient and excess-copper diets with DMN or AAF treatment and killed at monthly intervals for autopsy.

		Incidence (%)* of						
Experimental group	**Total no. of rats killed**	**Liver necrosis**	**Transitional nodule**	**Hepatomas**	**Hepatocellular Carcinomas**	**Metastase**	**Kidney neoplasm**	**Other neoplasm**
Copper deficient:								
Control	42	0.0	0.0	0.0	0.0	0.0	2.4	0.0
+ DMN	30	30.8	76.7	23.3	10.0	0.0	56.7	30.0
+ AAF	27	22.2	100.0	92.6	40.7	3.7	0.0	40.0
Excess-copper diet:								
Control	32	9.4	3.1	0.0	0.0	0.0	0.0	0.0
+ DMN	29	55.2	82.8	27.6	13.8	0.0	0.0	24.1
+ AAF	30	30.0	100.0	90.0	30.0	10.0	0.0	16.7

DMN – 50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water.
AAF – 0.06% acetylaminofluorene in the diet for 4-day periods alternating with control diet.
* Percentage of rats affected

To conclude

Liver: livers from excess-Cu control rats confirmed the occurrence of liver necrosis and transitional nodules in 3/32 and 1/32 animals, respectively. Neither of these lesions was found in the livers of animals fed a Cu-deficient diet. Exposure to DMN and AAF increased the incidence of liver necrosis and transitional nodules, and each induced a similar incidence of liver tumours in rats fed both the Cu-deficient and excess-Cu diets. It was concluded that the Cu level of the diet had no effect on the incidence of hepatic neoplasms.

Kidney: In the DMN group, 17/30 rats on the Cu-deficient diet had kidney tumours compared with 0/29 given excess Cu. There were no kidney tumours in the AAF-treated groups.

Other organs: The incidence of AAF-induced extra-hepatic tumours was apparently reduced by the excess-Cu diet (5/30, compared with 11/27 in the Cu-deficient group).

Reference: Burki, H.R. and Okita, G.T. (1969)
Guideline: No
GLP: No

A study was carried out to investigate the effects of oral CuSO4 on the incidence of 7,12-dimethylbenz(α)anthracene (DMBA)-induced ovarian tumours, tumours of the breast and lymphomas in C57BL/6J mice and of tumours of the lung in strain A mice. The study was divided into four separate experiments, designated A, B, C and D.
In all cases, CuSO4 was dissolved in drinking water at a concentration of 198 mg/l (equivalent to approximately 50 mg Cu^{2+}/l or 10 mg Cu/kg b.w/day). CuSO4-treated animals had access to the solution ad libitum over the entire experimental period.

Experiment A: CuSO4 was administered in the drinking water of 5 female mice (C57BL/6J) aged 4 – 6 months. Two weeks after commencement of copper treatment, the mice received an intravenous (i.v.) injection of 0.75 mg dimethylbenz(α)anthracene (DMBA), a known carcinogen. A second group of 5 mice received DMBA alone. Five untreated mice served as controls. The experiment was terminated 74 weeks after DMBA treatment.

Experiment B: CuSO4 was administered in the drinking water of 11 female mice (C57BL/6J) aged 12 – 15 weeks. After commencement of copper treatment, the mice received an i.v. injection of 0.75 mg DMBA.
A second group of 11 mice received DMBA alone. Ten untreated mice and 12 mice receiving CuSO4 served as controls. The experiment was terminated 44 weeks after DMBA treatment.

Experiment C: CuSO4 was administered in the drinking water of 9 female mice (strain A) aged 12 – 16 weeks. After commencement of the copper treatment, the mice received an i.v. injection of 0.75 mg DMBA and, 12 days later, an intraperitoneal (i.p.) injection of 0.5 mg DMBA. Ten other mice received 0.75 mg DMBA i.v., and 0.5 mg DMBA i.p only. Nineteen untreated mice and 12 mice receiving CuSO4 served as controls. The experiment was terminated 33 weeks after the first DMBA treatment.

Experiment D: CuSO4 was administered in the drinking water of eighteen pseudopregnant C57BL/6J female mice (i.e. virgins housed with vasectomised males), each of which also received 6 dermal applications of 0.5 ml of a 0.5% DMBA solution in olive oil at biweekly intervals. A separate group of 19 pseudopregnant females received dermal applications of DMBA, but did not receive CuSO4 in their drinking water. Eleven untreated mice and 17 pseudopregnant mice receiving CuSO4 served as controls. The experiment was terminated 50 weeks after the first DMBA treatment.

Animals in all experiments were observed daily. All mice found dead and those sacrificed were subject to post-mortem evaluation. Sections of the liver, lung, kidney, spleen, thymus, ovaries and all tumour-like structures were fixed in 10% formalin in phosphate buffer at pH 7.4. Specimens were embedded in wax, sectioned for light microscopy and stained by haematoxylin and eosin. Vaginal smears were also taken and stained with Wright's stain.

Experiments A and B: The incidences of ovarian tumours in Experiment A after 76 weeks were 0/5, 4/5, and 0/5 in the untreated controls, DMBA-treated mice and DMBA plus Cu-treated mice, respectively. The incidences of these tumours in Experiment B after 46 weeks were 0/10, 0/12, 11/11 and 6/11 in the untreated controls, copper-treated mice, DMBA-treated mice and DMBA/copper treated mice respectively. The results of these two experiments suggested that CuSO4 may inhibit DMBA-induced tumour development.

The incidences of lymphomas in Experiment A were 0/5, 1/5, and 5/5 in the untreated controls, DMBA-treated mice and DMBA plus Cu treated mice respectively. Although these results implied that incidence of lymphomas were greater in DMBA plus CuSO4-treated mice than in those receiving DMBA only, this finding could not be repeated in Experiment B (incidences of lymphoma 1/10, 2/12, 3/11 and 3/11 in the untreated controls, Cu-treated mice, DMBA-treated mice and DMBA plus Cu-treated mice, respectively). It was therefore concluded that CuSO4 had no effect on the induction of lymphomas by DMBA.

Experiment C: Tumour incidence in the 12 mice given CuSO4 alone (1 breast tumour, 2 lymphomas and no lung or ovarian tumours) was similar to that in the 19 untreated controls (2 lymphomas, no breast, lung or ovarian tumours). CuSO4 had no effect on the incidence of DMBA-induced lung adenomas (incidence 4/9 in DMBA plus Cu-treated mice and 4/10 in mice treated with DMBA only), although it appeared to prolong the survival of DMBA-treated mice (mean survival 28 weeks compared with 19 weeks in mice treated with DMBA only), and to slightly reduce the total number of tumours seen, as compared with mice given DMBA only.

Experiment D: No information was given on the tumour incidence in mice given CuSO4 alone. However, mice given DMBA plus CuSO4 had a greater number of mammary tumours (9 tumours amongst an original group of 18) than those given DMBA alone (5 tumours amongst an original group of 19). This increase was attributed to the greater longevity of Cu-treated mice.
No toxic effects were observed in otherwise untreated mice fed CuSO4 at the concentration used in these four experiments.

DMBA was injected or administered by skin paintings to C57BL/6J and to strain A female mice kept on a diet supplemented with CuSO4. It was found that CuSO4 had no effect on the incidence of DMBA-induced adenomas of the lung, lymphomas and breast tumours. CuSO4 did not prevent the induction of pre-cancerous lesions in the ovary, but may have delayed the development of granulosa cell tumours.

4.8.1.2 Carcinogenicity: inhalation

No data available.

4.8.1.3 Carcinogenicity: dermal

No data available.

4.8.2 Human information

In the VRA, a number of epidemiological studies have investigated the health hazards, including cancers (most frequently lung cancer) and non-malignant diseases, associated with occupational exposures in the copper mining, copper smelting and refining, and copper alloy industries. Most of these studies are confounded by numerous factors including co-occurring exposures to known carcinogenic compounds, such as arsenic; lack of consideration of individual exposures; failure to consider smoking status; and the use of biomarkers of copper status, such as serum copper levels, that are altered by the disease state. None of the available studies provide convincing evidence that copper plays an aetiological role in the development of cancer in humans.

Copper mining

Two cohort mortality studies of the same population of over 7000 copper miners in Tongling, China have addressed the risks associated with copper mining (Chen *et al,* 1993; 1995). In these studies, lung cancer mortality was found to be significantly increased, for underground miners and for drilling miners (both $p<0.01$). Cigarette smoking was a partial contributor to this excess mortality. Other cancers (oesophagus, stomach and liver) were also studied, but did not demonstrate a statistically significant increase. Although these were large, well-conducted epidemiological studies, the Chinese miners and smelters are subject to different genetic and environmental influences. Chinese workers are also likely to be subject to very different occupational exposures than European copper miners, such as those in Sweden. Although limited information regarding occupational exposures to dust and ionizing radiation was included in the two Chinese studies, exposure specifically to copper compounds was not measured. The results should therefore be extrapolated to European workers with caution. No well-designed epidemiological studies of European copper miners were available for review.

Copper smelting

The majority of the epidemiological studies have reported on large populations of copper smelter workers in the USA, at Anaconda in Washington State (Welch *et al,* 1982; Viren and Silvers, 1994), Tacoma in Montana (Enterline *el al,* 1995) and the Gila basin region of Arizona (Marsh *et al,* 1997; 1998). Additionally, the cancer risk of environmentally exposed residents in Arizona has been investigated by the latter authors. Other reports have described occupationally exposed populations in China (Chen *et al,* 1995), Japan, Sweden, (Welch *et al,* 1982; Viren and Silvers, 1994) and at a nickel copper smelter in Finland (Karjalainen *et al,* 1992).

Ten studies of copper smelters were identified which predominantly studied lung cancer mortality in populations of smelter workers, in most cases, focussing on the association with arsenic exposure. Potential involvement of copper in cancer mortality did not feature in any of these studies. Most of these studies demonstrated a statistically significant increase in lung cancer mortality. Of these, four out of five found a linear increase in the excess relative risk of respiratory cancer with increasing exposure to airborne arsenic (Pinto *et al,* 1978; Welch *et al,* 1982; Viren and Silvers, 1994; Lubin *et al,* 2000; Enterline *et al,* 1995). Four of these five studies were from populations in the USA, the other study analysed two published cohort studies from the USA and Sweden. It is notable that none of the available studies present exposure data for copper or other pollutants (apart from nickel exposure at a nickel/copper smelter in the study by Karjainen *et al,* 1992).

Community-based studies have reported some positive evidence for the association between lung cancer risk and reported copper smelter related employment, however there was little evidence of a positive association between lung cancer mortality and residential exposure to smelter emissions (Marsh *et al,* 1997; 1998).

Several studies have also examined mortality from other cancers. Results reported show little concordance. Some studies demonstrated no statistically significant increased mortality for other cancers (Pinto *et al,* 1978), while others demonstrated statistically significant increases in mortality due to other causes (Chen *et al,* 1995; Welch *et al,* 1982; Lubin *et al,* 2000; Enterline *et al,* 1995). There is little consistency between studies with respect to sites of excess non-respiratory cancers; urinary tract cancer (Welch *et al,* 1982), cancer of the large intestine and bone cancer (Enterline *et al,* 1995).

Copper refining

A single study has been published on mortality among 4,802 workers in nine US copper and zinc refineries with the aim of determining whether any excess mortality was associated with specific refining operations (Logue *et al* 1982). As 74% of the study population were employed in copper refining only, causes of mortality were separately analysed for this group, involving 335 decedents. [In the study report, this group is misleadingly referred to as the "cohort exposed only to copper"]. In this cohort, statistically significant increases were demonstrated for all cancers (63 observed; 61.01 expected; SMR 128) and for cancer of the digestive tract (20 observed; 15.71 expected; SMR 157). The significant excess mortality due to all cancers, including respiratory cancers, among this cohort was largely attributable to one plant which unlike the other study plants had its refinery adjacent to a smelter. A number of workers at this refinery had transferred from the smelter. It is therefore possible that previous occupational exposure could have contributed to the excess cancer mortality. This study provides no qualitative or quantitative exposure data, or data on smoking history. Consequently, association between exposure and the excess cancer mortality reported cannot be explored.

Copper alloys

A single cohort study of mortality in 347 copper cadmium alloy workers has been reported, focussing on the relationship between cadmium oxide exposure and mortality from lung cancer and non-malignant diseasea of the respiratory system (Sorahan *et al* 1995). This study showed a statistically significant increased risk of mortality from chronic, non-malignant respiratory disease in workers exposed to cadmium oxide fume, but found no increase in risk for lung cancer.

Serum copper levels and cancer

Several studies have investigated the possible association between serum copper concentrations and cancer risk. However these investigations are complicated by the fact that alterations in serum copper concentration may be related to the disease-state. Therefore epidemiological studies investigating serum copper levels only following diagnosis of cancer provide little useful information regarding the possible causal role of copper in cancer (Cavallo *et al*, 1991, Dabek *et al*, 1992, Prasad *et al*, 1992).

In the few prospective studies where copper serum levels were measured prior to diagnosis, there is no convincing evidence that dietary intake or serum-copper levels play an aetiological role in carcinogenisis. For example, Coates *et al* (1989) investigated serum copper levels in a cohort diagnosed with a range of cancers up to 10 years prior to diagnosis with cancer. This study found that there was only a positive correlation between copper levels and cancer risk in cases diagnosed fewer than 2-4 years after blood draw. In cases diagnosed more than 2-4 years after collection of the blood sample there was no statistically significant relationship. Cancer is a complex multistage process generally regarded to take many years to develop clinical features. If elevated copper serum levels were truly a risk factor for cancer, an association between copper serum levels and subsequent disease would have been expected to be maintained in cases where blood samples where taken many years prior to diagnosis. A single cohort study of over 5000 healthy women from Guernsey, studied between 1968 and 1975 investigated the influence of hormonal and other factors on breast cancer (Overvad *et al*, 1993). This study reported an association between raised serum copper levels and the risk of developing breast cancer. However, the authors concluded that elevated serum copper was probably disease mediated or an incidental association, rather than causal.

In summary, although serum copper appears to be elevated in some cancer patients and may be a potential marker of disease-state there is little or no convincing evidence that dietary copper plays an aetiological role in human cancer.

Genetic diseases in human:

Two rare genetic diseases of copper in the human provide evidence that copper is not carcinogenic following systemic absorption. These are Wilson's disease (WD) and Menkes' disease (MD). The following data were extracted from the Draft Assessment Report of copper compounds.

Wilson's disease is a defect in the ATPase for copper transport ATP7B (or WND), expressed mainly in the liver, resulting in faulty copper transport, impaired incorporation of copper into ceruloplasmin, impaired copper biliary excretion, and copper accumulation in the liver and brain. Hepatic copper levels range from 200 to 800 µg/g dry weight (normal range 20 to 50 µg/g), and patients present with hepatic cirrhosis and fatty infiltration of the liver. Urinary copper is much higher than normal (as in rats given sufficiently high oral doses to cause liver toxicity). Treatment is by chelation therapy using D-penicillamine, such that intestinal absorption is reduced, and chelated copper complexes are excreted in the urine, and liver and body levels are kept below levels at which liver disease occurs. Zinc therapy (orally as zinc sulphate) acts to induce excess metallothionein in the intestinal cells. Metallothionein has a stronger affinity for copper than zinc. The copper remains bound in the gut cells, which are then sloughed off, and the copper is lost. In the second or third decade of the disease, neurological symptoms can occur. Copper accumulation in the brain causes degeneration of the basal ganglia, resulting in defective movement, slurred speech, difficulty in swallowing, facial and other muscular spasms, dystonia and poor motor control. Depression and schizophrenia have been reported. Copper may also be deposited in the cornea (Kayser-Fleischer rings).

Menkes disease is an X-linked copper deficiency disease that is usually fatal in early childhood.
The symptoms result from a defect in the MNK protein, producing an inability to export copper from cells, particularly from the basal membrane of the small intestine, where copper is absorbed. This leads to very high concentrations of copper in sloughed intestinal cells, but the failure to export the "absorbed" copper to the bloodstream results in an effective copper deficiency for the rest of the body. The disease shows progressive mental retardation, hypothermia, seizures, poor muscle tone, feeding difficulties, jaundice, diarrhoea and a general failure to thrive. There are abnormalities of connective tissue with deformities of the skull, long bones and ribs. The hair is abnormal with a wiry texture and a spiral twist.

Both diseases result from genetic defects where the subject is unable to produce respectively the copper ATPases ATP7B and ATP7A. These are members of the human cation-transporting P-type ATPase family. The P-type ATPases are a large group of membrane proteins that utilise the energy of ATP hydrolysis to transport various ions across cell membranes. During the catalytic cycle the γ-phosphate of ATP is transferred to the invariant aspartic acid residue within the nucleotide-binding site of ATPase with the formation of acylphosphate intermediate: this property distinguishes the P-type ATPases from other cation-transporting pumps. Over 100 Ptype ATPases have been described. The loci of the encoding genes have been identified for both WD and MD. Both pump copper across cell membranes. The MD pump (ATP7A) is the pump that actually moves copper through the basal membrane of the intestinal epithelial cells so that copper enters the hepatic portal system where it binds to albumin, transcuprein and histidine to reach the liver. In the MD

subject, ATP7A is inactive, and copper from the diet accumulates in the intestinal epithelial cells, bound to induce metallothionein. The presence of copper within the cell induces the production of more metallothionein, and the copper-metallothionein complex accumulates during the life of the cell. When the cells are sloughed off into the intestinal lumen, as is the normal course of events, the cells and the copper within them are excreted in the faeces, and the copper is lost to the body. Subjects with Menkes' disease can still absorb small amounts of copper. Copper accumulates in fibroblasts and in the kidney of Menkes' disease subjects, but there is no evidence of increased incidence of cancer in these tissues either. Menkes' disease is effectively a disease of copper deficiency. In terms of risk assessment of copper in the normal human, the accumulation of copper in the intestinal epithelium on Menkes' subjects can be considered as the equivalent of an excessive oral dose of copper to the epithelial cells.

Carcinogens of the intestine may act by irritation or some other means to cause proliferation of the intestinal epithelium that eventually results in hyperplasia and tumour formation. MD subjects do not suffer from increased incidence of cancer of the intestine. This shows conclusively that excess copper in the intestinal cells does not cause cancer or long-term toxicity in that tissue. Wilson's disease (WD) involves the other ATPase previously referred to, ATP7B. In normal humans, this enzyme is primarily active in hepatocytes. It is involved in the trans-Golgi network (TGN). Copper absorbed by the hepatocyte via the inbound membrane pump hCTR1 (human copper transporter protein 1) and is bound to metallothionein within the cell. It may be bound by ATP7B to ceruloplasmin (a protein that binds up to 6 copper ions tightly and transports them to various tissues for use, including the brain. If there is excess copper in the hepatocyte, ATP7B is induced to traffic to vesicular compartments (lysosomes) and directly to the apical membrane, where copper is secreted from the cell bound to a trypsin-independent fragment of ceruloplasmin and excreted in the bile. In WD, ATP7B is inactive and the absorbed copper accumulates in the hepatocytes bound to metallothionein. The bile of WD subjects does not contain copper. In the hepatocyte, excess copper may accumulate in mitochondria, in the cytoplasm and in lysosomes, bound to metallothionein. Eventually the cell's copper storage capacity is exceeded.

Mitochondrial damage occurs and eventually the hepatocyte dies, whence the cell contents are released to the circulation, depositing copper in extrahepatic tissues. Wilson's disease thus leads to massive accumulation of copper in the liver. The disease usually manifests in late adolescence, and is ultimately fatal if not treated, but death is from liver failure, not from cancer. Treatment involves administration of penicillamine, which forms a copper complex capable of urinary excretion. There is no evidence of increased incidence of liver cancer in WD subjects. This shows that even massive accumulation of copper in the target organ, the liver, does not result in cancer in the human. Accumulation of copper leads to cell death, but this is only in the presence of excessive copper concentrations, brought about by a genetic condition resulting in the disruption of the natural homeostatic mechanisms for copper.

It should be noted that Wilson's disease is genetic, and the accumulation of copper and resulting liver failure occur under the natural levels of copper in the diet, not as a result of exposure to excessive levels of copper in the environment. However, the accumulation of copper in the liver may be taken as a model for accumulation of excess copper in a toxicity study, and the conclusion drawn that chronic high liver levels do not result in increased incidence of cancer.

Vineyard sprayer's lung: an occupational disease

Reference: Pimentel, J.C. and Marques, F. (1969)
Guideline: No
GCP: No.

Case reports of two male rural workers, whose main occupations were spraying vineyards using 'home-made' Bordeaux Mixture (solution of copper sulphate neutralised with hydrated lime) and/or cleaning the tartar from wine presses, admitted to the Thoracic Surgery Centre for investigation. In both cases tuberculosis had been diagnosed some months previously and had been treated. In one case there was improvement but not complete clearing and as his sputum was persistently negative for tubercle bacilli surgical lung biopsy was proposed. Similarly in the other case after improvement with treatment the symptoms reappeared on his return to work and lung biopsy was performed. The paper notes that the Bordeaux Mixture used to be applied to vines up to 14 times a season. The preparation of Bordeaux Mixture on the farm, from copper sulphate and lime is not relevant to the purchase of factory-prepared materials, as the home-made preparation is imprecisely neutralised, leading to excess of either copper sulphate or lime in the preparation. The home-made preparation was also applied by relatively primitive methods, e.g. by hand using a rush broom, or manual sprayers. Such practices should not be taken into account when assessing the application of modern commercial formulations with modern machinery at the significantly lower application rates (approx. 8 kg/Ha compared to >24 kg/Ha historically). The paper also describes an inhalation study in guinea pigs.

In Case 1, lung lesions had a focal distribution and corresponded to three distinct patterns; a varying number of alveoli filled with desquamated macrophages, granulomas in the alveoli septa and fibro-hyaline nodules which seemed to be the scars of the granulomas. Copper was found in the granular material contained in the intra-alveolar macrophages. Similar findings were present in Case 2. In a separate experimental study using guinea pigs, similar findings were reproduced.

This investigation showed the need for protective measures for workers while spraying and that lung biopsy was required for the correct identification of this type of condition. The fact that the condition has not been reported in the recent literature indicates that the condition was primarily associated with uncontrolled use of 'home-made' product without any protective measures, and that modern application techniques for copper products are not associated with the condition. It does highlight the need for respiratory protection.

Reference: Pimentel, J.C. and Menezes, A.P. (1975)
Guideline: No
GCP: No.

Three cases of death were examined, one was an alcoholic and all were rural workers involved with spraying vineyards using Bordeaux Mixture, a copper sulphate solution neutralised with hydrated lime (referred to in this summary as "home-made" Bordeaux mixture). All had characteristic pulmonary lesions described previously for vineyard sprayers using 'home-made' Bordeaux Mixture. Livers were examined histopathologically either at necropsy or from percutaneous biopsy material. Various staining techniques for the sections were used, including histochemically for copper. The sections were also viewed using ordinary and polarised light.

In all cases hepatic changes were found consisting of proliferation and diffuse swelling of Kupffer's cells and the formation of well defined histiocytic or sarcoid-type granulomas all with inclusions of copper. These lesions were always found near the portal tracts. The identification of copper within the lesions characterises the nature of these granulomas. The lesions were different from those observed in conditions such as primary biliary cirrhosis in which copper deposits can be found in hepatocytes; granulomas containing copper are never found. In the present condition, copper deposits were never found in the hepatocytes.

The occupational exposure to 'home-made' Bordeaux Mixture, the characteristic pulmonary lesions of vineyard sprayer's lung and the presence of copper in the liver of these patients define this new variety of hepatic granulomatosis.

Reference: Pimentel, J.C. and Menezes, A.P. (1977)
Guideline: No
GCP: No

The livers of 30 rural workers who sprayed vineyards with Bordeaux Mixture (solution of copper sulphate with hydrated lime) for periods that varied from 3 to 45 years were studied. The paper states that spraying was carried out from 15 to 100 days per year, and 600 litres of mixture were sprayed each day by each worker. As has been observed previously, these practices from more than 25 years ago, using home-made Bordeaux mixture and primitive application techniques and significantly higher application rates should not be used in a risk assessment of factory-produced copper plant protection products, applied using modern engineering equipment and protective clothing, at modern (lower) application rates.

The spleens of four of cases were also examined. All cases with other possible causes of liver damage, such as hepatitis, alcoholism etc were excluded. Several stains were used for sections including those for histochemical localisation of copper. Various light forms including conventional, polarised, phase contrast and interference microscopy were used. Normal livers were used as controls.

The pathological findings were varied and included diffuse and focal swelling and proliferation of Kupffer cells, (diagnostic, and present in all cases), histiocytic and sarcoid-like granulomata (7 cases) fibrosis of variable degree in the perisinusoidal, portal and subcapsular areas (8 cases), accompanied by atypical proliferation of the sinusoidal lining cells, one case of liver angiosarcoma, micronodular cirrhosis (3 cases) and idiopathic portal hypertension (2 cases). Abundant deposits of copper were revealed, by histochemical techniques, within pulmonary and hepatic lesions. These cases were characterised by long-term exposure. The single case of angiosarcoma was in a man who had sprayed vineyards with 'copper sulphate' from the age of 18 to 53 (35 years). The average exposure in the cases of fibrosis was 29 years, and the two cases of cirrhosis followed exposure for 28 and 30 years.

The presence of abundant deposits of copper within the liver suggest a relationship between the occupational exposure and liver disease. This is explored further in following summaries.

Reference: Villar, T.G. (1974)
Guideline: No
GCP: No

Description of 15 consecutive patients admitted to Lisbon University Hospital, and review of earlier papers (cited above). Patients were 35 to 76 years of age, average 54 years. Patients had all been exposed to Bordeaux Mixture. The periods of exposure were not stated for all subjects, but some had been exposed for over 20 years. Most had used 'manual pulverizers carried on their backs', although one subject had used a rush broom. Seven of the patients smoked, one had been exposed to pigeon droppings and another to wood dust. Lung x-rays, biopsies, autopsies (where deceased) and histopathology were performed.

The initial diagnosis was Vineyards Sprayer's Lung (VSL) in three cases, pigeon fancier's lung in one case, tuberculosis in five cases, and pulmonary granulomatosis in two cases. In all cases, VSL was subsequently noted. The paper noted that in some cases, the condition remained clinically "silent" until a bronchiopulmonary bacterial or viral infection , or exposure to some other dust triggered further progression of the disease. It is interesting that the authors made an association between lung cancer and VSL, both in the Abstract (describing it as 'remarkable') and in several places in the paper, ignoring the relationship between lung cancer and cigarette smoking. The paper contained no information as to which of the patients had smoked, only that seven of the fifteen had smoked.

Fifteen patients suffering from VSL were in some cases initially misdiagnosed, but all followed chronic exposure to Bordeaux Mixture. The authors noted that three patients also showed lung cancer, and that seven patients had smoked cigarettes, although the paper gave no information as to the smoking habits of the patients with lung cancer, preferring to emphasise a "remarkable incidence" of lung cancer in patients with VSL.

Reference: Villar, T.G. and Nogueira, T. (1980)
Guideline: No
GCP: No

The study cites a review of 20,000 autopsies of (presumably Portuguese) rural workers. Vineyard Sprayer's Lung (VSL) was identified in 832 cases (retrospectively), corresponding to 4% of all autopsies and 20% of those with respiratory symptoms.

The paper also cites 33 patients admitted to Lisbon University Hospital. There is no information in the paper to determine if some of these patients had been described previously in an earlier paper (5.9.2/04). It is worthy of note that the description of the single female in this study matches closely the single female in the previous study, and it is reasonable to assume that the fifteen cases in the earlier paper have been included in this paper. Where possible, lung function tests were performed, as were biopsies, autopsies, and histopathology.

The age range of the patients was 35 to 76 years, average 53 years. Twenty-four percent were stated to be medium to heavy smokers (8 of the 33 cases), although number of non-smokers was not stated. The single female in the study was stated to have sprayed vines from the ages of 10 to 14, and to have suffered pneumonia at the age of 50, during which she developed diffuse progressive fibrosis. She then presented with lung disease and was diagnosed with VSL. There were seven cases of lung cancer. The paper is seriously compromised in that there are no data to correlate smoking, which is known to be associated with lung cancer, and exposure to Bordeaux Mixture and VSL.

The author repeats an earlier conclusion that VSL is associated with high incidence of lung cancer, but ignores any possible association with cigarette smoking.

Reference: Plamenac, P. Santic, Z., Nikulin, A. and Serdarevic, H. (1985)
Guideline: No
GCP: No

Study of workers in the former Yugoslavia (Listica, Herzegovina) using "home-made" Bordeaux Mixture prepared by neutralising copper sulphate solution with lime. Unlike previous studies in Portugal, the study also recorded the smoking habits of the workers examined. The author

performed some particularly stomach-churning sputum analyses in workers professionally exposed to regular inhalation of Bordeaux Mixture, who at the time of investigation showed no sign of pulmonary or any other disease. Sputum specimens were obtained from 52 exposed rural workers and 51 unexposed rural workers, from the same region who did not work in vineyards and did not come into contact with copper. These acted as controls. Sputum samples were obtained by morning cough on three consecutive days. Only expectorated material containing pulmonary macrophages was accepted as sputum. Sputa samples were fixed in 75% alcohol, embedded in paraffin and sections stained with H & E. These were then tested for iron (Turnbull stain)and for copper with rubeanic acid and benzidine.

Smokers produced sputa containing abnormal columnar cells in all cases. Macrophages containing copper granules in the cytoplasm were found in 64% of workers engaged in vineyard spraying, compared to none in the control group. Sputum specimens were evaluated for eosinophils, respiratory spirals, respiratory cell atypia and squamous metaplasia. Abnormal findings were more frequent in smokers than non-smokers. Atypical squamous metaplasia was observed in 29% of smokers who were vineyard workers, but only in 5% of cases in the non-smoking vineyard sprayers. There was enhanced expectoration of sputum in a high percentage of vineyard sprayers and in smoking controls, indicating that exposure to copper and cigarette smoke affects the respiratory epithelium.

Exposure to (home-made) Bordeaux Mixture in vineyard spraying affects the sputum. Smoking appears to exacerbate the effects.

Reference: Menzes, A.P., and Pimentel, J.C. (1996)
Guideline: No
GCP: No

Abstract only. Summarises changes seen in liver of patients with Vineyard Sprayer's Lung, and notes that similar liver lesions have been recorded in the livers of workers exposed to other pathogenic dusts (cement, cork, fur, mica and wood).
The foreign material could be identified within the lesions, using appropriate histological and histochemical techniques. It would appear that inhaled particulates can be transported to the liver, and can cause liver changes.

The authors conclude that the identification of foreign materials stored by the liver can be an important diagnostic tool in inhalatory disease.

4.8.3 Other relevant information

Reference: Stoner, G.D., Shimkin, M.B., Troxell, M.C., Thompson, T.L. and Terry, L.S. (1975)
Guideline: No
GLP: No

Cupric acetate (one of several metallic compounds investigated) in 0.85% sodium chloride solution was administered by intra-peritoneal injection to groups of 10 male and female Strain A/Strong mice at dose levels of 36, 90 and 180 mg/kg body weight. The injections were given three times a week for eight weeks (24 injections). Similar groups of mice were given 0.85% sodium chloride solution (24 injections), a single injection of urethan (positive control at 20 mg/animal) or remained untreated. The mice were weighed every 2 weeks during the injection period and at monthly

intervals thereafter. They were killed 30 weeks after the first injection and their lungs removed and fixed in Tellyesniczky's fluid. After 1 to 2 days milky-white nodules on the lungs were counted; a few nodules were examined histopathologically to confirm the adenoma. Other selected organs (liver, intestines, thymus, kidney, spleen, salivary and endocrine glands) were examined histopathologically. Statistical analyses were performed.
Mean numbers of lung tumours in the vehicle and untreated control mice were similar indicating that occurrence was not significantly affected by the injections (table below). In the positive control the results demonstrated that the strain A was suitable for the induction of lung tumours. In mice treated with cupric acetate there was no statistically significant response to the numbers of tumours produced although the high dose produced a mean of 2.0. This result was based on only five surviving animals.

Table 37: Measurement of lung tumours

Treatment	Dose level (mg/kg)	Number of survivors	Animals with lung tumours (%)	Mean number lung tumours/animal
0.85% NaCl solution	NA	19/20	37	0.42
Urethan 20 mg	NA	18/20	100	21.6
Untreated	NA	19/20	31	0.28
Cupric acetate	180	5/20	60	2.00
	90	18/20	50	0.56
	36	15/20	27	0.40

The average numbers of tumours per lung increased in a dose-dependent manner but was not stastically significant. There was no evidence for any other tumors in the limited number of organs investigated. However, this study presented some deficiencies to assess carcinogenicity properties as the term of exposure, the inadequate numbers of animals, inappropriate exposure route and limited histopathological investigation.

4.8.4 Summary and discussion of carcinogenicity

Copper has been administered orally to rats in long term studies up to two years in duration. None of the studies presented below meets exactly the requirements of the International Guidelines, but they do show conclusively that copper has no carcinogenic activity.

Three types of studies have been performed:
- investigative toxicity studies demonstrating the long-term effects of very high dose levels (Haywood S., 1980 and 1985; Haywood S. and al., 1985),
- co-administration with known carcinogens to demonstrate that copper is effective at reducing the incidence and delaying the onset of tumours (Howell, J.S., 1958; Burki, H.R. and al. 1969; Carlton W.W. and al. 1973) and
- a two-year dietary administration study (Harrisson J.W.E., 1954).

