Committee for Risk Assessment

RAC

Annex 1

Background document

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

Difenacoum (ISO); 3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl) -4-hydroxycoumarin

EC number: 259-978-4
CAS number: 56073-07-5

CLH-O-0000003392-78-03/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

14 March 2014

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PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

Substance Name: DIFENACOUM

EC Number: 259-978-4

CAS Number: 56073-07-5

Index number: 607-157-00-X

Submitted by:	**Finland**
Date:	**November 2012**
Version:	**2**

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BACKGROUND TO THE PROPOSAL

Difenacoum has been reviewed as an existing active substance under both the Biocidal Products Directive (98/8/EC) and the Plant Protection Products Directive (91/414/EEC). It was included in Annex I to Directive 98/8/EC in April 2010 and in Annex I to Directive 91/414/EEC in January 2010. The hazards of difenacoum have been assessed by the Finnish Competent Authorities as part of these regulatory programmes. These assessments were discussed and agreed by the appropriate European technical committees under each review programme.

In accordance with Article 36(2) of Regulation EC 1272/2008 on classification, labelling and packaging of substances and mixtures, difenacoum should be considered for harmonised classification and labelling. This CLH report presents a classification and labelling proposal based on the information presented following the assessment of difenacoum under Directive 98/8/EC. The Assessment Report prepared, along with documents II-A of the Competent Authority Reports, are provided in section 13 of the IUCLID file.

Difenacoum is only used as an active ingredient in plant protection and biocidal products to kill rodents. It is not a REACH registered substance under the REACH Regulation (1907/2006), therefore no registration dossiers are available (last date for checking 01/10/2012).

History of the previous classification and labelling and previous CLH proposal

Difenacoum was first listed in Annex I to Directive 67/54/EEC in the 15th adaptation to technical progress (Commission Directive 91/632/EEC) classified with T+; R28 and T; R48/25 and labelled with T+: R:28-48/25 and S: 36/37-45. In the 19th adaptation to technical progress (Commission Directive 93/72/EEC) S-phrases were slightly altered to S: (1/2-)36/37-45. Environmental classification N, R50-53 was added in the 25th adaptation to technical progress (Commission Directive 98/98/EC) and consequently the S-phrases were altered to S: (1/2-)36/37-45-60-61.

New classification proposals were submitted to the Technical Committee on Classification and Labelling of Dangerous Substances (TC C&L) in August 2005 and in February 2006 and discussed in the Groups on Environmental and Human Health effects, respectively.

Because of a common mechanism of action anticoagulant rodenticides were discussed as a group. As for classification for human health the substance was discussed by the Specialised Experts (SE) for Reproductive Toxicity (September 2006) as well as at two meetings in TC C&L (November 2006 and May 2007) and in written follow up periods to these meeting. At the TC C&L Meeting in November 2006, a provisional reproductive toxicity classification for developmental effects with R61 was agreed, but without a final decision on the category to be used (Repr. Cat 1 or Repr. Cat 2). However, the discussion on the developmental classification was not finalised. As for other human health end-points the following classification was agreed in November 2006: T+: R26/27/28 and T; R48/23/24/25. A general discussion on specific concentration limits for acute and repeated dose toxicity for all anticoagulant rodenticides took place at the May 2007 meeting and at the same meeting the specific concentration limits for acute and repeated dose toxicity according to Directive 67/548/EEC for difenacoum were agreed. (Follow-up V, May 2007).

The environmental classification proposed to TC C&L (ECBI/97/04 Add. 3) was the same as the current proposal in this classification report namely adding specific concentration limits

corresponding an M-factor of 10 to the R50-53 classification. The decision was, however, postponed to wait for the discussion in the Technical Meeting for Biocides (ECBI/48/05).

Short summary of the scientific justification for the CLH proposal

Difenacoum is currently classified as T+; R28 according to Directive 67/548/EEC. Translation of the existing classification resulted in minimum classification as Acute oral toxicity 2*; (H300) according to the CLP Regulation. Due to the different classification criteria between DSD and CLP, and based on the available data on difenacoum (rat oral LD_{50} 1.8 mg/kg), it is now proposed to update the CLP classification as Acute oral toxicity 1 (H300).

Classification via other acute toxicity routes is also proposed. The acute inhalation LC_{50} values ranged between 3.65-5.85 µg/L/4 h, therefore classification of difenacoum as Acute Tox. 1; H330 is proposed. The LD_{50} value for acute dermal toxicity was < 5 mg/kg bw. This LD_{50} value fulfils the criterion for Acute Tox. 1 (< 50 mg/kg) under CLP Regulation, therefore classification of difenacoum as Acute Tox. 1; H310 is proposed.

Difenacoum is classified for repeated dose toxicity as T; R48/25 according to Directive 67/548/EEC via oral route and as STOT RE 1; H372** according to CLP. Classification for other exposure routes (dermal, inhalation) is proposed based on route-to-route extrapolation. Therefore, classification as T; 48/23/24/25 according to the Directive 67/548/EEC is proposed. In the CLP classification the two asterisks were given because the existing classification was translated from Directive 67/548/EEC with a general hazard statement not specifying the route of exposure as the necessary information was not available. It is now proposed that the asterisks be removed from the hazard statement H372 because no route of exposure can be excluded. In the present proposal specific concentration limits are also set.

Classification for reproductive toxicity is proposed because of teratogenicity. Developmental toxicity data on difenacoum is equivocal, however it is a coumarin derivative like warfarin, which is classified as Repr. 1A; H360D*** according to the CLP Regulation. Since also the mode of action causing vitamin K deficiency is the same, and the maternal vitamin K deficiency is the underlying reason for teratogenicity, it is proposed to classify difenacoum as Repr. 1A;H360D based on read-across from warfarin. Specific concentration limit should be set for difenacoum and it is to be specified at a later stage together with the other AVKs.

The current environmental classification for difenacoum according to CLP is Aquatic Acute 1 - H400 and Aquatic Chronic 1 - H410 and according to DSD N, R50-53. M-factor of 10 is proposed to be added for both acute and long-term classifications according to the 2nd ATP of CLP and corresponding specific concentration limits are proposed to be added to N, R50-53 classification.

The lowest acute toxicity value is LC50 (96 h) of 0.064 mg/l for fish *Oncorhynchus mykiss.* According to the criteria the substance is classified Aquatic Acute I - H400, M=10 in CLP.

Difenacoum is not readily/rapidly degradable and it has a potential to bioaccumulate (log K_{ow} > 4). There is only one chronic NOEC available for difenacoum and consequently chronic classification is assessed using two approaches: NOErC (72 h) of 0.13 mg/l for the algae *Scenedesmus subspicatus* would give Aquatic Chronic 2 - H411 classification whereas LC50 (96 h) of 0.064 mg/l for the fish *Oncorhynchus mykiss* gives, considering that the substance is bioaccumulative and not rapidly degradable, Aquatic Chronic - H410 classification with M-factor of 10. According to the criteria the most stringent outcome is chosen.

According to DSD difenacoum is classified N, R50-53 with specific concentration limits:

R50-53: C ≥2.5%

R51-53:0.25%≤C≤2.5%
R52-53: 0.025% C ≤ 0.25%

PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

Substance Name: Difenacoum

EC Number: 259-978-4

CAS number: 56073-07-5

Annex VI Index number: 607-157-00-X

Registration number(s): Not registered under REACH

Purity: ≥ 960 g/kg

Impurities: Confidential; None of (eco)toxicological concern

Table 1: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation	**Directive 67/548/EEC (Dangerous Substances Directive; DSD)**
Current entry in Annex VI, CLP Regulation	Acute Tox. 2* - H300 STOT RE 1 – H372** Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410	T+; R28 T; R48/25 N; R50-53
Current proposal for consideration by RAC	Acute Tox. 1 – H300 Acute Tox. 1 – H310 Acute Tox. 1– H330 Repr. 1A – H360D STOT RE 1 – H372 C ≥ 0.1%: STOT RE 1[a] 0.01% ≤ C < 0.1% : STOT RE 2[a] A SCLfor reprotoxicity: to be specified at a later stage together with the other AVKs M-factor: 10	T+; R26/27/28 Repr. Cat. 1; R61 T; R48/23/24/25 C ≥ 0.25%: T+; R26/27/28 0.025% ≤ C < 0.25%; T; R23/24/25 0.0025% ≤ C < 0.025%: Xn; R20/21/22 C ≥ 0.025%: T; R48/23/24/25[a] 0.0025% ≤ C < 0.025%: Xn; R48/20/21/22[a] C ≥ 2.5 %: N; R50-53 0.25 % ≤ C < 2.5 %: N; R51-53 0.025 % ≤ C < 0.25 %: R52-53
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Acute Tox. 1 – H300 Acute Tox. 1 – H310 Acute Tox. 1– H330 Repr. 1A – H360D STOT RE 1 – H372 Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410 C ≥ 0.1% : STOT RE 1 [a] 0.01% ≤ C < 0.1% : STOT RE 2[a] M-factor: 10	T+; R26/27/28 Repr. Cat. 1; R61 N; R50-53 C ≥ 0.25%: T+; R26/27/28 0.025% ≤ C < 0.25%; T; R23/24/25 0.0025% ≤ C < 0.025%: Xn; R20/21/22 C ≥ 0.025%: T; R48/23/24/25[a] 0.0025% ≤ C < 0.025%: Xn; R48/20/21/22[a] C ≥ 2.5 %: N; R50-53 0.25 % ≤ C < 2.5 %: N; R51-53 0.025 % ≤ C < 0.25 %: R52-53

[a]The proposed SCLs for repeated dose toxicity are of different magnitude due to different approach in setting the SCLs between CLP and DSD.

Proposed labelling

Directive 67/548/EEC:

R-phrases: R26/27/28-48/23/24/25-50/53-61

Symbol(s) : T+; N

S-phrases : S(1/2-)36/37-45-53-60-61.

Regulation EC 1272/2008:

Pictograms: GHS06, GHS08, GHS09

Signal word: Danger

Hazard statement codes: H300, H310, H330, H360D, H372, H410

As precautionary statements are not included in Annex VI of Regulation EC 1272/2008, no proposal is made.

Proposed notes (if any):

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JUSTIFICATION

1 IDENTITY OF THE SUBSTANCE AND PHYSICAL AND CHEMICAL PROPERTIES

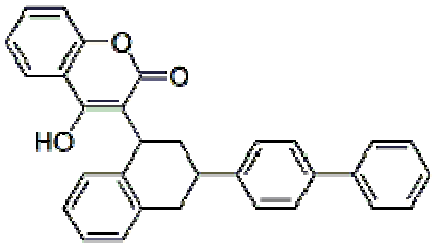
1.1 Name and other identifiers of the substance

CAS Name:	2H-1-benzopyran-2-one, 3-(3-[1,1'-biphenyl]-4-yl-1, 2,3,4-tetrahydro-1-naphthalenyl)-4-hydroxy-
EC Name:	3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphtyl)-4-hydroxycoumarinCAS
Number:	56073-07-5
IUPAC Name:	3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-napthyl)-4-hydroxycoumarin

1.2 Composition of the substance

CAS Name:	2H-1-benzopyran-2-one, 3-(3-[1,1'-biphenyl]-4-yl-1, 2,3,4-tetrahydro-1-naphthalenyl)-4-hydroxy-
EC Number:	259-978-4
CAS Number:	56073-07-5
IUPAC Name:	3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-napthyl)-4-hydroxycoumarin
Molecular formula:	$C_{31}H_{24}O_3$

Structural formula:



Isomers: Isomeric mixture of *trans* isomer (CAS N. 151986-16-2, CA Index Name: 2H-1-Benzopyran-2-one, 3-(3-[1,1'-biphenyl]-4-yl-1,2,3,4-tetrahydro-1-naphthalenyl)-4-hydroxy-, *trans*-) and *cis* isomer (CAS N. 151986-15-1, CA Index Name: 2H-1-Benzopyran-2-one, 3-(3-[1,1'-biphenyl]-4-yl-1,2,3,4-tetrahydro-1-naphthalenyl)-4-hydroxy-, *cis*-). The range of *cis* isomer is 50-80%. Both diastereomers are toxicologically active.

Molecular weight: 444.5 g/mol

Typical concentration (% w/w): ≥ 96.0 %

Concentration range (% w/w): 96.0-99.5 %

Impurities	There are 1-5 impurities present at concentrations ≤ 0.5- 4 %. None of these impurities affect classification of difenacoum. The impurities are considered confidential and are therefore not given in this report but only in the IUCLID file and flagged confidential.

1.3 Physico-chemical properties

No classification proposed.

Table 2: Summary of physico-chemical properties

Property	Value	Reference
Physical state at 20°C and 101.3 kPa	Solid powder	Doc II-A[1] (A3.1.1/01)
Melting/freezing point	211 – 215 °C (Purity: 98.7% w/w) An endotherm at 226.3 °C, melting is proposed. (99.7% w/w) 216.3 – 226 °C, melting (with signs of degradation) (99.7% w/w) 221.8 °C (99.4% w/w)	Doc II-A[1] (A3.1.1/01) Doc II-A[3] (A3.10) Doc II-A[3] (A3.1.1) Doc II-A[2] (A3.1.1/01)
Boiling point	No boiling point before start of decomposition (96.5%) The substance decomposes at 290 °C before boiling (99.4% w/w) No boiling point detected In tests up to the temperature of 250 °C. (99.7% w/w)	Doc II-A[1] (A3.10/01) Doc II-A[2] (A3.1.1/01) Doc II-A[3] (A3.1.1) Doc II-A[3] (A3.10)
Relative density	1.27 g/cm^3 at 20.5 °C (98.7% w/w) 1.35 g/cm^3 at 21.5 °C (99.4% w/w) 1.14 (1.1363) g/cm^3 at 20 °C (>99% w/w)	Doc II-A[1] (A3.1.1/01) Doc II-A[1] (A3.1.3/01) Doc II-A[2] (A3.1.3/01) Doc II-A[3] (A3.1.3)
Vapour pressure	1.9 x 10^{-11} Pa, with total error of x 352.5, at 25 °C (98.7%), (computer-based estimation). This can be expressed also as a range of 6.7 x 10^{-9} – 5.4 x 10^{-14} Pa. p (20°C) ≤ 1.0 × 10^{-5}Pa p (25°C) ≤ 1.0 × 10^{-5}Pa p (50°C) ≤ 1.0 × 10^{-5}Pa (99.4%) < 5 x 10^{-5} Pa at 45 °C (99%), an estimation.	Doc II-A[1] (A3.1.1/01) Doc II-A[2] (A3.2/01) Doc II-A[3] (A3.2)
Surface tension	Not applicable.	
Water solubility	< 0.05 mg/l [20°C, at pH 4] 1.7 mg/l [20°C, at pH 7] 61.0 mg/l [20°C, at pH 9] 0.0025 mg/l [20°C, at pH 4.0] 0.06 mg/l [20°C, at pH 7.1] 1.24 mg/l [20°C, at pH 9.0] ≤ 4.82 x 10^{-2} mg/l (4.82 x 10^{-5} g/l) [20°C, at pH 5.1] 0.483 mg/l [20°C, at pH 6.5] 3.72 mg/l [20°C, at pH 8.9]	Doc II-A[1] (A3.1.1/01) Doc II-A[2] (A3.5/01) Doc II-A[3]

Partition coefficient n-octanol/water (log value)	7.6 (estimated using a computer atom/fragment contribution method) log K_{ow} 6.09–6.13 at 20 °C (at pH 6.5) (> 96.09%) 7.62 (a QSAR estimation) The experimental information available on difenacoum suggest that it may be beyond the performance ranges of the experimental tests for log K_{ow}.	Doc II-A[1] (A3.9/01) Doc II-A[2] (A3.9/01) Doc II-A[3] (A3.9 (2))
Flash point	Not applicable.	
Flammability	Not highly flammable	Doc II-A[2] (A3.11/01)
Explosive properties	Not explosive	Doc II-A[3] (A3.15) Doc II-A[2](A3.15/01) Doc II-A[1] (A3.15/01)
Self-ignition temperature	No self-ignition up to the melting point	Doc II-A[1] (A3.11/01)
Oxidising properties	Not oxidizing.	Doc II-A[3] (A3.16/(1)) Doc II-A[3] (A3.16/(2)) Doc II-A[2] (A3.16/01) Doc II-A[1](A3.16/1) Doc II-A[1](A3.16/02)
Stability in organic solvents and identity of relevant degradation products	Not applicable.	
Dissociation constant	pKa value 4.84 (96.2% w/w) pKa value 4.5± 1.00 (a QSAR estimation)	Doc II-A[1](A3.6/01) Doc II-A[3] (A3.6)
Viscosity	Not applicable.	
Reactivity towards container material	Based on long experience no reactivity to UN packaging materials. Experience in use indicates no reactivity of difenacoum towards container materials, including polyethylene, high density polyethylene, polypropylene, lacquered tin plate, steel and stainless steel. Difenacoum is never stored in bulk as technical. Based on long experience no reactivity is expected in glass or HDPE jars.	Doc II-A[1] Doc II-A[2] Doc II-A[3]
Thermal stability	Temperature of decomposition >300°C (96.5%) Stable up to at least 290°C. (99.4%)	Doc II-A[1] (A3.10/01) Doc II-A[2] (A3.1.1./01) Doc II-A[3] (A3.10)

	No thermal events shown below 150 °C. The results indicate adequate thermal stability under conditions of practical handling and use.	
Other		

1.4 Derivation of n-octanol/water partition coefficient

Although experimental study reporting (see 4.3.1) n-octanol/water partition coefficient for difenacoum was available in the data submitted for the biocide assessment, that value could not be used (see below for details). Therefore, estimated data was used for this CLH assessment. Two very similar estimated logarithmic values of the partition coefficient (log K_{ow}), 7.6 and 7.62, were reported in separate studies (Table 2).)):

One of the studies (Doc II-A[1] (A3.9/01)) used a computer estimation package (an atom/fragment contribution method) *[Ref: J Pharm Sci, 84: 83-92, 1995]* in which chemical structures are entered using SMILES notation. Using a training set of 2351 chemicals with 125 groups and 230 correction factors, a correlation coefficient of 0.98 was found between the estimated and experimental values of log K_{ow}. The method was evaluated using a validation set of 6055 chemicals with a resulting correlation coefficient of 0.94 and a mean error of 0.31. The log K_{ow} estimate for difenacoum was 7.6.

Another log K_{ow} estimation study (Doc II-A[3] (A3.9 (2)), described as "EPIWIN model" in the biocide assessment, gave a log K_{ow} estimate of 7.62 for difenacoum. For the present CLH assessment, the dossier submitter (DS) performed a QSAR log K_{ow} estimation using the model KOWWIN (version 1.67) using EPI Suite 4.0) and the same result (log K_{ow} 7.62) was obtained as reported in the study by Doc II-A[3] (A3.9 (2)). Furthermore the DS verified the applicability of KOWWIN model and the following observations were done:

-the model includes in its training set fragments relevant to difenacoum
-all of the structural features of difenacoum are represented by the training set compounds
-the number of instances of any of the fragments in difenacoum does not exceed the maximum number among the training set compounds
-the molecular weight of difenacoum (444.53) is within the range of the training set compounds used for the KOWWIN model

Therefore, the DS concludes that KOWWIN model is applicable for the estimation of log K_{ow} for difenacoum. The estimated log K_{ow} result is used for the estimation of bioaccumulation of difenacoum under CLP regulation (see Chapter 4.3).

The estimation concerns the undissociated species of difenacoum.

One experimental log K_{ow} study (Doc II-A[2] (A3.9/01)) was available. The study was conducted using the OECD 117 method (HPLC method). Although that study was considered acceptable this experimentally derived value could not be used for the evaluation because the technical equivalence of difenacoum used for the study could not be clarified. Nevertheless this experimentally derived value (6.09-6.13 at 20 °C, at pH 6.5) gives further support for the estimated log $K_{ow.}$

2 MANUFACTURE AND USES

Difenacoum is used as an active substance in biocidal products and plant protection products (rodenticides). Difenacoum concentration in representative products ranges from 0.005-0.0075 %.

3 CLASSIFICATION AND LABELLING

3.1 Classification in Annex VI of Regulation 1272/2008

Table 3: Current Annex VI Table 3.1 classification and labelling

Classification		Labelling		Specific Conc. Limits, M-factors
Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	
Acute Tox. 2 * STOT RE 1 Aquatic Acute 1 Aquatic Chronic 1	H300 H372** H400 H410	GHS06 GHS08 GHS09 Dgr	H300 H372 ** H410	

Table 4: Current Annex VI Table 3.2 classification and labelling

Classification	Labelling	Concentration limits
T+; R28 T; R48/25 N; R50-53	T+; N R: 28-48/25-50/53 S: (1/2-)36/37-45-60-61	-

3.2 Self classification(s)

The existing harmonised classification is notified by practically all notifiers to the Classification and Labelling Inventory. Only one notifier had in addition classified the substance to Acute Toxicity Category 1 with hazard statements H310 and H330 in addition to H300.

4 ENVIRONMENTAL FATE PROPERTIES

Difenacoum has been evaluated according to Directive 98/8/EC. The evaluation was based on the dossiers of three different applicants and a brief overview of the environmental fate properties are given here.

4.1 Degradation

4.1.1 Stability

Hydrolysis

Difenacoum is hydrolytically stable over an environmentally relevant pH range of 4-9 (Table 5). The half-life is predicted to be greater than 1 year at 25°C.

