Committee for Risk Assessment

RAC

Annex 1

Background document

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

acetamiprid (ISO); (1*E*)-*N*-[(6-chloropyridin-3-yl)methyl]-*N'*-cyano-*N*-methylethanimidamide; (*E*)-N^1-[(6-chloro-3-pyridyl)methyl]-N^2-cyano-N^1-methylacetamidine

EC Number: -

CAS Number: 135410-20-7; 160430-64-8

CLH-O-0000006797-57-01/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

4 May 2020

CLH report

Proposal for Harmonised Classification and Labelling

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

International Chemical Identification:

acetamiprid (ISO); (1*E*)-*N*-[(6-chloropyridin-3-yl)methyl]-*N'*-cyano-*N*-methylethanimidamide; (*E*)-N^1-[(6-chloro-3-pyridyl)methyl]-N^2-cyano-N^1-methylacetamidine

EC Number: -

CAS Number: **135410-20-7; 160430-64-8**

Index Number: **608-032-00-2**

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Version number: 3 **Date: October 2018**

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON ACETAMIPRID (ISO); (1*E*)-*N*-[(6-CHLOROPYRIDIN-3-YL)METHYL]-*N'*-CYANO-*N*-METHYLETHANIMIDAMIDE; (*E*)-N^1-[(6-CHLORO-3-PYRIDYL)METHYL]-N^2-CYANO-N^1-METHYLACETAMIDINE

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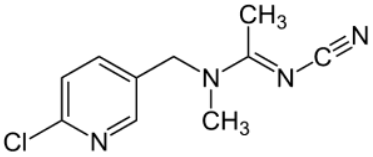
ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON ACETAMIPRID (ISO); (1*E*)-*N*-[(6-CHLOROPYRIDIN-3-YL)METHYL]-*N'*-CYANO-*N*-METHYLETHANIMIDAMIDE; (*E*)-N^1-[(6-CHLORO-3-PYRIDYL)METHYL]-N^2-CYANO-N^1-METHYLACETAMIDINE

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	(1*E*)-*N*-[(6-chloropyridin-3-yl)methyl]-*N'*-cyano-*N*-methylethanimidamide; (*E*)-N^1-[(6-chloro-3-pyridyl)methyl]-N^2-cyano-N^1-methylacetamidine
Other names (usual name, trade name, abbreviation)	
ISO common name (if available and appropriate)	Acetamiprid
EC number (if available and appropriate)	Not allocated
EC name (if available and appropriate)	
CAS number (if available)	135410-20-7; 160430-64-8
Other identity code (if available)	CIPAC No. 649
Molecular formula	$C_{10}H_{11}ClN_4$
Structural formula	
SMILES notation (if available)	CC(=NC#N)N(C)CC1=CN=C(C=C1)Cl
Molecular weight or molecular weight range	222.68 g/mol
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	
Description of the manufacturing process and identity of the source (for UVCB substances only)	
Degree of purity (%) (if relevant for the entry in Annex VI)	≥99%

1.2 Composition of the substance

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	**Concentration range (% w/w minimum and maximum in multi-constituent substances)**	**Current CLH in Annex VI Table 3.1 (CLP)**	**Current self-classification and labelling (CLP)**
Acetamiprid CAS number 135410-20-7; 160430-64-8	≥99%	Acute Tox. 4* (H302) Aquatic Chronic 3 (H412)	Acute Tox. 4 (H302) / Acute Tox. 3 (H301) Aquatic Chronic 3 (H412)

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
Confidential				Not relevant

Table 4: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	Function	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The additive contributes to the classification and labelling
Confidential					Not relevant

Table 5: Test substances (non-confidential information) (this table is optional)

Identification of test substance	Purity	Impurities and additives (identity, %, classification if available)	Other information	The study(ies) in which the test substance is used

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 6:

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	608-032-00-2	acetamiprid (ISO); (*E*)-N^1-[(6-chloro-3-pyridyl)methyl]-N^2-cyano-N^1-methylacetamidine		135410-20-7	Acute Tox. 4* Aquatic Chronic 3	H302 H412	GHS07 Wng	H302 H412			
Dossier submitters proposal	608-032-00-2	acetamiprid (ISO); (1*E*)-*N*-[(6-chloropyridin-3-yl)methyl]-*N'*-cyano-*N*-methylethanimidamide; (*E*)-N^1-[(6-chloro-3-pyridyl)methyl]-N^2-cyano-N^1-methylacetamidine		135410-20-7; 160430-64-8	**Retain** Aquatic Chronic 1 **Modify** Acute Tox. 3 **Add** Carc. 2 Repr. 2 Aquatic Acute 1	 H301 H351 H361d H400 H410	 GHS06 GHS08 GHS09 Dgr	 H301 H351 H361 H410		 M = 10 M = 100	
Resulting Annex VI entry if agreed by RAC and COM	608-032-00-2	acetamiprid (ISO); (1*E*)-*N*-[(6-chloropyridin-3-yl)methyl]-*N'*-cyano-*N*-methylethanimidamide; (*E*)-N^1-[(6-chloro-3-pyridyl)methyl]-N^2-cyano-N^1-methylacetamidine		135410-20-7; 160430-64-8	Acute Tox. 3 Carc. 2 Repr. 2 Aquatic Acute 1 Aquatic Chronic 1	H301 H351 H361d H400 H410	GHS06 GHS08 GHS09 Dgr	H301 H351 H361 H410		 M = 10 M = 100	

Table 7: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	Hazard class not assessed in this dossier	No
Flammable gases (including chemically unstable gases)	Hazard class not assessed in this dossier	No
Oxidising gases	Hazard class not assessed in this dossier	No
Gases under pressure	Hazard class not assessed in this dossier	No
Flammable liquids	Hazard class not assessed in this dossier	No
Flammable solids	Hazard class not assessed in this dossier	No
Self-reactive substances	Hazard class not assessed in this dossier	No
Pyrophoric liquids	Hazard class not assessed in this dossier	No
Pyrophoric solids	Hazard class not assessed in this dossier	No
Self-heating substances	Hazard class not assessed in this dossier	No
Substances which in contact with water emit flammable gases	Hazard class not assessed in this dossier	No
Oxidising liquids	Hazard class not assessed in this dossier	No
Oxidising solids	Hazard class not assessed in this dossier	No
Organic peroxides	Hazard class not assessed in this dossier	No
Corrosive to metals	Hazard class not assessed in this dossier	No
Acute toxicity via oral route		Yes
Acute toxicity via dermal route	Hazard class not assessed in this dossier	No
Acute toxicity via inhalation route	Hazard class not assessed in this dossier	No
Skin corrosion/irritation	Hazard class not assessed in this dossier	No
Serious eye damage/eye irritation	Hazard class not assessed in this dossier	No
Respiratory sensitisation	Hazard class not assessed in this dossier	No
Skin sensitisation	Hazard class not assessed in this dossier	No
Germ cell mutagenicity	Hazard class not assessed in this dossier	No
Carcinogenicity		Yes
Reproductive toxicity		Yes
Specific target organ toxicity-single exposure	Hazard class not assessed in this dossier	No
Specific target organ toxicity-repeated exposure	Hazard class not assessed in this dossier	No
Aspiration hazard	Hazard class not assessed in this dossier	No
Hazardous to the aquatic environment		Yes
Hazardous to the ozone layer	Hazard class not assessed in this dossier	No

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

Acetamiprid is approved as an active substance in plant protection products since January 1, 2005 (2004/99/EC) and expired on April, 30 2017 (1197/2012). It has been extended until April 2018 (EU 2016/2016). The review report for the active substance acetamiprid by the European Commission (EC, 2004) is dated June 16, 2004 and provides endpoints agreed during the first inclusion evaluation (Appendix II to the Review Report). The EFSA conclusion from October 2016 is available for acetamiprid (http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2016.4610/epdf).

A reasoned opinion on the review of the existing maximum residue levels (MRLs) for acetamiprid according to Article 12 of Regulation (EC) No 396/2005 from 2012 is available in (EFSA, 2011).

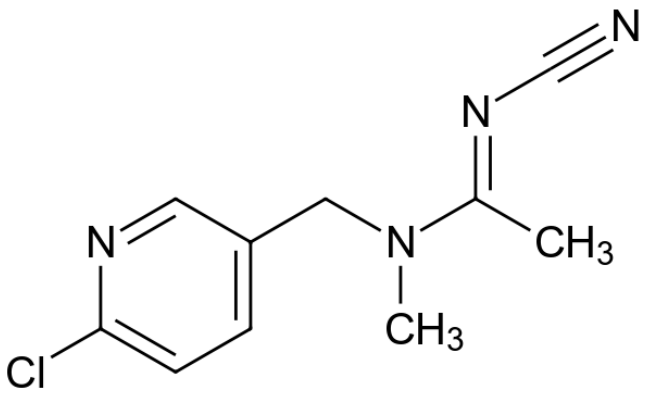
Acetamiprid is currently included in Annex VI of Regulation (EC) No 1272/2008 with Index Number 608-032-00-2 (CAS Number 135410-20-7) and classified as Acute Tox 4* (H302) and Aquatic Chronic 3 (H412).

A Renewal Assessment Report was prepared by the Netherlands EC November 2015 with Spain as co-RMS. A public consultation of the RAR was performed by ECHA with a deadline of February 2016. The final version was the updated RAR of August 2016.

The evaluation of acetamiprid under the Biocidal Product Regulation (Regulation (EU) No 528/2012) is currently ongoing (PT18). BE is the RMS for this evaluation.

RAC general comment

Acetamiprid is a pyridyl-methylamine insecticide registered for various applications, which has been evaluated in the context of both the Biocidal Products Regulation (BPR) (EU) 528/2012 (ECHA, 2017) and the Plant Protection Products (PPP) Regulation (EC) 1107/2009 (EFSA, 2012). Acetamiprid acts by affecting the central nervous system of insects, causing paralysis and death. Acetamiprid is a neonicotinoid insecticide, which acts on harmful organisms by contact and ingestion.



Acetamiprid is already included in Annex VI of the CLP Regulation (EC) No 1272/2008 with Index Number 608-032-00-2 (CAS Number 135410-20-7) and classified as Acute Tox. 4*; H302 and Aquatic Chronic 3; H412. The proposed change in the existing entry is due to the presence of a minimum classification and to some new data presented in the Renewal Assessment Report (RAR) on the renewal of the approval of the active substance acetamiprid as a PPP. In addition, the EFSA Pesticide Peer Review (PPR) no. 146 of 2016 concluded with a recommendation of classification as Carc. Cat. 2 on the basis of the two-year rat study.

A new DNT study was submitted for the BPC review and was part of the RAR for the PPP renewal (Anon., originally dated 2003, [2008 (revised report)]). No reproductive or developmental toxicity classifications were proposed during the previous reviews (PPP or BPR). The DS proposed Repr. 2 (H361) for developmental effects based on existing studies. A classification proposal for Carc. 2 was made during the PPP peer review 2016 but not during the BPR review (CAR 2018).

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

[B.] Justification that action is needed at Community level is required.

Reason for a need for action at Community level:

Change in existing entry due to the presence of a minimum classification and new data: more information on the acute toxicity, reproductive toxicity, specific target organ toxicity repeated exposure, and aquatic toxicity has become available in recent years which warrants a more severe classification of some of the mentioned endpoints.

5 IDENTIFIED USES

Acetamiprid is used in agriculture as an insecticide to control herbivorous (sucking and biting) insects and is applied as a foliar spray on crops.

6 DATA SOURCES

The Renewal Assessment Report (RAR) on the renewal of the approval of the active substance acetamiprid of the EU was used (EC, 2016). Data on acetamiprid were collected from publically available data through a search using several databases including PubMed and ToxNet. Acetamiprid is not registered under REACH.

7 PHYSICOCHEMICAL PROPERTIES

Table 8: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Pure a.s. (99.9%): white fine powder, with no characteristic odour Technical a.s. (99.9%): very pale yellow fine powder, with no characteristic odour	EC (2016)	measured
Melting/freezing point	98.9°C (99.7%)	EC (2016)	measured
Boiling point	Not relevant	EC (2016)	measured
Relative density	Specific gravity (20°C/20°C): 1.330 (99.7%)	EC (2016)	
Vapour pressure	$1.73x10^{-7}$ Pa at 50°C (>99%). Expected $<1x10^{-6}$ Pa at 25°C	EC (2016)	measured
Surface tension	1 g/L solution: 70.9 mN/m at 19.5°C	EC (2016)	measured
Water solubility	In distilled water: 4.25 g/l at 25°C (>99%) pH 5: 3.48 g/l at 25°C (>99%) pH 7: 2.95 g/l at 25°C (>99%) pH 9: 3.96 g/l at 25°C (>99%)	EC (2016)	measured
Partition coefficient n-octanol/water	log P_{ow}= 0.80 at 25°C (>99%)	EC (2016)	measured, shake flask
Flash point	Not relevant, as the test substance is a solid with a melting point above 40°C.	EC (2016)	expert statement
Flammability	Not highly flammable	EC (2016)	measured
Explosive properties	Non-explosive	EC (2016)	measured
Self-ignition temperature	No self-ignition temperature was determined up to 450°C	EC (2016)	measured
Oxidising properties	No oxidising properties	EC (2016)	expert statement
Granulometry	Not provided		
Stability in organic solvents and identity of relevant degradation products	Not provided		
Dissociation constant	pKa: 0.7 at 25°C	EC (2016)	measured
Viscosity	Not provided		

8 EVALUATION OF PHYSICAL HAZARDS

Not evaluated in this dossier.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Not evaluated in this dossier.

10 EVALUATION OF HEALTH HAZARDS

Acute toxicity

10.1 Acute toxicity - oral route

One new acute oral toxicity study (Anonymous, 2002) was submitted for the renewal of acetamiprid since the previous evaluation in DAR 2001.

In the study by Anonymous (1997i), 5 Crj: CD (SD) male rats/dose were treated with a single oral dose of 100, 150, 230, 340, 510 or 760 mg/kg b.w. of acetamiprid suspended in ion-exchanged water. 5 Crj: CD (SD) female rats/dose were treated with 70, 110, 150, 190, 230, 340, 510 or 760 mg/kg b.w.. All rats were observed for 14 days post-dosing for toxic signs and mortality. Body weight was recorded prior to dosing, and on days 1, 2, 3, 7 and 14 post-dosing, at termination or when an animal was found dead. All animals, both surviving till the end of the observation period and those found dead during the observation period, were subjected to gross necropsy. Note: the doses tested for each sex were determined in a range finding study.

Mortality rates are shown in Table 9, all deaths occurred within one day post administration. Clinical signs noted in the treated rats shortly after administration were lacrimation (one male from 100 mg/kg bw group), mydriasis (all dose groups, except females dosed 110 mg/kg bw), tremor (males dosed ≥ 230 mg/kg bw; females dosed 150 mg/kg and ≥ 250 mg/kg bw), clonic convulsion (both sexes, dosed ≥230 mg/kg bw), prone position (males dosed ≥ 340 mg/kg bw; females dosed ≥ 230 mg/kg bw) and lateral position (one male from 760 mg/kg bw group and that rat died on day of administration). Their incidence reached maximum at 1 or 3 hours post dosing while almost no sign was evident one day after administration. A body weight transient decrease was noted on day 1 in males dosed with 340 mg/kg. Body weight gains by all other surviving animals during the study were unaffected. No abnormality was observed during the gross necropsy of all animals.

The acute oral LD_{50} value for acetamiprid was calculated to be 417 mg/kg b.w. (95% confidence interval of 273-640 mg/kg b.w.) for male rats, and 314 mg/kg b.w. (95% confidence interval of 239-432 mg/kg b.w.) for female rats.

Table 9: Acute oral toxicity of acetamiprid in rats by Anonymous (1997i)

Sex	Dose (mg/kg)	Mortality incidence
Male	100	0/5
	150	0/5
	230	0/5
	340	3/5
	510	2/5
	760	5/5
Female	70	0/5
	110	0/5
	150	0/5
	190	0/5
	230	1/5
	340	4/5
	510	4/5
	760	5/5

In the study by Anonymous (2002), 5 Crj: CD (SD) rats/sex/dose were treated with a single oral dose of 140, 200, 280, 400 or 560 mg/kg b.w. of acetamiprid suspended in corn oil (homogeneity was not mentioned). All rats were observed for 14 days post-dosing for toxic signs and mortality. Body weight was recorded prior to dosing, and on days 1, 2, 3, 7 and 14 post-dosing, at termination or when an animal was found dead. All animals, both surviving till the end of the observation period and those found dead during the observation period, were subjected to gross necropsy. The selection of tested doses was based on the acute oral toxicity study of Anonymous (1997i) of acetamiprid suspended in water (Anonymous, 1997i).

Mortality rates are shown in Table 10, all deaths occurred within one day post administration. Clinical signs noted in all treated rats shortly after administration were mydriasis and tremor. Clonic convulsion was observed in males from 200 mg/kg b.w. and in females from 280 mg/kg b.w.. The incidence of these signs reached maximum at 1 or 3 hours post dosing while almost no sign was evident one day after administration. A transient decrease in body weight was noted on day 1 in survived males dosed with 200 and 280 mg/kg b.w.. Body weight gain in all other surviving animals during the study was unaffected. During the gross necropsy, dilated pelvis was observed in one male at the 140 mg/kg b.w., while no other abnormality was noted in any of the survived animals or those found dead during the study.

The acute oral LD_{50} value for acetamiprid in corn oil was found to be 195 mg/kg b.w. (95% confidence interval of 141-249 mg/kg b.w.) for male rats, and between 140 and 200 mg/kg b.w. for female rats.

Table 10: Acute oral toxicity of acetamiprid in corn oil in rats by Anonymous (2002)

Dose (mg/kg)	Mortality incidence	
	Males	Females
140	0/5	0/5
200	4/5	5/5
280	4/5	5/5
400	5/5	5/5
560	5/5	5/5

In the study by Anonymous (1998a), 5 Crj:CD (SD) rats were allocated without conscious bias to cages within the treatment group (five males and five females per dose group). Acetamiprid was prepared in ion-exchange water and administered at a volume of 10 mL/kg bw by oral gavage using a stomach tube. The day of dosing was designated Day 0.

In the main study, rats were dosed at 100, 150, 230, 340 and 510 mg/kg bw in both sexes. In an additional study, female rats were dosed at 80, 100, 120, 140 and 160 mg/kg bw. After administration, animals were observed for 14 days. The animals were observed for one hour and at three hours after administration and thereafter at least once a day. The animals were weighed prior to administration and after 1, 2, 3, 7 and 14 days and at death. Gross necropsy was performed on all animals.

As shown in Table 11, no mortality occurred in rats of both sexes treated at 100 mg/kg bw, and in females at 80 and 100 mg/kg bw. At higher dosages mortality ranged between 20 to 100% in both males and females, and occurred on days 1-2. No toxic signs were noted in males treated at 100 mg/kg bw and in females at 80 mg/kg bw. In males receiving 150-340 mg/kg bw, and in females receiving 100-230 mg/kg bw most animals showed crouching for 3 hours to one day after administration. At 150 to 510 mg/kg bw for males, and at 100 to 510 mg/kg bw for females, most rats showed tremors for 3 hours to one day after administration. A few rats showed low sensitivity, lateral position, prone position, salivation, urine incontinence and ataxia for 60 minutes to one day after administration. All toxic signs disappeared within 2 days after administration. In surviving male rats at 150 and 230 mg/kg bw, and in surviving females at 150 and 160 mg/kg bw, body weights were decreased on day 1 after administration, but recovered thereafter and there were no differences in body weight gains from day 2 onwards among all the treated groups. Three rats out of the 37 which died presented dark-reddish lungs at necropsy. No macroscopic abnormalities were observed for either for the other decedent rats, or animals killed on day 15.

Using the Probit method, the acute oral median lethal dose (LD_{50}) for acetamiprid was calculated to be 217 mg/kg bw (95% confidence limits: 167-282 mg/kg bw) for male rats and 146 mg/kg bw (95% confidence limits: 133-164 mg/kg bw) for female rats.

Table 11: Acute oral toxicity of acetamiprid to rats by Anonymous (1998a)

Dose (mg/kg bw)	Toxicological results*	Duration of signs	LD_{50} (mg/kg bw)
	male rats		
100	0/0/5	-	
150	1/4/5	1-3 h	217
230	2/5/5	1 h – 1 d	(95% confidence limits:
340	5/5/5	30 min – 3 h	167-282)
510	5/5/5	1-3 h	
	female rats		
80	0/0/5	-	
100	0/9/10	1-3 h	
120	1/2/5	3 h	
140	1/4/5	3 h	146
150	4/5/5	3 h – 1 d	(95% confidence limits:
160	3/5/5	1 h – 1 d	133-164)
230	5/5/5	3 h	
340	5/5/5	1-3 h	
510	5/5/5	30 min – 3 h	

* number of animals which died/number of animals with clinical signs/number of animals used

Table 12: Summary table of animal studies on acute oral toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels, duration of exposure	Value LD_{50}	Remarks (Klimisch score)*	Reference
Acute oral toxicity study in rats In compliance with the EEC B.1	5 Crj: CD (SD) male rats/dose Crj: CD (SD) female rats/dose	NI-25 (acetamiprid) Purity: 99.9% Vehicle: ion-exchanged water	Males: 100-760 mg/kg bw Females: 70-760 mg/kg bw 14 days	Males: 417 mg/kg bw Females: 314 mg/kg bw	1 (reliably without restriction)	Anonymous (1997i) (in RAR 2015)
Acute oral toxicity study in rats In compliance with the EEC B.1	5 Crj: CD (SD) male rats/dose Crj: CD (SD) female rats/dose	Acetamiprid Purity:>99.9% Vehicle: corn oil Homogeneity not mentioned	140-560 mg/kg bw 14 days	Males: 195 mg/kg bw Females: 140-200 mg/kg bw	1 (reliably without restriction)	Anonymous (2002) (in RAR 2015)
Acute oral toxicity study in rats In accordance with OECD 401	5 Crj: CD (SD) male rats/dose Crj: CD (SD) female rats/dose	NI-25 (acetamiprid) Purity: 99.46% Vehicle: ion-exchange water	Males: 100-510 mg/kg bw Females: 80-510 mg/kg bw 14 days	Males: 217 mg/kg bw Females: 146 mg/kg bw	1 (reliably without restriction)	Anonymous (1998a) (in RAR 2015)

* Klimisch et al. (1997)

10.1.1 Short summary and overall relevance of the provided information on acute oral toxicity

Three acute oral toxicity studies report LD_{50}-values of acetamiprid dissolved in ion-exchanged water or corn oil (see Table 12). The lowest calculated LD_{50} value in the studies using ion-exchanged water is 146 mg/kg bw. The lowest calculated LD_{50} value in the study using corn oil as vehicle is 140 mg/kg bw.

10.1.2 Comparison with the CLP criteria

Substances can be allocated to one of four toxicity categories based on acute toxicity by the oral, dermal or inhalation route according to the numeric criteria shown in Annex I, Table 3.1.1 in Regulation (EC) 1272/2008 on CLP. Following this table, both lowest calculated LD_{50} values are 50-300 mg/kg bw, resulting in classification as Acute Tox 3.

10.1.3 Conclusion on classification and labelling for acute oral toxicity

Acetamiprid should be classified as Acute Tox 3, H301 "Toxic if swallowed".

10.2 Acute toxicity - dermal route

Not evaluated in this dossier.

10.3 Acute toxicity - inhalation route

Not evaluated in this dossier.

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

The DS proposed a change to the Annex VI classification of Acute Tox. 3 on the basis of new study results presented in the RAR (2016). There are now 3 studies available as shown below:

Table 1: *Summary of the Acute oral toxicity studies*

Method, guideline, deviations if any	Test substance,	Dose levels, duration of exposure	Value LD_{50}	Reference
NEW Study In compliance with the EEC B.1 (equivalent to OECD TG 401) 5 Crj: CD (SD) male rats/dose Crj: CD (SD) female rats/dose	Acetamiprid Purity: >99.9% Vehicle: corn oil Homogeneity not mentioned	140-560 mg/kg bw 14 days	Males: 195 mg/kg bw Females: 140-200 mg/kg bw	RAR (2015)
In compliance with the EEC B.1 (equivalent to	NI-25 (acetamiprid) Purity: 99.9%	Males: 100-760 mg/kg bw Females: 70-760	Males: 417 mg/kg bw Females: 314	RAR (2015)

OECD TG 401) 5 Crj: CD (SD) male rats/dose Crj: CD (SD) female rats/dose	Vehicle: ion-exchanged water	mg/kg bw 14 days	mg/kg bw	
In accordance with OECD TG 401 5 Crj: CD (SD) male rats/dose Crj: CD (SD) female rats/dose	NI-25 (acetamiprid) Purity: 99.46% Vehicle: ion-exchange water	Males: 100-510 mg/kg bw Females: 80-510 mg/kg bw 14 days	Males: 217 mg/kg bw Females: 146 mg/kg bw	RAR (2015)

The lowest calculated LD_{50} value in the studies using ion-exchanged water as vehicle is 146 mg/kg bw. The lowest calculated LD_{50} value in the study using corn oil as vehicle is 140 mg/kg bw and the DS proposed classification as Acute Tox. 3.

Comments received during public consultation

A number of Member State Competent Authorities (MSCAs) supported the proposed change from Acute Tox. 4 to Acute Tox. 3. There was no disagreement.

Assessment and comparison with the classification criteria

According to the CLP criteria, substances with LD_{50} values falling within the range $50<LD_{50}\leq300$ mg/kg bw/day should be classified as Acute Tox. Category 3 (H301: Toxic if swallowed). There are two studies for acetamiprid in which the lowest LD_{50} value was within this range for female animals; 146 mg/kg bw when ion-exchange water was the vehicle and 140 mg/kg bw when corn oil was the vehicle. Cat. 3 is therefore the appropriate category for acetamiprid.

The proposal of the DS is supported by the RAC. Overall, RAC considers a classification as Acute Tox. 3; H301; with an ATE of 140 mg/kg bw is warranted for acetamiprid.

10.4 Skin corrosion/irritation

Not evaluated in this dossier.

10.5 Serious eye damage/eye irritation

Not evaluated in this dossier.

10.6 Respiratory sensitisation

Not evaluated in this dossier.

10.7 Skin sensitisation

Not evaluated in this dossier.

10.8 Germ cell mutagenicity

Not evaluated in this dossier. However, a short summary of the RAR regarding mutagenicity is presented since it may be of importance for the classification of carcinogenicity.

Table 13: Summary table of *in vitro* studies on genotoxicity from RAR 2015

Test substance	Type of study		Result		Reference
	Indicator cells	Endpoint	without activation	with activation	
Acetamiprid	B: *S. typh.* TA 98 TA 100 TA 1535 TA 1537 *Escherichia coli* WP2 *uvrA.*	 point mut. point mut. point mut. point mut.	negative	negative	Kanaguchi (1997b) from RAR 2015
acetamiprid	Chinese hamster ovary (CHO) cells	gene mutations (HGPRT)	negative	negative	Adams (1998) from RAR (2015)
acetamiprid	Chinese hamster ovary (CHO) cells	chromosome aberration	Positive at concentra-tions with medium cytotoxicity	positive at concentra-tions with medium cytotoxicity	Kanaguchi (1997a) from RAR (2015)
acetamiprid	Primary rat hepatocytes	Unscheduled DNA synthesis	negative		Ham (1998) from RAR (2015)

Table 14: Summary table of *in vivo* studies on genotoxicity

Test substance	Type of study		Result	Reference
	Species	Endpoint		
acetamiprid	Mouse, CD-1, 10/sex/dose	Mutagenicity (micronuclei)	Negative	Anonymous (1998d) from RAR (2015)
acetamiprid	Rat, Sprague-Dawley CD 5/sex/dose	Chromosomal aberration (bone marrow)	Negative	Anonymous (1998e) from RAR (2015)
acetamiprid	Rat, Sprague-Dawley, 3 males/dose	Unscheduled DNA synthesis	Negative	Anonymous (1997j) from RAR (2015)

10.8.1 Short summary and overall relevance of the provided information on genotoxicity

The genotoxic potential of acetamiprid has been investigated in a comprehensive range of *in vitro* and *in vivo* assays. The results of these assays are summarised in Table 2.6.4-1.

Conclusively from the genotoxicity evaluation of various studies, acetamiprid was found to be positive in chromosomal aberrations *in vitro* in CHO cells but this was found to be not relevant for the *in vivo* situation with a negative mouse micronucleus assay and metaphase analysis in rat bone marrow.

Although a positive result was obtained in an *in vitro* chromosome aberration test, further *in vitro* (i.e. UDS *ex vivo/in vitro* using primary rat hepatocytes) and *in vivo* studies (i.e. a mouse micronucleus in which mortalities occurred at the highest tested dosage of 80 mg/kg bw, a bone marrow metaphases analysis in rats in which mortalities as well as reduction of mitotic index, compared to controls, were observed at the highest tested dosage of 250 mg/kg bw, and an *in vivo* UDS) failed to confirm any genotoxic effect *in vivo*. Information available for metabolites of acetamiprid also suggest they are not genotoxic RAR (2015).

10.8.2 Conclusion

In conclusion, the overall weight of evidence from the *in vitro* and *in vivo* studies indicates acetamiprid is not genotoxic.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

Germ cell mutagenicity was not assessed in the CLH dossier and it was also not in the scope of consultation. However, it was briefly described in the CLH dossier in support of the assessment for the classification of carcinogenicity.

The genotoxic potential of acetamiprid was investigated in a comprehensive range of GLP and OECD guideline compliant *in vitro* and *in vivo* assays. The results of these assays were summarised in Tables 13 and 14 of the CLH report.

Acetamiprid did not induce point mutations in bacteria *in vitro*. The mammalian gene mutation assay was also negative. Acetamiprid was found to be positive in the chromosomal aberration in vitro assay in CHO cells. Although a positive result was obtained in the *in vitro* chromosome aberration test, further *in vitro* (i.e. UDS *ex vivo/in vitro* using primary rat hepatocytes) and *in vivo* studies (i.e. a mouse micronucleus, [mortalities at the highest tested dosage of 80 mg/kg bw], a bone marrow metaphases analysis in rats, [mortalities as well as reduction of mitotic index, compared to controls observed at the highest dose of 250 mg/kg bw], and an *in vivo* UDS) failed to confirm any genotoxic effect *in vivo*. Information available for metabolites of acetamiprid also suggest they are not genotoxic RAR (2016).

Comments received during public consultation

Hazard class not within the scope of consultation.

No comments received during consultation

Assessment and comparison with the classification criteria

Not discussed at RAC.

10.9 Carcinogenicity

Table 15: Summary table of animal studies on carcinogenicity

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Two Year Dietary Toxicity and Oncogenicity Study in Sprague Dawley rats, GLP	NI-25 (Acetamiprid, 99.38%)	NOAEL adenocarcinoma mammary gland: 7.1 mg/kg bw/day	Anonymous (1999e)
18-Month Dietary Oncogenicity Study in Mice, GLP	NI-25 (Acetamiprid, 99.38%)	No carcinogenic effects were observed.	Anonymous (1999a)

Two year dietary toxicity and oncogenicity study in rats

In the study by Anonymous (1999e) 341 Crl:CD (SD) BR rats/sex (4 weeks old) were obtained from Charles River Laboratories, (Michigan, U.S.A) and acclimated during a 14-day period before being dosed with 0, 160, 400 and 1000 ppm of acetamiprid (in the diet). 60 rats/group/sex were used in each group and 10 were euthanized at 12 months of study. Surviving animals were sacrificed after 2 years. Animals were examined for morbidity, mortality and toxicity twice daily and clinical observations were conducted weekly on all rats throughout the study. Body weight was measured pre-test, and together with food consumption was measured weekly for the first 14 weeks, and on a 2-week interval basis for the rest of the study. Water consumption was measured for 1 week every 6 months. Ophthalmoscopic examination was conducted on each rat once during the pre-test period and at 6, 12 and 24 months of study. Haematological, biochemical and urological analysis were conducted on 10 randomly selected rats/sex/group at 3, 6, 12, 18 and 24 months of study on the same animals when possible or replacement rats as needed. Blood samples were taken for rats euthanized in extremis when possible or at least a blood smear was made at necropsy. Blood samples were taken for overnight fasted animals (water available). Urine was collected during the fasting period. After 12 (interim) or 24-month (final) sacrifices, all rats received a complete post-mortem examination. Organ weights on protocol designated organs were recorded at necropsy and representative samples of protocol-designated organs and tissues were processed and prepared for microscopic examination.