The investigative toxicity studies, which were up to 52 weeks in duration, showed that dietary dose levels equivalent to 250 mg Cu/kg bw/day were associated with initial (week 6) liver damage including hypertrophied hyperchromatic parenchymal cells, necrosis and marked inflammatory

reaction, and kidney damage to the proximal convoluted tubule. Both liver and kidney showed complete recovery between 9 and 15 weeks of continued copper administration, through to scheduled termination at 52 weeks. Subsequently, these animals were able to tolerate even higher doses of copper, up to 300 mg/kg bw/day, even though this dose was lethal to naïve rats. There were no indications of pre-cancerous changes, and no tumours, up to 52 weeks administration (scheduled termination). The studies investigated high doses only; there was no attempt to derive no-effect levels.
The co-administration study was designed to show effects of copper when administered with a known liver carcinogen to two strains of rats for up to 19 months, and is one of several in the literature. The study showed that co-administration of copper significantly reduced the incidence and onset of liver tumours, which occurred at very high incidence in groups receiving the carcinogen without additional copper, and at control incidence in some groups receiving the carcinogen and additional copper. Thus copper has apparently a beneficial effect on liver cancer induction by a known carcinogen. It can also be concluded that copper has no activity as a cocarcinogen, or promoter (if copper had been a promoter, the liver tumours would have arisen earlier in the rats exposed to the carcinogen plus copper).
The two-year dietary study compared the administration of copper as sulphate or as gluconate with copper as potassium sodium copper chlorophyllin. The study showed that there was no increase in incidence of any tumour type after two years dietary administration of potassium sodium copper chlorophyllin at 3% dietary inclusion (approximately 80 mg Cu/kg bw/day).
But these studies suffered of real insufficiencies.

Copper is an essential nutrient, naturally present in almost all foodstuffs. Humans are exposed to copper in the diet from weaning as an essential micronutrient. Most western diets contain between 1 and 2 mg Cu/person/day. As such the population is exposed to copper in the diet every day. The various natural mechanisms for regulating copper in humans were described previously.

There are genetic abnormalities which lead to accumulation of copper in the liver, kidney and in the brain (Wilson’s disease), and in the intestinal epithelium, kidney and fibroblasts (Menkes’ disease). Both diseases can be fatal if not treated, but there is no evidence for increased incidence of cancer in victims of either Wilson’s or Menkes’ disease, despite the chronic high tissue copper levels.

The condition known as Vineyard Sprayer’s Lung (VSL) has been reported in several papers, mostly from Portugal, but also from the former Yugoslavia. The condition is characterised by lung lesions with a focal distribution corresponding to three distinct patterns; a varying number of alveoli filled with desquamated macrophages, granulomas in the alveoli septa and fibro-hyaline nodules which appear to be the scars of the granulomas. Hepatic changes included proliferation and diffuse swelling of Kupffer’s cells and the formation of well defined histiocytic or sarcoid-type granulomas all with inclusions of copper. These lesions were always found near the portal tracts. The identification of copper within the lesions characterised the nature of these granulomas. Copper deposits were never found in hepatocytes. The papers describe the preparation on-site of Bordeaux Mixture, as a copper sulphate solution neutralised with hydrated lime, and primitive application techniques at higher rates than those used in modern agriculture, where Bordeaux Mixture is formulated under controlled conditions in dedicated factories, and applied using modern machinery by workers wearing appropriate protective equipment. Most of the published findings date from the 1970s and 1980s. Some of the papers were compromised because the authors did not adequately describe the smoking habits of the subjects, only noting that certain subjects were heavy smokers. The Yugoslav paper surveyed smoking and non-smoking rural workers, including those which did

and those which did not use home-made Bordeaux mixture, and found that there were indications of adverse effects in users of Bordeaux Mixture that were exacerbated by smoking.

Bordeaux Mixture is a highly complex mineral mixture. If the reaction of the lime and copper sulphate is not strictly controlled, the resulting mixture may not be sufficiently neutralised, and may contain significant amounts of plaster and gypsum, in a form that if inhaled, may result in lung disease. One paper also notes that similar liver lesions to those in VSL have been recorded in workers exposed to other pathogenic dusts (cement, cork, fur, mica and wood), where the inhaled dust has been transported, presumably by macrophages, to the liver.

In theses epidemiological data analysis different confusing situation were identified (smoking, wood dust, arsenic, etc…). On the other hand, the IPCS publication (IPCS, 1998) on epidemiological studies excluded a link between Lung cancer and copper compound inhalation exposure.

Based on the limited information available in epidemiological studies, the link between Vineyard Sprayers Lung and lung cancer cannot be established.

The weight of evidence in humans and rats is that copper is not carcinogenic.

4.8.5 Comparison with criteria

1) Criteria in the CLP classification :

A substance shall be classified in category 2 for carcinogenic endpoint if the substance is suspected as human carcinogen. The placing of a substance in this category is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in category 1, based on strength of evidence together with additional consideration.

2) Comparison with criteria:

For copper compounds, no increase incidences of tumors were observed in the different animal studies by oral route. Moreover, there are two genetic conditions in human (Wilson's disease and Menkes' disease) that result in major alterations in copper absorption, distribution and excretion. Wilson's disease (where copper is absorbed in the intestine but cannot be pumped out of the liver to bile) leads to accumulation of copper in the principal target organ, the liver, and also in the kidney, brain and the cornea. People with Menkes' disease (where copper is absorbed by intestinal cells but cannot be pumped out of these cells to the hepatic portal system) can only absorb minimal amounts of copper, and show chronic accumulation of copper in the intestinal epithelium and high levels in kidney and in fibroblasts. Human subjects with these conditions may die of the condition itself (if untreated), but they do not show any increased incidence of cancer. If abnormally high levels of copper are present over long periods in an organ or tissue, yet there is no association between the high copper levels and cancer in these organs or tissues, in chronic disease, then it is reasonable to conclude that copper is not carcinogenic in these tissues.

4.8.6 Conclusions on classification and labelling

In this context, the available data do not support a classification for the carcinogenic endpoint.

RAC evaluation of carcinogenicity

Summary of the Dossier submitter's proposal

No data on tetracopper hexahydroxide sulphate are available in the CLH report. However, in light of the proposal to read-across between the different copper compounds for systemic endpoints (see section "RAC general comment" above), the dossier submitter referred in the CLH report to several long-term animal studies with other copper compounds and to human data on copper exposure.

Several animal studies administering copper compounds in either drinking water or diet of rats and mice for various periods of time (up to two years) are presented. However, none meet the guidelines for carcinogenicity testing and several have shortcomings when it comes to evaluating carcinogenicity, such as short duration. None of the studies showed an indication of carcinogenic potential of copper administered systemically. Co-administration of copper with known carcinogens appeared to lower the risk of tumour formation in some cases.

Several cohort or epidemiological studies in humans exposed to copper through copper mining, smelting and refining are briefly summarised in the CLH report. The dossier submitter concluded that they provide little evidence for increased risk of cancer with exposure to copper compounds. Reference is also made to reports of the occupational disease Vineyard Sprayer's Lungs (VSL) associated with exposure to home-made Bordeaux Mixture. Due to poor reporting and possible confounders such as smoking, the dossier submitter concluded that a link between lung cancer and VSL cannot be established. There are two rare genetic diseases of copper in humans (Wilson's disease and Menkes' disease), but there is no evidence of increased incidences of cancer in patients with either disease, despite the chronic high tissue copper levels.

The dossier submitter concluded that the weight of evidence in humans and animals is that copper is not carcinogenic and that therefore no classification for carcinogenicity is warranted for copper compounds, including tetracopper hexahydroxide sulphate.

Comments received during public consultation

No comments were received during the public consultation.

Assessment and comparison with the classification criteria

RAC notes that no data are available on tetracopper hexahydroxide sulphate. The CLH report contains some data on other copper compounds (among which copper sulphate pentahydrate), from which the dossier submitter proposed to read-across to tetracopper hexahydroxide sulphate. In view of the considerations presented in the section "RAC general comment", RAC has not pursued the aspect of grouping any further. RAC concludes that in the absence of relevant data no proposal for classification for carcinogenicity can be made for tetracopper hexahydroxide sulphate.

4.9 Toxicity for reproduction

Table 38: Summary table of relevant reproductive toxicity studies

Method	Results	Remarks	Reference
Fertility			
2-generation study Sprague-Dawley rats 30/sex/group Oral, diet Copper sulphate pentahydrate 0, 100, 500, 100 or 1500 ppm equivalent to in actual doses (P1-F1): 0, 1.53-2.65, 7.7-13.3, 15.2-26.7, and 23.6-43.8 mg/kg body weight/day	*Parental toxicity* No treatment related effect on mortality, clinical signs, bw gain, food consumption, food efficiency in either sex in any generation. At 1500 ppm: ↓ spleen weight in female. ↓ liver iron concentration in P1 females at 1500 ppm. *Fertility effects* No adverse effects on fertility, general reproductive performance or offspring viability and growth. *Offspring effects* At 1500 ppm: ↓ spleen weight in F1and F2 male and female weanlings. ↑ Brain copper concentration in F1 females and F1 and F2 male weanlings. ↓ plasma iron concentration in F2 male and female weanlings. ↑ liver copper concentration in F1 female. 1000ppm: ↑ liver copper concentration of F1 males and F1 and F2 male and female weanlings. The majority of effects are reported in weanlings and in dams at the end of lactation - the food intake and compound consumption data show that both of these “populations” were consuming significantly higher amounts of diet than towards the end of the pre-mating maturation periods, and that the spleen effects are not seen in males at termination, when compound consumption is much lower. From this it may be concluded that the spleen effects may be transient even at high doses, and that when the dietary intake i.e. dose level is reduced, the spleen effect diminishes.	OECD 416 GLP	Mylchreest, E. (2005)
Fertility (cross mating) Wistar rats 20 females/groups Gavage Copper gluconate 0, 3 or 30 mg/kg/day	No differences between treated and control groups in any of the parameters studied (pregnancy rate, implantation, resorption, live foetuses, gross fetal anomalies, duration of gestation, litter size, number of live young, gross anomalies, litter and mean pup weight throught the weaning.	No GLP	De la Iglesia F. W. *et al* (1973)
Fertility/	No adverse effects on mating performance and pregnancy rate.	No GLP	Lecyk, M.

teratology Mouse Copper sulphate 0, 500, 1000, 1500, 2000, 3000 or 4000 ppm 4000ppm correspond approximately to 570 mg/kg bw/d		Deviations: No detail given on the size of the groups. The study did not measure maternal bw gains or maternal liver histology or copper content.	(1980)
Developmental toxicity			
Teratology NZW Rabbit 22 females/group Gavage Copper hydroxide 0, 6, 9 or 18 mg/kg/day	<u>Maternal toxicity</u> 3 deaths and 2 abortions (subsequently sacrified) at 18 mg/kg/day. Animal found dead showed diarrhoea, red staining, weakness and irregular respiration. Marked initial weight loss at and above 9 mg/kg bw/d. Mean weight gain was 31% and 72% at 9 and 18 mg/kg bw/d, respectively. Marked inappetance during the initial part of the treatment period. <u>Developmental effects</u> ↓ mean foetal bw at 18 mg/kg bw/d (9% lower than control). 3 treated foetus and 1 control animal have malformations. These malformations were considered spontaneous and unrelated to treatment.↑ Incidence of foetal skeletal findings at 9 and 18 mg/kg/day.	OECD 414 GLP Purity: 61.14% w/w	Munley, S. (2003a to d)
Teratology Rat Gavage Copper gluconate 0, 0.1, 3 or 30 mg/kg/day	No maternal or developmental effects	No GLP Deviations: Partial summary. Treatment duration too short (day5-15 of pregnancy). Size of the groups not given.	De la Iglesia F. W. *et al* (1972a)
Teratology Swiss Mice Gavage Copper gluconate 0, 0.1, 3 or 30 mg/kg/day	No maternal effects. Litter parameters were not adversely affected by treatment.	No GLP Deviations: The treatment duration is too short, the methodology suffers of insufficiencies, and there was no information in the summary on examination for visceral and skeletal defects. Size of the groups not given	De la Iglesia F. W. *et al* (1972b)

Teratology Cu^{2+} as copper wire (Intra uterine device) Rat Wistar Developing foetuses were exposed to intrauterine copper from days 9 to 21 of pregnancy	There was no significant increase in the incidence of congenital malformations or growth retardation in foetuses from uterine horns containing copper coils, when compared with those from unoperated horns, sham-operated horns, or horns containing stainless-steel coils. But there were significant increases in fetal brain, fetal liver, placenta and uterine copper levels in comparison with rats containing steel coils or no coils.	No guideline No GLP Purity = 99.9% Investigation of the effects of intrauterine exposure to copper IUDs and prenatal development in the rat	Barlow, S.M., Knight, A.F. and House, I. (1981)
Teratology Cu^{2+} as copper wire (Intra uterine device) **Rat:** Holtzman strain. **Hamster:** Not stated. **Rabbit:** New Zealand White. **Rat and Hamster:** approximately 2.75 µg per day **Rabbit:** approximately 5.50 µg per day **Rat/hamster:** From day 6 of pregnancy until sacrifice of parent **Rabbit:** From day 7 of pregnancy until sacrifice of parent	No adverse effects (teratogenicity or growth and development) attributable to the exposure of parent females to copper were seen in F_1 or F_2 animals.	No guideline No GLP Purity = 99.9% Investigation of the effects of intrauterine exposure to copper IUDs and prenatal development	Chang, C.C. And Tatum, H.J. (1973)
Teratology Wistar Rat 14 mated females/group Copper acetate Oral (drinking water) 0.185% w/v (approximately 65 mg Cu/kg body weight per day). Duration: 7 weeks immediately prior to mating	Histology of maternal liver and kidney showed changed consistent with toxicity. Foetal liver and kidney histologically normal, with some delays to ossification of skeleton.	No guideline (published paper) No GLP	Haddad, D.S., Al-Alousi, L.A. and Kantarjian, A.H. (1991)

4.9.1 Effects on fertility

4.9.1.1 Non-human information

Reference: Mylchreest, E. (2005)
Guideline: OECD 416
GLP: Yes
Deviations: Yes

- Testicular histopathological examinations are not fully described

Copper sulphate pentahydrate was selected as a representative form of copper for investigation of gonadal function, effects of conception, parturition and growth/development of rats over two generations. One set of litters were produced in each generation. Five groups of 30 male and 30 female Sprague-Dawley (Crl:CD (SD)IGS) rats were given copper sulphate pentahydrate in diet (by direct admixture – no vehicle included in test substance/diet mixture) at dose concentrations of 0, 100, 500, 1000 or 1500 ppm (equivalent to 0, 1.53-2.65, 7.7-13.3, 15.2-26.7, 23.6-43.8 mg/kg body weight/day). Animals in the P1 generation were dosed for at least 70 days prior to mating, continuing through to sacrifice on test day 109-113 (males) or day 21 postpartum (females). The F1 generation were given treated diet at same test substance concentrations from day 21, for at least 70 days prior to mating and then continuing to sacrifice on test day 119 (F1 males) or day 21 post-partum (F1 female dams) or the day of weaning for F1 or F2 pups.

Fresh treated diet was prepared for each group at weekly intervals throughout the study. The untreated diet, fed to controls, was a standard rodent breeder diet – certified Rodent LabDiet 5002. Diets were sampled for assessment of homogeneity and stability at room temperature for 7 or 14 days, and under refrigerated and/or frozen storage for periods of 7, 14 or 21 days. Drinking water and standard diet were sampled for analysis of copper concentration.

For the P1 generation, 165 male and 165 female rats were obtained at approximately 8 weeks of age and in a weight range of 262-332g (males) or 166-231g (females). The rats were non-siblings. The rats were housed individually in suspended stainless steel mesh cages except for mating when males and females were housed as breeding pairs. After completion of cohabitation phase the females were individually housed in polycarbonate pans (if no evidence of copulation), or, if pregnant, returned to stainless steel mesh cages for gestation and then transferred to polycarbonate pans from day 20 of gestation and through lactation. Food and water were provided ad libitum through out the study.

For the P1 generation, the obtained rats were ranked by weight after a suitable acclimation period and then allocated to study groups using a stratified randomisation procedure to ensure group mean initial bodyweights were not statistically different. For the F1 litters, offspring were randomly selected on day 21 postpartum, one rat/sex/litter where possible, for allocation as parents for the F2 generation.

During the study cageside observations for assessment of clinical signs or evidence of moribundity/death were completed at least once daily and a full clinical examination (including handling and examination for abnormal appearance and/or behaviour) was completed weekly during pre-mating, gestation and lactation phases. Bodyweights were recorded at weekly intervals, pre-mating, and weekly thereafter for males and for females without evidence of copulation, or that did not deliver a litter. For the F1 generation, additional weights were recorded on achievement of developmental landmarks (vaginal

patency or preputial separation). During gestation and lactation the dams were weighed on days 0, 7, 14 and day 21. Food consumption was recorded, and reported weekly during the 70 day pre-mating phase for both P1 and F1 generations (values for food efficiency and daily test substance intake were derived from food consumption during this phase of the study). Food consumption was also recorded for pregnant P1 and F1 dams on days 0, 7, 14 and 21 of gestation and 0, 7 and 14 of lactation.

After approximately 10 weeks exposure to treated diet, the rats were pair-housed for breeding (1:1 with non-sibling mate), remaining together for up to two weeks or until evidence of copulation was observed. Vaginal lavage samples were analysed for oestrous cycling from all females beginning 3 weeks prior to mating period up to end of cohabitation or time of mating. Additional samples were collected at terminal sacrifice. Sperm parameters (number of motile sperm and abnormal sperm per 200 cells per animal, sperm count per cauda epididymis and per gram epididymis, spermatid count per testis and per gram testis) were evaluated from the left testis for males of both parental generations at terminal sacrifice. The right testis was preserved in Bouin's fluid for traditional histopathology.

From Day 20 of gestation, after transferring dams to polycarbonate pans, the females were examined twice daily for signs of delivery/offspring. During lactation, on days 0, 4, 7, 14 and 21, pups were handled and examined for abnormal behaviour and appearance. On day 0, live and dead pups were counted and live pups were sexed and weighed. Litters were culled to 4/sex (where possible) on day 4 when pups were again weighed and counted. Pups were weighed again on days 7, 14 and 21.

For the F1 generation, offspring (1 rat/sex/litter) from the F1 litters were selected. Developmental landmarks (vaginal patency, preputial separation) were checked.

Terminal procedures for all P1 and F1 parents involved macroscopic examination and examination of uteri for presence and number of implantation sites. Blood samples were collected from ten animals of each group. Tissues (males: testis, epididymides, prostate, seminal vesicles, coagulating glands; females: ovaries, uterus, vagina, cervix; both sexes: brain, liver, gross abnormalities, kidneys, pancreas, femur, intestines, heart.) were collected from each adult and preserved for possible histopathology.

Pups found dead during lactation and those surviving to termination were subject to gross pathological examination and the carcass preserved. From the pups culled on lactation day 4, six of each sex were selected per group and samples of brain and liver collected and stored deep frozen.

For the F1 and F2 weanlings - all showing gross abnormalities or clinical signs were subject to gross pathological examination; one pup/sex/litter was also subject to necropsy. Gross lesions and tissue from potential target organs (brain and liver) were preserved and microscopic examination of these tissues completed for F1 and F2 high dose and control pups. Blood samples were collected from ten rats of each sex from F1 and F2 males and females. Tissue samples (brain, liver, kidney, pancreas, femur, intestine and heart) were collected from the same pups and stored, after freezing in liquid nitrogen, for possible chemical analysis or microscopic evaluation.

Organ weights were collected for P1 and F1 adults males: testes, epididymides, right cauda epididymis, seminal vesicles, prostate; females: ovaries, uterus; both sexes: liver, brain, kidneys, spleen, adrenal, pituitary and thyroid. Final bodyweight data were used for calculation of relevant organ/weight ratios. No organ weights were recorded for nursing pups but liver, brain, spleen and thymus weights were recorded for one pup/sex/litter for F1 and F2 weanlings.

Tissues designated for histopathological examination included: reproductive organs, gross abnormalities, liver and brain for P1 and F1 adults – only high dose and control groups examined. In addition, reproductive organs were examined for all mated animals failing to produce a litter.

No microscopic examinations were completed for nursing offspring.
Liver, brain and gross abnormalities were examined from one pup/sex/litter for F1 and F2 weanlings of control and high dose groups only.
Quantitative assessment of primordial and growing ovarian follicles was completed for ten lactating F1 females from control and high dose groups only.

Analytical findings:

Stability evaluation indicated the test substance was stable in diet for the study duration. The test substance stability analysis indicated the test material was stable for the duration of the assay.
Homogeneity analyses indicated that the mixing procedures were adequate for the study.
Concentration assessment indicated that the nominal target dose levels had been achieved. The mean copper content of control diet was 13.7 ppm. The mean copper concentration added to test diet diets was in the range of 25 to 382 ppm (100 to 1500 ppm copper sulphate pentahydrate). Copper concentration in drinking water, analysed on two occasions during the study were 0.014 and 0.024 ppm.
Test substance achieved intake is tabulated in table 39, for the various phases of the study and for each generation.

Table 39: Summary of achieved test substance intake (mg/kg bw/day)

Group/study phase:	**Dose level (nominal ppm concentration)**			
	100	500	1000	1500
P1 males – pre-mating	1.53	7.7	15.2	23.6
P1 females – pre-mating	1.92	9.6	19.1	29.5
P1 females – gestation	1.67	8.6	17.0	26.2
P1 females – first two weeks of lactation	3.39	17.7	33.8	55.7
F1 males – pre-mating	2.25	11.5	23.5	36.1
F1 females – pre-mating	2.65	13.3	26.7	43.8
F1 females – gestation	1.69	8.5	17.1	26.5
F1 females – first two weeks of lactation	3.27	17.6	35.2	55.4

There were no clinical reactions to treatment throughout the study for the P1 male rats. The P1 females showed no clinical reaction to treatment during pre-mating, gestation or lactation at any of the four dose concentrations. Similarly there were no clinical signs of reaction to treatment for the F1 males or F1 females at any dose level or at any stage of the study.

There were no effects, considered attributable to treatment with copper sulphate pentahydrate, on either body weight or body weight gain in comparison with controls, for the males and females of the P1 generation. Occasional statistically significant increases (males) or decreases (females) were small in magnitude, of sporadic occurrence or showing no dose relationship and were considered spurious findings.
Similarly, for the F1 generation adults, there were no treatment related effects on bodyweight or weight gain in either sex at any of the dose concentrations.

While there were occasional statistically significant differences in food consumption and food utilisation efficiency (tables 40 and 41) between treated and control groups in both sexes in the P1 and F1 adult groups, these were either small in magnitude or showed no dose relationship. In summary, there were no consistent effects on food consumption of food conversion efficiency to indicate an effect of treatment for the males in either generation nor for the females, either pre-mating or during gestation/lactation.

Table 40: Food consumption P1 adults

Week	**Males (ppm)**					**Females (ppm)**				
	0	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Pre-mating (g/day)										
0-7	25.3 [0.235]	26.5 [0.233]	25.4 [0.243]	25.5 [0.241]	27.1 [0.206] *	18.6 [0.174]	20.0* [0.143]	19.4 [0.154]	19.7 [0.147]	19.7 [0.154]
7-14	25.2 [0.190]	25.9 [0.190]	25.8 [0.205]	25.3 [0.206]	26.4 [0.169]	18.7 [0.109]	19.9 [0.103]	19.4 [0.097]	18.4 [0.089]	18.6 [0.075]
14-21	25.7 [0.180]	26.2 [0.161]	26.8 [0.166]	26.7 [0.165]	26.8 [0.161]	19.1 [0.064]	20.2 [0.079]	20.6* [0.072]	19.8 [0.099]	19.5 [0.094]
21-28	27.0 [0.156]	26.6 [0.140]	27.1 [0.148]	27.0 [0.158]	26.6 [0.149]	20.4 [0.107]	21.0 [0.109]	20.6 [0.072]	19.8 [0.10]	20.0 [0.083]
28-35	27.4 [0.136]	26.9 [0.126]	28.0 [0.139]	27.4 [0.131]	27.7 [0.121]	19.9 [0.053]	20.6 [0.053]	20.6 [0.084]	20.2 [0.067]	20.7 [0.074]
35-42	27.6 [0.108]	27.1 [0.114]	27.5 [0.115]	26.9 [0.109]	26.7 [0.113]	19.6 [0.047]	20.3 [0.031]	19.2 [0.002]*	19.4 [0.041]	20.1 [0.027]
42-49	27.4 [0.106]	26.9 [0.104]	27.3 [0.111]	26.6 [0.102]	26.3 [0.115]	18.6 [0.051]	19.3 [0.062]	18.5 [0.079]	18.6 [0.044]	19.4 [0.064]
49-56	27.2 [0.074]	27.1 [0.075]	27.3 [0.067]	26.9 [0.069]	27.2 [0.062]	18.7 [0.037]	19.5 [0.063]	18.9 [0.045]	19.1 [0.043]	19.4 [0.018]
56-63	26.4 [0.074]	27.6 [0.087]	28.2* [0.087]	27.4 [0.077]	27.3 [0.039]	18.6 [0.043]	18.9 [0.052]	18.9 [0.048]	19.5 [0.062]	19.8 [0.067]
63-70	26.3 [0.059]	27.0 [0.061]	28.0* [0.062]	27.8* [0.073]	27.6 [0.070]	18.8 [0.042]	19.6 [0.047]	19.5 [0.023]	20.2 [0.043]	20.3* [0.026]
During gestation (g/day)										
0-7						23.1 [0.218]	23.7 [0.214]	23.9 [0.212]	23.3 [0.215]	24.7 [0.209]
7-14						24.1 [0.170]	25.4 [0.160]	25.8 [0.170]	26.0 [0.175]	25.6 [0.171]
14-21						23.6 [0.428]	23.9 [0.400]	25.0 [0.409]	24.4 [0.413]	25.4 [0.428]
0-21						23.5 [0.272]	24.3 [0.257]	24.9 [0.264]	24.6 [0.265]	25.2* [0.270]
During lactation (g/day)										
0-7						35.9 [0.059]	38.3 [0.071]	40.2 [0.079]	37.8 [0.045]	42.7* [0.059]
7-14						49.7 [-0.002]	53.5* [0.008]	56.8* [0.009]	53.1 [0.007]	58.7* [0.006]
0-14						42.8 [0.025]	45.9 [0.035]	48.5* [0.037]	45.5 [0.025]	50.7* [0.028]
[ ] food conversion efficiency {grams weight gain/grams food consumed}										

* Statistically significantly different from controls p < 0.05

Table 41: Food consumption F1 adults

Week	Males (ppm)					Females (ppm)				
	0	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Pre-mating (g/day)										
0-7	14.7 [0.427]	14.9 [0.434]	15.8 [0.413]	14.9 [0.410]	15.3 [0.400]	14.1 [0.384]	13.6 [0.397]	14.1 [0.392]	13.4 [0.380]	14.1 [0.360]
7-14	19.9 [0.401]	20.8 [0.394]	22.1* [0.379]	21.1 [0.397]	21.9 [0.377]	18.1 [0.325]	18.3 [0.319]	19.5 [0.310]	19.3 [0.315]	20.8 [0.287]*
14-21	23.1 [0.362]	24.2 [0.352]	25.3* [0.333]*	24.3 [0.352]	25.3* [0.333]*	19.7 [0.239]	21.1 [0.221]	22.4* [0.219]	21.0 [0.243]	21.8* [0.221]
21-28	25.4 [0.344]	26.4 [0.336]	26.8 [0.335]	27.2 [0.326]	26.9 [0.309]*	19.7 [0.178]	20.3 [0.159]	20.4 [0.158]	20.6 [0.175]	22.2* [0.150]
28-35	27.2 [0.295]	28.5 [0.282]	28.6 [0.272]*	28.8 [0.258] *	29.6 [0.253]*	19.4 [0.153]	21.5 [0.153]	21.8* [0.142]	21.4 [0.151]	22.0* [0.127]
35-42	27.3 [0.240]	29.1 [0.230]	28.7 [0.228]	28.8 [0.228]	29.9 [0.204]	20.2 [0.124]	21.8 [0.122]	21.5 [0.132]	21.5 [0.123]	23.4 [0.114]
42-49	28.8 [0.186]	29.0 [0.189]	29.2 [0.183]	29.5 [0.171]	29.3 [0.177]	20.8 [0.117]	22.2 [0.099]	21.4 [0.099]	22.3 [0.094]	23.4 [0.090]
49-56	28.5 [0.156]	28.9 [0.162]	28.9 [0.148]	30.2 [0.160]	29.0 [0.145]	20.8 [0.079]	21.8 [0.105]	21.4 [0.089]	21.1 [0.075]	21.8 [0.069]
56-63	28.0 [0.124]	29.1 [0.140]	28.5 [0.126]	29.1 [0.120]	29.3 [0.128]	21.4 [0.083]	21.4 [0.063]	22.0 [0.080]	23.9 [0.089]	24.0 [0.066]
63-70	27.6 [0.112]	28.8 [0.126]	28.5 [0.122]	29.3 [0.117]	28.9 [0.119]	21.4 [0.069]	21.3 [0.072]	20.2 [0.060]	20.6 [0.054]	21.1 [0.057]
During gestation (g/day)										
0-7						23.1 [0.229]	23.5 [0.213]	23.9 [0.212]	23.4 [0.223]	23.8 [0.219]
7-14						24.3 [0.176]	24.3 [0.164]	24.6 [0.162]	25.5 [0.182]	25.0 [0.165]
14-21						25.0 [0.430]	24.1 [0.458]	25.1 [0.445]	24.4 [0.422]	24.5 [0.447]
0-21						24.1 [0.280]	23.9 [0.278]	24.5 [0.270]	24.4 [0.276]	24.4 [0.277]
During lactation (g/day)										
0-7						35.8 [0.047]	37.3 [0.078]	42.4* [0.070]	40.5 [0.060]	45.9* [0.062]
7-14						52.0 [0.019]	50.3 [-0.029]	54.7 [-0.007]	54.7 [-0.027]	54.8 [-0.018]
0-14						43.9 [0.032]	43.8 [0.018]	48.5* [0.028]	47.6 [0.014]	50.3* [0.026]

[ ] food conversion efficiency {grams weight gain/grams food consumed}
* Statistically significantly different from controls p < 0.05

There were no treatment-related effects on any of the sperm parameters investigated for males in either the P1 or F1 generation.

The mean percent number of days in oestrus, dioestrus or proestrus were unaffected in either the P1 or F1 generations. The total mean cycle length was similarly unaffected by treatment with copper sulphate pentahydrate. The total number of days spent in oestrus was slightly higher for the P1 females dosed at 1000 or 1500 ppm (47 and 40% respectively) in comparison with controls (30%) but since there were no effects on mean oestrous cycle length nor any adverse reproductive changes, this minor change was not considered to be biologically significant.
At termination the distribution of oestrous cycle stages was similar for P1 and F1 females and no treatment effect was postulated.

For the P1 and F1 generations there were no treatment-related effects on any of the reproductive indices investigated at any of the four dose concentrations (tables 42 and 43). These included precoital interval length, mating and fertility indices, gestation length, the number of implantation sites and the implantation efficiency.

Table 42: P1 adult reproductive performance

Group	**1**	**2**	**3**	**4**	**5**
Treatment (ppm)	**Control**	**100**	**500**	**1000**	**1500**
Males: n	30	30	30	30	30
Number mating	27	30	28	28	29
Mating index%	90.0	100	93.3	93.3	96.7
Females: n	30	30	30	30	30
Number pregnant	25	29	27	25	27
Fertility index (%)	92.6	96.7	96.4	89.3	93.1
Mean gestation length (days)	22.2	22.4	22.3	22.3	22.4
Total resorption	0	0	0	0	0
Mean number of implantation sites per pregnant female	14.5	14.1	13.9	14.0	13.8
Number of pregnant females	25	29	27	25	27
Implantation efficiency (%)	93.3	92.4	91.6	93.2	91.7
Mean number of pups born per litter	13.6	13.2	13.0	13.1	13.6

Table 43: F1 adult reproductive performance

Group	**1**	**2**	**3**	**4**	**5**
Treatment (ppm)	**Control**	**100**	**500**	**1000**	**1500**
Males: n	30	30	30	29	30
Number mating	29	30	30	29	30
Mating index%	96.7	100	100	100	100
Females: n	30	30	30	29	30
Number pregnant	28	30	28	25	30
Fertility index (%)	96.6	100	93.3	86.2	100
Mean gestation length (days)	22.2	22.2	22.2	22.2	22.3
Total resorption	0	0	0	0	0
Mean number of implantation sites per pregnant female	15.0	14.5	14.7	14.0	14.2
	28	30	27	25	30

Number of pregnant females					
Implantation efficiency (%)	94.7	95.8	93.8	95.6	91.7
Mean number of pups born per litter	14.2	13.9	13.8	13.3	13.2

Treatment with copper sulphate pentahydrate had no effect on the number of pups born, the number of liveborn pups or the numbers of pups surviving to 4, 7, 14 or 21 days post-partum (tables 44 and 45). In either generation, F1 or F2 offspring, there were any treatment-related effects on the sex ratio within litters, or survival indices during lactation at any of the dose concentrations tested.