Table 5: Hydrolysis

Guideline/ Test method	pH	Temp. (°C)	Initial conc. (mg/l)	Reaction rate constant, K_h ($1/s \times 10^5$)	Half-life, DT_{50} (year)	Reference
OECD 111	4	25	1.0	-	> 1	Doc II-A[3] (A7.1.1.1.1)
	7	25	1.0	-	> 1	
	9	25	1.0	-	> 1	
OECD 111	4	60 ± 0.7	0.002	Not determinable, difenacoum is hydrolytically stable	–	Doc II-A[2] (A7.1.1.1.1/01)
	7	60 ± 0.7	0.02	Not determinable, difenacoum is hydrolytically stable	–	
	9	60 ± 0.7	0.2	Not determinable, difenacoum is hydrolytically stable	–	
OECD 111	7	50	0.05		> 1 year	Doc II-A[1](A.7.1.1.1.1)
	9	50	25.6		> 1 year	
EPA Guidelines (subdivision N, 161-1)	5	25 ±1	0.02		Not hydrolysed	Doc II-A[1] (A7.1.1.1.1/02)
	7	25 ±1	0.1		*ca.* 1000 days	
	9	25 ±1	0.1		*ca.* 80 days	

Photolysis in water

Difenacoum undergoes rapid phototransformation in water (half-life about 8 hours or less) (Table 6). In one of the two studies study (Doc II-A[1] (A7.1.1.1.2/01)), individual transformation products were formed less than 10% of the active substance added and the transformation products were not identified. In the other study (Doc II-A[3] (A7.1.1.1.2 (1)), two breakdown products above 10% were detected, but were not identified. Because the photodegradation is regarded as a minor removal process for difenacoum and the exposure to water is low no further characterization of metabolites was deemed necessary.

Table 6: Photolysis of difenacoum in water

Guideline/ Test method	**pH**	**Initial conc. (mg/l)**	**Total recovery of test substance (% of appl. a.s.)**	**Photolysis rate constant (K^c_p)**	**Direct photolysis sunlight rate constant (K_{pE})**	**Reaction quantum yield (ϕcE)**	**Half-life ($t_{½E}$)**	**Reference**
EPA: OPPS 835-2210 (Test site located at a latitude of 52°N)	not reported	1.55	-	4.98 d^{-1} (5 hours exposure)	13.1 d^{-1} (summer) 2.2 d^{-1} (winter) 10.2 d^{-1} (spring)		summer: 0.053 d (38 min.*) winter: 0.315 d (227 min.*) spring: 0.068 d (49 min.*)	Doc II-A[3] (A7.1.1.1.2 (1))
EPA Pesticide Assessment Guidelines, Subdivision N, Paragraph 161-2 (October 1982). *Acetonitrile used as a co-solvent.*	5	0.02	Mean 88%	0.21 h^{-1} *(cis: 0.18 h^{-1} trans: 0.37 h^{-1}*	Not determined	Not determined	3.26 h*(cis 3.87, trans 1.86)*	Doc II-A[1] (A7.1.1.1.2 /01) Doc II-A[2] (A7.1.1.1.2 /01)
	7	0.10	Mean 84%	0.09 h^{-1} *(cis: 0.07 h^{-1} trans: 0.29 h^{-1})/*	Not determined	Not determined	8.05 h *(cis 9.86, trans 2.35*	
	9	0.10	Mean 91%	0.09 h^{-1} (cis: 0.06 h^{-1} trans: 0.14 h^{-1})/	Not determined	Not determined	7.32 h (*cis 12.03, trans 5.03)*	

*assuming a 12 hour day.

Photodegradation in air

Difenacoum has the potential for rapid photo-oxidative degradation. Photodegradation characteristics of the active substance have been estimated using the EPIWIN v. 3.12 program. The indirect photolysis half-life of difenacoum with OH radicals is 2.08 hours and 2.015 hours with ozone (Table 7). Photodegradation is considered to be of limited relevance in view of the limited exposure to air, resulting from the extremely low volatility of difenacoum (vapour pressure 6.7 x 10^{-9} to 5.4 x 10^{-14} Pa at 25^{o}C).

Table 7: Photo-oxidation of difenacoum in air

Guideline / Test method	Initial molar TS concentration	Total recovery of TS (% of appl. a.s.)	OH radical rate constant (k_{OH})	Ozone rate constant (k_{Ozone})	Half-life, reaction with OH-radicals, $t_{½}$ (•OH)	Half-life, reaction with ozone, $t_{½}$ (Ozone)	Reference
SAR estimation	Not applicable	Not applicable	61.71 × 10^{-12} cm^3/molecule × s	13.65 × 10^{-17} cm^3/molecule × s	12-hour day, 1.5 10^6 × OH/cm^3: 0.173 d (≡ 2.08 h) 24-hour day, 5.0 × 10^5 OH/cm^3: 0.26 d (≡ 6.24 h)	0.084 d (≡2.015 h)	Doc II-A[2] (A7.3.1/01)

4.1.2 Biodegradation

4.1.2.1 Biodegradation estimation

Experimental data is available and therefore estimation is not needed.

4.1.2.2 Screening tests

The relevant biodegradation screening tests available for difenacoum include four ready biodegradability tests and one inherent biodegradability test (Table 8). The four ready biodegradability tests were performed according to OECD 301B, 301D, and 301F guidelines with CO_2 evolution, dissolved oxygen, and oxygen consumption as test parameters, respectively. Sludge from sewage treatment was used as inoculum. Initial test substance concentration was between 2 and 100 mg/l in the experiments. Incubation period was 28 days and the degree of degradation was between 0% and 31%, which is below the ready biodegradability pass levels of 60% or 70%.

An inherent biodegradation test was conducted according to an OECD 302D draft guideline (OECD 2001) with CO_2 evolution as test parameter and sewage sludge as inoculum. Inoculum was adapted for difenacoum. The degradation was 3% after 56 days incubation, indicating that difenacoum is not inherently biodegradable.

Table 8: Biodegradability of difenacoum

Guideline/ Test method	Test type	Test para-meter	Inoculum			Additional substrate	TS conc.	Degradation		Reference
			Type	Conc.	Adap-tation			Incub.-period (d)	Degree (%)	
OECD 301B	R	CO_2 evolution	Domestic sewage sludge	Not stated	No		18.2 mg/l	28	31	Doc II-A[3] (A7.1.1.2.1)
OECD 301 D	R	O_2 concentration	Activated sludge	3.12×10^4 cells/ml (average for all test vessels)	No	Toxicity control:, 1 mg/l benzoic acid	2 mg/l	28	9.7	Doc II-A[2] (A7.1.1.2.1/02
OECD 301D	R	Theoretical Oxygen Demand (ThOD)	Activated sludge	3 mg/l of supernatent after allowing solids to settle for at least 30 minutes.	No	None	2.34 mg/l.	28	0	Doc II-A[1] (A7.1.1.2.1/01)
OECD 301 F	R	O_2 consumption.	Activated sludge	Suspended solids concentration: 28.7 mg dry mass/ l	No	Toxicity control: 100 mg/l benzoic acid	100 mg/l (2.56 mg ThOD/mg difenacoum)	28	0	Doc II-A[2] (A7.1.1.2.1/03)
OECD 302D**	I	CO_2 evolution	Domestic sewage sludge	1g/l soil in mineral medium	Yes		21-25mg/l	56	3 max	Doc II-A[3] (A7.1.1.2.2)

***** R=ready biodegradability; I= inherent biodegradability
**Only a draft guideline is available (OECD 2001).

4.1.2.3 Simulation tests

Difenacoum degrades slowly under aerobic conditions in soil, with a measured DT50 of 439 (Table 9). Degradates were not characterized; however, it was shown that at the end of the 108-day incubation period degradates did not exceed 10% of the applied radioactivity.

Table 9: Aerobic degradation in soil

Guideline / Test method	Soil type	% Organic carbon	Application rate of test substance ([14C]-difenacoum)	Sampling time points	DT_{50}	Degradation products	Reference
BBA guidelines, Part IV, 4-1, December 1986.	Speyer 2.1; Speyer 2.2	1.4 (Speyer 2.1); 2.8 (Speyer 2.2)	0.2mg/$kg_{dry\ weight\ equivalent\ of\ soil}$	0, 2, 4, 8, 16, 64 and 108 days after application	results for Speyer 2.1 soil due to the very low recovery are not valid for determination of DT50-value. 439 days for Speyer 2.2 soil	<8% of applied dose at 108 days.	Doc II-A[1] (A7.2.1/01)

4.1.3 Summary and discussion of persistence

Difenacoum is not readily or inherently biodegradable. In the ready biodegradability tests according to OECD 301B, OECD 301D, and OECD 301F guidelines, level of degradation was 0-31%, being therefore below the ready biodegradability pass levels of 60 or 70%. In the inherent biodegradation test according to OECD 302D draft guideline, the degradation was 3%. Difenacoum degrades slowly in soil with DT50 of 439 days

Difenacoum does not hydrolyse at the environmentally relevant conditions. Difenacoum undergoes rapid phototransformation in water (half-life about 8 hours or less) and in air. However, phototransformation in air does not have high environmental significance due to the low volatility of difenacoum (see 4.2.2).

4.2 Environmental distribution

4.2.1 Adsorption/desorption

Difenacoum has a strong adsorption tendency. The QSAR Koc value of 1.8 x 10^6 (Doc II-A[1], Doc II-A[2]) was determined for difenacoum, because the experimentally derived Koc values were regarded as unreliable.

The experimental Koc (Doc II-A[1] (A7.1.3/01)) values were determined with the HPLC method and although the studies per se were regarded valid, the test method appeared to be unsuitable for difenacoum. The HPLC method (OECD 121) is not an actual study with measurements in real soil, but only an estimation based on the comparison of test substance to reference substances under artificial system, and hence there may be more uncertainties than in the adsorption/desorption batch-test (OECD 106). The experimentally derived Koc values were inversely related to pH, so that high values were obtained in acidic conditions (Koc of 426 579 at pH 3-4) and low values in neutral or alkaline conditions (17-165 at pH 7-8.5) (Doc II-A[1] (A7.1.3/01)). The experimentally derived Koc values are not supported by the physical and chemical properties of difenacoum. Difenacoum is a large aromatic molecule with two polar groups which can potentially ionize at environmental relevant pH. Difenacoum has also a low water solubility and a high log K_{ow}. The HLPC-method gives quite low Koc value suggesting that ionized form of difenacoum will not have great affinity to organic matter. Although difenacoum is a weak acid with probably two dissociable

sites, it might not be in ionized form with low adsorption in natural environment, or ionizable form might behave like a neutral form if the charge is shielded by the large molecule size. Also comparison to similar anticoagulant molecules supports the expert view that due to the intrinsic properties of these molecules the adsorption to particles is probable.

The low mobility of difenacoum in soil is further supported by experimental data (Table 10) showing that concentrations in leachate from column leaching studies conducted with both the active substance and the product were non-determinable.

Table 10: Leaching study conducted with the rodenticidal product containing 50 mg/kg difenacoum

Guideline / Test method	Endpoint / Type of test	Test Material/Exposure	Results	Remarks	Reference
BBA Guidelines, Merkblatt No 37 (1973). This study pre-dates the requirements of GLP. Soil columns were prepared using three soil types (Speyer 2.1, pH 7.6 and 2.2, pH 6.2 (coarse sand), Speyer 2.3m pH 7.5 (loamy coarse sand). The test material was added to the soil columns and 380ml deionised water (equivalent to 200mm rain) was applied to the top of each column.	Leaching of formulated product in soil.	Cereal-based pellet product containing a nominal 0.005% w/w difenacoum. The duration of the test was 48 hours.	Residues of difenacoum in the leachate were less than 0.006µg difenacoum/ml, which was the limit of determination.	The active substance difenacoum, formulated as wheat-based pellets, was not leached through 30cm of soil by 200mm rain.	Doc II-A[1] (B.7.1)

4.2.2 Volatilisation

Difenacoum is not expected to volatilise to air in significant quantities. The vapour pressure of difenacoum is very low (6.7 x 10-9 Pa) (Table 2). Henry's law constant 1.75 x 10^{-6} Pa m^3/mol (based on water solubility of 1.7 mg/l) indicates a very weak tendency for volatilization from an aqueous solution.

4.2.3 Distribution modelling

Not relevant for this type of dossier.

4.3 Bioaccumulation

4.3.1 Aquatic bioaccumulation

Difenacoum has a considerable bioaccumulation potential in aquatic organisms. However, there is no valid experimental bioaccumulation data available for aquatic organisms. One bioaccumulation test on Rainbow trout (*Onchorhynchus mykiss*) is available (Doc II-A[1](A7.4.3.3.1)) but is not considered acceptable due to lack of measured concentrations in water, absence of steady-state, and high mortality at the higher difenacoum concentration. Nevertheless the test indicated accumulation of difenacoum in fish. The conclusion in the biocide evaluation was that it is not technically possible to conduct a valid bioaccumulation test with difenacoum.

4.3.1.1 Bioaccumulation estimation

Bioaccumulation is assessed using an estimated log K_{ow} value of 7.6 (Table 2) (see Chapter 1.4 for derivation of low K_{ow}) because no valid experimental bioaccumulation data is available. The log K_{ow} of 7.6 indicates a potential to bioaccumulate.

4.3.1.2 Measured bioaccumulation data

No valid quantitative experimental bioaccumulation data was available (see 4.3.1). The available experimental bioaccumulation data nevertheless showed an indication of bioaccumulation of difenacoum in fish.

4.3.2 Terrestrial bioaccumulation

Not relevant for classification purpose.

4.3.3 Summary and discussion of bioaccumulation

Difenacoum has a potential to bioaccumulate. The estimated log K_{ow} of difenacoum (7.62) is above the cut-off values of log $K_{ow} \geq 4$ (CLP) and log $K_{ow} \geq 3$ (DSD).

4.4 Secondary poisoning

Not relevant for classification purpose.

5 HUMAN HEALTH HAZARD ASSESSMENT

Introduction to vitamin K function and mode of action of anticoagulant rodenticides

Anticoagulant rodenticides (AVKs) including warfarin are 4-hydroxycoumarin derivatives which act by inhibiting vitamin K recycling in the body. Vitamin K is needed for posttranslational modification of blood clotting proteins (FII, prothrombin; FVII, FIX, Christmas factor; FX, Stuart factor and proteins S and C; Brenner et al. 2009). Vitamin K deficiency impairs normal blood clotting mechanism by preventing the carboxylation of essential glutamic acid residues in several blood-clotting proteins. The AVKs thereby increase bleeding tendency and eventually induce profuse hemorrhages and death.

Vitamin K functions as a coenzyme in carboxylation of glutamate residues into γ-carboxyglutamate (Gla) which are essential for procoagulant activity of these coagulating factors. Vitamin K is the group name for a number of related compounds (incl. subtypes), K_1, K_2 and K_3 being the basic forms which all can serve as essential nutrients. Vitamin K from dietary sources must first be enzymatically activated before it can act as a cofactor. The activation is carried out by vitamin K epoxide reductase (VKOR). The resulting vitamin K hydroquinone (KH_2) is the active coenzyme, and its oxidation to vitamin K 2,3-epoxide (KO) provides the energy for the carboxylation reaction. The epoxide (KO) is then recycled back to its reduced form in two reduction steps mediated by the vitamin K epoxide reductase (VKOR) (Figure 1A).

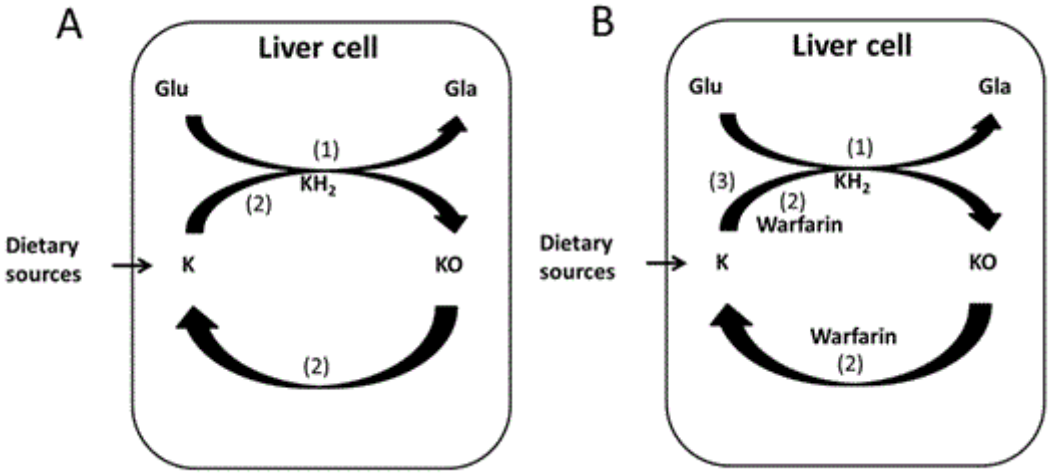


Figure 1: Vitamin K cycle in the liver cell in the absence (A) or presence (B) of warfarin

Vitamin K from dietary sources is converted to vitamin K hydroquinone (KH2) by vitamin K epoxide reductase (VKOR) (2). The carboxylation of glutamate residues (Glu) to into γ-carboxyglutamate (Gla) in vitamin K-dependent proteins is carried out by γ-glutamyl carboxylase (1) in a KH2 -dependent manner. KH2 is concomitantly oxidized to KO which in turn undergoes reductive recycling first to K and then to KH2. Warfarin inhibits the activity of VKOR leading to buildup of KO. In liver cells NAD(P)H-dependent quinone reductase (3) can bypass the inhibitory action of warfarin and thus provide the KH2 substrate for the carboxylase enzyme (1). However, it cannot convert the epoxide KO back to K. (Modified from Shearer and Newman, 2008).

Vitamin K epoxide reductase (VKOR) is the target enzyme for coumarin anticoagulants. The blocking of the VKOR leads to rapid exhaustion of the supply of vitamin K hydroquinone (KH_2), and thus to a prevention of the formation of Gla coagulation factor precursors in the liver. The mechanism of causing vitamin K hydroquinone (KH_2) deficiency in other tissues is based on the same enzyme inhibition. Vitamin K hydroquinone (KH_2) is also an essential cofactor in the posttranslational γ-carboxylation of glutamic acid residues of other vitamin K dependent proteins, e.g., in cartilage, bone and nervous systems. Vitamin K -dependent proteins in bone metabolism include osteocalcin and matrix Gla protein (MPG). Their primary function is to prevent overcalcification of the bone and cartilage, and defects in these proteins can lead to early calcification of the cartilage and thus can cause reduced or abnormal growth of cartilage and subsequent abnormal bone development.

In the liver, there is an alternative pathway for vitamin K reduction that may be induced by high levels of vitamin K quinone (dietary vitamin K). A hepatic (NAD(P)H-dependent quinone reductase(s) insensitive to warfarin action can bypass the inhibition of VKOR to provide KH_2 and thus overcome the inhibitory action in the liver (Figure 1B). However, this enzyme is not able to convert KO back to K and therefore continuous vitamin K administration is needed. Thus, dietary vitamin K supplementation allows maintaining a blood coagulation system. However, extrahepatic vitamin K deficiency cannot be compensated and therefore administration of vitamin K can overcome warfarin antagonism only in the liver but not e.g. in bone (Shearer and Newman 2008).

RAC general comment
Difenacoum belongs to a group of compounds known as anticoagulant rodenticides, i.e. those with an anti-vitamin K mode of action (sometimes abbreviated to AVK) which are used mainly as active substances in biocidal products for pest control of rats, mice and other rodents. Some of the substances had an existing harmonised classification. However, only Warfarin is currently classified for toxicity to reproduction in category 1A. The eight substances were previously discussed by the Technical Committee on Classification and Labelling of Dangerous Substances (TC C&L) of the European Chemicals Bureau (ECB) (2006 – 2008). However, the work was referred to be continued at ECHA and to that end Member State Competent Authorities (MSCAs) were requested to prepare CLH proposals. CLH proposals for eight AVK rodenticides, Coumatetralyl (Denmark), Difenacoum (Finland), Warfarin (Ireland), Brodifacoum (Italy), Flocoumafen (The Netherlands), Difethialone (Norway) Chlorophacinone (Spain) and Bromodialone (Sweden), were submitted by eight different Dossier Submitters (DS). The dossiers were handled as a group but the Committee for Risk Assessment (RAC) proceeded to evaluate the proposals on a substance by substance basis comparing the human data available for Warfarin (and other AVKs) and relying on a weight-of–evidence approach as required by Regulation 1272/2008 (CLP). Endpoints for which no classification was proposed by the dossier submitter have not been assessed by RAC.

5.1 Toxicokinetics (absorption, distribution, metabolism and elimination)

Absorption

Oral

Orally administered difenacoum is rapidly absorbed from the gastrointestinal tract, as shown by peak blood concentrations occurring approx. 4–24 hours after dosing. Absorption after oral intake of a single dose ranges between 68% and 82%. No major differences were found in absorption between males and females.

Inhalation

No data available.

Dermal

According to *in vitro* studies with human skin, difenacoum is not effectively absorbed through skin. Absorption within 24h was below the analytical limit of quantitation (<1.34% of applied dose for absorbed dose) in studies where 0.5 % w/w difenacoum liquid concentrate or 50 ppm difenacoum pellet baits were used as test material. On the basis of these studies, total absorption of <2.23% of the applied dose could be estimated. For risk characterization 3% dermal absorption was used for pellets and grains. According to another *in vitro* study with human skin, the dermal absorption of difenacoum from wax block bait containing 0.005% difenacoum was 0.047% of the applied dose during 24 h after 8 h exposure.