Table 16: Mean of acetamiprid intake during the study (mg/kg b.w./day)

	Sex Dose (ppm)	160	400	1000
Drug intake (mg/ kg bw/day)	Male	7.1	17.5	46.4
	Female	8.8	22.6	60.0

No test article-related effect on survival was observed. During the study, 6 animals died following blood collection and 142 animals were either euthanized in extremis or found dead. Clinical signs in males

included hunched posture, labored breathing and red/brown material around the nose in the mid- and high-dose. An increase in the incidence of rales was noted also during weeks 66-104. No gross or microscopic lesions consistent with rales were observed. Clinical signs in females included hunched posture, labored breathing in the mid- and high- dose. The inflammation, sporadically observed in both male and female rats, at the skin (ulceration, abrasion, scabbed area) and the other organs and tissues (kidney, urinary bladder, prostate), was not considered as treatment-related.
The body weights of the 1000 ppm males and females, and 400 ppm females (until week 100) were statistically significantly lower than control during the study. In females of the 160 ppm group, the body weights were also slightly lower than controls during the study, however statistical significance was attained only for two weeks period. Group mean body weights at 104 weeks are presented in the Table 15.

Table 17: Mean body weights at 104 weeks

	Sex Dose (ppm)	**0**	**160**	**400**	**1000**
Mean body weights (g)	Male	667 (100)	668 (100)	673 (101)	578 (87)**
	Female	433 (100)	453 (105)	338 (90)	367 (85)*

Number in parentheses indicates percentage relative to control.
*P<0.05. ** P<0.01. compared to control by Multiple Comparison.

Statistically significant decreases of food consumption values (per animal) were noted in both sexes of the 1000 ppm groups and in females of the 400 ppm group. Food consumption values (g/kg/day) of the 1000 ppm males and females were increased during most of the study periods, except week 1 (in which palatability may have caused a low value).
No dose-related change was noted in water consumption (g/kg/day). Upon Ophthalmoscopy, no dose-related change was noted.
Statistically significant increase by 6.1% was observed on the MCHC at 24 month in the 1000 ppm group male animals.
At 12 and 18 month and terminal intervals, females had lower triglycerides values at 1000 ppm compared to controls. These differences were statistically significant at 12 and 18 months and were considered to be possible effects to test article administration. Total bilirubin value was statistically significantly different than controls, however this change was not dose related effect. Other statistically significant changes were found sporadically, however these were not considered to be biologically significant nor dose-related (Table 16).

Table 18: Statistically significant effects of acetamiprid on clinical chemistry findings after chronic administration on rats.

End-point	**Dietary level (ppm)**							
	males				**females**			
	0	160	400	1000	0	160	400	1000
Clinical chemistry								
Total bilirubin (12 M#)	ns	ns	ns	ns	0.21	0.156* (-26%)	0.156* (-26%)	0.125** (-41%)
BUN (6 M)	ns	ns	ns	ns	12	13	13	15** (25%)
Triglycerides (12 M)	ns	ns	ns	ns	190	165	126	71** (62.6%)
Triglycerides (18 M)	ns	ns	ns	ns	237	205	151	87* (63.3%)
Globulin (18 M)	ns	ns	ns	ns	3.6	3.6	3.7	4.0* (11.1%)
A/G ratio (18 M)	ns	ns	ns	ns	1.1	1.0	1.1	0.8* (27.3%)

Percentage relative to control in brackets.

#M = Months
*p<0.05; ** P<0.01. compared to control by Multiple Comparison.

The urinalysis examination revealed a slight increase of osmotic pressure in males at 1000 ppm. These values were within normal range of biological variation and were not considered to reflect acetamiprid toxicity.
Necropsy did not reveal gross abnormalities related to treatment.
Statistically significant changes in the mean body weight were seen in the terminal sacrifice in both sexes at 1000 ppm. Mean absolute heart weight was decreased in both sexes at 1000 ppm. Mean absolute kidney weight was decreased in males at 1000 ppm and mean relative to body liver and lung weight was increased in males at 1000 ppm. Mean absolute prostate weight was decreased in males at 1000 ppm. Mean absolute thymus weight was decreased in females at 1000 ppm. Variations in organ weights were related to the decreased bodyweight in both males and females at the top-dose and were not considered to be associated to the treatment (Table 17).

Table 19: Statistically significant variations in organ weight at terminal sacrifice after 2 year administration of acetamiprid on rats.

Dose	**0 ppm**		**160 ppm**		**400 ppm**		**1000 ppm**	
Sex	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Terminal body weight (g)	663	429	671	450	676	385	576**	362*
Brain								
% body weight (x10)	3.32	4.90	3.22	4.56	3.30	5.32	3.74*	5.56
Heart								
absolute (g)	1.97	1.42	1.91	1.44	1.91	1.33	1.81**	1.28*
Kidney								
absolute (g)	5.36	3.85	5.14	3.31	5.00	3.23	4.49**	3.10
Liver								
% body weight	3.34	3.69	3.33	3.59	3.37	3.89	3.65*	4.07
Lung/mainstem bronchi								
% body weight (x10)	3.69	4.63	3.56	4.39	3.87	4.83	4.31**	5.63
Prostate								
absolute (g)	0.88	-	0.74	-	0.76	-	0.65**	-
Spleen								
absolute (g)	1.15	0.78	1.10	0.69	0.94*	0.71	0.99	0.75
% body weight (x10)	1.76	1.89	1.66	1.59	1.41**	1.91	1.72	2.06
Thymus								
absolute (g)	0.34	0.32	0.38	0.31	0.39	0.22	0.30	0.20*

* p< 0.05; **. p< 0.01

Microscopic observation on animals sacrificed or found dead after 1 year revealed an increased incidence of trace to mild centrilobular hepatocellular hypertrophy in males from 400, and in females at 1000 ppm. All males sacrificed after 1 year presentedhepatocyte vacuolation in the 400 and 1000 ppm groups whereas females were not affected. Other findings were not considered to be treatment related (Table 18).

Table 20: Non neoplastic lesions observed in the 12 months interim sacrifice after chronic administration on rats.

Sex		**Male**				**Female**			
Organs /lesions	**Dose (ppm)**	**0**	**160**	**400**	**1000**	**0**	**160**	**400**	**1000**
Liver: hypertrophy		0/10	0/10	4/10*	10/10**	0/10	0/10	0/10	4/10*
hepatocyte vacuolation		2/10	4/10	10/10**	10/10**	1/10	0/10	0/10	1/10
Kidney : chronic progressive		6/10	4/10	4/10	1/10	0/10	1/10	0/10	0/10

nephropathy								
microconcretion, papilla	1/10	2/10	1/10	2/10	3/10	4/10	5/10	3/10
mineralisation tubular, trace	0/10	0/10	0/10	0/10	4/10	5/10	4/10	5/10
Lung : medial calcification	6/10	9/10	6/10	8/10	6/10	8/10	5/10	6/10
Heart : inflammation, subacute trace	5/10	4/10	8/10	4/10	2/10	0/0	0/0	2/10
Testis : vascular mineralisation, trace	0/10	0/10	0/10	1/10	-	-	-	-
degeneration, seminiferous tubules, severe	0/10	1/10	0/10	1/10	-	-	-	-
mineralisation, tubular, trace	0/10	0/10	0/10	1/10	-	-	-	-
Mammary gland : hyperplasia	-	-	-	-	3/10	1/10	2/10	2/10
galactocele	-	-	-	-	1/10	1/10	7/10**	4/10
Pituitary : cyst. trace	0/10	2/10	1/10	1/10	0/10	0/10	0/10	2/10
hyperplasia, trace	1/10	2/10	2/10	1/10	3/10	5/10	3/10	2/10

P<0.05, ** P<0.01, compared to control by Fisher's exact test.

There were no treatment related neoplastic lesions in the 1-year sacrifice animal groups.
Non neoplastic microscopic changes associated with treatment were detected at final sacrifice in the kidney of the 1000 ppm male (microconcretions of the renal papilla) and in the liver of the 400 and 1000 ppm male groups (hypertrophy and hepatocyte vacuolation). In the 1000 ppm female treatment group, mammary gland hyperplasia was increased in terminal sacrifice animals. Representative non neoplastic microscopic observations on final sacrifice animals are presented in the Table 19.

Combined microscopic non-neoplastic observations for all animals on study (interim and final sacrifice, and all animals dead or sacrificed at unscheduled dates) did not reveal treatment related effects other than observations identified in the liver (hypertrophy at 400 ppm in males and 1000 ppm in males and females; and hepatocyte vacuolation in males at 400 and 1000 ppm) and in the kidney (microconcretion in the papilla in males at 1000 ppm). The bone marrow hyperplasia in femur observed in the low and mid dose male rats, is not considered to be treatment-related, since it does not appear in the high dose group. For further justification, see the mice carcinogenicity study below.

When all animals in the study were combined, a significant trend increase of mammary gland adenocarcinoma ($p<0.05$) was observed with the Cochran Armitage Trend Test and the Peto test. Nevertheless, such incidence was not statistically significant with the Fisher Exact test, and was within historical control data (28.3% high dose in this study, compared to 14.0% to 28.6% for historical control data (n=6, same laboratory and same period). Mammary gland observations are summarised in table 20.
There was no increase of mammary tumors when all types of tumors were considered, and no increase was observed in multiplicity of tumors nor in mammary tumors causing deaths. Nevertheless, a continuum is observed between hyperplasia (significant at high dose) and increased adenocarcinoma incidence in the mammary gland (see Table 20). These findings are treatment related with a NOAEL of 7.1 mg/kg bw/day.

Table 21: Non neoplastic lesions observed on final sacrifice after chronic administration on rats.

Sex	**Male**				**Female**			
Dose (ppm)	**0**	**160**	**400**	**1000**	**0**	**160**	**400**	**1000**
Liver: hypertrophy	0/30	0/40	15/33**	32/40**	0/23	0/26	1/29	2/29
hepatocyte vacuolation	5/30	3/40	15/33*	27/40**	2/23	4/26	4/29	2/29
altered foci. clear cell	13/30	21/40	17/33	11/40	6/23	6/26	5/29	3/29
cystic degeneration	10/30	9/40	9/33	3/40	0/23	3/26	2/29	3/29
Kidney:microconcretion, papilla	9/30	18/40	17/33	31/40**	17/23	25/26*	27/29	25/29
chronic progressive nephropathy	29/30	37/40	31/33	30/40	8/23	7/26	8/29	8/29
Adrenal cortex: hypertrophy	6/30	6/40	8/33	7/40	5/23	0/5	0/2	8/29
hyperplasia	7/30	6/40	1/33	3/40	4/23	0/5	0/2	3/29
cystic degeneration	4/30	1/40	11/33	8/40	21/23	4/5	2/2	28/29

Sex	**Male**				**Female**			
Dose (ppm)	**0**	**160**	**400**	**1000**	**0**	**160**	**400**	**1000**
Adrenal medulla : hyperplasia	10/30	13/40	7/33	5/40	2/23	0/4	0/1	1/29
Bone marrow, femur: hyperplasia, myeloid	8/30	26/40**	20/33**	13/40	8/23	8/26	16/29	12/29
Bone marrow, Sternum : hyperplasia, myeloid	9/30	19/40	11/33	13/40	7/23	9/26	16/29	13/29
Brain : mineralisation	3/30	0/1	0/0	7/40	1/23	0/0	0/0	1/29
Epididymis : aspermia	1/30	0/1	1/2	3/40	-	-	-	-
lumenal debris, cellular	0/30	0/1	1/2	6/40*	-	-	-	-
Eye : cataract	0/30	6/40**	2/33	0/40	0/23	0/5	0/6	1/29
keratitis	0/30	3/40	2/33	3/40	3/23	0/5	1/6	1/29
retinal atrophy	1/30	2/40	0/33	3/40	3/23	0/5	2/6	2/29
uveitis	0/30	0/40	0/33	2/40	2/23	0/5	0/6	1/29
optic nerve degeneration	2/30	5/38	1/33	1/40	3/23	1/3	2/5	1/29
Harderian gland : hypertrophy	3/30	20/40**	16/33**	7/40	3/23	0/0	0/0	7/29
pigment, porphyrin	11/30	20/40	12/33	13/40	3/23	0/0	0/0	4/29
Heart : Inflammation. subacute	10/30	10/40	13/33	19/40	9/23	0/0	0/0	4/29
myocardial fibrosis	8/30	18/40	14/33	7/40	5/23	0/0	0/0	1/29
Lung : medial calcification	26/30	34/40	27/33	36/40	16/23	22/26	16/29	20/29
alveolar macrophages	5/30	8/40	7/33	9/40	3/23	2/26	3/29	11/29*
cholesterol clefts, trace	1/30	5/40	1/33	7/40	2/23	0/26	0/29	1/29
Inflammation, chronic	4/30	5/40	4/33	7/40	3/23	0/26	0/29	4/29
Lymph node, mesenteric**:** angiectasis	1/30	0/0	0/1	5/40	0/23	0/0	0/0	2/28
Mammary gland : hyperplasia	0/1	0/1	0/1	0/0	5/23	10/26	10/29	18/29**
galactocele	0/1	1/1	1/1	0/0	11/23	15/26	19/29	17/29
Nerve, Sciatic: degeneration nerve fiber, trace	0/30	2/40	1/33	3/39	8/23	0/0	0/0	11/29
Pancreas : atrophy	3/29	6/39	9/33	6/40	0/22	4/26	7/29*	2/29
Pituitary : hyperplasia	7/30	13/40	12/33	14/40	5/23	7/26	0/29	1/29
Spleen : extramedullary hematopoiesis	4/30	0/1	0/1	9/40	8/23	0/0	0/0	10/29
Testis : medial calcification	10/30	9/40	10/33	13/40	-	-	-	-
degeneration, seminiferous tubules	1/30	12/40**	3/33	3/40	-	-	-	-
Thyroid : C-cell hyperplasia	6/30	4/40	3/33	6/40	7/23	4/26	3/29	13/29

* P<0.05, ** P<0.01, compared to control by Fisher's exact test.

Table 22: Mammary gland observations after chronic administration on rat.

	dose levels ppm			
Microscopic observations	**0**	**160**	**400**	**1000**
fibroadenoma	17/59	15/60	10/60	15/60
adenoma	1/59	0/60	4/60	3/60
benign tumor (adenoma or fibroadenoma)	18/59	15/60	14#/60	18/60
adenocarcinoma	10/59	11/60	16/60	17/60
any mammary tumor	24/59	21/60	24/60	29/60

This incidence is reported as 12/60 in the table B.6.5.1-7 in the RAR.

Conclusion

Test-article related pathologic changes included trace to mild centrilobular hepatocellular hypertrophy in males from 400 ppm and in females at 1000 ppm. Trace to moderate hepatocellular vacuolation was observed in males from 400 and trace to severe microconcretions in the renal papillae were observed in males at 1000 ppm. The liver in males and females and the kidney in males were identified as target organs.

The NOAEL is set at 160 ppm, equivalent to 7.1 mg/kg b.w./day and 8.8 mg/kg b.w./day for males and females, respectively, based on body weight gain reductions and increased incidence of adenocarcinoma in the mammary gland in females, and histopathological changes in the liver at 400 ppm in males.

Long-term oral toxicity/carcinogenicity in the mouse

In the study by Anonymous (1999a) 60 mice/group/sex were dosed with 0, 130, 400 or 1200 ppm acetamiprid in the diet from which 10/sex/group were sacrificed after 1 year. Surviving animals were sacrificed after 18-months of treatment. Observations of morbidity, mortality and toxicity were performed 3 times a day during working days and twice daily on weekends and holidays. Clinical examinations were performed weekly. Body weight and food consumption were recorded weekly for the first 16 weeks of study and monthly thereafter. Haematological analysis was performed after 12 and 18 months on 10 randomly selected mice/sex/group and on animals sacrificed in extremis when possible. A complete post-mortem examination was conducted on all animals sacrificed and absolute organ weights were recorded together with bodyweight. Representative samples of protocol designated organs and tissues were processed and examined microscopically from all animals sacrificed or dead on study when possible.
The mean values of acetamiprid intake during the study are presented in the Table 21.

Table 23: Mean values of acetamiprid intake during chronic administration on mice.

	Sex Dose (ppm)	**130**	**400**	**1200**
Drug intake (mg/kg bw/day)	**Male**	20.3	65.6	186.3
	Female	25.2	75.9	214.6

Clinical signs were limited to the week period 1-13 and comprised an increased incidence of decreased defecation for male and female mice at the 1200 ppm dosage level, which was correlated to the decreased food consumption observed during the first 20 weeks of the study. Other observations were randomly observed among groups and were not related to the treatment. There was no tendency of increased mortality with dose. The inflammation, sporadically observed in both male and female rats, at the skin (ulceration, abrassion, scabbed area) and the other organs and tissues (kidney, urinary bladder, prostate), was not considered as treatment-related.

The body weight of the 1200 ppm animals was statistically significantly lower than controls during the study, being 15.8% and 17.6% lower than the respective control value at the end of the study. In both sexes of 400 ppm group, the body weight was also slightly lower than controls and a statistical significance was attained sporadically. No treatment-related effects were observed at the dose level of 130 ppm. Body weight change was calculated for the early study periods of 0-1, 0-2, 0-4, 0-8, 4-13 and 0-13 week, and thereafter for 13-48, 48-78 and 0-48 and 0-78 week. There was an early and consistent decrease in bodyweight gain at 1200 ppm for males and females. The early statistically significant decrease observed for the period 0-4 (and consequently 0-13 week) at 130 ppm in females was not considered to be toxicologically relevant, as it was not observed at 400 ppm, and also was not observed in males or at the end of the study. The mean body weight value (g) at week 78 is shown on Table 22.

Table 24: Mean body weight (g) of mice at week 78 after acetamiprid administration.

	Sex Dose (ppm)	**0**	**130**	**400**	**1200**
Mean body weights (g)	**Male**	38 (100)	38 (100)	36 (95)	32 (84)**
	Female	34 (100)	34 (100)	33 (97)	28 (82)**

Number in parentheses. percentage relative to control.
** P<0.01. compared to control by Multiple Comparison.

Decreases of food consumption values (g/kg) were noted in both sexes of the 1200 ppm group during the first 20 weeks of the study. At week 24, the values increased over control group and remained increased

until the end of the study. No significant change was noted in other groups. Food consumption, when expressed in g/animal/day, was significantly lower, mainly in male high dose treated group, the entire study period when compared to the control group. Food efficiency values were variable with no obvious trend.
Upon haematology, there were no dose-related nor statistically significant changes in any parameters among groups after 12 or 18 months.
Variations in organ weights, regarding decreases in absolute and relative heart and kidney weight, increase in relative pituitary weight and decrease in prostate absolute weight, were mainly observed in high dose treated males. These changes were considered as related to the decreased body weight in both males and females at the top-dose and not associated to the treatment. Statistically significant variations in organ weight at the interim sacrifice (1-year) were observed in the high dose treated animals and are shown on Table 23.

Table 25: Statistically significant variations in organ weight at the interim sacrifice (1-year) after chronic administration of acetamiprid on mice.

Dose	**0 ppm**		**130 ppm**		**400 ppm**		**1200 ppm**	
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Body weight	37	34	36	33	36	33	31**	28**
Heart								
Absol. w.	0.22	0.18	0.21	0.18	0.21	0.18	0.18*	0.16
Rel.w. (o/br.)	0.44	0.35	0.42	0.35	0.43	0.36	0.37*	0.32
Kidney								
Absol. w.	0.90	0.62	0.89	0.61	0.86	0.57	0.68**	0.48**
Rel.w. (o/br.)	1.8	1.2	1.7	1.1	1.7	1.1	1.4**	1.0
Pituitary								
Rel.w. (o/b.)	5.62	12.01	7.16*	11.77	5.01	10.76	12.07*	12.03
Prostate								
Absol. w.	0.031	-	0.026	-	0.023	-	0.019*	-

*Significantly different from control group, p<=0.05
**Significantly different from control group, p<=0.01

Statistically significant variations in organ weight at the terminal sacrifice are shown on Table 24. These variations regarded increase in relative liver weight at 1200 ppm male group and from 400 ppm in females, increase in relative kidney weight in females at 1200 ppm, decrease in relative adrenal weight in females from 400 ppm, increase in relative spleen weight in males from 400 ppm.

At 2 year macroscopic observation, an increased incidence of skin/ear portion missing in female mice administered 1200 ppm was noted. This finding was not considered toxicologically significant as the alteration is believed to occur as a common incidental finding in mice of this strain and was not associated with increased skin ulcers or abrasions. All remaining lesions were distributed across dosage groups and were of the type commonly observed in laboratory maintained mice of this strain. Macroscopic observations at final sacrifice are presented in the Table 25.

Microscopic observation after 1 year of administration revealed significant centrilobular hepatocellular hypertrophy both in males and females treated with 1200 ppm acetamiprid, at all observation intervals (Table 26). Statistically significant increased incidence of myeloid hyperplasia was observed in the bone marrow of the femur in the 1200 ppm males euthanised at the interim sacrifice. This increase was also observed in males at 12 month sacrifice from 130 ppm. However, the incidences were small and not significant for the total number of mice sacrificed after 1 year, for the animals found dead or sacrificed *in extremis* (Table 26). This finding is not considered as toxicologically significant, since:

- No dose response relationship was observed between acetamiprid administration and bone

marrow hyperplasia. In addition, the incidences of bone marrow hyperplasia in the sternum or femur in the control group are above all historical control data.
- Hematology values were not affected in a way related to bone marrow hyperplasia.
- Chronic administration of the test substance did not increase the severity or the incidence or the effect on bone marrow and there was no progression of bone marrow hyperplasia, as observed at postmortem examination of the animals at the end of the study.

The incidence of hyperplasia of the bone marrow in the sternum was not statistically significantly increased (Table 27) when all combined animals were considered together at the final sacrifice. Additionally, according to the information provided by the notifier, individual animal data from the acetamiprid chronic toxicity study shows a strong association between evidence of severe inflammatory reactions and the bone marrow hyperplasia.

A correlation between bone marrow hyperplasia and localized active inflammation mainly associated with ulceration was found, since few animals in each group showed inflammatory lesions without bone marrow hyperplasia. The probability for the presence of both observations (bone marrow hyperplasia and severe inflammation), when tested with the 2-tail Fisher's exact test, was statistically significant in both sexes for each study group.

Furthermore, even though the incidences of bone marrow hyperplasia in the sternum or femur in the control group in acetamiprid studies are above the historical control data of this laboratory, they are still within the range of spontaneous incidence from other laboratories. That comparison shows a high variability in incidences of bone marrow hyperplasia within studies, thus incidences observed in all groups in the acetamiprid study are considered to reflect a normal spontaneous background.

For these reasons, this change is not considered to be treatment-related.

The above justification concerning bone marrow hyperplasia in mice is also applicable to the rat carcinogenicity study (see above), where the low and mid dose male groups reached statistical significance.

Table 26: Statistically significant variations in organ weight in mice at the terminal sacrifice, after chronic administration of acetamiprid.

Dose	**0 ppm**		**130 ppm**		**400 ppm**		**1200 ppm**	
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Body weight	38	33	38	34	36	32	32**	28**
Adrenal								
Absol. w.	10.1	14.3	10.2	14.6	9.9	12.3*	8.9	10.1**
Rel. (o/br.w.)	19.08	27.14	20.21	27.43	19.33	23.52*	18.26	19.79**
Rel. (o/b.w.)	2.67	4.35	2.73	4.34	2.76	3.82*	2.83	3.59**
Brain								
Absol. w.	0.52	0.53	0.50	0.53	0.52	0.53	0.49**	0.51**
Rel. (o/b.w..)	13.9	16.1	13.5	16.0	14.4	16.6	15.4**	18.4**
Heart								
Absol. w.	0.23	0.19	0.23	0.20	0.23	0.20	0.21	0.17*
Rel. (o/b.w..)	6.07	5.89	6.16	5.94	6.35	6.42	6.60*	6.27
Kidney								
Absol. w.	0.90	0.58	0.87	0.60	0.82*	0.59	0.74**	0.53*
Rel. (o/b.w..)	2.39	1.75	2.32	1.78	2.27	1.83	2.35	1.90*
Liver								
Rel. (o/b.w..)	5.93	5.92	5.78	6.20	6.25	6.57**	6.90**	7.01**

Ovary								
Absol. w.	-	38.6	-	42.3	-	38.9	-	31.8*
Pituitary								
Absol. w.	2.45	3.18	2.52	3.52	2.42	3.38	2.08*	2.81
Prostate								
Absol. w.	0.031	-	0.036	-	0.026	-	0.024*	-
Spleen								
Rel. (o/b.w..)	2.80	4.04	3.39	4.79	3.80*	4.64	4.21*	4.73
Testis								
Rel. (o/b.w..)	5.72	-	5.54	-	5.96	-	6.39*	-

*Significantly different from control group. p<=0.05
**Significantly different from control group. p<=0.01

Table 27: Macroscopic observations at 2-year final scheduled sacrifice.

SEX	**Male**				**Female**			
Dose (ppm)	**0**	**130**	**400**	**1200**	**0**	**130**	**400**	**1200**
No. of animals	38	42	38	39	38	42	38	43
Skin, ear, partly missing	17 (45)	22 (52)	19 (50)	27 (69)*	6 (16)	8 (19)	5 (13)	22 (51)**
Liver, mass(es)	5 (13)	2 (5)	0*	1 (3)	0	1 (2)	1 (3)	0
Lung, nodule(s)	8 (21)	8 (19)	2 (5)*	3 (8)	3 (8)	11 (26)*	8 (21)	6 (14)

Percentages in parentheses. * P<0.05, **P<0.01, compared to control by Fisher's exact test.

Table 28: Microscopic observations (amyloidosis) in male mice at terminal sacrifice

	0 ppm	**130 ppm**	**400 ppm**	**1200 ppm**	**HCD**
Adrenal cortex amyloidosis	0/37	3/42	5/37*	7/39**	19.35% - 25.0%
Kidney amyloidosis	0/38	3/42	5/38*	7/39**	22.50% - 24.32%
Nonglandular stomach amyloidosis	0/38	0/0	0/0	5/39*	0.0%- 3.23%
Jejunum amyloidosis	1/38	0/0	0/0	7/39*	16.13% – 27.03%
Liver amyloidosis	0/38	3/42	3/38	5/39*	6.45% – 21.62%
Testis amyloidosis	0/38	2/42	2/38	5/39*	6.45% - 13.51%
Thyroid amyloidosis	0/38	3/42	3/38	5/39*	15.0% - 21.62%

*p≤0.05, **P≤0.01

Table 29: Microscopic observations in mice after acetamiprid administration.

Microscopic observation in mice after acetamiprid administration				
Interim (0-12 month) sacrifice				
Dose	**0 ppm**	**130 ppm**	**400 ppm**	**1200 ppm**

Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Bone marrow Femur: Myeloid hyperplasia	0/10	0/10	1/10	1/10	2/10	0/10	4/10 [1]	0/10
Liver: Centrilobular hypertrophy	0/10	0/10	0/10	0/10	1/10	0/10	8/10 [2]	8/10 [2]
Terminal (12 month-termination) sacrifice								
Bone marrow Femur: Myeloid hyperplasia	0/38	0/38	5/42 [1]	5/42 [1]	7/38 [2]	4/38	6/39 [1]	6/43 [1]
Bone marrow Sternum: Myeloid hyperplasia	0/38	0/38	6/42 [1]	6/42 [1]	7/38 [2]	4/38	6/39 [1]	6/43 [1]
Liver: Centrilobular hypertrophy	0/38	0/38	0/42	0/42	0/38	3/38	23/39 [2]	16/43 [2]
Kidney: Chronic progressive nephropathy	32/38	21/38	35/42	24/42	33/38	21/38	24/39	35/43 [1]
Lung: Epithelial hyperplasia	3/38	0/38	4/42	4/42	2/38	1/38	3/39	5/43 [1]

1: Statistically different from control, $p<0.05$
2: Statistically different from control, $p<0.01$

In males, the incidence of amyloidosis in the terminal sacrifice was increased in a number of organs at the top-dose and in the adrenal cortex and kidney from 400 ppm. Nevertheless, those incidences did not exceed historical control data (except for the nonglandular stomach) and when all mice were considered together, these incidences were not statistically significant.

Epithelial hyperplasia in the lung was statistically increased in females at the top-dose level (Table 27). This lesion is generally considered a preneoplastic lesion that may progress to alveolar bronchiolar adenoma or carcinoma. However, this finding was not considered to be treatment-related, as the incidence of lung tumors was similar among all groups.

Chronic progressive nephropathy in statistically significant grade was noted in high dose treated females at interim and terminal observation (interim and final sacrifices with all decedent or unscheduled sacrificed animals). This lesion was considered to remain within the incidence of the historical control data and remained of trace severity, thus it was not considered to be test article-related.
There were no neoplasms at a statistically significantly increased incidence noted at any dose, when the observation of malignant and benign tumours had shown that acetamiprid is not oncogenic to the mouse when administrated in the diet for 78 weeks.

Table 30: Statistically significant incidences of non-neoplastic lesions in mice for all combined animals on study (interim and final sacrifices with all decedent or unscheduled sacrificed animals).

Sex	Male				Female			
Dose (ppm)	**0**	**130**	**400**	**1200**	**0**	**130**	**400**	**1200**
Liver: hypertrophy centrilobular	0/60	0/60	2/60	37/60**	0/60	0/60	4/60	26/60**
Kidney: Chronic progressive nephropathy	47/60	45/60	45/60	31/60	33/60	33/60	33/60	48/60**
Bone marrow, Sternum: hyperplasia, myeloid	11/60	10/60	14/60	14/60	4/60	11/60*	7/60	8/60

Denominators, number of animals examined.
P<0.05, ** P<0.01, compared to control by Fisher's exact test.

Conclusion
The liver was identified microscopically as the target organ in both sexes at the top-dose with increased liver-to-bodyweight ratio, associated with increased incidences of centrilobular hepatocellular hypertrophy, considered to be a response of liver hyperfunction. Up to 1200 ppm (at least 186 mg/kg b.w./day) giving 16% and 18% of body weight reduction in males and females respectively. Acetamiprid is not oncogenic to the mouse when administrated in the diet for 78 weeks.
The NOAEL is set at 130 ppm, equivalent to 20.3 and 25.2 mg/kg b.w/day for males and females respectively, based on transient decreased body weight observed at 400 ppm in males and increased liver weight in females.