Table 44: Litter data for F1 pups

Group	**1**	**2**	**3**	**4**	**5**
Treatment (ppm)	**Control**	**100**	**500**	**1000**	**1500**
Number of pregnant females	25	29	27	25	26
Mean litter size – birth	13.6	13.2	13.0	13.1	13.6
Mean number live born	13.6	13.1	12.7	12.9	13.5
Mean number of pups pre-culling on day 4	13.4	12.9	13.2	12.8	13.4
Mean number of pups per litter post day 4 culling	7.8	7.8	7.9	7.9	8.0
Mean number of pups per litter on day 7	7.8	7.8	7.9	7.9	8.0
Mean number of pups per litter on day14	7.8	7.8	7.9	7.9	8.0
Mean number of pups per litter on day 21	7.8	7.8	7.9	7.9	8.0
Sex ratio (% males)	52	48	53	49	50
Gestation index (% litters with at least one live pup)	100	100	100	100	100
Mean percent born alive	99.5	98.9	95.2	98.9	99.5
Viability Day 0-4 (%)	98.6	98.8	99.5	98.9	99.2
Lactation index	99.5	100	99.5	100	100
Litter survival (% litters with at least one pup alive at day 21)	100	100	100	100	100
Mean pup weight (g) –					
Day 0	6.6	6.7	6.7	6.5	6.7
Day 4 pre-culling	10.7	11.3	11.0	10.7	11.1
Day 4 post-culling	10.7	11.3	11.0	10.8	11.1
Day 7	17.3	18.5	18.0	17.2	17.8
Day 14	34.8	36.4	36.2	34.7	35.7
Day 21	57.8	59.5	59.0	55.7	57.0

Table 45: Litter data for F2 pups

Group	**1**	**2**	**3**	**4**	**5**
Treatment (ppm)	**Control**	**100**	**500**	**1000**	**1500**
Number of pregnant females	28	30	27	24	30
Mean litter size – birth	14.2	13.9	13.8	13.3	13.2
Mean number live born	14.1	13.7	13.7	13.2	13.1

Mean number of pups pre-culling on day 4	13.9	13.6	13.3	13.0	13.0
Mean number of pups per litter post day 4 culling	8.0	8.0	7.8	8.0	7.8
Mean number of pups per litter on day 7	8.0	8.0	7.8	8.0	7.8
Mean number of pups per litter on day14	7.9	8.0	7.8	8.0	7.8
Mean number of pups per litter on day 21	7.9	7.9	7.8	8.0	7.7
Sex ratio (% males)	49	53	56	49	51
Gestation index (% litters with at least one live pup)	100	100	100	100	100
Mean percent born alive	99.5	98.9	99.7	99.0	99.3
Viability Day 0-4 (%)	98.3	99.3	96.2	98.5	99.4
Lactation index	99.6	99.2	100	99.5	99.6
Litter survival (% litters with at least one pup alive at day 21)	100	100	100	96.0	100
Mean pup weight (g) – Day 0 Day 4 pre-culling Day 4 post-culling Day 7 Day 14 Day 21	 6.3 10.2 10.2 16.8 34.2 56.0	 6.4 10.9 10.9 17.7 35.3 58.1	 6.5 10.7 10.6 17.6 36.5 58.4	 6.4 11.0 10.9 17.7 35.1 57.3	 6.6 10.8 10.9 17.5 35.4 56.7

Clinical signs were noted among the pups of the F1 or F2 generation but at low incidence and showing no dose-relationship. The clinical observations were not considered to be treatment-related or toxicologically significant.

An increase in mean pup weight in F1 litters dosed at 100 ppm (low dose) on lactation day 7 was not treatment-related since there were no other dose correlations. There were no treatment-related effects on pup weight at any dose levels for the F1 or F2 offspring.

There were no treatment-related effects on preputial separation for F1 males at any dose level. For the F1 females the mean age at vaginal opening was increased for the high dose group (1500 ppm) in comparison with concurrent controls – 33.6 versus 32.1 days. However, the historical control data for this parameter indicates a mean vaginal opening time of 32.3 days and a minimum and maximum range of 31.3 to 33.9 days. Hence the difference was small (1.5 days) and well within the range of historical control data. The apparent slight delay in vaginal opening was not considered an effect of treatment with copper sulphate pentahydrate.

Pathology findings:

There were no significant differences between the high dose (1500 ppm) and control groups in respect of total numbers for primordial and pre-antral follicles.

There were no test-substance related deaths during the course of the study. Of the 120 P1 and 120 F1 males, only one was sacrificed *in extremis* with a fractured nose (killed on day 14). From the same number of P1 and F1 females, only three rats died during the study. One was sacrificed *in extremis* on day 119 due to dystocia; one was found dead on day 17 – the cause

of death being pyelonephritis and one was sacrificed *in extremis* on day 119 but the cause of morbidity was not established.

For the adult P1 rats there was a small decrease in mean absolute and relative spleen weight (circa 9% reduction compare with controls) in the high dose group (1500 ppm). The effect was statistically significant for females. While there were no significant differences among the males, the trend for a slight reduction in spleen weight at higher doses was evident. None of the other organs weighed for P1 animals showed any effect of treatment. Results are summarised in table 46.

For the F1 weanlings of the high dose group (1500 ppm), small decreases in absolute (9%) and relative (10-11 %) spleen weight were apparent in comparison with controls. None of the other organs weighed for F1 weanlings showed any effect of treatment. Results are summarised in table below.
There were no changes in organ weight among the F1 adults that were considered attributable to treatment
For the F2 weanlings of the high dose group (1500 ppm), small decreases in absolute (10% males, 15% females) and relative (10% males, 15% females) spleen weight were apparent in comparison with controls. The high dose group effects were significantly lower than the controls. None of the other organs weighed for F2 weanlings showed any effect of treatment. Results are summarised in table below.

Table 46: Summary of spleen weights for males and females in P1, and for F1 and F2 weanlings

	Males					**Females**				
Dose concentration (ppm)	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
P1 adults										
Final body weight	595.4	600.1	603.9	599.5	586.8	328.8	332.2	335.8	333.3	331.9
Absolute spleen weight [g]	0.866	0.887	0.892	0.881	0.841#	0.643	0.629	0.639	0.605	0.586#
Relative spleen weight [g/100g bw]	0.146	0.148	0.148	0.147	0.143	0.195	0.190	0.190	0.182	0.177*
F1 adults										
Final body weight	593.5	619.2	600.0	598.5	584.7	326.0	328.2	335.0	332.9	329.2
Absolute spleen weight [g]	0.897	0.887	0.867	0.900	0.841	0.624	0.641	0.632	0.642	0.612
Relative spleen weight [g/100g bw]	0.151	0.143	0.145	0.150	0.145	0.192	0.195	0.189	0.193	0.186
F1 weanlings										
Final body weight	58.3	60.1	60.9	56.6	58.7	54.5	56.8	56.2	53.5	55.3
Absolute spleen weight [g]	0.256	0.290	0.280	0.238	0.232#	0.245	0.283*	0.265	0.236	0.223#
Relative spleen weight [g/100g bw]	0.439	0.477	0.460	0.417	0.394	0.449	0.498*	0.470	0.429	0.401
F2 weanlings										
Final body weight	56.9	59.3	59.2	59.8	57.3	54.6	56.8	56.8	55.3	54.7
Absolute spleen weight [g]	0.253	0.269	0.254	0.252	0.227#	0.254	0.265	0.252	0.243	0.217*

Relative spleen weight [g/100g bw]	0.440	0.451	0.430	0.421	0.397*	0.462	0.465	0.444	0.440	0.396*

considered to be a treatment-related effect (decreased weight).
* Statistically significantly different from controls $p < 0.05$

The small decrease in spleen weight for F1 and F2 weanlings was considered an effect of treatment although weanling spleen weights are highly variable (e.g statistically significant increase in absolute weight (+16 %) for the low dose F1 females). The effect could be considered adverse, in the absence of any confirmatory microscopic examinations. However, the effect may reflect a transient physiological change such as a marginal decrease in sinusoidal dilatation. The pathologist's review of data indicated the ranges for the high dose spleen weights were similar to control ranges. Of 111 weanlings in the high dose group, only 4 had spleen weights that were lower than the control range. There were no treatment-related effects on thymus weight to indicate a test substance related effect on the lymphoid system. Extramedullary haematopoiesis in the livers of control and high dose weanlings was normal suggesting the haematopoietic system was unaffected by treatment.
The other ogan weights showed no changes that were considered attributable to treatment with copper sulphate pentahydrate.

There were no treatment-related changes apparent during necropsy of the P1 adult rats, F1 adults or F1 and F2 weanlings or F1 and F2 pups.
All macroscopic observations in the adult rats, P1 or F1, were within the range of normal background lesions. Among the F1 and F2 weanlings the incidence of gross lesions was low and observations were randomly distributed across control and treated groups. For the F1 and F2 pups, the observations of non-expanded lungs or no milk spot in stomach were considered non-specific lesions that are commonly observed among stillborn pups and were therefore not considered to be an effect of treatment with copper sulphate pentahydrate.

All microscopic findings seen in the P1 adults, F1 adults or F1 and F2 weanlings were considered to be incidental and common background lesions for the strain of rat used in the study. There were no treatment-related histopathological changes in liver, brain or reproductive organs.

Eighteen P1 and nine F1 pairs failed to produce litters. The cause of reproductive failure in 22 of these pairs was not determined. One F1 female had dystocia and in three of the P1 females there was an absence of recent corpora lutea in the ovaries. None of the breeding failures were considered attributable to test substance administration.

Tissue metal concentrations:

Specifically assessments of copper, iron, manganese and zinc concentrations were investigated in liver and brain and plasma for each subset of animals. Results were as follows.

For the P1 males there were no test-substance related changes in copper, iron, manganese and zinc concentrations at any dose level. Plasma samples were not obtained for these animals. There was a decreased in liver iron concentration in the high dose group but this was not considered a treatment effect due to high inter-individual variability and a lack of consistency with the female response and an absence of any dose relationship.

The P1 females dosed at 1500 ppm had a treatment-related increase in liver copper concentration and a decrease in liver iron concentration. Copper and iron levels in the brain

were unaffected and there were no changes in manganese or zinc concentrations in liver, brain or plasma.

For the F1 adult males, liver copper concentration was increased in groups dosed at 1000 or 1500 ppm. Compared with the magnitude of similar changes seen in the P1 generation, the effects in F1 males were small but in comparison with controls, some individuals showed a 2-3 fold increase and the effect was considered attributable to treatment. Copper concentrations in brain or plasma were unaffected by treatment at any dose level. There were no treatment-related changes in iron, manganese or zinc concentrations in liver, brain or plasma at any dose level.

For the F1 adult females, liver and brain copper concentrations were increased at 1500 ppm. Copper concentration in plasma was not affected at any dose level. There were no treatment-related changes in iron, manganese or zinc concentrations in liver, brain or plasma at any dose level.

For the F1 and F2 weanlings, there was a treatment-related increase in liver copper concentration for males and females dosed at 1000 and 1500 ppm in each generation. Brain copper concentrations were slightly increased for the males (but not females) dosed at 1500 ppm in each generation. No plasma data were available for the F1 weanlings and there were no changes in plasma copper concentration for the F2 weanlings. A treatment-related decrease in plasma iron concentration was evident for the male and female F2 weanlings dosed at 1500 ppm. Changes in manganese and zinc concentrations in liver, brain or plasma were all considered to be spurious since they showed no dose relationship, had high inter-individual variability or the changes were small in magnitude.

In summary, (tables 47, 48, 49 and 50), the concentration of copper in the liver of F1 males and F1 and F2 male and female weanlings dosed at 1000 and 1500 ppm was increased. The concentration of copper in the liver of P1 and F1 females dosed at 1500 ppm was also increased. Copper concentrations in the brain were increased for F1 females and F1 and F2 male weanlings dosed at 1500 ppm. The concentration of iron in the liver of P1 females dosed at 1500 ppm was decreased and plasma iron concentration was decreased in F2 male and female weanlings in the 1500 ppm dose group.

Table 47: Summary of tissue concentrations of copper, iron, manganese and zinc in brain, liver or plasma for males and females P1

	Males – P1					**Females – P1**				
Dose concentration (ppm)	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Copper (ppm)										
Liver	6.44	4.47	5.20	5.60	5.98	4.76	5.30	5.46	5.67	8.73*
Plasma	--	--	--	--	--	1.43	1.36	1.35	1.48	1.38
Brain	3.27	3.46	431	4.98	3.26	3.17	3.41	3.58	2.93	3.38
Iron (ppm)										
Liver	158	143	155	143	128*	150	151	138	150	107*
Plasma	--	--	--	--	--	2.96	2.90	3.24	3.17	3.32
Brain	22.4	24.4	26.2	22.8	24.9	20.4	21.0	20.8	19.9	18.9
Manganese (ppm)										
Liver	2.45	2.26	2.62	2.75	2.34	3.46	3.49	3.20	3.52	3.56
Plasma	--	--	--	--	--	--	--	--	--	--

Brain	0.451	0.525	0.534	0.459	0.573	0.419	0.438	0.411	0.433	0.422
Zinc (ppm)										
Liver	33.1	31.6	33.8	32.4	28.7	28.8	28.5	29.4	30.5	29.2
Plasma	--	--	--	--	--	1.94	1.90	1.93	1.86	1.72
Brain	17.5	16.6	16.8	16.3	16.6	14.7	14.1	14.7	15.4	13.4

* Statistically significantly different from controls $p < 0.05$

Table 48: Summary of tissue concentrations of copper, iron, manganese and zinc in brain, liver or plasma for males and females F1 adults

	Males – F1 adults					**Females – F1 adults**				
Dose concentration (ppm)	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Copper (ppm)										
Liver	4.56	4.87	6.16	7.36*	7.53*	5.70	5.16	5.36	5.35	15.3*
Plasma	1.24	1.38	1.28	1.51	1.44*	1.49	1.52	1.34	1.50	1.37
Brain	2.59	2.64	2.83	3.11*	2.80	2.89	2.93	3.00	3.23	3.49*
Iron (ppm)										
Liver	121	133	124	143	110	149	149	163	116	133
Plasma	2.41	2.27	2.72	2.35	2.88	3.46	4.00	4.01	3.56	4.12
Brain	18.5	18.4	16.5	17.2	17.5	18.5	18.4	21.2	20.6	19.9
Manganese (ppm)										
Liver	1.93	2.00	2.20	2.14	1.82	3.46	3.18	3.28	3.06	3.64
Plasma	--	--	--	--	--	--	--	--	--	--
Brain	0.355	0.350	0.343	0.376	0.319	0.368	0.394	0.405	0.412	0.438*
Zinc (ppm)										
Liver	26.7	27.8	32.4*	28.3	25.8	31.9	30.7	32.6	27.9	32.2
Plasma	0.971	0.916	0.989	1.019	1.080	1.93	1.87	2.03	1.54*	1.80
Brain	13.3	13.4	14.1	14.0	12.1	14.6	15.1	15.0	15.1	15.4

* Statistically significantly different from controls $p < 0.05$

Table 49: Summary of tissue concentrations of copper, iron, manganese and zinc in brain, liver or plasma for males and females F1 weanlings

	Males – F1 weanlings					**Females – F1 weanlings**				
Dose concentration (ppm)	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Copper (ppm)										
Liver	14.7	24.2	25.2	50.0*	82.7*	21.5	22.8	23.5	53.1*	86.8*
Plasma	--	--	--	--	--	--	--	--	--	--
Brain	2.26	2.27	2.28	2.44	2.59*	2.43	2.35	2.43	2.40	2.60
Iron (ppm)										
Liver	33.9	33.0	32.0	37.4	36.1	33.6	36.1	38.3	39.5	37.4
Plasma	--	--	--	--	--	--	--	--	--	--
Brain	11.1	15..3	13.1	11.5	11.0	16.6	12.4	13.7	11.3	12.8
Manganese (ppm)										
Liver	2.01	1.95	2.01	2.08	2.27	2.08	2.13	1.96	2.18	2.23
Plasma	--	--	--	--	--	--	--	--	--	--
Brain	0.500	0.503	0.527	0.561	0.565	0.562	0.505	0.522	0.539	0.629
Zinc (ppm)										

Liver	31.4	31.5	35.2	37.4*	36.8*	32.5	31.8	34.3	39.7*	37.3*
Plasma	--	--	--	--	--	--	--	--	--	--
Brain	13.8	13.6	14.1	14.2	14.5	14.1	13.9	14.5	15.4	14.4

* Statistically significantly different from controls $p < 0.05$

Table 50: Summary of tissue concentrations of copper, iron, manganese and zinc in brain, liver or plasma for males and females F2 weanlings

	Males – F2 weanlings					**Females – F2 weanlings**				
Dose concentration (ppm)	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Copper (ppm)										
Liver	16.4	30.2	28.0	47.6*	64.3*	24.9	21.5	27.9	38.8*	53.5*
Plasma	0.526	0.533	0.582	0.543	0.554	0.581	0.550	0.587	0.573	0.543
Brain	2.55	3.12	2.35	2.49	3.24*	2.59	2.63	2.52	2.41	2.78
Iron (ppm)										
Liver	33.8	37.0	37.1	36.8	29.8	41.8	38.1	39.1	42.2	35.2
Plasma	3.20	3.98	2.78	2.73	1.55	3.21	3.54	3.19	2.54	1.41*
Brain	11.1	11.4	11.8	11.0	10.0	11.6	11.1	12.5	11.4	10.7
Manganese (ppm)										
Liver	2.04	2.03	2.03	2.06	2.24	2.12	1.92	2.21	2.03	2.30
Plasma	--	--	--	--	--	--	--	--	--	--
Brain	0.490	0.535	0.465	0.510	0.570*	0.479	0.555	0.524	0.521	0.570*
Zinc (ppm)										
Liver	30.9	30.3	33.3	29.8	31.2	34.2	27.3*	31.6	33.2	31.7
Plasma	2.07	2.30	2.07	2.42	2.04	2.38	2.19	2.15	1.95	2.18
Brain	14.7	15.2	14.8	14.7	16.5*	15.6	15.1	15.1	15.0	14.5

* Statistically significantly different from controls $p < 0.05$

Overall summary of findings.

There were no effects considered to be related to copper sulphate treatment on the following parameters at any concentration (100 to 1500 ppm):

- Mortality and clinical signs of toxicity in P1 and F1 males and females
- Body weights, weight gain, food consumption, food efficiency in P1 and F1 males and females
- Sperm and estrous cycle parameters in P1 and F1 males and females
- Mating, precoital interval, fertility, gestation length, number of implantation sites, and implantation efficiency in the P1 and F1 generations
- Number of pups born, born alive, alive on day 4, 7, 14, or 21, sex ratio, and survival indices during the lactation period in F1 and F2 litters
- Body weights and clinical observations in F1 and F2 litters during lactation
- Age at preputial separation in F1 males and vaginal opening in F1 females
- Ovarian follicle counts in F1 females
- Weight of testes, epididymides, right cauda epididymis, seminal vesicles, prostate, ovaries, uterus, thyroid gland, brain, liver, adrenal glands, kidneys and pituitary in P1 and F1 males and females; Weight of liver, brain and thymus in F1 and F2 weanlings; Weight of the spleen in P1 males and F1 males and females
- Gross observations in P1 and F1 adults and F1 and F2 weanlings

- Microscopic observations in the liver, brain and reproductive organs in P1 and F1 adults
- Microscopic observations in the liver and brain in F1 and F2 weanlings.

Potentially adverse effects considered to be related to copper sulphate treatment were limited to the 1500 ppm groups and were comprised of:

- Decreased spleen weight in P1 adult females, and F1 and F2 male and female weanlings.

Under the conditions of this study there were no treatment-related effects in either generation (P1 and F1 adults or F1 and F2 offspring) on reproduction parameters or indications of systemic toxicity at any of the dose concentrations used (doses of 100 to 1500 ppm). There were no adverse effects of treatment at up to 1500 ppm on fertility, general reproductive performance or offspring viability and growth at any dose level (dietary levels 0, 100, 500, 1000 and 1500 ppm $CuSO_4$). Dietary intake varied with stage of maturation and effects observed at the high dose may reflect changes in food intake and test substance consumption for the different populations within the study. Actual dosed values were 1.53-2.65, 7.7-13.3, 15.2-26.7 and 23.6-43.8 mg/kg body weight/day, for the 100, 500, 1000 and 1500 ppm groups, respectively. However since young rats consume more diet the mg/kg bw/day exposure is greater at weaning and at the beginning of each maturation phase. Pregnant and lactating females also consume more diet and are subject to a greater mg/kg bw/day exposure.
The concentration of copper was increased in the liver of F1 males and F1 and F2 male and female weanlings at 1000 and 1500 ppm and in P1 and F1 females at 1500 ppm. Brain copper concentration was increased in F1 females and F1 and F2 male weanlings at 1500 ppm. The concentration of liver iron was decreased in P1 females at 1500 ppm. The concentration of plasma iron was decreased in F2 male and female weanlings at 1500 ppm. There were decreased spleen weight in P1 adult females, and F1 and F2 male and female weanlings.
The majority of effects are reported in weanlings and in dams at the end of lactation - the food intake and compound consumption data show that both of these “populations” were consuming significantly higher amounts of diet than towards the end of the pre-mating maturation periods (the food intake of the weanlings is virtually the same as in the first week of the F1 maturation period, when compound consumption of F1 males is 58 mg Cu/kg/day, for example, and during lactation when the adult females are eating lots to feed their young), but that the spleen effects are not seen in males at termination, when compound consumption is much lower (22.9 mg Cu/kg/day in F1 males). From this it may be concluded that the spleen effects may be transient even at high doses, and that when the dietary intake i.e. dose level is reduced, the spleen effect diminishes. However, while the iron effects and the brain copper effects at 1500 ppm are also probably temporary and related to high dietary intakes (in that the male weanlings showed the finding, but when those weanlings grew older they did not), there is insufficient evidence to support 1500 ppm as a NOAEL.

From these results, the no-observed-effect level (NOEL) for reproductive toxicity was 1500 ppm, the highest concentration tested. The systemic NOEL for P1 and F1 rats and F1 and F2 offspring during lactation was 1000 ppm, based on reduced spleen weight in P1 adult females, and F1 and F2 male and female weanlings at 1500 ppm. The dietary concentration of 1000 ppm was equivalent to mean daily intakes of copper of 15.2 - 23.5 mg/kg body weight/day for male rats during pre-mating and 17.0 - 26.7 mg/kg body weight/day for female rats during pre-mating and gestation.

Reference: De la Iglesia F. W. (1973)
Guideline: No. Cross-mating fertility study
GLP: No

Three groups of 20 female Wistar rats were given copper gluconate orally by gavage at 0, 3 or 30 mg/kg/day for two weeks prior to mating through to either day 20 of pregnancy or day 21 post partum. Females were paired (1m:2f) with untreated males. In a parallel study, two groups of 10 males received copper gluconate at 3 mg/kg/day for 60 days prior to pairing (1m:2f) with either untreated females or females that had received copper gluconate at 3 mg/kg/day for 60 days prior to mating. A further group of 10 males and 20 females were maintained untreated for 60 days and allowed to mate. Parameters investigated included pregnancy rate (percentage of pregnancies), day 20 litter parameters including implantations, resorptions, live foetuses, gross foetal anomalies; litter parameters included duration of gestation, litter size, number of live young, gross anomalies, litter and mean pup weights through to weaning.

There were no significant differences between treated and control groups in any of the parameters studied.

Copper gluconate did not affect the fertility of either the male or female rat, following oral administration. This is discussed further at the end of this section.

Reference: Lecyk, M. (1980)
Guideline: No
GLP: No
Deviations: Yes (from OECD 414)

- Housing and feeding conditions of test animals,
- information on the age and weight of test animals,
- no detail given on the size of the groups,
- in several dose groups, the number of pregnant animals was smaller than recommended by the guideline (16 animals).
- in the absence of information on the weight of test animals and the weight of treated diet consumed, it was not possible to accurately determine the dose received on a mg/kg bodyweight basis,
- no information on maternal toxicity was presented in the report,
- no post-mortem information was presented in the report for dams,
- no information was presented on: the weight of gravid uteri; the number of corpora lutea; degrees of resorption of dead foetuses,
- the sex ratio of live foetuses was not reported,
- no justification is provided for use of mouse whereas the preferred rodent species is rat for this study,
- males were fed the appropriate test diet prior to mating and no information on male toxicity was then reported,
- the study did not measure maternal bw gains or maternal liver histology or copper content.

Copper sulphate was administered to groups of male and female mice, strains C57BL and DBA, by admixing the aqueous solution with the diet at dose levels of 0, 500, 1,000, 1,500, 2,000, 3,000 and 4,000 ppm corresponding approximately to 0, 71, 142, 214, 285, 427 and 570 mg/kg bw/day . The feed was granulated and dried before administering to the animals. The males and females were paired after one month of treatment and the day of mating (appearance of a vaginal plug) was designated Day 0 of gestation. On Day 19 of gestation the females were killed and foetuses (living and dead) were counted and weighed. One half of the foetuses in each group was examined for visceral abnormalities (Wilson technique) and the other half was cleared and stained with alizarin for skeletal examination.

Although the paper does not give details of group size and pregnancy rate, from the numbers of pregnant females (particularly at 4000, 3000 ppm), pregnancy rate was not adversely affected by dietary administration of copper at up to 4000 ppm for one month prior to mating (table below).

In both strains of mice, there was no effect on the embryonic growth at the lower doses, 2,000 ppm and below. The authors claimed a slight stimulation indicated by lower % foetal mortality and slightly higher weights of the foetuses than the controls at doses up to 2000 ppm. A treatment-related effect was noted at higher levels, at 3,000 and 4,000 ppm, where decreased foetal weights and a higher mortality were recorded (table 51). It should be noted that mean litter size was smaller than normal for the mouse in all groups. Various development malformations were observed in both these groups in both strains, although there was no consistent pattern of type. Abnormalities classed by the authors as malformations at 3000 ppm (3 foetuses in total) were last lumbar vertebra included in sacrum (one foetus) and unilateral fused rib (two foetuses); at 4000 ppm, hernia of the thoracic wall, hydrocephalus and fusion of thoracic ribs and vertebrae, (each one foetus, two foetuses with encephalocoel and two foetuses with (last lumbar) hemivertebra as part of sacrum. However, as no information was presented in this study regarded maternal toxicity, the possibility that the effects on embryonic development were secondary to maternal toxicity cannot be excluded.

Table 51: Mouse embryonic development

	Dose level (ppm)						
	0	**500**	**1000**	**1500**	**2000**	**3000**	**4000**
C57BL mice							
Number of pregnant females	21	10	18	7	10	22	18
Number of live foetuses (%)	65 (83.1)	46 (89.2)	81 (86.5)	31 (87.1)	42 (78.6)	55 (72.8)	35 (71.5)
Number of dead foetuses (%)	11 (16.9)	5 (10.8)	11 (13.5)	4 (12.9)	9 (21.4)	15 (27.2)	10 (28.5)
Mean litter size	3.09	4.60	4.50	4.42	4.20	2.50	1.94
Mean foetal weight (g)	1.10	1.35	1.22	1.14	1.25	1.00	0.99
Abnormal foetuses (%)	-	-	-	-	-	1 (1.8)	3 (8.5)
DBA mice							
Number of pregnant females	17	10	10	14	10	18	20
Number of live foetuses (%)	76 (84.3)	54 (90.8)	51 (88.3)	58 (82.8)	41 (83.0)	56 (75.0)	45 (70.4)
Number of dead foetuses (%)	12 (15.7)	5 (9.2)	6 (11.7)	10 (17.2)	7 (17.0)	14 (25.0)	16 (29.6)
Mean litter size	4.47	5.40	5.10	4.14	4.10	3.11	2.70
Mean foetal weight (g)	0.96	1.24	1.19	1.17	1.13	1.11	1.09
Abnormal foetuses (%)	-	-	-	-	-	2 (3.7)	4 (7.4)

Dietary administration of 3,000 and 4,000 ppm copper as sulphate (approximately equivalent to dose levels of 430 and 570 mg/kg bw/day, using the US FDA conversion factor of 7 for mice) for one month prior to pairing did not adversely affect mating performance or pregnancy rate but caused an increase in foetal mortality, decreases in foetal weights and slight increase in incidence of malformations.. It should be noted that the study did not measure maternal bodyweight gains, or maternal liver histology or copper content. The NOEL for fertility effects was greater than 4,000 ppm (approximately 570 mg/kg bw/day) and the NOEL for foetal effects was 2,000 ppm (approximately 285 mg/kg bw/day).

4.9.1.2 Human information

Reference: Ralph, A. and McArdle, H. (2001)
Guideline: No
GCP: No
The publication is a review of data on copper metabolism and toxicity during pregnancy and lactation, with emphasis on the human.

The review considers the following aspects:

Fertilisation: Copper metal is known to interrupt implantation and development of the blastocyst when present in the uterus as an intra-uterine contraceptive device (IUD), but once implantation has taken place, IUDs do not show adverse effects on maintenance of pregnancy.

Maternal serum copper levels and ceruloplasmin levels rise steadily throughout pregnancy, and fall significantly at parturition. The concentration in the mother is higher than in the foetus, which establishes a concentration gradient from the mother to the foetus. The rise in plasma concentration may be due to either enhanced uptake from food or decreased biliary excretion. It is induced by oestrogen. Various studies have shown that copper requirements of pregnant humans are up to one third greater than non-pregnant human females. Copper and ceruloplasmin are present in amniotic fluid, but uptake from amniotic fluid by the foetus is small. The placenta has been shown to take copper from the maternal blood as both ceruloplasmin and by lower-weight complexes (albumin, histidine), but that delivery by ceruloplasmin is more efficient. Ceruloplasmin is not itself passed across the placenta, but ceruloplasmin and histidine may deliver copper to the placental cells via specific cell surface receptors. The placenta has a regulatory role on the transfer of copper from mother to baby, as infant serum concentrations of copper do not correlate with those of the mother. This has been demonstrated in both human and rat. Women with Wilson's disease can give birth to healthy babies if the condition is well managed (zinc sulphate therapy). Pregnant women with untreated Wilson's disease tend to have spontaneous abortions. In the Brewer study (2000), of 26 pregnancies in 19 women who were on zinc therapy throughout their pregnancy, 24 new-borns were normal, one had a heart defect (corrected by surgery) and another showed anencephaly. Anencephaly has also been associated with very low maternal copper serum levels, and there have been two reported cases of anencephaly where an IUD was used (Graham et al. 1980).

Foetal development: copper accumulates in the placental layers and is transferred to the foetus by an active process driven by foetal needs; it is thought to be incorporated in the foetal liver into foetally synthesised ceruloplasmin. Copper is present in the foetal circulation in ceruloplasmin, albumin, α-fetoprotein, transcuprein and low molecular weight ligands. The human foetus accumulates copper at a rate of 50 μg/kg/day during the latter half of pregnancy, and 50% of it is stored as metallothionein in the liver. The ratio of copper in the liver of newborn infants to adults is 15:4. There are no reports of adverse effects of acute toxicity of copper in human pregnancy. Foetal copper accumulation occurs in the third trimester, and premature and low-weight babies are at risk of copper deficiency. Studies indicate that the capacity of pre-term infants to utilise copper from the diet is limited; most of the ingested copper is present in the stool, indicating either ineffective absorption or limited ability to retain and store copper.

Parturition. Serum maternal plasma level returns to normal in the human within two to five weeks. The timing of the return to normal may be influenced by the duration of breast-feeding.

Lactation. Ceruloplasmin occurs in the milk of humans and other mammals, concentrations being higher in the early stages of lactation. Approximately 20-25% of copper in human milk is present as ceruloplasmin. Breast-feeding supplies up to 60 μg/kg/day, and is approximately 24% bioavailable. Maternal copper blood levels are under hormonal control (e.g. oestrogen, see above), but alterations in maternal copper intake through dietary supplementation, or elevated blood levels through other factors, such as severe infections, and even Wilson's disease, do not alter copper content of breast milk. It is likely that there are homeostatic mechanisms that regulate mammary gland uptake of copper and its secretion in milk, but these have not been explained. In human breast milk, approximately 75% of the copper is in the whey, bound to soluble albumin or low molecular weight ligands. Another 15-20% is in lipids, bound to the outer fat globule membrane, and about 5% is in insoluble form, possibly bound to casein. Differences in composition of other milks (cow, soy) affect the

bioavailability to the human baby. Absorption and retention rates from formula milks are very low, although toxicity has been observed where infants have been given substantial amounts of cow's milk boiled in untinned copper vessels. Awareness of the disease in India [Indian Childhood Cirrhosis] and Austria [Idiopathic Copper Toxicosis] has resulted in use of other containers, and the incidence has fallen. Human milk, unsurprisingly, contains the most bioavailable copper for the human baby. Healthy infants fed exclusively on cow's milk for 6 months became copper deficient, but the condition reversed on weaning to solid foods.

Growth and development. Neonatal humans have high concentrations of copper in the liver and low concentrations of serum copper and ceruloplasmin. Newborn humans also show high concentrations of metallothionein that decrease after birth. Copper in the new-born's liver appears to provide much of the copper requirements of the infant while it is breast-fed, until weaning at 4-6 months. However, milk must provide a significant contribution, as mice showing 'toxic milk mutation' die if they are kept on mother's milk, because the mother cannot secrete the normal amounts of copper into the milk, and the pups die of copper deficiency. Premature birth restricts the hepatic storage of copper (as the mother's supply via the placenta is no longer available), and milk formulae for premature infants contains additional copper to compensate for this. Low copper levels at this time may have neurological implications during the critical period of brain growth. Excess copper in drinking water at concentrations of approximately 8 mg/L showed chronic toxicity in adults but not in children under 6 years of age. As the infant grows, levels of ceruloplasmin increase. Studies in rats show that copper absorption is high during the neonatal period, but decreases by weaning, as more is retained in the intestinal mucosa. With increasing postnatal age, more is transported to the liver and less is bound to the intestine. There is evidence in rats that during lactation, intestinal copper absorption occurs by diffusion and solvent drag, and only after weaning does a saturable (adult, see Section B.6.1) copper transport system become evident. Children require higher levels of copper in the diet than adults, especially during periods of rapid growth. Girls aged 6-10 were fed on diets of copper ranging from 1.1 to 3.8 mg/day. At intakes under 2 mg/day, copper balance was negative. A positive copper balance was achieved on a vegetarian diet with a copper intake of over 2.8 mg/day. It was suggested that an intake of 1.3 mg/day was sufficient for equilibrium, but that 2.5 mg/day was necessary for growth. Serum of normal children reaches a peak of 1.57 mg/L between 6 and 11 years and falls to 1.1 mg/L in adults between 22 and 75 years.

Intake: the review found no evidence of copper toxicity from customary dietary intake, unless the food had been accidentally contaminated with copper during preparation e.g. acid fruit such as apples, were stewed in a copper vessel, or there was repeated ingestion of milk heated in copper vessels. A study of three cities in the US state of Massachusetts showed no incidence of ill-health in adults or children under 6 years of age, despite drinking water concentrations of over 8 mg/L. Most dietary intakes are below the 10-12 mg/adult/day set by international organisations.