Distribution

Difenacoum is widely distributed in the tissues both after single and repeated doses. The main site of accumulation is the liver, the target organ. The concentration in the liver ranges between approx. 20 % and 40 % of the administered dose. The next highest concentrations have been found in pancreas or skeletal muscle depending on the study. Minor quantities are found in other tissues throughout the body. After repeated dosing, the concentrations in tissues have been found to be 3 – 10 fold higher than after single dosing, i.e., accumulation in the organism takes place.

The concentration in fat after exposure is relatively low indicating that although difenacoum is highly lipophilic, that property does not significantly affect tissue distribution because difenacoum has a high affinity for specific binding sites in tissues such as the liver.

Metabolism

Difenacoum is rapidly and extensively metabolised in rats. In one study, 60 % of the radioactivity in the faeces was present in the form of metabolites. The faecal metabolites have been found to account for 21% to 39% of the administered dose (range from different dossiers). Four major metabolites have been found in faeces and 2 to 5 in liver. In rats, metabolites have been identified as hydroxylated difenacoums and glucuronide conjugates. Metabolites in the liver have been found to account for 35–53 % of the radioactivity within one day. Unchanged difenacoum accounted for 42 % of the activity in liver after one day.

The metabolism of difenacoum is postulated to occur mainly by glucuronidation of the 4-hydroxy group of the coumarin ring and also by hydroxylation of the aromatic rings. The presence of the 4-hydroxy coumarin moiety and a highly lipophilic side chain are the key requirements for potent anticoagulant activity.

Elimination

Elimination from the body is slow. The main elimination route in rats is faeces, urine being only a minor route. During seven days after dosing, 37% to 55% is eliminated in faeces and approx. 2% in urine. During a five-day sampling, elimination half-lives of 31 to 55 hours were detected depending on dose level. Difenacoum is partly excreted in faeces as metabolites (around 60% of the eliminated radioactivity). Neither the extent of faecal excretion nor the proportion of metabolites in faeces is dose dependent. Exhalation of the test substance as CO_2 is insignificant.

Elimination from tissues is biphasic. An initial rapid phase during the first few days after dosing is followed by a very slow phase. The halflife of elimination of radioactivity during the rapid phase (days 1-8) was 3 days, while for the slower phase (days 28-182) it was 118 days. The slow elimination from tissues is consistent with the slow faecal elimination found in excretion studies.

5.2 Acute toxicity

5.2.1 Acute toxicity: oral

Table 11: Summary of oral acute toxicity studies

Method **Guideline**	**Species, strain, sex, no./group**	**Dose levels** **duration of exposure**	**Value** **LD50/LC50**	**Remarks**	**References**
OECD Guideline No. 401. GLP	Rat, Wistar Male: 5 (five doses)	1.20; 1.47; 1.80; 2.20; 2.69 mg/kg bw Acute exposure; 14 d post-exposure period	1.8 mg/kg bw (95% conf. limits 1.5-2.1) No. of deaths: 0/5, 2/5, 2/5, 4/5, 5/5 on days 5-8 No signs of toxicity until day 5		Doc II-A[1], 3.2 (A6.1.1/01) Key study
OECD Guideline No. 401. GLP	Rat, Sprague Dawley Female: 5 (four doses) Male: 5 (one dose)	1.4; 1.8; 2.33; 3.0 mg/kg bw (females); 2.5 mg/kg bw (males) Acute exposure; 14 d post-exposure period	2.6 mg/kg bw (95% conf. limits 2.1-3.7) No. of deaths 0/5, 0/5, 1/5, 4/5 on days 5-7 No deaths in males No overt signs of toxicity until day 5		Doc II-A[1], 3.2 (A6.1.1/02)
OECD Guideline No. 401. GLP	Rat, Sprague Dawley Male: 5 (three doses) Female: 5 (three doses)	cis: (0); 0.75; 1.25; 2.0 mg/kg bw trans: (0); 4.0; 8.0; 12.0 mg/kg bw Acute exposure; 14 d post-exposure period	cis: 1.2 mg/kg bw, male No. of deaths 0/5, 3/5, 5/5 1.6 mg/kg bw, female No. of deaths 0/5, 0/5, 5/5 trans: 7.3 mg/kg bw, male No. of deaths 0/5, 3/5, 5/5 6.0 mg/kg bw, female No. of deaths 0/5, 5/5, 5/5	LD50s for females are estimations	Doc II-A[1], 3.2 (A6.1.1/04)

			Deaths on days 4-9		
OECD Guideline No. 423 (ATC method) GLP	Rat, Sprague Dawley Male: 3 (two doses) Female: 3 (two doses)	5; 25 mg/kg bw Observation time: 14 d	20.5 mg/kg bw, male No. of deaths 0/3, 2/3 32.5 mg/kg bw, female No. of deaths 0/3, 1/3 Combined: 25 mg/kg bw Deaths on days 4-14		Doc II-A[2], 3.2 (A6.1.1/01)
OECD Guideline No. 423 GLP	Rat, Wistar Female: 3 (three groups; two low dose groups)	5; 50 mg/kg bw Observation time: 14 d	Between 5 and 50 mg/kg bw No. of deaths: 0/6, 3/3 Deaths on days 5-8		Doc II-A[3], 3.2 (A6.1.1)
OECD Guideline No. 401. GLP	Mouse, MFI Male: 5 (three doses) Female: 5 (three doses)	cis: (0); 0.30; 0.60; 0.90 mg/kg bw (males); (0); 0.50; 1.50; 2.50 mg/kg bw (females) trans: (0); 1.25; 1.75; 2.25 mg/kg bw (males); (0); 2.0; 4.0; 6.0 mg/kg bw (females) Acute exposure 14 d post-exposure period	cis: 0.45 mg/kg bw, male No. of deaths 0/5, 5/5, 5/5 1.0 mg/kg bw, female No. of deaths 0/5, 5/5, 5/5 trans: 1.2 mg/kg bw, male No. of deaths 3/5, 4/5, 5/5 2.8 mg/kg bw, female No. of deaths 1/5, 5/5, 5/5 Deaths on days 4-11	Only LD50 for trans isomer in females is a calculated value	Doc II-A[1], 3.2 (A6.1.1/04)
No guideline study given, but study conducted in accordance with the scientific principles accepted at the time (1973). Non-GLP	Mouse, LAC Male: 10 (seven doses) At the top dose 5 animals	0.2; 0.5; 1; 2; 5; 10; 20 mg/kg bw Acute exposure; 21 d post-exposure period	0.8 mg/kg bw No. of deaths 1/10, 5/10, 6/10, 8/10, 9/10, 10/10, 5/5 Deaths on days 3-10	Reliability 3 This study can be used only as supplementary data	Doc II-A[1], 3.2 (A6.1.1/03)

The LD_{50} values determined for difenacoum range from 1.8 to < 50 mg/kg bw for the rat and are around 1 mg/kg bw for the mouse. Deaths resulted from profuse haemorrhage due to anticoagulation at 3-14 days after ingestion of the dose. A study with separate *cis* and *trans* isomers of difenacoum revealed that the *cis* isomer is somewhat more toxic (approx. 2-5 times depending on species) than the *trans* isomer (Doc II-A[1], 3.2 (A6.1.1/04)).

5.2.2 Acute toxicity: inhalation

Table 12: Summary of acute toxicity studies via inhalation

Method Guideline	Species, strain, sex, no./group	Dose levels duration of exposure	Value LD_{50}/LC_{50}	Remarks	References

OECD Guideline No. 403 GLP	Rat, Sprague Dawley 4 groups of 10 animals (5 males and 5 females in each group)	0; 3.65; 5.85; 10.14 µg/l 4 hour exposure; 14 day post-exposure period Particle size: MMAD 0.55 to 0.70 µm Aerosol Vehicle: acetone	Between dose levels 3.65 and 5.85 µg/l/4h Not calculable due to the nature of the dose-response curve No deaths in the low dose group and only one survivor among the rest of animals. Deaths on days 4-7		Doc II-A[1], 3.2 (A6.1.3/01) Key study
OECD Guideline No. 403 GLP	Rat, strain not stated Male: 5 (3 doses) Female 5 (3 doses)	3.28; 7.52; 20.33 µg/l/4h 4 hour exposure; 14 day post-exposure period Particle size: MMAD 0.78, 0.86 and 0.89 µm + GSD 2.74, 2.41 and 3.15 µm Aerosol Vehicle: acetone	Males: 20.74µg/l/4h (95% confidence limits 12.03-39.76) Females:16.27 µg/l/4h (95% confidence limits 10.03-26.24) No. of deaths: 0/5, 0/5, 2/5 (males) and 0/5, 0/5, 4/5 (females) Killed in extremis on days 4-6		Doc II-A[3], 3.2 (A6.1.3)

MMAD: mass median aerodynamic diameter
GSD: geometric standard deviation

The LC_{50} values obtained in the rat were 3.65-5.85 µg/l/4 h in one study (Doc II-A[1], 3.2 (A6.1.3/01)) and 16-21 µg/l/4 h in another study (Doc II-A[3], 3.2 (A6.1.3). The test substance was dosed as an aerosol in acetone. The mass median aerodynamic diameter (MMAD) of the aerosol droplets was less than 1 µm in both available studies; this is well within the respirable range of the rat (up to 5 µm).

5.2.3 Acute toxicity: dermal

Table 13: Summary of dermal acute toxicity studies

Method Guideline	**Species, strain, sex, no./group**	**Dose levels duration of exposure**	**Value LD_{50}/LC_{50}**	**Remarks**	**References**
OECD Guideline No. 402. GLP	Rat, Sprague Dawley Female: 5 (four doses) Male: 5 (one dose)	49; 60; 73; 90 mg/kg bw (females); 60 mg/kg bw (males) 24 hour exposure Post-exposure period: 14 days	63 (95% conf. limits 34-85) mg/kg bw No. of deaths Females: 2/5, 1/5, 3/5, 5/5 Males: 2/5 All deaths on days 6-12 No signs of toxicity in any animal on days 1-5	Powder on moistened skin Males not found to be more sensitive than females	Doc II-A[1], 3.2 (A6.1.2/01)

OECD Guideline No. 402 GLP	Rat, Sprague Dawley Female: 5 (2 groups) Male: 5 (2 groups)	5; 25 mg/kg bw 24 hour exposure Post-exposure period: 14 days	< 5 mg/kg bw Death of all animals on days 5-14	Administration of test substance in sesame oil.	Doc II-A[2], 3.2 (A6.1.2/01) Key study
OECD Guideline No. 402 GLP	Rat, Wistar Female: 5 (three doses) Male: 5 (one dose)	20; 55; 155 mg/kg bw (females); 20 mg/kg bw (males) 24 hour exposure Post-exposure period: 21 days (females); 15 days (males)	Female: 52 mg/kg bw No. of deaths Females: 0/5, 3/5, 5/5 Males: 2/5 All deaths on days 6-14	The test substance was used undiluted with no vehicle Males proved to be more sensitive than females	Doc II-A[3], 3.2 (A6.1.2)

The LD_{50} values obtained in the three studies available with rats were 63 mg/kg bw (95% confidence limits 34-85 mg/kg bw), 52 mg/kg bw and <5 mg/kg bw. One of these values is well below the threshold value for classification as very toxic according to Directive 67/548/EEC and as Acute Tox. Category 1 according to Regulation EC 1272/2008 (threshold in both legislations ≤ 50 mg/kg) while the other two are around the threshold value. The difference between the obtained LD_{50} values may, to some extent, be explained by the fact that difenacoum was applied in a sesam oil matrix in the study that yielded the lowest LD_{50} value whereas pure powder was used in the other tests.

5.2.4 Acute toxicity: other routes

Not applicable.

5.2.5 Summary and discussion of acute toxicity

Difenacoum is acutely toxic by the oral and inhalation routes. Furthermore, it is justified to consider difenacoum toxic also by the dermal route due to overall mortality in one study and because the lower confidence limit of the result of the acute toxicity test is below the threshold for classification even in the study yielding the highest LD_{50} value.

Comparison with classification criteria

LD_{50} values for difenacoum ranged from 1.8 mg/kg to < 50 mg/kg in rat and 0.8 mg/kg in mouse. Difenacoum is currently classified as T+, R28 according to Directive 67/548/EEC where the limit for classification as very toxic via oral route is ≤ 25 mg/kg, therefore no change in classification is proposed. The classification as Acute Tox. 2(*); H300 according to the CLP Regulation is the minimum classification arising from the translation of the classification in Annex I to Directive 67/548/EEC. Due to the different classification criteria between DSD and CLP, it is proposed to re-classify difenacoum in a more severe category according to CLP where the limit for classification as Acute Tox. 1 via oral route is ≤ 5 mg/kg. Therefore classification of difenacoum as Acute Tox. 1; H300 is warranted.

The acute inhalation LC_{50} values ranged between 3.65-5.85 µg/L/4 h. The limit for classification as Acute Tox.1 via inhalation route is ≤ 0.05 mg/l/4h for dusts and mists under CLP Regulation, therefore classification of difenacoum as Acute Tox. 1; H330 is proposed. The limit for classification as very toxic via inhalation route is ≤ 0.25 mg/l/4h for aerosols under Directive 67/548/EEC, therefore classification of difenacoum as T+; R26 is proposed.

The LD_{50} value for acute dermal toxicity was < 5 mg/kg bw. This LD_{50} value is < 50 mg/kg which is the limit for classification as Acute Tox. 1 under CLP Regulation, and limit for classification as very toxic under Directive 67/548/EEC via the dermal route. Therefore classification of difenacoum as Acute Tox. 1; H310 is proposed under the CLP Regulation and as T+; R27 under Directive 67/548/EEC.

Specific concentration limits

Specific concentration limits (SCLs) suggested according to Directive 67/548/EEC:

C ≥ 0.25%	T+; R26/27/28
0.025% ≤ C< 0.25%	T; R23/24/25
0.0025% ≤ C < 0.025%	Xn; R20/21/22

Basis for calculations:

Rat acute oral LD_{50} = 1.8 mg/kg/bw (T+; R28). Cut off value for R28 is 25 mg/kg; 25/1.8 = 13.9 . The general conc. limit is 7% therefore SCL is 7/13.9 = 0.5% for R28. For R25 = 0.07%; for R22 = 0.007%

Rat acute dermal LD_{50} = 5 mg/kg bw (T+; R27). Cut off value for R27 is 50 mg/kg. SCL for R27 is 0.7 %; for R24= 0.1%; for R21= 0.01%

Rat acute inhalation LC_{50} between 0.0036 mg/l/4h and 0.0058 mg/l/4h. Cut off for R26 is 0.25 mg/l/4h. The SCL for R26 is 0.1%-0.2%; for R23 0.01%-0.03%; for R20 0.001-0.003%

To avoid too many different SCL it was decided that the SCLs should be set 1-2 orders of magnitude lower than the GCLs for all three acute toxicity endpoints. The set SCLs reflect the concensus proposal.

These SCLs for acute toxicity are proposed as agreed y TC C&L (Technical Committee on Classification and Labelling) in May 2007 (Follow-up V, May 2008).

Specific concentration limits are not applicable for acute toxicity under the CLP Regulation.

Conclusions on classification

- DSD: Difenacoum is currently classified as T+; R28. It is now proposed to classify it also as T+; R26 and T+; R27. The resulting classification would be **T+: R26/27/28** (Very toxic by inhalation, in contact with skin and if swallowed).

Proposed SCLs:

$C \geq 0.25\%$: T+; R26/27/28

$0.025\% \leq C < 0.25\%$: T; R23/24/25

$0.0025\% \leq C < 0.025\%$: Xn; R20/21/22

- CLP: Difenacoum is currently classified as Acute Tox. 2(*); H300. It is now proposed to re-classify it in a more severe hazard category via oral route, as Acute Tox. 1; H300 (Fatal if swallowed). Classifications also via dermal and inhalation routes are proposed in the most severe hazard categories. The resulting classifications would be **Acute Tox. 1; H300** (Fatal if swallowed), **Acute Tox. 1; H330** (Fatal if inhaled) and **Acute Tox. 1; H310** (Fatal in contact with skin).

In this context it shall be noted that due to the legal text in the CLP Regulation, no SCLs are set for acute toxicity for any substance. Regarding the classification and labelling of mixtures, different outcome might be reached under the DPD and CLP. This is a matter of different methods used in classification of mixtures for acute toxicity under these legislations. A practical consequence of this is that the hazard communication may in some cases be hampered under the CLP Regulation even if there was a need to pass information on hazard due to a highly toxic ingredient in a mixture.

RAC evaluation of acute toxicity

Summary of the Dossier submitter's proposal

Difenacoum is currently classified for acute oral toxicity as Acute Tox. 2* (H300) according to CLP. Due to the different classification criteria between DSD and CLP and based on the available data on Difenacoum, it is now proposed to remove the minimum classification (*) and update the CLP classification for acute oral toxicity 1; H300.

The dossier submitter (DS) also proposed additional classifications for acute toxicity via other routes of exposure i.e. acute inhalation toxicity 1; H330 and acute dermal toxicity 1; H310.

Comments received during public consultation

Three Member States supported the proposed classification.

Assessment and comparison with the classification criteria

Acute toxicity: oral

There are five studies in rats and two in mice performed according to OECD Test Guideline (TG) 401 except for one of the two studies in mice.

The LD_{50} values ranged from 1.8 mg/kg bw to < 50 mg/kg bw in rats and are around 1 mg/kg bw for mice. Deaths resulted from haemorrhages due to anticoagulation occurring at 3-14 days after ingestion of the dose. A study with separate *cis* and *trans* isomers of Difenacoum revealed that the *cis* isomer is somewhat more toxic (approx. 2-5 times depending on species) than the *trans* isomer.

In two studies that were performed according to OECD TG 401 the LD_{50} values in rats were 1.8 mg/kg bw and 2.6 mg/kg bw. These values fall within the criteria for classification for acute toxicity 1; H300 (CLP criterion; $LD_{50} \leq 5$ mg/kg bw).

RAC agreed with the DS that Difenacoum should be classified via oral route as acute toxicity 1, H300.

<u>Acute toxicity: inhalation</u>

In two studies performed according to OECD TG 403, the acute inhalation LC_{50} values in

rats ranged between 4-6 µg/L/4hr and 16-21 µg/L/4hr. The limit for classification for acute toxicity 1 via inhalation route is ≤ 0.05 mg/L/4hr for dusts and mists, therefore RAC agreed with the DS that Difenacoum should be classified for acute toxicity 1; H330.

Acute toxicity: dermal
Three studies in rats are available, performed according to OECD TG 402. In one of these studies the LD_{50} values are well below the threshold value for classification for acute toxicity 1, H310 under CLP Regulation. Besides all animals died on days 5-14.

In the other two studies the values are around the threshold value which is ≤ 50 mg/kg bw. In one of these studies Difenacoum was applied on moistened skin and in the other one the substance was used undiluted with no vehicle. Corresponding to OECD TG 402 the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. RAC is of the opinion that the difference between the obtained LD_{50} values can be explained definitely by the fact that Difenacoum was applied in a sesame oil matrix in the study that yielded the lowest LD_{50} values whereas pure powder was used in the other studies.

Consequently RAC agreed with the DS that Difenacoum should be classified as acute toxicity 1; H310 via the dermal route.

In conclusion, RAC supported the classification for acute toxicity 1 for all three routes of exposure.

5.3 Irritation

No classification is included for this hazard class in Annex VI, Tables 3.1 and 3.2, CLP Regulation and no classification is currently proposed as also agreed by the TC C&L in 2006/2007 and based on information in CAR.

5.4 Corrosivity

No classification is included for this hazard class in Annex VI, Tables 3.1 and 3.2, CLP Regulation and no classification is currently proposed as also agreed by the TC C&L in 2006/2007 and based on information in CAR.

5.5 Sensitisation

No classification is included for this hazard class in Annex VI, Tables 3.1 and 3.2, CLP Regulation and no classification is currently proposed as also agreed by the TC C&L in 2006/2007 and based on information in CAR.

5.6 Repeated dose toxicity

5.6.1 Repeated dose toxicity: oral

Table 14: Summary of oral repeated dose toxicity studies

Route Guideline	Duration of study	Species, strain, sex, no./group	Dose levels, frequency of application	Results	LO(A)EL	NO(A)EL	References
Oral (dietary) OECD Guideline No. 408 GLP	90 d	Rat, Sprague Dawley 10 males, 10 females per group	0; 0.03; 0.1; 0.3/0.2 mg/kg bw/day, daily The highdose reduced on day 20 onwards Coagulation parameters were measured at the end of the study	Low dose: no significant effects Medium dose: 2/10 males died; increase of TT and PTT in males and females, signs of toxicity High dose: 7/10 males and 4/10 females died; increase of TT and PTT in females, signs of toxicity	0.1 mg/kg bw/day	NOAEL 0.03 mg/kg bw/day	Doc II-A[2], 3.5 (A6.4.1/01) Key study
Oral (gavage) OECD Guideline No. 408 non-GLP (Individual data not available due to shut up of the testing facility)	90 d	Rat, Wistar 8 males, 8 females per group	0; 0.01; 0.02; 0.03; 0.06 mg/kg bw/day	No clinical signs of toxicity at any dose. Slight increase in KCT at 0.06 mg/kg bw/day, apparently in both sexes. In histology, sporadic cases of haemorrhage observed in various organs at all dose levels, with no dose-response. A somewhat higher dose range would have been desirable to induce clearer toxic effects. Reliability 3 due deficiencies in performance and reporting of the study. Can be used only as supplementary data.	-	A suggestive NOAEL is 0.03 mg/kg bw/day for both sexes, based on increased KCT and supported by histological findings	Doc II-A[3], 3.5 (A 6.4.1) Supportive study
Oral (dietary) OECD Guideline No. 408 Rangefinding study GLP	28 d	Rat, Sprague Dawley 5 males, 5 females per group	0; 0.01; 0.03/0.3; 0.1/1 mg/kg bw/day Increased doses from day 20 onwards Coagulation parameters were measured on d 16 and d 28	Low dose: no effects Medium dose: increase of TT and PTT High dose: 8/10 animals died, increase of TT and PTT	Not feasible to set a value due to change of doses during the study	NOAEL 0.1 mg/kg bw/day	Doc II-A[2], 3.5 (A6.3.1/01) Supportive study
Oral (Via gelatin capsules) Guideline not quoted but study conducted	6 weeks	Dog, Beagle 1 male and 1 female in each dose group	0; 0.01; 0.025; 0.05; 0.1; 0.2 mg/kg bw/day 5 or 7 days per week not stated Coagulation parameters	Low dose: 0.01 mg/kg bw/day: changes in clotting times Other doses: 0.025, 0.05, 0.1 and 0.2 mg/kg bw/day: the animals were killed for humane reasons in Weeks 3, 2, 2 or 1 respectively, when	LOAEL 0.01 mg/kg bw/day for the effect on blood coagulati		Doc II-A[1], 3.5 (A6.4.1/01) Supportive study

following principles of OECD Guideline No. 409 Range finding study Non-GLP			were measured weekly	PT exceeded the set criteria and/or because of clinical evidence of internal haemorrhage	on		

KCT: kaolin-cephalin time
PT: prothrombin time
PTT: partial thromboplastin time
TT: thrombin clotting time

Repeated oral administration of difenacoum to rats resulted in marked increase in clotting time and haemorrhage in a wide range of tissues, with treatment related deaths due to massive haemorrhaging.