10.9.1 Short summary and overall relevance of the provided information on carcinogenicity

Two studies investigated the long-term oral toxicity/carcinogenicity of acetamiprid in rats and mice. There was an increase in adenocarcinoma of the mammary gland in female rats. This increase in incidence was significant in a trend test, but not in a pair-wise comparison with the controls. The increase in the highest dose (28.3%) was just within the historical control range of the laboratory of 14.0% - 28.6%. In addition, an increase in mammary gland hyperplasia (see Table 29) was observed at the highest dose which was statistically significant increased compared to the controls. No carcinogenicity was observed in the mice study.

Table 31: Incidence of mammary gland hyperplasia in rats at different sacrifices.

Dose (ppm)	0	160	400	1000
1-year interim	3/10	1/10	2/10	2/10
2-year final	5/23	10/26	10/29	18/29 (62%)*
Unscheduled (determined by calculation)	9/26	2/24	4/21	6/21
Total	17/59	13/60	16/60	26/60 (43%)*

*: P<0.01

HCD: 58.6%, 8.9% and 20% (unclear whether this includes interim sacrifices)

10.9.2 Comparison with the CLP criteria

Substances can be allocated to one of two categories based on strength of evidence and additional considerations (weight of evidence) as described in Annex I, Table 3.6.1 in in Regulation (EC) 1272/2008 on CLP. Classification in category 1A is not appropriate as there is no human data. Classification in category 1B is not considered appropriate as there is only one study in one species in which a possible increase in carcinoma was observed. Classification in category 2 should be considered.

Originally no classification was proposed in the RAR by the RMS. However, a majority of the member states in the EFSA meeting considered the increase in mammary gland adenocarcinoma as substance related as there was a continuum between hyperplasia (significant at high dose) and increase incidence of adenocarcinoma. Overall, the increase in adenocarcinoma of the mammary gland of female rats could be questioned as the increase was significant in a trend test but not in the pair-wise comparison with the controls, and no decrease in latency period was observed. Also the incidence at the highest dose remained within the historical control range although border line. However, the increase in mammary gland hyperplasia after two years of exposure (but within the historical control range of the performing laboratory of 8.9 – 58.6%) could be seen as supportive for classification.

The fact that the increase in mammary tumors was only observed in one species does not reduce the concern because the proposal is for classification in category 2. Also, the fact that the increase was observed in one sex does not reduce the concern because it is considered likely that there a difference in susceptibility between the sexes for these types of tumors due to differences in function of this tissue between males and females. Multi-site is regarded as an indication that classification in category 1B should be considered in case of a single positive study. However, the absence of a multi-site response is not considered to further reduce the concern for a single site carcinogen. Progression to malignancy is observed but only in one species. Therefore, this also supports category 2.

Mammary tumors in female SD rats are known to occur with a high spontaneous incidence (CLP guidance 5.0). In such cases the CLP guidance suggests a comparison with the historical control data. As discussed above the observed incidence in adenocarcinoma is just within the available historical control data of the performing laboratory. Also, it is unclear whether the stated HC data include the results of the interim section. Therefore, comparison with the historical control is not considered conclusive.

Further, no excessive toxicity was observed. There was no substance related effect on mortality and the reduction in body weight and body weight gain at the highest dose level was in the range of the MTD. On the contrary, feed restriction reduces the occurrence of fibroadenoma in female SD rats (Keenan et al., 1995). This may also explain the absence of an increase in benign mammary tumors as part of the continuum between hyperplasia and adenocarcinoma.

As a continuum is observed between hyperplasia (significant at high dose) and an increased incidence of adenocarcinoma in the mammary gland, these findings are treatment related. Therefore, classification in category 2 is proposed based on a treatment related increase in mammary adenocarcinoma.

10.9.3 Conclusion on classification and labelling for carcinogenicity

Acetamiprid should be classified as Carc. 2, H351 “Suspected of causing cancer”.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The dossier presents two long-term toxicity/carcinogenicity studies, which have been evaluated under both the Plant Protection Product and Biocidal Product regulations. Classification for carcinogenicity was not proposed initially by the evaluating competent authority but was proposed during the EFSA Peer Review consultation and therefore presented in the Annex by the DS for discussion by the RAC.

Rat study

In a guideline study, Crl:CD (SD) BR rats/sex (4 weeks old at start) received 0, 160, 400 and 1000 ppm of acetamiprid (in the diet). 60 rats/group/sex were used in each group and 10 were euthanized at 12 months of study. The dietary intake was calculated to deliver 7.1, 17.5 and 46.4 mg/kg bw/day in males and 8.8, 22.6, and 60.0 mg/kg bw/day in female rats. Survival was not affected by treatment. Non-specific clinical signs of toxicity were noted in mid and high dose rats. Mean body weights were statistically significantly reduced in rats at 1000 ppm (and at 400 ppm for females only) at both interim and terminal sacrifice. Triglycerides and A/G ratio were significantly reduced in females at 1000 ppm at 12 and 18 months. Mean relative liver weight was increased in the high dose animals (statistically significant in high dose males). Variations in other organ weights were related to the decreased bodyweight in both males and females at the top-dose and were not considered to be associated to the treatment. Combined microscopic non-neoplastic observations for all animals on study (interim and final sacrifice, and all animals dead or sacrificed at unscheduled dates) did not reveal treatment related effects other than observations identified in the liver (hypertrophy and hepatocyte vacuolation from 400 ppm in males only at terminal sacrifice).

A significant trend increase of mammary gland adenocarcinoma ($p<0.05$) was observed with the Cochran Armitage Trend Test and the Peto test when all animals in the study were combined. Nevertheless, such incidence was not statistically significant with the Fisher Exact test and was within the values for historical control data (28.3% high dose in this study, compared to 14.0% to 28.6% for historical control data (n=6, same laboratory and same period). The incidences are presented in Table 2 (as originally presented by the DS with the 50 animals from the main study added to the 10 animals from the interim sacrifice groups):

Table 2*: Rat mammary gland observations at terminal sacrifice, n=60.*

Microscopic observations	**dose levels ppm**			
	0	**160**	**400**	**1000**
	Neoplastic lesions			
Fibroadenoma	17/59	15/60	10/60	15/60
Adenoma	1/59	0/60	4/60	3/60
Benign tumour (adenoma or fibroadenoma)	18/59	15/60	14#/60	18/60
Adenocarcinoma	10/59	11/60	16/60	17/60
Any mammary tumour	24/59	21/60	24/60	29/60

This incidence is reported as 12/60 in the table B.6.5.1-7 in the RAR.

Additional historical control data (HCD) were presented in the RAR and was considered supportive of the RMS opinion that the finding at the top dose was within the background and

not treatment related. This additional information shows that the historical control ranges for mammary adenocarcinomas in female Sprague Dawley (CD) rats are:

- MPI laboratory: 13.3 – 28.6%
- Charles River Laboratories: 0 – 58.3%
- WIL Laboratories: 0 – 37.2%
- Covance Laboratories: 2.2 – 40%

The information (*see also revised data by RAC under Additional Key Elements*) shows that this is a common tumour in female rats, with a large range of normal variability. In the 2-year study in rat with acetamiprid, mammary gland adenocarcinoma was found at 16.9% (18.4%)[1] for the control group, 18.3% (20.4%)[1] for the 160 ppm group, 26.7% (31.9%)[1] for the 400 ppm group and 28.3% (34.7%)[1] for the 1000 ppm group. All these values are within or close to the historical control ranges reported for this tumour type in this strain of rat in the MPI Laboratory (13.3 - 28.6%) where this 2-year study with acetamiprid was performed. The study with acetamiprid was run between 1 Oct. 1991 and 1 Oct. 1993. RAC notes that the HCD for the MPI Lab only included 6 studies which started between 16 Oct. 1991 and 28 July 1994 and ended between 22 Oct. 1993 and 31 July 1996. RAC notes the CRL labs HCD[2] is a more robust database with 24 studies and a range from 0 – 58.3%, with a mean of 24.2% incidence, the initiation dates varied from 1991 to 1996.

The DS concluded that since the HCD came from the same laboratory and were from the correct time period, the reported HCD were appropriate and acceptable. Therefore, the slight increase observed at the top dose level was not considered to be treatment related.

Overall, the increase in adenocarcinoma of the mammary gland of female rats was questioned as it was significant in a trend test but not in the pair-wise comparison with the controls, and no decrease in latency period was observed. In addition, the incidence at the highest dose remained within the higher end of the historical control range.

Hyperplasia in the mammary gland was increased significantly in the top dose level females. HCD were provided for this finding, indicating an incidence range of 0.00 – 58.57%. The finding at the top dose level is just outside this range (i.e. 62%) but was interpreted as supportive for the discussion on classification.

Table 3: *Incidence of mammary gland hyperplasia in rats at different interim intervals.*

Dose (ppm)	**0**	**160**	**400**	**1000**
1-year interim	3/10	1/10	2/10	2/10
2-year final	5/23	10/26	10/29	18/29 (62%)*
Unscheduled (determined by calculation)	9/26	2/24	4/21	6/21
Total	17/59	13/60	16/60	26/60 (43%)*

* $p < 0.01$

The mammary tumours were seen in one species and one sex and in the absence of substance related mortality. The high dose was in the range of the MTD. It was suggested that the reduced weight and body weight gain may have been responsible for the absence of an increase in adenoma as feed restriction has been associated with a reduction in the occurrence

[1] Revised data by RAC, see "Additional Key Elements"

[2] Giknis & Clifford, March 2001. Compilation of Spontaneous Neoplastic Lesions and Survival in Crl:CD (SD) BR Rats from Control Groups.

of fibroadenoma in female SD rats (Keenan *et al.*, 1995).

The DS proposed classification in category 2 based on a treatment-related increase in mammary adenocarcinoma and the possibility that the statistically significant increase in incidence of hyperplasia may represent a continuum indicating, a transition into neoplasia.

Mouse study

An 18-month, guideline compliant study was evaluated in which 60 mice/group/sex were dosed with 0, 130, 400 or 1200 ppm acetamiprid in the diet from which 10/sex/group were sacrificed after 1 year. Surviving animals were sacrificed after 18-months of treatment.

Mortality was not adversely affected and clinical signs of toxicity were non-specific. Mean body weight and mean body weight gains were statistically significantly reduced in animals at the high dose of 1200 ppm. There were some overall reductions in food consumption in high dose males. Haematology parameters were unaffected. Variations in organ weights, regarding decreases in absolute and relative heart and kidney weight, increase in relative pituitary weight and decrease in prostate absolute weight, were considered related to the decreased body weight in both males and females at the top-dose and not associated with treatment.

A statistically significant increase in hepatocellular hypertrophy was seen in mice of the high dose at interim sacrifice. In addition, a statistically significant increase in myeloid hyperplasia of the femoral bone marrow was seen in males at this dose. This was not considered to be treatment-related because of the following:

- No dose response relationship was observed between acetamiprid administration and bone marrow hyperplasia.
- Haematology values were not affected in a way related to bone marrow hyperplasia.
- Chronic administration of the test substance did not increase the severity or the incidence or the effect on bone marrow and there was no progression of bone marrow hyperplasia, as observed at post-mortem examination of the animals at the end of the study.

Table 4a: *Microscopic observation in mice after acetamiprid administration*

Interim (0-12 month) sacrifice								
Dose	**0 ppm**		**130 ppm**		**400 ppm**		**1200 ppm**	
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Bone marrow Femur: Myeloid hyperplasia	0/10	0/10	1/10	1/10	2/10	0/10	4/10 [1]	0/10
Liver: Centrilobular hypertrophy	0/10	0/10	0/10	0/10	1/10	0/10	8/10 [2]	8/10 [2]
Terminal sacrifice (18 month-scheduled)								
Bone marrow Femur: Myeloid hyperplasia	0/38	0/38	5/42 [1]	5/42 [1]	7/38 [2]	4/38	6/39 [1]	6/43 [1]
Bone marrow Sternum: Myeloid hyperplasia	0/38	0/38	6/42 [1]	6/42 [1]	7/38 [2]	4/38	6/39 [1]	6/43 [1]

Liver: Centrilobular hypertrophy	0/38	0/38	0/42	0/42	0/38	3/38	23/39 [2]	16/43 [2]
Kidney: Chronic progressive nephropathy	32/38	21/38	35/42	24/42	33/38	21/38	24/39	35/43 [1]
Lung: Epithelial hyperplasia	3/38	0/38	4/42	4/42	2/38	1/38	3/39	5/43 [1]

*p < 0.05, ** p < 0.01, compared to control by Fisher's exact test

Table 4b*: Statistically significant incidences of non-neoplastic lesions for all combined animals on study (interim and final sacrifices with all decedent or unscheduled sacrificed animals).*

Sex	**Male**				**Female**			
Dose (ppm)	**0**	**130**	**400**	**1200**	**0**	**130**	**400**	**1200**
Liver: hypertrophy centrilobular	0/60	0/60	2/60	37/60**	0/60	0/60	4/60	26/60**
Kidney: Chronic progressive nephropathy	47/60	45/60	45/60	31/60	33/60	33/60	33/60	48/60**
Bone marrow, Sternum: hyperplasia, myeloid	11/60 S:1/48 D:10/12	10/60 S:7/52 D:3/8	14/60 S:9/48 D:5/12	14/60 S:11/49 D:3/11	4/60 S:0/48 D:4/12	11/60* S:7/52 D:4/8	7/60 S:4/48 D:3/12	8/60 S:6/53 D:2/7
Bone marrow, Femur: hyperplasia, myeloid	10/60 S:0/48 D:10/12	9/60 S:6/52 D:3/8	13/60 S;9/48 D:4/12	13/60 S:10/49 D:3/11	4/60 S:0/48 D:4/12	10/60 S:6/52 D:4/8	7/60 S:4/48 D:3/12	8/60 S:6/53 D:2/7

D=premature death, S=sacrificed
*p < 0.05, ** p < 0.01, compared to control by Fisher's exact test.

Note: the CLH report refers to HCD for this finding (p. 18). This data was considered in the re-review (RAR) when the applicant was required to submit additional HCD on hyperplasia. These were from 3 studies performed from April 1989 – October 1990, January 1991 – July 1992 and June 1991 – December 1992. The 18-month study in mice performed with acetamiprid was performed from October 1991 to April 1993. Therefore, the HCD are from the appropriate time period and from the same laboratory.

The HCD data show that the incidence of hyperplasia in bone marrow of the femur was 0 - 1 in 50 animals (males) and 0/50 (females). For hyperplasia in bone marrow of the sternum the incidence was 0 – 2 in 50 animals (males) and 0/50 in females. The overall combined findings in Table 30 of the CLH report show that in the control groups, myeloid hyperplasia in the sternum was 11/60 (males) and 4/60 (females), which is indeed above the HCD.

The incidence of hyperplasia of the bone marrow in the sternum was **not** statistically significantly increased (CLH Table 29) when all combined animals were considered together at the final sacrifice.

In addition, in males, the incidence of amyloidosis in the terminal sacrifice was increased in several organs at the top-dose and in the adrenal cortex and kidney from 400 ppm. Nevertheless, those incidences did not exceed HCD (except for the nonglandular stomach) and when all mice were considered together, these incidences were not statistically significant.

In conclusion, there were no neoplasms at a statistically significantly increased incidence noted at any dose and it was concluded that acetamiprid is not carcinogenic to the mouse when administrated in the diet for 18 months.

Comments received during public consultation

Three MSCAs commented in support of the proposal to classify as Carc. 2 on the basis of a possible increase in mammary tumours in association with a significant increase in hyperplasia.

One manufacturer and two downstream user companies also commented and did not support the proposed classification. These parties disagreed with the DS argument that a continuum between the observed statistically significant mammary hyperplasia and the increase in mammary adenocarcinoma (statistically significant trend) can be the basis for classification for carcinogenicity. Their rational being;

- A continuum has not been demonstrated as adenoma are not increased.
- Hyperplasia was not increased in the interim kill.
- Hyperplasia is a non-neoplastic change, is not evidence of carcinogenicity, and is not appropriate for carcinogenicity classification.
- The HCD incidence of hyperplasia is highly variable at the test facility.
- Mammary gland hyperplasia or any other mammary pathology was not seen in other species tested.

In addition, mammary adenocarcinoma has a high background in SD female rats (see Guidance on the Application of the CLP Criteria) and the incidence of mammary tumours in this study remained within the historical control range for this specific test facility.

Acetamiprid is not genotoxic, there is no apparent mode of action and it is negative in the USEPA ToxCAst ER bioactivity model (no evidence of ED disruption).

Additional key elements

Rat study

Note 1: The argument was made that the reduction in body weight/weight gain may be associated with a reduced occurrence of fibroadenoma. However, this is not relevant to the argument relating to the continuum from hyperplasia to adenocarcinoma as fibroadenoma is not part of this continuum. Fibroadenomas are a discrete separate entity from adenoma/adenocarcinoma, which do not progress to malignancy in humans or in rats (Russo, J., 2015. Significance of rodent mammary tumours for human risk assessment. Toxicol Pathol. 2015 February; 43(2); 125-170).

Note 2: Analysis of the hyperplasia incidence reveals that while there was an apparent increase in incidence at the high dose, there was no increase in severity (table 5). In addition, the severity was trace/mild in almost all cases.

Table 5*: Incidence of hyperplasia of the mammary gland from animals in the 24 month groups (not including those from the 12 month interim sacrifice).*

Dose level (ppm)	0 (control)	160	400	1000
No. of animals examined	49	49	47	49
Mammary gland:				
Hyperplasia	14	12	14	24*
-trace	9	10	10	17
-mild	5	2	3	6
-moderate	0	0	1	1

*significantly different from control; p≤0.01

It is noted that an increased incidence of 'trace' mammary hyperplasia is not a strong supporting argument for classification as a carcinogen. According to the RMS (RAR), HCD were provided for this finding as well, indicating an incidence of 0.00 – 58.57% (no further detail given). The RAC adjusted finding at the top dose level is well within this range (49% found).

Revised Incidence data

RAC reassessed (table 6) the incidence of mammary gland findings; however, the conclusions remain unchanged. Table 2 should have presented the tumour incidence from the whole life study animals and not included the 10 animals sacrificed at 12 months. Such treatment underestimates the true incidence of tumours because the interim sacrifice animals have not been allowed to live out the entire duration over which the treatment with compound is being assessed. In addition, the data from the most appropriate Charles River Laboratories report has also been revised (Giknis & Clifford, 2001). The data from 24 studies conducted between 1991 and 1996 gave a mean incidence of 24.2% for female rat mammary gland adenocarcinoma with a range from 0% (2 studies) to a maximum of 58.3%, a clear indication that this is a common tumour in female rats, with a large range of normal variability.

Table 6: *Rat% mammary gland incidence at terminal sacrifice, n=50****.***

Microscopic observations	**dose levels mg/kg bw/day**			
	0	**8.8**	**22.6**	**60.0**
fibroadenoma	34.7	30.6	21.3	28.6
adenoma	2.0	0	8.5	4.1
benign tumour (adenoma / fibroadenoma)	36.7	30.6	29.8	32.7
adenocarcinoma	**18.4**	**20.4**	**31.9**	**34.7**

Assessment and comparison with the classification criteria

Classification in category 1A is not appropriate in this case as there is no human data. Classification in category 1B is also not considered appropriate as there is only one study in which a possible increase in carcinoma was observed.

Classification in category 2 is therefore considered as evidence was presented which was obtained from a single animal study, and 'which was not sufficiently convincing to place the substance in Cat. 1A or 1B based on the strength of evidence together with additional

considerations'.

Two studies investigated the long-term oral toxicity/carcinogenicity of acetamiprid in rats and mice.

The argument for classification is based on a statistically significantly increased incidence in mammary adenocarcinoma in the rat 2-year study.

There was an increase in adenocarcinoma of the mammary gland in female rats. This increase in incidence was significant in a trend test, but not in a pair-wise comparison with the controls. The increase in the highest dose (34.7%) was outside the historical control range of the performing laboratory (MPI) of 13.3% - 28.6% and in addition, the incidence on the concurrent control, low and mid groups were 18.4%, 20.4% and 31.9%, respectively. Moreover, an increase in mammary gland hyperplasia (49%) was observed at the highest dose which was statistically significantly increased compared to the concurrent control. HCD were also provided for this finding, indicating an incidence of 0.00 – 58.57%. The finding at the top dose level however was within this range.

Following the EFSA technical peer review, the increase in mammary gland adenocarcinoma was considered to be substance-related due to what was described as a continuum between hyperplasia and increased tumour incidence. This may be supported by the observation of increased mammary hyperplasia in the highest dose groups. Progression from cell damage to proliferation and then followed by progressive stages from hyperplasia, dysplasia to benign and malignant tumour *in situ* has been demonstrated for mammary epithelia cells e.g., following exposure to 7,12-dimethylbenz[a]anthracene, Al-Dhahrei *et al.*, 2008.

However, while the incidence of mammary tumours is statistically significant in a trend test, the groups are not statistically different by pairwise comparison.

It should be noted that the increased incidence is just outside the range of the HCD for the test house at the appropriate time interval and several other sources of HCD indicate this type of tumour is highly variable in incidence. The in-house (MPI) HCD was limited in that few studies (6) were available. Nevertheless, more comprehensive external collections of HCD were also available where it could be verified that the incident values are all within the historical control ranges for this tumour type in this strain of rat (e.g. CRL laboratory data published in 2001: 0 - 58.3%). The CRL data was more robust, even though originating from different laboratories, and it shows how variable and common this tumour type actually is and prompting the question whether it would be possible to determine if a substance related effect on mammary gland tumours could ever be reliably detected in this strain of rat (figure 1).

This tumour is a common finding in SD rats which is noted in the CLP Guidance where it is stated that "even a statistically significant increase within the historical control range may not be providing reliable evidence of treatment-related carcinogenicity". Additional HCD from other test facilities support this observation.

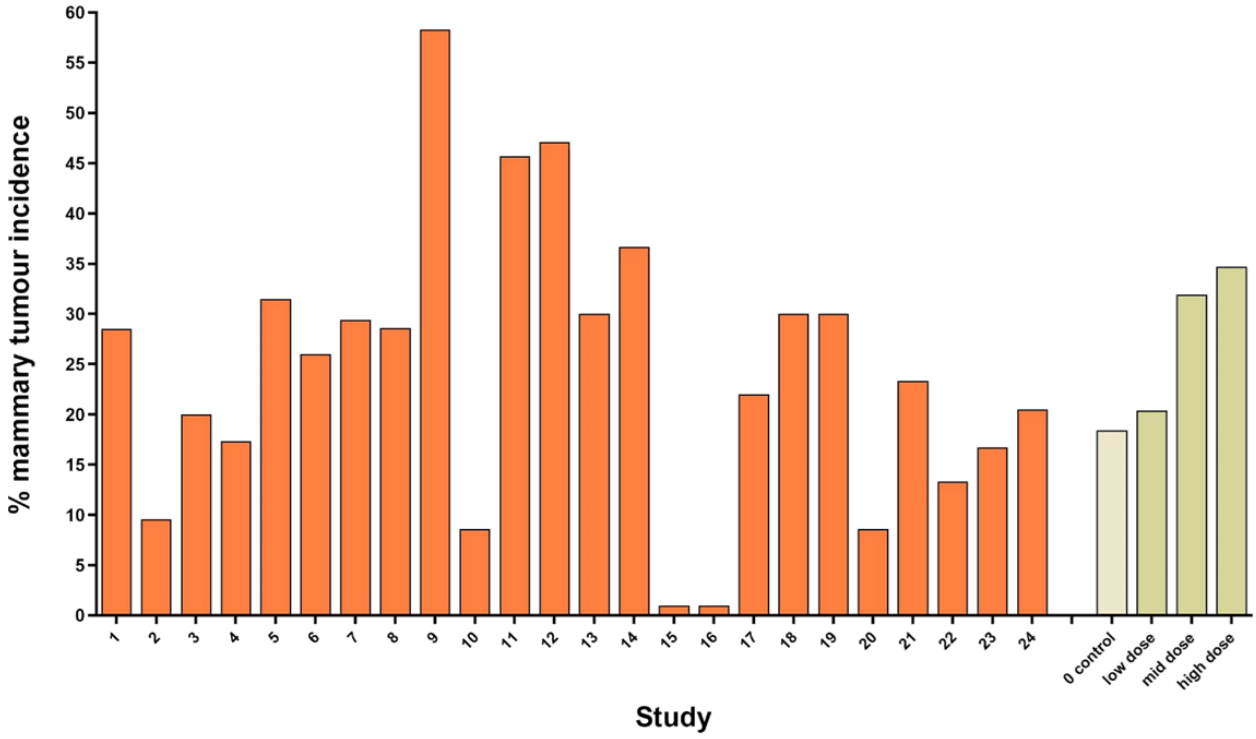


Figure 1: CRL study historical control data taken from Giknis & Clifford, March 2001. Compilation of Spontaneous Neoplastic Lesions and Survival in Crl:CD (SD) BR Rats from Control Groups. This graph illustrates the high background incidence of this tumour type in this strain of rat. 24 studies, initiation from 1992-1996, mean 24.2%: 0 – 58.3%, 1729 animals in total.

No mode of action was apparent from the data presented and the substance was not shown to be genotoxic. In addition, acetamiprid has been assessed in the US EPA ToxCast ER bioactivity model which is considered highly relevant in the assessment of endocrine disruption potential via oestrogenic activity. Acetamiprid shows no oestrogenic activity (US EPA EDSP21).

There was an apparent increased incidence of mammary epithelia hyperplasia but no increase in severity was observed at interim or at terminal sacrifice. The occurrence of mild hyperplasia at terminal sacrifice is not consistent with a continuum from a normal state to malignancy. There was no increase in adenoma. There was no evidence of the expected progression as described by Al-Dhahrei *et al.* (2008), there was no progression from hyperplasia to dysplasia to benign and malignant tumour *in situ* with a clear treatment related effect. The argument that reduced body weight is associated with a reduced incidence of mammary fibroadenoma is not relevant to the observation that increased incidence of adenoma was not observed; these are unrelated pathological entities – fibroadenoma is not part of the continuum referred to above.

The observed tumours were also confined to a single species. No carcinogenicity was observed in the mouse carcinogenicity study.

In conclusion, **RAC considers the carcinogenic evidence to be insufficient for classification.**

10.10 Reproductive toxicity

10.10.1 Adverse effects on sexual function and fertility

10.10.1.1 In vivo

In the study by Anonymous (1999d), acetamiprid was administered dietary to 26 rats/sex/group (F0 generation) at 0, 100, 280 or 800 ppm for at least 10 weeks prior to mating and throughout mating, gestation, and lactation periods. Pregnant females were allowed to deliver and F1 litters were culled to 4 male and female pups on day 4 post-partum when possible. 26 male and female from each F1 litter were randomly selected to produce an F2 generation under the same conditions. The study was terminated after weaning the F2 generation. Animals (parents and pups) were sacrificed at the end of the weaning period (except for pups culled on day 4). Survival (twice a day), clinical signs and physical examination were recorded. Animals were examined periodically for body weight changes and food consumption. Oestrous cycles were determined for parental females and the reproductive abilities of both generations were assessed in mating trials. During lactation, litters were monitored for growth and development. At termination, reproductive capacity evaluations (ovarian follicle count, sperm motility, total sperm count and sperm morphology) were performed on F0 and F1 adults. Necropsy was performed on parents and pups from both generations and selected tissues were preserved. Designated tissues, including reproductive organs were microscopically evaluated from the adult animals in control and high-level groups and selected animals in low- and mid-level groups. Organ weights were collected from F0 and F1 parent and offspring animals. In this two-generation study, a complete reproductive assessment was not obtained for the high-dose (800 ppm) F0 males, due to a technical error. In order to meet this limitation, a reproductive assessment, supplementary to the two-generation study, was conducted (Appendix 45 of the Final Report). Acetamiprid was administered dietary to 26 male rats/group at 0 or 800 ppm for at least 20 weeks. The males were monitored for clinical signs, body weight changes, food consumption and general health and provided data on testicular sperm counts, epididymal sperm counts, and sperm morphology.

The mean achieved dosages (mg/kg b.w./day) for both the F1 and F2 generations for each specific study period are given in the Table 30. During lactation, mean maternal test material compound values were increased for all groups and for both generations. These differences are attributed to the beginning of feed consumption and wastage by the pups that may have confounded accurate measurement of maternal ingestion of the test article during lactation.

Table 32: Mean achieved doses of acetamiprid (mg/kg/day) for the F1 and F2 generations

	Males/pre-mating			**Females/pre-mating**			**Gestation**			**Lactation**		
Dose levels (ppm)	100	280	800	100	280	800	100	280	800	100	280	800
	mg/kg bw/day											
F0	6.5	17.9	51.0	7.6	21.7	60.1	6.8	18.5	50.9	13.2	37.5	108.1
F1	7.5	21.0	63.3	8.4	23.8	72.6	6.6	18.5	55.2	13.7	40.3	105.5

Premating period: week 0 to 10 for F0, and week 0 to 13 for F1 generation. Gestation period: day 0 to 20, lactation period: day 0 to 14. (Rounded values).

F0 generation

One F0 male of the control group and one F0 female of the 100 ppm group died during week 2 and on lactation day 18 respectively. No death or clinical signs were attributed to treatment.

Lower mean body weight values and food consumption for the F0 parent animals were limited to the 800 ppm groups throughout the study. Transient significant decreases in mean body weight gain and/or food

consumption occurred for the 280 ppm males during the first 2 weeks of the growth phase. It must be mentioned that the mean maternal food consumption values were significantly decreased for all dose levels throughout gestation. However, taking into consideration the fact that the values for the low-and mid- level groups were similar to historical control ranges recorded in the specific laboratory, only the significant decreases at 800 ppm were clearly treatment related.

Mating performance, fertility and reproduction parameters, oestrous cycles for the F0 generation were unaffected by treatment. Viability and weaning indices were unaffected by treatment (Table 31). In the 800 ppm group mean numbers of live pups/litter with live pups were significantly decreased on days 14 and 21. The range of the % number of females with stillborn pups as well as the values of the livebirth index observed at all dose levels was 3.8-26.9 and 97-100 respectively. Both ranges are within the historical control data ranges (0-58 and 92-100 respectively). Furthermore in-utero and post-natal growth of the 800 ppm F1 pups were significantly decreased throughout lactation (Table 31).

Table 33: Litters indices for the F0 and F1 females

Dietary level (ppm)	0	100	280	800
Pup Livebirth Indices				
F0-F1 phase	100	99	98	97
F1-F2 phase	99	97	97	98
Pup Viability Indices				
F0-F1 Phase	95	99	98	96
F1-F2 Phase	94	90	95	66**
Pup Weaning Indices				
F0-F1 Phase	96	99	99	94
F1-F2 Phase	98	94*	97	73**

* Significantly different from control; $p < 0.05$; ** Significantly different from control; $p < 0.01$

Landmark data for preputial separation and vaginal opening (mean age in days of pups in a litter reaching the criterion) were significantly increased and correlated with the decreased body weights for 800 ppm F1 pups. The mean age of reaching preputial separation was also increased for the 280 ppm male F1 pups and correlated with a significant decrease in mean body weight gain for this group during their early growth phase (week 0-1).

Necropsy examinations did not reveal compound-related changes in F0 adult animals or F1 pups. Organ weight changes comprised increased mean brain-to-body weight percentage and decreased kidney-to-brain weight ratio of the 800 ppm F0 females. No changes were observed for male (prostate, testis, epididymis total, epididymis, cauda (left), seminal vesicle) or female (ovary, uterus) reproductive organs (Table 32).

For F1 pups at 800 ppm, increased mean brain-to-body weight percentages in males and females, increased thymus-to-body weight percentage in males, decreased spleen-to-brain weight ratios in males and females and decreased thymus-to-brain weight ratio in females were observed (Table 33).