There is no evidence for adverse effects of oral exposure through customary diets worldwide (which includes countries where copper is used in agriculture) for any adverse effects of copper on pregnancy, parturition, lactation or growth and development in the human. There is evidence of toxicity particularly to neonates repeatedly exposed to milk heated in copper vessels, or exposure to acid fruit stewed in copper vessels.

4.9.2 Developmental toxicity

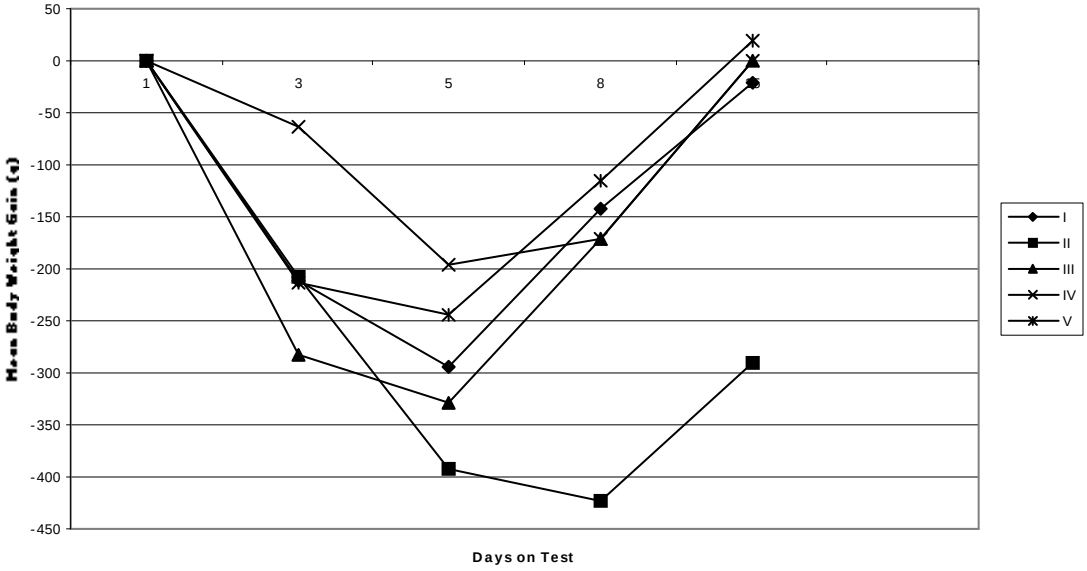
4.9.2.1 Non-human information

Reference: Munley S. M. (2003a)
Guideline: No. Range-finding study designed to assess relative tolerance of five technical copper substances in the rabbit.
GLP: Yes

Five technical materials, copper hydroxide (batch number 380-71-05, copper content 60.1% w/w), copper oxychloride (batch number 27003B, copper content 57% w/w), Bordeaux Mixture (batch number 1/170), copper content 26.38% w/w), tribasic copper sulphate (batch number 471/2002, copper content 31.12% w/w), and copper (I) oxide, (batch number 280802, copper content 87% w/w) were given orally by gavage as suspension in 0.5% aqueous methyl cellulose in deionised water to non-pregnant female Hra:(NZW)SPF rabbits. The animals were approximately 6 to 6.5 months old and weighed from 3382 g to 4116 g on the day after arrival. Animals were quarantined for at least 5 days before dosing. Rabbits were housed individually, and were given 150 g laboratory rabbit diet each day, and tap water *ad libitum*. Food consumption and bodyweight were recorded daily, clinical observations were recorded daily and post dosing. Dose formulations were prepared daily at a dose volume of 1 mL/kg bw, based on that day's bodyweight. Any animals found dead were necropsied. At termination, all animals were given a gross external and visceral examination. Lesions were retained in an appropriate fixative. In the first part of the study, groups of two rabbits were dosed with each technical material for up to 14 days. Concentrations were calculated to give 30 mg as copper /kg bw/day. In view of the moderate toxicity seen at 30 mg Cu/kg bw/day, doses of 50 mg Cu/kg bw/day were given to a further group of 2 rabbits per technical substance, to assess tolerance to a higher dose. Mortalities occurred after the first dose and surviving rabbits were given 40 mg Cu/kg bw/day for the remaining six days of administration.

Animals at 30 mg Cu/kg bw/day showed bodyweight loss during the first half of the treatment period, followed by recovery during the second week of treatment (Figure 6.7.3.1.1.). There were no marked differences between the five technical substances. Food consumption reflected bodyweight changes; during the first week of dosing animals showed marked reductions in food consumption, and in the second week the animals generally resumed eating.

Figure 1: Bodyweight change with five forms of copper at 30 mg/kg/day

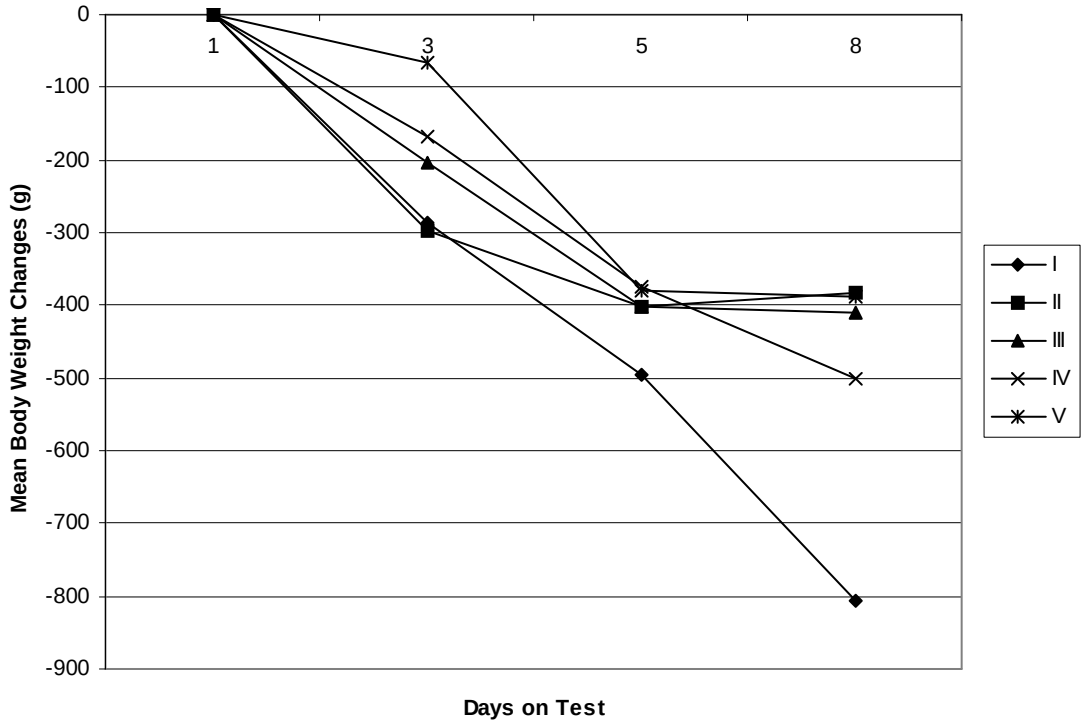


I: copper hydroxide; II: copper (I) oxide; III: copper oxychloride; IV: tribasic copper sulphate; V: Bordeaux mixture

There were no deaths among animals treated with copper hydroxide, Bordeaux mixture, tribasic copper sulphate or copper oxide. One animal dosed with copper oxychloride was found dead on day 2. There were no indications of any adverse effects of treatment, or of dosing error, and this animal was replaced by a similar animal from the same batch. A second animal dosed with oxychloride died on day 11. The animal showed no remarkable necropsy findings other than fur staining. During the study, it was discovered that the two animals dosed with tribasic copper sulphate were underdosed by approximately 40%, because of a calculation error. These animals were also replaced by two similar animals, and the food/bodyweight data from the underdosed animals was not used in the comparison of the five substances. Three animals were inadvertently sacrificed prematurely in the second week of treatment. Necropsy revealed various stomach findings, including ulceration, red or dark discolouration, and haemorrhagic areas in one animal dosed with copper hydroxide, both animals dosed with copper oxide, and three of the four animals dosed with tribasic copper sulphate.

At 50 mg Cu/kg bw/day, one of the two animals died after the first dose in each group except tribasic copper sulphate. The Study Director immediately reduced the dose concentration to 40 mg Cu/kg bw/day (i.e. from day 2) and there were no further deaths. All decedents showed either stomach ulceration or dark discolouration and thickening of the non-glandular portion of the stomach. Survivors at 40 mg Cu/kg bw/day showed weight loss (Figure 6.7.3.1.2) and reduced food consumption. At termination, all survivors showed stomach ulcerations.

Figure 2: Bodyweight change with five forms of copper at 50/40 mg/kg/day



I: copper hydroxide; II: copper (I) oxide; III: copper oxychloride; IV: tribasic copper sulphate; V: Bordeaux Mixture

The general pattern and degree of inappetance and weight loss followed by recovery, and the observation of stomach ulceration at necropsy was considered sufficient to show that there were no major differences in the sensitivity of the rabbit to the five copper substances. Doses greater than 30 mg Cu/kg bw/day were considered unsustainable for repeat dosing studies. As there were no major differences between the five substances, further preliminary investigations would be performed on only one substance, copper hydroxide.

Reference: Munley S. M. (2003 b)
Guideline No. Range-finding study designed to assess effect of treatment equal in duration to a teratology study in the non-pregnant rabbit.
GLP: Yes

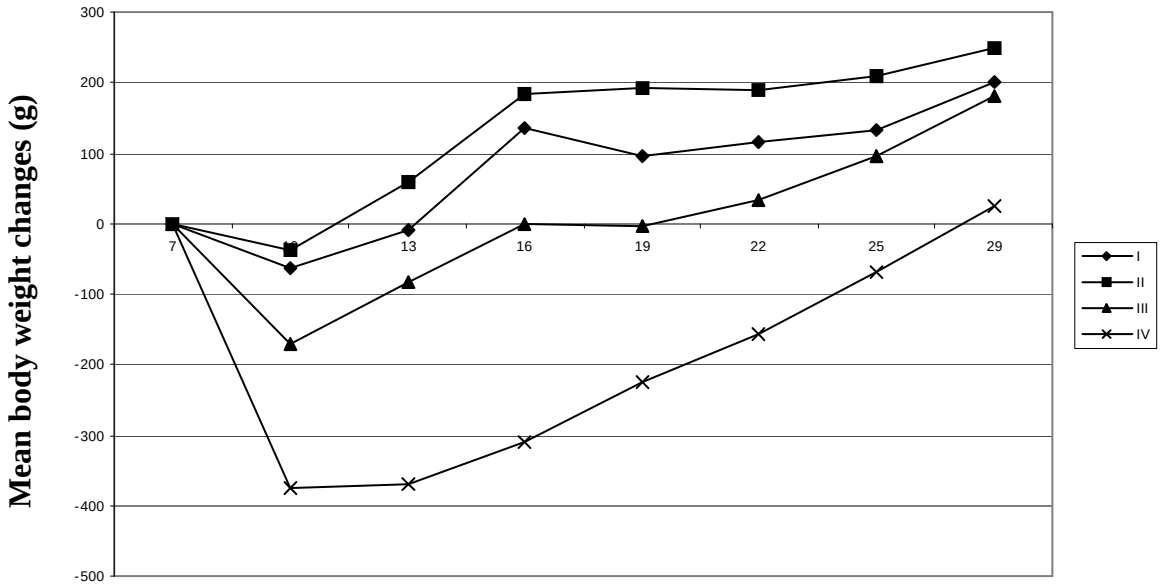
Technical copper hydroxide (batch number 380-71-05, copper content 60.1% w/w) was given orally by gavage as suspension in 0.5% aqueous methyl cellulose in deionised water to groups of five non-pregnant female Hra:(NZW)SPF rabbits for 23 consecutive days. Dose levels were 0, 7.5, 15 or 30 mg Cu/kg bw/day. Analysis of dose formulations confirmed stability, homogeneity and verified the accuracy of formulation. The animals were approximately 6 to 6.5 months old and weighed from 2936 g to 3748 g on the first day of dosing. Animals were quarantined for at least 5 days before dosing. Rabbits were housed individually, and were given 150 g laboratory rabbit diet each day, and tap water *ad libitum*. Food consumption and bodyweight were recorded daily, clinical observations were recorded daily and post dosing. Dose formulations were prepared daily at a dose volume of 1 mL/kg bw, based on that day's bodyweight. Any animals found dead were necropsied. At termination on day 24, all animals

were given a gross external and visceral examination. Lesions were retained in an appropriate fixative.

There were two deaths at 30 mg Cu/kg bw/day, on day 2 and 3 respectively. The latter animal showed lethargy, weakness and abnormal gait or mobility prior to death. Both decedents showed haemorrhages and/or discolouration of the stomach lining. There were no deaths at 15 mg Cu/kg bw/day. Two animals at 7.5 mg Cu/kg/bw/day died on days 2 or 5 due to intubation errors. Necropsy findings included punctured lung tissue.

Bodyweights and food consumption at 30 and 15 mg Cu/kg bw/day were lower than controls from the start of treatment. There was a group mean bodyweight loss during the first week of treatment, with recovery to initial mean values by day 19 in both groups (Figure 6.7.3.2.1). Food consumption and bodyweight gains at 7.5 mg Cu/kg bw/day were not adversely affected by treatment.

Figure 3: Bodyweight change of females



I: Control; II: 7.5 mg/kg/day; III: 15 mg/kg/day; IV: 30 mg/kg/day

Necropsy findings in animals surviving to termination were limited to haemorrhages and/or discolouration of the stomach lining in one animal at 30 mg Cu/kg bw/day.

Treatment at 30 and 15 mg Cu/kg bw/day was associated with initial inappetance and bodyweight loss, followed by recovery. There were two deaths at 30 mg Cu/kg bw/day. Necropsy findings in decedents and one animal at 30 mg Cu/kg bw/day included to haemorrhages and/or discolouration of the stomach lining. There were no adverse effects of treatment at 7.5 mg Cu/kg bw/day.

Reference: Munley S. M. (2003c)
Guideline: No. Range-finding study in the pregnant rabbit.
GLP: Yes

Technical copper hydroxide (batch number 021121/1, copper content 61.14% w/w) was given orally by gavage as suspension in 0.5% aqueous methyl cellulose in deionised water to groups of five time-mated female Hra:(NZW)SPF rabbits during days 7 to 28 of pregnancy. Dose levels were 0, 7.5, 15 or 30 mg Cu/kg bw/day. Analysis of dose formulations confirmed stability, homogeneity and verified the accuracy of formulation. The animals were approximately 6 to 6.5 months old and weighed from 2885 g to 4330 g on the day of mating, which was defined as day 0 of pregnancy. Initial group size was five, but intubation errors resulted in deaths in treated groups; the dead animals were replaced with similar time-mated does from the same supplier. Group sizes were thus 5, 8, 9 and 8. Animals were quarantined for at least 5 days before dosing. Rabbits were housed individually, and were given 150 g laboratory rabbit diet each day, and tap water *ad libitum*. Food consumption and bodyweight were recorded daily from day 4. Clinical observations were recorded daily and post dosing. Dose formulations were prepared daily at a dose volume of 1 mL/kg bw, based on that day's bodyweight. Any dams dying prior to planned termination were necropsied and pregnancy status was assessed. At termination on day 29, all surviving animals were given a gross external and visceral examination. Lesions were retained in an appropriate fixative. The gravid uterus was weighed, and corpora lutea were counted. Numbers of live and dead foetuses, early and late resorptions were recorded. Live foetuses were euthanased, weighed and examined externally. Any dams dying prior to planned termination were necropsied and pregnancy status was assessed.

There were two deaths at 30 mg Cu/kg/ bw/day. One dam was sacrificed *in extremis* on day 9. Necropsy revealed stomach haemorrhages. Subsequent histopathology indicated a haemolytic event that resulted in haemoglobin nephropathy and probable renal failure, consistent with acute copper toxicity. The second female was found dead on day 26; necropsy revealed a small liver and moderate autolysis.

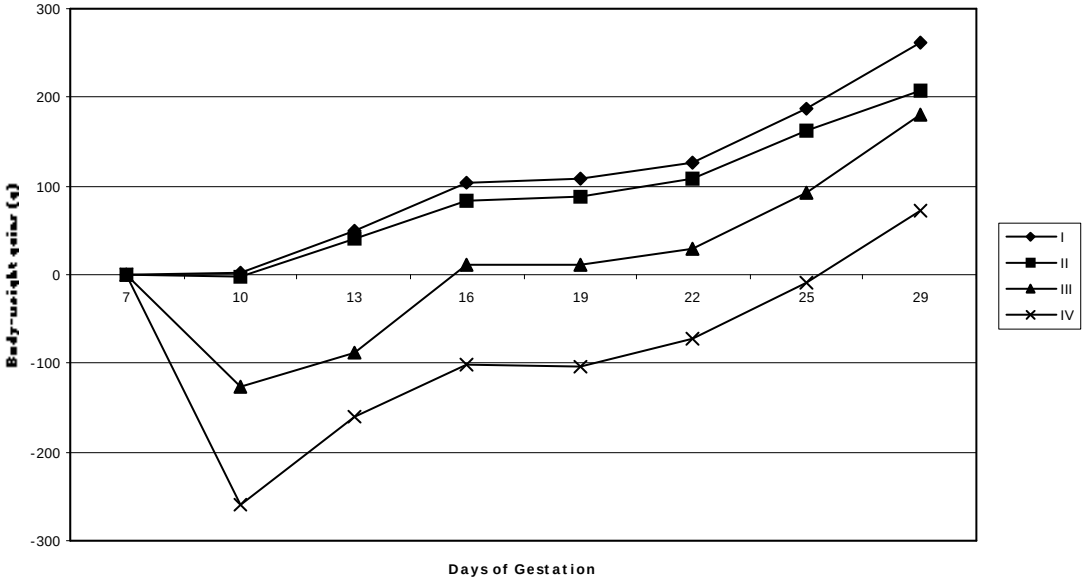
Five other animals (two each at 7.5 and 15 mg Cu/kg/ bw/day, and one at 30 mg Cu/kg/ bw/day) were either accidentally killed or were found dead as a result of intubation injuries. These deaths were not considered treatment-related. These animals were replaced on study with similar time-mated does from the same supplier.

Clinical observations were limited to low incidence of diarrhoea that was considered not to be related to treatment.

There were clear bodyweight losses and reduced food consumption at 15 and 30 mg Cu/kg/ bw/day (Figure 6.7.3.3.1). At 30 mg Cu/kg/ bw/day, overall bodyweight gain during the treatment period was reduced by 88% relative to the control group. Food consumption was also markedly reduced, being 44% lower than controls. At termination, mean bodyweight was 9% lower than controls. Similar but less pronounced effects were noted at 15 mg Cu/kg/ bw/day, where overall bodyweight gain and food consumption were 11% and 22% lower than controls, respectively.

Bodyweight gain and food consumption at 7.5 mg Cu/kg/ bw/day were not adversely affected by treatment.

Figure 4: Bodyweight change of dams



I: Control; II: 7.5 mg/kg/day; III: 15 mg/kg/day; IV: 30 mg/kg/day

Mean foetal weight at 30 mg Cu/kg/ bw/day was reduced by 12% relative to the control group.
Incidence of total resorptions was slightly increased (1.3 ±0.5 versus 0.3 ±0.5 in controls) and there were four foetuses (2 from 2 litters) with onphalocoele. These foetuses tended to be very low weight and immature (e.g. the two foetuses from one dam weighed only 28.95 g and 20.86 g respectively, compared to mean control foetal weight of 41.28 g). Omphalocoele is protrusion of the intestines at the umbilicus. During development, the intestines are contained within the membranes of the peritoneum and amnion. As the foetus matures, the body wall gradually encloses the abdominal cavity and the membrane-bound intestines effectively withdraw into the body, until by late gestation, the body wall has reached the umbilicus. Omphalocoele can occur in low-weight foetuses as a consequence of foetal immaturity secondary to marked maternal weight loss.
Litter parameters at 15 mg Cu/kg/ bw/day were similar to controls, and there were no malformed foetuses. There was one foetus at 7.5 mg Cu/kg/ bw/day with anasarca, domed head and short tail.

Treatment at 30 mg Cu/kg/ bw/day was associated with death and necropsy findings consistent with acute copper toxicity, marked maternal bodyweight loss and reduced food consumption, reduced mean foetal weight and foetal defects consistent with immaturity. Treatment at 15 mg Cu/kg bw/day was also associated with maternal bodyweight loss and reduced food consumption, but litter parameters were not adversely affected by maternal treatment. There were no adverse effects of treatment at 7.5 mg Cu/kg bw/day.

Reference: Munley S. M. (2003d)
Guideline: OECD 414
GLP: Yes
Deviations None

Technical copper hydroxide (batch number 021121/1, copper content 61.14% w/w) was given orally by gavage as suspension in 0.5% aqueous methyl cellulose in deionised water to groups

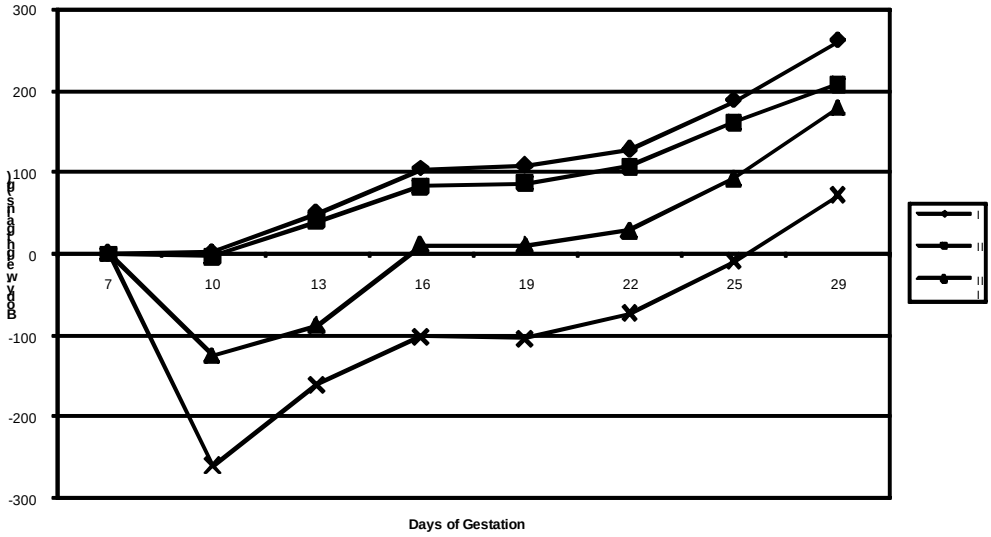
of 22 time-mated female Hra:(NZW)SPF rabbits during days 7 to 28 of pregnancy. Dose levels were 0, 6, 9 or 18 mg Cu/kg bw/day. Analysis of dose formulations confirmed stability, homogeneity and verified the accuracy of formulation. The animals were approximately 5 months old and weighed from 2988 g to 4412 g on the day of mating, which was defined as day 0 of pregnancy. Animals were quarantined for at least 5 days before dosing. Rabbits were housed individually, and were given 150 g laboratory rabbit diet each day, and tap water *ad libitum*. Food consumption and bodyweight were recorded daily from day 4, clinical observations were recorded daily and post dosing. Dose formulations were prepared daily at a dose volume of 1 mL/kg bw, based on that day's bodyweight. Any dams dying prior to planned termination were necropsied and pregnancy status was assessed. At termination on day 29, all surviving animals were given a gross external and visceral examination. Lesions were retained in an appropriate fixative. The gravid uterus was weighed, and corpora lutea were counted. Numbers of live and dead foetuses, early and late resorptions were recorded. Live foetuses were euthanased, weighed and examined for external and visceral alterations. The eyelids of each foetus were removed to allow examination of the eyes. Foetal sex was recorded during visceral examination. The skull was part-sectioned between the parietal and frontal bones to allow inspection of the brain. After examination, foetuses were eviscerated, fixed in alcohol and stained with Alizarin red S for skeletal examination.

There were three deaths and two females with abortion (subsequently sacrificed) at 18 mg/kg bw/day. The dead animals were found on days 9, 10 and 16, and the aborted animals were killed on day 22 of pregnancy. One of the animals found dead showed diarrhoea, red staining of under-cage board, weakness and irregular respiration prior to death. The other two animals appeared normal prior to death, but all three showed necropsy findings including stomach haemorrhage and/or ulceration, dark discolouration or mottling o flung tissue, pale liver, gelatinous tan rectal discharge and brown liquid in the chest cavity. One of the animals showing abortion had diarrhoea. Necropsy of the other aborted animal showed red discoloured stomach lining. Abortion in mid to late pregnancy is observed in rabbits that show marked inappetance and weight loss. One other female at 18 mg/kg bw/day was killed following intubation injury on day 15 of pregnancy. Necropsy findings included stomach haemorrhage and evidence of intubation injury to lung tissue.

There were no substance-related deaths among animals dosed at 9 or 6 mg/kg bw/day. One female at 6 mg/kg bw/day aborted on day 27of pregnancy. This was not considered to be related to treatment, as there were no abortions at 9 mg/kg bw/day, and the abortion occurred later than those at 18 mg/kg bw/day. Single instances of abortion in late pregnancy are not uncommon in groups of pregnant rabbits.In addition to clinical observations noted previously for decedents, occasional animals in all groups showed alopecia. This was not considered treatment-related. One control animal, and 6, 2 and 7 animals at 6, 9 and 18 mg/kg bw/day showed one or more daily records of diarrhoea.

Group mean bodyweight data showed marked initial weight losses at 18 and 9 mg/kg bw/day during the initial part of the treatment period, followed by part-recovery during middle and late pregnancy (Figure 6.7.3.4.1.). At termination, mean weight gain of animals at 9 mg/kg bw/day was 31% lower than controls, and mean weight gain of animals at 18 mg/kg bw/day was 72% lower than controls. Group mean bodyweight gains at 6 mg/kg bw/day were marginally lower than controls.

Figure 5: Bodyweight change of dams



I: Control; II: 6 mg/kg/day; III: 9 mg/kg/day; IV: 18 mg/kg/day

Group mean food consumption was consistent with bodyweight data: animals at 9 and 18 mg/kg bw/day showed marked inappetance during the initial part of the treatment period. At 18 mg/kg bw/day animals showed reduced food consumption throughout the remainder of the study, but at 9 mg/kg bw/day, food consumption during the latter half of the study was only slightly below controls. Total food consumption at 9 and 18 mg/kg bw/day was 17 and 30% lower than in the control group. Food intake at 6 mg/kg bw/day was marginally lower than controls.

Pregnancy rate was high. The number of litters available for examination was lower at 18 mg/kg bw/day because of the deaths and animals with abortion. One female at 6 mg/kg bw/day showed total resorption, but as this was a single incidence, and there were no similar findings at higher dose levels, this is considered unrelated to treatment (table below).

Table 52: Summary of adult performance

Number of females:	**Dose level (mg/kg bw/day)**			
	0	**6**	**9**	**18**
Mated	22	22	22	21
Pregnant	21	21	21	21
Aborted (killed)	0	1	0	2
Found dead	0	0	0	3
Intubation error (killed)	0	0	0	1
Total resorptions	0	1	0	0
With live young	21	19	21	15

The number of foetuses, and numbers of early and late embryonic deaths were not adversely affected by maternal treatment (table below). Mean foetal weight was slightly lower at 18 mg/kg bw/day (9% lower than controls). The difference from control was considered treatment-related, but it was not statistically significant.

Table.53: Group mean litter data

Group Mean Litter parameter	Dose level (mg/kg bw/day)			
	0	6	9	18
Corpora lutea	10.0	10.2	9.1	10.1
Number of implantations	8.8	9.0	7.8	9.0
Early embryonic death	0.8	0.7	0.2	0.4
Late embryonic death	0.1	0.3	0.0	0.2
Total embryonic death	1.0	1.0	0.2	0.6
Number of live young	7.9	8.0	7.6	8.4
Percent males in litter	48	58	50	48
Mean foetal weight (g)	42.95	41.71	43.93	38.91
Number with malformations	1	1	0	2

There was a total of four foetuses with malformations: one control foetus showed fused ribs, one foetus at 6 mg/kg bw/day showed ectopic kidney, and two foetuses (from separate litters) at 18 mg/kg bw/day showed hemivertebra (table below). These malformations were considered spontaneous and unrelated to treatment.

Table 54: Incidence of foetal variations

Variation	Dose level (mg/kg bw/day)			
	0	6	9	18
Number examined	165	152	159	126
Developmental				
External	0	0	0	0
Visceral	0	0	0	0
Head	0	0	0	0
Extra rib (%)	105 (64)	102 (67)	127 (80)	110 (87)
Fused sternebrae	1	2	0	0
Retardation				
Kidney small papilla	2	6	6	2
Ossification mandible	0	0	0	1
Ossification pelvis	0	1	1	2
Ossification skull	0	0	1	5
Ossification sternebrae	65	60	76	51
Ossification vertebrae	1	0	0	0
Total (%) with retardation	68 (41)	67 (44)	80 (50)	57 (45)
Total (%) with variations	125 (76)	124 (82)	143 (90)	118 (94)

Percentage values calculated from group totals, not from means of individual litter percentages

There was a slight increase in incidence of foetuses at 18 mg/kg bw/day with retarded ossification of skull and pelvic bones. However, there was no correlation with foetal weight, and the biological significance of such a slight increase is uncertain, as there was no increase in the incidence of retarded sternebral ossification. Retarded sternebral ossification is a more common indicator of foetal immaturity. Rib alterations occurred at a very high incidence across all groups in this study; almost all litters were affected. The biological significance of an increase in incidence of a very common finding is uncertain.

Administration of copper to pregnant rabbits at 18mg/kg bw/day was associated with marked initial bodyweight loss, inappetance, abortion and death. Pups in litters from surviving dams showed slightly lower mean foetal weight, and slightly increased incidence of a common skeletal variant. Maternal treatment at 9 mg/kg bw/day was associated with initial bodyweight loss and inappetance; pups also showed slightly increased incidence of a common skeletal

variant, but mean foetal weights were not adversely affected. Maternal administration of copper hydroxide was not associated with increased incidence of foetal malformations, pre-implantation losses, or foetal (embryonic) deaths. The maternal and foetal no observed effect level was 6 mg/kg bw/day, based on maternal weight loss, inappetance, and an increased incidence of a common skeletal variant in foetuses at 9 mg/kg bw/day.

Comments:

- Copper hydroxyde appears to be more toxic in rabbits than in any other animal species.
- The study suffered of some events, including errors of intubation.
- The nutrition of the rabbit depends on bacterial digestion of cellulose, where the vegetation which forms the bulk of the diet is broken down by bacteria in the caecum to form sugars. These are ejested as soft faeces, and immediately eaten, a process known as refection or copography. Copper is known to have bacteriostatic/bactericidal activity, and oral administration of copper will affect the activity of the caecal bacteria, compromising the efficiency of the digestive process and effectively reducing the calorific intake of the rabbits, resulting in nutritional impairment. Metabolism studies show that copper is excreted in the bile, and copper from biliary excretion and any unabsorbed copper are excreted in faeces. Copper is an element; it is stable. Copper present in faeces will be taken in orally during coprophagy , so that the rabbit will have an extra dose of a stable material such as copper from its own faeces.

It is therefore impossible to quantify the actual daily oral dose, because the total oral intake is significantly more than the administered dose, and the study NOEL is too conservative. It can be concluded that the rabbit NOEL is not appropriate for establishing human risk assessment endpoints for copper.

Reference: De la Iglesia F. W. et al. (1972a)
Guideline: No
GLP: No
Deviations: Yes

- Partial summary,
- treatment duration too short (day5-15 of pregnancy),
- size of the groups not given.

Three groups of pregnant female Wistar rats were given copper gluconate orally by gavage at 0, 0.1, 3 or 30 mg/kg/day from days 5 to 15 of pregnancy. Bodyweight and food intake were recorded weekly. Day of sacrifice not stated in FAO summary, but presumed day 20. Litter parameters (corpora lutea, implantation sites, implantation losses, resorptions, numbers of live foetuses, foetal weight, crown-rump length) were recorded. Foetuses were examined for visceral and skeletal defects.

Maternal body weights and food intake were similar in all groups. Litter parameters were not adversely affected by treatment The incidence of skeletal and visceral abnormalities was not affected by maternal treatment. The NOEL was 30 mg/kg/day.

Copper as gluconate was stated to be not embryotoxic or teratogenic when administered orally to rats during the period of organogenesis.

Reference: De la Iglesia F. W. et al. (1972 b)

Guideline: No
GLP: No
Deviations: Yes

- The treatment duration is too short, the methodology suffers of insufficiencies, and there was no information in the summary on examination for visceral and skeletal defects,
- size of the groups not given.

Three groups of pregnant female Swiss mice were given copper gluconate orally by gavage at 0, 0.1, 3 or 30 mg/kg/day from days 6 to 14 of pregnancy. Bodyweight and food intake were recorded weekly. Day of sacrifice not stated in FAO summary, but presumed day 20. Litter parameters (corpora lutea, implantation sites, implantation losses, resorptions, numbers of live foetuses, foetal weight, crown-rump length) were recorded. There was no information in the summary on examination for visceral and skeletal defects.

Maternal body weights and food intake were similar in all groups. Litter parameters were not adversely affected by treatment. The NOEL was 30 mg/kg/day.

Copper as gluconate was stated to be not embryotoxic or teratogenic when administered orally to mice during the period of organogenesis.

Reference: Barlow, S.M., Knight, A.F. and House, I. (1981).
Guideline: No
GLP: No
Deviations: Yes

- IUDs were implanted on Day 9, and not prior to implantation
- Group sizes are smaller than recommended by the guideline
- The number of dose levels is fewer than recommended
- Levels of food consumption are not reported.
- Foetal sex is not reported.