In a key study (Doc II-A[2], 3.5 (A6.4.1/01)), feeding rats at a dietary dose of up to 0.2 mg/kg bw/day for 90 days gave rise to clinical, haematological, biochemical and pathological findings indicative of toxic effects related to anticoagulation. No other adverse effects were observed. The lowest dose used causing treatment related deaths was 0.1 mg/kg bw/day. The NOAEL value could be established at 0.03 mg/kg bw/day.

In another 90 day rat study a slight increase in KCT was seen at dose 0.06 mg/kg however no clinical signs of toxicity was seen (Doc II-A[3],3.5 (A 6.4.1)). In histopathological examination haemorrhages were observed in various organs however with no obvious dose-response relationship. This study is considered supportive.

In a 28 day rat range finding study (Doc II-A[2],3.5 (A6.3.1/01)) a dose-related increase in TT and PTT was observed at dose 0.3 mg/kg and statistically significantly at dose 1.0 mg/kg, measured on test day 28. NOAEL was 0.1 mg/kg. This study is acceptable as a supportive range finding study but not as a stand-alone 28 day study.

A range finding 90 day dog study (Doc II-A[1],3.5 (A6.4.1/01)) only lasted for 42 days due to premature sacrification of the animals when the coagulation times increased to 40/100 seconds. However clear dose-dependency was seen in the prolonged PT and KCT-values in both male and female dogs. Prolonged PT and KCT were also time-dependent effects, and were observed at the lowest administered dose 0.01 mg/kg which caused prolongation of PT and KCT from day 30. This time-dependence of the increase in PT and KCT is probably a sign of the effect of accumulation of difenacoum. A LOAEL of 0.01 mg/kg was obtained based on prolonged PT and KCT. The study is accepted as supportive information regarding clinical and haematological effects on blood coagulation. However, the only shortcoming of this study is the low number of experimental animals (2 dogs/dose).

5.6.2 Repeated dose toxicity: inhalation

Repeated dose studies are available *via* oral route only. However, due to similar effects seen in acute oral, dermal and inhalation toxicity studies, it is considered justified to conclude that

difenacoum causes a similar concern for serious damage to health by prolonged exposure also via dermal and inhalation routes**.**. Thus, based on the results of the acute dermal and inhalation toxicity studies and route-to-route extrapolation, classification as T, R48/23/24/25 according to Directive 67/548/EEC is therefore justified. According to Regulation EC 1272/2008, difenacoum is currently classified as a specific target organ toxicant in category 1 irrespective of the route of application; no change in this classification is proposed.

5.6.3 Repeated dose toxicity: dermal

See section 5.6.2.

5.6.4 Other relevant information

There is a rabbit 22 day reproductive toxicity study on difenacoum where some coagulation parameters have been measured (Doc II-A[3], 3.8.1 (A6.8.1 (2))). Based on the increased maternal PT and PTT, a LOAEL of 0.01 mg/kg can be derived. In a similar rabbit 13 day reproductive toxicity study a maternal LOAL of 0.015 mg/kg is derived based on increased PT and KCT (Doc II-A[1],3.8.1 (A6.8.1/02)).

These LOAEL values are considered relevant when setting specific concentration limits for repeated dose toxicity. Since the effects on coagulation parameters (PT and KCT) are manifested already at an early stage of treatment with difenacoum, the total length of the study does not play a significant role in this case.

Studies on pregnant women and pregnant rats have shown that the blood coagulation parameter prothrombin time (PT) is unaffected or slightly decreased during pregnancy even if other changes in blood clotting and hemodynamics may take place (De Rijk et al. 2002; Honda et al. 2008; Urasoko et al. 2009; Hui et al. 2012). In one report it is claimed that PT is increased during rat pregnancy however the increase was slight (from 16.1 to 19.0 s) and took place between gestation days 12-15 whereafter it declined to 15.2 s at day 20 (Papworth and Clubb 1995). In rabbit, PT time has been reported to increase during pregnancy, however the increase was very slight and a matter of milliseconds, from 6.4 ± 0.1 s (non-pregnant) to 6.7 ± 0.3 s (pregnant) at GD13 (Mizoguchi et al. 2010).

No changes in PT values during the course of pregnancy were seen in the pregnant control rabbits or pregnant control rats in the reproductive toxicity studies reviewed in this CLH report, however the PT was significantly increased in difenacoum- treated pregnant rabbits. Therefore, we consider it relevant to use the derived LOAEL values for PT from reproductive toxicity studies on rabbit in setting specific concentration limit for repeated dose toxicity.

5.6.5 Summary and discussion of repeated dose toxicity

Difenacoum is currently classified as R48/25 "Toxic: danger of serious damage to health by prolonged exposure if swallowed" according to Directive 67/548/EEC. Due to similar effects seen in acute oral, dermal and inhalation toxicity studies, a route-to-route extrapolation is justified and classification as very toxic *via* all the three routes for repeated dose toxicity is considered justified. Classification as T; R48/23/24/25 “Toxic: Danger of serious damage to health by prolonged exposure through inhalation, in contact with skin and if swallowed” according to Directive 67/548/EEC was agreed by the TC C&L in November 2006 (Follow-up V, May 2007).

According to Regulation EC 1272/2008, difenacoum is currently classified as a Category 1 specific target organ toxicant in repeated exposure (STOT RE 1) with hazard statement H372** "Causes damage to organs through prolonged or repeated exposure" irrespective of the route of application. The two asterisks were given because the existing classification was translated from Directive 67/548/EEC with a general hazard statement not specifying the route of exposure as the necessary information was not available. It is now proposed that the asterisks be removed from the hazard statement H372 because no route of exposure can be excluded on the basis of current data.

Comparison with classification criteria

The oral LOAEL obtained from the 90 day repeated dose toxicity study in rat (0.1 mg/kg bw/day) is 100 times lower than the limit of 10 mg/kg bw /day for STOT RE 1 according to the CLP Regulation and 50 times lower than the limit of 5 mg/kg bw/day for classification for T; R48/25 according to Directive 67/548/EEC.

The mechanism of toxicity (interfering with the recycling of vitamin K) is unaffected by route of exposure. Due to similar effects seen in acute oral, dermal and inhalation toxicity studies, a route-to-route extrapolation is justified and classification via all the three routes for repeated dose toxicity is considered justified.

Specific concentration limits

Specific concentration limits (SCLs) for repeated dose toxicity based on rat data LOAEL of 0.1 mg/kg according to Directive 67/548/EEC were agreed by TC C&L (Technical Committee on Classification and Labelling) in May 2007 (Follow-up V, May 2008) as follows:

$C \geq 0.25\%$: T; R48/23/24/25

$0.025\% \leq C < 0.25\%$: Xn; R48/20/21/22

However, we are of the opinion that the rat LOAEL-value should not be used to derive the SCL because it seems that in the studies on difenacoum, rat has not been the most sensitive species in terms of blood clotting parameters. Instead, a rabbit LOAEL of 0.01 mg/kg based on maternal toxicity in a reproductive toxicity study and a dog LOAEL of 0.01 mg/kg based on repeated dose toxicity study should be the basis to derive the SCLs. Grounds for this are explained in the following.

In the 90 day rat repeated dose toxicity study (Doc II-A[2], 3.5 (A6.4.1/01) a LOAEL of 0.1 mg/kg was obtained. A LOAEL of the same level was obtained from rat reproductive toxicity studies, 0.09 mg/kg, based on prolonged KCT.

However, a LOAEL of 0.01 mg/kg was derived in the 42 day repeated dose toxicity dog study based on prolonged PT and KCT. This study was ranked with index 3 as a full subchronic study. However, this range finding study is considered acceptable for classification for the following reasons: even if this study does not cover the full requirements of a repeated toxicity study, the results regarding the effects on blood clotting are nevertheless not compromised. Clear dose- and time responses were obtained in the prolonged PT and KCT values, which are relevant parameter with anticoagulants. Also, the given difenacoum doses were accurate since they were orally administered as gelatin capsules. The study was in principal conducted according to the OECD TG 409 however this was not stated in the study summary. The only important shortcoming in this study protocol is the low number of experimental animals (2 dogs/dose).

Also, there is a rabbit 22 day reproductive toxicity study on difenacoum where some coagulation parameters (PT and PTT) have been measured in the control group and in the highest difenacoum dose group (22 rabbits/group) (Doc II-A[3],3.8.1 (A6.8.1 (2))). Based on the increased maternal PT and PTT, a LOAEL of 0.01 mg/kg was derived. In a similar rabbit 13 day reproductive toxicity study PT and KCT were measured in all treatment groups (20 rabbits in each treatment group; parameters measured on day 8 from all animals, and on days 14 and 20 from 12 pre-designated dams from other groups and from all high dose group dams). A maternal LOAEL of 0.015 mg/kg was derived based on increased PT and KCT (Doc II-A[1],3.8.1 (A6.8.1/02). As already explained in section 5.6.4, pregnancy itself does not affect the PT parameter, therefore we consider it relevant to use the LOAEL values from the reproductive toxicity studies.

It seems that rabbit and dog are the most sensitive species in the studies reviewed in this CLH report and the obtained rabbit and dog LOAEL values are of the same magnitude and 10-fold smaller than that of rat. Therefore it is considered that LOAEL of 0.01 mg/kg should be used for setting the specific concentration limits for repeated dose toxicity.

Calculation for specific concentration limits according to the ECHA's Guidance on the Application of the CLP criteria:

$$SCLCat1 = \frac{ED}{GV1} \cdot 100\% = 0.1\%$$

$$SCLCat2 = \frac{ED}{GV2} \cdot 100\% = 0.01\%$$

ED - Effective Dose: LOAEL 0.01 mg/kg bw/day based on an increase in prothrombin time after oral application (dog, repeated toxicity study and rabbit, reproductive toxicity study)
GV1 - Guidance Value 1: 10 mg/kg bw/day
GV2 - Guidance Value 2: 100 mg/kg bw/day

Calculation for specific concentration limits using the rabbit/dog LOAEL of 0.01 mg/kg and the same underlying approach as for the previously proposed repeated dose toxicity SCLs under Directive 67/548/EEC :

The cut off classification of a substance as T; R48/25 is 5 mg/kg. The LOAEL of 0.01 mg/kg is 500-times lower than the cut-off. The general concentration limit for T; R48/25 is 10%, thus the calculated SCL would be 10/500 = 0.02 %. Then, the SCL for Xn; R48/22 would be 0.002%. The SCL for dermal and inhalation routes would be the same as for oral toxicity. To avoid too many different SCL and to adjust with the other SCLs for other end-points, the SCLs would be:

$C \geq 0.025\%$: T; R48/23/24/25

$0.0025\% \leq C < 0.025\%$: Xn; R48/20/21/22

Conclusions on classification:

- According to Directive 67/548/EEC, difenacoum is currently classified as T; R48/25 via the oral route. It is now proposed to classify difenacoum for repeated toxicity also via inhalation and dermal routes and thus classifications T; R48/23 and T; R48/24 are proposed. The resulting classification would be **T; R48/23/24/25**.

  Proposed SCLs:

  $C \geq 0.025\%$: T; R48/23/24/25

  $0.0025\% \leq C < 0.025\%$: Xn; R48/20/21/22

- According to Regulation EC 1272/2008, difenacoum is currently classified as STOT RE 1 with hazard statement H372** "Causes damage to organs through prolonged or repeated exposure" irrespective of the route of application. The two asterisks on the hazard statement were given because the existing classification was translated from Directive 67/548/EEC with a general hazard statement not specifying the route of exposure as the necessary information was not available. It is now proposed that the asterisks be removed from the hazard statement H372 because based on the route-to-route extrapolation, no route of exposure can be excluded. The resulting classification would be: **STOT RE 1; H372.**

  Proposed SCLs:

  STOT RE 1: $C \geq 0.1\%$

  STOT RE 2: $0.01\% \leq C < 0.1\%$

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier submitter's proposal

Difenacoum is currently classified as STOT RE 1; H372**. In Annex VI of CLP, the existing entry contains two asterisks with a general hazard statement not specifying the route of exposure as the necessary information was not available when the entry was translated from DSD to CLP. The DS proposed to remove the asterisks from the hazard statement H372 because no route of exposure can be excluded.

The DS proposed to derive SCLs using the rabbit LOAEL of 0.01 mg/kg bw/day based on maternal toxicity in a reproductive toxicity study and the dog LOAEL of 0.01 mg/kg bw/day based on anticoagulation effects after repeated exposure.

Comments received during public consultation

Three Member States supported the proposed classification. One of them supported specifically the proposed SCLs by the DS.

Assessment and comparison with the classification criteria

Repeated dose toxicity: oral

There are three studies conducted in rats (two 90 day and one 28 day) according to Guideline OECD No. 408. Each of them shows that repeated oral administration of Difenacoum resulted in marked increase in clotting time and haemorrhages in a wide range of tissues, with treatment related death due to the massive haemorrhages.

The lowest dose used causing treatment-related death was 0.1 mg/kg bw/day. This value is 100 times lower than the limit of 10 mg/kg bw/day for classification for STOT RE 1 according to the CLP Regulation.

Besides there is one 90 day dog study following principles of OECD TG 409 that only lasted for 42 days due to premature sacrifice of the animals when prothrombin time (PT) exceeded the CLP criteria (PT increased to 40/100 seconds). In this study, a clear dose-dependency was seen in the prolonged PT and kaolin-cephalin time (KCT) values in both male and female dogs. Furthermore prolonged PT and KCT were time-dependent effects, and were observed at the lowest administered dose 0.01 mg/kg bw/day which caused prolongation of PT and KCT from day 30. This time-dependence of the increase in PT and KCT is probably a sign of Difenacoum accumulation. A LOAEL of 0.01 mg/kg bw/day was obtained based on prolonged PT and KCT in dogs. The only shortcoming of this study is the low number of experimental animals (2 dogs/dose).

In addition, in two reproductive toxicity studies in rabbits maternal LOAELs were derived based on increased clotting time: a LOAEL of 0.01 mg/kg bw/day was derived based on increased PT and PTT (partial thromboplastin time) in a 22 day study and a LOAEL of 0.015 mg/kg bw/day was derived based on increased PT and KCT in a similar 13 day study.

RAC agreed on the classification for STOT RE 1 according to CLP.

Repeated dose toxicity: inhalation and dermal

Repeated dose studies are available via the oral route only. However, due to similar effects seen in acute oral, dermal and inhalation toxicity studies, a route-to-route extrapolation is reasoned and classification via all three routes for repeated dose toxicity is justified.

RAC therefore supports not specifying exposure routes in the hazard statement. The effect levels are well below the guidance value of 10 mg/kg bw/day warranting classification with STOT RE category 1; H372 (Causes damage to the blood through prolonged or repeated exposure).

Setting specific concentration limits (SCLs):
SCLs based on the rat LOAEL of 0.1 mg/kg bw/day were agreed by TC C&L (Technical Committee on Classification and Labelling) in May 2007 (Follow-up V, May 2008) but were not inserted into Annex VI of CLP.

However, it seems that dogs are more sensitive in terms of change in blood clotting parameters in the studies reviewed in the CLH report. Therefore RAC proposed to derive SCLs based on a dog LOAEL of 0.01 mg/kg bw/day from the repeated dose toxicity study.

Using Haber's law, the effect level derived at day 30 is recalculated into an equivalent 90-day effect level of 0.003 mg/kg bw/day (0.01 mg/kg/day x 30 days / 90 days). Based on the guidance for setting SCL for repeated dose toxicity, an effect level of 0.003 mg/kg/day results in a SCL of 0.03% for STOT RE 1. The SCL value should, according to the guidance, be rounded down to nearest preferred value of 1, 2, or 5, resulting in a SCL of 0.02% for STOT RE 1, and 0.002% for STOT RE 2.

5.7 Mutagenicity

No classification is included for this hazard class in Annex VI, Tables 3.1 and 3.2, CLP Regulation and no classification is currently proposed as also agreed by the TC C&L in 2006/2007 and based on information in CAR.

5.8 Carcinogenicity

There is no data available. For risk assessment, performance of carcinogenicity studies were not scientifically or ethically justified. There is no indication of carcinogenic potential of difenacoum from any other available studies or data on structurally related warfarin. The overall conclusion is that no classification for carcinogenicity is warranted.

5.9 Toxicity for reproduction

5.9.1 Effects on fertility

Table 15: Summary of the fertility study

Route of exposure **Test type Method Guideline**	**Species Strain Sex no/group** **Exposure Period**	**Doses**	**Critical effects**	**NO(A)EL F0 parental**	**NO(A)EL F1** **Parental/ offspring**	**NO(A)EL F2 offspring**	**Reference**
				m & f	**m & f**	**m & f**	
Oral gavage Multigeneration reproduction toxicity study	Rat, Wistar Male and Female 25/sex/dose level P and F1:	Start with 0; 20; 40; 80 µg/kg bw/day On day 20: 80 reduced to 60 µg/kg bw/day	Deaths at 20 µg/kg bw/day and on-wards caused by general haemorrhagic diathesis. No clear effects on fertility, although signs of changes in oestrus cycle at 10, 20 µg/kg bw/day in both generations and	0.01 mg/kg bw/day (due to deaths) NOAEL No	20 µg/kg bw/day NOAEL (no clear effects on fertility) No	20 µg/kg bw/day NOAEL (no effects on postnatal devel-opment)	Doc II-A[3], 3.8.2 (A6.8.2)

OECD 416 GLP	10 weeks prior to mating and 2 weeks of mating, gestation and lactation	From day 40: 0; 10; 20 µg/kg bw/day because of the death of all male animals dosed with 60 µg/kg	ovarian cysts at 60 µg/kg bw/day, slightly increased post-implantation loss in F1, decreased total sperm count in F0 generation at 20 µg/kg bw/day, slightly prolonged precoital period in F0 generation.	NOEL for changes in oestrus cycles	NOEL for changes in oestrus cycles		

Effects on fertility have been studied in a rat multigeneration study (Doc II-A[3], 3.8.2 (A6.8.2))). In this study, dose levels had to be lowered twice during the course of the study due to extensive mortality. Regardless of the very low doses, it can be concluded that difenacoum does not have clear effects on fertility. However, there were indications of disturbed estrous cyclicity perhaps due to ovarian hormonal disturbance. Main findings related to fertility (irregular estrous cycles in treated animals in both generations and ovarian cyst at maternally toxic dose of 0.06 mg/kg bw/day in F0 females) did not affect the fertility index. No severe increase in postimplantation loss was observed. In the literature, there are no indications of adverse fertility effects associated to vitamin K deficiency or the better-known coumarin-derived human reproductive toxicant warfarin. It is considered that classification for fertility effects is not warranted for difenacoum and the possible effects on ovarian function are adequately covered by the repeated dose toxicity classification.