Table 34: Summary of statistically significant variations in organ weight data for F0 animals

	0 ppm		100 ppm		280 ppm		800 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Terminal body weight	532.0	316.3	529.0	308.4	524.7	317.7	513.0	291.5*
(g) (SD)	(40.9)	(29.7)	(40.0)	(22.8)	(35.1)	(24.5)	(38.0)	(23.7)
			Brain					
(%) to bw	0.413	0.628	0.421	0.667*	0.428	0.629	0.428	0.691*
			Kidney					
brain ratio	1.600	1.169	1.567	1.149	1.526	1.159	1.569	1.083*

*, $p < 0.05$; SD standard deviation

Table 35: Summary of statistically significant variations in organ weight data for F1 pups

	0 ppm		100 ppm		280 ppm		800 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Terminal body weight (g)	49.5	47.3	48.3	45.2	48.7	46.6	39.1*	38.9*
(SD)	(5.6)	(4.4)	(7.0)	(5.9)	(5.6)	(4.1)	(6.5)	(5.8)
			Brain					
absolute (g)	1.52	1.48	1.52	1.48	1.50	1.46	1.39*	1.36*
(%) to bw	3.110	3.151	3.207	3.309*	3.120	3.146	3.632*	3.564*
			Spleen					
absolute (g)	0.24	0.23	0.24	0.23	0.24	0.24	0.18*	0.19*
brain ratio	0.159	0.158	0.158	0.154	0.157	0.161	0.131*	0.141*
			Thymus					
absolute (g)	0.24	0.26	0.25	0.25	0.26*	0.26	0.21*	0.22*
(%) to bw	0.486	0.543	0.510	0.537	0.536*	0.568	0.529*	0.552
brain ratio	0.158	0.173	0.162	0.165	0.173*	0.182	0.149	0.158*

*, $p < 0.05$; SD standard deviation

No histopathological changes were attributed to treatment and no effect on testes or ovaries were reported. Due to the loss for technical reasons of the high-dose epididymes, sperm count and sperm morphology were not investigated on the 800 ppm males. A supplementary assessment (Appendix 45) was conducted to evaluate total testicular and epididymal sperm counts and sperm morphology for the animals receiving 800 ppm acetamiprid where no compound-related effects were observed.

F1 generation

Two 100 ppm and five 800 ppm F1 females experienced total litter deaths whereas pup observations such as thin pale weak and no visible milk in stomach were noted more frequently for litters with offspring mortality. However these findings were not dose dependent.

Mean body weights were consistently lower in F1 parents and correlated with lower food consumption values in the 800 ppm animals during all study phases. Body weight changes were consistently lower in male and female F1 parent animals at 800 ppm during the first 13 weeks of treatment. A transient significant decrease in mean body weight gain occurred for the 280 ppm males during the first week of the growth phase.

Mating performance, fertility and oestrous cycles were unaffected by treatment. Limited to the 800 ppm group, in-utero growth, post-natal growth, body weight throughout lactation, viability and weaning indices were significantly decreased following treatment of F2 pups (Table 30, Table 34).

Table 36: *In Utero*/Pup Survival and Covariate –Adjusted Pup Mean Weights at Days 0 and 21

Dietary level (ppm)		0	100	280	800
Mean No. Live Pups/Litter with Live Pups					
Day 0					
F0-F1 Phase		13.46	13.68	13.20	12.65
F1-F2 Phase		12.60	13.96	14.50	12.04
Day 21					
F0-F1 Phase		7.96	7.92	7.64	7.35*
F1-F2 Phase		7.00	7.86	7.75	6.11
Covariate adjusted mean for pup weight/litter					
Day 0					
F0-F1 phase	Males	6.50	6.41	6.57	6.03**
	Females	6.11	6.06	6.24	5.73**
F1-F2 phase	Males	6.31	6.42	6.49	5.97*
	Females	5.97	5.98	6.11	5.57*
Day 21					

F0-F1 phase	Males	51.10	49.67	50.27	39.89**
	Females	48.67	47.25	47.74	39.45**
F1-F2 phase	Males	48.47	48.29	46.79	41.22**
	Females	45.37	45.81	44.66	40.27

*, $p < 0.05$; **, $p < 0.01$. Litter size was used as covariate in the statistical analysis.

The mean age of 800 ppm F2 litters pups (in days) reaching eye opening was significantly increased and correlated with their decreased body weights. At 800 ppm the mean age in days of all pups in a litter reaching pinna unfolding was higher than that of the control group, although not significantly.
Necropsy of F1 adult and pup animals did not reveal compound-related changes.
For F1 adult animals, mean liver-to-body weight percentages (both sexes), brain-, spleen-, and uterus-to-body weight percentages (females), and spleen-to-brain weight ratio (females) were significantly increased at 800 ppm (Table 35).
In the 800 ppm F2 pup groups, increased brain-to-body weight percentages in both sexes, increase thymus-to-body weight percentages and decrease spleen-to-brain weight ratio in males were observed (Table 35).
No histological changes were attributed to treatment in the F1 animals, and no compound-related effects were observed on reproductive organs or functions, including ovarian follicle-count data, evaluation of sperm motility, testicular and epididymal sperm count and sperm morphology of the 800 ppm animals.

Table 37: Summary of statistically significant variations in organ weight data for F1 animals

	0 ppm		100 ppm		280 ppm		800 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Terminal body weight	593.4	344.1	583.5	344.7	581.4	336.5	534.8*	302.8*
(SD)	(51.5)	(27.7)	(53.3)	(32.7)	(53.3)	(25.8)	(60.7)	(34.1)
			Brain					
absolute (g)	2.30	2.07	2.25	2.09	2.22	2.07	2.07*	1.95*
(%) to bw	0.390	0.604	0.388	0.611	0.384	0.620	0.391	0.648*
			Spleen					
absolute (g)	0.86	0.60	0.87	0.65	0.82	0.64	0.77*	0.64
(%) to bw	0.146	0.176	0.150	0.190	0.142	0.190	0.145	0.213*
brain ratio	0.376	0.292	0.388	0.312	0.370	0.307	0.374	0.329*
			Uterus					
(%) to bw	-	0.198	-	0.202	-	0.204	-	0.241*
			Kidney					
absolute (g)	4.07	2.55	4.06	2.50	4.06	2.47	3.56*	2.28*
			Liver					
(%) to bw	3.432	3.685	3.424	3.707	3.544	3.676	3.657*	3.975*
			Thymus					
absolute (g)	0.49	0.36	0.41	0.42	0.50	0.37	0.38*	0.33
			Adrenal					
absolute (g)	0.066	0.092	0.062	0.079	0.066	0.088	0.057*	0.074
			Testis					
absolute (g)	3.84	-	3.71	-	3.80	-	3.53*	-
			Epididymis, cauda (left)					
absolute (g)	0.40	-	0.37	-	0.40	-	0.34*	-

*, $p < 0.05$; SD, standard deviation.

Table 38: Summary of statistically significant variations in organ weight data for F2 pups

	0 ppm		100 ppm		280 ppm		800 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Terminal body weight (SD)	48.0	45.0	48.1	45.0	46.0	43.6	39.4*	39.6*
	(6.4)	(6.8)	(5.9)	(5.7)	(6.9)	(6.1)	(7.5)	(6.7)
			Brain					
absolute	1.48	1.43	1.50	1.43	1.46	1.41	1.38*	1.35*

(%) to bw	3.141	3.245	3.157	3.217	3.216	3.275	3.602*	3.481*
			Spleen					
absolute	0.23	0.22	0.23	0.22	0.22	0.22	0.19*	0.20*
brain ratio	0.154	0.153	0.157	0.157	0.153	0.153	0.139*	0.145
			Thymus					
absolute	0.24	0.25	0.24	0.23	0.25	0.24	0.22*	0.22
(%) to bw	0.508	0.548	0.492	0.511*	0.536	0.546	0.549*	0.567

*, $p < 0.05$

In conclusion, the dietary exposure of rats to acetamiprid throughout two generations did not result in effects on reproductive performance or fertility. The NOAEL of this study was 100 ppm (6.5 mg/kg b.w./day) based on minimal transient effects on body weight and/or food consumption in the 280 ppm males during the growth phase. The NOAEL for pup development was 280 ppm (17.9 mg/kg b.w./day) based on reduced postnatal survival of F2 pups and consistent effects on body weights of adults and pups of both generations in the 800 ppm groups. The NOAEL for reproductive performance or fertility was 800 ppm (51 mg/kg b.w./day) as no treatment related effects were observed over 2 generations.

Repeated dose toxicity studies

In the study by Anonymous (1997f), CD™ (SD) rats (10 rats/sex/group) of 6 weeks old were dosed with 0, 50, 100, 200, 800, 1600 ppm of acetamiprid, via a dietary mixture, study termination after 13 weeks. At the highest dose group significant increase in relative testis weight was observed. This increase was probably secondary to the decrease in body weight. (This study is described in more detail in "10.10.4 Adverse effects on development".)

In the study by Anonymous (1999a) 60 mice/group/sex were dosed with 0, 130, 400 or 1200 ppm acetamiprid in the diet from which 10/sex/group were sacrificed after 1 year. The decrease in prostate absolute weight, was mainly observed in high dose treated males and is considered as related to the decreased body weight at the top-dose and not associated to the treatment.

In the study by Anonymous (1992), CD-1™ (ICR) mice, seven weeks old at initiation of treatment, obtained from Charles River Japan Inc. (Shiga) were used for the following doses groups (10/group/sex): 0, 400, 800, 1600 and 3200 ppm of acetamiprid incorporated in the diet, study termination was as 12-13 weeks. At the highest dose group significant increase in relative testis weight and a decrease in absolute and relative ovary weight were observed, although the relative weight increases and absolute weight decreases were attributed to reduced body weight.

In the study by Anonymous (1997a) New Zealand White Rabbits (Hra :[NZW]SPF) (five/sex/group) were treated on the dorsal intact skin clipped free of hair at the following doses: 0, 100, 500, and 1000 mg/kg b.w./day of Acetamiprid (5/sex/dose). Testis was examined microscopically but no compound-related effects were observed.

In a non-GLP, non-guideline study by Zhang et al. (2011) the effect of acetamiprid on the reproductive function of male mice and to study the role of oxidative stress in acetamiprid-induced damage to the testes was examined.
Kunmin male mice weighing 25-30 g were supplied by the Chonging Academy of Chinese Materials Medica. The animals were housed in rooms under a controlled temperature (22±2°C) with 50-60% relative humidity and a 12 h L/12 h D photoperiod, with ad libitum access to water and food pellets. The animals were acclimatized to laboratory conditions for up to 7 d prior to the gavages. According to a preliminary test, the dose of acetamiprid (purity >97%, from Shanghai Yongyuan Chem. Ltd. (Shanghai, China) was 30 mg/kg bw. Fifty male mice were randomly allocated into five groups (n=10 per group). The groups were as follows: (I) control; (II) blank (peanut oil); (III) 30 mg/kg acetamiprid; (IV) 30 mg/kg acetamiprid + 20 mg/kg vitamin E; and (V) 20 mg/kg vitamin E. Both acetamiprid and vitamin E were dissolved in 0.1 mL

peanut oil and delivered orally every day for 35 d. At 36 d after the start of treatment, all mice were sacrificed.

Haematological biochemical analysis and hormone assay
Blood samples were taken from the eye sockets of mice under anaesthesia using a 1 mL syringe before they were sacrificed. Blood samples were centrifuged at 5,000 r/min for 4 min and the serum samples were stored at 4°C for haematological biochemical analysis or 70°C until hormone analyses were performed. The activities of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were determined using an automatic chemistry analyser. Serum hormone concentrations were assayed using ELISA, according to the kit instructions. The sensitivity was 0.1 ng/mL.

Sperm collection and analysis of sperm output
The excised left epididymis was weighed and the sperm collected. The sperm were collected by centrifugation with saline-merthiolate-triton (SMT). The number of sperm was measured using a hemocytometer Epididymal sperm counting results were expressed as the number of sperms per gram of epididymis. 100 sperm from each epididymis were assayed for viability and malformations. Sperm viability was assessed by the eosin Y stain and the motility of sperm was assayed by the number of sperm that could move in a line. The percentage of viable sperm and the motility of sperm were calculated. The rate of sperm malformation was assayed by the motility of sperm and whether the acrosome was intact. The integrity of the acrosome was assessed using the Wright-Giemsa stain.

Ultrastructure of the Leydig cells and histological structure of the testis and epididymis
Samples of testis and epididymis were immersion-fixed in Bouin's solution for histopathology and embedded in paraffin. Serial sections (5 μm thick) were cut and stained with haematoxylin and eosin (H&E). The sections were mounted with dextran plasticizer xylene (DPX) and examined using Leica light microscopy. Leydig cells were quantified in the interstitium between seminiferous tubules stained by H&E.

The ultrastructure of Leydig cells were analysed. Samples of testes were cut into 2-mm-thick slices and fixed in ice-cold fixative consisting of 4% paraformaldehyde, 0.25% glutaraldehyde and 0.15 mol/L Hepes-KOH buffer (pH 7.4) for 30 min. Samples were post-fixed in 2% osmium tetroxide, dehydrated and embedded in Araldite 502. Ultra-thin sections (70-90 nm thick) of the blocks were picked up on copper grids, sections were stained with uranyl acetate and lead citrate and analysed under transmission electron microscope (TEM) at 80 kV.

Antioxidant enzyme activities and oxidative stress assays
Homogenization procedure of testes tissue was carried out for 2 min at 12 861×g in 5 mL of ice-cold Tris-HCl buffer (0.01 mol/, pH 7.4) containing 0.01% EDTA-2Na, 0.01 mol/L saccharose and 0.8% NaCl. All procedures were performed at 4°C. Homogenate, supernatant and extracted samples were prepared to determine the activities of CAT, GSH-Px, T-SOD, malondialdehyde (MDA) and NO.

Western blot analysis
Protein was isolated from testicular tissue using SDS PAGE and Western blot analyses. After blocking in PBS that contained 2% Tween-20 and 3% bovine serum antigen (BSA), membranes were incubated in a 1:200 dilution of primary antibody (anti-p38) and in a 1:1000 (antiphospho-p38) dilution of primary antibody in 5% phosphate-buffered saline-Tris (PBST) at 4°C overnight. The membranes were washed three times and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2500) at room temperature for 1 h. Reactive bands were visualized by SuperSignal® West Pico chemiluminescent substrate and the membranes were then subjected to X-ray autoradiography. The Western blot X-ray films were scanned using Uniscan A688 and Smart Panel Scan software. Band intensities were determined by Quantity One software. The densitometry value of the phospho-p38 (p-p38) signal was divided by the value of the total p38 in the same lane in order to normalize the value to the protein load.

Total residues of acetamiprid in testes and livers

Testes or liver tissues (0.2 g) were ultrasonically extracted for 1 h with anhydrous sodium carbonate (1 g) and diamine methane (5 mL) using a homogenizer and centrifuged for 5 min at 12 861×g. The sample was dried before acetonitrile (20%) was added. Different concentrations of standard acetamiprid (2, 5, 10, 50 and 100 ng/mL) were used and detected in duplicate. The volume of each sample was 50 μL. High performance liquid chromatography (HPLC) was performed with a C18 column, methanol for the mobile phase A and 1% acetic acid water for the mobile phase B. The flow rate was 0.3 mL/min. Mass spectrometry (MS) conditions were: selected reaction monitoring (SEM) acquisition, the parent ion was 223.054, the qualitative ion was 73.054 m/z and the quantitative ion was 126.047 m/z. We took the mass concentration (ng/mL) for the horizontal and the peak area for the vertical coordinates, then drew the standard curve to enable the regression equation to be calculated. Tissue residues were calculated by the following formula:
Residues (ng g-1) =[(A×C×V)/As×m]×n
Where, A was the sample size, C was the standard preparation concentration, V was the volume size, n was the dilution multiple, as was the standard peak area and m was the sample quality.

Results

Effect of acetamiprid on body weight gain and the weight of testis, epididymis, seminal vesicles and prostate gland

Compared to the controls, acetamiprid decreased body weight gain (-38%) and the relative weight of the testis and epididymis (-17%), seminal vesicles and prostate gland (-17%) ($P<0.05$). Vitamin E significantly ameliorated the effect of acetamiprid on body weight and testis, epididymis, seminal vesicles and prostate gland, compared to the acetamiprid only group ($P<0.05$). The body weight and testosterone-responsive organs were not affected in the blank (peanut oil) and vitamin E groups ($P>0.05$).

Acetamiprid negatively affected sperm output and quality

Compared to the control group, acetamiprid decreased sperm number (-76%), viability (13%) and motility (52%) ($P<0.05$), while increased the rate of acrosome deformity ($P<0.05$). Vitamin E reduced these adverse effects of acetamiprid by increasing sperm count, viability and sperm motility ($P<0.05$) and decreasing the rate of spermatic malformations ($P<0.05$). Compared to the control group, administration of peanut oil and vitamin E had no effect on sperm count, viability, motility and intact acrosome rate ($P>0.05$).

Table 39: Effect of acetamiprid on body weight gain and the weight of testis, epididymis, seminal vesicles, and prostate gland in mice

Group	Body weight gain	Testis and epididymidis/Body weight×1 000	Seminal vesicles and prostate/Body weight×1 000
Control	15.140±2.649 a	4.513±0.549 a	8.954±1.391 a
Blank (peanut oil)	13.087±0.919 a	4.379±0.403 a	8.866±0.915 a
Acetamiprid	9.386±2.175 b	3.713±0.629 b	7.423±1.042 b
Acetamiprid+Vitamin E	12.286±2.047 a	4.336±0.506 a	8.829±0.571 a
Vitamin E	15.138±4.479 a	4.421±0.383 a	8.871±1.062 a

The values denoted by different letters within same column represent significant differences ($P<0.05$). The same as below.

Table 40: Effect of acetamiprid on sperm count and quality in mice

Group	Sperm count (×108 g-1 epididymis)	Viability (%)	Sperm motility (%)	Rate of intact acrosomes (%)
Control	6.34±0.57 a	96.21±2.62 a	69.75±6.95 a	80.83±9.01 a
Blank (peanut oil)	5.80±0.65 a	95.60±2.53 a	67.50±6.36 a	78.12±8.82 a
Acetamiprid	1.52±0.22 c	83.73±7.91 b	33.50±2.12 c	37.83±3.75 b
Acetamiprid+Vitamin E	3.95±0.56 b	93.12±1.94 a	49.33±5.69 b	68.00±5.29 a
Vitamin E	5.94±0.50 a	93.91±2.93 a	69.00±7.07 a	78.83±5.62 a

Effect of acetamiprid on serum testosterone concentration
Compared to the controls, serum testosterone level decreased in the acetamiprid only group ($P<0.05$). Vitamin E increased the concentration of testosterone compared to the acetamiprid only group. Peanut oil and vitamin E had no effect on testosterone concentration ($P>0.05$).

Effect of acetamiprid on histological structure of the testis and epididymis
Testes from the control group were in various stages of spermatogenesis; Leydig cells were abundant in the interstitium. In the acetamiprid only group, there was vacuolization of the seminiferous tubules and the number of spermatids and interstitial Leydig cells were obviously decreased. Moreover, some cells sloughed from the lumen of the seminiferous tubules, some primary spermatocytes vacuolized and the interstitium got widened. In the acetamiprid with vitamin E group, some spermatozoa remained within the seminiferous tubules, the number of spermatids and interstitial Leydig cells increased and the interstitial space was smaller in comparison to the acetamiprid only group. Peanut oil and vitamin E had no obvious effect compared to the controls. In the control group, sperms were numerous in the lumens of the epididymis. In the acetamiprid only group, there was almost no sperm in the lumens of the seminiferous tubules. Vitamin E increased the number of sperm in comparison to the acetamiprid group. Peanut oil and vitamin E had no effect on the epididymis.

Effects of acetamiprid on the ultrastructure of Leydig cells
In the control group, Leydig cells had normal endoplasmic reticulum (ER) and mitochondrial profiles, the cytoplasmic organelles were abundant, chromatin distribution was normal and the structure of chromatospherite and the boundary of the nuclear membrane were clear. In the acetamiprid only group, a large number of mitochondria were swollen. Vitamin E appeared to prevent these structural changes to some degree. Organelles were abundant and structure of mitochondria was normal, but the chromatin was slightly aggregated. Furthermore, the structure of local endoplasmic reticulum was unclear when compared to the control. The administration of peanut oil and vitamin E had no effect on the ultrastructure of Leydig cells.

Effect of acetamiprid on oxidative stress
Acetamiprid increased MDA and NO concentrations compared to the controls ($P<0.05$). Vitamin E ameliorated the effect of acetamiprid and MDA and NO concentrations were lower in acetamiprid group that received vitamin E than in acetamiprid only group. Compared to the controls, peanut oil and vitamin E had no effect on the concentrations of MDA and NO.

Acetamiprid decreased the activity of antioxidant enzymes
In the acetamiprid group, the activity of CAT, GSH-Px and T-SOD was reduced compared to the control ($P<0.05$). Compared to acetamiprid only group, vitamin E increased the concentrations of CAT, GSH-Px and T-SOD ($P<0.05$). Compared to the control, peanut oil and vitamin E had no effect on antioxidant enzymes ($P>0.05$).

Effect of acetamiprid on p38 activity
Compared to the controls, the concentration of antiphospho-p38 protein was elevated with acetamiprid treatment and vitamin E prevented this elevation. Peanut oil and vitamin E had no effect on p38 activity.

Effect of acetamiprid on serum enzymes
Compared to the controls, acetamiprid increased the activity of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) ($P<0.05$). Vitamin E prevented this increase of these serum enzymes in comparison to acetamiprid only group. Peanut oil and vitamin E had no effect on the activity of ALT, AST and ALP ($P>0.05$).

Acetamiprid residue in the testes and livers

Acetamiprid was not detected in the testes and liver of the control, peanut oil and vitamin E groups. Compared to the controls, the amount of acetamiprid residue in the testes was increased in acetamiprid only group ($P<0.05$). Additionally, the concentration of acetamiprid in the liver was higher than that in the testes ($P<0.05$). Vitamin E reduced the amount of acetamiprid residue in the liver and testes in comparison to acetamiprid only group ($P<0.05$).

10.10.1.2 In vitro

In a non-GLP, non-guideline study by Gu et al. (2013) *in vitro* effects of acetamiprid (purity >96%, Shanghai Pesticide Research Institute (Shanghai, China)), imidacloprid (purity >96%, Shanghai Pesticide Research Institute (Shanghai, China)) and nicotine (>99%, Sigma-Aldrich (St Louis, MO)) on mammalian reproduction was performed. The study included an integrated testing strategy for reproductive toxicology, where sperm quality, sperm penetration into oocytes and preimplantation embryonic development were determined and compared.

Animals
6–8 weeks old female B6D2F1 (C57BL/6xDBAx2) strain mice were used as oocyte donors and 10–15 weeks old male B6D2F1 mice were used as semen donors. All mice were housed under controlled light conditions (12 h light: 12 h dark) in the Laboratory Animal Services Facility and were fed a standard mouse diet and water ad libitum.

Experimental design
To investigate the effect of the three test materials on fertilization and embryonic development, concentrated acetamiprid, imidacloprid and nicotine were prepared as medium supplements. DMSO was used at final concentration ≤0.1% and this vehicle was used as control to investigate the potential effect of the solvent. In the preliminary experiment, nicotine exposure at 500 µM for 30 minutes did not impair either motility and fertilization capability of mouse spermatozoa. Concentrations of 5 mM were then adopted for the sperm exposure experiment.

In sperm exposure experiment, mouse spermatozoa were placed in acetamiprid, imidacloprid or nicotine-containing (500 µM or 5 mM) HTF medium supplemented with bovine serum albumin for 30 min first, then washed by and incubated in fresh HTF-BSA medium for another 60 min until capacitation finished, followed by normal IVF procedure. Control spermatozoa were processed with the same procedure except the exposure of chemicals.

To study their effects on the development of early embryos that skipped the stage of fertilization or the first cleavage, zygotes with two pronuclei as well as 2-cell stage embryos by natural insemination were cultured in acetamiprid, imidicloprid or nicotine-added KSOM medium (500 µM) to observe how chemicals worked at subsequent developmental stage. Furthermore, the consecutive exposure process from fertilization to blastocyst formation was monitored with exposure concentration of 500 µM both in HTF medium for fertilization and KSOM medium for embryo culture. Concentrations of pesticides were limited to 500 µM because the preliminary experiments indicated that oocytes and embryos with higher than 500 µM of nicotine would induce massive fragmentation or death the next day.

Collection of Spermatozoa
Caudal epididymides were isolated, gently squeezed out and placed in a 2 ml eppendorf tube with HTF-BSA. 'Swim-up' spermatozoa were obtained after incubation at 37ºC for 10 min.

Collection of Oocytes and Embryos
Mature female mice were superovulated with 10 IU of pregnant mare serum gonadotropin (PMSG) and 5 IU of human chorionic gonadotropin (HCG) at 48 h intervals. 14–16 h after HCG administration, cumulus oocyte complexes (COCs) were collected from the removed oviducts and then maintained in human tubal

fluid medium supplemented with 10% human serum albumin at 37ºC in an atmosphere of 5% CO2 in air until use.

With regard to the recovery of the naturally fertilized zygotes and embryos, several female mice were mated with males and examined 12–18 h after HCG injection for the presence of copulation plugs. Fertilized oocytes and 2-cell embryos were recovered by flushing the oviducts 24 h and 40 h later after the HCG injection, respectively. The cumulus of oocytes were dispersed with 0.1% hyaluronidase and washed in several changes of HCZB medium. Fertilized oocytes (identified by the presence of a second polar body and two pronuclei) and 2-cell embryos were then placed in potassium chloride supplemented simplex optimized medium, which was designed for culture of implantation stage embryos and previously equilibrated in a humidified atmosphere of 5% CO2 in air at 37ºC.

Sperm Motility Assay
The control droplet consisted of an equivalent volume of DMSO in treated groups. After incubation, a 15 µl aliquot of the treated and control samples was transferred into each of two compartments on a glass cannula slide for computer-assisted sperm analysis (CASA) using the integrated visual optical system (IVOS) motility analyser. Thirty frames were acquired at a frame rate of 60 Hz. The operational settings of the IVOS were as follows: minimum contrast (40) and size (four pixels), gate thresholds 0.38/1.65 for intensity and 0.42/2.34 for size, static elongation 0/75, progressive minimum path velocities of sperm (VAP) 50 µm/sec, straightness threshold 50% and magnification 0.82.

Sperm Chromatin Dispersion (SCD) Assay
Generally, SCD assay was developed as the HalospermH kit instructed. An aliquot of each semen sample was diluted to 5–10 million/ml in PBS. The unfixed suspensions were mixed with 1% low-melting-point aqueous agarose (to obtain a 0.7% final agarose concentration) at 37ºC. Aliquots of 20 µl mixture were pipetted onto a glass slide precoated with 0.65% standard agarose, covered with a coverslip (22 x 22 mm) and left to solidify at 4ºC for 5 min. Then coverslips were carefully removed and slides immediately incubated with freshly prepared acid denaturation solution for 7 min (RT) in the dark to generate restricted single-stranded DNA (ssDNA) motifs from DNA breaks. The denaturation was then stopped, followed by incubation with lysing solution for 23 min (RT). Slides were thoroughly washed in deionized water for 5 min, dehydrated in sequential 70%, 90% and 100% ethanol baths (2 min each) and air dried. Afterwards, cells were stained with modified Wright-Giemsa stain for bright-field microscopy and a minimum of 400 spermatozoa per sample were evaluated under the x 40 objective of the light microscope. After staining, four SCD patterns were established: sperm heads with (i) large size halos, whose halo width was similar or larger than the minor diameter of the core, (ii) medium size halos, whose halo size was between those with large and with small halo, (iii) small size halos, whose halo width was similar or smaller than one third of the minor diameter of the core and (iv) without a halo or degraded sperm cells, the latter ones were weakly or irregularly stained. The spermatozoa without DNA damage showed nucleoids with large- or medium-sized halos of spreading DNA loops whereas those with fragmented DNA appeared with a small or no halo. Finally, the percentage of sperm (iii) and (iv) was considered as DNA fragmentation index (DFI) for each semen sample. In this study, spermatozoa pre-incubated in acetamiprid, imidacloprid or nicotine-added HTF medium (5 mM or 500 µM) for 30 min were analysed for DNA integrity.

In vitro Fertilization and Preimplantation Embryonic
IVF procedure was performed as previously described (Wakayama et al, 2009). HTF medium was equilibrated in a 37ºC, 5% CO2 incubator one day before experiment. Next day, caudal epididymides were collected from adult male mice. A dense sperm mass was squeezed out and then incubated in HTF-BSA medium for 60–90 min at 37ºC to develop their fertilization potential (capacitation). A small volume of capacitated sperm suspension was added to a drop of 200 µl HTF-BSA medium containing freshly ovulated oocytes to achieve a final sperm concentration of 106/ml. Four to six hours later, fertilized oocytes at pronuclear stage were washed and cultured in KSOM for in vitro development to morula/blastocyst stages in 5% CO2 in air. Oocytes were observed for male and female pronucleus formation (fertilization) at 6 h after

the initiation of culture and the number of 2-cell embryos, 4-cell embryos, morulae and blastocysts after 24, 48, 72 and 96 h in culture were checked and recorded.

Results

Influences of Chemical Exposure on Sperm Function

With CASA, objective and quantitative descriptions of changes in sperm kinematic parameters were obtained in response to exogenous toxicant. Treated with 500 μM or 5 mM of acetamiprid or imidacloprid for 30 minutes, motility of spermatozoa showed no obvious difference from that of control. Any toxicant-induced reproductive hazards associated with sperm DNA lesion were then investigated using SCD assay. All treated groups displayed a minor increase in average percentage of DNA fragmented spermatozoa compared with those of control groups without reaching a significant difference ($P<0.05$), as shown in Figure 1.

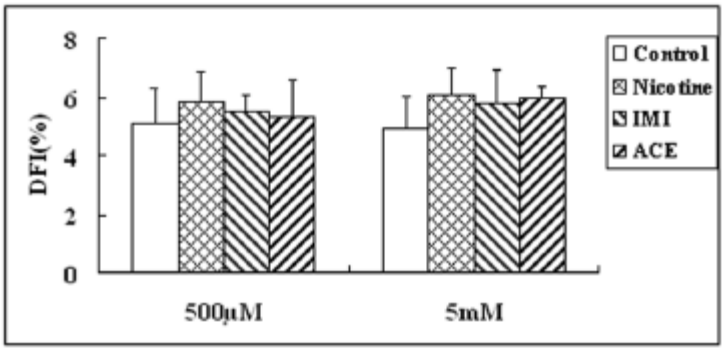


Figure 1: The SCD results of toxicants exposure upon sperm DNA integrity

With respect to the difference between 500 μM and 5 mM of each test material, the response toward exogenous compounds at current differential concentration was not obvious, as similar effects on sperm motility and DFI were observed.