These deficiencies do not, however, compromise the validity of the data reported.

A study was carried out to investigate the potential for intrauterine copper IUDs to affect prenatal development in the rat.

Female Wistar rats aged about 12 weeks and weighing 200-250 g were used in this study. For 2 weeks before mating and throughout the experiment they were held at 21-24°C under reversed lighting conditions (12 h red light, 12 h white light). Food and water were fed *ad libitum*. At the beginning of the experimental period, female rats were housed in groups of 3 and a male was introduced into each cage in the morning. Males were removed in the evening and vaginal smears taken. The day on which spermatozoa were found in the smear was designated Day 1 of pregnancy. Rats were weighed daily from Days 1 to 21 of pregnancy.

On Day 9 of pregnancy, rats were assigned randomly to treatment groups. Animals receiving IUDs were anaesthetized and one uterine horn exposed through an incision in the flank. A coil was inserted between each implantation site by making an incision in the uterus with an intravenous cannula with cutting needle. The other horn was left unoperated as a control. To control for the physical presence of devices in the uterus, some animals had

similar-sized coils of stainless-steel wire inserted into one horn, leaving the other unoperated. To control for the stress of the operation and other factors such as loss of uterine fluid, other animals were sham-operated, with no IUDs inserted. Animals in another group were left unoperated. Rats were returned to the animal room until sacrifice on Day 21.

This study was reported in terms of three separate experiments. The details of Experiments 1 and 2 are shown in the following table:

Table 55: Details of experiment 1 and 2

Experiment	Group	No. of animals	Uterine horn*
1	1 (COPPER IUD)	9	A OPERATED (9) B unoperated (8)
	2 (SHAM-OPERATED)	10	A operated (10) B unoperated (9)
	3 (No operation)	10	Unoperated (20)
2	4 (Copper IUD)	13	A operated (13) B unoperated (13)
	5 (Steel IUD)	14	A operated (14) B unoperated (13)
	6 (No operation)	7	Unoperated (14)

* Figures in parentheses indicate number of horns containing implantation sites.

Experiment 3 was carried out to determine whether copper released from IUDs penetrated into foetuses. Pregnant rats were treated as follows: on Day 9 of pregnancy, copper IUDs were inserted between each embryo in both uterine horns of 2 rats (Group 7). In another 2 rats, steel IUDs were inserted in both horns (Group 8). One rat was left as an unoperated control. Test animals were killed on Day 22 of pregnancy, and samples of maternal liver and uterus, all foetal brains, foetal livers and placentae were removed for copper analysis.

Rats were anaesthetised on Day 21 of pregnancy and a maternal blood sample taken for copper analysis. After sacrifice, the uterus was exposed and opened up. In IUD-bearing animals, copper or steel coils were removed, washed and weighed. The number and position of live and full-term dead foetuses (no signs of maceration), late resorptions (maceration, death occurring at the foetal stage), and early resorptions (death occurring at the embryonic stage) were noted. Numbers of corpora lutea in each ovary were also noted. Foetuses were weighed and examined for gross external abnormalities. They were then either fixed in Bouin's fluid for examination of soft tissues by the slicing technique of Wilson or in alcohol and stained with Alizarin red S for skeletal examination.

Experiment 1 results: Gravimetric analysis of the IUDs before insertion on Day 9 and after removal on Day 21 of pregnancy showed a mean ± s.e.m copper loss of 48 ± 3 µg (about 4 µg/coil/day). Maternal plasma copper levels (mean ± s.e.m.) on Day 21 of pregnancy were 203 ± 5 (*n* = 9), 208 ± 12 (*n* = 10) and 200 ± 5 (*n* = 10) µg/100 ml in Groups 1, 2 and 3 respectively. Differences between the groups were not significant. Two rats had unilateral pregnancies, the remainder were bilateral. The only significant differences in comparisons of the 5 sub-groups of uterine horns were between resorptions in Group lA and Group 2A or 2B ($P < 0.015$) and between Group lA and Group 3 ($P = 0.03$). There were no significant differences between the sub-groups in either overall incidence of abnormal foetuses or specific abnormalities and anomalies.

Table 56: Outcome of pregnancy in rats carrying coiled copper IUDs from days 9 to 21 of pregnancy (experiment 1)

Group	No. of rats	Uterine horn	No. of implantation sites	Fetuses		Resorptions		Mean ± s.e.m. fetal wt (g)
				Live	Dead	Early	Late	
1 (copper IUD)	9	A Operated (9)	63	51	0	9	3	2·95 ± 0·12
		B Unoperated (8)	42	39	0	2	1	3·02 ± 0·10
2 (sham-operated)	10	A Operated (10)	57	55	0	1	1	2·97 ± 0·10
		B Unoperated (9)	47	47	0	0	0	2·85 ± 0·07
3 (no operation)	10	Unoperated (20)	126	117	0	9	0	3·12 ± 0·05

Figures in parentheses indicate number of horns containing implantation sites.

Experiment 2 results: Gravimetric analysis of the IUDs before insertion on Day 9 and after removal on Day 21 of pregnancy showed a mean ± s.e.m copper loss/coil of 74 ± 4 µg, i.e. about 6 µg/coil/day. No significant reduction in weight of the steel coils was found between insertion and removal. Mean ± s.e.m. copper levels in maternal plasma on Day 21 of pregnancy were 207 ± 6 (n = 13), 194 ± 9 (n = 12) and 208 ± 14 (n = 7) µg/100 ml in Groups 4, 5 and 6, respectively. The differences are not significant. There was a significant increase in the incidence of resorptions in Groups 4A and 5A in comparison with Groups 4B and 5B ($P < 0.005$). There was no significant difference between Groups 4A and 5A. There were no significant differences in the overall incidence of foetal abnormalities. The only significant difference in the incidence of specific soft tissue abnormality was an excess of tracheobronchomegaly in Group 4A compared with Group 4B ($P < 0.02$). However, the difference between Group 4A and Group 6 was not significant. The only significant difference in the incidence of skeletal anomalies was a slight excess of extra 14th rib in foetuses from Group 4B in comparison with Group 6 ($P < 0.05$).

Table 57: Outcome of pregnancy in rats carrying coiled copper IUDs from days 9 to 21 of pregnancy (experiment 2)

Group	No. of animals	Uterine horn	No. of implantation sites	Fetuses		Resorptions		Mean ± s.e.m. fetal wt (g)
				Live	Dead	Early	Late	
4 (copper IUD)	13	A Operated (13)	75	57	0	16	2	2·96 ± 0·08
		B Unoperated (13)	95	91	0	4	0	3·04 ± 0·07
5 (steel IUD)	14	A Operated (14)	98	75	0	13	10	2·83 ± 0·08
		B Unoperated (13)	110	108	0	2	0	2·86 ± 0·07
6 (no operation)	7	Unoperated (14)	102	102	0	0	0	2·79 ± 0·11

Figures in parentheses indicate number of horns containing implantation sites.

Experiment 3 results: Foetal brain and liver and placental copper levels were significantly elevated in Group 7 animals, compared with those from Group 8 or the unoperated control. Variance in foetal copper levels in Group 7 was low, suggesting relatively uniform exposure of embryos and foetuses. Maternal liver levels of copper were not elevated in Group 7 (5.0 and 6.8 µg/g) compared with Group 8 (4.9 and 5.2 µg/g) or the unoperated control (4.5 µg/g). Uterine copper levels were considerably elevated in Group 7 (33.1 and 21.3 µg/g) compared with values in Group 8 (2.0 and 2.1 µg/g) and the control animal (1.8 µg/g).

Examination of the offspring for structural abnormalities confirmed that copper had no significant teratogenic or growth-retarding effect in the rat. The incidence of major malformations was low in all groups and the minor disturbances that were seen in all groups are known to be common spontaneous malformations in the strain of rat used. The copper ions released from intrauterine wire were insufficient to elevate maternal plasma copper levels. Copper levels in the rat maternal liver were not elevated, but the copper released from the IUDs did penetrate the foetus. Foetal brain copper levels were increased by 65% and foetal liver levels by more than 100% in copper-exposed offspring compared with those from mothers with steel IUDs or no IUDs. The lack of teratogenicity of copper released from IUDs cannot therefore be attributed to lack of exposure of the conceptuses. Moreover, the embryos were exposed to copper throughout organogenesis. The IUDs were inserted on the morning of Day 9 of pregnancy, which corresponds to the primitive-streak stage marking the onset of organogenesis, and is well before the time of neural tube closure on Days 10-11.

Intrauterine mortality rates of 19 and 24% in copper IUD horns were significantly higher than in sham-operated (4%) or untreated controls (0 and 8%), but were no higher than in horns carrying inert steel IUDs (25%). These results suggest that the deaths were probably due to trauma from the insertion and the physical presence of devices in the uterus, rather than to any specific effect of copper.

Reference: Chang, C.C. and Tatum, H.J. (1973).
Guideline: No
GLP: No
Deviations: Yes (from OECD 414)

- The toxicity of copper to reproduction / teratogenicity was assessed only after implantation of embryos in the Parent females. No copper was administered to males,
- only a single 'dose level' was used. The dose received by parent females was estimated, not measured,
- F_1 and F_2 generations were not exposed to copper during their growth, mating and reproduction,
- test and control groups generally contain fewer animals than recommended,
- effects on the oestrus cycle were not assessed,
- sperm parameters were not assessed.

These deficiencies do not, however, necessarily compromise the validity of the data generated.

A study was carried out to determine whether copper wire, placed within the uterus after implantation and kept in situ throughout pregnancy, produced any teratogenic effects on the embryo, or altered in any way the development and subsequent growth of the offspring of rats, hamsters and rabbits. The potential for adverse effects on the fertility of treated animals was also assessed.
Nulliparous female rats of the Holtzman strain, adult cycling female hamsters and adult albino New Zealand female rabbits were used.

In rats and hamsters, positive matings were verified by the presence of sperm in vaginal smears. The day of insemination was designated as Day 1 of pregnancy. In rabbits, visual observation only was used to confirm copulation, and that day was designated as Day 0 of pregnancy.
Copper wire (99.9% pure) was inserted into the endometrial cavities of both uterine horns of rats and hamsters on Day 6 of pregnancy. It was estimated that the rate of dissolution of the wire used in the cycling rat was approximately 2.75 µg per day.
In rabbits, the wire was inserted into the uterine horns on Day 7 of pregnancy. The amount of copper released in 24 hrs from the wire was estimated to be approximately 5.50 µg on the assumption that the rate of dissolution of the wire used in the rabbit is similar to that in the rat.

The wire was left *in situ* during pregnancy and lactation, and the gestation period was recorded. The mothers were sacrificed at the time of weaning and the ovaries, uteri and adrenals were fixed with Bouin's solution for histological examination. The number and sex of the pups of rats and hamsters were recorded at birth and the offspring were observed for gross abnormalities. The body weight of F_1 generation rats was recorded at 5-day intervals from the age of 5 days through 60 days. The offspring of rats and hamsters were weaned at the age of 25 days and the number of surviving F_1 generation was recorded. In the meantime, the females were separated from the males and maintained in separate cages to raise F_2 and F_3 generations. In rabbits, laparotomy was done on Day 15 of pregnancy and the number of implantation sites was recorded. The offspring were weaned when 30-35 days of age. Some of the F_1 generation rabbits were sacrificed at the age of either 3 or 6 months.
When the F_1 generation rat and hamster females reached the age of 90 days and the males 120 days, each female was cohabited with one fertile male and each male with 2 virgin cycling females for 10 days. The fertility of the F_1 generation animals was evaluated by the following regimens: a) the ratio of the animals mated over the animals used and b) the number of implantation sites or the number of pups delivered. Some of the animals delivered by the F_1 generation were eliminated at the time of weaning and examined for gross malformations. The remaining animals were used for fertility testing when they reached maturity. The fertility of F_2 generation animals was tested in a manner similar to that described for the F_1 generation.

At autopsy, the body weight and the weights of the following organs were determined: ovaries, uteri and adrenals in the females; testes, seminal vesicles, epididymus (in the rabbit only), ventral prostate and adrenals in the males.

Rats: There was no difference in gestation periods between the mothers bearing the wire in the uterus and controls. All copper wire treated and control mothers delivered normally. However, a comparison of the average number of pups delivered from treated mothers to those from untreated rats showed that the copper wire treated females delivered 6.5 ± 0.7 pups, a number significantly lower than that of the untreated controls (8.6 ± 0.6) at the 5% confidence level. It is considered likely that the incidence of fewer pups in the treated group was due to manipulation of the uterus and damage to the embryos at or near the site when the copper wire was inserted.

No teratogenic effects were evident in offspring. No abnormalities were observed at birth, at weaning or at the time of the fertility test. There was no effect of copper wire on survival rates of the F_l generation animals at the time of weaning. Survival rates of the descendants of treated and untreated mothers indicates that lactation was not interrupted by the wire. F_l generation animals of both sexes grew normally, as evidenced by the increases in body weight. There were no significant differences in fertility of offspring of copper treated and untreated mothers of either sex in the F_1 generation. There were no significant differences in organ weights of offspring of copper wire treated and untreated mothers in either sex of the F_1 generation.

There were no significant differences in fertility among F_2 generation descendants of copper wire treated and untreated animals. There were no significant differences in body weights or organ weights in either sex of the F_2 generation.

At autopsy, there were no gross anatomical deformities noted in Parent, F_1 or F_2 generations. Histological examination of the ovaries, uteri and adrenals of Parent females, and of female and male tissues of F_1 and F_2 generations did not show deviations from normal.

Hamsters: There was no difference in the average number of pups born between the group bearing copper wire and the control group. The gestation period for treated animals was not different from the controls. Lactation in treated mothers was considered to be normal, based on the average body weights of pups and the percentage lost at weaning. No teratogenic effects were observed in the F_1 generation animals at birth and at weaning. Histological examination of the ovaries, uteri and adrenals of mothers with copper wire showed no deviation from normal.

There was no apparent effect on the fertility of offspring of treated and untreated mothers in either sex of the F1 generation. There were no significant differences in organ weights of offspring of copper wire treated and untreated mothers in either sex of the F1 generation. There were no significant differences in fertility among F2 generation descendants of copper wire treated and untreated animals. However, the average number of pups delivered in BB females (descendants of control Parent) and AA males (descendants of copper treated Parent) was significantly lower than that of normal animals (2.0±1.0 vs 7.8±0.9) for female parents, 3.1±0.6 vs 7.9±0.8 for mal parents). The cause for this difference in the F2 generation is not known.There were no significant differences in body weights or organ weights in either sex of the F2 generation, other than an unexplained increase in the adrenal weights of control males. At autopsy, there were no gross anatomical deformities noted in Parent, F1 or F2 generations. Histological examination of the ovaries, uteri and adrenals of Parent females, and of female and male tissues of F1 and F2 generations did not show deviations from normal.

Rabbits: At the time of insertion of the copper wire (Day 7 of pregnancy), there was no difference in the average number of implantation sites between the animals which were to be exposed to copper wire and the controls. However, at laparotomy on Day 15 of pregnancy, the number of implantation sites was significantly less than that observed on Day 7 of pregnancy. The number of pups subsequently delivered from these animals was reduced as compared to that in the control animals. This difference was thought to be due to manipulation of the uterus at the time of insertion of the copper wire.

There were no gross anatomical deformities noted in Fl generation at birth, at weaning or at autopsy. The fertility of F1 generation was normal.

The Parent females were autopsied after weaning. Histological examination of the ovaries, uteri and adrenals showed no deviations from normal.

No teratogenic effects were observed in F1 generation animals and their growth rate was normal. There were no significant differences in body weight and organ weights between the Fl generation animals of either sex from copper treated and untreated mothers. Histological examination of the female and male reproductive tissues of Fl generation animals showed no deviations from normal.

Reference: Haddad *et al.(*1991)
Guideline: No
GLP: No.

Water loaded with copper acetate was administered to Wistar albino rats at increasing stepwise concentration of the copper acetate to 0.185% over a period of seven weeks (approximately 65 mg Cu/kg body weight per day). A group of control animals received demineralised water. At the end of seven weeks 7 rats from each group were sacrificed to serve as non-pregnant controls. The remaining rats were mated singly. The pregnant females were divided into three groups. The first group with 7 controls and 14 experimental rats were sacrificed at 11.5 days of gestation; the second group with 7 controls and 14 experimental rats were sacrificed at 21.5 days of gestation and the third group with 7 controls and 14 experimental rats were allowed to litter. Blood samples were collected for the measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase levels.

Histopathology was performed on liver and kidneys, including staining for copper and iron. Samples of liver were subjected to atomic absorption spectrophotometry for copper levels. Embryos from the dams killed after 11.5 days were examined for growth and development and 21.5 day foetuses and newborn pups were counted, weighed and examined for external malformations. Two foetuses and two newborn pups (from each litter) were processed and examined for visceral malformations. Histopathological examination was performed on sections of liver and kidney from one foetus and one newborn pup. The remaining foetuses and newborn pups were processed for skeletal assessment. Statistical analyses were performed.

General observations: There were no treatment related clinical signs throughout dosing and maternal weight gains for the treated animals were similar to those in the controls. Pregnancy rate was not adversely affected by maternal treatment.

Duration of gestation: There was no difference in the duration of gestation between the controls and the copper loaded group.

Clinical chemistry: There were no differences in the serum AST, ALT and alkaline phosphatase activities between the control and the copper loaded groups (Table below).

Table 58: Clinical chemistry parameters

Parameter	**Controls**	**Copper loaded**
AST (IU/L)	27.3	25.9
ALT (IU/L)	14.2	17.3
Alkaline phosphatase (IU/L)	11.8	9.6

Histopathology: Liver and kidney sections from the control animals showed normal histology with no copper deposits. Liver sections from the copper loaded rats showed copper deposition in the hepatocytes and to a lesser extent in the Kupffer cells; copper was present as clusters or granules in the cytoplasm. Analysis of copper content showed that copper levels of treated rats was higher than controls (207.7 μg/g dry weight in treated compared to 23.4 μg/g dry weight in controls). Lesions included hypertrophy of the hepatocytes with cloudy eosinophilic cytoplasm, areas of focal necrosis surrounded by inflammatory foci of polymorph and lymphocyte infiltration, the presence of sinusoidal dilatation and the appearance of cytoplasmic vacuolation. In the kidneys, copper deposition was present in the proximal convoluted tubules. Lesions were confined to the proximal convoluted tubules, characterised as cloudy swelling due to hydropic degeneration and obliteration of the lumen with occasional desquamation of the epithelial cells. Liver and kidney sections stained for iron showed no deposits. These findings are similar to those seen in papers by Haywood et.al (Section B.6.5.2.3 –B.6.5.2.5), where liver damage and kidney changes were recorded at high levels of dietary copper administration. The histological changes indicated that the levels of copper loading were in excess of the maximum tolerated dose, although actual analysed liver levels of copper were lower than where analysed by Haywood (Section B.6.5.2.4). Foetal and newborn liver and kidney sections showed a normal histological pattern with no copper deposits.

Foetal and newborn examinations: At 11.5 days gestation, overt embryonic development was similar in most parameters analysed. However, there were minor changes in mean somite number, mean crown-rump length and mean yolk sac diameter were slightly decreased when compared with the controls (Table below). These changes indicated a slight delay in development for time of gestation, although the small sample size, and the imprecise nature of the parameters measured must be taken into account.

Table 59: Growth and development of 11.5 day old embryos

Parameter	**Controls**	**Copper loaded**
Number of dams examined	14	6
Number of embryos examined	56	95
Number (%) of embryos showing:		
Presence of heart beat	56 (100)	95 (100)
Presence of fused allantois	56 (100)	94 (99)

Normally closed anterior neuropore	56 (100)	92 (96)
Normally closed posterior neuropore	53 (94)	80 (84)
Presence of normal turning	54 (96)	87 (91)
Presence of forelimb buds	56 (100	95 (100)
Presence of normal optic vesicle	56 (100)	92 (96)
Presence of normal otic vessel	56 (100)	93 (97)
Mean somite number	23.48	22.03*
Mean crown-rump length in mm	2.98	2.71*
Mean yolk sac diameter in mm	4.56	3.98*

* $P < 0.005$

The number of offspring per litter and the mean foetal weights of the treated animals were stated to be similar to controls. Similarly, external and visceral examination revealed no differences. Skeletal examination showed reduction in the number of ossified centres in almost all the ossification centres examined, which was significant generally in 21.5 day old foetuses but significant only in cervical vertebrae, caudal vertebrae and hindlimb phalanges in newborn pups (Table below). These ossification findings are generally considered transient, in that they reflect the stage of the ossification process, and it is significant that the incidence was much lower in the new-born pups than in the day 20.5 foetuses. It should be noted the presence or absence of an ossification centre is not the same as the presence or absence of the feature itself; absence means that the feature has not yet ossified i.e. it is still cartilage. The differences may reflect maternal copper-calcium balance, leading to slightly reduced availability of calcium to the foetus.

4.9.3 Other relevant information

Table 60: Summary of investigative studies (data from literature)

Method **Species**	**Exposure conditions and doses**	**Observations and remarks**
Pregnancy Marois (1972) No GLP Rat and Rabbit	Sub cutaneous (rat) ; i.v. (rabbit) Copper acetate 10 or 15 mg/kg with or without progesterone to rat, 8 mg/kg copper acetate only to rabbit	Copper acetate alone terminated pregnancy in 3/6 rats; copper acetate + progesterone did not. Authors state that copper interrupted CNS control of pregnancy in rat.
Post-implantation embryo *in vivo* and *in vitro* O'Shea (1979) No GLP Mouse 6 mated females/group	i.v. *in vitro* phase in culture bath Copper sulphate 4 mg Cu/kg bw i.v. *In vitro* phase 0.332, 1.60 or 3.2 µg copper/mL culture bath	Injection on early day 7 of pregnancy killed all embryos. Injection on day 8 showed a high incidence of neural tube, cranial and heart defects, injection on day 9 showed fewer anomalies. Embryoculture showed similar malformations
Fertility Auerlich et al. (1982) No GLP Mink 12/sex/group	Diet Copper sulphate 0, 25, 50, 100 or 200 ppm (approximately 3, 6, 12 or 24 mg Cu/kg body weight per day)	Reduced offspring survival at 100 and 200 ppm. Reduce kit weight was observed at and above 100 ppm. Elevated copper levels in liver and plasma in

	Duration: 9 months before mating and 3 months after mating	mink. No information was provided on developmental malformations.
Post implantation embryo development Ferm (1974) No GLP Golden Hamster 10 mated females/ groups.	i.v. Copper sulphate 2.13, 4.25, 7.50 or 10.0 mg Cu/kg bw or copper citrate complex 0.25-1.50, 1.80, 2.20, or 4.9 mg Cu/kg bw	High doses maternally lethal, near-lethal doses increased resorption and embryos with neural tube, cranial, tail, thoracic wall and heart malformation. Sulphate much better tolerated than citrate complex.
Post implantation embryo development Di Carlo(1979) No GLP Golden Hamster 12 -17 mated females/groups	i.p. Copper citrate complex 2.7 mg Cu/kg bw	5% of embryos with heart defects
Antitesticular effect Kamboj (1963) No guideline applicable Swiss albino rats 6 males/group Swiss albino mice 3 males/group	Cupric sulphate Rat single intratesticular injection to the left testis of 0.02, 0.04 and 0.08 mM/kg Rat and mice subcutaneous injection 0.08 mM/kg Killed after 2-7 days in rats And after 30 days in mice Note: the route of administration is not appropriate for risk assessment purposes.	Intratesticular injection in rats caused dose-related degrees of degeneration of the seminiferous epithelium and the interstitium. Single subcutaneous injection was ineffective. Continuous administration to mice caused slight weight change but no histopathological changes. There were no changes following subcutaneous administration.

In these studies single high doses of copper have been administered parentally, via intravenous, sub cutaneous or intra peritoneal injection. These studies appear to have been performed in order to study induction of foetal malformations, and the routes of administration were chosen because it is not possible to engender similar malformations by oral administration. The studies are not strictly relevant to the classification of copper hydroxide but they are known to the regulatory authorities.

4.9.4 Summary and discussion of reproductive toxicity

- Non human data

Fertility

Effects on fertility were investigates in a two generation study in rat. In this study, no treatment related effects were observed on reproduction parameters or systemic toxicity.

In the four other fertility studies (non guideline, not GLP), there were no differences from controls in any of the parameters studied.

Development

Developmental toxicity of copper has been investigated in a well-conducted GLP study in rabbit. In this study, no malformation of concern was noted and the observed developmental effects were considered to be secondary non specific consequence of the maternal toxicity and not a direct effect on development.

Moreover the extensive data on the absorption and excretion of copper in the human, in livestock and in laboratory animals, show that there is no potential for any cumulative effect over several generations.

- Human data

There is a comprehensive review of copper in pregnancy and childhood in the human. This review identified risk to neonates fed cows milk boiled in untinned copper vessels. In most of the reported studies, there were no indications of any adverse effects on pregnancy, birth or growth that were associated with exposure to copper.

However, Graham *et al.* (1980) reported two cases of anencephaly in women where an intra-uterine contraceptive device (IUD) was used. Anyway, copper released from these devices significantly increases copper concentrations only in the intrauterine fluid in the first 12 months of utilization, but it do not increase serum copper or caeroplasmin concentrations (Gosden et al., 1977). In addition, the mean daily release of copper by the IUD corresponds at only 1% of the mean daily copper intake by the alimentation. Moreover, others more recent studies reported that the IUD does not increase the risk of congenital abnormalities (Pasquale 1996; Weissmann-Brenner et al. 2007).

4.9.5 Comparison with criteria

Reprotoxic substances can be toxic to the development of the unborn child or can cause impairment of fertility in male and female subjects.

Reprotoxic substances are divided into 2 groups;

- Effects on male or female fertility, including adverse effects on libido, sexual behaviour, any aspect of spermatogenesis or oogenesis, or on hormonal activity or physiological response.
- Developmental toxicity, including any effect interfering with normal development before and after birth.

1) Criteria in the CLP classification :

- Fertility and developmental toxicity

Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects.

2) Comparison with criteria:

- ⇨ As in a rat two-generation study (guideline and GLP) and in four other fertility studies (non guideline, not GLP) there were no differences from controls in any of the parameters studied, no classification is proposed for copper concerning fertility and reproduction
- ⇨ As no malformation of concern was noted in a well-conducted GLP study in rabbit, no classification is proposed for copper concerning developmental toxicity reproduction

4.9.6 Conclusions on classification and labelling

Based on all the available data and the weight of evidence on the impact of copper on developmental toxicity, no classification is required for copper compounds concerning reproductive and developmental effects.

RAC evaluation of reproductive toxicity

Summary of the Dossier submitter's proposal

No data on tetracopper hexahydroxide sulphate are available in the CLH report. However, in light of the proposal to read-across between the different copper compounds for systemic endpoints (see section "RAC general comment" above), the dossier submitter included in the CLH report several animal studies investigating the reproductive toxicity of other copper compounds, as well as some human data.

Fertility – Effects of copper sulphate pentahydrate on fertility were examined in a 2-generation study conducted according to OECD TG 416 (Mylchreest, 2005). No treatment-related effects were seen on any of the fertility and litter parameters investigated. Two other non GLP studies conducted with copper gluconate (De la Iglesia *et al.*, 1973) and copper sulphate (Lecyk, 1980), included as supporting evidence, also showed no effects on fertility.

Development – An OECD TG 414 compliant rabbit developmental toxicity study conducted with copper dihydroxide (Munley, 2003d) showed some slightly increased incidences in common skeletal variants that were considered secondary non-specific consequences of maternal toxicity. Two other non-guideline studies exposing rats and mice to copper gluconate via gavage (De la Iglesia *et al.*, 1972) did not reveal treatment-related effects on developmental parameters. Another non-guideline compliant study with copper acetate administered to rats via drinking water (Haddad *et al.*, 1991) showed some delayed ossification in foetuses but not in new-borns. In addition, two studies exposing pregnant rats, rabbits and hamsters to intra-uterine copper wire (to mimic exposure to intra-uterine contraceptive device (IUD)) showed no teratogenic or growth-retarding effects in the offspring (Barlow *et al.*, 1981; Chang & Tatum, 1973).

Human exposure – Copper in the uterus (as IUD) is known to prevent implantation of the blastocyst, but once implantation takes place the foetus develops normally. The CLH report mentions that although two cases of anencephaly after use of IUD have been reported (Graham *et al.*, 1980), more recent reports indicated that IUD did not increase the risk of congenital abnormalities (Pasquale, 1996; Weissmann-Brenner *et al.*, 2007). No further details on any of these publications were however presented. Dietary exposure to copper does not appear to result in adverse effects on pregnancy, birth or growth and development (Ralph & McArdle, 2001).

Based on the available data and the weight of evidence, the dossier submitter concluded that no classification for reproductive and developmental effects is warranted for copper compounds, including tetracopper hexahydroxide sulphate.

Comments received during public consultation
No comments were received during the public consultation.

Assessment and comparison with the classification criteria
RAC notes that no data on tetracopper hexahydroxide sulphate are available. The CLH report contains data on other copper compounds (among which copper sulphate pentahydrate), from which the dossier submitter proposed to read-across to tetracopper hexahydroxide sulphate. In view of the considerations presented in the section "RAC general comment", RAC has not pursued the aspect of grouping any further. RAC concludes that in the absence of relevant data no proposal for classification for reproductive toxicity can be made for tetracopper hexahydroxide sulphate.

4.10 Other effects

In the review of copper toxicity database of Stern et al. (2007), no new information was available in human or animals.

4.10.1 Non-human information

4.10.1.1 Neurotoxicity

Table 61: Neurotoxicity study results

Method Species	Exposure conditions and doses	Observations and remarks
Malhotra (1982) No guideline applicable No GLP 12 male/groups treated for 21 days	Group 1: lead acetate 100mg/Pb/L Group2: i.p cupric chloride at 2 mg cu/kg Group 3: lead acetate (100mg/L) in dreaking water + cupric chloride (2 mg cu/kg) i.p. Group 4: control: sodium acetate 100mg/L in drinking water	Copper treatment alone had no effect on the levels of copper in the brain mitochondria, and did not affect enzyme activities in mitochondria. The presence of copper reduced the levels of lead and the adverse effects of lead.
Murthy *et al.*, (1981) No guideline applicable No GLP 6 male rats	Dietary administration of 250 mg/kg Cu (as copper (II) sulphate in pentahydrate) for 30 days, equivalent to about 20 mg/kg bw/day	No affect their locomotor activity, learning ability or re-learning capacity and memory. But analysis of biogenic amines in the brain revealed an increase in the dopamine and norepinephrine levels of animals receiving the protein-adequate diet. Furthermore, the administration of Cu was associated with decreased levels of calcium and zinc in the brains of rats fed both the low- and high-protein diets. The Cu content of the brain was elevated in all Cu-treated animals (+174 and +172% for low- and high-protein diets, respectively). The neurotoxicological significance of these findings is unclear, given that there were no associated effects on behaviour.

Summary and discussion of neurotoxicity
The limited amount of evidence indicates that excess copper does not adversely affect function of brain mitochondria. In the many toxicity studies on animals, there have been no

indications that copper is selectively neurotoxic. However, in humans with the genetic condition Wilson's disease, where the copper transporter WND protein is inactive, copper progressively accumulates in the liver and in the brain, and subjects in the later stages of the disease, which is fatal through liver failure if not treated, show signs of neurotoxicity. In genetically normal humans, and in normal laboratory animals, the natural homeostatic mechanisms that regulate copper prevent any accumulation in brain and neural tissues, such that copper is never neurotoxic.

Acute, short term and long term studies where copper has been administered in the diet to laboratory animals have not shown any neurotoxic signs, and histopathology of neural tissues have not shown any adverse effects associated with copper administration.

4.10.1.2 Immunotoxicity

Table 62: Immunotoxicity study results

Method **Species**	**Exposure conditions and doses**	**Observations and remarks**
Immune response Pocino, M (1991) No standard guideline C57BL/6 mice Males and females	$CuSO_4$ Drinking water 50, 100 and 200 ppm 3-10 weeks	100 or 200 ppm: depressed levels of all of the four immunological parameters investigated, including both cellular and humoral immune responses. Other studies in humans have shown that the nausea threshold for copper as sulphate in drinking water is 6 – 8 µg/L.

4.10.1.3 Specific investigations: other studies

Table 63: Complementary studies

Method **Species**	**Exposure conditions and doses**	**Observations and remarks**
Johansson, A (1983&1984) No standard guideline 8 male rabbits	$CuCl_2$ Inhalation $0.6mg/m^3$	No effects on alveolar type II cells, alveolar macrophages, and no increased pulmonary phospholipids
Acute toxicity of copper to mink Auerlich (1982) No guideline applicable dark mink 6animals groups	Copper sulphate intraperitoneal injection 3.1, 6.2, 9.4, 12.5 and 25.0 mg/kg Copper acetate 5, 10 and 20 mg/kg	LD_{50} of copper sulphate was 7.5 mg/kg, and the LD_{50} of copper acetate was 5.0 mg/kg.

4.10.1.4 Human information

No data available

4.10.2 Summary and discussion

Neurotoxicity

The limited amount of evidence indicates that excess copper does not adversely affect function of brain mitochondria. In many toxicity studies on animals, there have been no indications that copper is selectively neurotoxic.

Immunotoxicity

In a drinking water study in mice, concentrations of copper sulphate as high as 200 ppm were associated with inhibition of the immune response, although the authors indicated that the effects were similar to zinc deficiency immune inhibition, as excess copper can cause zinc deficiency through induction of metallothionein, which removes both metals. A NOAEL of 50 ppm was demonstrated.No data on food or water consumption or on bodyweights were present in this paper, so it was impossible to assess either the dose administered or to quantify a NOEL in terms of actual intake of copper

Other studies

A series of studies was performed on salts of copper, cadmium and cobalt, to determine if rabbit alveolar type II cells and alveolar macrophages showed similar changes to those induced in earlier studies with nickel. Rabbits were exposed 6 hours/day, daily for 4 to 6 weeks to 0.6mg Cu/m3 as CuCl2 but findings were generally similar to controls.