5.9.2 Developmental toxicity

Table 16: Summary of teratogenicity studies

Route of exposure Test type Method Guideline	Species Strain Sex no/group	Exposure Period	Doses	Critical effects dams fetuses	NOEL maternal toxicity	NOEL Terato-genicity Embryo-toxicity	Refe-rence
Oral gavage Develop-mental toxicity OECD 414 GLP	Rabbit, New Zealand White Female, 22/group	Days 7-28 post insemi-nation	0; 0.001; 0.003; 0.010 mg/kg bw/day	Maternal toxicity at all dose levels with clinical signs of toxicity, pathological alterations related to anticoagulant effect and deaths. Significantly increased coagulation time (PT and PTT) at the high dose (parameters measured only at high dose and control groups at the end of the study). Higher incidence of skeletal variations at two dose levels compared to controls, but without dose dependence. In conclusion, clear developmental toxicity was not observed.	< 0.001 mg/kg/day (LOAEL, based on clinical signs of toxicity and pathologic al alterations)	< 0.01 mg/kg/day (NOEL/ NOAEL)	Doc II-A[3], 3.8.1 (A6.8.1 (2))
Oral gavage Develop-mental Toxicity OECD 414 GLP	Rabbit, New Zealand White Female 20/group	Days 8-20 (inclusive) postmating (day 0)	0; 0.001; 0.005; 0.015 mg/kg bw/day	Maternal toxicity at 0.015 mg/kg bw (increased PT and KCT, measured at GD 8, 14 and 20). No maternal toxicity at lower doses. Foetal effects observed in both test and control groups and included defects not previously seen in this strain or laboratory, but the effects were not dose related. In conclusion, clear developmental toxicity was not	0.005 mg/kg bw/day (NOEL/ NOAEL	0.015 mg/kg/day (NOEL/ NOAEL)	Doc II-A[1], 3.8.1 (A6.8.1/0 2)

				observed.			
Oral gavage Develop-mental Toxicity OECD 414 GLP	Rat, Wistar derived Female, 24/group	Days 7-16 (inclusive) postmating (day 0)	0; 0.01; 0.03; 0.09 mg/kg bw/day	Maternal toxicity at 0.09 mg/kg bw (vaginal bleeding and increased KCT measured on days 6, 11, 14 and 17) but no foetal effects even in dams prematurely culled.	0.03 mg/kg bw/day (NOEL/ NOAEL)	0.09 mg/kg bw/day (NOEL/ NOAEL)	Doc II-A[1], 3.8.1 (A6.8.1/01)
Oral gavage Develop-mental toxicity OECD 414 GLP	Rat, Wistar Female, 20/group	Days 7-16 post mating	0; 0.01; 0.03; 0.09 mg/kg bw/day	Maternal toxicity at 0.09 mg/kg bw/day (clinical signs indicating bleeding, three dams killed in extremis). However, no effects on coagulation times. At 0.09 mg/kg bw/day, one foetus with microphthalmia, one foetus with discoloured adrenals and some minor skeletal effects in foetuses. In conclusion, developmental toxicity was not observed.	0.03 mg/kg bw/day (NOEL/ NOAEL)	0.09 mg/kg bw/day (NOEL/ NOAEL)	Doc II-A[3], 3.8.1 (A6.8.1 (1))

PT: prothrombin time
KCT: kaolin-cephalin time

Teratogenicity tests have been performed in two species, rat and rabbit according to the OEDC 414 test guideline.

In the rabbit, the lowest LOAEL value for maternal toxicity is 0.001 mg/kg bw/day based on the increased haemorrhages in the kidneys (no NOAEL could be set; Doc II-A[3], 3.8.1 (A6.8.1 (2)). A higher maternal LOAEL value of 0.015 mg/kg bw/day was obtained in another rabbit developmental toxicity study based on prolongation of prothrombin time (Doc II-A[1], 3.8.1 (A6.8.1/02)). In this study the maternal NOEL/NOAEL value was 0.005 mg/kg bw/day. Dose range and spacing are comparable in these studies. The main difference is the length of the exposure period, 22 days compared to 13 days, the longer exposure period leading to the typical adverse effects at a lower dose. This may be an indication of accumulation of difenacoum. Also the slightly different toxicokinetics and different acute toxicity potencies of the isomers (cis, trans) may have contributed to the slightly different results. In both studies, foetal effects (mainly skeletal) were observed but not considered treatment related. After a longer exposure period, higher incidences of skeletal variations were observed at two dose levels compared to controls, but the incidences were not dose dependent. After 13-day exposure, foetal effects (mostly vertebral and rib effects) were observed in both test and control groups including defects not previously seen in this strain or laboratory, but these effects were not dose related. The NOEL/NOAEL value for developmental toxicity is 0.015 mg/kg bw/day after 13-day exposure and 0.01 mg/kg bw/day after 22-day exposure.

In the rat, the NOEL/NOAEL for maternal toxicity is 0.03 mg/kg bw/day (Doc II-A[1], 3.8.1 (A6.8.1/01) and Doc II-A[3], 3.8.1 (A6.8.1 (1)). There was no evidence of embryotoxic or teratogenic potential following oral exposure of pregnant rats at 0.09 mg/kg bw/day (=NOEL/NOAEL for developmental toxicity).

In conclusion, clear developmental toxicity was not observed in rabbits or rats.

5.9.3 Human data

For difenacoum, there is no human data available.

5.9.4 Other relevant information

Anticoagulant rodenticides, including difenacoum, are structurally similar to warfarin. The mode of action, i.e. interference with vitamin K recycling is also the same. The teratogenicity of warfarin in humans has been demonstrated through case reports after administration of warfarin as a medical substance. Warfarin is classified for developmental toxicity (CLP: Repr. 1A; H360D ***/DSD: Repr. Cat. 1, R61; Annex VI to CLP Regulation).

Coumarin embryopathy and other coumarin-induced fetal effects

In humans, warfarin and other coumarin derivatives may cause a syndrome characterized by nasal hypoplasia, vertebral and epiphyseal stippling (chondrodysplasia punctata), hypoplasia of the extremeties or other skeletal anomalies (warfarin embryopathy or coumarin embryopathy) after exposure during the first trimester (Pauli et al. 1987, Driel van et al., 2002). Exposure to coumarins during 2^{nd} and 3^{rd} trimesters may cause bleeding which has been associated with several brain and central nervous system malformations (Ville et al. 1993; Howe and Webster 1994; Driel van et al. 2002).

The risk for warfarin embryopathy has been estimated to be 2.4% (Blickstein and Blickstein, 2002), but also much higher risks have been reported such as 6 % (23 cases out of 394, Driel van et al. 2002) and 12 % (5 cases out of 41, Soma-Pillay et al. 2011). The risk for warfarin embryopathy has not been related to the maternal warfarin dosage and embryopathy occurs also with low-dose warfarin (Driel van et al. 2002; Soma-Pillay et al. 2011).

Increased risks of spontaneous abortion and premature delivery have also been reported in association with warfarin treatment. The risks for spontaneous abortion and premature deliveries have been estimated to be 24 and 14%, respectively (Blickstein and Blickstein, 2002). The risks for fetal loss and stillbirth is significantly increased with increasing doses of warfarin (Soma-Pillay et al. 2011; Basude et al. 2012).

Vitamin K hydroquinone (KH_2) deficiency

4-Hydroxycoumarin derivates are vitamin K antagonists. Second-generation anticoagulants (such as difenacoum) are even more potent vitamin K-antagonists than warfarin; the dissociation of enzyme/inhibitor complexes is expected to be extremely low. Their use as rodenticide is based on the inhibition of the vitamin K-dependent step in the synthesis of a number of blood coagulating factors. Vitamin K epoxide reductase is the target enzyme for coumarin anticoagulants. The blocking of the vitamin K epoxide reductase leads to rapid exhaustion of the supply of vitamin K hydroquinone, and thus to an prevention of the formation of Gla coagulation factor precursors in the liver. The mechanism of causing vitamin K hydroquinone deficiency in other tissues such as bone and cartilage is based on the same enzyme inhibition.

Embryos and foetuses are dependent on maternal supply of vitamin K and the vitamin K_1-dependent regulatory pathways appear to be critical for proper embryogenesis (Saxena et al., 1997; Driel van et al., 2002). Any reason causing vitamin K deficiency may harm developing embryos. Vitamin K deficiency in embryos and foetuses may be caused, e.g. by exogenous compounds such as warfarin or phenytoin, by genetic disorders or by maternal malnutrition or malabsorption due to physiological disorders (Pauli, 1997, Menger et al., 1997, Jaillet et al. 2005). The developmental

anomalies caused by vitamin K deficiency are very similar irrespective of the underlying cause for vitamin K deficiency.

On the fetal side, vitamin K levels are very low during embryonic development. Thus, embryos are very sensitive to even small changes in vitamin K balance, human embryos being even more sensitive than rodent embryos (Howe and Webster, 1994, 1997). Vitamin K_1 and K_2 (MK-4) pass through the placenta only in small quantities (Hiraike et al., 1988; Kazzi et al., 1990; Iioka et al., 1991 and 1992). Vitamin K_1 requires a high gradient (approximately 10 times higher maternal concentrations), but vitamin K_2 is actively incorporated into the placenta and then gradually released into foetal blood. Concentrations of vitamin K_2 on maternal and foetal side are similar. Thus, foetal blood levels of vitamin K_1 and K_2 follow maternal blood levels of these vitamins.

Relevance of the OECD414 test for AVKs

The teratogenicity of warfarin is not easily demonstrated in animal studies designed according to the conventional OECD 414 protocol. The teratogenic properties have only been confirmed in rat by using a study design where high doses of warfarin were given with co-exposure to vitamin K to achieve a net extrahepatic vitamin K deficiency. This approach preserved the vitamin K-dependent processes of the liver and thus there were no signs of haemorrhage (Howe and Webster, 1990, 1992; Howe et al., 1992). In addition, the exposure period was adjusted to correspond the critical periods in rat for the observed effects in humans (nasal and skeletal development). In rat, the nasal and skeletal development takes place during late fetal and early postnatal life, therefore warfarin was given postnatally starting on the day of birth. Without vitamin K supplementation and an adapted study protocol results from warfarin have been equivocal.

Warfarin and other AVKs have been discussed in the The Commission Working Group of Specialized Experts for Reproductive Toxicity (September 2006; ECBI/31/07). The experts unanimously agreed on read-across from warfarin and stated that all anti-vitamin K rodenticides should collectively be regarded as human teratogens and classified as Reprotoxic Category 1, R61. Classification of all the coumarin anticoagulant rodenticides as Repr. Cat. 1; R61 or Repr. Cat. 2; R61 according to Directive 67/548/EEC was provisionally agreed by the TC C&L in November 2006. The conclusion of Specialized Experts is cited below:

Conclusions Anticoagulant Rodenticides (ECBI/31/07):

Warfarin is an established human teratogen classified as Repr. Cat. 1; R61. It is uncertain whether teratogenicity of warfarin can be detected in pre-natal developmental toxicity studies (including OECD guideline 414). The teratogenic mechanism of warfarin is likely to involve maternal Vitamin K depletion and/or direct effects on embryo/foetus via transplacental exposure. Given the vitamin K inhibition, there is concern that other anti-vitamin K (AVK) compounds could cause similar teratogenic effects as warfarin in humans.

The other AVK rodenticides have not shown teratogenic effects in conventional rat and rabbit developmental studies and there is no data in humans. Given the uncertainties surrounding the ability of the standard pre-natal developmental toxicity studies to detect warfarin teratogenicity the predictive value to humans of these studies is uncertain.

On the basis of currently available data, there are no convincing arguments that other AVKs including the second generation compounds could not pass the placenta. Both the mechanism of action and the possible placental passage give reason for concern of possible teratogenicity in human.

Considering all the available information the Specialised Experts unanimously agreed that the AVK rodenticides should collectively be regarded as human teratogens. Therefore the other AVK rodenticides should be classified as Repr. Cat. 1; R61.

Since the expert conclusion described above further rat studies on developmental toxicity of warfarin and on placental transfer of warfarin and flocoumafen have been conducted. Results from the new OECD 414 guideline study with warfarin (Kubazky 2009) performed by the CEFIC Rodenticide Data Development Group have been provided to DSs of AVKs and are summarized below. The study of placental transfer of warfarin and flocoumafen has not been available to the DS of difenacoum.

Summary of the new OECG 414 guideline study with warfarin sodium (Kubazky 2009)

Reference	:	Kubaszky (2009)	Exposure	:	day 6-15 (TP 1) or day 6-19 (TP 2)
Type of study	:	Teratogenicity	Doses	:	0, 0.125, 0.150, 0.200, 0.250 mg/kg bw per day
Year of execution	:	2007	Vehicle	:	aqueous CMC
Test substance	:	Warfarin sodium	GLP statement	:	Yes
Route	:	Oral by gavage	Guideline	:	OECD 414
Species	:	Rat Crl:(Wi) BR-Wistar	Acceptability	:	Acceptable
Group size	:	25 dams per group, except high dose group 12 dams/group	$NOAEL_{mat}$	:	0.125 mg/kg bw per day
			$NOAEL_{dev}$	:	< 0.125 mg/kg bw per day

The study was performed according to the OECD test guideline No. 414: Prenatal Development Toxicity Study. No Vitamin K supplementation was used. There were two treatment regimens in the study: warfarin at dose levels of 0, 0.125, 0.150 and 0.200 mg/kg /day were given orally by gavage to one set of groups of female Wistar rats at days 6-15 post coitum (TP 1) and at the same dose levels to a second set of groups at days 6-19 post coitum (TP 2). Each dose group consisted of 25 animals. Two additional extra high dose (0.250 mg/kg /day) groups were added at a later stage of the study in order to demonstrate clear maternal toxicity. These groups consisted of 12 animals each. No extra control group was added to match the extra high dose groups.

Maternal mortality was observed in TP 1 at doses 0.150 mg/kg and higher. There was one moribund dam in TP 2 at dose 0.150 mg/kg and dead or moribund dams at dose 0.250 mg/kg. Animals died or they were sacrificed on grounds on moribundity between days 14 and 17 of gestation and in one case on day 19. Clinical signs recorded for the dead or sacrificed animals included piloerection, paleness, reduced activity and vaginal bleeding and open vaginal orifice. In some surviving animals at doses 0.150 mg/kg and higher vaginal bleeding, open vaginal orifice, paleness, hemorrhage or piloerection were recorded. At necropsy, blood filled uterine horns or uteri, pale organs, bloody secretion in stomach and in intestine were observed in dead or surviving dams. The mortality and clinical signs were attributed to the warfarin-treatment.

There was no difference in the number of corpora lutea, pre- or postimplantation losses or numbers of implantations or fetuses between the experimental groups including controls.

In foetuses, internal and external hemorrhages of different sizes were recorded in all treatment groups when compared to controls however these effects were not dose-dependent but still attributed to the warfarin-treatment.

A malformation manifested as yellowish discolouration in the lens (central cataract) was observed in TP 1 in 1/99 foetuses at dose 0.200 mg/kg, and in TP 2 in 2/124 fetuses at dose 0.150 mg/kg and in 4/132 foetuses at dose 0.200 mg/kg. This malformation is considered rare and treatment-related since it has not been recorded in the historical data of the laboratory or in the database of the supplier of the Wistar rats.

There were no clear indications for warfarin-related skeletal malformations in the foetuses. However, in TP 1, in one litter at dose 0.150 mg/kg, 4 out of 7 foetuses had short nose and wide frontal bone. These affected fetuses had also abnormally high body weights, ranging from 3 to 6 grams. This litter was excluded from the statistical analysis on the grounds that it might have been a day older than the remaining litters and therefore at a different developmental stage. The study author did not exclude the effects being treatment-related.

Discussion by the DS of difenacoum:

This study showed that warfarin causes hemorrhages and cataract in the foetus. The incidence of cataract is considered as a manifestation of teratogenicity of warfarin (e.g. Driel van et al. 2002). However, the skeletal malformations typical for humans were not convincingly observed in this study.

With regard to the doubt whether a standard OECD 414 test can detect coumarin-specific developmental effects, this study shows that some of the developmental effects induced in humans by warfarin are also detectable in rats, but others are not. It seems that the TP 2 protocol was more sensitive since the incidence of cataract was higher in this regimen than in the TP 1 regimen. However, even in TP 2 the timing of exposure is not optimal in view of skeletal development of rat. The rat teratogenicity studies with difenacoum have been performed with a protocol where difenacoum was given on days 7-16 of gestation, resembling the TP 1 protocol of warfarin. However, no signs of cataract were observed with difenacoum. No study with difenacoum applying the TP 2 regimen has been carried out in rats. It is therefore impossible to draw any conclusions whether difenacoum would be able to induce cataract in rat if equivalent similar treatment protocol to warfarin was used. As regards to the skeletal and facial defects typical for warfarin in humans, most of these have been demonstrated in rats in studies where warfarin has been given postnatally since the nasal and skeletal development in rat takes place during late fetal and early postnatal life (Howe and Webster, 1990; Howe and Webster, 1992). Therefore it is of no surprise that these malformations were not detected in the new warfarin study covering only the prenatal period.

Case reports on maternal vitamin K deficiency and consequent fetal effects

There is increasing evidence showing that any underlying reason causing maternal vitamin K deficiency may harm the developing foetus. There are several recent case reports showing that persistent vomiting during pregnancy (hyperemesis gravidarum) may cause maternal vitamin K deficiency and consequential defects and bleeding in the foetus. In one case intractable vomiting began at 7 weeks of gestation, resulting in weight loss from 70 kg to 55 kg by week 11. Metabolic screening revealed decreased prothrombin level (42%). The vomiting continued and the mother was hospitalized at week 12, the prothrombin level was now 27% and factor V level 126% suggesting vitamin K deficiency. The mother received intravenous vitamin K supplementation and the prothrombin time was normalized. At week 24 an ultrasound scan was carried out revealing normal fetal biometry with reduced nasofrontal angle. No other anomalies were found. The child was born at week 37 and 5 days. She had a flat nasal bridge and hypoplasia of the distal phalanges. (Alessandri et al. 2010).

In another case a mother suffering from persistent vomiting was referred to hospital at week 9 of gestation. Her body weight had decreased by 5 kg from the baseline. At week 14 her prothrombin time (PT) dropped to the nadir (28%; normal range 80-125%). After daily intravenous vitamin K administration PT normalized however vomiting persisted. At week 17ultrasonographic tomography showed enlarged biparietal diameter and hydrocephalus which was aggravated with advancing gestational age. The mother chose induced abortion. Autopsy revealed subarachnoid haemorrhage, hemosiderin deposits to the choroid plexus near the Foramen of Luschka and on the surface of the brain stem. The hemosiderin deposits probably blocked the pathway of cerebrospinal fluid absoption. (Kawamura et al. 2008).

In a third case a pregnant woman was admitted to hospital 3 times during the pregnancy due to recurrent vomiting. The vomiting occurred 5 to 10 times per day and had started at gestation week 16. She did not receive extra vitamin supplements. Ultrasonographic examinations showed normal

fetal size and adequate fetal growth. At week 32 there were decreased fetal movements and decreased fetal heart rate, therefore an emergency caesarean section was performed. The child was pale, not breathing, bradycardic and hypotonic. He was resuscitated and transferred to neonatal intensive care unit. He did not have nasal hypoplasia. There was a hematoma in the palm of the left hand. Coagulation tests (PT, PTT, coagulation factor levels) revealed coagulopathy and he received vitamin K treatment. At 40 min of age cranial ultrasound examination revealed massive intracranial haemorrhage including subdural, intraparenchymal and intraventricular haemorrhage. Repeated cranial CT at 14 days of life revealed gradual resorption of blood. (Eventov-Friedman et al. 2009).

Biliary lithiasis in early pregnancy has been related to abnormal development of facial and distal limb bones, vitamin K deficiency being the ultimate cause. The absorption of exogenous vitamin K by the intestinal cells requires the presence of biliary salts. A pregnant woman started vomiting and had abdominal pain during her early pregnancy and at 9 weeks of gestation she was hospitalized. Abdominal ultrasonography revealed biliary lithiases in the gall bladder. Two weeks later vitamin K deficiency was confirmed due to changes in PT and clotting factors (II, VII, X, V). Intravenous vitamin K at 11 weeks and 5 days normalized the prothrombin time. The child was born at term. Hypoplasia of the nasal bones was noted. Clinical examination showed dysmorphic features suggestive of Binder syndrome (a low anterior hairline, epicanthal folds, depressed nasal bridge with short upturned nose, a thin upper lip and high arched palate). She also had short and drumstick like distal phalanges of the hands and feet. (Jaillet et al. 2005).

5.9.5 Summary and discussion of reproductive toxicity

With regard to reproductive toxicity of difenacoum, read-across to warfarin is justified due to structural similarity and the common mode of action through Vitamin K deficiency. See chapter 5.9.4 for the scientific justification.

Effects on fertility

In analogy to teratogenicity and developmental toxicity, read-across to warfarin data is justified. Warfarin has not been classified as toxic to fertility. In literature, there are no indications of adverse fertility effects associated to warfarin or vitamin K deficiency.

Study on reproductive toxicity shows that no clear effects on reproduction were observed. There was a tentative effect on oestrus cycle and the total sperm count, but results could be obtained only from two very low dose levels. Overall toxicity leading to premature deaths and lowering of the doses during the study deteriorate the overall validity of the study. Possible effects on reproduction seem to be overwhelmed by lethality.

In conclusion, based on the current knowledge of absence of fertility effects of analogous compounds and vitamin K deficiency, difenacoum **should not be classified as toxic to fertility**.

Developmental toxicity

Clear developmental toxicity in response to difenacoum was not observed in rabbits or rats. However, we are of the opinion that the studies performed were not suitable for the determination of developmental toxicity of difenacoum. There are grounds for believing that difenacoum is indeed developmentally toxic. Namely, difenacoum contains the same chemical moiety responsible for the

teratogenicity of warfarin, and it has the same mode of action (inhibition of vitamin K cycle leading to vitamin K deficiency) that is a known mechanism of teratogenicity in humans. Warfarin is classified for developmental toxicity (CLP: Repr. 1A; H360D ***/DSD: Repr. Cat. 1, R61).

The teratogenicity of warfarin in humans has been demonstrated through case reports after administration of warfarin as a medical substance. However, results of warfarin studies in rats have been equivocal and without vitamin K supplementation and an adapted study protocol teratogenicity of warfarin has not been easily demonstrated. It is assumed that vitamin K supplementation along with an adaptation of the study protocol would have been needed also to reveal the teratogenic effects of difenacoum.