When IVF was performed with the spermatozoa pretreated with test materials at the concentration of 500 μM or 5 mM, all fertilized oocytes survived without evident changes in cell morphology. Fertilized oocytes were judged normal by extrusion of second polar body and the presence of two pronuclei, which represents success of fertilization. The preliminary data indicated that treatment with 500 μM of chemicals for 30 min did not induce any significant adverse effect on fertilization potential of the spermatozoa and subsequent embryo development. However, during the culture process, embryos originated from spermatozoa pretreated with 5 mM of chemicals were more inclined to encounter failure of the first cleavage, wherein some of them exhibited various degrees of cellular fragmentation and asymmetry. Finally, when these embryos were cultured in vitro up to 96 h, with fragmentation, loss of cytoplasm or decrease in cytoplasmic clarity, part of them would arrest or degenerate during the developmental progression. In the presence of 5 mM toxicant in HTF medium, all treated spermatozoa retained their potential to fertilize oocytes. However, in nicotine and imidacloprid-exposed groups, rates of pronucleus formation (fertilization), the first cleavage and morula/blastocyst formation were remarkably decreased, compared to those of non-treated control ($P<0.05$). In the ACE-exposed group, the first cleavage of zygote and blastocyst formation process were also impaired (91.2% and 58.5%, respectively), compared to those of the control group (98.5% and 74.6%, respectively) whereas the fertilization rate was slightly lower than that of control without reaching a statistical significance. Thus, ACE appeared to pose much weaker adverse effects on mouse spermatozoa, at least in terms of fertilization process in vitro. During embryo culture, embryo fragmentation, a process where portions of the embryo's cells have broken off, was noteworthy to the author. It is preferable to have little or no fragmentation when evaluating a normal embryo, while nicotine and imidacloprid exposure remarkably elevated the fragmented embryo percentage compared to the control. Comparisons were also made among treated groups, it was observed that ACE exerted a significantly moderate effect, whereas nicotine exposure showed the most severe reproductive hazard.

Influences of Chemical Exposure on Fertilization and Subsequent Embryonic Development in vitro
In consecutive exposure experiments with chemical exposure in HTF and KSOM medium, the concentration of supplementation was constricted to 500 μM in both of media, which allowed the fertilized oocytes to proceed to blastocysts without inducing excessive fragmented or dead embryos. It was shown that a mixture of oocytes and spermatozoa in chemical-added HTF maintained normal fertilization capacity compared with controls (ratio of oocytes with pronuclei formation, $P<0.05$), while the percentages of 2-cell embryo and morula/blastocyst formation decreased significantly ($P<0.05$). Among the test materials, ACE appeared weakest in the effect on the first cleavage (ratio of 2-cell embryo formation), with no significance found.

Influences of Chemical Exposure on Preimplantation
Considering that exposure of 1 mM chemicals would cause considerable embryo fragmentation or even death in the preliminary experiments, 500 μM was used in culture medium. Exposure of the test materials caused a moderate decline in the percentage of 2-cell embryo formation and a drastic impact on morula/blastocyst formation derived from normal zygotes, compared with controls ($P<0.05$). When comparison was made among the test materials, exposure of imidacloprid or acetomiprid retarded embryonic development to a more moderate degree than nicotine but no significant difference existed between the imidacloprid and acetamiprid groups. Taken together, the adverse effects exerted on the development of zygotes are in the order of nicotine>imidacloprid>acetamiprid.

Incubated with 500 μM of each chemical, the development of naturally fertilized 2-cell embryos was also monitored. Under these conditions, treatment with the test materials did not show significant adverse effects on 2-cell embryos ($P<0.05$), with less extent of effects than those observed in zygotes. These results collectively revealed that the doses up to 500 μM of each chemical used in the study did not exert toxicity at the onset of 2-cell embryo, but fertilization or zygote as well as the subsequent developmental procedure with preceding chemical exposure.

Discussion
Despite of lower affinity to mammalian nAChRs, neonicotinoids have been illustrated to impair mammalian reproduction by recent animal studies. In the present study, a set of in vitro models of reproductive toxicology were used and examined the direct effects of imidacloprid and acetomiprid, on spermatozoa, fertilization procedure and preimplantation embryo development. Sperm quality, such as motility and DNA integrity, are important in male fertility and in the particular contribution to early embryonic development, which is also a sensitive and quick testing strategy for reproductive toxicology. In vitro exposure of nicotine to human semen was reported to be able to cause human sperm DNA damage and motility decrease (1 mM for 20 min). However, motility and DNA integrity were not significantly affected by a high exposure dosage (5 mM for 30 min) of chemicals, even with nicotine, which may result from the difference in the experimental objects, i.e. mouse spermatozoa versus human semen. When IVF process was introduced, subtle differences among the spermatozoa caused by pretreatment with the different test materials could still be detected through the procedures of fertilization and subsequent embryonic development.

The IVF procedure includes sperm-egg binding, zygote formation and the first cleavage to form 2-cell embryo. After being transferred into KSOM medium, 2-cell embryo could conduct multiple cleavages to successively form 4-cell embryo, morula then blastocyst in vitro. In order to determine the specific embryo developmental stages that the test materials could affect, a mixture of spermatozoa and eggs for fertilization, naturally fertilized zygotes and 2-cell embryos were separately prepared and consecutive chemical exposure with a concentration of 500 μM was conducted until blastocyst formation. Exposure to these materials during fertilization could adversely affect 2-cell formation and subsequent embryo development, normal zygotes with chemical exposure could impair subsequent 4-cell embryo formation and the following procedure, whereas there was no significant adverse effect on subsequent development when normal 2 cell embryos were treated with these chemicals. Compared with the effects on fertilization procedure or zygotes, the results suggested that 2-cell embryos were most resistant to exposure of 500 mM nicotine, imidacloprid or acetamiprid, which is consistent with the previous study of 2-cell embryos toxicity with nicotine.

Although it may cause human reproduction disorder, nicotine could show in vitro detriments only with a concentration much higher than the exposure level in an 'average' smoker, suggesting that nicotine might adversely affect spermatozoa or embryos in an indirect way. These results supported previous reports and imply that imidacloprid and acetamiprid may work with a similar mechanism to nicotine. Studies indicate that acute or chronic exposure of nicotine will cause oxidative stress in animal and human body, which could do harm to reproductive organs. Several studies reported that oxidative stress caused by testicular tissue and lymphocyte in semen will impair sperm parameters, suggesting that owing to lack of lymphocytes around, 'swim-up' mouse spermatozoa in this study are more resistant to the exposure of nicotine than human spermatozoa in semen. Human exposure to neonicotinoids is very limited (12.8–350 ng/ml in the urine of farm workers, with or without protection) and this study indicated that, at exposure levels, imidacloprid and acetamiprid do not show adverse effects on mouse sperm functions and early embryo development in vitro. However, recent animal studies showed that imidacloprid and acetamiprid could cause oxidative stress in the body and even chronic exposure of imidacloprid with a low concentration could result in oxidative stress in tissues, which suggests that low level of neonicotinoid exposure over a long period may also exert impact on human reproduction especially for professional populations.

Taken together in a reproductive toxicity study, several in vitro tests were integrated and reported in this and previous studies conducted by the authors, which covered sperm quality, sperm penetration into oocytes, process of oocyte in vitro maturation and preimplantation embryonic development. The results indicated that, at high levels, direct exposure of nicotine, imidacloprid or acetamiprid had harmful effects on sperm function and embryonic development and stages mainly at fertilization, zygote formation and first cleavage of zygote, with the extent in an order of nicotine>imidacloprid>acetamiprid. These results elucidated the reproductive toxicities of two neonicotinoids on mammals from a new prospective, which evaluated the direct effects of pesticides on gametes, fertilization and embryonic development.

Table 41: Summary table of animal studies on adverse effects on sexual function and fertility

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Result on general toxicity	Results on sexual function and fertility	Remarks (Klimisch score)*	Reference
Two-Generation Reproduction Study with NI-25 in Sprague-Dawley Rats 26 rats/sex/group (F0 generation) In compliance with EEC B.35 method	NI-25 (Acetamiprid), purity 99.9% 0, 100, 280 or 800 ppm for at least 10 weeks prior mating. Study was terminated after weaning the F2 generation.	Reduced body weight and food consumption	No effects on reproductive performance or fertility were observed. NOAEL: 800 ppm (51 mg/kg b.w./day) Reduced absolute testis and epididymis weights at 800 ppm in F1 males.	1 (reliably without restriction)	Anonymous (1999d)
Thirteen week (90 days) subchronic toxicity study in 10 rats/group/sex (6 weeks old) In compliance with EEC B.26	NI-25 (Acetamiprid) Purity: 99% ww 0, 50, 100, 200, 800, and 1600 ppm Oral Sacrificed after 13 weeks (surviving animals)	Several symptoms of general toxicity in 800 ppm and 1600 ppm males and females. (NOAEL set at 200 ppm)	Significant increase in relative testis weight at 800 ppm and 1600 ppm males, not accompanied by any macroscopic or microscopic lesions.	1 (reliably without restriction)	Anonymous (1997f)

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Result on general toxicity**	**Results on sexual function and fertility**	**Remarks (Klimisch score)***	**Reference**
18-Month dietary oncogenicity study in 60 mice/group/sex In compliance with EEC B.33	NI-25 (Acetamiprid) Purity: 99.38% 0, 130, 400, and 1200 ppm Oral Sacrificed after 1 year or after 18 months (surviving animals)	Decreased body weight in 1200 ppm males	Decrease in prostate absolute weight in 1200 ppm male group, not accompanied by any macroscopic or microscopic lesions. Decreased absolute ovary weight at final sacrifice at 1200 ppm; no effect on ovary weight at interim sacrifice.	1 (reliably without restriction)	Anonymous (1999a)
Thirteen week (90 days) subchronic toxicity study in 10 mice/group/sex In compliance with EEC B.26	NI-25 (Acetamiprid) Purity: 99.2% 0, 400, 800, 1600, and 3200 ppm Oral Sacrificed after 13 weeks (surviving animals)	Several symptoms of general toxicity in 1600 ppm and higher groups	Increase in testis and ovary weight at 3200 ppm groups, not accompanied by any macroscopic or microscopic lesions.	1 (reliably without restriction)	Anonymous (1992)
21-Day dermal toxicity study in 5 rabbits/sex/group In compliance with EEC B.9	NI-25 (Acetamiprid) Purity: 99.9% 0, 100, 500, and 1000 mg/kg bw/day	No systemic toxicity observed	No compound-related effects on testis.	1 (reliably without restriction)	Anonymous (1997a)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Result on general toxicity	Results on sexual function and fertility	Remarks (Klimisch score)*	Reference
Fifty adult Kunmin male mice (25-30 g) were divided into five groups (n=10 per group), i.e., control, blank, acetamiprid alone, acetamiprid and vitamin E and vitamin E alone. All groups were treated for 35 d.	Acetamiprid (>97% pure, Shanghai Yongyuan Chem. Ltd.) Gavage, daily at 35 mg/kg for 35 days. Groups were as follows: (I) control; (II) blank (peanut oil); (III) 30 mg/kg acetamiprid; (IV) 30 mg/kg acetamiprid + 20 mg/kg vitamin E; and (V) 20 mg/kg vitamin E.	Significant decrease in body weight in all groups	The results showed that acetamiprid significantly decreased the body weight (-38%) and the weight of testosterone responsive organs, such as the testis and epididymis (-17%), and seminal vesicle and prostate (-17%). Furthermore, acetamiprid also significantly reduced the serum testosterone concentration and decreased sperm count (-76%), viability (-13%), motility (-52%) and the intactness of the acrosome ($P<0.05$ for each parameter). The mice treated with acetamiprid had damaged seminiferous tubules and Leydig cells based on the histological structure of testes; there was degeneration of the mitochondria and endoplasmic reticulum of Leydig cells.	2 (reliable with restriction)	Zhang et al. (2011)

* Klimisch et al. (1997)

Table 42: Summary table of *in vitro* studies on adverse effects on sexual function and fertility

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Remarks (Klimisch score)*	Reference
Direct chemical exposure (500 µM or 5 mM) on spermatozoa during capacitation was performed and in vitro fertilization (IVF) process, zygotes and 2-cell embryos were respectively incubated with chemical-supplemented medium until	Acetamiprid (>96% pure) Addition to culture media. 5 mM, 500 µM,	Culture experiments in the presence of chemicals in medium showed that the fertilization process and zygotes are adversely affected by direct exposure ($P<0.05$), in an order of	3 (not reliable)	Gu et al. (2013)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Remarks (Klimisch score)*	Reference
blastocyst formation to evaluate the reproductive toxicity of these chemicals and monitor the stages mainly affected. Non-GLP, Non guideline	Sperm and oocytes were exposed for 30 minutes. Concepti were exposed for 96 h.	nicotine>IMI>ACE, whereas developmental progression of 2-cell stage embryos was similar to controls (P<0.05).		

* Klimisch et al. (1997)

10.10.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

Only one study on sexual function and fertility is available (Anonymous, 1999d). Dietary exposure of rats to acetamiprid throughout two generations did not result in effects on reproductive performance or fertility (NOAEL 800 ppm (51 mg/kg b.w./day). Five repeated dose studies indicate effects on sexual organs following exposure to ≥1000 ppm, in general in presence of general toxicity. The methods as described in the study by Zhang *et al.* are well described and may indicate that effects on male reproductive function occur following exposure to 30 mg/kg bw/day (although the used acetamiprid has a purity of >97%), e.g. a clear reduction in sperm count was observed. Although the *in vitro* study of Gu *et al.* is a well described study it is an in vitro study not relevant to actual gamete exposures, relevance of concentrations tested not justified and way in excess of in vivo exposures, (with the lower concentration being defined as that for nicotine which caused massive fragmentation and death the next day) and exposure to neat material rather than metabolites as would occur in vivo. The *in vitro* study is superseded by existing *in vivo* data.

10.10.3 Comparison with the CLP criteria

For the purpose of classification for reproductive toxicity, substances are allocated to one of two categories. As shown in Annex I, Table 3.7.1 (a) in Regulation (EC) 1272/2008 on CLP, within each category, effects on sexual function and fertility, and on development, are considered separately. Acetamiprid is not a known or presumed human reproductive toxicant (category 1), neither a suspected human reproductive toxicant, resulting in no classification.

Classification for adverse effects on sexual function and development in category 1A is based on human data (CLP Annex I table 3.7.1(a). As there is no human data available, classification in category 1A is not warranted.

Classification for adverse effects on sexual function and development in category 1B is based on adverse effects on fertility or on reproductive organs as observed in animal studies in the absence of general toxicity or in the presence of general toxicity but not considered secondary to the general toxicity. As no effects on fertility were observed in the available 2-generation study and the observed effects on the reproductive organs in the repeated dose toxicity studies were observed in the presence of general toxicity and was limited to effects that can be expected to be secondary to the observed maternal toxicity, classification in category 1B is not applicable.

Classification for adverse effects on sexual function and fertility in category 2 is based on adverse effects on fertility or on reproductive organs as observed in animal studies in the presence of general toxicity but it is unknown whether it is secondary to the general toxicity or there are limitations in the study. No effects on fertility were observed in the available 2-generation study. Effects on reproductive organs were sometimes observed in the 2-generation study and in the repeated dose studies. These effects were mostly limited to reductions in absolute organs weights such as testis and epididymis in the presence of reduced body weights (see Table 38). As no such effects were observed on relative organ weights and no histopathological effects were observed, the reduced absolute organ weights are considered secondary to the general toxicity and do not warrant classification. The only exception is the in vivo study by Zhang in which adverse effects on testis weight, histopathology and sperm parameters were observed in the presence of reduced body weights in

mice after exposure by gavage for 35 days. However, the results in this study are clearly different from the 13-week repeated dose diet study in mice at dose levels up to approximately 450 mg/kg bw/day (Anonymous, 1992). In this study only an increase in relative testis weight and reduced body weights was observed at dose levels of 230 and 450 mg/kg bw/day. Also no such effects were observed in the 18-months study in mice (Anonymous, 1999a). A likely explanation for the differences between these two studies is the purity/impurity of the tested acetamiprid. The Zhang study was performed with acetamiprid with a purity of > 97% from Shanghai Yongyuan Chem. Ltd. whereas the Anonymous (1992) study used acetamiprid with a purity of >99% from the applicant. The last purity is in line with the required purity of minimally 99%. Therefore, it considered likely that the results obtained by Zhang are due to different impurities and are not representative for the form of acetamiprid marketed in Europe. Therefore, the results of this study are not taken into account for classification and labelling. Overall, the available data do not warrant classification in category 2. Therefore, no classification for effects on sexual function and fertility is proposed.

10.10.4 Adverse effects on development

10.10.4.1 Rats

In the preliminary study by Anonymous (1997f), mated rats were treated with acetamiprid (purity 99.46%) at dose levels of 0, 18, 35 and 70 mg/kg b.w./day. Treatment-related reductions in parental body weight and food consumption were observed at 35 and 70 mg/kg b.w./day. No offspring anomaly was noted at any dose levels. The no effect level was considered to be 18 mg/kg b.w./day. From these results, the following dose levels were selected: 5, 16, 50 mg/kg b.w./day.

The test substance was administered orally by gavage at doses of 0, 5, 16 and 50 mg/kg b.w./day to groups of 24 mated females rats/group, for a period of 10 days from 6 to 15 days of gestation. Control animals received vehicle (0.2 ml/100 g/day) only, in the same procedure as treated animals. During the administration period, the clinical signs were examined daily and body weight was measured on days 0, and 6-21. Daily food consumption was also measured on days 6-21. The rats were sacrificed by exsanguination on day 21 and their organ weights (liver, spleen, kidney, adrenal and ovary) were measured. Viable fetuses were examined for external and visceral malformations and variations, and their body weights and placental weights were recorded.

Maternal observations

No mortality or toxic sign occurred in any treated animal.

Statistically significant maternal body weight gain depression and a decrease in food consumption were noted in dams from 50 mg/kg b.w./day group (Table 41). The body weight gain during days 6 to 15 of gestation was significantly ($P<0.01$) decreased at 50 mg/kg b.w./day (24.8 g) compared to control (42.1 g).

Statistically significant increases of liver weights and their body weight ratios and kidney/body weight ratios were observed in the high dose group. No treatment-related changes were noted in dams from other dose groups.

The numbers of total implantation, corpora lutea, live fetuses were not altered by the administration. The number of resorbed fetuses was significantly increased at 16 mg/kg b.w./day but this finding was not related to dose.

Table 43: Summary of findings (Dams)

Dose (mg/kg b.w./day)	**0**	**5**	**16**	**50**
No. of females/group	24	24	24	24
Clinical sign				
No. of death	0	0	0	0

Body weight change (g)	days 0 - 6	29.9	28.6	30.5	30.1
	days 6 - 15	42.1	43.8	39.8	24.8**
	days 15 - 21	84.4	90.3	86.0	95.3
	days 0 - 21	156.4	162.7	156.3	150.2
No. of pregnancy and percentage		23 (95.8)	23 (95.8)	24 (100)	23 (95.8)
Necropsy	Spleen, Hypertrophy	0	0	0	1 (4.3)
	Spleen and peritoneum, Adhesion	0	0	1 (4.2)	0
Organ weights (g)					
Liver weight	Absolute ratios				1.11*
	Relative to body weight ratios				1.11**
Kidney (L)	Relative to body weight ratios				1.07*
Uterine examination					
No. of dams examined		23	23	24	23
No. of corpora lutea/dam		20.7	19.4	20.8	20.1
No. of live fetuses/dam		15.0	14.8	14.8	14.1
No. of dead/resorbed fetuses and percentage		12 (3.4)	16 (4.5)	25 (6.6)*	18 (5.3)
Mean pre-implantation loss (%)		23.6	19	21.8	26.2
Mean post-implantation loss (%)		3.4	4.4	6.7*	7.3

Vacant column, Normal; Number in parentheses, percentage.
*Significantly different from control, $P<0.05$
**Significantly different from control, $P<0.01$

Fetal variations

Visceral and skeletal variations and skeletal malformations were sporadically found in the dose and control groups. These were considered to be spontaneous because there was no statistical significance between the control and dose groups. The main malformations and variations noted are listed in Table 42.

Statistical significance was observed at 50 mg/kg b.w./day, concerning the shortening of the thirteenth rib compared to the control group. Dose dependence incidence of this finding was observed after analysis of all doses but not after analysis of the low and intermediate doses only.

Table 44: Summary of findings (Fetuses)

Dose (mg/kg bw/day)	**0**	**5**	**16**	**50**
Mean Fetus weights (g) M	5.277	5.363	5.121	5.211
F	4.954	5.098	4.946	4.930
Sex ratio (M/F)	0.9	1.0	0.8	1.2
External observation				
No. of foetuses examined	345	340	355	324
Placental haemorrhage [v] (%)	0 (0)	0 (0)	0 (0)	3 (0.9)[c]
Subcutaneous haemorrhage [v] (%)	2 (0.6)	1 (0.3)	1 (0.3)	5 (1.5)
Short tail [m] (%)	1 (0.3)	0 (0)	0 (0)	0 (0)
Internal observation				
No. of foetuses examined	174	175	180	162
Dilatation of renal pelvis [v] (%)	13 (7.5)	8 (4.6)	19 (10.6)	10 (6.2)
Thymic remnant in the neck [v] (%)	8 (4.6)	7 (4.0)	5 (2.8)	3 (1.9)
Skeletal observation				
No. of foetuses examined	171	165	175	162
Variation/delayed ossification				
Bilobed shape, thoracic vertebral body	7 (4.1)	12 (7.3)	8 (4.6)	11 (6.8)
Splitting, cervical vertebral body	10 (5.8)	10 (6.1)	10 (5.7)	11 (6.8)
Shortening of the 13th rib	1 (0.6)	6 (3.6)	1 (0.6)	15 (9.3)***[c]
Wavy ribs	1 (0.6)	0 (0)	1 (0.6)	1 (0.6)
14th rudimental rib	8 (4.7)	5 (3.0)	2 (1.1)	0 (0)[c]
Asymmetry of the sternebrae	11 (6.4)	16 (9.7)	21 (12.0)	15 (9.3)
Skeletal malformations				
Partial hypertrophy of ribs	1 (0.6)	0 (0)	1 (0.6)	1 (0.6)
Fusion and bifurcation of the ribs	1 (0.6)	0 (0)	0 (0)	0 (0)

Defect of postcervical vertebrae	1 (0.6)	0 (0)	0 (0)	0 (0)
Fusion of the sternebrae	0 (0)	0 (0)	1 (0.6)	0 (0)

[v] Variation, [m] malformation; Number in parentheses, percentage;
[C] $p<0.05$, Cochran-Amitage trend test (all doses).
*** $p<0.01$, Fisher's exact test

Conclusion
The NOAEL of the test substance when administered to pregnant rats was 16 mg/kg b.w./day with regard to systemic maternal toxicity (i.e., inhibition of body weight growth, decrease in food consumption and increase in liver weights at 50 mg/kg b.w./day). Based on the increased finding of shortening of the 13th rib in the high-dose group, the foetal NOAEL is also 16 mg/kg b.w./day. There was no teratogenicity in fetuses or fetotoxicity even at 50 mg/kg b.w./day, the highest dose level employed.

In the developmental neurotoxicity study by Anonymous (2008), pregnant female Sprague-Dawley rats (25/group) were exposed by gavage from gestation day 6 through lactatation day 21 daily exposed to 0, 2.5, 10 or 45 mg/kg/day. F0 maternal toxicity was expressed at the dose level of 45 mg/kg/day by a single mortality and reductions in body weight gain and food consumption. F1 developmental toxicity was expressed at the dose level of 45 mg/kg/day by early postnatal mortality and reduced post-weaning body weights. Deficits in auditory startle response occurred in the 45 mg/kg/day group F1 males and females without concomitant effects in other functional endpoints (FOB), neuropathology or brain morphometry. Based on the results of this study, the NOAEL (no-observed-adverse-effect level) for maternal toxicity, developmental toxicity and developmental neurotoxicity was considered to be 10 mg/kg/day (conclusions study report). In agreement with the member states experts, the final NOAEL for development neurotoxicity was set at 2.5 mg/kg bw/day (see Confidential Annex section 2 for more details and discussions).

As mentioned before (study by Anonymous (1999d) (see 10.10.1.1 In vivo)), the two-generation study shows that the F2 pups of the 800 ppm group showed a significant decrease on in-utero growth, post-natal growth, body weight throughout lactation, viability and weaning indices. Based on the reduced postnatal survival of F2 pups, the developmental NOAEL is 280 ppm.

10.10.4.2 Rabbits

In the preliminary study by Anonymous (1997e), 20 males and 70 females NZW rabbits, about 5 months old, from Kitayama Labes Co., Ltd, (Ina, Nagano), were used. Pregnant rabbits (4 animals/group) were administered acetamiprid via gastric intubation at dose levels of 0, 5, 13, 30 and 75 mg/kg b.w./day. Dosing was performed during days 6 to 18 of gestation. All high-dose animals died by day 14. Treatment-related reductions in parental body weight and food consumption were observed at 13 and 30 mg/kg b.w./day. No external abnormalities were noted in fetuses at any dose levels. From these results, the following dose levels were selected: 7.5, 15 and 30 mg/kg b.w./day.
Subsequently, the test substance was administered orally by gavage at doses 0, 7.5, 15 and 30 mg/kg b.w./day to groups of 17 mated females/dose. Dosing was performed during days 6 to 18 of gestation. Control animals received vehicle only, in the same procedure as treated animals (gastric intubation). The mated females were observed for clinical signs once a day from the first mating to necropsy. Individual body weights (on gestation days 0, 6, 7, 8, 10, 12, 14, 16, 18, 19, 20, 22, 24 and 28) and daily food consumption (on days 6, 7, 8, 10, 12, 14, 16, 18, 19, 20, 22, 24 and 28) were measured. On gestation day 28, all mated females were sacrificed and their organ weights (liver, spleen, kidney, adrenal and ovary) were measured. The number of corpora lutea was counted. After weighing the gravid uterus, the numbers of total implantations, viable, dead and resorbed fetuses were counted. Viable fetuses were examined for external malformations and variations, and their body weights and placental weights were recorded. After fixation with ethanol, each fetus was used for visceral and skeletal evaluation.

Maternal observations

Treatment related maternal body weight depression and a statistically significant decrease of food consumption were noted in females from 30 mg/kg b.w./day group. The body weight gain during days 6 to 19 of gestation was decreased (not statistically significant) at 30 mg/kg b.w./day (-7.3 g) compared to control (35.2 g). No treatment-related changes were noted in females from other dose groups.
Food consumption values on days 7 and 8 were significantly lower ($P<0.01$) in the 30 mg/kg b.w./day group than those in the control group.
In females, there were no toxic signs, deaths nor organ weight changes in any dose group. The numbers of total implantations, corpora lutea, live fetuses were not altered by administration. The main malformations and variations noted are listed in Table 43.

Table 45: Summary of findings (Dams)

Dose (mg/kg b.w./day)		**0**	**7.5**	**15**	**30**
No. of females/group		*17*	*17*	*17*	*17*
Clinical signs					
No. of deaths		0	3[$] (17.6)	2[$] (11.8)	1[$] (5.9)
Body weight changes (g)	days 0 - 6	84.8	77.3	121.6	73.6
	days 6 - 19	35.2	40.3	66.3	-7.3
	days 19 - 28	174.9	71.1	0.2	166.5
	days 0 - 28	294.9	188.7	188.1	232.8
No. of pregnancies and percentages		12 (70.6)	15 (88.2)	14 (82.4)	14 (82.4)
Uterine examination					
No. of dams examined		12	12	12	13
No. of implantation sites/dam		8.8	9.1	9.2	7.2
No. of corpora lutea/dam		11.0	11.8	11.2	9.3
No. of live fetuses/dam		7.8	8.3	7.8	6.6
No. of fetuses dead or resorbed and percentage		12 (11.4)	10 (8.6)	16 (13.5)	8 (7.3)

Vacant column, Normal; Number in parentheses, percentage. [$] Death by inadequate administration.

Fetal observations
No statistical significant effect of NI-25 treatment was evident on the sex ratios, fetal and placental weights. The incidences of underdeveloped (fetal body weight less than 60% of the control body weight) fetuses (13.1%, 7.4% and 7.0% at doses 7.5, 15 and 30 mg/kg b.w./day respectively) in the treated groups were higher than the control (3.2%), but there was no statistical significance.
External, visceral and skeletal variations and/or malformations were sporadically found in dose and control groups. These were considered to be spontaneous because there was no statistical significance or dose dependency between the control and dose groups. Furthermore the most frequent variation was the appearance of the thirteenth rib. The incidence of this finding was reported by Morita et al. (1987) to be higher in New Zealand White rabbits than in the other rabbit strains. The incidence of shortened thirteenth rib in the 7.5 mg/kg b.w./day group (16.2%) was significantly higher than the control (3.2%), but this was not considered to be related to NI-25 treatment because of no dose-response relationship ($P=0.68$, not significant). The main malformations and variations noted are listed in Table 44.

Table 46: Summary of findings (Fetuses)

Dose (mg/kg b.w./day)		**0**	**7.5**	**15**	**30**
Mean Fetus weights (g)	M	42.91	41.59	39.38	42.13
	F	40.11	40.18	37.01	40.51
Sex ratio (M/F)		1.1	1.0	1.0	1.2
External observation					
No. of fetuses examined		93	99	94	86
Open eyelid and manus varus [m]					1 (1.2)
Internal observation					

Dose (mg/kg b.w./day)	**0**	**7.5**	**15**	**30**
No. of fetuses examined	93	99	94	86
Malformations				
Right subclavian artery, abnormal origin	1 (1.1)			
Bifid apex of heart		1 (1.0)		
Microphthalmia				1 (1.2)
No of fetuses with variation	3 (3.2)	3 (3.0)	5 (5.3)	2 (2.3)
Skeletal observation				
No. of fetuses examined	93	99	94	86
Variation/delayed ossification				
Splitting of sternebra	3 (3.2)		2 (2.1)	1 (1.2)
13 th rib	62 (66.7)	63 (63.6)	42 (44.7)**	60 (69.8)
Shortened 13 th rib	3 (3.2)	16 (16.2)**	6 (6.4)	4 (4.7)
Floating rib	14 (15.1)	12 (12.1)	10 (10.6)	4 (4.7)*
Skeletal malformations				
Fusion of thoracic vertebral arch and fusion of ribs				2 (2.3)
Fusion of sternebrae	3 (3.2)	3 (3.0)	1 (1.1)	2 (2.3)
Absence of thoracic vertebral arch	1 (1.1)			
Absence of thoracic vertebral body and fusion of ribs			1 (1.1)	
Absence of thoracic vertebral arch and bifurcation of ribs		1 (1.0)		
Absence of lumbar vertebral arch			1 (1.1)	

Number in parentheses, percentage; Vacant column, Normal. [m] malformation. *P<0.05, **P<0.01, compared to control by Chi-square test.

Conclusion

The main effects of acetamiprid, when administered to pregnant rabbits were inhibition of body weight gain and decrease in food consumption at dose 30 mg/kg b.w./day. The NOAEL of acetamiprid was 15 mg/kg b.w./day with regard to systemic adult toxicity. There was no teratogenicity or fetotoxicity in fetuses even at dose level of 30 mg/kg b.w./day, the highest level tested.

10.10.4.3 Supportive studies

Two additional studies were mentioned in the RAR that may be of value for classification. In one acute neurotoxicity study with rats dosed orally via gavage, the animals (3/sex/dose) were subjected to a functional observational battery prior to treatment and at 6 hours post dosing (time of peak effects) and on day 7 and 14 post dosing (Anonymous, 1997c). Motor activity of each animal was also quantitatively assessed at the same intervals. Clinical signs, body weight and food consumption was monitored. At the end of the observation period, animals were killed using whole body perfusion and examination was confined to designated tissues of the nervous system including microscopy.

Effects on the functional observational battery and locomotor activity were confined to the day of dosing:

1) At 100 mg/kg, there were observations of tremor and chewing movements of the mouth. Males were difficult to handle. Pupils were dilated. Animals felt cold to touch. Males showed increased urination. Gait and posture were affected: hunched posture, walking on toes, slipping of hindlimbs. Rectal temperature was statistically significantly reduced. Forelimb grip strength was increased among males and landing foot splay was decreased among females. Locomotor activity was statistically significantly reduced in both sexes.