In an acute intra peritoneal study on mink, the LD50 of copper sulphate was 7.5 mg/kg and the LD50 of copper acetate was 5.0 mg/kg.

4.10.3 Comparison with criteria

In many toxicity studies on animals, there have been no indications that copper is selectively neurotoxic. No classification under Regulation (EC) 1272/2008 is proposed. No classification or SCLs are considered necessary.

Excess copper is associated with inhibition of the immune response in mice. However, this may be an indirect effect of copper-induced zinc deficiency rather than a direct effect of copper. Thus, immune system is not a primary target organ of toxicity for copper.

4.10.4 Conclusions on classification and labelling

Copper compounds should not be classified. No SCL is considering necessary.

5 ENVIRONMENTAL HAZARD ASSESSMENT

The environmental fate properties assessment of Copper is based on the Draft Assessment Report, Addenda to the Draft Assessment Report and the Conclusion on Pesticide Peer Review of Copper compounds (2008) for the environmental behaviour and based on the EU Voluntary Risk Assessment (Existing Substances Regulation) of Copper, Copper II sulphate pentahydrate, Copper (I) oxide, Copper (II) oxide, Dicopper chloride trihydroxide (2008), and the Assessment Report for Copper (II) Oxide for Biocidal Product PT08 (2010) for the aquatic toxicity.

5.1 Degradation

In soil

Copper is an inorganic compound that cannot be degraded in soils. It is therefore not possible and not relevant to define a route and a rate of degradation in soils as usually made for organic compounds.

However, copper can be present under different forms, most of which are strongly bound to inorganic and organic ligands contained within soil and sediments; while a marginal fraction of copper is present as various species in the soil solution. The fate and behaviour of copper, as its bio availability, strongly depend on the distribution of these different forms.

The distribution and equilibrium between the different forms of copper in soil depend on many factors, such as soil pH, texture and organic matter content. If the mobile, active and toxicologically significant substance is mainly the free copper ions Cu^{2+} present in the soil solution, it is not possible to predict how much this form will represent from the total applied amount of copper. The activity of the free copper ion will steadily increase with decreasing pH for instance, while the contribution of complex species will decrease. The binding affinities of Cu^{2+} with organic or inorganic matter are also dependent on the presence of competing metal ions and inorganic anions.

In water

Metals are indeed natural elements and are therefore, by definition, not degradable. In water, copper cannot be transformed into related metabolites or degradation products and consequently hydrolysis and bio-degradation processes in water will have no action on copper in this respect. Although unable to degrade, copper are subject to chemical transformation processes with a wide array of materials so that the vast majority of copper in aquatic systems is rapidly bound to mineral particles, precipitated as insoluble inorganic salts, or bound to organic matter. In pure water very low levels of the free Cu^{2+} ion are present in solution, with amounts governed by the propensity of the metal cation to hydrolysis in water, as shown in the following equation:

$$Cu(H_2O)_6^{2+} + H_2O \leftrightarrow CuOH(H_2O)_5^{+} + H_3O^{+}$$

The reaction is pH dependent with a distribution constant equal to 6.8. Therefore, below pH 5.8 the predominant form will be $Cu(H_2O)_6^{2+}$, whilst above pH 7.8, the predominant form will be $CuOH(H_2O)_5^{+}$. This latter form of copper is an inorganic complex for which a wide range of other possible types could be formed in natural water, with either cupric or cuprous ions and a range of inorganic ligands (e.g. OH^-, HCO_3^-/CO_3^{2-}, $H_2PO_4^-/HPO_4^{2-}$, Cl^-, SO_4^{2-} and S^{2-}) and organic ligands (e.g. humic and fulvic acids) associated with dissolved organic matter. In natural water, the solubility of copper is regulated primarily by the formation of malachite ($Cu_2(OH)_2CO_3$) at pH < 7 and by tenorite (CuO) at pH > 7. The concentration of Cu^{2+} ions in solution will be higher at low pH, however the exact concentration will depend considerably on the type and concentration of ligands presenting the water.

Copper entering a water body is rapidly bound to material in the water phase resulting in very low levels of free Cu^{2+} ion in solution. In a water-sediment system, total copper was re-distributed from the surface water to the sediment, at a worst case dissipation rate of 30.5 days (considered as a DT50 for the water column), calculated using first-order kinetics. The majority of the applied copper in the sediment is bound to solid matter. Therefore, in a complex environment, total or even dissolved copper levels are not appropriate to assess bio-

available copper exposure. Within the soluble water phase, complexation process reduces the actual amount of copper, available for uptake by biological organism.

In the Guidance to Regulation (EC) No 1272/2008 Classification, Labelling and Packaging of substances and mixtures (metal annex), it is stated that 'Environmental transformation of one species of a metal to another species of the same does not constitute degradation as applied to organic compounds and may increase or decrease the availability and bioavailability of the toxic species. However as a result of naturally occurring geochemical processes metal ions can partition from the water column. Data on water column residence time, the processes involved at the water – sediment interface (i.e. deposition and re-mobilisation) are fairly extensive, but have not been integrated into a meaningful database. Nevertheless, using the principles and assumptions discussed above in Section IV.1, it may be possible to incorporate this approach into classification. This approach will be accepted if a laboratory/mesocosm study is available to validate the following principles

1) Soluble metal concentration are shown to have decreased by > 70% in 28 days

2) The absence of remobilization is verified

3) Demonstration that changes in the sediment redox will not result in release of the metal.

In the sediment compartment, copper binds to the sediment organic carbon (particulate and dissolved) and to the anaerobic sulphides, resulting in the formation of CuS. CuS has a very low stability constants/solubility limit (LogK=-41 (Di Toro *et al.*,1990) – see section *adsorption/desorption*) and therefore the 'insoluble' CuS keeps copper in the anaerobic sediment layers, limiting the potential for remobilization of Cu-ions into the water column. Simpson et al (1998) and Sundelin and Erikson (2001).

In order to demonstrate removal from the water column to assess the "persistence" or lack of degradation of metal ions, responsible for the toxicity of metals and metal compounds (> 70% removal within 28 days), the copper Task force provided the following study (Rader (2013)).

EXECUTIVE SUMMARY

Introduction

In line with the GHS guidance, "rapid degradation" for metals requires one to demonstrate not only rapid loss from the water column, but also limited remobilization potential from sediment.

The two main objectives of this work are:

- Simulate copper removal from the water column of a generalized lake environment using a transport and fate model, TICKET-UWM, to see if the rapid removal benchmark of 70% removal of dissolved copper in 28 days is met and assess the extent to which copper deposited to sediment is remobilized to the water column.
- Assess the predictive capabilities of the TICKET-UWM by testing it against data from several laboratory and field datasets including a lake, a reservoir, a large enclosure in a lake, and laboratory microcosms.

The Tableau Input Coupled Kinetics Equilibrium Transport Unit World Model for Metals in Lakes (hereafter referred to simply as the TICKET-UWM) (Farley et al., 2007; Farley et al., 2008; Farley et al., 2010) was developed to address the complexities of metal speciation and its influence on the fate and effects of metals in the environment. Processes considered by the

model include complexation by aqueous inorganic and organic ligands such as dissolved organic carbon (DOC), adsorption to particulate phases such as particulate organic carbon (POC) and iron/manganese oxides, binding to biological receptors (biotic ligands), dissolution kinetics of metals powders, and cycling of organic matter and sulfide production in lakes.

Generalized Lake Simulations

The model was used in time-variable mode to simulate copper removal following a single short-term addition to the water column of a generalized lake based upon the EUSES model lake (RIVM, 2004; ECHA, 2010). The initial copper concentrations used for the simulations were set at the pH-specific chronic ERVs (Table 1), 0.1 mg/L, and 1 mg/L. The water chemistries for three pH values (6, 7, and 8) were based upon directives in Annex IV of the Guidance on the Application of the CLP Criteria (ECHA, 2011) and Annex 10 of the *Globally Harmonized System of Classification and Labelling of Chemicals* (GHS) (United Nations, 2011) (Tables 2 and 3).

Table 1. Summary of Initial Copper Concentrations Used for TICKET-UWM Simulations.

Metal	pH 6		pH 7		pH 8	
	Chronic	Acute	Chronic	Acute	Chronic	Acute
Copper	20	25	7	35	11	30

All concentration in units of µg/L

Table 2. TICKET Unit World Model Input Parameters for EUSES Model Lake

Parameter		Value	
Simulation time, days		28	
Time step, days		0.1	
Volume, m^3		3.6×10^9	
Surface area, km^2		1200	
Depth, m		3	
Residence time, yr		0.110	
Settling rate, m/d		2.5	
Burial rate, cm/yr		0.3	
Resuspension rate , cm/yr		2.44 [a]	
Diffusive exchange, cm/day		0.24 [b]	
Sediment f_{oc}		0.05	
Sediment solids conc., g/L		500	
Active depth, cm		3	
AVS, µmol/g_{dry}		0.77 [c]	
Initial Cu conc., µg/L as Cu		29	
Initial Cu conc., µmol/L as Cu		0.46	
POC, mg/L		1.5	
DOC, mg/L		2.0	
pH [d]	6.09	7.07	8.00
Alkalinity, mg/L as $CaCO_3$	3.85	7.47	37.2
Calcium, mg/L	8.0	32.1	80.1
Magnesium, mg/L	1.2	4.9	12.1
Sodium, mg/L	1.8	3.4	18.0
Potassium, mg/L	0.3	1.2	3.02
Sulfate, mg/L as SO_4	4.8	19.2	47.9
Chloride, mg/L	14.5	57.8	145

[a] Calculated using the settling velocity, suspended solids concentration, sediment bulk solid concentration, and the burial (net sedimentation) rate shown in table using a solids balance (Chapra, 1997).

[b] EUSES pore water side mass transfer coefficient. Based on Di Toro et al. (1981) mass transfer resistance is all in the sediment.

[c] 10th percentile value from the Flanders dataset (Vangheluwe, 2005; additional information from: http://echa.europa.eu/copper-voluntary-risk-assessment-reports [environment/Risk Characterization/Chapter 3.3.7.1.3.])

[d] This is the pH of the water column and the sediment.

Table 3. Distribution Coefficients, Fraction Particulate Values, and Maximum Rapid Removal Depths for Different pH Values[a]

pH	log K_D	Fraction Particulate (f_{Part})	Max Depth with 70% Removal [b] in 28 days, m
6.09	6.29 (6.17 – 6.30)	0.967 (0.957 – 0.968)	53
7.07	6.18 (6.01 – 6.19)	0.958 (0.939 – 0.959)	52
8.00	5.60 (5.57 – 5.60)	0.857 (0.847 – 0.857)	47

[a] Average over 28 days is indicated with range shown in parentheses. Initial Cu concentration was set at the pH-specific acute ERV (Table 2-1)

[b] Based on total copper

These parameters were determined to be close to the 10-90th percentile ranges observed in EU surface waters (Table 4), probably with the exception of the concentration of chlorides (not a critical parameter in the assessment).

Table 4. ARCHE overview of physico-chemical characteristics of EU surface waters

	Median of 10th percentiles	Median of 50th percentiles	Median of 90th percentiles
Calcium	17.22 mg/L	49.92 mg/L	89.99 mg/L
Magnesium	3.29 mg/L	7.81 mg/L	17.57 mg/L
Sodium	3.81 mg/L	8.25 mg/L	19.91 mg/L
Potassium	1.03 mg/L	2.23 mg/L	5.11 mg/L
Chloride	4.67 mg/L	13.47 mg/L	34.82 mg/L
Sulfate	8.28 mg/L	22.08 mg/L	81.07 mg/L
Hardness (as $CaCO_3$)	68.01 mg/L	161.88 mg/L	327.45 mg/L
DOC	1.67 mg/L	3.01 mg/L	5.63 mg/L
TOC	3.66 mg/L	9.32 mg/L	15.45 mg/L
Susp.solids	6.15 mg/L	16.00 mg/L	41.66 mg/L
pH	7.06	7.66	8.03

For the generalized lake removal calculations, copper adsorption onto suspended solids was described by means of two different approaches: 1) using empirical distribution coefficient values (log K_D) from the copper risk assessment (Cu RA) document (Heijerick et al., 2005) for the water column and sediment; and 2) using the speciation models within TICKET-UWM to calculate "instantaneous" distribution coefficients based upon water chemistry and the concentration of particulate sorbents (e.g., particulate organic carbon, POC). Based on the description of the rapid removal definition in Annex IV, removal was evaluated by comparing the concentration of dissolved copper at a particular time to the initial concentration (Figures 1 and 2).

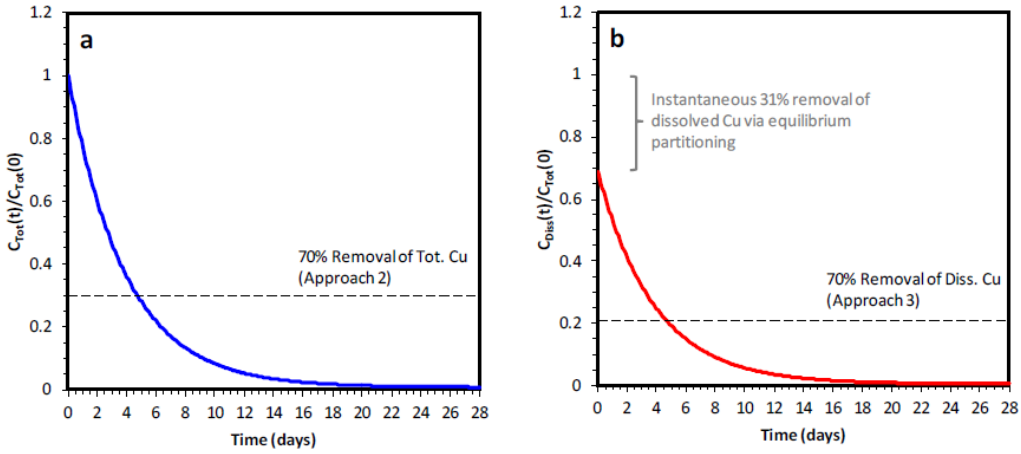


Figure 1. a) Total and b) dissolved copper (Cu) removal from the water column using EUSES model parameters and the linear partitioning method. The initial total copper concentration in the water column, *C*Tot(0), is 35 µg/L. The horizontal dashed lines represents a) *C*Tot(t)/*C*Tot(0) = 0.3 (70% removal of total copper) and b) *C*Diss(t)/*C*Diss(0) = 0.3 (70% removal of dissolved copper).

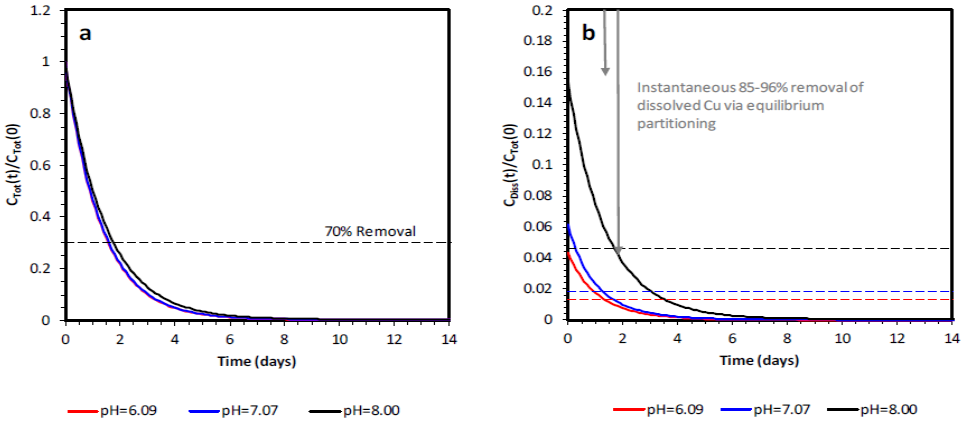


Figure 2. a) Total and b) dissolved copper removal from the water column under different pH conditions. Copper speciation (including binding to POC) was calculated using WHAM V in TICKET-UWM. The initial total copper concentration in the water column, *C*Tot(0), was set at the pH-specific acute ERV values (Table 2-1). The horizontal dashed lines represents a) *C*Tot(t)/*C*Tot(0) = 0.3 (70% removal of total copper) and b) *C*Diss(t)/*C*Diss(0) = 0.3 (70% removal of dissolved copper). Note for b) the color of the dashed line corresponds to the pH of the simulation to which it applies

Using the empirical distribution coefficient, copper removal was rapid: 31% of the copper initially added to the system was removed immediately via equilibrium partitioning onto particles, and the remaining 39% left the water column within 3.3 days. In an alternate, more conservative approach in which adsorbed copper was considered equally bioavailable to dissolved copper, *total* copper was compared to the initial concentration and the rapid removal benchmark was met 4.7 days after copper addition.

Using the speciation calculation approach, model-estimated distribution coefficients at the three pH values were higher than the empirical value from the Cu RA document. As a result, 70% removal of dissolved copper occurred instantaneously via initial partitioning for most test cases. The time required for 70% removal of total copper varied between 1.5 and 3.2 days. **Therefore, for a generalized lake environment, copper removal from the water column satisfies the definition for rapid removal of 70% dissolved copper removal in 28 days.**

Various water column sensitivity analyses were conducted. These examined the effect of different loadings (from the chronic ERVs up to 1 mg/L), increased DOC concentration (from 2 to 15 mg/L) (Table 5 and Figure 3) and lowered settling velocity (from 2.5 to 0.24 m/d). The sensitivity analyses provided additional support that copper is rapidly removed from the water column (70% within 28 days).

Table 5. Distribution Coefficients and Fraction Particulate Values for Different pH Values at a DOC Concentration of 15 mg/L[a]

pH	log K_D	Fraction Particulate (f_{Part})
6.09	5.46 (5.36 – 5.46)	0.810 (0.773 – 0.813)
7.07	5.32 (5.19 – 5.33)	0.757 (0.701 – 0.761)
8.00	4.74 (4.72 – 4.74)	0.450 (0.441 – 0.451)

[a] Average over 28 days is indicated with range shown in parentheses. Initial Cu concentration was set at the pH-specific acute ERV (Table 2-1)

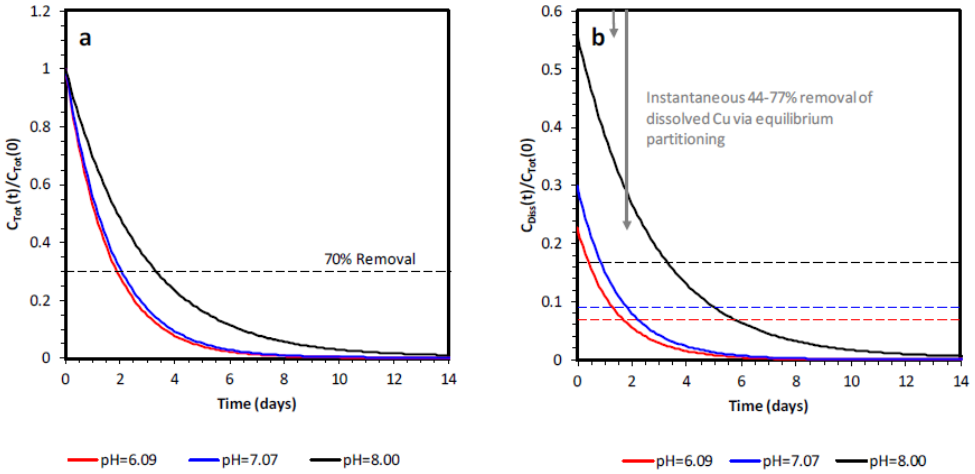


Figure 3. a) Dissolved and b) total copper removal from the water column under different pH conditions for a DOC concentration of 15 mg/L. Copper speciation (including binding to POC) was calculated using WHAM V in TICKET-UWM. The initial total copper concentration in the water column, *C*Tot(0), was set at the pH-specific acute ERV (Table 2-1). The horizontal dashed line represents *C*(t)/*C*Tot(0) = 0.3 or 70% removal of copper.

To examine the potential for remobilization of copper from sediments, a series of 1-year simulations were made. These focused on resuspension, diffusion, and burial to/from the sediment layer and their net effect on copper concentrations in the water column. For the base case scenario, the default EUSES parameters, pH 7 water chemistry and acute ERV loading of 35 µg/L were used. Sediment bulk and porewater chemistry was specified based on data from Besser et al. (2010), Flemish waterways (Vangheluwe et al., 2000), and sediment monitoring data from 1995 (Personal communication with M. Vangheluwe, 2010). Rates of resuspension, diffusion, and burial were set to EUSES model lake values. Remobilization from the sediment was evaluated by examining the water column copper concentration response with and without feedback from the sediment. Simulations were made with an oxic sediment layer (Figure 4) as well as with an anoxic sediment layer (Figure 5) (with varying concentrations of AVS) and varying resuspension rates (up to 10 times the default EUSES model lake value).

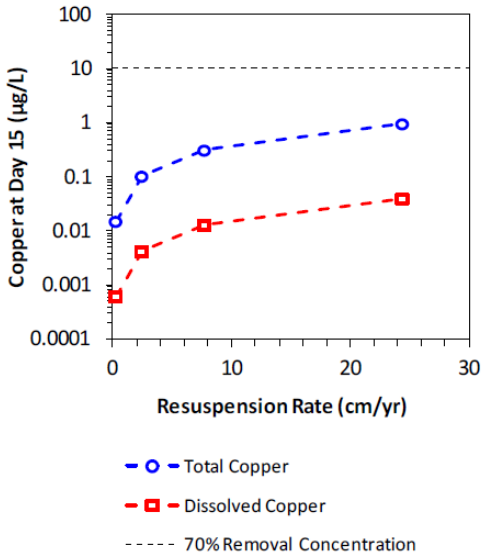


Figure 4. Effect of resuspension rate on total and dissolved copper concentration at day 15 in oxic sediment

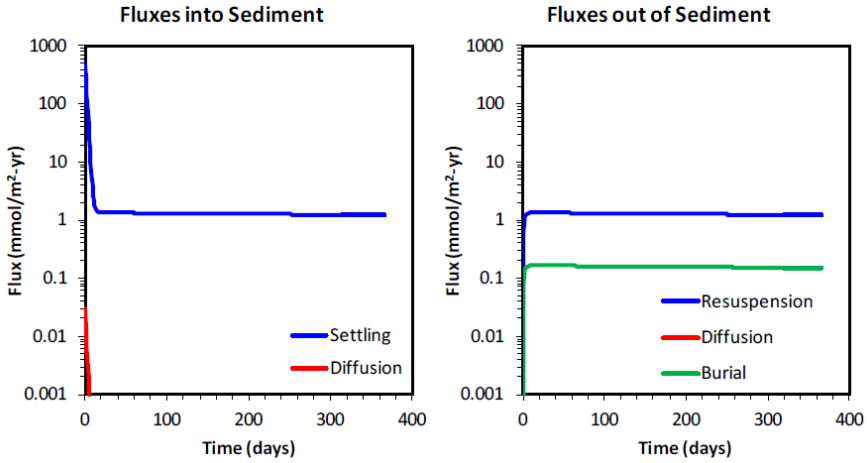


Figure 5. Flux time series plots in anoxic sediment.

For the oxic case, sulfide production and metal sulfide precipitation were not included. Metals can sorb to POC, HFO, and HMO in the sediment and precipitate as carbonates and/or hydroxides (Figure 6). For the anoxic case, metal binding to HFO and HMO was not considered (Figure 7). Metals can sorb to POC and precipitate as sulfides, carbonates, and/or hydroxides. A model run was also made with empirical distribution coefficients from the Cu RA document.

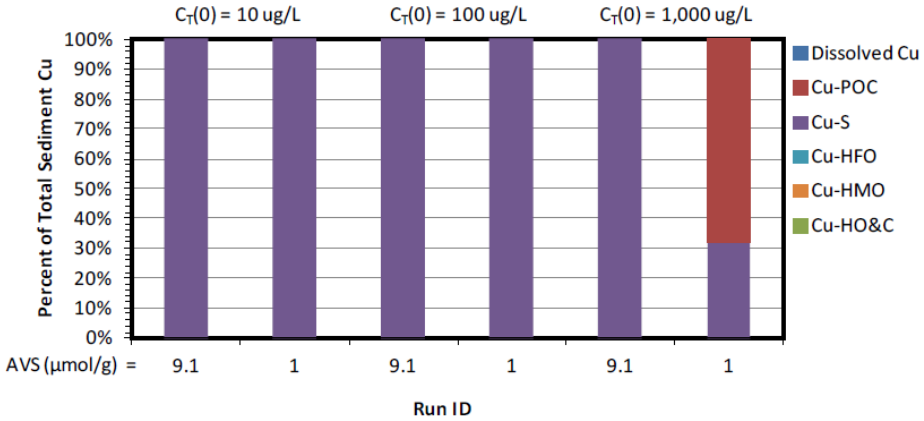


Figure 6. Summary of day 20 sediment copper speciation for initial copper concentrations of 10, 100, and 1,000 µg/L, in oxic sediment

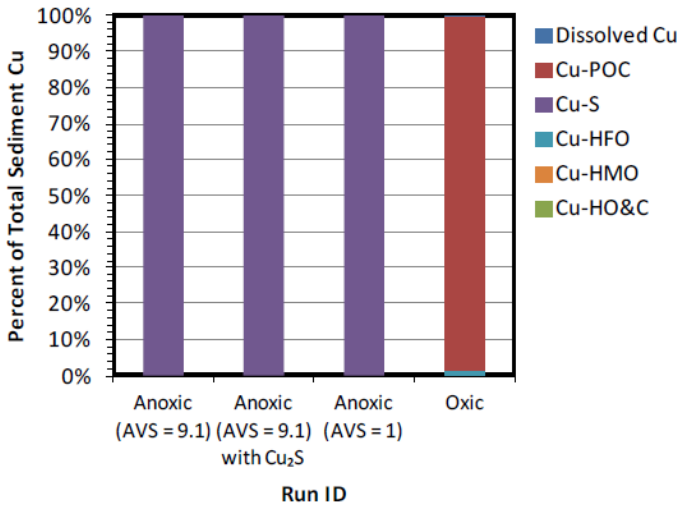


Figure 7. Summary of day 20 sediment copper speciation in anoxic sediment

In the simulations without sediment feedback (i.e., no resuspension or diffusion), water column total and dissolved copper concentrations decreased rapidly and within 20 days of copper addition were more than 4 orders of magnitude below the concentration corresponding to 70% removal (Figure 8). With feedback, the water column copper concentrations leveled off within 50 days of addition as the resuspension and settling fluxes set up a pseudo steady-state in the water column. For the remainder of the simulation time, copper was slowly depleted out of the water column / active sediment layer domain via the effect of burial. In simulations with AVS present, copper in the sediment was precipitated as insoluble copper sulfide solid (CuS or Cu_2S). In simulated sediments with AVS present in excess of copper, essentially all sediment copper was present as copper sulfide precipitate. As a result of this strong binding, the sediment log K_D greatly exceeded the water column log K_D and the net diffusive flux of copper was directed into the sediment. For all cases considered, the pseudo steady-state total and dissolved copper concentrations were lower than the concentration corresponding to 70% removal. Research (Simpson et al., 1998; Sundelin and Eriksson, 2001) suggests that the potential for copper release from sulfides and other sediment binding phases is limited. This supports the idea that additional metal immobilization capacity afforded by sulfides in sediment will be long-lived. **This indicates that the potential for copper remobilization from sediment is limited.**

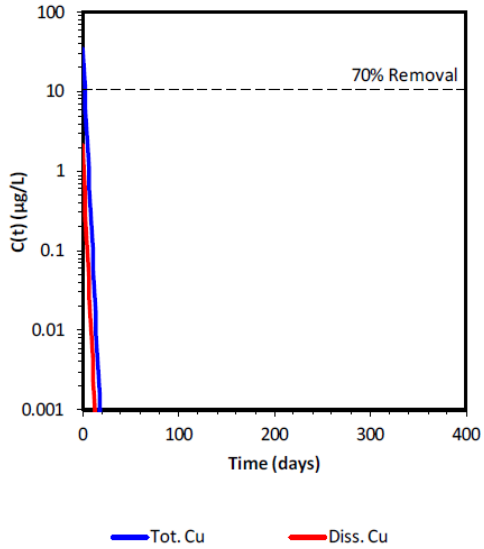


Figure 8. TICKET-UWM simulations with water column isolated from the sediment (i.e., no feedback/remobilization) for initial total copper equal to 35 µg/L. The dashed line is at a concentration corresponding to 30% remaining (70% removal) based on the initial total concentration.

Various sediment sensitivity analyses were conducted (Table 6). These examined the effect of different loadings (0.01, 0.1 and 1 mg/L), varying pH values in the water column (6-8) and sediment (7-7.5), varying hardness (factor of 2 variation), and decreased sediment solids concentration (500 to 150 g/L). The sensitivity analyses provided additional support that the potential for copper remobilization from the sediment is limited.

Table 6. Copper Sediment Sensitivity Analysis Runs

Removal Approach and Output Quantity	Sensitivity Analysis Run						
	Base Case [a]	WC pH = 6.094 Sed pH = 7	WC pH = 7.073 Sed pH = 7	WC pH = 8.002 Sed pH = 7.5	Sediment solids = 150 g/L_{bulk}	Hardness × 2	Hardness ÷ 2
Tot. Cu Range, µg/L [b]	0.252 - 0.279	0.250 - 0.277	0.252 - 0.280	0.282 - 0.313	0.905 - 1.00	0.252 - 0.279	0.252 - 0.279
Diss. Cu Range, µg/L [b]	0.0104 - 0.0116	0.00831 - 0.00918	0.0105 - 0.0116	0.0403 - 0.0447	0.0378 - 0.0417	0.0104 - 0.0116	0.0104 - 0.0116
Total Settling IN, tonnes	636	636	636	636	1350	636	636
Total Resusp. OUT, tonnes	277	277	277	277	994	277	277
Total Diffusion NET, tonnes [c]	0.0397	0.0280	0.0352	0.108	0.0699	0.0397	0.0397
Total Burial OUT, tonnes	34.0	34.1	34.0	34.0	33.8	34.1	34.0
Water column log K_D, L/kg [b]	6.19	6.29	6.19	5.60	6.18	6.19	6.19
Sediment log K_D, L/kg [b]	13.8	13.8	13.8	13.8	14.4	13.8	13.7
Time for 70% Removal (Approach 2), days	1.72	2.10	2.13	2.33	1.73	1.72	1.72
$[0.3\times C_T(0)]$/Max QSS C_T [d]	107	108	107	98.5	30.0	107	107

[a] Select simulation parameters: water column pH 7.07; sediment pH 7.56; anoxic sediment with AVS = 1 µmol/g, settling velocity 2.5 m/d; initial Cu concentration = 0.1 mg/L; Cu_2S is the potential copper sulfide precipitate

[b] Ranges and average are based on data from the quasi-steady state period of the simulation.

[c] This number is the diffusive flux integrated over the *entire* 365-day simulation. Negative diffusive flux values are directed out of the sediment and positive diffusive flux values are directed into the sediment.

[d] This quantity is the ratio of the total Cu concentration representing 70% removal ($0.3\times C_T(0)$) to the maximum total concentration during the quasi-steady-state period (Max QSS C_T). This is meant to give an indication of where sustained water column concentrations lie relative to the 70% removal benchmark.

TICKET-UWM Testing with Laboratory and Field Datasets

The ability of the TICKET-UWM to described copper removal in laboratory and field systems was evaluated using data from

1. Two shallow lakes in the Limousin region of France: Lake Courtille and the Saint Germain les Belles Reservoir.
2. A mesocosm study using large enclosures in Lake Baldegg (Lucerne, Switzerland).
3. A microcosm study conducted at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME).

Lake Courtille and the Saint Germain les Belles Reservoir were dosed with copper sulfate ($CuSO_4 \cdot 5H_2O$) to control the algae population and the copper concentrations in the water column were monitored (Van Hullebusch et al., 2002a, 2002b, 2003a, 2003b, 2003c). **Observed dissolved and total copper removal from the two waterbodies was rapid. For Lake Courtille, 70% removal of dissolved and total copper occurred 15 and 17 days after copper addition, respectively (Figure 9). For the Saint Germain, 70% removal of dissolved and total copper occurred 1.5 and 7 days after copper addition, respectively (Figure 10).**

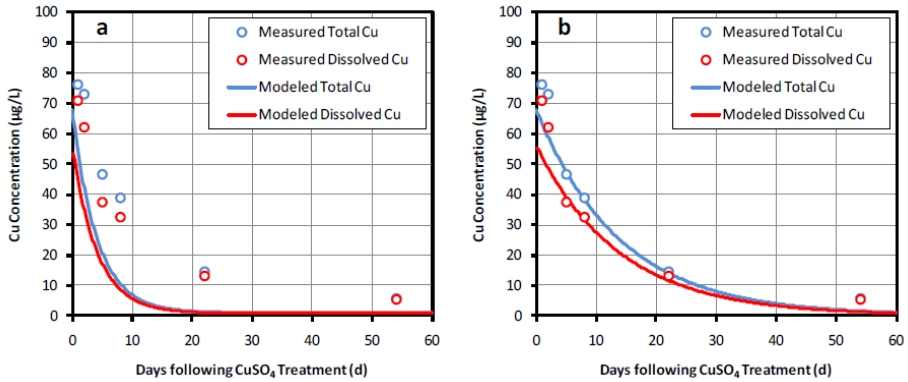


Figure 9. Comparison of TICKET-UWM output (lines) and measured data (points) for copper in the water column of Lake Courtille: EUSES scenario. Model results are from a) the EUSES scenario (water column K_D $10^{4.48}$ L/kg; sediment K_D = $10^{4.39}$ L/kg, and settling velocity = 2.5 m/d) and b) the EUSES scenario with the settling velocity reduced to 0.70 m/d.