Furthermore, it must be taken into account that human foetuses are much more vulnerable to vitamin K (hydroquinone) deficiency than rodents (Howe and Webster, 1994; Howe et al., 1997). This may be related to the fact that human foetuses have very low blood vitamin-K concentrations, with a mid-gestation mean value of 30 pg/ml and a maternal level of 395 pg/ml, compared with plasma levels of 8,600 pg/ml in 20 day rat foetuses and maternal rat levels of 22,000 pg/ml (Howe and Webster,1990). In humans this 13 times difference in vitamin K level between mother and foetus may explain why teratogenic effects are observed in foetuses at dose levels that are not toxic to the mother. In contrast, the difference in vitamin K levels is only 2.5 between mother and foetus in the rat. Hence, the dose causing adverse effects in the foetus are most likely closer to the maternal lethal dose in rats than in humans.

More generally, our view is that without an adjusted protocol including vitamin K supplementation (or other methods to prevent maternal bleeding), rodents are not good models for studying developmental effects of coumarin-derived compounds. Due to this, and taking into account the conclusions reached by TC C&L (see section 5.9.4), we have been compelled to omit the results from standard OECD 414 studies and instead use read across for the classification of difenacoum as a developmental toxic agent.

On these grounds, especially due to the human experience with warfarin, difenacoum is considered to be teratogenic and developmentally toxic and **classification as a reproductive toxicant in category 1A is proposed under CLP Regulation and in category 1 under Directive 67/548/EEC**.

Specific concentration limits

To account for the high potency of difenacoum (low ED on blood clotting parameters) for disturbing the vitamin-K balance of exposed mammalian species setting a specific concentration limit (SCL) for developmental toxicity should be considered. However, no numerical value is proposed for difenacoum at the moment. Instead, we consider it important to collect all available and relevant information on the anticoagulant rodenticides together in order to make an extensive potency comparison of the individual substances in one go.

It is recognised that since applicable substance specific data does not exist, the SCL cannot be set based on data on difenacoum itself. According to the draft revised ECHA guidance (Guidance on the application of the CLP criteria) potency determination of individual substance within a group of substances using non-testing methods could be possible in some cases. In this particular case the proposed reproductive toxicity classification is based on read across from human data on warfarin. The read across and common mode of action of all anticoagulant rodenticides is further supported by the numerous examples of other reasons causing imbalance of vitamin-K leading to the typical developmental effects. See section 5.9.4 of the CLH report for the details.

In conclusion, a SCL for warfarin is proposed following the principles introduced in the draft revised CLP guidance. Whether the SCL for difenacoum could be based on read across from warfarin should be discussed together with the other AVKs. Eventually, a common approach for setting the SCLs would be chosen for all AVKs following the principles of the available guidance. We believe that based on acute toxicity, data from repeated dose toxicity and toxicokinetic studies there is enough information on the properties of difenacoum to judge upon the level of SCL. However, a comprehensive comparison between the individual substances is necessary before finally concluding on the numerical value for difenacoum. The potency differences of individual substances should be taken into account in arriving in the numerical values as much as possible. We welcome a general discussion regarding the strength of evidence for the potency of reproductive toxicity of the other AVKs compared to that of warfarin. Meanwhile, based on the known common mode of action, toxicokinetics and the ED for blood clotting parameters we support at least the same SCL for difenacoum as proposed for warfarin.

Conclusions on classification

Read-across rationale

According to the REACH Regulation (1907/2006), information on intrinsic properties on substances by other means than tests may be generated. According to the ECHA guidance "Information requirements and chemical safety assessment, Chapter R.6: QSARs and grouping of chemicals", structural similarity and similar properties may be used as a basis for read-across. This principle is equally relevant both for risk assessment and classification and labeling, and there is also a reference to the relevant REACH Annex XI in CLP, i.e. Article 5(1), point c regarding identification and examination of available information on substances.

Difenacoum is an anticoagulant that is used for rodent control. Difenacoum is structurally related to warfarin and other AVKs (Figure 2). Anticoagulant activity is associated with the 4-hydroxy coumarin moiety that forms part of the chemical structure of AVKs and with the large aromatic substituent at 3-position which varies between AVKs. In warfarin this 3-position substituent is a simple phenyl group. The more potent second generation AVKs, including difenacoum, have larger lipid-soluble substituents at the 3-position. The potency of second-generation anticoagulants can be partly explained by their highly lipophilic nature, which enables them to bind strongly to biological membranes. It is to be expected that the dissociation of enzyme/inhibitor complexes will be extremely slow. There is no species specificity of the inhibitors. Any species-dependent differences which might be found *in vivo* will presumably be brought about by a different pharmacokinetic or pharmacodynamic behaviour in these species.

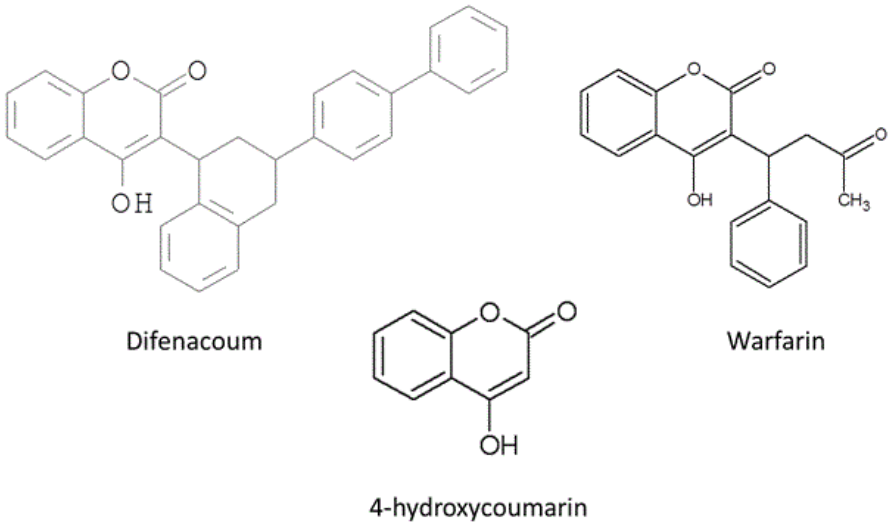


Figure 2: The chemical structures of difenacoum, warfarin and 4-hydroxycoumarin

The use of AVK rodenticides is based on the inhibition of the vitamin K-dependent step in the synthesis of a number of blood coagulation factors. AVKs interfere with the vitamin K recycling by blocking VKOR activity which leads to exhaustion of the supply of KH_2. Difenacoum possesses a toxicologically similar mode of action with warfarin in causing vitamin K deficiency. For warfarin, there is substantial evidence of toxicity for reproduction following administration of the substance in humans as an agent in anticoagulant therapy, see section 5.9.4. The apparent mode of action of warfarin in causing developmental defects is via vitamin K deficiency. The teratogenicity of warfarin using the standard OECD 414 test has not been convincingly shown suggesting that without an adjusted protocol the teratogenicity of warfarin is difficult to prove. The negative results from the OECD 414 test on difenacoum can very likely be explained by similar inappropriate study design.

Taken together, since warfarin is an established teratogen based on human evidence, it is justified to read-across the classification of difenacoum from warfarin on the basis of similar chemical structure and similar mode of action.

It is proposed to classify difenacoum as

- **Repr. 1A; H360D** "May damage the unborn child" according to Regulation EC 1272/2008. SCL should be set in analogy with the other AVKs.

- **Repr. Cat. 1, R61** "May cause harm to the unborn child" according to Directive 67/548/EEC. SCL should be set in analogy with the other AVKs.

RAC evaluation of reproductive toxicity
Summary of the Dossier submitter's proposal Difenacoum is an anticoagulant that is structurally related to warfarin and other antivitamin K anticoagulants rodenticides (AVKs). Classification for reproductive toxicity is proposed by the DS because of teratogenicity. A rat multi-generation study high toxicity and premature death were observed and dose

levels administered during the study had to be lowered. Sign of changes in oestrus cycle and decreased total sperm count were observed from two very low dose levels but they did not affect the fertility. However, effects on reproduction may have been masked due to the excessive mortality

The DS concluded that based on the current knowledge of absence of fertility effects of analogous compounds and vitamin K deficiency, Difenacoum should not be classified as toxic to fertility.

Developmental toxicity data on Difenacoum are equivocal. Clear developmental toxicity was not observed. However, Difenacoum is a coumarin derivative like warfarin which is classified as Repr. 1A according to the CLP Regulation. Since also the mode of action causing vitamin K deficiency is the same, and the maternal vitamin K deficiency is the underlying reason for teratogenicity, it is proposed to classify Difenacoum as a reproductive toxicant in category 1A; H360D.

The DS argued that SCLs should be set together with the other AVKs. No numerical value was proposed but the DS supported setting SCLs for Difenacoum at least equal to these proposed for Warfarin.

Comments received during public consultation

Four Member States agreed with the DS proposal to classify Difenacoum as Repr. 1A; H360D based on the human evidence of developmental toxicity of Warfarin.

One Member State pointed out that SCLs for reprotoxicity are necessary for Difenacoum. Furthermore the same Member State suggested to harmonise the SCLs between the other AVK anticoagulants (Warfarin, Flocoumafen, Difethialone, Coumatetralyl, Brodifacoum, Bromadiolone, Chlorophacinon).

Six industry organisations disagreed with the proposed classification for Repr. 1A. They provided two statements from an expert toxicologist to demonstrate that the basis for read-across for developmental toxicity from Warfarin to Difenacoum is invalid.

Assessment and comparison with the classification criteria

Fertility:

A rat multi-generation study conducted according to OECD TG 416 showed excessive mortality. Dose levels had to be lowered twice during the course of the study. Deaths occurring at 0.020 mg/kg bw/day and above were caused by general haemorrhagic diathesis.

There were irregular oestrous cycles in treated animals in both generations and ovarian cyst at maternally toxic dose of 0.06 mg/kg bw/day in F0 females perhaps due to ovarian hormonal disturbance. The fertility index was not affected and no severe increase in post-implantation loss was observed. In addition, there are no indications of adverse fertility effects associated to vitamin K deficiency in the literature.

RAC agreed with the DS that Difenacoum should not be classified as toxic to fertility based on the current knowledge of absence of fertility effects of analogous compounds and vitamin K deficiency.

Developmental toxicity:

Two rat and two rabbit teratogenicity studies performed according to the OECD TG 414 are available.

In the rat studies the NOEL/NOAEL for maternal toxicity was 0.03 mg/kg bw/day. There was no evidence of embryotoxic or teratogenic potential following oral exposure of pregnant rats at 0.09 mg/kg bw/day (= NOEL/NOAEL for developmental toxicity).

In a 22-day rabbit study the LOAEL value for maternal toxicity was 0.001 mg/kg bw/day

based on increased haemorrhages in the kidneys. In this study no NOAEL could be set. In the second rabbit study (13 day study) the LOAEL value for maternal toxicity based on prolongation of prothrombin time was 0.015 mg/kg bw/day. The maternal NOEL/NOAEL value was 0.005 mg/kg bw/day. The longer exposure period lead to typical adverse effects at lower dose. This could be due to accumulation of Difenacoum. Also the slightly different toxicokinetics and different acute toxicity potencies of the cis- and tans-isomers may have contributed to the small difference in the results.

In both rabbit studies foetal effects (mainly skeletal) were not dose but time-dependent. Concerning the 13=day rabbit study it has to be pointed out that many of the vertebral and rib defects were atypical, i.e. not recorded previously in the testing laboratory. However, no clear developmental toxicity was observed in rabbits. The NOEL/NOAEL value for developmental toxicity was 0.015 mg/kg bw/day after 13 days and 0.01 mg/kg bw/day after 22 days of exposure.

Relevance of the OECD TG 414 test for AVKs:
The OECD guideline study on Warfarin (Kubazky, 2009) performed by the CEFIC Rodenticide Data Development Group indicates that Warfarin caused haemorrhages and cataract in the foetus. The incidence of cataract was considered as a manifestation of teratogenicity of Warfarin (Driel van et al., 2002). The skeletal malformations typical for humans were not convincingly observed in the study. However, most of the skeletal and facial defects typical for Warfarin in humans have been demonstrated in rats in studies where Warfarin has been given postnatally since the nasal and skeletal development in rat takes place during late foetal and early postnatal life (Howe and Webster, 1990; Howe and Webster, 1992).

In summary the Kubazky study showed that some of the developmental effects induced in humans by Warfarin were also detectable in rats, but others were not. There is no study on Difenacoum that could be compared to the above mentioned Kubazky study. Therefore it is not possible to draw any conclusions whether Difenacoum would be able to induce cataract in rat if equivalent treatment protocol to Warfarin was used.

It remains doubtful whether a standard OECD TG 414 test can detect coumarin-specific developmental effects.

Overall conclusion on classification for developmental toxicity
Based on the known developmental toxicity of the AVK rodenticide Warfarin in humans (Repr. 1A), the reproductive toxicity of Difenacoum has been analysed in detail. It is acknowledged that the animal developmental toxicity studies on Warfarin were weakly positive and that the animal developmental toxicity studies on Difenacoum were negative. However, in comparison with Warfarin, Difenacoum and other 2nd generation AVKs have higher acute and repeated dose toxicity, steeper dose-response curves, and much longer half-lives in the exposed organisms, making the evaluation of developmental effects of all 2nd generation AVK rodenticides difficult. Thus to avoid maternal toxicity and lethality, relatively low doses in repeated exposure during gestation were used which hindered the detection of developmental toxicity effects.

As there are no data on the outcome of maternal exposure to Difenacoum in humans, classification in category 1A is not considered to be applicable for Difenacoum.

Based on the assumption that all AVK rodenticides, including Warfarin and other anticoagulant coumarin pharmaceuticals (see below) share the same mode of action (MoA), namely inhibition of vitamin K epoxide reductase (VKOR), the assessment of Difenacoum includes consideration of the total data base for the AVKs. A weight of evidence assessment resulted in the conclusion that Difenacoum has the capacity to adversely affect the human in utero development. Therefore a classification with cat 1B was proposed with the reasoning given below.

The reasons for this presumption were:

- Difenacoum shares the same MoA as expressed by other anticoagulant AVK rodenticides and coumarin pharmaceuticals (inhibition of vitamin K epoxide reductase, an enzyme involved with blood coagulation and foetal tissues development, including bone formation, CNS development and angiogenesis)
- Warfarin and 2 other coumarin pharmaceuticals (Acenocoumarol, Phenprocoumon) have been shown to cause developmental toxicity in humans.
- One of the 2nd generation AVK rodenticides (Brodifacoum) has been shown to cause foetal effects in humans, possibly after one or a few exposures.
- For AVK rodenticides with a long half-life in the body, even single exposures might suffice to trigger developmental effects. However, such studies are normally not conducted and effects of single dose exposure cannot be detected in standard OECD TG 414 test where the repeated exposure may lead to maternal mortality with steep dose-response.

The standard animal studies will not pick up all developmental toxicity effects of the AVK rodenticides, most notably the face and CNS malformations that are characteristic for Warfarin and other AVK coumarin pharmaceuticals.

The most sensitive window for face malformations in humans is the first trimester of pregnancy. Thus, also if some AVK rodenticides may have a lower degree of placental transfer than Warfarin, this will not affect the face malformation hazard as the placenta is not yet fully developed during the first trimester.

Not all steps of the MoA in the target tissues liver and bone have been proven, thus introducing some uncertainty in the assessment. However, the RAC was of the opinion that the uncertainty is not sufficiently big to warrant a category 2 classification.

Reliable evidence of an adverse effect on reproduction in humans, which is required for Repr. 1A, was not available for Difenacoum, but a potential for human developmental toxicity is presumed based on the above stated weight of evidence assessment. Thus RAC proposed to classify Difenacoum as Repr. 1B; H360D, i.e. "presumed human reproductive toxicant", instead as Repr. 1A; H360D as proposed by the DS.

Setting specific concentration limits (SCLs):
Regarding SCLs for Difenacoum, it is acknowledged that the specific data on developmental toxicity of Difenacoum is too scarce to guide in setting the SCLs.

However, for Warfarin there is sufficient data to set a SCL for developmental toxicity. Thus, based on human data, doses of 2.5-5 mg/person/day (equivalent to 0.04-0.08 mg/kg bw/day) may cause developmental toxicity and could perhaps be regarded as an ED_{10} level. This human ED_{10} value would, if using the guidance for setting SCLs based on animal data, belong to the high potency group (< 4 mg/kg bw/day). The guidance states that for an ED_{10} <4 mg/kg bw/day, the SCL is 0.03%, and for ED_{10} below 0.4 mg/kg bw/day the SCL becomes 0.003%. Also if starting from an ED_{10} value obtained from animal studies (0.125 mg/kg bw/day; Kubaszky et al., 2009), it would qualify Warfarin for the high potency group and result in a SCL of 0.003%. Thus, RAC concluded on a SCL of 0.003% for the developmental toxicity of Warfarin.

As the other AVK rodenticides are equally or more toxic than Warfarin, it was not considered appropriate to apply the generic concentration limit (GCL) for these substances (0.3%), but rather to base the SCLs on the SCL proposed for Warfarin. Thus, RAC was of the opinion that the SCL for Warfarin can be used as a surrogate SCL for other AVK rodenticides resulting in a SCL of 0.003% for Difenacoum and the AVK rodenticides Flocoumafen, Defethialone, Coumatetralyl, Brodifacoum, Bromadiolone and Chlorophacinon.

Supplemental information - In depth analyses by RAC

Difenacoum is a second generation AVK-rodenticide, having the same MoA as Warfarin (EHC, 1995). Warfarin is known to cause death of embryos or foetuses and malformations, mainly nasal hypoplasia in humans. Since deformation of the naso-maxial part of the face is very specific, it is also referred to as human "Warfarin embryopathy", and Warfarin was consequently classified as a known human developmental toxicant in Reprotoxicity category 1A (H360D).

In addition to skeletal malformation, Warfarin may cause spontaneous abortion, stillbirth, neonatal death, premature delivery, and ocular atrophy, among which spontaneous abortion and stillbirth being the most frequent one (ca. 27% of pregnancies), and naso-maxial hypoplasia being the most frequent among live births (ca. 5% of pregnancies). Substitution of Warfarin by heparin during first trimester of pregnancy removes the risk of naso-maxial hypoplasia.

Hydroxycoumarin derivates are vitamin K antagonists. Second-generation anticoagulants (e.g. Difenacoum, Brodifacoum, Bromodiolone) are even more potent vitamin K antagonists than Warfarin. They inhibit vitamin K epoxide reductase and vitamin K reductase. Vitamin K is necessary for proper functioning of carboxylases needed for both blood coagulation and bone development. Effects on blood coagulation are shared between all AVKs. As vitamin K also is involved in bone formation, effects on bone formation is expected but only proven for some AVK rodenticides (Acenocoumarol, Phenprocoumon, Brodifacoum).

Considering the same MoA for Difenacoum and Warfarin, the question is whether they will have similar developmental toxicological effects in humans. There are no human data for Difenacoum. However, there are human evidences of developmental toxicity not only for Warfarin but also for the AVK coumarins, Brodifacoum, Acenocoumarol and Phenoprocoumon, making it plausible that also Difenacoum may be a human teratogen.

Another question is whether the developmental studies for Difenacoum have a predictive value for effects on humans. Clear developmental toxicity in response to Difenacoum was neither observed in rats nor in rabbits. This could either be because of no such inherent toxicity or because the animal studies are not sufficiently predictive for effects in humans.

Human Warfarin embryopathy may involve foetotoxicity (e.g., spontaneous abortion and stillbirth), ocular atrophy, and skeletal malformations. In some rat studies, Warfarin was indicated to cause foetotoxicity, foetal haemorraghes, and ocular effects. With very specific design of the studies,the bone-related malformations were detected in rats (Howe and Webster, 1990; Howe and Webster, 1992).

<u>Haemorrhages</u>
In a rat OECD TG 414 guideline study with Warfarin (Kubaszky, 2009) increased incidence (without clear dose-response) of foetal haemorrhages were observed. However, it should be noted that small foetal haemorrhages are not easily detected, and in the reporting of the Kubaszky study (2009) it was stated specifically that clinical observations were made "*with special attention to external signs of haemorrhages*". Considering the lack of dose-response, it can be questioned if the haemorrhages were substance-related. On the other hand, one may not expect a very clear dose-response considering the small dose spacing in this study (0.125-0.25 mg/kg bw/day).

It seems that haemorrhages can be picked up in an OECD TG 414 study. However, it is not clear how severe they need to be or if special attention is needed to note them, i.e. whether or not they would normally be detected in a standard OECD TG414 study.

No foetal haemorrhages were reported in the rat and rabbit studies on Difenacoum.

Bone effects
Human Warfarin embryopathy includes effects on bone formation, typically in the nose region. There were equivocal indications of disturbed ossification in skull bones (in foetuses from one mid-dose litter) in the Kubaszky study (2009). The finding of malformed skulls only concerned one single litter from the mid dose, with malformations in 2 out of 7 pups, indicating that a relationship with treatment was not likely. The critical period for nasal and skeletal development is not the same for humans (during the first trimester) and rats (late foetal/early postnatal period), and it was concluded that this malformation can therefore not be picked up by a standard rat/rabbit OECD TG 414 study.

Difenacoum data: In a 22 day rabbit study higher incidence of skeletal variations at two dose levels compared to controls without dose dependence were recognised. In a similar 13 day rabbit study foetal effects were observed in both test and control groups included defects not previously seen in this strain or laboratory. However, the effects were not dose related.

Maternal toxicity
In the Warfarin study by Kubaszky (2009) on rats maternal toxicity (vaginal bleeding, open vaginal orifice) and mortality was observed in test protocol 1 at doses 0.150 mg/kg bw/day and higher.

Maternal toxicity on Diefenacoum was observed at all dose levels (0.001; 0.003; 0.010 mg/kg bw/day) in a 22 day rabbit study and at 0.015 mg/kg bw/day in a similar 13 day rabbit study. In two rat studies maternal toxicity has been indicated at 0.09 mg/kg bw/day. Increased haemorrhages as well as increased coagulation times were noted.