2) At 30 mg/kg, there was evidence of tremor among males (one animal showed continuous tremor), one female showed tail tremor. There was also an indication of increased urination among males.

3) No effects were observed at 10 mg/kg.

There were no effects on the functional observational battery and locomotor activity on day 7 or 14. There was no evidence of irreversible neuropathology. There were no apparent effects on sensory systems. In summary, clinical signs (behavioural changes in both sexes) and reduced locomotor activities in both sexes

at the high dose and in males only at the medium dose were observed on the day of treatment. The NOAEL was considered to be 10 mg/kg.

In the second study, rats were repeatedly dosed acetamiprid (0, 100, 200, 800 or 1600 ppm) orally via the diet for 13 weeks (Anonymous, 1997d). All of the animals (10/sex/dose) were subjected to functional observational battery prior to treatment and after 4, 8, 13 weeks of treatment. Motor activity of each animal was quantitatively assessed at the same intervals. Clinical signs, body weight and food consumption was monitored.
There were no mortalities and any clinical signs associated with acetamiprid administration. Bodyweights of males and females treated at 800 and 1600 ppm were statistically significantly lower than controls (Table B.6.7.1-4). Cumulative food intake was also statistically significantly lower among males and females treated from 800 ppm. Food utilisation was impaired for males at 1600 ppm and for females from 800 ppm. Cumulative food consumption in rats treated with acetamiprid was statistically significant lower among males and females at 800 and 1600 ppm over 13 weeks. Statistically significant lower values for forelimb grip strength were recorded during week 4 among females from 800 ppm, during week 8 for all treated female groups (although there was no apparent dose relationship) and during week 13 for males at 1600 ppm. There were also records of statistically significant lower values for hindlimb grip strength among males from 800 ppm. There were no other behavioral changes observed which could be attributed to neurotoxicity. There was no evidence of neuropathology from the histopathological examination of the central and peripheral nerve system.
In summary, The NOAEL for systemic toxicity was established at 200 ppm (14.8 and 16.3 mg/kg/day for males and females respectively) based on food consumption and body weight effects. There were no neuropathological or other findings to indicate changes in motor function and co-ordination except for the observation of lower grip strength values of the limbs (mainly for males at 1600 ppm). Taking into consideration all these factors, the lower grip strength values were not considered to be indicative of any irreversible neurotoxic effect.
Limitations: The frequency of clinical observations and functional tests were not in compliance with OECD 424 guideline.
The RMS from the RAR (2015) noted that in the original report, no NOAEL for neurotoxicity in this study was derived. Since the original assessment concluded that the lower grip strength found was not considered to be indicative of any irreversible neurotoxic effect, the RMS proposed a neurotoxicity NOAEL of 1600ppm (118 and 134 mg/kg bw/day).

Table 47: Summary table of animal studies on adverse effects on development

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Remarks (Klimisch score)***	**Reference**
Two-Generation Reproduction Study with NI-25 in Sprague-Dawley Rats 26 rats/sex/group (F0 generation) In compliance with EEC B.35 method	NI-25 (Acetamiprid), purity 99.9% 0, 100, 280 or 800 ppm for at least 10 weeks prior mating. Study was terminated after weaning the F2 generation.	NOAEL pup development: 280 ppm (17.9 mg/kg b.w./day), based on reduced postnatal survival of F2 pups and consistent effects on body weights of adults and pups of both generations in the 800 ppm groups.	1 (reliably without restriction)	Anonymous (1999d)
Teratogenicity Study in Charles River CD (SD) strain Rats Range-finding study	NI-25 (Acetamiprid), purity 99.46% Vehicle 5% Arabic gum and 0.01% Tween 80 in water 0, 18, 35 and 70 mg/kg b.w./day	Treatment-related reductions in parental body weight and food consumption observed at 35 and 70 mg/kg b.w./day. No offspring anomaly was noted at any dose levels. NOAEL: 18 mg/kg b.w./day	1 (reliably without restriction)	Anonymous (1997f)

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Remarks (Klimisch score)***	**Reference**
Teratogenicity Study in Charles River CD (SD) strain Rats 24 mated females/group In compliance with EEC B.31 method	NI-25 (Acetamiprid), purity 99.46% Vehicle 5% Arabic gum and 0.01% Tween 80 in water 0, 5, 16, 50 mg/kg b.w./day During day 6-15 of gestation	NOAEL pregnant rat: 16 mg/kg b.w./day based on reduced body weight and food intake and an increase in liver weight. NOAEL fetus: 16 mg/kg bw/day based on increased incidence of shortening of the 13th rib.	1 (reliably without restriction)	Anonymous (1997f)
Oral developmental neurotoxicity study in Sprague-Dawley rats 25 rats/group (F0 generation) In compliance with guideline OPPTS 870.6300	Acetamiprid, purity :99% Vehicle 5% gum Arabic with 0.01% Tween 80 0, 2.5, 10 or 45 mg/kg/day daily from gestation day 6 through lactation day 21 daily	NOAEL for maternal toxicity, developmental toxicity: 10 mg/kg/day NOAEL developmental neurotoxicity of 2.5 mg/kg bw/day	1 (reliably without restriction)	Anonymous (2008)
Teratogenicity Study in NZW Rabbits 4 pregnant rabbits/group Range-finding study	NI-25 (Acetamiprid), purity 99.46% Vehicle 5% Arabic gum and 0.01% Tween 80 in water 0, 5, 13, 30 and 75 mg/kg b.w./day During day 6-18 of gestation	All high-dose animals died by day 14. Treatment-related reductions in parental body weight and food consumption were observed at 13 and 30 mg/kg b.w./day. No external abnormalities were noted in fetuses at any dose levels.	1 (reliably without restriction)	Anonymous (1997e)
Teratogenicity Study in NZW Rabbits 17 mated females/dose In compliance with EEC Method B.31	NI-25 (Acetamiprid), purity 99.46% Vehicle 5% Arabic gum and 0.01% Tween 80 in water 0, 7.5, 15 and 30 mg/kg b.w./day During day 6-18 of gestation	NOAEL systemic adult toxicity: 15 mg/kg b.w./day. , based on decreased body weight gain and food consumption. At the highest dose tested (30 mg/kg b.w./day) no teratogenicity or fetotoxicity in fetuses was observed.	1 (reliably without restriction)	Anonymous (1997e)
Other supportive studies				
Acute oral neurotoxicity study in Crl :CD BR rats (3/sex/dose group). GLP and in compliance with OECD TG 424	Acetamiprid, Purity 99.9%. vehicle: 0.5% sodium carboxymethylcellulose dosed at 0, 10, 30, 100 mg/kg b/w	Behavioural changes including reduced locomotor activity at 100 mg/kg bw/day and reduced temperature. NOAEL 10 mg/kg bw/day	1 (reliably without restriction)	Anonymous (1997c) from RAR 2015
Sub-chronic (13 weeks) oral neurotoxicity study with rats (10/sex/dose group). GLP but not in compliance with an OECD guideline	Acetamiprid, Purity 99.9%, administered via the diet at 0, 100, 200, 800 or 1600 ppm (0, 7.4, 14.8, 59.7, 118 mg/kg bw/day for males and 0, 8.5, 16.3, 67.6 and 134 mg/kg bw/day for females)	Reduced cumulative food consumption and decreased body weight at >800 ppm No evidence of neurotoxicity NOAEL 14.8 mg/kg bw/day and LOAEL 59.7 mg/kg bw/day	2 (reliably without restriction)	Anonymous (1997d) from RAR 2015

* Klimisch et al. (1997)

10.10.5 Short summary and overall relevance of the provided information on adverse effects on development

The possible adverse effects of acetamiprid on development were studied in rats as well as in rabbits. In both rats and rabbits, no teratogenicity or fetotoxicity in foetuses at the highest dose tested inducing some maternal toxicity was observed (50 and 30 mg/kg b.w./day, respectively). In the teratogenicity study by Anonymous (1997f), in the 50 mg/kg bw/day foetuses shortening of the 13th rib was observed. In the 2-generation study in rats by Anonymous (2008)a reduction in post-natal survival (viability index and weaning index) was observed in the F2 high dose animals in the presence of maternal toxicity in the form of reduced body weights. Pup body weights were reduced in both F1 and F2 pups with accompanying delays in preputial separation, vaginal opening, eye opening and pinna unfolding. In the developmental neurotoxicity study, post-natal survival was also reduced mainly in the early part of the lactation period at the high dose level (45 mg/kg bw/day). In addition, a reduction in post-weaning body weights was observed. The NOAEL for maternal toxicity and developmental toxicity is 10 mg/kg bw/day. However, the NOAEL for developmental neurotoxicity is 2.5 mg/kg bw/day based on a reduced startle response at 10 and 45 mg/kg bw/day. It could not be concluded that reduced auditory startle responses in offspring at 10 mg/kg bw were not related to treatment. The effects at 45 mg/kg bw/day were observed in the presence of maternal toxicity based on reduced body weight gain and food consumption. As the reduction in post-natal survival was observed in a test with exposure until day 21 of lactation, it is unknown whether this is an effect due to in utero exposure or via lactation.

10.10.6 Comparison with the CLP criteria

For the purpose of classification for adverse effects on development, substances are allocated to one of two categories. Following Annex I, Table 3.7.1 (a) in Regulation (EC) 1272/2008 on CLP, there is some evidence from experimental animals, resulting in classification as Repr. Cat. 2 H361.

Classification of substances for effects on development in category 1A is based on human data. As there is no human data for acetamiprid, classification in category 1A is not warranted.

Classification of substances for effects on development in category 1B is based on animal studies showing clear evidence for an effect on development in the absence of maternal toxicity or considered not to be secondary to the observed maternal toxicity. The only developmental effect observed in the absence of maternal toxicity was the reduced startle response at 10 mg/kg bw/day in the developmental neurotoxicity study. However, this was not a clear effect as it was concluded that it could not be excluded that the effect was substance-related. Therefore, this effect does not justify classification in category 1B. Reduction in body weight and related delays in development such as preputial separation and vaginal opening were seen in the presence of maternal toxicity in the form of reduced body weight (gain) and food consumption. Reduced maternal body weights normally result in a reduction in pup body weights, therefore it cannot be concluded that these effects on development are not secondary to the maternal toxicity. These effects do not warrant classification in category 1B. The most adverse developmental effect is the reduced post-natal survival of the pups as observed in the F2 pups of the 2-generation study and in the developmental neurotoxicity study but not in the F1 pups of the 2-generation study. These developmental effects were observed in the presence of maternal toxicity including reduced maternal body weight (gain) and food consumption. It is considered unclear whether the developmental effects are secondary to the maternal toxicity. Therefore, classification in category 1B is not warranted based on the reduced post-natal survival.

Classification of substances for effects on development in category 2 is based on animal studies showing clear evidence for an effect on development in the presence of maternal toxicity and where it cannot be determined whether the observed developmental effects are secondary to the maternal toxicity or where the observed effects are not clear or the provided studies show deficiencies.

The following effects were observed that warrant a classification in category 2:

- A decrease in postnatal survival was observed in the developmental neurotoxicity study at the high dose of 45 mg/kg bw/day at postnatal day 0-1. Three dams in this high dose group had total litter loss, of which in one litter most pups (13 out of 15) were missing and most likely eaten by the dam. These three total litter losses decreased the total postnatal survival in this high dose group by 16% compared to the control. In this high dose group maternal toxicity was observed with significantly lower body weight and body weight gains (around -15% compared to control), including a body weight loss during gestation days 6 to 9.
- A decrease in postnatal survival was observed in the F2 pups of the 2-generation study in the high dose of 800 ppm (51 mg/kg bw/day); this effect was not observed in the F1 pups. For the F2 pups, the viability index (nr of alive pups at D4 pre-culling / nr of live born pups) was significantly decreased at 800 ppm and also the weaning index (nr of alive pups at weaning / nr of alive pups at day 4 post-culling) was significantly decreased. Five females of this high dose group experienced total litter loss, where pup observations showed thin pale and weak appearance and no visible milk was found in the stomach. At this high dose level maternal toxicity was observed with a significantly reduced body weight (86% of control) and body weight gain (72% of control during days 0-7) during gestation; during lactation actual body weight loss was seen during days 0-4. In addition, several relative organ weights were significantly increased at this high dose adult animals, including the brain, spleen and uterus. No histological changes were found in the adult animals of the 800 ppm group. The parental NOAEL in this study was set at 280 ppm based on decreased body weight gain and food consumption at the high dose of 800 ppm.
- A decrease in startle response in the developmental neurotoxicity study was observed. The startle response was significantly decreased at the high dose of 45 mg/kg bw/day. At this dose maternal toxicity was evident by a significantly lower body weight and body weight gain (about 85% of control) and in addition a body weight loss was observed in this dose group at gestation days 6 to 9. At the mid-dose of 10 mg/kg bw/day, no maternal toxicity was observed. At this 10 mg/kg bw/day a decrease in startle response was observed in the pups, although this difference was not statistically significant. In an EFSA scientific opinion (EFSA Journal 2013;11(12):3471) it was proposed to lower the developmental (neurotoxicity) NOAEL to 2.5 mg/kg bw/day, thus taking into account this decrease seen at 10 mg/kg bw/day. This proposal was made based on the fact that the study missed certain study data (motor activity, learning and memory evaluation) therefore no firm conclusion could be taken. As a conservative approach it was proposed to lower the NOAEL to 2.5 mg/kg bw/day until such time as new and scientifically sound evidence is provided. The Netherlands as RMS for this active substance decided to follow the proposal from this EFSA scientific opinion.

The effect of food restriction of pregnant dams resulting in reduced maternal body weights on post-natal mortality is described differently between studies. According to Wolterbeek et al. (2004) food restriction in Wistar rats increases pup mortality during day 1-4 post-natal. However, no such effects were reported by Carney et al. (2004) in CD (SD derived) rats. Seen the limited effect on maternal body weight and the inconsistent reports on the effect of food restriction on post-natal mortality, it is unclear whether the observed effects (in CD rats) are secondary to the maternal toxicity or a direct effect of the substance. Therefore, classification in category 2 is justified.

10.10.7 Adverse effects on or via lactation

As mentioned before, in the study by Anonymous (1999d), acetamiprid was administered dietary to 26 rats/sex/group (F0 generation) at 0, 100, 280 or 800 ppm for at least 10 weeks prior to mating and throughout mating, gestation, and lactation periods. Pregnant females were allowed to deliver and F1 litters were culled to 4 male and female pups on day 4 post-partum when possible. 26 male and female from each F1 litter were randomly selected to produce an F2 generation under the same conditions. The study was terminated after weaning the F2 generation. Animals (parents and pups) were sacrificed at the end of the weaning period (except for pups culled on day 4). Survival (twice a day), clinical signs and physical examination were recorded. Animals were examined periodically for body weight changes and food consumption. Oestrous

cycles were determined for parental females and the reproductive abilities of both generations were assessed in mating trials. During lactation, litters were monitored for growth and development. Post-natal growth of the 800 ppm F1 and F2 pups (highest dose tested) were significantly decreased throughout lactation. As mentioned before, a reduction in post-natal survival (viability index and weaning index) was observed in the F2 high dose animals in the presence of maternal toxicity in the form of reduced body weights. Pup body weights were reduced in both F1 and F2 pups with accompanying delays in preputial separation, vaginal opening, eye opening and pinna unfolding.

In the developmental neurotoxicity study (Anonymous, 2008), exposure from gestation day 6 until lactation day 21 resulted in post-natal effects including reduced survival, reduced body weight gain, reduced body weight at gaining vaginal patency and effects on the startle response at the highest dose in the presence of maternal toxicity. A possible effect on the startle response was also observed at 10 mg/kg bw/day.

However, as the post-natal effects were observed in tests with exposure until day 21 of lactation or later, it is unknown whether this is an effect due to in utero exposure or via lactation.

In a study by Anonymous (1999b), lactating Holstein cows (562 to 688 kg) were orally dosed once daily for 28 consecutive days with encapsulated acetamiprid (purity 99.9%). The nominal dose rates based upon acetamiprid/kg feed intake on a dry matter basis were 6, 18 and 60 ppm. When animals were exposed to acetamiprid for 28 consecutive days, acetamiprid residue concentrations in whole milk showed to be dose-dependent and exhibited rapidly increasing concentrations until a plateau was reached in about 1 day. Residue concentrations of the metabolite N-desmethyl-acetamiprid (IM-2-1) in whole milk were both dose- and time-dependent, reaching a plateau at about 7 days. IM-2-1 was the predominant residue in milk. The sum of both residues in milk at the highest tested dose level of 60 ppm in dry matter cow food was approximately 1 mg/kg milk.

Lactating goats (US EPA, 2002) were orally dosed twice daily for 7 days with encapsulated [pyridine-2, 6-14C]-acetamiprid at dietary equivalent levels of 1.0 ppm or 8.6 ppm per day. At the end of the 7-day dosing period, the goats were sacrificed 22 hours after the last administration.
Most of the administered radioactivity (AR) was excreted via urine or faeces (about 95–99% AR) and less than 1% AR in milk (reaching a plateau after about 3 days). In tissues, radioactivity did not exceed 1.6% AR and in milk, about 94–96% TRR was found in the whey with about 3–5% TRR occuring in milk fat and precipitated milk proteins.
The predominant residue in milk, liver and kidney was the IM-2-1 metabolite (70–89% TRR) and in muscle, the major residue was IM-2-2 (about 50% TRR), with the IM-2-3 and IM-2-4 metabolites also being found at 6% and 13% TRR respectively. Acetamiprid (parent) was only found in milk, at less than 10% TRR and < 0.005 mg/kg.

Table 48: Summary table of animal studies on effects on or via lactation

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Remarks (Klimisch score)*	Reference
Two-Generation Reproduction Study with NI-25 in Sprague-Dawley Rats 26 rats/sex/group (F0 generation) In compliance with EEC B.35 method	NI-25 (Acetamiprid), purity 99.9% 0, 100, 280 or 800 ppm for at least 10 weeks prior mating. Study was terminated after weaning the F2 generation.	Decreased in-utero and post-natal growth of F1 and F2 pups exposed to 800 ppm (51 mg/kg b.w./day). In addition, post-natal mortality was increased in the F2 pups.	1 (reliably without restriction)	Anonymous (1999d)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Remarks (Klimisch score)*	Reference
Oral developmental neurotoxicity study in Sprague-Dawley rats 25 rats/group (F0 generation) In compliance with guideline OPPTS 870.6300	Acetamiprid, purity :99% Vehicle 5% gum Arabic with 0.01% Tween 80 0, 2.5, 10 or 45 mg/kg/day daily from gestation day 6 through lacatation day 21 daily	Reduced pup body weight in both F1 and F2 highest dose, unknown whether this is an effect due to lactation	1 (reliably without restriction)	Anonymous (2008)
Feeding study in lactating Holstein cows	Acetamiprid, purity 99.9% 6, 18 and 60 ppm for 28 days	Dose-dependent concentration acetamiprid in whole milk. Metabolite IM-2-1 concentration both dose- and time dependent.	2 (reliable with restrictions)	Anonymous (1999b)
Twice daily oral exposure lactating goats	1.0 and 8.6 ppm, twice daily for 7 days	Less than 1% was excreted via milk	2 (reliable with restrictions)	(US EPA 2002)

* Klimisch et al. (1997)

10.10.8 Short summary and overall relevance of the provided information on effects on or via lactation

Although no effects on lactation were mentioned, a decreased post-natal growth of F1 and F2 rat pups was observed (Anonymous, 1999d). In addition, dose-dependent concentrations acetamiprid and its metabolite IM-2-1 were observed in whole milk of exposed cows (Anonymous, 1999b), clearly indicating transfer into milk. However, in goats it was shown that mainly the metabolite of acetamiprid was found in milk.

10.10.9 Comparison with the CLP criteria

For the purpose of classification for effects on or via lactation, substances are allocated to a separate hazard category and should be ascribed to a substance irrespective if it classified in any other category for reproductive toxicity or not. Following Table 3.7.1 (b) in Guidance to Regulation (EC) 1272/2008 on CLP, results of one or two generation studies in animals which provide clear evidence of adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk, can result in classification as Lact. H362. However, it is unclear whether the observed effects were due to exposure via milk or due to in utero exposure. The fact that the body weights were already reduced at day 0 post-natal suggests that the in utero exposure at least contributed to the reduced post-natal weight reduction. In the study by Anonymous (2008) it is stated that the body weight gain of pups was comparable but a reduction was already observed on day 1. Although some exposure via milk is suggested by the available milk residue studies, the amount of acetamiprid is limited and the contribution of the metabolite to the toxicity could even be lower as the acute oral toxicity of this metabolite is lower than acetamiprid. Therefore, it cannot be shown that the observed post-natal effects are due to exposure to acetamiprid or its metabolites via milk. Classification is therefore not warranted.

10.10.10 Conclusion on classification and labelling for reproductive toxicity

There is some evidence from animal studies on adverse effects on development resulting in classification as Repr. Cat. 2 H361d.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Acetamiprid was evaluated in a guideline and GLP compliant two-generation study (OPPTS 870.3800, approximating OECD TG 416, 1983; Anon., 1999d) in SD rats in order to assess its effects on sexual function and fertility. A supplementary study to the main 2-generation study further assessed reproduction in male rats at the top dose to address a lack of male sperm investigations due to technical errors in the primary two-generation study. The effects of acetamiprid on development following exposure during pregnancy were tested in guideline (OECD TG 414; 1981) and GLP compliant pre-natal developmental toxicity studies in rats (Anon., 1997f) and rabbits (Anon., 1997e). Preliminary dose range finding developmental studies in rat and rabbits were also available and briefly described by the DS. A developmental neurotoxicity study in rats (Anon., 2008), with exposure from gestation day 6 until lactation day 21 was also available. The DS also described two peer reviewed journal publications: a repeat dose, non-GLP, non-guideline study by Zhang *et al.* (2011) investigating the effect of acetamiprid on the reproductive function of male mice and also a non-GLP, non-guideline study by Gu *et al.* (2013) on the *in vitro* effects of acetamiprid on spermatozoa, fertilization and preimplantation embryo development.

Effects on sexual function and fertility

Rat 2-gen Study

In a 2-generation reproduction study, acetamiprid (purity 99.9%) was administered to Sprague-Dawley rats (26/sex/group) at concentrations of 100, 280 or 800 ppm (table 7), for at least 10 weeks prior to mating, during mating, throughout gestation and lactation until weaning of the F2 pups. At termination, reproductive capacity evaluations (ovarian follicle count, sperm motility, total sperm count and sperm morphology) were performed on F0 and F1 adults. Necropsy was performed on parents and pups from both generations. Minimal maternal toxicity was evident from a lack of effects on body weight and food consumption parameters.

Table 7: *Mean achieved doses of acetamiprid (mg/kg bw/day) for the F0 and F1 generations*

	Males/pre-mating			**Females/pre-mating**			**Gestation**			**Lactation**		
Dose levels (ppm)	**100**	**280**	**800**	**100**	**280**	**800**	**100**	**280**	**800**	**100**	**280**	**800**
F0	6.5	17.9	51.0	7.6	21.7	60.1	6.8	18.5	50.9	13.2	37.5	108.1
F1	7.5	21.0	63.3	8.4	23.8	72.6	6.6	18.5	55.2	13.7	40.3	105.5

Premating period: week 0 to 10 for F0, and week 0 to 13 for F1 generation. Gestation period: day 0 to 20, lactation period: day 0 to 14. (Rounded values).

General toxicity – Parental toxicity

F0 parents

Mortality and clinical signs: One F0 male of the control group and one F0 female from the low dose group (100 ppm) died during week 2 and on lactation day 18,

respectively. No death or clinical signs were attributed to treatment.

Body weight & body weight gain: Lower mean body weight values and food consumption were limited to the top dose groups (800 ppm) throughout the study. Body weight was less than approximately 8% relative to controls at week 10 (premating period) for both sexes. Decreases in mean body weight gain (approximately 8%) and food consumption (approximately 20%, significant) were noted for females in the high dose group over gestation days 0-20.

Organ weights & histopathology: Necropsy examinations did not reveal compound-related changes in F0 adult animals or F1 pups. Organ weight changes comprised increased mean brain-to-body weight percentage (about 10% relative to controls) and decreased kidney-to-brain weight ratio (7.5%) of the 800 ppm F0 females. No changes were observed in the reproductive organs.

No histopathological changes were attributed to treatment and no effect on testes or ovaries were reported. The reproductive assessment of high dose males in the 2-generation study was not concluded because the epididymides removed from these animals autolysed due to an error in thawing prior to analyses. A supplemental study coincident with the 2-generation study was then conducted where 26 males were fed either control chow or chow containing 800 ppm acetamiprid for at least 20 weeks. No compound-related effects were observed on total testicular and epididymal sperm counts or on sperm morphology.

F1 parents

Mortality and clinical signs: Two low dose group (100 ppm) and five top dose group (800 ppm) females experienced total litter deaths. No death or clinical signs were noted in adults of either sex throughout all study phases.

Body weight & body weight gain: Mean body weights were consistently lower in F1 parents and correlated with lower food consumption values in the 800 ppm animals during all study phases. Body weight was less than 12-14% relative to controls at week 10 (premating period, males and females respectively) for both sexes. Decreases in mean body weight gain (approximately 8%) and food consumption (approximately 13%, significant) were noted for females in the high dose group over gestation days 0-20.

Organ weights & histopathology: Necropsy of F1 adult and pup animals did not reveal compound-related changes. No histological changes were attributed to treatment in the F1 animals, and no compound-related effects were observed on reproductive organs or functions, including ovarian follicle-count, evaluation of sperm motility, testicular and epididymal sperm count and sperm morphology of the top dose animals.

Reproductive effects

The DS noted that mating performance, fertility, reproduction parameters and oestrous cycles for both the F0 and F1 adult generations were unaffected by treatment.

Offspring effects

F1 pups

Viability and weaning indices were unaffected by treatment up to the top dose of 800 ppm, and values were well within HCD for the performing laboratory. However, at the top dose of 800 ppm, mean numbers of live pups/litter with live pups showed a very small but significant reduction on days 14 and 21, this was of questionable biological significance. Pup body weights were affected: in-utero to a small extent (< 7% reduction relative to controls) but post-natal growth of the 800 ppm were significantly decreased throughout lactation, showing reductions of between 11-24% across both sexes relative to the mean control bodyweight.

Landmark and Pubertal attainment

Anogenital distance was not recorded for F1 pups. Landmark data for preputial separation and vaginal opening (mean age in days of pups in a litter reaching the criterion) were described as significantly increased but by how much was not included in the RAR. The DS considered that these effects correlated with the decreased body weights of the pups. The mean age of reaching preputial separation was also significantly increased for the 280 ppm male F1 pups. Necropsy examinations did not reveal compound-related changes in F0 adult animals or F1 pups.

F2 pups

Viability and weaning indices were seriously affected by treatment at the top dose of 800 ppm (see Table 33, CLH report); the values were outside historical controls for the performing laboratory. Pup mortality (not including those culled) from PND 0-21 was greatly increased. Male and female pup body weights were also affected in the second generation in-utero to a small extent (approximately a 7% reduction relative to controls) but post-natal growth of the 800 ppm F2 pups were significantly decreased throughout lactation, showing reductions of between 15-22% across both sexes relative to the mean control bodyweight.

Landmark and Pubertal attainment

Anogenital distance was not affected by treatment. Landmark data for preputial separation and vaginal opening was unavailable because the F2 pups were sacrificed on LD21. The DS noted in the 800 ppm group, in-utero growth, post-natal growth, and body weight throughout lactation, were significantly decreased following treatment. The mean age for reaching eye opening in the 800 ppm F2 pups (in days) was significantly increased and correlated with their decreased body weights. Necropsy of F1 adult and F2 pup animals did not reveal compound-related changes.

Conclusion

The DS concluded dietary exposure of rats to acetamiprid throughout two generations did not result in effects on reproductive performance or fertility. The DS did not propose classification.

Repeat dose studies and two studies from the peer reviewed public literature.

The DS noted several repeated dose studies which indicated limited effects on sexual organs

following exposure to ≥ 1000 ppm in the presence of general toxicity. No specific information was presented on how fertility or mating might be affected, just that there were increases in relative testis weight in rats; decreases in prostate absolute weight in mice; significant increases in relative testis weight and a decrease in absolute and relative ovary weights in an additional mouse study:

- 90-day rat oral toxicity study: ↑ relative testis weight (+13%/+19% at 800/1600 ppm), not significant. ↓ body weight relative to controls was -14%/-13% at 800/1600 ppm), significant (Anon. 1997f).
- 90-day mouse oral toxicity study: ↑ relative testis weight (+37% at 3200 ppm, significant. ↓ in absolute and relative ovary weight was -65%/-52% at 3200 ppm / 466 mg/kg bw/day), significant (Anon., 1992).
- 18-month mouse dietary carcinogenicity study: ↓ prostate absolute weight in the interim and terminal sacrifice groups (-39%/-23% at 1200 ppm / 186 mg/kg bw/day), significant (Anon., 1999a).

<u>Two studies from the peer reviewed public literature</u>

Two studies from the public literature were included in the acetamiprid RAR and described in detail by the DS in the CLH report.

1. Oxidative stress

Role in acetamiprid-induced impairment of the male mouse reproductive system – Zang *et al.*, (2011)Non-guideline and non-GLP study.

The method and the results in the study by Zhang *et al.* (2011) are well described and indicate that effects on male reproductive function may occur following gavage exposure to 30 mg/kg bw/day (acetamiprid purity >97%) to groups of 10 Kunmin male mice per treatment over 35 days.

Compared to the controls, acetamiprid decreased body weight gain (-38%) and the relative weight of the testis and epididymis (-17%), seminal vesicles and prostate gland (-17%) ($p < 0.05$). Compared to the controls, serum testosterone level decreased in the acetamiprid only group ($p < 0.05$). Vitamin E significantly ameliorated these effects.

Compared to the control group, acetamiprid decreased sperm number (-76%), viability (13%) and motility (52%) ($p < 0.05$), with a significant increased rate of acrosome deformity ($p < 0.05$). Vitamin E reduced these adverse effects.

Histological investigations showed various stages of spermatogenesis in testes of the control group mice; Leydig cells were abundant in the interstitium. In the acetamiprid only group, there was vacuolization of the seminiferous tubules and the number of spermatids and interstitial Leydig cells were clearly decreased. There was degeneration of the mitochondria and endoplasmic reticulum within the Leydig cells.

Acetamiprid increased malondialdehyde (MDA) and nitric oxide (NO) concentrations compared to the controls ($p < 0.05$). Vitamin E also ameliorated these effects.

While these results suggest a possible mode of action, they are not definitive and the DS did not consider their impact with regard to sexual function and fertility classification. The DS concluded the results in this study were different from the 13-week repeated dose diet study in

mice, which tested at much higher dose levels up to approximately 450 mg/kg bw/day (Anonymous, 1992). According to the DS, the results obtained by Zhang *et al.* were due to different impurities and were not representative for the form of acetamiprid marketed in Europe.

2. **Reproductive effects of two neonicotinoid insecticides on mouse sperm function and early embryonic development *in vitro* – Gu *et al.*, (2013). PLoS one July 2013, Volume 8, Issue 7.** Non-guideline and non-GLP study.

The DS described a series of *in vitro* mechanistic studies investigating and comparing the effects of acetamiprid, imidacloprid and nicotine (at concentrations of 500 µM and 5 mM), on fertilization and embryonic development up to the pre-implantation stage. Female B6D2F1 (C57BL/6xDBAx2) strain mice were used as oocyte donors and male B6D2F1 mice were used as semen donors.