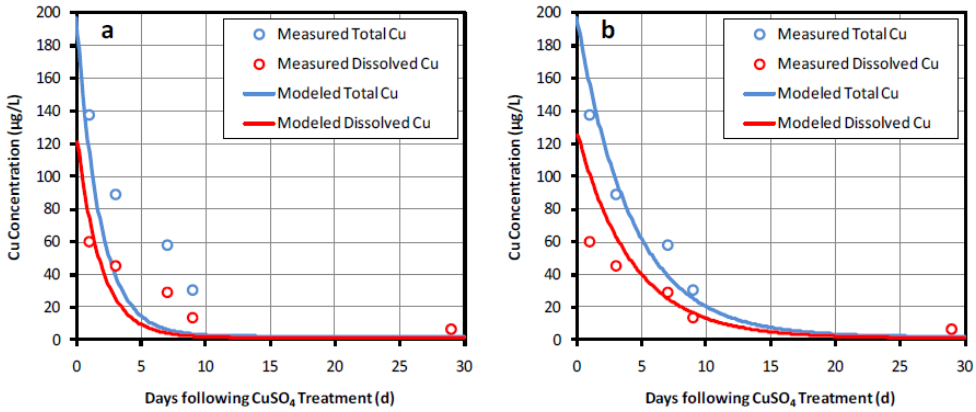


Figure 10. Comparison of TICKET-UWM output (lines) and measured data (points) for copper in the water column of the Saint Germain les Belles Reservoir: EUSES scenario. Model results are from a) the EUSES scenario (water column K_D $10^{4.48}$ L/kg; sediment K_D = $10^{4.39}$ L/kg, and settling velocity = 2.5 m/d) and b) the EUSES scenario with the settling velocity reduced to 1.02 m/d.

For the model testing, physical and chemical parameters serving as input for the TICKET-UWM were specified based on measurements in Van Hullebusch et al. (2002a, b; 2003a, b, c). TICKET-UWM input parameters not directly measured in the studies, such as settling velocity and burial rate, were set to regional values from the EUSES model lake. Copper partitioning to suspended solids was described using the two approaches discussed above as well as using an observed log K_D based upon data from the actual sites. While the settling velocity was initially set at the EUSES model lake value of 2.5 m/d, it was adjusted as necessary to optimize the model fit to the measured data.

Key findings from model testing with the Lake Courtille and the Saint Germain les Belles Reservoir datasets include the following:

- The Cu RA log K_D was more consistent with observed values than log K_D values resulting from TICKET-UWM speciation calculations. These tended to overestimate the extent to which copper binds to particles;
- Predicted copper removal rates with the EUSES settling velocity value of 2.5 m/d were notably higher than observed; and
- Reasonable model fits to the data were achieved with settling velocities between 0.68 and 1.02 m/d. These values are within the settling velocity ranges for organic particles indicated by Burns and Rosa (1980) and O'Connor (1988).

The MELIMEX (MEtal LIMnological EXperiment) study was undertaken to study the effects of increased metal loading (relative to natural levels) on lacustrine biota and investigate the speciation, distribution and fate of added metals (Gächter, 1979). The experiment was conducted in Lake Baldegg (Lucerne, Switzerland) using large enclosures called limno-corrals (12 meters in diameter and 10 meters deep) to isolate portions of the lake water column and sediment for study. Copper was added continuously to the limno-corrals and periodically the water column was sampled at several depths. The samples were analyzed for several water quality parameters including total and dissolved copper (Figure 11).

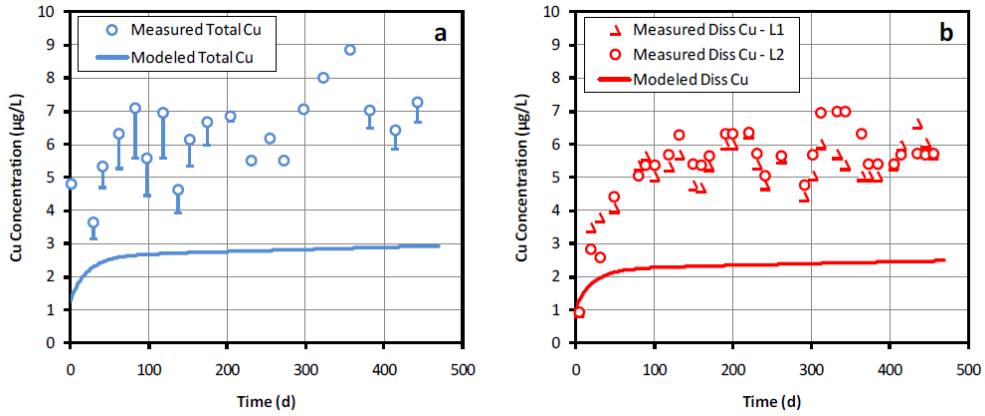


Figure 11. Comparison of TICKET-UWM output (lines) and measured data (points) for a) total and b) dissolved copper in the water column of the MELIMEX limno-corral(s): EUSES scenario (water column K_D 104.48 L/kg; sediment K_D = 104.39 L/kg, and settling velocity = 2.5 m/d). Observed epilimnetic total copper values from limno-corral L2 are indicated with points while the estimated concentration over the entire water column are denoted with whiskers. Dissolved values are averaged over the entire water column of L1 and L2.

This study involved continuous copper addition to enclosures and the associated response was an increase in copper in the water column. The performance of the TICKET-UWM was evaluated based upon its ability to reproduce the copper increase in the water column. To address the rapid removal benchmark, additional TICKET-UWM simulations (referred to as post-loading simulations) were made in the absence of copper loading. The initial copper concentration for these simulations was the final model-predicted concentrations from the continuous load runs.

For model testing, physical and chemical parameters serving as input for the TICKET-UWM were specified based on measurements from the study itself. TICKET-UWM input parameters not directly measured in the studies were set to regional values from the EUSES model lake. Baccini et al (1979a) estimated a log K_D value and settling velocity of 4.12 and 0.2 m/d, respectively, for the limno-corrals.

Key findings from model testing with the MELIMEX study dataset include the following:

- Using the above log K_D and settling velocity, a reasonable fit to the observed copper data was achieved (Figure 12);
- As was the case for Lake Courtille and the Saint Germain les Belles Reservoir, the experimental data were well-described using settling velocities markedly lower than the EUSES default value of 2.5 m/d;
- TICKET-UWM speciation calculations tended to overestimate the log K_D; and
- For many of the post-loading simulations, the rapid removal benchmark was not met (Figure 13).

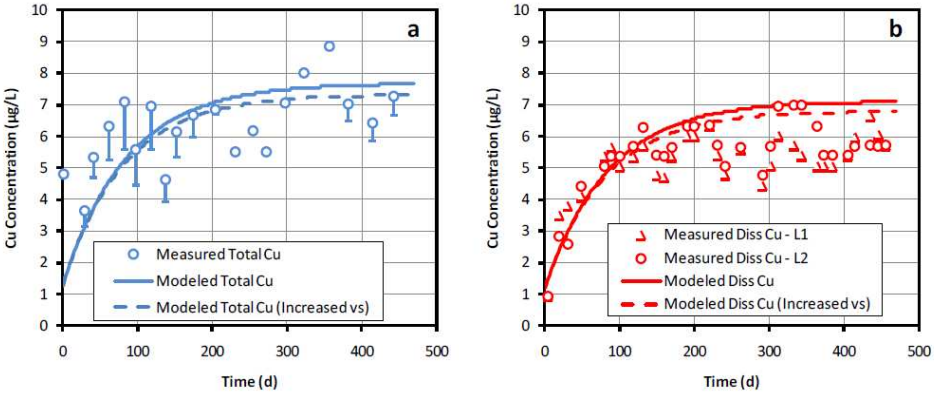


Figure 12. TICKET-UWM output (lines) and measured data (points) for a) total and b) dissolved copper in the water column of the MELIMEX limno-corral(s): Observed *K*D and settling velocity scenario. Model simulations use experimentally-estimated values for the water column *K*D (104.12 L/kg) and settling velocity (0.2 m/d) are indicated with solid lines. Model output with increased settling velocity of 0.28 m/d are shown with dashed lines. Observed epilimnetic total copper values from limno-corral L2 are indicated with points while the estimated concentration over the entire water column are denoted with whiskers. Dissolved values are averaged over the entire water column of L1 and L2.

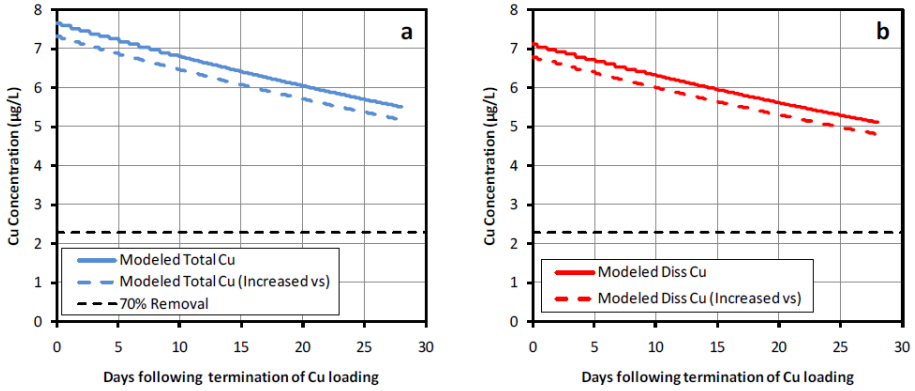


Figure 13. a) Total and b) dissolved copper TICKET-UWM results from post-loading simulations for the observed K_D and settling velocity scenario. Model simulations use the experimentally-estimated values for the water column K_D (104.12 L/kg) and settling velocity (0.2 m/d). The blue and red dashed lines refer to simulations with the settling velocity increased to 0.28 m/d. The horizontal black dashed line denotes 70% copper removal.

However, because of the low settling velocity, low distribution coefficient, and low suspended solids concentration (relative to the EUSES value), this field test case is more representative of a "worst-case" scenario for copper removal from the water column and therefore not necessarily an appropriate field test case to compare to the rapid removal definition.

A microcosm study was undertaken at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) to study the effects of continuous copper exposure on aquatic organisms (Schäfers, 2003). Microcosms were filled with water and sediment collected from a manmade pond near Schmallenberg-Oberkirchen, Germany and dosed to achieve six different nominal concentrations: 5, 10, 20, 40, 80, and 160 µg/L. Model testing was performed using

dissolved copper data sampled 1, 24, and 48 hours after copper addition and total copper data sampled 24 hours after addition. **Based on half-lives calculated from the measured dissolved copper data, the time required for 70% copper removal (relative to the initial nominal copper concentration), is between 2.4 and 7.6 days. This is consistent with the definition for rapid removal.**

For the model testing, physical and chemical parameters serving as input for the TICKET-UWM were specified based on measurements from the study itself. TICKET-UWM input parameters not directly measured in the studies were set to regional values from the EUSES model lake. Initial copper was specified in the model by 1) setting the initial total copper concentration to the nominal copper concentration for each microcosm, and 2) setting the initial total copper to produce the initial dissolved concentration extrapolated from measured values at 1, 24, and 48 hours after copper addition.

Key findings from model testing with the IME microcosm study dataset include the following (Figure 14):

- By optimizing settling velocities in the simulations for the 80 and 160 µg/L microcosms, general agreement between model-predicted and experimental dissolved copper removal rates was observed in each of the examined partitioning scenarios and initial copper specification approaches.
- For the 5, 10, 20, and 40 µg/L microcosms, optimization of the model fit to the data when the initial total copper was set at the nominal values was complicated by measured total copper concentrations above the nominal value.
- With initial dissolved copper, $C_D(0)$, specified in TICKET-UWM by extrapolation from measured data, agreement between model-predicted and experimental dissolved copper removal rates was observed for all microcosms once the settling velocity was optimized.
- Optimized settling velocities for simulations using the log K_D calculated from experiment data and the log K_D obtained from TICKET-UWM speciation calculations were 0.67 and 0.89 m/d, respectively. These values are consistent with the range observed in the Lake Courtille and Saint Germain les Belles (0.68 – 1.02 m/d) and the range associated with POC (Burns and Rosa, 1980);
- Unlike model applications to Lake Courtille, Saint Germain les Belles Reservoir, and the MELIMEX mesocosms, TICKET-UWM speciation calculations for the IME microcosms tended to underestimate the log K_D;
- Both model simulations (i.e., with Cd(0) specified) and measured data indicate rapid removal of dissolved copper; and
- The relatively shallow depth of the microcosms (75 cm) favors rapid removal.

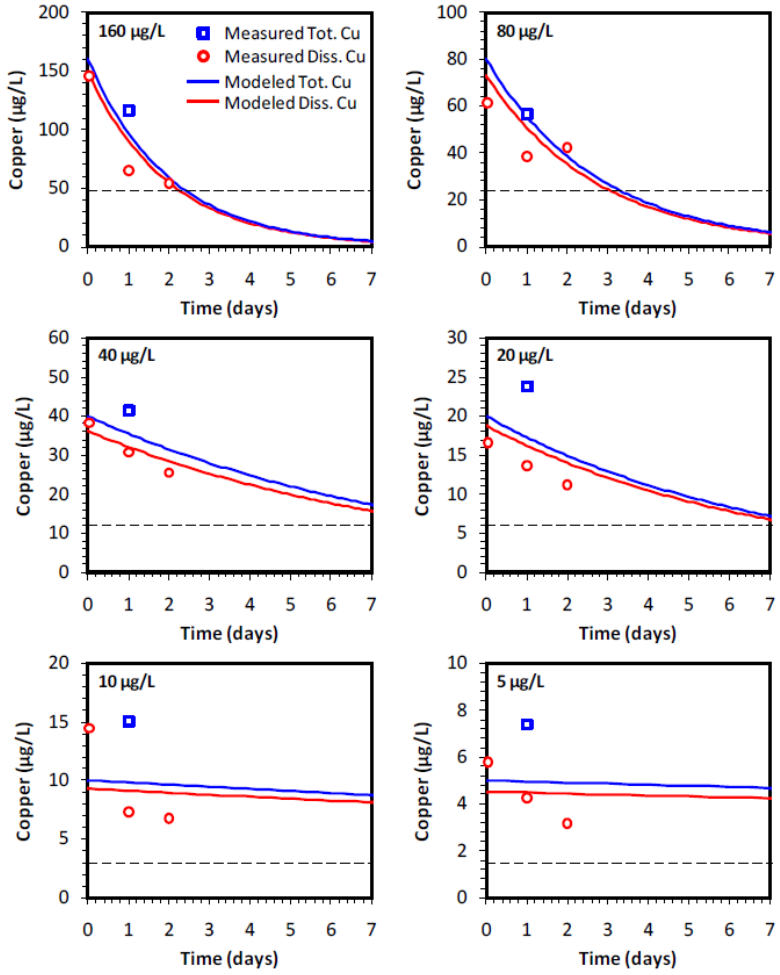


Figure 14. Comparison of TICKET-UWM output (lines) to measured data (points) for total copper (blue squares and lines) and dissolved copper (red circles and lines) in the water column of the IME microcosms for the EUSES scenario with optimized settling velocities. The horizontal black dashed line denotes 70% dissolved copper removal relative to the nominal value.

Conclusions

In this study, simulations with the TICKET-UWM were made for a generalized lake environment (EUSES model lake) and for four different surface water systems including a lake, a reservoir, a large enclosure in a lake, and laboratory microcosms. The aims of these analyses were to assess the removal of copper relative to the rapid removal definition and to test the ability of the TICKET-UWM to describe copper dynamics in the water column of lake systems to confirm its use as a screening level copper risk assessment tool.

The conclusions from this work are as follows:

- For a generalized lake environment consisting of the EUSES model lake parameters, copper removal from the water column satisfies the definition for rapid removal of 70% dissolved copper removal in 28 days;
- For all sediment remobilization scenarios tested the pseudo steady-state copper concentrations resulting from sediment feedback are markedly lower than that

corresponding to 70% removal suggesting that the sediment copper remobilization potential is limited;

- In simulations with AVS present, copper in the sediment was precipitated as an insoluble copper sulfide solid (CuS or Cu_2S). In simulated sediments with AVS present in excess of copper, essentially all sediment copper was present as copper sulfide precipitate. As a result of this strong binding, the sediment log K_D greatly exceeded the water column log K_D and the net diffusive flux of copper was directed into the sediment. Research (Simpson et al., 1998; Sundelin and Eriksson, 2001) suggests that the potential for copper release from sulfides and other sediment binding phases is limited. This supports the idea that additional metal immobilization capacity afforded by sulfides in sediment will be long-lived.
- The above findings for the generalized lake environment support ”rapid removal of copper-ions,” equivalent to “biodegradation of organic substances”
- For the whole-lake spike addition studies (Lake Courtille and Saint Germain les Belles Reservoir), TICKET-UWM results, in concert with the measured data indicate rapid removal of copper (i.e. greater than 70% in 28 days);
- For the relatively shallow IME microcosms, both model simulations (i.e., with the initial dissolved copper specified) and measured data indicate rapid removal of dissolved copper.
- Hypothetical TICKET-UWM simulations modeling the removal of copper in the MELIMEX limno-corrals following termination of copper loading indicate relatively slow copper removal. However, because of low settling velocity, low distribution coefficient, and low suspended solids concentration (relative to the EUSES value), this field test case is more representative of a “worst-case” scenario for copper removal from the water column and therefore not necessarily an appropriate field test case to compare to the rapid removal definition.
- In most cases, the EUSES settling velocity produced more rapid copper removal that observed.
- The TICKET-UWM (with linear partitioning calculations) provided satisfactory descriptions of copper dynamics in the water column of Lake Courtille, the Saint Germain les Belles Reservoir, and the IME microcosm study with calibrated settling velocities ranging from 0.67 to 1.02 m/d. This range is consistent with the range associated with POC (Burns and Rosa, 1980). For systems lacking information on settling rates, use of a settling velocity within this range may be more justified than the EUSES settling velocity.
- With the exception of the IME microcosm study, TICKET-UWM speciation calculations using WHAM V and surface complexation model overestimated the log K_D for copper binding to suspended solids. Additional work is necessary to 1) modify the TICKET-UWM codebase to allow for flexibility in the humic/fulvic acid composition of particulate organic carbon (POC), 2) determine a set of model input parameter guidelines (e.g. HA/FA acid composition, fraction active, etc.) that allows for more accurate calculations of the distribution coefficient in WHAM V.
- The study confirms that a relatively simplistic model (e.g. one water column cell and one sediment layer) can be used to simulate copper fate in the water column of lakes in a reasonably accurate manner. The critical issue is accurate parameterization of the

characteristics and processes associated with the water body, particularly metal partitioning and particle settling velocity.

RMS opinion:

The principle of rapid removal is based on the hypothesis that metal speciation transformation in sediment leads to less or non-toxic forms. Speciation of copper in sediment is indeed well known, but there might however remain uncertainty on copper toxicity towards sediment-dwelling organisms as no toxicity thresholds are set for classification, and no comparison can be done with aquatic organisms for endpoints are expressed in different units. For information, in the biocide RAR, the HC_5 based on a data set of NOEC values for sediment-dwelling organisms was 19 mg/kg sed.

Apart from that general comment, RMS considers the model TICKET-UWM as globally well designed and tested. Indeed, several simulations were conducted in order to assess the sensitivity of parameters, such as suspended solids, DOC, pH, K_D... AVS in sediments could be a matter for discussion, for an increase of AVS amount leads a lower toxicity to aquatic organisms. However, a low amount of AVS was considered in the model (0.77 µmol/g, see Table 2). It can therefore be considered that a worst case was assessed.

RMS is therefore of the opinion that copper fulfils the criteria of rapid removal, as more than 70% of copper is removed from the water column within 28 days.

Moreover, it is also demonstrated in the study that the potential for copper remobilization from sediment is limited in oxic and anoxic conditions.

5.2 Environmental distribution

5.2.1 Adsorption/Desorption

The adsorption studies submitted were reviewed in the EU monograph and were not accepted, as they are not believed to be relevant. Therefore, no information about the sorption properties of copper could be used.

Column leaching studies were performed on four German soils with an applied dose equivalent to 18.1 kg copper/ha. Soils were leached with 393 ml of de-ionised water over 48h at 20°C. Levels of copper measured in leachates were not significantly different between control and treated soils. Results showed that most of applied copper remained in the top 6 cm of the soils.

In the EU Voluntary Risk assessment of Copper compounds, it was stated that adsorption of copper to soil, sediment, colloids and suspended particles plays an important role for the behaviour of copper in the environment. Inorganic particles such as clay minerals and iron, manganese and aluminium oxides, as well as organic materials, constitute the principal adsorbents for copper in water, sediment and soil (Landner and Lindeström 1999).

pH and organic matter are the most important abiotic factors affecting the adsorption of copper. Copper adsorption increases with pH. Organic matter restricts heavy metal movement and availability, even under very acidic conditions (Tyler and McBride 1982).

5.2.2 Volatilisation

Not relevant for copper.

5.2.3 Distribution

According to the EU Voluntary Risk assessment of Copper compounds, the most important parameters determining the distribution of copper in the aquatic and soil compartments is adsorption onto solid materials and therefore the copper partitioning coefficients. From the literature overview, the following partitioning coefficients have been derived for Cu metal and Cu compounds:

Partition coefficient in suspended matter

Kpsusp = 30,246 l/kg (log Kp (pm/w) = 4.48) (50th percentile)

Partition coefficient in sediment

Kpsed = 24,409 l/kg (log Kp(sed/w) = 4.39) (50th percentile)

Partition coefficient in soil

Kpsoil = 2 120 l/kg (log Kp (soil/w) = 3.33) (50th percentile)

5.3 Aquatic Bioaccumulation

5.3.1 Aquatic bioaccumulation

5.3.1.1 Bioaccumulation estimation

Based on its log Pow of 0.44, no concern over any potential for bioaccumulation could be concluded for copper compounds. No study is therefore available to determine bioconcentration factors in fish.

Because of homeostasis of metals in vertebrates, BCF values are not indicative of potential bioaccumulation.

The copper Risk Assessment Report (2008) provided detailed information on (1) the essentiality of copper; (2) the homeostatic control of copper; (3) the mechanisms of action of copper-ions; (4) the comparison between copper toxicity from dietary versus waterborne exposures.

These data demonstrate that:

- Copper is an essential nutrient for all living organisms
- Copper ions are homeostatically controlled in all organisms and the control efficiencies increase with trophic chain. As a consequence,
  - copper BCF/BAF values
    - decrease with increasing exposure concentrations (water and food)
    - vary depending on nutritional needs (seasonal, life stage, species dependent)
    - vary pending on “internal detoxification” mechanisms
  - Copper BMFs values are < 1
- Water-borne exposure (not diet borne exposure) is the exposure route critical to copper toxicity

5.3.1.2 Measured bioaccumulation data

None

5.3.2 Summary and discussion of aquatic bioaccumulation

Taking into account homeostasis phenomenon, neither bioaccumulation nor biomagnification are expected for copper compounds.

5.4 Aquatic toxicity

For data provided from the pesticide monograph, all the aquatic toxicity studies of copper compounds were performed on GLP and according to OECD guidelines. Then, the reliability factor is 1. Some studies were conducted without analytical measurements and were considered only as indicative in the pesticide monograph. They are identified with a reliability factor of 2 in the first table in 5.5.

As long as copper compounds dissociate in water, all acute tests were conducted with the salt of concern, when the chronic studies were conducted with other salts but considered relevant. All endpoints are expressed as copper.

This section is based on the information available under the EU Voluntary risk assessment of Copper compounds.

A proposal for classification was carried out within the framework of the EU Voluntary risk assessment of Copper compounds. This work is detailed in “Appendix K1: classification: Acute and chronic ecotoxicity data on soluble copper species” of the EU-VRAR (Existing Substances Regulation) of Copper, Copper II sulphate pentahydrate, Copper (I) oxide, Copper (II) oxide, Dicopper chloride trihydroxide (2008). However, this work has never been discussed in a technical group competent for classification.

A large copper database was taking into account to determine the proposal of classification. Data were selected using both reliability and relevance criterion.

When more than one acceptable test is available for the same species the geometric mean of the toxicity values was used as representative toxicity value for that species. Considering the crucial importance of pH of the test media on the copper solubility and ecotoxicity, for the acute and chronic toxicity endpoints, 3 pH categories were distinguished within the acute and chronic ecotoxicity database: pH 5.5-6.5, >6.5-7.5 and >7.5-8.5.

The lowest species mean-specific acute L(E)C_{50} and chronic NOEC was selected as final hazard classification entry at the three pH levels. The endpoints are expressed as dissolved copper.

5.4.1 Fish

5.4.1.1 Short-term toxicity to fish

The relevant endpoints for short-term toxicity to fish extracted from the pesticide monograph are presented in the table below:

Test substance	Species	Test system	Endpoint (mg Cu/L)	Reference

Tribasic Copper Sulphate SC	*O. mykiss*	Static	> 13.2	total (mean)	Ellgehausen (1986)
	C. carpio	Flow-through	> 19.3	total (mean)	Wüthrich (1992a)
Tribasic Copper Sulphate	*O. mykiss*	Static	0.095	total (nominal)	Buccafusco (1977a)
		Static	1.696	total (nominal)	Buccafusco (1977b)

According to the EU Voluntary risk assessment of Copper compounds, 249 individual data points for fish were selected for 5 standard species (*Oncorhynchus mykiss, Pimephales promelas, Lepomis macrochirus, Brachydanio rerio* and *Cyprinus carpio).* When evaluating the high quality L(E)C_{50} values at the three pH classes, sufficient data for the 3 pH classes were found for 3 fish species (*O. mykiss, P. promelas, L. macrochirus*).

As expected, an increased LC_{50} with increasing pH was noted for these fish species. The lowest recorded geomean LC_{50} value (0.0081 mg Cu/L) was recorded for *P. promelas* tested in ecotoxicity media with low pH (between 5.5 and 6.5).

The results are presented in the table here below:

Test organism	**L(E)C_{50} (mg/L)**			
	pH: 5.5-6.5	**pH: >6.5-7.5**	**pH: >7.5-8.5**	**All pHs**
Oncorhynchus mykiss				
n	6	19	28	33
Min	0.0042	0.0028	0.0095	0.0028
Max	0.0820	0.8900	0.5160	0.8900
Geometric mean	0.0290	0.0594	0.1030	0.0734
Brachydanio rerio				
n	/	3	1	4
Min	/	0.0350	0.1490	0.0350
Max	/	0.1200	0.1490	0.1490
Geometric mean	/	0.0740	0.1490	0.0880
Cyprinus carpio				
n	/	/	2	2
Min	/	/	0.8000	0.8000
Max	/	/	0.8100	0.8100
Geometric mean	/	/	0.8049	0.8049
Pimephales promelas				
n	2	32	170	204
Min	0.0044	0.0059	0.0124	0.0044
Max	0.0150	1.4000	1.0600	1.4000
Geometric mean	***0.0081***	0.2140	0.2181	0.1793
Lepomis macrochirus				
n	1	2	3	6
Min	0.7100	1.0000	4.2500	0.7100
Max	0.7100	1.1000	9.1505	9.1505
Geometric mean	0.7100	1.0488	5.5093	2.2524

5.4.1.2 Long-term toxicity to fish

The relevant endpoints for long-term toxicity to fish extracted from the pesticide monograph are presented in the tables below:

Test substance	Species	Test system	NOEC (mg Cu/L)	Reference
Copper Hydroxide WP	*O. mykiss*	Flow-through (ELS)	0.0155 total (=nominal)	Schäfers (2000)
Tribasic Copper Sulphate SC	*O. mykiss*	Flow-through 21 days	0.97 total (=nominal)	Wüthrich (1992c)

Toxicity to fish embryo

Test substance	Species	Test system	NOEC (mg Cu/L)	Reference
Tribasic copper sulphate	*D. rerio* (embryo)	Static/48 hours	76.8 total (=nominal)	Schäfers (2002c)

According to the EU Voluntary risk assessment of Copper compounds, 29 individual data points for fish were selected for 3 standard species (*Oncorhynchus mykiss*, *Pimephales promelas* and *Salvelinus fontanilis*). No chronic toxicity values for fish were gathered at pH 5.5-6.5.

The results are presented in the table here below:

Test organism	NOEC (mg/l)			
	pH: 5.5-6.5	pH: >6.5-7.5	pH: >7.5-8.5	All pHs
Oncorhynchus mykiss				
n	/	4	1	5
Min	/	0.0022	0.0160	0.0022
Max	/	0.0450	0.0160	0.0450
Geometric mean	/	0.0161	**0.0160**	0.0161
Pimephales promelas				
n	/	5	10	15
Min	/	0.0048	0.0145	0.0048
Max	/	0.0106	0.3380	0.3380
Geometric mean	/	0.0077	0.0419	0.0239
Salvelinus fontanilis				
n	/	9	/	9
Min	/	0.0070	/	0.0070
Max	/	0.0490	/	0.0490
Geometric mean	/	0.0161	/	0.0161

5.4.2 Aquatic invertebrates

5.4.2.1 Short-term toxicity to aquatic invertebrates

The relevant endpoints for short-term toxicity to aquatic invertebrates extracted from the pesticide monograph are presented in the table below:

Test substance	Species	Test system	NOEC (mg Cu/L)	Reference
Tribasic copper sulphate	*D. magna*	Static/48 hours	0.047 total (nominal)	LeBlanc and Surprenant (1978)

According to the EU Voluntary risk assessment of Copper compounds, 91 individual data points for aquatic invertebrates were selected for 2 standard species (*Ceriodaphnia dubia and Daphnia magna).* Sufficient data for the 3 pH classes were found for these 2 invertebrate species.

The results are presented in the table here below:

Test organism	$L(E)C_{50}$ (mg/L)			
	pH: 5.5-6.5	**pH: >6.5-7.5**	**pH: >7.5-8.5**	**All pHs**
Daphnia magna				
n	7	11	52	70
Min	0.0338	0.0070	0.0098	0.0070
Max	0.3600	0.7920	0.5290	0.7920
Geometric mean	0.0657	0.1056	0.0550	0.0620
Ceriodaphnia dubia				
n	4	4	13	21
Min	0.0095	0.0280	0.0085	0.0085
Max	0.0560	0.0840	0.2000	0.2000
Geometric mean	0.0344	***0.0473***	***0.0298***	0.0344

5.4.2.2 Long-term toxicity to aquatic invertebrates

The relevant endpoints for long-term toxicity to aquatic invertebrates extracted from the pesticide monograph are presented in the table below:

Test substance	Species	Test system	NOEC (mg Cu/L)		Reference
Copper oxychloride	*D. magna*	Semi-static	0.0076	total (mean)	Bellmann (1993)
	D. magna	Semi-static	0.059	total (=nominal)	Noack (2001)
Tribasic Copper Sulphate SC	*D. magna*	Semi-static	0.057	total (mean)	Wüthrich (1992d)

According to the EU Voluntary risk assessment of Copper compounds, 19 individual data points for aquatic invertebrates were selected for 2 standard species (*Ceriodaphnia dubia and Daphnia magna*). Sufficient data for the 3 pH classes were found for these 2 invertebrate species.

The results are presented in the table here below:

Test organism	NOEC (mg/L)			
	pH: 5.5-6.5	**pH: >6.5-7.5**	**pH: >7.5-8.5**	**All pHs**
Ceriodaphnia dubia				
n	1	4	5	10
Min	0.0200	0.0040	0.0063	0.0040
Max	0.0200	0.0190	0.1220	0.1220
Geometric	**0.0200**	**0.0074**	0.0259	0.0151

Test organism	NOEC (mg/L)			
	pH: 5.5-6.5	pH: >6.5-7.5	pH: >7.5-8.5	All pHs
mean				
Daphnia magna				
n	2	1	6	9
Min	0.0215	0.1810	0.0126	0.0126
Max	0.0280	0.1810	0.1060	0.1810
Geometric mean	0.0245	0.1810	0.0455	0.0463

5.4.3 Algae and aquatic plants

The relevant endpoints for toxicity to aquatic plants extracted from the pesticide monograph are presented in the table below:

Test substance	Species	Test system	Endpoint (mg Cu/L)		Reference
Tribasic Copper Sulphate SC	*Ps. Subcapitata*	Static (72 h)	E_bC_{50} > 12.3	total (mean)	Wüthrich (1992b)
Tribasic Copper Sulphate	*S. carpricornutum*	Static (72 h)	E_bC_{50} = 0.033 ErC_{50} = 0.173	total (nominal) total (nominal)	Mallett (2001)

According to the EU Voluntary risk assessment of Copper compounds, 17 individual acute data points for algae were selected for 1 standard species (*Raphidocelis subcapitata).* Sufficient data for the 3 pH classes were found for this species.

The results are presented in the table here below:

Test organism	$L(E)C_{50}$ (mg/L)			
	pH: 5.5-6.5	pH: >6.5-7.5	pH: >7.5-8.5	All pHs
Raphidocelis subcapitata				
n	2	3	12	17
Min	0.1520	0.0320	0.0129	0.0129
Max	0.1940	0.1631	0.2453	0.2453
Geometric mean	0.1717	0.0760	0.0618	0.0723

According to the EU Voluntary risk assessment of Copper compounds, 28 individual chronic data points for algae were selected for 2 standard species (*Raphidocelis subcapitata* and *Chlorella vulgaris).* Sufficient data for the 3 pH classes were found for these 2 species.