These results are in line with RAC members´ previous comments that the dose causing foetal toxicity in rodents is close to the dose inducing significant maternal toxicity.

Toxicokinetics and transplacental transfer:
The AVK rodenticides have different physico-chemical characteristics (e.g. a range of 0.7-6.3 for the log Kow and 292-542 for the molecular weight) which lead to differences in kinetics, mainly expressed as different half-lives. This affects the potency, but a comparison of the toxicity profiles shows much smaller differences than indicated by the 5-6 orders of magnitude difference in lipophilicity.

It is noted that the AVK-drugs Acenocoumarol and Phenprocoumon exhibit teratogenicity despite having different pharmacokinetics (half-lives) than Warfarin. Half-lives of 2-8 hours were reported for Acenocoumarol, 30-45 hours for Warfarin and 156-172 hours for Phenprocoumon (Rane and Lindh, 2010). It seems that the MoA is more important than half-life as determinant for developmental toxicity.

Due to differences in physico-chemical properties and toxicokinetics (metabolism, liver accumulation, etc.) the transplacental transfer might differ between the various AVKs. Only one study investigated the transplacental transfer of AVKs in rats. Johnson (2009; see CLH report on Flocoumafen) studied the transplacental transfer of Warfarin and Flocoumafen in rats, at a stage when the placenta is fully developed (GD 19). From this study it appeared that both Warfarin and Flocoumafen can cross the maternal-foetal placental barrier in rats. However, in the rat there was a lower foetal availability of Flocoumafen than of Warfarin (the normalized Flocoumafen plasma concentration was 7-fold lower than that of Warfarin), but the concentration of Flocoumafen was higher in the foetus than in the dam, whereas the opposite was true for Warfarin. Although, it is not known what this difference in concentrations means, it seems important to mention it.

Other AVK anticoagulants have also been shown to cross the placenta in humans, e.g.,

Acenocoumarol and Phenindione (Hoyer, 2010). There are no data on Difenacoum.

It is concluded that all AVK rodenticides are expected to cross the placenta, and although there might be some quantitative differences, the toxicokinetic aspects are are expected to be qualitatively similar between Warfarin and Difenacoum in humans making it not possible to exclude similar effects of Warfarin and Difenacoum in humans.

5.10 Other effects

Not applicable.

5.11 Derivation of DNEL(s) or other quantitative or qualitative measure for dose response

Not relevant for this type of dossier.

6 HUMAN HEALTH HAZARD ASSESSMENT OF PHYSICO-CHEMICAL PROPERTIES

Not relevant for this type of dossier.

7 ENVIRONMENTAL HAZARD ASSESSMENT

7.1 Aquatic compartment (including sediment)

7.1.1 Toxicity test results

7.1.1.1 Fish

In total four acute tests are available on two different fish species. All tests are considered valid; they were carried out under GLP and according to standard test guidelines. Difenacoum concentrations declined during the test period in all tests, but this was considered acceptable, because the test results were based on the measured concentrations. Adsorption of difenacoum to fish or walls of aquaria and photolytic degradation may have contributed to the declined concentrations. Time dependent tendency of precipitation of difenacoum was also reported. Three of four tests showed rather similar toxicity to fish (0.258-0.557 mg/l), but one of the tests resulted in a lower LC50 of 0.064 mg/l. The test is considered valid despite the lack of dose related mortality. Taking also the other toxic symptoms into account the effects increased with the concentration. These effects were cough frequency, swimming position, abnormal swimming and lying on the bottom of the tank. The test is described in more detail below. The lack of dose response was also observed in avian dietary tests and was explained by the mode of action of difenacoum: it inhibits the blood clotting, but does not induce haemorrhages itself. All studies are summarised in the Table 17.

Reference: Doc II-A[1], 4.2.,1 A7.4.1.1 Difenacoum: Acute Toxicity to *Oncorhynchus mykiss*

The acute toxicity of Difenacoum (purity 96.3 %) to *Oncorhynchus mykiss* (Rainbow trout) was determined in a 96 h semi static test with renewal of the test media after 48 h. The study was carried out in accordance with requirements of Annex 5 (92/69/EEC) to EC Commission Directive 92/32/EEC: C.1. Acute Toxicity to Fish, and in compliance with GLP.

Seven fish were placed in each test vessel (15 l aquaria) and one vessel per concentration level was used in the test. Tanks were covered with perspex lids to minimize dust contamination and evaporation loss. Due to low water solubility of difenacoum dimethyl sulfoxide (DMSO) was used as a solvent when preparing the test solutions providing nominal concentrations: 0.0039, 0.009, 0.019, 0.04, 0.09 and 0.2 mg/l. Both control test and solvent control test were included in the test. Analytical samples were taken at the start of the test (0 h), at 48 h (before and after changing the test medium), and at 96 h. The actual test concentrations were: 0.001, 0.002, 0.008, 0.019, 0.054, and 0.145 mg/l. An accurate mean measured concentration for the nominal 0.0039 mg/l treatment could not be determined since the majority of samples analyzed were below the limit of quantification, and was therefore assigned an approximate value of 0.001 mg/l. Measured concentrations were 22-72 % of the nominal and they declined with time. Test temperature range was 13.6-15.6 °C, dissolved oxygen was 85-100 % of air saturation value and pH varied 7.4-7.7 during the test.

Mortality did not increase consistently with the concentration, as it was observed at the highest concentration (0.145 mg/l) and at the third highest concentration (0.019 mg/l). When sublethal symptoms were taken into account, the effects were dose related. The sublethal symptoms were an increased cough frequency, swimming position compared to controls, abnormal swimming or lying on the bottom of the tank. LC_{50} (96 h) value was determined to be 0.064 mg/l based on the measured concentrations which were calculated as arithmetic means of two geometric mean values (aged solution and renewed solution).

Table 17: Short-term toxicity to fish

Guideline GLP	Species	Endpoint	Exposure design	Exposure duration	Results LC_{50} mg/l	Remarks	Reference
OECD 203 GLP	Rainbow trout *Oncor-hynchus mykiss*	Mortality	Semi-static	96 h	0.064	Key study Mortality did not increase consistently with the concentration. Result is based on the measured concentrations which are calculated as arithmetic means of two geometric mean values (aged solution and renewed solution). Measured concentrations ranged from 22 to 73% of the nominal values. Dimethyl sulfoxide used as solvent.	Doc II-A[1], 4.2.1 (A7.4.1.1)
OECD 203 GLP	Bluegill sunfish *Lepomis macro-chirus*	Mortality	Semi-static	96 h	0.258	Dose related mortality. Result is based on the measured concentrations. Concentrations declined below 80% of the nominal concentrations.	Doc II-A[1], 4.2.1
OECD 203 GLP	Rainbow trout *Oncor-hynchus mykiss*	Mortality	Semi-static	96 h	0.33	Dose related mortality. Result is based on the measured concentrations that were 73-105% of nominal concentrations. Dimethylformamide used as solvent.	Doc II-A[3], 4.2.1 (A7.4.1.1)
OECD 203 GLP	Rainbow trout *Oncor-hynchus mykiss*	Mortality	Static	96 h	0.557	Dose related mortality. Result is based on the measured concentrations that were 27-36% of the nominal concentrations at the end of the test. Acetone used as solvent.	Doc II-A[2], 4.2.1 (A7.4.1.1)

No long-term fish tests are available.

7.1.1.2 Aquatic invertebrates

Table 18: Short-term toxicity to aquatic invertebrates

Guideline GLP	Species	Endpoint	Exposure design	Exposure duration	Results EC_{50} mg/l	Remarks	Reference
OECD 202 GLP	*Daphnia magna*	Immobility	Static	48 h	0.52	Dose related immobility. Result is based on the measured concentrations that ranged from 91 to 103% of the nominal values. Dimethylformamide used as solvent.	Doc II-A[1], 4.2.1 (A7.4.1.2)
OECD 202 GLP	*Daphnia magna*	Immobility	Static	48 h	0.61	Result is based on the measured concentrations.	Doc II-A[1], 4.2.1
OECD 202 GLP	*Daphnia magna*	Immobility	Static	48 h	0.705	Dose related immobility. Result is based on the measured concentrations that were 23-59% of the nominal concentrations at the end of the test. Acetone used as solvent.	Doc II-A[2], 4.2.1 (A7.4.1.2)
OECD 202 GLP	*Daphnia magna*	Immobility	Semi-static	48 h	0.91	Dose related immobility. Result is based on the measured concentrations that were 54-98% of nominal concentrations. Dimethylformamide used as solvent.	Doc II-A[3], 4.2.1 (A7.4.1.2)

No long-term toxicity tests to aquatic invertebrates are available.

7.1.1.3 Algae and aquatic plants

Table 19: Growth inhibition of algae

Guideline GLP	Species	Exposure design	Exposure duration	Results NOE_rC mg/l	Results E_rC_{50} mg/l	Remarks	Reference
OECD 201 GLP	*Selenastrum capricornutum*	Static	72 h	0.13	0.51	Dose related growth inhibition. Result is based on the measured concentrations that were 59-91% of nominal concentrations. Dimethylformamide used as solvent.	Doc II-A[3], 4.2.1 (A7.4.1.3)
OECD 201 GLP	*Selenastrum capricornutum*	Static	72 h	0.25	0.80	Dose related growth inhibition. Result is based on the measured concentrations that were 71-96 % of nominal concentrations. Dimethyl sulfoxide used as solvent.	Doc II-A[1], 4.2.1 (A7.4.1.3)

Guideline GLP	Species	Exposure		Results		Remarks	Reference
		design	duration	NOE_rC mg/l	E_rC_{50} mg/l		
OECD 201 GLP	*Desmo-desmus sub-spicatus*	Static	72 h	1.3	4.73	Dose related growth inhibition from 2.56 mg/l onwards, no significant inhibition at lower concentrations. Result is based on the measured concentrations that were 23-59% of the nominal concentrations at the end of the test. Acetone used as solvent.	Doc II-A[2], 4.2.1 (A7.4.1.3)

7.1.1.4 Sediment organisms

Not relevant for this type of dossier.

7.1.1.5 Other aquatic organisms

Not relevant for this type of dossier.

7.1.2 Calculation of Predicted No Effect Concentration (PNEC)

Not relevant for this type of dossier.

7.2 Terrestrial compartment

7.2.1 Toxicity test results

7.2.1.1 Toxicity to soil macro organisms

Not relevant for this type of dossier.

7.2.1.2 Toxicity to terrestrial plants

Not relevant for this type of dossier.

7.2.1.3 Toxicity to soil micro-organisms

Not relevant for this type of dossier.

7.2.1.4 Toxicity to other terrestrial organisms

Toxicity to birds

Toxicity to other above ground organisms

7.2.2 Calculation of Predicted No Effect Concentration (PNEC_soil)

Not relevant for this type of dossier.

7.3 Atmospheric compartment

Not relevant for this type of dossier.

7.4 Microbiological activity in sewage treatment systems

7.4.1 Toxicity to aquatic micro-organisms

Not relevant for this type of dossier.

7.4.2 PNEC for sewage treatment plant

Not relevant for this type of dossier.

7.5 Calculation of Predicted No Effect Concentration for secondary poisoning (PNEC_oral)

Not relevant for this type of dossier.

7.6 Conclusion on the environmental classification and labelling

Comparison with criteria

Table 20: Comparison of difenacoum data with criteria for environmental hazards

Endpoint	Results	Comparison with classification criteria
Degradation	Difenacoum is **hydrolytically stable** at environmentally relevant conditions. Difenacoum is not **readily biodegradable** under test conditions, as indicated by the ready and inherent biodegradability tests as well as soil biodegradation test. In the ready biodegradability tests according to OECD 301 guidelines, level of degradation was 0-31%, being therefore below the ready biodegradability pass level of 60 or 70%. In the inherent biodegradation test, the degradation was 3%. Difenacoum degrades slowly in soil with DT50 of 439 days	According to CLP and DSD criteria, **difenacoum is not readily/rapidly degradable in the environment**, based on ready and inherent biodegradation in water as well as biodegradation in soil. According to both regulations, a substance is regarded as readily/rapidly degradable if biodegradation level of of 70% is reached in a ready biodegradability test, fulfilling the 10-day window or if there is other convincing scientific evidence to demonstrate that the substance can be degraded in the aquatic environment to a level > 70 % within a 28-day period.
Bioaccumulation	Log K_{ow} 7.62 (estimation using Kowwin model, version 1.67) Log K_{ow} 7.6 (estimation using an atom/fragment contribution method)	The estimated log K_{ow} value is above the two classification criteria: log K_{ow} < 4 (CLP) and log K_{ow} < 3 (DSD). Therefore, according to CLP and DSD criteria, **difenacoum has potential to bioaccumulate.**
Acute hazard and long-term hazard	*Oncorhynchus mykiss* LC50 = 0.064 mg/l	Difenacoum fulfills the criteria for **R50-53** classification according to Directive 67/548/EEC (DSD) and the criteria for the proposed classification as **H400** according to Regulation EC 1272/2008 (CLP) (namely L(E)C50 ≤ 1 mg/l). In the case of the H400 classification according to CLP, a **M-factor of 10** is applicable based on 0.01 < L(E)C50 ≤ 0.1 mg/l. In the case of DSD classification, a specific concentration limits shall be applied: **R50-53: C ≥ 2.5%** **R51-53: 0.25% ≤ C ≤ 2.5%** **R52-53: 0.025% C ≤ 0.25%**
	Chronic toxicity data was available for algae only but not for other trophic levels (fish and crustacea). NOEC has been derived from the 72 h algae study. *Scenedesmus subspicatus* NOECr (72 h) = 0.13 mg/l	No adequate chronic data is available for all three trophic levels, thus the classification of difenacoum into the chronic category assessed using two approaches according to CLP (2nd ATP): 1. In the case of non-rapidly degradable substances for which there are adequate chronic toxicity data available **H411** classification is applicable for difenacoum based on 0.1 < NOEC < 1 mg/l. 2. In the case of non-rapidly degradable substances for which adequate chronic toxicity data are not available classification is based on the combination of acute aquatic toxicity data and environmental fate data; **H410** classification is applicable for difenacoum based on 96 h LC50 (for fish) ≤ 1 mg/l and the log K_{ow} ≥ 4. **M-factor of 10** derived for acute aquatic hazard classification is also applied to the chronic aquatic hazard classification. The most stringent outcome shall be chosen and therefore difenacoum shall be classified as **H410 with M-factor of 10** according to Regulation EC 1272/2008.

Conclusion of environmental classification according to Regulation EC 286/2011 (2nd ATP to EC 1272/2008)

Based on the CLP Regulation, difenacoum should be classified as:

Classification categories	Aquatic **acute category 1, M factor 10** Aquatic **chronic category 1, M factor 10**	
Hazard Statement	**H400**	'Very toxic to aquatic life',
	H410	'Very toxic to aquatic life with long lasting effects'

Conclusion of environmental classification according to Directive 67/548/EEC

Difenacoum should be classified Dangerous for the Environment with the following risk and safety phrases:

N	Dangerous for the Environment
R50-53	Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
S60	This material and its container must be disposed of as hazardous waste
S61	Avoid release to the environment. Refer to special instructions/safety data sheet

Specific concentration limits shall be applied:
R50-53: C ≥ 2.5%
R51-53: 0.25% ≤ C ≤ 2.5%
R52-53: 0.025% C ≤ 0.25%

RAC evaluation of environmental hazards

Summary of Dossier submitter's proposal

There is a current entry in Annex VI for Difenacoum with an environmental classification as Aquatic Acute 1 (H400) and Aquatic Chronic 1 (H410) with no M factors. The DS proposed to add to the current entry M-factors of 10 for both Aquatic Acute 1 and Aquatic Chronic 1.

Degradation
Degradation was studied in four hydrolysis tests, two photolysis tests in water, four ready biodegradability tests, one inherent biodegradation test and one degradation test in soil.

The DS considered Difenacoum as hydrolytically stable (DT_{50} > 1 year, pH =7, 25ºC) and rapidly photodegradable with an experimental half-life about 8 hours at pH 7. It was

degraded rapidly in the atmosphere by reaction with OH radicals, although the presence of this compound in air is not expected due to its low vapour pressure.

Difenacoum is not readily or inherently biodegradable under test conditions. In the ready biodegradability tests according to OECD TG 301B, OECD TG 301D, and OECD TG 301F, the level of degradation was between 0-31%, being therefore below the ready biodegradability pass levels of 60 or 70%. In the inherent biodegradation test according to OECD TG 302D draft guideline, the degradation was 3%.

Difenacoum showed a very slow degradation under aerobic conditions in soil with a DT_{50} of 439 days.

The DS concluded based on the available data that Difenacoum is not rapidly degradable.

Bioaccumulation
The estimated log K_{ow} of Difenacoum is 7.62, which is above the cut-off value of log $K_{ow} \geq 4$ in CLP. Furthermore, a bioaccumulation test on *Oncorhynchus mykiss* is available, and although it is not considered as a valid study due to the lack of measured concentrations in water, absence of steady-state and high mortality at the higher Difenacoum concentration, the test indicated accumulation of Difenacoum in fish.

In conclusion, since the log K_{ow} indicated high potential for bioaccumulation, the DS concluded that Difenacoum has potential for bioaccumulation.

Aquatic toxicity
Four acute toxicity studies in fish (*Oncorhynchus mykiss* and *Lepomis macrochirus*) with LC_{50} values between 0.064 and 0.557 mg/L, four tests in invertebrates (*Daphnia magna*) with EC_{50} = 0.52-0.91 mg/L and three studies in algae (*Pseudokirkneriella subcapitata* and *Desmodesmus subspicatus)* with E_rC_{50} = 0.51-4.73 and NOE_rCs = 0.13-1.3 mg/L were reported by the DS. No long-term tests in fish and invertebrate are available but the three algae tests can be also considered chronic tests. All the toxicity values for these tests were based on mean measured concentrations.

Fish (*Oncorhynchus mykiss*) was the most sensitive taxonomic group in acute tests, with LC_{50} value of 0.064 mg/l, while in chronic tests the most sensitive algae species was *Pseudokirkneriella subcapitata,* with a NOE_rC value of 0.13 mg/l. However, no adequate chronic data is available for all trophic levels, and in this case the surrogate approach from fish shall be chosen as the most stringent outcome to propose the aquatic chronic classification, taking into account that the substance is no rapidly biodegradable, the log $K_{ow} \geq 4$ and the LC_{50} (for fish) ≤ 1 mg/l (EC_{50} = 0.064 mg/L).

Comments received during public consultation

Three Member States supported the environmental classification proposed by the DS. One Member State agreed with the aquatic acute classification and the M-factor of 10 but asked if this M-factor was also for aquatic chronic classification.

In their post public consultation response, the DS confirmed that the M-factor of 10 was proposed for both, aquatic acute and aquatic chronic toxicity.

RAC assessment and comparison with criteria

Degradation
RAC agreed that Difenacoum can be considered hydrolytically stable and rapidly photodegradable based on the information provided in the CLH report.

RAC also agreed that Difenacoum is not readily or inherently biodegradable under test conditions. Furthermore, in an aerobic soil study Difenacoum showed a very slow degradation rate (DT_{50} = 439 days), therefore, based on these data, RAC agreed with the DS that Difenacoum should be considered **not rapidly degradable** according to CLP.

Bioaccumulation

The estimated log K_{ow} for Difenacoum was 7.62 which is above the cut-off values of log $K_{ow} \geq 4$ (CLP), therefore RAC agreed with the DS that Difenacoum has **high potential for bioaccumulation**.

Aquatic toxicity

The acute hazard classification should be based on the lowest acute toxicity value, i.e. LC_{50} of 0.069 mg/l (*Oncorhynchus mykiss,* OECD TG 203). Since this value is ≤ 1 mg/l, RAC agreed with the DS to classify Difenacoum as Aquatic Acute category 1 (H400) with an M-Factor of 10.

Regarding chronic toxicity, no adequate chronic data was available for all three trophic levels. Only chronic information from algae were submitted in the CLH report and according to the lowest NOEC of 0.13 mg/L a classification as Aquatic Chronic 2 (H411) could be applied for Difenacoum. However, the surrogate approach must also be applied for chronic toxicity due to the lack of chronic data for fish and invertebrates. Taking into account that the substance is not rapidly degradable, the log $K_{ow} \geq 4$ and the LC_{50} (fish) ≤ 0.1mg/L (0.069 mg/L), which was the highest acute toxicity between invertebrates and fish, classification as Aquatic Chronic 1 (H410) with an M- factor of 10 is justified.

In conclusion, RAC agreed with the DS's proposal to classify Difenacoum according to CLP criteria as Aquatic Acute 1 (H400) with an M-factor of 10 and Aquatic Chronic 1 (H410) with an M-factor of 10.

JUSTIFICATION THAT ACTION IS REQUIRED ON A COMMUNITY-WIDE BASIS

Difenacoum is an active substance in the meaning of Directive 91/414/EEC and Directive 98/8/EC and therefore subject to harmonised classification and labelling (Regulation EC 1272/2008 article 36(2)).

OTHER INFORMATION

REFERENCES

The classification proposal is based on the Assesment Report by the Finnish Competent Authority and on three separate Document II-As provided by the three applicants. Each Document II-A are referred to in the study summary tables and in the text as follows:

Doc II-A[1] (Competent Authority Report, Document II-A, Difenacoum, Sorex Limited, 10.3.2008)

Doc II-A[2] (Competent Authority Report, Document II-A, Difenacoum, Hentschke & Sawatzki KG, 10.3.2008)

Doc II-A[3] (Competent Authority Report, Document II-A, Difenacoum, the Activa/PelGar Brodifacoum and Difenacoum Task Force, 23.06.2009)

In the reference list below the different studies can be found on the basis on the applicant (Source) and the BPD ID Section No in question.