Test 1: Effect of chemical exposure on sperm function. The motility of spermatozoa showed no obvious difference from that of controls. The percentage of DNA fragmented spermatozoa was similar across all treatment groups.

Test 2: *In vitro* fertilization performed with the pre-treated spermatozoa. In the presence of toxicant at levels up to 5 mM, all treated spermatozoa retained their potential to fertilize oocytes. All fertilized oocytes survived without evident changes in cell morphology. However, during the culture process, embryos originating from spermatozoa pre-treated with the highest level of test substance, adversely decreased the rates of pronucleus formation (fertilization), the first cleavage and morula/blastocyst formation, compared to those of non-treated controls. Acetamiprid had the weakest effect compared with the other tested compounds. *In vitro* fertilisation performed with normal untreated spermatozoa where the test substances were included in the incubation medium showed similar effects. Direct exposure of nicotine, imidacloprid or acetamiprid had harmful effects in the order of nicotine > imidacloprid > acetamiprid.

In conclusion, the DS did not consider this study to be of much value. The main criticism centred around the *in vitro* concentrations used in the experiment which were considered to be of no relevance to actual *in vivo* gamete exposures.

DS Conclusion on sexual function and fertility

No effects on fertility were observed in the available 2-generation study. Effects on reproductive organs were sometimes observed in the 2-generation study and in the repeated dose studies. These effects were mostly limited to reductions in absolute organ weights such as testis and epididymis in the presence of reduced body weights. As no such effects were observed on relative organ weights and no histopathological effects were observed, the reduced absolute organ weights were considered secondary to the general toxicity and did not warrant classification. The *in vivo* study by Zang *et al.* (2011) was considered to conflict with the results from the 90-day and 18-month studies in mice. The DS questioned whether the active substance used was representative for the form of acetamiprid marketed in Europe. The *in vitro* studies by Gu *et al.* (2013) were not considered relevant from an *in vivo* exposure point of view. No classification for effects on sexual function and fertility was proposed by the DS.

Developmental toxicity

As regards the two-generation study, the DS concluded (see above) that the developmental effects noted in the two-generation reproduction toxicity study (reduced body weight during and at the end of lactation period in two generations of both sexes) did not provide sufficient evidence for classification for developmental toxicity. However, the reduced survival index in the offspring at the highest dose level was recognised as a factor in a weight of evidence for developmental classification.

Developmental toxicity was primarily investigated in the rat and the rabbit in GLP and OECD TG 414 (1981) guideline compliant studies.

Rat preliminary study

In a rat preliminary gavage dosing study (Anon., 1997f), mated rats were treated with acetamiprid (purity 99.46%) at dose levels of 0, 18, 35 and 70 mg/kg bw/day. Treatment-related reductions in parental body weight and food consumption were observed at 35 and 70 mg/kg bw/day. No offspring anomaly was noted at any dose level. Based on these results the following dose levels were selected for the main study: 5, 16, 50 mg/kg bw/day.

Rat main developmental study

The test substance was administered orally by gavage at doses of 0, 5, 16 and 50 mg/kg bw/day to groups of 24 mated female rats/group, for a period of 10 days from GD6 to 15. No treatment-related changes were noted in dams from dose groups with less than 50 mg/kg bw/day.

Maternal toxicity

There was no mortality or clinical signs associated with treatment. There were reductions in the body weight gain during days 6 to 15 of gestation at the highest dose level; this was significantly ($p < 0.01$) decreased at 50 mg/kg bw/day (24.8 g) compared to control (42.1 g). There was no effect on body weight gain after GD15.

Statistically significant but small increases of liver weights and their body weight ratios and kidney/body weight ratios were also observed in the high dose group.

The number of total implantations, corpora lutea and live foetuses were not affected by treatment. The number of resorbed foetuses was significantly increased at 16 mg/kg bw/day but this finding did not show a clear dose-related trend.

Table 8: *Summary of findings in the Dams (based on Table 43, CLH report).*

Dose (mg/kg bw/day)	**0**	**5**	**16**	**50**
No. of females/group	24	24	24	24
No. pregnant (%)	23 (95.8)	23 (95.8)	24 (100)	23 (95.8)
Uterine examination:				
No. of dams examined	23	23	24	23
No. of corpora lutea/dam	20.7	19.4	20.8	20.1
No. of live foetuses/dam	15.0	14.8	14.8	14.1

No. of dead/resorbed foetuses and percentage	12 (3.4)	16 (4.5)	**25 (6.6)***	18 (5.3)
Mean pre-implantation loss (%)	23.6	19	21.8	26.2
Mean post-implantation loss (%)	3.4	4.4	6.7*	7.3

*Significantly different from control, $p < 0.05$
**Significantly different from control, $p < 0.01$

Foetal anomalies

Visceral findings: There were no test article related effects.

External findings: There were no test article related malformations. In the high dose group, there were 3 and 5 incidences of the variations placental and subcutaneous haemorrhage.

Skeletal findings: There were no skeletal malformations linked to acetamiprid exposure. Statistical significance was observed at 50 mg/kg bw/day, concerning the shortening of the thirteenth rib (variation). There was no clear dose response for this effect. All other anomalies occurred at incidences similar to controls.

The DS concluded there was no evidence for teratogenicity or foetotoxicity in the rat even at the highest dose of 50 mg/kg bw/day (findings summarised in Table 44 of the BD).

Rat developmental neurotoxicity study

In the developmental neurotoxicity study by Anon. (1999, report final revision date 2008), pregnant female Sprague-Dawley rats (25/group) were exposed by gavage from gestation day 6 through lactation day 21 daily. The dose groups were 0, 2.5, 10 and 45 mg/kg bw/day. Maternal toxicity was observed at the dose level of 45 mg/kg bw/day by a single mortality and reductions in body weight gain and food consumption (around 15% and 12% respectively, when compared to control).

The DS noted the following toxicologically relevant and non-relevant effects:

1. A decrease in pup postnatal survival (45 mg/kg bw/day, PND 0-1);
   three dams in this high dose group had total litter loss, this significantly decreased the total postnatal survival in this group by 16% compared to the control.

2. During PND 0-1, pups that were found dead, euthanized in extremis or counted as dead due to the death of the dam (dams numbered 7, 16, 4 and 59 in the control, 2.5, 10 and 45 mg/kg bw/day dose groups, respectively) were reported incorrectly in the BD and recalculated here by RAC.

3. A decrease in the pup startle response was observed. The maximum response amplitudes (Vmax) and the average response amplitudes (Vave) in the 45 mg/kg bw/day group males were significantly reduced compared to the control group values on PND 20 and 60. Maternal toxicity was evident by a significantly lower body weight and body weight gain. A reduction (not statistically significant), was also observed in the mid-dose group of 10 mg/kg bw/day (where no maternal toxicity was observed).

4. When the entire post-weaning period (PND 21-72) was evaluated, mean body weight gain in the 45 mg/kg bw/day group males and females were reduced (less than 10%) and statistically significant compared to the control group values.

5. Time to preputial separation was not affected by the test article at any dose level. The mean days of acquisition were PND 43.6, 43.7, 43.1 and 43.9 for the control, 2.5, 10 and 45 mg/kg bw/day groups, respectively.

6. Time to vaginal opening was not affected by the test article at any dose level. The mean days of acquisition were PND 32.2, 32.3, 32.7 and 32.5 for the control, 2.5, 10 and 45 mg/kg bw/day groups, respectively.

Deficits (attenuation) in the auditory startle response occurred in the top dose F1 males and females without concomitant effects in other functional endpoints, neuropathology or brain morphometry. The only adverse effect observed in the absence of maternal toxicity was the reduced startle response at 10 mg/kg bw/day, although this difference was not statistically significant.

The DS, in annex I to the CLH report, considered the effects on Vmax and Vave in the 10 mg/kg bw/day group as toxicologically significant. The DS agreed with the position stated in the EFSA Peer Review report EFSA (2016) and acknowledged that the EFSA PPR panel took a precautionary and conservative approach when determining the NOAEL (2.5 mg/kg bw/day) for the study. The DS considered the evidence from this study relevant in its opinion on supporting classification for development.

<u>Rabbit preliminary study</u>

In a rabbit preliminary gavage dosing study (Anon., 1997e), pregnant does (4 animals/group) were administered acetamiprid at dose levels of 0, 5, 13, 30 and **75** mg/kg bw/day. Dosing was performed during days 6 to 18 of gestation. All high-dose animals died by day 14. Treatment-related reductions in parental body weight and food consumption were observed at 13 and 30 mg/kg bw/day. No external abnormalities were noted in foetuses at any dose level. Based on these results, the following dose levels were selected for the main study: 7.5, 15 and 30 mg/kg bw/day.

<u>Rabbit main developmental study</u>

The test substance was administered orally by gavage at doses 0, 7.5, 15 and 30 mg/kg bw/day to groups of 17 mated females/dose (Anon., 1997e). Dosing was performed during days 6 to 18 of gestation. Control animals received vehicle only. Based on the results of the preliminary study, the maximal tolerated dose was considered to be 30 mg/kg bw/day.

Maternal toxicity

There was no substance related mortality, no toxic signs or organ weight changes in any dose group (Anon., 1997e). The numbers of total implantations, corpora lutea and live-foetuses did not show any treatment related response. Maternal body weight depression and a statistically significant decrease of food consumption were noted in females from the 30 mg/kg bw/day group. The body weight gain during days 6 to 19 of gestation for the 30 mg/kg bw/day dose group was negative (-7.3 g). Although not statistically significant, the difference was substantial when compared to the control (+35.2 g).

No treatment-related changes were noted in females from other dose groups.

Table 9: *Summary of findings in the does (based on Table 45, CLH report).*

Dose (mg/kg bw/day)	**0**	**7.5**	**15**	**30**
No. of females/group	17	17	17	17
No. pregnant (%)	12 (70.6)	15 (88.2)	14 (82.4)	14 (82.4)
Uterine examination:				
No. of dams examined	12	12	12	13
No. of corpora lutea/dam	11.0	11.8	11.2	9.3
No. of live foetuses/dam	7.8	8.3	7.8	6.6
No. of dead/resorbed foetuses and percentage	12 (11.4)	10 (8.6)	16 (13.5)	8 (7.3)
Mean pre-implantation loss (%)	20.8	23.2	19.2	26.1
Mean post-implantation loss (%)	11.4	8.6	13.5	7.3

Foetal anomalies

Visceral findings: no test article related effects, though one foetus of the high dose group showed microphthalmia.

External findings: no test article related effects.

Skeletal findings: there were some sporadic, non-statistically significant differences in the type and incidence of skeletal anomalies in the test article treated groups compared to the control group. The DS considered these were spontaneous because there was no statistical significance or dose dependency between the control and dose groups.

The DS concluded there was no evidence for substance related teratogenicity or foetotoxicity.

Other studies

The DS briefly described two rat neurotoxicity studies, an acute oral neurotoxicity study and a sub-chronic (13 weeks) oral neurotoxicity study. There was little relevance to developmental toxicity. There was no evidence of neurotoxicity in the 90 day study which tested up to 1600 ppm (118 mg/kg bw/day and 134 mg/kg bw/day for males and females respectively). The acute study showed some behavioural changes including reduced locomotor activity at 100 mg/kg bw/day and reduced temperature.

DS Conclusion on development

The possible adverse effects of acetamiprid on development were studied in rats as well as in rabbits. In both rats and rabbits, in the presence of limited maternal toxicity, *no teratogenicity or foetotoxicity* was observed at the highest doses tested (50 and 30 mg/kg bw/day, respectively).

In the 2-generation study in rats a reduction in post-natal survival (viability index (PND0-4) and weaning index (PND4-21)) was observed in the F2 high dose animals in the presence of maternal toxicity in the form of reduced body weight. Pup body weights were substantially reduced in both F1 and F2 pups with accompanying delays in preputial separation, vaginal opening, eye opening and pinna unfolding.

In the developmental neurotoxicity study, post-natal survival was also reduced mainly in the early part of the lactation period at the top dose level (45 mg/kg bw/day). In addition, a reduction in post-weaning body weights was also observed. Reduced auditory startle responses in offspring at 10 mg/kg bw/day (observed in the absence of maternal toxicity), were assumed to be related to treatment.

The DS noted the following toxicologically relevant effects in **support of its proposal for Repr. 2, H361d**:

1. The decrease in postnatal survival observed in the F2 pups of the rat 2-generation study at the top dose of 800 ppm (51 mg/kg bw/day); this effect was not observed in the F1 pups.
2. The decrease in postnatal survival observed in the rat developmental neurotoxicity study at the top dose of 45 mg/kg bw/day at PND 0-1.
3. The decrease in startle response in the developmental neurotoxicity study, significant at the top dose of 45 mg/kg bw/day in males at PND 20 and 60 and observed but not statistically significant at the mid dose of 10 mg/kg bw/day.

Adverse effects on or via lactation

The DS discussed adverse effects on or via lactation in the context of effects seen in four studies:

1. Rat 2-generation study (Anon., 1999d): post-natal growth of the 800 ppm F1 and F2 pups (highest dose tested) were significantly decreased throughout lactation. In addition, a reduction in post-natal survival (viability index and weaning index (PND 4-21)) was observed in the F2 high dose animals in the presence of limited maternal toxicity. Pup body weights were reduced in both F1 and F2 pups with accompanying delays in preputial separation, vaginal opening and eye opening.
2. In the developmental neurotoxicity study (Anon., 2008), exposure from gestation day 6 until lactation day 21 resulted in post-natal effects including reduced survival, reduced body weight gain, reduced body weight ~~at gaining vaginal opening~~ and effects on the startle response at the top dose in the presence of limited maternal toxicity. A possible effect on the startle response was also observed at the mid dose of 10 mg/kg bw/day.
3. Lactating Holstein cows (Anon., 1999b), exposed to acetamiprid for 28 consecutive days showed a dose-dependent and rapid increase of acetamiprid concentration and its IM-2-1 residue in whole milk.
4. Lactating goats (US EPA, 2002) were orally dosed twice daily for 7 days with encapsulated [pyridine-2, 6-^{14}C]-acetamiprid. Less than 1% of the administered radioactivity was found in milk, less than 10% of this being parent compound.

The DS noted that it was unclear whether the observed effects in the 2-generation and developmental neurotoxicity studies were due to exposure via milk or due to *in utero* exposure. There was no cross-fostering study to substantiate effects via lactation. The body weights were already reduced at post-natal day 0, which suggested that the *in utero* exposure at least contributed to the reduced post-natal weight reduction. Some exposure via milk was suggested by the available milk residue studies, but the amount of acetamiprid was very limited and the contribution of the main metabolite IM-2-1 to the toxicity was presumed even lower as the acute oral toxicity for this metabolite was less than that of the parent compound.

The DS concluded no classification for effects on or via lactation because the available data was insufficient to determine if the observed post-natal effects were due to exposure *in utero* or exposure to acetamiprid or its metabolites via milk.

DS Conclusion for Reproductive Toxicity Classification

The DS considered there was sufficient evidence from animal studies on adverse effects on development to propose Repr. 2; H361d.

Comments received during general consultation

Seven comments in total were provided on reproductive toxicity during the general consultation. Four commenting MSCAs supported the proposal to classify acetamiprid as Repr. 2; H361d for effects on development although one of them expressed doubts on where to assign the effects observed.

One MSCA expressed doubts about the described effect on startle response being adverse with regard to further development and asked for additional details on the effect and post-natal mortality from the developmental neurotoxicity study. The DS supplied the details in an annex to the CLH report derived from the latest version of the DAR.

There was one comment from a company-manufacturer, they disagreed with classification for reproductive toxicity. They supplied two detailed reports – one originally targeted at the draft Renewal Assessment Report (RAR) where they requested that their position on the adequacy and interpretation of the developmental neurotoxicity study be represented by the DS (Li, 2015). The second was a report by Exponent (2019) outlining several points on reproductive toxicity and overlapping with points made by Li, (2015). These points were adequately addressed by the DS in the RCOM document and did not represent new data that had to be evaluated further.

There were two comments by company-downstream users. The first commenter stressed that the classification was erroneous because of the major impact by maternal toxicity on the findings. The DS disagreed with this interpretation. The second commenter provided an expert statement on Carcinogenicity and Reproductive Toxicity classification in a pdf document attachment. The DS referred back to its two previous responses on reproductive effects, no new data was submitted.

Assessment and comparison with the classification criteria

<u>Rat 2-generation study</u>

No effects on reproductive performance were observed in the available 2-generation study at dietary concentrations up to 800 ppm. Mating performance, fertility, most reproduction parameters and oestrous cycles for both the F0 and F1 adult generations were unaffected by treatment (tables 10a and 10b). Effects on reproductive organs were not observed in the 2-generation study but were observed at higher concentrations in the repeated dose studies. These effects were mostly limited to reductions in absolute organ weights such as testis and epididymis in the presence of reduced body weights. As no effects were generally observed on relative organ weights and no histopathological changes were observed, the reduced absolute organ weights are considered secondary to the general toxicity and do not warrant classification.

Table 10a: *Summary of F0 generation mating and F1 litter body weight and survival data (Mean ± SD).*

Dose (ppm)	**Control**	**100**	**280**	**800**
No of rats (male/female)	26/26	26/26	25/25	26/26
No. of pregnant females	26	25	25	26
Fertility index (%)	100	96	100	100
Gestation index	100	100	100	100
Gestation length (days)	22.0 ± 0.4	21.8 ± 0.4	22.0 ± 0.0	22.0 ± 0.2
Total no. of pups/litter	13.50 ± 2.10	13.84 ± 1.62	13.44 ± 3.01	13.08 ± 2.06
No. of live pups/litter	13.46	13.68	13.2	12.65
Sex ratio (%) of F1 pups (LD0)	48	52	45	46
Pup viability index during lactation (%)				
Live birth index (%)	100	99	98	97
Viability index (%)	95	99	98	96
Weaning index (%)	96	99	99	94
Pup mortality (not culled) LD0-21	22	5	10	26
No. of total litter losses	1	0	0	0
Male pup bw change				
Mean pup body weights day 0 (%)	100	101.7	102.9	**94.6***
Mean pup body weights day 4 (%)	100	93.5	92.7	**78.5****
Mean pup body weights day 7 (%)	100	95.2	90.7	**76.4****
Mean pup body weights day 14 (%)	100	98.3	94.1	**81.6****
Mean pup body weights day 21 (%)	100	99.6	96.5	**85.0****
Female pup bw change				
Mean pup body weights day 0 (%)	100	100.2	102.3	**93.3***
Mean pup body weights day 4 (%)	100	92.7	90.4	**77.5****
Mean pup body weights day 7 (%)	100	94.2	88.5	**78.4****
Mean pup body weights day 14 (%)	100	99.3	95.5	**84.8****
Mean pup body weights day 21 (%)	100	101.0	98.4	88.8

** $p \leq 0.01$

***Table 10b**: Summary of F1 generation mating and F2 litter body weight and survival data (Mean ± SD).*

Dose (ppm)	**Control**	**100**	**280**	**800**
No of rats (male/female)	26/26	26/26	26/26	26/26
No. of pregnant females	20	24	24	23
Fertility index (%)	77	92	92	88
Gestation index	100	100	100	100
Gestation length (days)	22.1 ± 0.3	22.0 ± 0.4	22.1 ± 0.3	21.8 ± 0.7
Total no. of pups/litter	12.70 ± 4.50	14.50 ± 3.51	14.92 ± 2.24	12.26 ± 4.11
No. of live pups/litter	12.60	13.96	14.50	12.04
Sex ratio (%) of F1 pups (LD0)	53	50	48	51
Pup viability index during lactation (%)				
Live birth index (%)	99	97	97	98
Viability index (%)	94	90	95	**66****
Weaning index (%)	98	**94***	97	**73****
Pup mortality (not culled[1]) LD0-21	20	33	22	**122[2]**
No. of total litter losses	0	2	0	5
Male pup bw change				
Mean pup body weights day 0 (%)	100	98.6	101.1	**92.8****
Mean pup body weights day 4 (%)	100	95.5	99.5	**84.6****
Mean pup body weights day 7 (%)	100	94.0	99.4	**79.9****
Mean pup body weights day 14 (%)	100	94.0	98.7	**78.4****
Mean pup body weights day 21 (%)	100	97.2	98.4	**78.1****
Female pup bw change				
Mean pup body weights day 0 (%)	100	99.2	102.1	**93.8****
Mean pup body weights day 4 (%)	100	95.6	98.2	**84.2****
Mean pup body weights day 7 (%)	100	94.7	99.1	**82.3****
Mean pup body weights day 14 (%)	100	94.2	98.8	**80.8****
Mean pup body weights day 21 (%)	100	97.1	98.1	**81.1****

** $p \leq 0.01$
[1] pup mortality excluding those pups that were intentionally culled according to the study design.
[2] 92 pups from LD0-4 including 1 entire litter loss; 30 pups from LD5-21 including 4 entire litter losses.
HCD range for pup mortality (20 studies, 1994-1998): LD0-4: 1-30; LD5-21: 0-5.

RAC noted statistically significant effects on (i) pup body weight (development, possible lactation effect), (ii) pup survival (development) and (iii) pubertal time of attainment (fertility and sexual function or development).

Maternal Toxicity: There was evidence of limited maternal toxicity. In F0 females there were decreases in mean body weight (8 to 10% relative to controls, significant, ~~P~~p ≤ 0.01) and body weight gain (approximately 8%) and food consumption (approximately 20%, significant p ≤ 0.01) in the high dose group over gestation days 0-20. In F1 females, there were decreases in mean body weight (12 to 14% relative to controls, significant, p ≤ 0.01) and body weight gain

(approximately 8%) and food consumption (approximately 13%, significant $p \leq 0.01$) in the high dose group over gestation days 0-20.

Effects on pup body weight

The effects on neonatal body weight were significant. F1 pup body weights were affected *in-utero* to a small but significant extent (< 7% reduction relative to controls) and postnatal growth at 800 ppm was significantly decreased throughout lactation, showing reductions of between 11-24% across both sexes relative to the mean control bodyweight. A similar effect on pup body weights was observed for the F2 generation. F2 pup body weights were affected *in-utero* to a small but significant extent (approximately a 7% reduction relative to controls) and postnatal growth at 800 ppm was significantly decreased throughout lactation, showing reductions of between 15-22% across both sexes relative to the mean control bodyweight. It is unclear whether the observed effects were due to exposure via milk or due to *in-utero* exposure. The fact that the body weights were already reduced at PND 0 suggests that the *in-utero* exposure may have at least contributed to the reduced postnatal weight reduction.

Effects on pup survival

Viability and weaning indices were clearly affected by treatment at the top dose of 800 ppm; these values were outside historical controls for the performing laboratory. Pup mortality (not including those culled) from LD0-21 was greatly increased and is clear evidence of an adverse developmental effect.

Effects on pubertal time of attainment

There was no treatment-related effect on anogenital distance; however, this data was only recorded for F2 pups. Landmark data for preputial separation and vaginal opening (mean age in days of pups in a litter reaching the criterion) were reported for F1 pups only (table 11a), and were significantly increased in the mid dose (males) and high dose groups (both sexes). The HCD from the performing laboratory was as follows:

- Preputial separation: mean = 41.7 days, range 41.1 – 42.4 days, 5 studies (1994-1998)
- Vaginal opening: mean = 32.9 days, range 31.7 – 34.3 days

The mean age for attainment of preputial separation in males was significantly delayed by nearly 5 days in the top dose group compared with the control group. This was outside the performing laboratory historical control values. Note: HCD from several other sources were reported in the original study report. The mean delay in preputial separation in the top dose group remained outside the upper bound limits of the HCD range in all cases.

The mean age of attainment of vaginal opening in females was significantly delayed by about 3 days in the top dose group. This was greater than the mean value for the historical control data but did not lie outside of the HCD range for this endpoint.

RAC notes that there was no effect on time of pubertal attainment in the rat developmental neurotoxicity study (Anon., 2008).

Table 11a*: Summary of F1 litter landmark and pubertal attainment (covariate adjusted mean ± SD).*

Dose (ppm)	Control	100	280	800
Preputial separation	41.56 ± 1.83	42.36 ± 1.05	**43.32* ± 2.54**	**46.48** ± 2.75**

Vaginal opening	31.08 ± 0.91	31.81 ± 1.46	31.80 ± 2.18	**33.98** ± 3.62**
Anogenital distance (mm)[1]	--	--	--	--

* p ≤ 0.05; ** p ≤ 0.01
[1] Not recorded for F1 pups

Note: day of attainment of other landmarks such as eye opening, incisor eruption and pinna unfolding were not affected by treatment with acetamiprid.

***Table 11b**: Summary of F2 litter landmark and pubertal attainment (covariate adjusted mean ± SD).*

Dose (ppm)	Control	100	280	800
Preputial separation[1]	--	--	--	--
Vaginal opening[1]	--	--	--	--
Eye opening	14.56 ± 0.69	14.65 ± 0.78	14.35 ± 1.10	**15.44* ± 0.92**
Anogenital distance (mm)				
males:	1.88 ± 0.22	1.85 ± 0.17	1.88 ± 0.19	1.84 ± 0.21
females:	0.70 ± 0.07	0.70 ± 0.09	0.69 ± 0.07	0.69 ± 0.08

* p ≤ 0.05;
[1] Not recorded for F2 pups, scheduled termination on LD21.

Note: day of attainment of other landmarks such as incisor eruption and pinna unfolding were not affected by treatment with acetamiprid.

Under CLP (Annex I: 3.7.1.3) it is recognised that adverse effects on sexual function and fertility include effects on the onset of puberty. This criterion would appear to be satisfied for acetamiprid, since it significantly delays the time of onset for preputial separation in males and to a lesser extent, vaginal opening in females. The criteria as specified under section 3.7.2 of CLP indicate classification with Repr. 2 may be considered when there is some evidence from animal studies "of an adverse effect on sexual function and fertility".

Reductions in body weight during postnatal development are known to cause delays in the onset of puberty. It is established in the scientific literature that growth rate is of greater importance than arrival at a particular fixed weight in determining the onset of puberty. For the top dose F1 female and male pups there is evidence of a significant delay in growth amounting to about a 15% reduction relative to concurrent controls by PND 21. This indicates a recovery by the pups as the maximum effects were observed during PND 4-14. RAC considers that the delayed pubertal effects seen in males may not only be a direct result of acetamiprid toxicity (and therefore indicative of a direct effect on pubertal attainment and thus fertility), but may also be indicative of a more generalised adverse effect on growth and thus support classification for developmental toxicity.

Rat Oral Developmental Neurotoxicity Study

This 1-generation study (compliant to US-EPA guideline OPPTS 870.6300 (1998)), was designed to determine the potential of the test article, acetamiprid, to induce functional and/or morphological changes to the nervous system, which may arise in the offspring from exposure of the mother during pregnancy and lactation. The test article was administered orally by gavage to three groups of 25 Crl:CD (SD)IGS BR rats once daily from gestation day 6 through lactation day 21, inclusively. Dosage levels were 0, 2.5, 10 and 45 mg/kg bw/day.

There were no significant differences among groups for pregnancy rates, gestation length and

parturition, the only exception being the high dose female, which died because of dystocia. At the scheduled necropsy of F0 females on lactation day 21, no internal findings were noted. The mean numbers of implantation sites, numbers of pups born and numbers of unaccounted sites recorded at the scheduled necropsy were unaffected by treatment.

Table 12: *Summary of reproductive performance*

Parameter	**Control**		**2.5 mg/kg bw/day**		**10 mg/kg bw/day**		**45 mg/kg bw/day**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Females on study	25		25		25		25	
Females that died	-		-		-		1	
Females allowed to deliver	25		25		25		25	
Non-gravid	2	8	2	8	2	8	-	-
Gravid	23	92	23	92	23	92	25	100
Females with total litter loss	-		-		-		3	12
Females with viable pups	23	100	23	100	23	100	21	84
Gestation length (days – mean value)	21.5		21.5		21.5		21.7	
Implantation sites (mean value)	15.4		15.7		16.2		15.9	
Number born (mean value)	15.1		15.3		15.5		15.5	
Unaccounted sites (mean value)	0.4		0.3		0.7		0.5	

Unlike the results from the rat 2-generation study, the mean numbers of days to acquisition of preputial separation and vaginal opening in the treated group males and females were not affected by F0 maternal test article administration (table 13). There was no evidence of effects on sexual function and fertility.

Table 13*: Summary of F1 litter pubertal attainment.*

Dose (mg/kg bw/day)	**Control**	**2.5**	**10**	**45**
Preputial separation	43.6 ± 1.01	43.7 ± 1.57	43.1 ± 1.51	43.9 ± 1.77
Vaginal opening	32.2 ± 0.71	32.3 ± 0.95	32.7 ± 1.40	32.5 ± 1.14

* $p \leq 0.05$; ** $p \leq 0.01$

Overall, RAC does not consider the changes in maternal body weight and body weight gain or food consumption as excessive maternal toxicity. The DS did not discuss the pubertal findings in much detail in the CLH report. RAC considers the male pubertal delay in the rat 2-generation study sufficient for discussion on classification for adverse effects on fertility and or development. Classification as reproductive toxicant in Cat. 2 for effects on fertility was proposed for plenary discussion, however it is also recognised that these effects can also reflect the adversity of significantly decreased growth and thus support relevant effects by developmental toxicity. The pubertal findings in the rat developmental neurotoxicity study were also discussed and may simply be a consequence of the different dosing strategy where exposure is after implantation. The effects on pup body weight and survival are best described under classification for development. RAC considered the delays in male puberty as caused by

a general developmental delay and thus supporting of the overall evidence for adverse effects on development and supportive of classification in category 2 for development.

Developmental toxicity was primarily investigated in the rat and the rabbit in GLP and OECD TG 414 (1981) guideline compliant studies. In both rats and rabbits, no teratogenicity or foetotoxicity was observed at the top doses tested (50 and 30 mg/kg bw/day, respectively).

In the developmental neurotoxicity study (Anon., 2008), exposure from gestation day 6 until lactation day 21 resulted in postnatal effects including reduced survival, reduced body weight gain, reduced body weight at time of vaginal opening (responses were within the HCD), and effects on the acoustic startle response at the highest dose. It is also noted by RAC that the effects on body weights and survival in these pups were similar to or at least supportive of those in the rat 2-generation study. An important question is whether the effects on auditory startle response (figure 2), represent specific neurotoxicity or developmental toxicity. Also, the sex-specificity of the response (males only), particularly at PND60 remains unexplained.