The results are presented in the table here below:

Test organism	NOEC (mg/L)			
	pH: 5.5-6.5	pH: >6.5-7.5	pH: >7.5-8.5	All pHs
Chlorella vulgaris				
n	5	7	4	16
Min	0.0875	0.0211	0.0225	0.0211
Max	0.3055	0.1097	0.1009	0.3055
Geometric mean	0.1867	0.0683	0.0557	0.0889

Test organism	NOEC (mg/L)			
	pH: 5.5-6.5	pH: >6.5-7.5	pH: >7.5-8.5	All pHs
Raphidocelis subcapitata				
n	1	3	8	12
Min	0.0947	0.0529	0.0157	0.0157
Max	0.0947	0.0655	0.1640	0.1640
Geometric mean	0.0947	0.0598	0.0345	0.0431

5.4.4 Other aquatic organisms (including sediment)

Test substance	Species	Test system	NOEC (nominal, mg Cu/L)	Reference
Tribasic Copper Sulphate SC	*C. riparius*	Static	NOEC = 0.50	Stäbler (2002b)
Copper hydroxide WP	Indoor microcosm study	6 applications at 10-d interval	NOEC =0.012 total (nom) = 0.00312 dissolved	Schäfers, C. (2000a)

5.5 Comparison with criteria for environmental hazards (sections 5.1 – 5.4)

The table below presents the comparison criteria for available data issued from the Pesticide monograph:

Organism	Test substance	Species	Test conditions	LC_{50}/ EC_{50} (mg Cu/L)[1]	NOEC (mg Cu/L) [1]	Reliability
Fish	Tribasic Copper Sulphate SC	*O. mykiss*	Acute static	> 13.2 total (mm)		1
		C. carpio	Acute Flow-through	> 19.3 total (mm)		1
	Tribasic Copper Sulphate	*O. mykiss*	Static	0.095		2
		Lepomis macrochirus	Static	1.696		2
	Copper Hydroxide WP	*O. mykiss*	Flow-through (ELS)		0.0155 total (nom)	1
	Tribasic Copper Sulphate SC	*O. mykiss*	Flow-through 21 days		0.97 total (nom)	1
	Tribasic copper sulphate	*D. rerio (embryo)*	48 hours		76.8 total (nom)	1
Invertebrates	Tribasic Copper Sulphate	*D. magna*	48 hours	**0.047 total (nominal)** [2]		2
	Copper oxychloride	*D. magna*	Semi-static-21d		0.0076 total (mm)	1
		D. magna	Semi-static - 21d		0.059 total (nom)	1
	Tribasic Copper Sulphate SC	*D. magna*	Semi-static – 21d		0.057 total (mm)	1
Algae	Tribasic Copper Sulphate SC	*Ps. subcapitata*	Static (72 h)	E_bC_{50} > 12.3 total (mm)		1
	Tribasic Copper Sulphate	*S. carpricornutum*	Static (72 h)	E_bC_{50} = 0.033 ErC_{50} = 0.173	total (nominal) total (nominal)	2
Sediment dwelling	Tribasic Copper Sulphate SC	*C. riparius*	Static		0.50	1

organisms						
Microcosm	Copper hydroxide WP	Indoor microcosm study	6 applications at 10-d interval		0.012 total (nom) 0.00312 dissolved	1

1: nom = nominal concentrations ; mm = mean measured concentrations
2: critical acute endpoint

The acute and chronic reference values for aquatic organisms issued from the EU risk assessment for Copper compounds are presented in the table below:

pH range	**Reference values**	
	L(E)C_{50} (mg/l)	**NOEC (mg/l)**
pH 5.5-6.5	0.0292	0.0200
pH >6.5-7.5	0.0473	0.0074
pH >7.5-8.5	0.0298	0.0160

Tribasic copper sulfate is considered as soluble copper species. The solubility product of Tribasic copper sulfate exceeds the L(E)C_{50} values for all organisms. Therefore ecotoxicity data obtained from tests carried out with soluble copper species were used directly for classification.

According to the recommendation of the Guidance on the Application of the CLP criteria dated on November 2012, it is important to ensure that the data point to be used as the justification for the classification is expressed in the weight of the molecule of the metal compound to be classified. So, the classification is based on the Acute $ERV^2_{compound}$ and chronic $ERV_{compound}$ calculated as follow:

Acute $ERV_{compound}$ = acute ERV of the metal compound = acute ERV of metal ion x (molecular weight of metal compound/(atomic weight of the metal x number of metal ions))

Chronic $ERV_{compound}$ = chronic ERV of the metal compound = chronic ERV of metal ion x (molecular weight of metal compound/ atomic weight of the metal x number of metal ions)

The table below summarises the acute and chronic ERV- Tribasic copper sulfate which should be taken into account for classification of Tribasic copper sulfate compound.

Source	**pH range**	**Environmental Reference values (ERV) for Tribasic copper sulfate**	
		Acute ERV- Tribasic copper sulfate (mg/l)	**Chronic ERV- Tribasic copper sulfate (mg/l)**
DAR		0.09	

[2] ERV : ecotoxicity reference value

Source	pH range	Environmental Reference values (ERV) for Tribasic copper sulfate	
		Acute ERV- Tribasic copper sulfate (mg/l)	Chronic ERV- Tribasic copper sulfate (mg/l)
DAR		0.09	
RAR	pH 5.5-6.5	0.05	0.036
	pH >6.5-7.5	0.09	0.013
	pH >7.5-8.5	0.05	0.029

(Molecular weight of Tribasic copper sulfate = 461.3, atomic weight of copper ion = 63.546)

5.6 Conclusions on classification and labelling for environmental hazards (sections 5.1 – 5.4)

Conclusion of environmental classification according to Regulation EC 1272/2008

Taking into account the recommendations of the Annex IV of the Guidance to Regulation (EC) No 1272/2008 Classification, Labelling and Packaging of substances and mixtures, a metal compound is considered as readily soluble if the water solubility is greater or equal to the acute ERV of the dissolved metal ion concentration. The water solubility of Tribasic copper sulfate is equal to 3.42 mg/L and 0.255 mg/L at pH 5.6 and 9.8 respectively. Therefore, this compound is considered as **ready soluble metal compound**.

For acute toxicity classification, the lowest ERV- Tribasic copper sulfate (0.05 mg/l) is below the trigger value of 1 mg/L which leads to the aquatic environmental hazard acute category 1, H400. An M-factor of 10 should also be applied.

For chronic toxicity classification, there is evidence of rapid removal from water column. The lowest chronic ERV- Tribasic copper sulfate (0.013 mg/L) is between the trigger values of 0.01 and 0.1 mg/L which leads to the aquatic environmental hazard chronic category 2, H411.

RAC evaluation of environmental hazards

Summary of the Dossier Submitter's proposal

The dossier submitter (DS) considered tetracopper hexahydroxide sulphate to be a form of copper sulphate and thus currently covered by the Annex VI entry for copper sulphate (Index No. 029-004-00-0). The DS's proposal specified an acute M-factor to be assigned to the existing harmonised classification as Aquatic Acute 1 and proposed to change the harmonised chronic classification from Aquatic Chronic 1 to Aquatic Chronic 2, based on the following arguments:

The water solubility of tetracopper hexahydroxide sulphate (3.42 mg/L and 0.255 mg/L at pH 6.2 at pH 9.8, respectively) exceeds the acute ERV of the dissolved metal ion. Taking into account the recommendations of the CLP guidance[3], this compound is considered to be a readily soluble metal compound for classification purposes.

[3] CLP Guidance… ECHA Guidance on the Application of the CLP criteria (version 4.0 November 2013)

For aquatic acute classification, the lowest acute Ecotoxicity Reference Value (acute $ERV_{Cu4(OH)6SO4½H2O}$ 0.05 mg/L) was considered to be below the trigger value of 1 mg/L, the DS concluded the classification as Aquatic Acute 1 (H400) is appropriate.

As the lowest acute $ERV_{Cu4(OH)6SO4½H2O}$ (0.05 mg/L) is above 0.01 mg/L but ≤0.1 mg/L, the DS proposed an acute M-factor of 10.

In order to demonstrate removal from the water column (> 70% removal within 28 days) to assess the "persistence" or lack of degradation of metal ions the DS considered information provided by the copper task force (Rader, 2013). Evidence of rapid removal from the water column was based on the TICKET-Unit World Model (UWM), which describes partitioning to dissolved organic carbon, particulates, etc., deposition and transformation to sulfides in sediment. Together with evidence from field studies, the dossier submitter considered that this provides a satisfactory description of copper ion dynamics, and was therefore of the opinion that more than 70% of dissolved copper (II) ions are removed from the water column within 28 days, i.e. that dissolved copper compounds are rapidly removed. The potential for copper remobilisation from sediment was expected to be limited in oxic and anoxic conditions.

For aquatic chronic classification, the DS proposed that rapid removal of tetracopper hexahydroxide sulphate from the water column can be demonstrated. The lowest chronic $ERV_{Cu4(OH)6SO4½H2O}$ (0.013 mg/L) is above 0.01 mg/L but ≤0.1 mg/L, hence the DS concluded that classification as Aquatic Chronic 2 (H411) is appropriate for a substance subject to rapid removal. A chronic M-factor is not applicable.

Comments received during public consultation

Five comments were submitted on the environmental part of the DS's proposal of which one commenter agreed with the proposal without further comment, one agreed but with some observations, one agreed but suggested an acute M-factor of 100, and four commenters provided extensive comments challenging the DS's proposal.

An industry association pointed to disagreements in the selection and interpretation of ecotoxicity data between the CLH report and the REACH dossier, but agreed with the proposal. Four MSCAs objected to the use of the TICKET-UWM, for several reasons. Among them the fact that the model is designed for shallow lakes (so is not representative of turbulent or flowing systems or circumstances where sediment is not present), it includes significant assumptions about transformation to sulfides, and uses default assumptions for factors (like concentration of the particulate matter) that may vary spatially and temporally. One MSCA pointed out that dissolution data for copper (II) oxide (CuO) show an increase in dissolved copper ion concentrations by a factor of four between day 7 and day 28 at a loading rate of 1 mg/L, which does not suggest rapid transformation to less soluble forms. The lack of an existing international agreement about how to apply the rapid removal concept was also highlighted (including by one other CA, although they did not object to the approach taken). These CAs therefore indicated that dissolved copper (II) ions should not be considered to be rapidly removed from the aquatic environment, and that the chronic classification should therefore be Aquatic Chronic 1 (M-factor of 1) rather than Aquatic Chronic 2. In response, the dossier submitter agreed that copper (II) ions cannot currently be considered to be rapidly removed from the water column, and proposed changes to the proposed classification accordingly.

In addition, in several comments, MSs requested changes to, or better justification of, the selection of the lowest ecotoxicity data values, since there appeared to be discrepancies between some of the source documents and the way the information was summarised in the CLH report. Some of the differences were related to the use of geometric means rather than the lowest value for a species, and in other cases it was due to uncertainties about whether the cited data referred to the compound itself or to the metal ion. Furthermore one CA pointed out that it may be appropriate to apply the

surrogate approach, since there is no chronic test result available for the most sensitive species (*Pimephales promelas*) in the acute tests. In addition, the same CA noted that there are data on other invertebrate species and it was not clear why these were not included in the CLH report. Moreover, considering the amount of ecotoxicological data available for copper, it was proposed to use the species sensitivity distribution (SSD) curve for each trophic level for both short and long-term effects.

Another MSCA suggested that an explicit statement should be included that nano-forms should be considered separately.

Additional key elements

The following additional details (Tables 1 to 3) were not included in the CLH report but were extracted by RAC from the voluntary Risk Assessment Report (vRAR, 2008[4]).

Table 1. Ecotoxicity information extracted from Table 3-11 in chapter 3.2 of the vRAR (2008).

Taxonomic group	**Non-normalised "species mean" NOEC values (µg Cu/L)**
Algae	43.1 (*Pseudokirchneriella subcapitata*, n=12; growth); 138.0 (*Chlorella vulgaris*, n=17; growth); 79.8 (*Chlamydomonas reinhardtii*, n=4; growth)
Higher plants	30.0 (*Lemna minor*, n=1; growth)
Rotifer	33.5 (*Brachionus calyciflorus*; n=4; intrinsic rate of growth)
Molluscs	8.0 (*Campeloma decisum*, n=2; mortality); 6.0 (*Juga plicifera*, n=1; mortality); 19.1 (*Villosa iris*, n=1; mortality); 18.3 (*Dreissenia polymorpha*, n=2; filtration rate)
Cladocerans	13.1 (*Ceriodaphnia dubia*, n=10; reproduction); 12.6 (*Daphnia magna*, n=1; growth); 14.5 (*Daphnia pulex*, n=9; mortality)
Insects	10.4 (*Clistoronia magnifica*, n=2; reproduction/mortality); 16.9 (*Chironomus riparius*, n=1; growth); 40.0 (Paratanytarsus parthenogeneticus, n=2; growth/reproduction)
Amphipods	11.0 (*Gammarus pulex*, n=1; reproduction); 50.3 (*Hyalella azteca*, n=6; mortality)
Fish	13.0 (*Ictalurus punctatus*, n=2; growth/mortality); 20.8 (*Oncorhynchus kisutch*, n=2; mortality); 11.6 (*Oncorhynchus mykiss*, n=4; growth); 14.0 (*Salvelinus fontinalis*, n=5; growth); 17.8 (*Pimephales promelas*, n=4; growth); 56.2 (*Pimephales notatus*, n=2; growth); 39.0 (*Perca fluviatilis*, n=1; growth); 120.0 (*Noemacheilus barbatulus*, n=1; mortality); 12.9 (*Catostomus commersoni*; n=1; growth/mortality); 34.9 (*Esox lucius*; n=1; growth/mortality)

1.

Table 2. Lowest recorded species-specific acute L(E)C_{50} for each taxonomic group and proposed reference values extracted from Appendix K1 of vRAR (2008).

Test organism	**L(E)C_{50} µg copper ion/L**

[4] The voluntary Risk Assessment Report for Copper and Copper compounds was prepared by the European Copper Institute (ECI) and discussed by EU Member State CAs under the Existing Substance Regulation ((EEC) No 793/93 (ESR)) (subsequently adopted for biocide applications and REACH CSRs).

	pH 6	pH 7	pH 8	pH All
Fish	29.2	35.0	97.4	35.0
Invertebrates	25.0	47.3	29.8	34.4
Algae/aq.plants	263.5	120.1	106.8	138.9
Proposed reference	*25.0*	*35.0*	*29.8*	*34.4*

Table 3. Lowest recorded species-specific chronic NOEC for each taxonomic group and proposed reference values extracted from Appendix K1 of vRAR (2008).

Test organism	NOEC µg copper ion/L			
	pH 6	pH 7	pH 8	pH All
Fish	23.0	7.7	11.4	15.6
Invertebrates	20.0	7.4	19.8	14.9
Algae/aq.plants	30.0	22.0	34.5	22.0
Proposed reference	*20.0*	*7.4*	*11.4*	*14.9*

Assessment and comparison with the classification criteria

Water solubility:

The CLH report does not present transformation/dissolution data for $Cu_4(OH)_6SO_4$½H_2O over different timescales, pH values or loading rates. RAC notes that such data do not exist according to the industry comments submitted during public consultation, so in its absence the available water solubility data have been used. Section 1 of the CLH report indicates that the water solubility value is 500 mg/L (280 mg/L as dissolved copper) at pH 5.6, < 3.42 mg/L (1.88 mg/L as dissolved copper) at pH 6.2 and ≤ 0.255 mg/L (≤ 0.141 mg/L as dissolved copper) at pH 9.8 (at 20 °C).

Degradability

Rapid removal: RAC considers that the TICKET-UWM provides a useful insight into key fate pathways for metal ions including copper in a model shallow lake system. This generic approach allows systematic comparisons to be made between metals. However, the choice of model default parameters has not (yet) been resolved, especially as some properties are likely to vary spatially and temporally. For example, comparison with monitoring data in the CLH report suggested that the model may overestimate the extent to which copper binds to particles, and may use a settling velocity that is higher than observed in reality. In addition, post-loading simulations for one field study that was claimed to be "more representative of a worst case scenario" (on the basis of settling velocity, distribution coefficient and a relatively low suspended solids concentration compared to model defaults) did not predict 70% removal from the water column after 28 days. As this was a natural lake, RAC does not agree that it should be dismissed as a "worst case". Since the concept of rapid degradation for organic substances is conservative and does not include sequestration by particulate matter (or other fate pathways such as volatility), it seems inconsistent to apply such approaches to metals.

The DS's proposal also relied heavily on the premise that copper (II) ions will partition rapidly to sediment, where they will be transformed at the surface to insoluble minerals (especially copper (II) sulfide) over a relatively short timescale so that binding to sediment is effectively irreversible. RAC notes that the DS's proposal did not describe the behaviour of copper (II) ions in aquatic systems with little or no sediment (e.g. rivers or lakes with sand or gravel substrates), high turbulence or sediment at depths substantially in excess of 3 metres. Even where sediment is present, the oxidation state of surface layers may not always favour sulfide formation, and the situation may also be complicated if there is a high level of existing metal contamination. RAC therefore does not consider that a convincing case has been made that copper (II) ions will always rapidly speciate to non-available forms, or that this process was demonstrated to be irreversible under all relevant circumstances. At a general level, RAC considers that

decisions about rapid removal could be based on observations from a standardised OECD Transformation/Dissolution test. In this case, T/D studies showed increasing concentrations of copper ions over 28 days (not a decline), indicating that copper (II) ions remained in solution under these test conditions.

In conclusion, RAC considers that copper (II) ions are not subject to rapid environmental transformation for the purposes of classification and labelling.

Bioaccumulation

The bioaccumulation behaviour of copper (II) ions in organisms should consider both essentiality and homeostatic mechanisms. The DS's proposal did not present a clear description of the available data for comparison with the CLP criteria. However, in view of the degradability conclusion, this end-point does not influence the determination of the chronic M-factor and so was not considered further.

Ecotoxicity

Choice of ecotoxicity data: The ecotoxicity database for copper (II) ions is extensive, with many studies of acute and chronic toxicity in fish, invertebrates and algae/higher plants using a variety of copper compounds at different pH values as well as hardness and dissolved organic carbon (DOC) levels. The two principal sources of information cited in the DS's proposal are the pesticide DAR and the vRAR (2008). RAC considers that the chronic ecotoxicity information in the vRAR is generally reliable for hazard assessment as it was evaluated in depth by the relevant industry experts and reviewed by the pre-REACH CAs[5]. However, Tables 1-3 in the section "Additional key elements" show that the presentation of ecotoxicity information in these sources is inconsistent (presumably due to differences in data aggregation as pointed out in the public comments). This is considered further below:

a) Given the large number of studies for individual species, the data in the CLH report were aggregated to present single values for each species in three different pH bands. The CLP Guidance for metals recommends transformation/dissolution testing at different pHs, so RAC agrees that grouping into pH bands is appropriate as there is a clear trend in toxicity that would be overlooked if all the data for a species were combined. However, the reasons for the choice of the actual pH bands were not explained, and the effects of hardness and DOC were not discussed.

b) The dossier submitter's proposal used geometric means even if there are only two data points for a species in a particular pH band. This is not consistent with the CLP Guidance (which indicates that at least four data points are preferred) or the REACH CSRs, and led to discrepancies between the data sets, which were noted during public consultation.

c) For invertebrates, data were presented for only two species of crustacean (*Daphnia magna* and *Ceriodaphnia dubia*). RAC notes that it is standard practice to consider all relevant data from reliable standard test guideline studies, and so the dossier submitter's proposal was not necessarily based on a comprehensive data set. The dossier submitter did not provide any additional information in response to the public consultation comments on this issue. However, RAC notes that the vRAR (2008) contains long-term toxicity data for several other invertebrate taxonomic groups (including molluscs and insects) as well as higher plants (*Lemna minor*). Further details are provided in the

[5] Italy has been acting as a reviewing Member State for the substance and the risk assessment report has been reviewed by the Technical Committee on New and Existing Substances (TC NES) according to standard operational procedures of the Committee.

section "Additional key elements".

i) In the vRAR (2008), all the reliable chronic NOEC data were compiled in a species sensitivity distribution, deriving a hazardous concentration for 5% of the species (HC_5) (with the 50th percentile confidence interval) of 7.3 µg/L (6.1-7.9 µg/L) based on the best fitting approach, or 6.1 µg/L (3.7-8.6 µg/L) using the log normal curve fitting. These values are very similar to the lowest NOEC in the dataset (6.0 µg/L for the mollusc *Juga plicifera*).

ii) Due to the variation in physico-chemical conditions used in the tests, in the vRAR (2008) the data were also 'normalised' using a biotic ligand model. The lowest normalised NOEC is 5.3 µg/L for the rotifer *Brachionus calyciflorus* (at pH 8.1, hardness of 165 mg/L $CaCO_3$ and DOC of 3.2 mg/L). The lowest HC_5-50 derived for an ecoregion is 7.8 µg/L (4.4-11.7 µg/L).

iii) RAC notes that the CLH report also mentioned a NOEC of 3.12 µg/L (as copper) from an indoor microcosm study using copper hydroxide, without specifying the measured end-point or study duration; it was also pointed out, in comments during the public consultation, that in the final EFSA conclusion a NOEC of 4.8 µg/L is cited which was used for the overall risk assessment for aquatic organisms. As it was not clear how this information would be used in hazard classification, it was not considered further.

In summary, the lowest long-term NOEC reported in the CLH report is 7.4 µg/L for *Ceriodaphnia dubia* at pH 6.5-7.5. The omission of data for other invertebrate groups from the DS's proposal does not appear to make a significant difference as the most sensitive data all lie in the range 1-10 µg/L.

Discrepencies in the ecotoxicity data as presented: The lowest acute toxicity value selected in the CLH report is 0.029 mg/L (29 µg/L) at pH 5.5-6.5, giving the source as the vRAR. The origin of this data point is unclear, but RAC assumes that it relates to data for *O. mykiss* (a similar value was obtained with *Ceriodaphnia dubia* at pH >7.5-8.5). However, the lowest geometric mean LC_{50} reported in the CLH report is 8.1 µg/L (as copper) for fathead minnow *P. promelas* at pH 5.5-6.5 (cited as coming from the vRAR – an actual study reference was not provided). This is based on two values, both for larval fish, 15.0 µg/L and 4.4 µg/L. One comment received during public consultation suggested that this latter value should be used for the acute ERV, which would in turn lead to an acute M-factor of 100. Further comments from industry during PC indicated that the test medium in the study which resulted in the lowest EC_{50} (cited as Erickson *et al.,* 1996) used a high flow-through rate, had low hardness (22 mg $CaCO_3$/L) and low DOC concentration (not stated), and used larvae that were less than 24 hours' old. Although not mentioned in the CLH report, in the original paper the lowest LC_{50} was determined at the minimum pH, i.e. 6.0. Industry therefore considered this test to represent a worst case, and suggested that the sensitivity of this species at pH 6 versus pH 7 was unexpected and may be related to insufficient adaptation to low pH conditions. The data were therefore not considered reliable and not used for classification in the REACH registrations as well as the vRAR. Nevertheless, RAC notes that other minimum acute fish LC_{50}s are of the same order of magnitude (e.g. *O. mykiss* at all pHs, and *P. promelas* at pH 6.5-7.5). The OECD TG 203 permits testing in waters with total hardness as low as 10 mg $CaCO_3$/L, and a preferred minimum pH of 6.0, so the conditions used in the Erickson (1996) study were within the validity criteria of the guidelines and cannot be considered a worst case. In addition, this species can tolerate poor conditions such as turbid, hot, poorly oxygenated, intermittent streams, which are unsuitable for most fishes (http://www.fishbase.org/Summary/speciesSummary.php?ID=4785&AT=fathead+minno

w). Further papers provided by industry stakeholders following public consultation (Mount, 1973 and Zischke *et al.*, 1983) indicate that *P. promelas* can survive at pHs as low as 4.5, so that a pH of 6.0 does not appear to be intolerable over short exposures. RAC also notes that the replacement test for acute fish toxicity (OECD TG 236) involves embryos, so the life stage argument was not considered relevant either. It is also unclear why the dossier submitter decided to include them in the CLH report if they had been previously rejected. RAC accepts that an acute toxicity test with fish larvae may be more sensitive than one with older fish if they were not properly acclimated, but does not find the other reasons for rejection convincing.

Data for other species show a trend of increasing acute fish toxicity with declining pH, presumably due to increasing bioavailability. The acute LC_{50} for *Danio rerio* at pH 6.5-7.5 (35 µg/L, n=3 so a geometric mean is not appropriate) is similar to that of *O. mykiss* at pH 5.5-6.5 (geometric mean 29 µg/L), implying that the sensitivity of *D. rerio* at the lower pH could be higher. Rather than ignoring the *P. promelas* data completely, the geometric mean LC_{50} of 8.1 µg/L is therefore considered to be relevant for hazard classification as it takes account of uncertainties about the sensitivity of fish at acidic pH, although this is a conservative approach given the life stages that were tested (N.B. if the most sensitive value of 4.4 µg/L were used the classification and acute M-factor would be 100 for tetracopper hexahydroxide sulphate). RAC has not considered how DOC or hardness affect the observed pattern in ecotoxicity data, as such an analysis was not presented in the CLH report.

As noted above, the lowest reported long-term NOEC in the CLH report is 7.4 µg/L for *Ceriodaphnia dubia* at pH 6.5-7.5, and this value is consistent with the large amount of chronic data presented in the vRAR (2008), including the HC_5. However, this is almost identical to the acute LC_{50} for *P. promelas* at pH 5.5-6.5, and there are no measured chronic toxicity data for any fish species in the pH range of 5.5-6.5. Consequently, the adequancy of the long-term study results was questioned. At first sight it might seem disproportionate to consider the whole long-term fish toxicity data set (n=29) as 'non-adequate'. However, the acute fish test data clearly show that for the three species for which data across the total pH range of 5.5-8.5 are available, the toxicity is the highest in the lowest pH range, i.e., 5.5-6.5. Therefore, despite the large number of fish studies used in the dossier submitter's proposal, RAC believes that it is appropriate to consider the surrogate method for the fish trophic group (as was suggested in one of the public consultation comments). [N.B. The CLP criteria and guidance do not address this specific issue, but Example D in Section 4.1.3.4.4 of the CLP guidance is comparable to some extent. It describes a substance with a large data set, for which acute as well as chronic toxicity data are available for all three trophic levels. For crustacea, chronic data are available for *Daphnia magna*, which is clearly the least sensitive of the invertebrate species for which acute data are available. Hence, according to the guidance, the chronic aquatic toxicity data for *D. magna* in this case should be considered not in conformity with the definition of 'adequate chronic data'.]

In addition, it was indicated in comments received during public consultation that in the DAR for copper hydroxide, a 92-d NOEC of 1.7 µg/L was obtained in a fish early life stage test for *O. mykiss* at pH 8.0 (cited as Schäfers, 2000). This result does not appear to have been taken into account in the data aggregation used in the dossier submitter's proposal. Another reliable chronic result for this species in the pH range > 7.5-8.5 was included in the CLH report (NOEC 16 µg Cu/L). Industry in their comments following the public consultation raised some issues about the reliability of the lower value of 1.7 µg/L (e.g. the reported copper concentrations were highly variable in this study and the test substance was a formulation containing 10% w/w dispersant and also an adhesive). Whilst toxicity was still likely to have been driven by copper ions, the composition might have had some influence. It was also sparingly soluble, rather than a soluble salt. This result was therefore not used directly but is considered by RAC as supporting information for chronic classification purposes.

ERV derivation: The lowest acute $L(E)C_{50}$ (as dissolved copper) presented in the CLH report is 8.1 µg/L for *P. promelas* at pH 5.5-6.5. The acute $ERV_{Cu4(OH)6SO4½H2O}$ is therefore equal to 0.015 mg/L [{acute ERV of metal ion x molecular weight of the metal compound/(atomic weight of the metal x number of metal ions)}, so 0.0081 x 461.3/(63.5 x 4)]. This is lower than the acute ERV proposed in the CLH report (0.05 mg/L), which is based on a different acute toxicity value.

The lowest long-term NOEC (as dissolved copper) presented in the CLH report is 7.4 µg/L for *Ceriodaphnia dubia* at pH 6.5-7.5. The chronic $ERV_{Cu4(OH)6SO4½H2O}$ is equal to 0.013 mg/L [{chronic ERV of metal ion x molecular weight of the metal compound/(atomic weight of the metal x number of metal ions)}, so 0.0074 x 461.3/(63.5 x 4)]. As noted under in Annex 1, other apparently reliable NOEC data exist that are lower than this value, but still in the range 1-10 µg/L (e.g. a normalised NOEC of 5.3 µg/L for the rotifer *Brachionus calyciflorus* at pH 8.1, hardness of 165 mg/L $CaCO_3$ and DOC of 3.2 mg/L). Similarly, it was suggested in comments received during public consultation to use the lowest chronic NOEC from the DAR derived for *Daphnia magna* of 5.7 µg Cu/L. These data will make only a very small difference to the chronic $ERV_{Cu4(OH)6SO4½H2O}$. However, there are no chronic toxicity data for the fish species that is acutely most sensitive at pH 5.5-6.5, so the surrogate method for the fish trophic group is therefore considered.

Acute aquatic hazard:
The water solubility (280 mg/L at pH 5.6, 1.88 mg/L at pH 6.2 and ≤0.141 mg/L at pH 9.8, all as dissolved copper) exceeds the acute ERV of the dissolved metal ion (0.0081 mg/L based on the *P. promelas* data), so the substance is considered to be a readily soluble metal compound. RAC agrees to classify tetracopper hexahydroxide sulphate **Aquatic Acute 1 (H400)** on the basis of the acute $ERV_{Cu4(OH)6SO4½H2O}$ (0.015 mg/L). As the lowest acute $ERV_{Cu4(OH)6SO4½H2O}$ is above 0.01 mg/L but ≤0.1 mg/L, the **acute M-factor is 10**.

Chronic aquatic hazard:
As the substance is considered to be a readily soluble metal compound, classification may be based on the lowest chronic $ERV_{Cu4(OH)6SO4½H2O}$ (0.013 mg/L based on data for *Ceriodaphnia dubia*). Since this is below 0.1 mg/L, classification as **Aquatic Chronic 1 (H410)** is appropriate for a substance not subject to rapid environmental transformation based on RAC conclusion on rapid removal from the environment. As the lowest chronic $ERV_{Cu4(OH)6SO4½H2O}$ is above 0.01 mg/L but ≤ 0.1 mg/L, the chronic M-factor would be 1 for a substance not subject to rapid environmental transformation. However, using the surrogate method for the fish trophic group, the **chronic M-factor** should be consistent with the acute M-factor, i.e. **10**.

In summary, RAC agrees with the DS's proposal to classify tetracopper hexahydroxide sulphate as **Aquatic Acute 1 (H400)** with an **acute M-factor** of **10** but considers that a more stringent chronic classification (**Aquatic Chronic 1 (H410), chronic M-factor 10**) is required than originally proposed (Aquatic Chronic 2 (H411)) because of the conclusion on rapid environmental transformation as well as the most sensitive fish toxicity data. The classification is based on a MW of 461.3 (based on the formula $Cu_4(OH)_6SO_4½H_2O$) and the presence of 4 copper atoms per molecule.

6 OTHER INFORMATION

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8 ANNEXES

ANNEX I: purity and impurity profile (confidential)

See separate file

ANNEX II: summary of copper compounds under review for classification

Substance		CAS number	Current harmonised classification	Proposed CLH (changes displayed in bold)	Regula-tory program
Copper sulphate (CAS : 7758-98-7)	Copper sulphate pentahydrate	7758-99-8	Acute Tox. 4 * - H302 Eye Irrit. 2 – H319 Skin Irrit. 2 – H315 Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410	Acute Tox. 4 - H302 **Eye Dam. 1 – H318** ~~**Skin Irrit. 2 – H315**~~ Aquatic Acute 1 – H400, **M=10** **Aquatic Chronic 2 – H411**	BPD
	Tribasic copper sulphate	12527-76-3	Acute Tox. 4 * - H302 Eye Irrit. 2 – H319 Skin Irrit. 2 – H315 Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410	Acute Tox. 4 - H302 ~~**Eye Irrit. 2 – H319**~~ ~~**Skin Irrit. 2 – H315**~~ Aquatic Acute 1 – H400, **M=10** **Aquatic Chronic 2 – H411**	PPP
Copper thiocyanate		1111-67-7	Salts of thiocyanic acid: Acute Tox. 4 * - H332 Acute Tox. 4 * - H312 Acute Tox. 4 * - H302 EUH32 Aquatic Chronic 3 – H412	**EUH 32** **Aquatic Acute 1 – H400, M=10** **Aquatic Chronic 2 – H411**	BPD
Basic copper carbonate		12069-69-1	None	**Acute Tox. 4 - H302** **Acute Tox. 4 - H332** **Eye Irrit 2 – H319** **Aquatic Acute 1 – H400, M=10** **Aquatic Chronic 2 – H411**	BPD
Copper hydroxide		20427-59-2	None	**Acute Tox. 4 - H302** **Acute Tox. 2 - H330** **Eye Dam. 1 – H318** **Aquatic Acute 1 – H400, M=10** **Aquatic Chronic 1 – H410, M=1**	BPD PPP
Copper (I) oxide		1317-39-1	Acute Tox. 4 * - H302 Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410	**Acute Tox. 4 - H302** **Acute Tox. 4 - H332** **Eye Irrit. 2 – H319** **Aquatic Acute 1 – H400, M=10** **Aquatic Chronic 1 – H410, M=1**	BPD PPP
Copper (II) oxide		1317-38-0	None	**Acute Tox. 2 - H330** **Aquatic Acute 1 – H400, M=10** **Aquatic Chronic 1 – H410, M=1**	BPD

Copper oxychloride	1332-40-7 or 1332-65-6	None	**Acute Tox. 3 - H301** **Acute Tox. 4 - H332** **Aquatic Acute 1 – H400, M=10** **Aquatic Chronic 2 – H411**	PPP
Coated copper flake	7440-50-8	None	**Acute Tox. 4 - H302** **Acute Tox. 3 - H331** **Aquatic Acute 1 – H400, M=10** **Aquatic Chronic 1 – H410, M=1**	BPD
Bordeaux mixture	8011-63-0	None	**Acute Tox. 4 - H332** **Eye Dam. 1 – H318** **Aquatic Acute 1 – H400, M=10** **Aquatic Chronic 2 – H411**	PPP