References for the studies reviewed in the Document II-As

Author(s)	**Year**	**Title.** **Source (where different from company)** **Company, Report No.** **(Un)Published**	**Data Protection Claimed (Yes/No)**	**Source**	**BPD ID Section No / Reference No**
Confidential	2003	Determination of the Melting Point and Boiling Point of Difenacoum Technical Chemex Environmental International Ltd., Report No. ENV5799/120139. GLP, Unpublished	Yes	Doc II-A[3]	A3.1.1
Confidential	1996	Difenacoum: Determination of Physico-chemical Properties. XXXXX, Report No: 355/7-1014. GLP, unpublished. [DF-959-0018]	Yes	Doc II-A[1]	A3.1.1/01
Confidential	2001	Difenacoum purified: thermal stability - melting point/ melting range - boiling point/ boiling range Siemens Axiva, Frankfurt, Germany, Report No.: 20011213.01 GLP, Not Published	Yes (New/First)	Doc II-A[2]	A3.1.1/01
Confidential	2001a	Difenacoum – Determination of the Relative Density ChemService S.r.l., Report No. CH-152/2000 GLP, Unpublished	Yes	Doc II-A[3]	A3.1.3

Author(s)	**Year**	**Title.** **Source (where different from company)** **Company, Report No.** **(Un)Published**	**Data Protection Claimed (Yes/No)**	**Source**	**BPD ID Section No / Reference No**
Confidential	1996	Difenacoum: Determination of Physico-chemical Properties. XXXXX, Report No: 355/7-1014. GLP, unpublished. [DF-959-0018]	Yes	Doc II-A[1]	A3.1.3/01
Confidential	2001	Difenacoum purified: relative density Siemens Axiva, Frankfurt, Germany, Report No.: 20011213.02 GLP, Not Published	Yes (New/First)	Doc II-A[2]	A3.1.3/01
Confidential	1997	Difenacoum - Determination of the Vapour Pressure ChemService S.p.A., Report No. CH-14/96-C-DIF GLP, Unpublished	Yes	Doc II-A[3]	A3.2
Confidential	2001	Difenacoum purified: vapour pressure Siemens Axiva, Frankfurt, Germany, Report No.: 20011213.03 GLP, Not Published	Yes (New/First)	Doc II-A[2]	A3.2/01
Confidential	2005	Difenacoum – Determination of Water Solubility SafePharm Laboratories Ltd., Report No. 1558/011 GLP, Unpublished	Yes	Doc II-A[3]	A3.5
Confidential	2002	Water Solubility of Difenacoum GAB & IFU, Niefern-Öschelbronn, Germany, Report No.: 20011378/01-PCSB GLP, Not Published	Yes (New/First)	Doc II-A[2]	A3.5/01
SafePharm Laboratories	2004	ACD/I- Lab Web Service (ACD/pKa 8.02) QSAR SafePharm Laboratories Ltd. Unpublished	No	Doc II-A[3]	A3.6
Confidential	2005	Physico-chemical testing with difenacoum: Estimation of dissociation constant and adsorption coefficient. XXXXX, Report No: 26059. GLP, unpublished. [DF-3.6-0386]	Yes	Doc II-A[1]	A3.6/01
Confidential	2006	Calculation of Partition-coefficient SafePharm Laboratories Ltd. Unpublished	No	Doc II-A[3]	A3.9(2)

Author(s)	**Year**	**Title.** **Source (where different from company)** **Company, Report No.** **(Un)Published**	**Data Protection Claimed (Yes/No)**	**Source**	**BPD ID Section No / Reference No**
Confidential	2002	Partition Coefficient of Difenacoum (HPLC Method) GAB & IFU, Niefern-Öschelbronn, Germany, Report No.: 20011378/01-PCPC GLP, Not Published	Yes (New/First)	Doc II-A[2]	A3.9/01
Confidential	1996	Difenacoum: Determination of Physico-chemical Properties. XXXXX, Report No: 355/7-1014. GLP, unpublished. [DF-959-0018]	Yes	Doc II-A[1]	A3.9/01
Confidential	2005	Determination of the Thermal Stability and Breakdown Products of Difenacoum Chemex Environmental International Ltd., Report No. ENV7063/120139 GLP, Unpublished	Yes	Doc II-A[3]	A3.10
Confidential	2000	Difenacoum: Evaluation of Thermal Properties by Differential Scanning Calorimetry. XXXXX, Report No: 355/50-D2141. GLP, unpublished. [DF-959-0078]	Yes	Doc II-A[1]	A3.10/01
Confidential	2005a	Physico-Chemical Properties Analysis on Difenacoum, XXXXX, Report No: GLP 13921R1V1/05 [DF-959-0173]	Yes	Doc II-A[1]	A3.11/01
Confidential	2001b	Difenacoum – Determination of the Explosive Properties ChemService S.r.l., Report No. CH-154/2000 GLP, Unpublished	Yes	Doc II-A[3]	A3.15
Confidential	2004	Explosivity of Difenacoum technical EBRC Consulting GmbH, Hannover, Germany, Report No.: HEN-040112-01 Not GLP, Not Published	Yes (New/First)	Doc II-A[2]	A3.15/01
Confidential	2005	Assessment of Potential Oxidising and Explosive Properties of Difenacoum, XXXXX, Report No: J13516R1V1/05 [DF-959-0176]	Yes	Doc II-A[1]	A3.15/1 A3.16/1

Author(s)	**Year**	**Title.** **Source (where different from company)** **Company, Report No.** **(Un)Published**	**Data Protection Claimed (Yes/No)**	**Source**	**BPD ID Section No / Reference No**
Confidential	2004	Oxidising properties of Difenacoum technical EBRC Consulting GmbH, Hannover, Germany, Report No.: HEN-040112-02 Not GLP, Not Published	Y (New/First)	Doc II-A[2]	A3.16/01
Confidential	2005b	Oxidising Properties on a Sample of Difenacoum, XXXXX, Report No: GLP14238R1V3/05 [DF-959-0364]	Yes	Doc II-A[2]	A3.16/02
Confidential	2001c	Difenacoum – Determination of the Oxidizing Properties ChemService S.r.l., Report No. CH-156/2000 GLP, Unpublished	Yes	Doc II-A[3]	A3.16/1
Confidential	2006	Difenacoum Technical – Determination of the Oxidizing Properties ChemService S.r.l., Report No. CH-267/2006 GLP, Unpublished	Yes	Doc II-A[3]	A3.16/2
Confidential	2004	Acute Oral Toxicity Study (Acute Toxic Class Method) of Test Item Difenacoum Technical in Rats XXXXX, Report No. 04/904-001P. GLP, Unpublished	Yes	Doc II-A[3], 3.2	A6.1.1
Confidential	1995a	Difenacoum: Acute Oral Toxicity Study in the Male Wistar Rat. XXXXX, Report no: 355/34-1032. GLP, unpublished. [DF-959-0011].	Yes	Doc II-A[1], 3.2	A6.1.1/01
Confidential	2002	Acute toxicity study of Difenacoum technical by oral administration to sprague-dawley rats XXXXX, GLP, Not Published	Yes (New/First)	Doc II-A[2], 3.2	A6.1.1/01
Confidential	1995c	Difenacoum – Acute Oral Toxicity Study in the Rat. XXXXX, Report No: 355/8-1032. GLP, unpublished. [DF-959-0006].	Yes	Doc II-A[1], 3.2	A6.1.1/02
Confidential	1973a	Acute Oral Toxicity of WBA 8107 to Male Albino Mice. XXXXX, Report No: RIC0943. Not GLP, unpublished. [C2.1/17].	Yes	Doc II-A[1], 3.2	A6.1.1/03

Author(s)	**Year**	**Title.** **Source (where different from company)** **Company, Report No.** **(Un)Published**	**Data Protection Claimed (Yes/No)**	**Source**	**BPD ID Section No / Reference No**
Confidential	1998	Acute Oral Toxicity (LD_{50}) Tests with Cis- and Trans-isomers and a Racemic Mixture of Difenacoum. XXXXX, Report No. 3175/2/2/98. GLP, unpublished. [DF-959-0065].	Yes	Doc II-A[1], 3.2	A6.1.1/04
Confidential	2004	Acute Dermal Toxicity Study of Test Item Difenacoum Technical in Rats XXXXX, Report No. 04/904-002P. GLP, Unpublished	Yes	Doc II-A[3], 3.2	A6.1.2
Confidential	1995d	Difenacoum: Acute Dermal Toxicity Study in the Rat. XXXXX, Report No: 355/9-1032. GLP, unpublished. [DF-959-0007].	Yes	Doc II-A[1], 3.2	A6.1.2/01
Confidential	2002	Acute toxicity study of Difenacoum technical in sprague-dawley rats by dermal administration XXXXX, GLP, Not Published	Yes (New/First)	Doc II-A[2], 3.2	A6.1.2/01
Confidential	1995	Difenacoum – 4-Hour Acute Inhalation Toxicity Study to the Rat, XXXXX, Report No MLS/9825. GLP, Unpublished	Yes	Doc II-A[3], 3.2	A6.1.3
Confidential	1996	Difenacoum: Single Dose Inhalation (Head-Only) Toxicity Study in the Rat. XXXXX, Report No: 355/11-1050. GLP, unpublished. [DF-959-0025].	Yes	Doc II-A[1], 3.2	A6.1.3/01
Confidential	2003	4-week dose range-finding study for a 90-day subchronic toxicity study of Difenacoum technical by repeated oral administration to sprague-dawley rats XXXXX GLP, Not Published	Yes (New/First)	Doc II-A[2], 3.5	A6.3.1/01
Confidential	1995	Difenacoum – 90-day Feeding Study in the Rat XXXXX, Report No. MLS/10016. GLP, Unpublished	Yes	Doc II-A[3], 3.5	A6.4.1

Author(s)	**Year**	**Title.** **Source (where different from company)** **Company, Report No.** **(Un)Published**	**Data Protection Claimed (Yes/No)**	**Source**	**BPD ID Section No / Reference No**
Confidential	1994c	Difenacoum: 6 Week Oral Toxicity Study In Dogs. XXXXX, Report No: TL/L/5738. Not GLP (uncompleted study), unpublished. [CTL/L/5738, SuppSeries].	Yes	Doc II-A[1], 3.5	A6.4.1/01
Confidential	2003	90-day subchronic toxicity study of Difenacoum technical by repeated oral administration to CD rats XXXXX GLP, Not Published	Yes (New/First)	Doc II-A[2], 3.5	A6.4.1/01
Confidential	1995	Difenacoum – Development Toxicity to the Rat XXXXX, Report No. MLS/10013. GLP, Unpublished	Yes	Doc II-A[3], 3.8.1	A6.8.1 (1)
Confidential	2004	Teratology Study of the Test Item Difenacoum Technical in Rabbits XXXXX, Report No. 03/738-105N. GLP, Unpublished	Yes	Doc II-A[3], 3.8.1	A6.8.1 (2)
Confidential	1994a	Difenacoum: Developmental Toxicity Study in the Rat. XXXXX, Report No: CTL/P/4354. GLP, unpublished. [C2.5/01].	Yes	Doc II-A[1], 3.8.1	A6.8.1/01
Confidential	1994b	Difenacoum: Developmental Toxicity Study in the Rabbit. XXXXX, Report No: CTL/P/4245. GLP, unpublished. [C2.5/02].	Yes	Doc II-A[1], 3.8.1	A6.8.1/02
Confidential	2004	Two Generation Reproduction Toxicity Study of Test Item Difenacoum Technical in Rats XXXXX, Report 03/738-202P. GLP, Unpublished	Yes	Doc II-A[3], 3.8.2	A6.8.2
Confidential	1997	Difenacoum –Determination of Abiotic Degradation Hydrolysis as a Function of pH ChemService S.p.A., Report No. CH-15/96-B-DIF. GLP, Unpublished	Yes	Doc II-A[3]	A7.1.1.1.1
Confidential	2002	Abiotic degradation of Difenacoum - hydrolysis as a function of ph GAB & IFU, Niefern-Öschelbronn, Germany, Report No.: 20011378/01-PCHY GLP, Not Published	Yes (New/First)	Doc II-A[2]	A7.1.1.1.1/01

Author(s)	**Year**	**Title.** **Source (where different from company)** **Company, Report No.** **(Un)Published**	**Data Protection Claimed (Yes/No)**	**Source**	**BPD ID Section No / Reference No**
Confidential	1996	Difenacoum: Determination of Physico-chemical Properties. XXXXX, Report No: 355/7-1014. GLP, unpublished. [DF-959-0018]	Yes	Doc II-A[1]	A7.1.1.1.1/01
Confidential	1992a	Difenacoum: Hydrolysis Study. XXXXX, Report No: 7031. GLP, unpublished. [F4.1/01].	Yes	Doc II-A[1]	A7.1.1.1.1/02
Condifential	2004	Determination of the Direct Photolysis Rate in Water by Sunlight of Difenacoum Chemex Environmental International Ltd., Report No. ENV6767/120139. GLP, Unpublished	Yes	Doc II-A[3]	A7.1.1.1.2 (1)
Confidential	1992	Difenacoum: Photolysis in Buffered Aqueous Solutions. XXXXX, Report No: XXXXX. GLP, unpublished. [F4.1/02].	Yes	Doc II-A[1] Doc II-A[2]	A7.1.1.1.2/01
Confidential	2003	Determination of the Ready Biodegradability of Difenacoum Technical Chemex Environmental International Ltd. Report No. ENV5798/120139. GLP, Unpublished	Yes	Doc II-A[3]	A7.1.1.2.1
Confidential	2003	Difenacoum – Determination of Ready Biodegradability by the Closed Bottle Test. XXXXX, Report No: 21948. GLP, unpublished. [DF-959-0123].	Yes	Doc II-A[1]	A7.1.1.2.1/01
Confidential	2005	Assessment of the ready biodegradability of Difenacoum with the closed bottle test. GAB, Niefern-Öschelbronn, Germany, Report No.: 20011378/02-AACB, June 20/2005. GLP, Not Published	Yes (New/First)	Doc II-A[2]	A7.1.1.2.1/02

Author(s)	**Year**	**Title.** **Source (where different from company)** **Company, Report No.** **(Un)Published**	**Data Protection Claimed (Yes/No)**	**Source**	**BPD ID Section No / Reference No**
Confidential	2005	Manometric Respirometry Test (according to EC method C.4-D and OECD 301 F) – Test item: Difenacoum. Fraunhofer-Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany, Report no. XXXXX 001 / 3-15, May 17, 2005 (unpublished).	Yes (New/First)	Doc II-A[2]	A7.1.1.2.1/03
Confidential	2005	Evaluation of the Determination of the Inherent Biodegradability of Difenacoum Chemex Environmental International Ltd., Report No., ENV7148/120139. GLP, Unpublished	Yes	Doc II-A[3]	A7.1.1.2.2
Confidential	2002	Difenacoum: Physico-Chemical Testing with Difenacoum: Estimation of Adsorption Coefficient. XXXXX, Report No: 21677. GLP, unpublished. [DF-959-0117].	Yes	Doc II-A[1]	A7.1.3/01
Confidential	1992b	(^{14}C)-Difenacoum: A Study of the Degradation in Two Soils. XXXXX, Report No: 6927. GLP, unpublished. [F3.1/02].	Yes	Doc II-A[1]	A7.2.1/01
Confidential	2003	Estimation of the photochemical oxidative degradation rate in the atmosphere of Difenacoum EBRC Consulting GmbH, Hannover, Germany, Report No.: HEN-031114-01 Not GLP, Not Published	Yes (New/First)	Doc II-A[2]	A7.3.1/01
Confidential	2003a	The Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) of Difenacoum Technical XXXXX, Report No. ENV5794/120139. GLP, Unpublished	Yes	Doc II-A[3], 4.2.1	A7.4.1.1
Confidential	2002a	Acute toxicity testing of Difenacoum in rainbow trout *(Oncorhynchus mykiss)* (teleostei, salmonidae) XXXXX, Germany, Report No.: 20011378/01-AAOm GLP, Not Published	Yes (New/First)	Doc II-A[2], 4.2.1	A7.4.1.1/01

Author(s)	**Year**	**Title.** **Source (where different from company)** **Company, Report No.** **(Un)Published**	**Data Protection Claimed (Yes/No)**	**Source**	**BPD ID Section No / Reference No**
Confidential	1995b	Difenacoum: Acute Toxicity to *Oncorhynchus mykiss.* XXXXX, Report Number: 355/17-1018. GLP, unpublished. [DF-959-0030].	Yes	Doc II-A[1], 4.2.1	A7.4.1.1/01
Confidential	1995c	Difenacoum: Acute Toxicity to Lepomis macrochirus. XXXXX, Report Number: 355/23-1018. GLP, unpublished. [DF-959-0033].	Yes	Doc II-A[1], 4.2.1	A7.4.1.1/02
Confidential	2003b	The Toxicity to *Daphnia magna* of Difenacoum Technical XXXXX, Report No. ENV5793/120139. GLP, Unpublished	Yes	Doc II-A[3], 4.2.1	A7.4.1.2
Confidential	2002b	Assessment of toxic effects of Difenacoum on Daphnia magna using the 48h acute immobilisation test XXXXX, Germany, Report No.: 20011378/01-AADm GLP, Not Published	Yes (New/First)	Doc II-A[2], 4.2.1	A7.4.1.2/01
Confidential	1991	Difenacoum: Acute Toxicity to Daphnia magna. XXXXX, Report no:BL4314/B. GLP, unpublished. [G6.1/01D].	Yes	Doc II-A[1], 4.2.1	A7.4.1.2/02
Confidential	2003 c	The Growth Inhibition of the alga *Selenastrum capricornutum* by Difenacoum Technical XXXXX, Report - ENV5792/120139. GLP/Unpublished	Yes	Doc II-A[3], 4.2.1	A7.4.1.3
Confidential	2002	Testing of toxic effects of Difenacoum on teh single cell green alga Desmodesmus subspicatus XXXXX, Germany, Report No.: 20011378/01-AADs GLP, Not Published	Yes (New/First)	Doc II-A[2], 4.2.1	A7.4.1.3/01
Confidential	1995 d	Difenacoum: Inhibition of growth to the alga Selenastrum capricornutum. XXXXX, Report Number: 355/19-1018. GLP, unpublished. [DF-959-0032].	Yes	Doc II-A[1], 4.2.1	A7.4.1.3/01

Author(s)	**Year**	**Title.** **Source (where different from company)** **Company, Report No.** **(Un)Published**	**Data Protection Claimed (Yes/No)**	**Source**	**BPD ID Section No / Reference No**
Confidential	2004	The Bioconcentration potential of Difenacoum in Rainbow Trout (Oncorhynchus mykiss) under flow-through conditions. XXXXX. Report No. ENV6596/120139. GLP, Unpublished	Yes	Doc II-A[3]	A7.4.3.3.1
Confidential	1982	Difenacoum: Leaching of Formulated Material in Soil Columns. XXXXX, Report No: RJ 0266B. Not GLP, unpublished. [F3.2/03]	Yes	Doc II-A[1]	B 7.1/01

References from publicly available sources

Author(s)	Year	Title. Source (where different from company) Company, Report No. (Un)Published	Data Protection Claimed (Yes/No)	Source	BPD ID Section No / Reference No
Alessandri, J-L. D., Ramful, D. and Cuillier, F.	2010	Binder phenotype and brachytelephalangic chondrodysplasia punctata secondary to maternal vitamin K deficiency. Clin Dysmorphol 19: 85-87	No	Public	
Basude, S., Hein, C., Curtis, S.L., Clark, A. and Trinder, J.	2012	Low-molecular-weight heparin or warfarin for anticoagulatio in pregnant women with mechanical heart valves: what are the risks? a retrospective observational study. BJOG 119: 1008-1013	No	Public	
Blickstein, D. and Blickstein, I.	2002	The risk of fetal loss associated with Warfarin anticoagulation. Int J Gynecol Obst 78: 221-225	No	Public	
Brenner, B., Kuperman, A.A., Watzka, M. and Oldenburg, J.	2009	Vitamin K-dependent coagulation factors deficiency Semin Thromb Hemost 35: 439-446	No	Public	
De Rijk, E.P.C.T, Van Esch, E. and Flik, G.	2002	Pregnancy dating in the rat: Placental morphology and maternal blood parameters. Toxicol Pathol 30: 271-282	No	Public	
Driel Van, D., Wesseling, J., Sauer, P.J.J., Touwen, B.C.L., Veer Van Der, E. and Heymans, H.S.A.	2002	Teratogen update: fetal effects after in utero exposure to coumarins Overview of cases, follow-up findings, and pathogenesis Teratology 66: 127-140	No	Public	
Eventov_Friedman, S., Klinger, G. and Shinwell, E.S.	2009	Third trimester fetal intracranial haemorrhage owing to vitamin K deficiency associated with hyperemesis gravidarum. J Pediatr Hematol Oncol 31: 985-988	No	Public	

Author(s)	Year	Title. Source (where different from company) Company, Report No. (Un)Published	Data Protection Claimed (Yes/No)	Source	BPD ID Section No / Reference No
Hiraike, H., Kimura, M. and Itokawa, Y.	1988	Distribution of K vitamins (phylloquinone and menaquinones) in human placenta and maternal and umbilical cord plasma. Am J Obstet Gynecol 158: 564-569	No	Public	
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