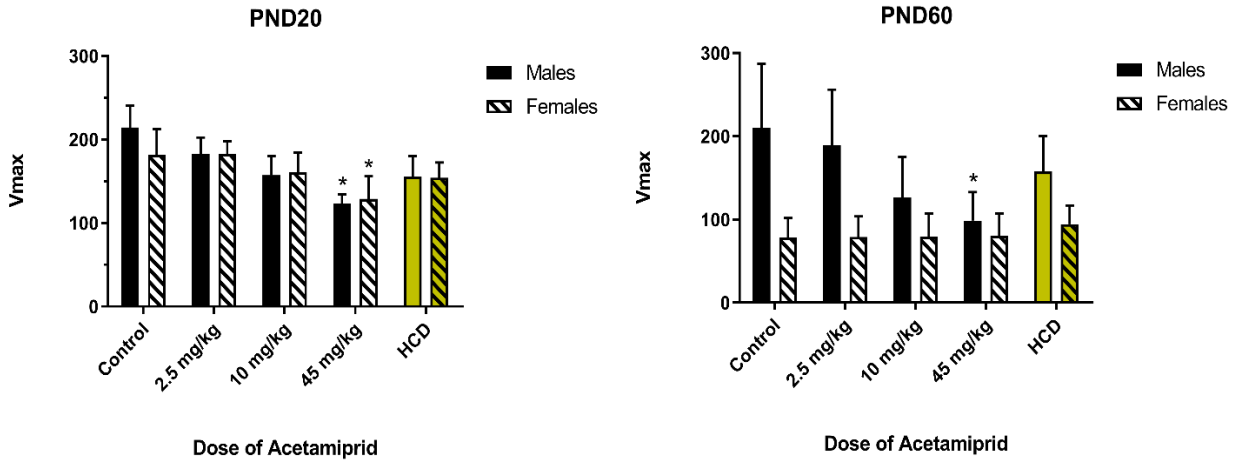


Figure 2: The acoustic startle reflex at PND20 and PND60. Each test session consisted of 5 blocks of 10 trials. There were 10 rats/sex/dose group. Vmax and Vave essentially provide identical information. The PND20 HCD was derived from 19 studies (Oct. 1999 – Jan. 2005). The PND60 HCD was from 22 studies (Oct. 1999 – Jan. 2005). Acetamiprid inhibition of the startle reflex was statistically different from controls in the top dose group only. Error bars are ± 1 s.d.

Note: the acoustic startle response is muscular activity produced by reflex in response to a sudden loud sound (figure 2). At 10 mg/kg bw/day, the startle response amplitude (i.e. how strong the response was to the stimuli) appeared to be diminished. At this dose, the difference in startle amplitude was not statistically different from concurrent controls and remained within the laboratory's HCD range. In addition, there were no concomitant neurodevelopmental delays in any of the other functional assessments at 10 mg/kg bw/day (motor activity, learning and memory assessments), nor any organic changes in the brain as exemplified by a lack of neuropathology or morphometry findings at any dose level. The lowering by EFSA of the NOAEL to 2.5 mg/kg bw/day, was over-precautionary, and admitted as such by the technical

committee during peer-review but may be considered toxicologically relevant.

In summary, the decrease in startle response in the developmental neurotoxicity study, while significant at the top dose of 45 mg/kg bw/day in males at PND20 and 60 and observed but not statistically significant at the mid dose of 10 mg/kg bw/day may not be an indicator of developmental toxicity, it does however suggest an effect such as diminution of motor function. It remains unclear since no recovery group was used in the rat developmental neurotoxicity study to show if the startle response returns to control levels following cessation of exposure. The histopathological investigation failed to show any treatment related changes in the brain.

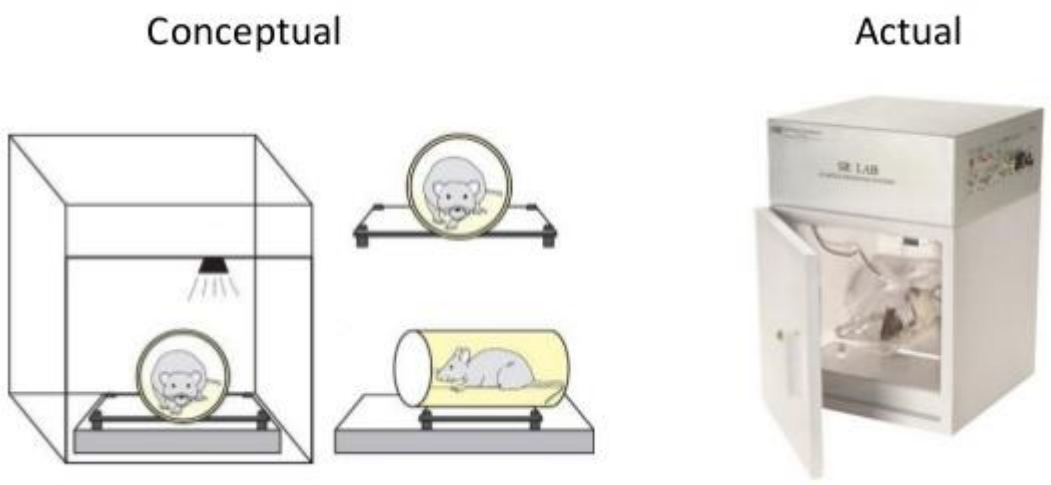


Figure 2: Setup apparatus for measurement of the acoustic startle reflex in rats.

RAC concurs with the DS in supporting classification in category 2 for developmental effects based on:

- The statistically significant reduction in postnatal pup body weights in the top dose group (800 ppm; 105-108 mg/kg bw/day lactation phase) for both sexes during PND 0-21.
- The decrease in postnatal survival observed in the F2 pups of the rat 2-generation study at the top dose of 800 ppm; this effect was not observed in the F1 pups. This is the most adverse developmental effect noted.
- The decrease in postnatal survival observed in the rat developmental neurotoxicity study at the top dose of 45 mg/kg bw/day on PNDs 0-1.

Overall, RAC considers the reductions in pup body weight, postnatal survival and delayed male rat pubertal attainment sufficient for classification as **Repr. 2; H361d** for adverse effects on development.

Adverse effects on or via lactation

RAC supports the assessment of the DS noting that it was unclear whether the observed effects in the 2-generation and the neurotoxicity developmental toxicity studies were due to exposure via milk or due to *in utero* exposure. There was no cross-fostering study to substantiate effects via lactation. Clear evidence of an adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk could not be demonstrated. **Therefore, no classification for effects on or via lactation is warranted.**

10.11 Specific target organ toxicity-single exposure

Not evaluated in this dossier.

10.12 Specific target organ toxicity-repeated exposure

Not evaluated in this dossier.

10.13 Aspiration hazard

Not evaluated in this dossier.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

11.1 Rapid degradability of organic substances

Table 49: Summary of relevant information on rapid degradability

Method	Results	Remarks	Reference
OECD Guideline 301B Ready biodegradability	27% of the initially applied dose was degraded in 28 days	Not ready biodegradable	Anonymous (1999c)
OECD Guideline 111 Hydrolysis	stable at environmentally relevant conditions		Gomyo and Kobayashi (1993)
EPA Guideline 161-2 Photochemical degradation	Half-life: 34 days	Degradation product detected at >10% of the applied dose	Hausmann and Class (1998)
OECD Guideline 309 Surface water simulation	Half-life: 3.9-8.5 days	Mayor metabolite detected with increasing concentrations up to 81.5%	Möndel (2014)
Aquatic-sediment simulation study performed according to Directive s95/36/EC and 91/414/EEC	Half-lives for the whole system: 23.1-31.6 days	Mayor metabolites detected for which half-lives could not be determined	McMillan-Staff and Austin (2001) and Jarvis and Montesano (2014)

11.1.1 Ready biodegradability

The following study was available in the original registration dossier (EC, 2004), the description below is based on the summary in the RAR.

Ready biodegradability of acetamiprid has been assessed by Anonymous (1999c)in a GLP study following the OECD 301B guideline. Activated sewage sludge was washed three times and used to assess biodegradation in a culture medium with 10.5 mg acetamiprid/L. Incubation was performed in sealed vessels and the CO2 produced was captured in two vessels with 0.05 M NaOH solution. Sampling of the first vessel was performed 17 times over the 29 day incubation. The second vessel was only sampled at test initiation and test termination. Over 28 days of incubation the degradation of the initial dose was 27% and therefore the substance is considered not readily biodegradable.

11.1.2 BOD_5/COD

Information of oxygen demand is not available.

11.1.3 Hydrolysis

In the original registration dossier (EC, 2004) on hydrolytic degradation is acetamiprid was available, the description below is based on the summary in the RAR.

Hydrolysis of radiolabelled acetamiprid was tested in a GLP study by Gomyo and Kobayashi (1993) in three buffer solutions of pH 4, 5, 7 and 9 and at temperatures of 22, 35 and 45 °C. The hydrolysis was performed

in the dark and duplicate samples were taken at day 0, 1, 2, 5, 9, 15, 22 and 35 days. At pH 4, 5 and 7 the substance was stable at all test temperatures. At pH 9 the substance became instable higher temperatures with half-lives of 52.9 days and 13 days for temperatures of 35 and 45°C. On the basis of this, it can be concluded that the substance is stable at environmentally relevant conditions.

11.1.4 Other convincing scientific evidence

Other scientific evidence is not available.

11.1.4.1 Field investigations and monitoring data (if relevant for C&L)

Field investigations and monitoring data relevant for classification purposes is not available.

11.1.4.2 Inherent and enhanced ready biodegradability tests

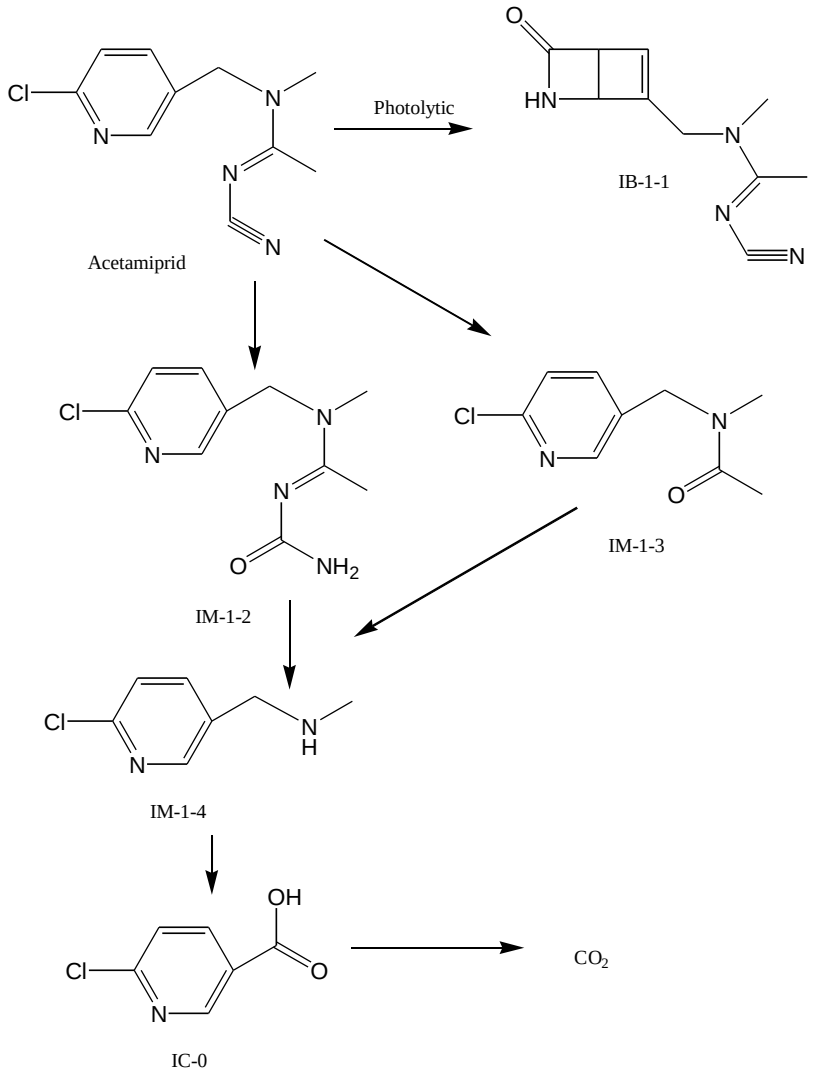
Inherent and enhanced ready biodegradability tests are not available.

11.1.4.3 Water, water-sediment and soil degradation data (including simulation studies)

In the dossier for the original registration (EC, 2004) a water/sediment simulation study was available, this study has been reassessed for the RAR. In the renewal dossier, a new study on the aerobic mineralisation in surface water is also available, the descriptions below are based on the summaries in the RAR.

McMillan-Staff and Austin (2001) examined the biodegradation of radiolabelled acetamiprid in a water-sediment system with sediment from two different locations (Manningtree and Ongar, both Essex, UK). Incubation was performed in flasks and lasted for 115 days mainly in the dark at a temperature of 20 °C. For analysis flasks were removed after 0, 1, 6, 24 and 48 hours and after 7, 14, 30, 62, 100 and 155 days. Analysis of the samples was performed with HPLC. CO_2 in the air from each flask was trapped in potassium hydroxide solutions and analysed with LSC. The overall recovery was 97.3% of the applied dose. After 155 days, carbon dioxide and accounted for 10% and 28.3% of the applied dose in Manningtree and Ongar sediment systems, respectively. In Manningtree and Ongar waters the metabolite IM-1-4 reached maximum levels of 12.3% (day 30) and 9.6% (day 14) of the applied dose, respectively. IC-0 reached maximum levels of 8.3% (day 155) and 26.2% (day 62) of the applied dose, respectively. The metabolite IM-1-2 was formed in the water during the early part of the study at maximum levels of 11% (day 7) of the applied dose, at one sampling point, after which it declined. This metabolite was not considered as major. In Manningtree and Ongar sediments IM-1-4 was the major metabolite found at its highest levels of 30.7% (day 30) and 15.6% (day 100) of the applied dose, respectively. IC-0 did not exceed 10% of the applied dose in both sediments. Some minor metabolites were observed in both the sediment and water phases, none of which exceeded 5% of the applied dose in the total system.

In the DAR the following degradation pathway is presented:



The results of this study were reassessed by Jarvis and Montesano (2014) according to FOCUS Kinetics guidelines. For the whole system, single first order degradation was considered most appropriate and for acetamiprid half-lives of 23.1 and 31.6 days were calculated for the Manningtree and Ongar sediment systems respectively with a geometric mean of 27 days.

Despite some deviations to the FOCUS Kinetics model, the RMS finds the results of the simulation modelling acceptable. The RMS notes that only visual fits of the best model (according to the study authors) was presented in the report. RMS also performed both a SFO fit and FOMC fit for acetamiprid, for the dissipation for the whole system, in order to check the choice of the SFO model as best fit by the study authors. There is almost no difference in the result between the SFO and FOMC fit. RMS agrees with the choice of the study authors, that SFO provides the best fit. The geometric mean DT50 value for the whole system of 27 days can be used for classification purposes.

Table 50: Kinetic analysis of degradation of acetamiprid in the whole system

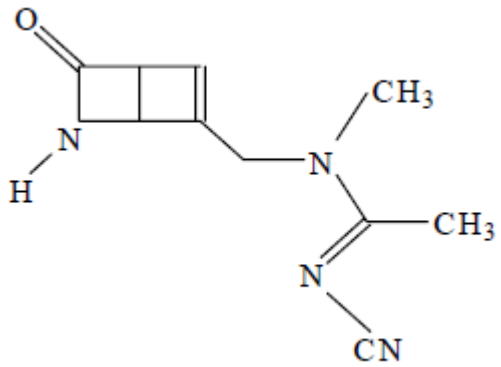
System	Model	DT_{50} (days)	DT_{90} (days)	χ^2 error (%)	t-test
Mannintree	SFO	23.1	76.7	7.6	1.91e-06
	FOMC	21.5	91.8	7.5	-
Ongar	SFO	31.6	104.9	6.7	3.98e-11
	FOMC	29.0	123.3	6.6	-

Möndel (2014) performed a GLP study on the aerobic degradation of radiolabelled acetamiprid in surface water from a natural pond. The degradation of two concentrations was tested in duplicate: 2 µg/L and 10 µg/L and a sterile control and a bio-control with benzoic acid was also performed. Samples were taken after 0, 1, 3, 7, 14, 28 and 59 days. Samples for the biocontrol were taken within 72 hr. Analysis was performed by HPLC after clean-up with a solid phase column. Volatiles were trapped in oil wetted quartz wool and soda lime and after extraction analysed with LSC. The total mass balance was between 97.5 and 101.6% of the applied radioactivity. Acetamiprid declined to 6.5% and 11.5% of the applied dose in the low and high dosed systems respectively. In all systems, one metabolite (IM-1-4) was detected with a concentration >10% of the applied dose and the concentration of this metabolite increased to 81.5 and 70.8% in the low and high dose respectively over the 59 days of the incubation. Other metabolites did not exceed 5% of the applied dose. Volatiles did not exceed 1% (≤0.57%). On the basis of single first order kinetics, half-lives for acetamiprid of 3.9 and 8.5 days were determined for the low and high dose respectively. In the DAR the RMS has re-evaluated the half-lives and concluded that bi-phasic models are more appropriate to fit the degradation curves, this resulted in half lives of 2.4 and 6.8 for the low and high dose respectively. For the main metabolite, no acceptable fit could be achieved and a half-life for this metabolite is not available.

11.1.4.4 Photochemical degradation

A GLP report on direct photochemical degradation by Hausmann and Class (1998) was present in the original registration dossier, the description below is based on the summary in the RAR.

Radiolabelled acetamiprid was dissolved in a buffer at pH 7 at a concentration of 10 mg/L. The solutions were exposed at 25°C for 30 days with 12 hour light per day. The light intensity was 690 W/m^2 and wavelengths <290 nm were filtered out. Samples were taken at day 0, 2, 6, 13, 20, and 30. A recovery of the radiolabels was achieved of 99.5%. After 30 days, only 0.1-0.2% of the applied doe was retrieved in one of the volatile traps and 54% of the parent compound was still present in the test solutions. The half-live of the parent was calculated to be 34 days. Degradation products were detected. One of these was present 35% (referred to as UK in the report) of the applied dose, other degradation products had a total of 7%. For the major metabolite (IB-1-1), the structure was clarified as:



11.2 Environmental transformation of metals or inorganic metals compounds

Not relevant

11.3 Environmental fate and other relevant information

Adsorption

In the RAR a GLP-compliant soil batch adsorption desorption study according to OECD TG 106 is reported (Flückiger, 1997). Radio-labelled ^{14}C-acetamiprid (purity not reported) was tested in five soils. Adsorption phase was 4 hour, which was shown during pre-study to be sufficient to reach equilibrium. Following

replacement of aqueous phase desorption was allowed for 4 hours and the desorption step was repeated once more. Aqueous phase and soil extracts were analysed by TLC and LSC. TLC analyses showed that the ^{14}C-acetamiprid remained stable during testing. Mass balance ranged 97.2-100.3% applied radioactivity (% AR). ^{14}C-acetamiprid ranged 66.3-90.4% AR in the aqueous phase, and 5.9-31.1% AR in the soils. The reported K_{oc} values were 71.09 (sandy loam), 71.38 (silt loam), 121.81 (silt loam), 129.98 (loamy sand) and 138.39 (sand) L/kg with Freundlich exponents ranging 0.825 to 0.907. The arithmetic mean Koc was reported to be 106.53 L/kg, i.e. log K_{oc} of 2.02. The study is considered reliable, and shows that acetamiprid does not strongly bind to soils.

Volatilisation

In the RAR data on fate and behaviour in air is presented, considering the low vapour pressure ($1.73x10^{-7}$ Pa at 50°C) of acetamiprid, this data is considered not relevant for classification purposes.

11.4 Bioaccumulation

Table 51: Summary of relevant information on bioaccumulation

Method	Results	Remarks	Reference
log Pow	0.80		DAR

11.4.1 Estimated bioaccumulation

Estimated data on the bioaccumulation of acetamiprid is not available in the RAR. Considering the low Pow of the substance, further estimation is also not considered relevant.

11.4.2 Measured partition coefficient and bioaccumulation test data

Acetamiprid has a log Pow of 0.80, therefore it is considered to have a low potential for bioaccumulation.

11.5 Acute aquatic hazard

Table 52: Summary of relevant information on acute aquatic toxicity

Method	Species	Test material	Results	Remarks	Reference
Fish					
Static GLP toxicity study according to OECD guideline 203	*Oncorhychus mykiss*	Acetamiprid Purity: 99.57%	LC50 > 100 mg/L (nominal)	Measured concentrations were close to nominal. Study is considered reliable (Ri=2)*	Anonymous (1997h)
Flow through toxicity study	*Lepomis macrochirus*	Acetamiprid Purity: >99%	LC50 > 119.3 mg/L (mean measured)	Study is considered reliable (Ri=2)*	Anonymous (1997b)
Static GLP toxicity study according to J MAFF guideline 2735	*Cyprinus carpio*	Acetamiprid (NI-25) Purity: 99.46%	LC50 >100 mg/L (nominal)	Study is considered unreliable (Ri=3)*	Anonymous (1995)
Flow through GLP study according to FIFRA	*Cyprinodon variegatus*	Acetamiprid Purity: >99.9%	LC50 = 92 mg/L (mean measured)	Study is considered reliable (Ri=2)*	Anonymous (1998c)

guideline 72-3					
Static non-GLP study	*Oreochromis mossambicus*	Acetamiprid	LC50 = 5.99 mg/L (nominal)	Study is considered unreliable (Ri=3)*	Anonymous (2015)
Invertebrates					
Static GLP study according to OECD guideline 202	*Daphnia magna*	Acetamiprid (NI-25) Purity: 99.9%	LC50 = 49.8 mg/L (mean measured)	Study is considered reliable (Ri=2)*	Saika (1997)
Static GLP study according to ASTM guideline E-729 and FIFRA 72-2	*Chironomus riparius*	Acetamiprid Purity: 99.3%	**LC50 = 20.7 µg/L** (mean measured)	Study is considered reliable (Ri=2)*	Putt (2003b)
Static GLP study according to OPPTS draft guideline 850-1020	*Gammarus fasciatus*	Acetamiprid Purity: 99.3%	LC50 = 100 µg/L (mean measured)	Study is considered reliable (Ri=2)*	Putt (2003a)
Static GLP study according to FIFRA 72-3 test guideline	*Mysidopsis bahia*	Acetamiprid Purity: 99.9%	EC50 = 66 µg/L (mean measured)	Study is considered reliable (Ri=2)*	Anonymous (1998b)
Static non-GLP study	*Gammarus pulex*	Acetamiprid	EC50 = 50 µg/L (nominal)	Study is considered not assignable (Ri=4)*	Beketov and Liess (2008)
Static non-GLP study	*Simulium latigonium*	Acetamiprid	EC50 = 3.73 µg/L (nominal)	Study is considered not assignable (Ri=4)*	Beketov and Liess (2008)
Algae/Aquatic Plants					
Static GLP study according to OECD guideline 201	*Scenedesmus subspicatus*	Acetamiprid Purity: 100%	EC50 > 98.3 mg/L (mean measured)	Study is considered reliable (Ri=2)*	Suteau (1996)
Static GLP study according to FIFRA guideline 122-2 and 123-3	*Anabaene flos-aquae*	Acetamiprid (N-25) Purity: 99.9%	EC50 > 1.3 mg/L (mean measured)	Study is considered reliable (Ri=2)*	Hoberg (1997a)
Static GLP study according to FIFRA guideline 122-2 and 132-3	*Lemna gibba*	Acetamiprid Purity: 99.9%	EC50 > 1.0 mg/L (mean measured)	Study is considered reliable (Ri=2)*	Hoberg (1997b)

* Reliability according to Klimisch et al. (1997), since assessment is based on summaries in the DAR, Ri=1 is not given.

11.5.1 Acute (short-term) toxicity to fish

Two additional studies on acute toxicity to fish were included in the renewal dossier for acetamiprid since the previous evaluation (EC, 2004). The descriptions below are based on the summaries in the RAR.

Anonymous (1997h) has tested *Oncorhynchus mykiss* at five exposure concentrations for 96 hours in a batch exposure according to OECD guideline 203. The acetamiprid had a purity of 99.57% and the five test concentrations were 25, 35, 50, 70 and 100 mg/L. The mean wet weight of the fish was 2.05 g and the mean length was 5.8 cm. Mortality was only observed in the highest test concentration. Test concentrations were verified by analysis and were 101 to 104% of nominal and were constant over the exposure period. Loss of equilibrium, darkened body and swelling of abdomen was observed in the 50, 70 and 100 mg/L exposure concentrations. Based on these observations, the LC50 was determined to be >100 mg/L and the NOEC was 35 mg/L. The endpoints are considered reliable and can be used for classification purposes.

Anonymous (1997b) has tested *Lepomis macrochirus* at five exposure concentrations for 96 hours in a flow through set up. The acetamiprid had a purity of >99% and the nominal test concentrations were 11.4, 20.6, 37, 66.7 and 120 mg/L. Analysis of the test concentrations was performed at the start and end of the test. These showed that initial concentrations were maintained throughout the test (93 to 99% of nominal), only for one value a recovery of 131% was reported. The mean measured concentrations were 11.8, 20, 35.4, 65 and 119.3 mg/L. No mortalities were observed in any of the concentrations but darkened pigmentation was observed for all exposure concentrations. Therefore, the LC50 was stated to be >119.3 mg/L and the NOEC <11.8 mg/L. The endpoints are considered reliable and can be used for classification purposes.

Anonymous (1995) has tested *Cyprinus carpio* in a static test set-up at three concentrations and a control for 96 hours according the Japanese MAFF guideline 2735. The acetamiprid had a purity of 99.46% and the nominal test concentrations were 10, 30 and 100 mg/L. The concentration of the test substance was not analytically verified and pH, hardness and dissolved oxygen was only reported for the initial test solution. The fish had a total length of 4.5 ± 0.3 cm and the test temperature was 24.5 - 25 °C. Mortality or signs of toxicity was not observed in any of the test concentrations. The endpoint from this test are considered unreliable because the test concentrations were not verified and it is unknown if the dissolved oxygen remained above 60% because only initial concentrations for dissolved oxygen were reported. Also, the temperature and weight of the test animals do not meet the validity criteria of the OECD 203 test guideline. The endpoint will not be used for classification purposes.

Anonymous (1998c) tested *Cyprinodon variegatus* at five exposure concentrations for 96 hours under flow through conditions according to FIFRA guideline 72-3. The acetamiprid tested had a purity of 99.9% and the test concentrations were 19, 32, 54, 90 and 150 mg/L. Test concentrations were verified initially and at test termination. The mean measured test concentrations were 17, 30, 55, 79 and 140 mg/L. 100% mortality was reported for the highest test concentration and 5% mortality for the 79 mg/L and for all surviving fish at this concentration sublethal effects were observed. For the remaining test concentrations, no mortality or sublethal effects were observed. The validity criteria of the OECD 203 guideline were met. In the report, the LC50 was reported to be 100 mg/L and the NOEC as 55 mg/L. In the RAR, the LC50 and NOEC were redetermined and reported as 92 mg/L and 79 mg/L respectively. The values recalculated in the RAR will be used for classification purposes.

One additional study was retrieved from the public literature that was not included in the renewal dossier. Anonymous (2015) reported a 96 h LC50 of 5.99 mg/L for the fish *Oreochromis mossambicus*. The publication does however contain only limited information of the test set-up because of which the reliability of the study cannot be assessed. Furthermore, from the publication is concluded that the test concentrations are not verified by analysis. Therefore the endpoint is considered unreliable and will not be used for classification purposes.

11.5.2 Acute (short-term) toxicity to aquatic invertebrates

Three additional studies on acute toxicity to aquatic invertebrates were included in the renewal dossier for acetamiprid since the previous evaluation (EC, 2004). The descriptions below are based on the summaries in the RAR.

Acute toxicity of acetamiprid to *Daphnia magna* was tested in a static test by Saika (1997) according to OECD guideline 202. The test compound had a purity of 99.9% and five concentrations were test of 12.5, 25, 50, 100 and 200 mg/L. The test concentrations were measured over the test period and were close to nominal. In the RAR only ranges for the measured concentrations are given (12.9-13.3, 24.2-25.3, 49-50.6, 94.3-96.4 and 189.4-195.6 mg/L). After 48 hours immobilisation was observed in the 25 mg/L solutions and higher and death was observed in the two highest exposure groups. The EC50 based on mean measured concentrations was 49.8 mg/L, the NOEC based on nominal concentration was 12.5 mg/L. The EC50 will be used for classification purposes.

Acute toxicity to *Chironomus riparius* was determined by Putt (2003b) in a static test according to ASTM guideline E-729 and FIFRA guideline 72-2. The following summary is taken over from the original DAR and RAR and adapted for classification purposes:

Characteristics

Reference	:	Putt, A. E., 2003a	Species	:	*Chironomus riparius*
Type of study	:	Acute toxicity	Exposure duration	:	48 h
Year of execution	:	2003	Nominal dose		0, 6.3, 13, 25, 50 and 100 µg/L
GLP statement	:	yes	Dosing method	:	Static
Guideline	:	ASTM Guideline E-729, FIFRA 72-2	Acceptability	:	Acceptable
Test substance	:	Acetamiprid technical (Lot/Batch no. 2050183)	LC50	:	20.7 µg/L (mean measured)
a.s. content	:	99.3% pure			

Methods
Based on the results of preliminary testing, the nominal test concentrations were: 6.3, 13, 25, 50 and 100 µg/L. A total of 140 organisms (5 per replicate, 4 replicates per concentration) were exposed to five concentrations of the test substance, a dilution water control and a solvent control for 48 hours under static conditions. Test animals were 4 days old at test initiation. A 1.0 mg/mL stock solution was prepared by placing 0.25180 g (0.25004 as active ingredient) of acetamiprid in a 250 mL volumetric flask and bringing to volume with acetone (0.10 mL/L).
Mortality was recorded at 0, 24 and 48 hours of exposure. Biological observations and observations of the physical characteristics of each replicate test solution were also made and recorded at 0, 24 and 48 hours. Temperature, pH and dissolved oxygen concentration were measured at 0, 24 and 48 hours in one replicate of each treatment level and the controls. At test initiation and test termination, one sample was removed from each test solution and the control for analysis of test substance concentration. All samples were analysed by high performance liquid chromatography with ultraviolet detection (HPLC/UV).
If at least one concentration caused mortality of greater than or equal to 50 % of the test population, then a computer program (TOXSTAT® Version 3.5) was used to calculate the LC50 values and the 95 % confidence intervals. Four methods, Spearman-Karber Estimate, Probit analysis, Logit Analysis and Log-Log Analysis were used in the computer program. If two or more statistical methods produced acceptable results, then the method which yielded the smallest 95 % confidence interval was selected (here: Log-Log Analysis).
Results
Analytical results showed a mean recovery from 92 to 110% (mean measured concentrations: 6.0, 14, 26, 46 and 110 µg/L). Results were expressed in mean measured concentrations, which is acceptable.
The cumulative percentage mortality is presented in Table 53.

Table 53: Mean cumulative mortality of acetamiprid to *Chironomus riparius*

Mean measured concentration (µg/L)	Mean cumulative mortality of organisms (%)	
24 hours	**48 hours**	
Control	0	0
Solvent control	0	0
6.0	0	5
14	0	10
26	0	65
46	90	100
110	100	100

Conclusion
Based on mean measured concentrations, the 48-hour LC50 value for acetamiprid technical and Chironomus riparius was determined to be 24 µg/L, with a 95% confidence interval of 21 to 27 µg/L. The No-Observed-Effect-Concentration (NOEC) was determined to be 14 µg/L
Guidelines and limitations
- The GLP status of the laboratory was checked by RMS
- The required guideline according to 1107/2009 is OECD 235.
- The validity criteria according to OECD 235 are met:
- RMS checked the LC50 using TOXRAT 2.10 and calculated an LC50 based on the mean measured concentration of 20.7 µg/L. This is in line with the study result.
The study is acceptable, the results as mentioned under Conclusion will be used for classification purposes. The Dossier Submitter notes that the current study setup contains no sediment, thus exposure occurs only via the water.

Acute toxicity to *Gammarus fasciatus* was determined by Putt (2003a) in a static test according to OPPTS draft guideline 850.1020. The test compounds had a purity of 99.3% and the nominal test concentrations were 9.4, 19.0, 38.0, 75.0 and 150 µg/L in four replicates. Exposure lasted for 96 hours and the test concentrations were determined at the start and initiation of the test. The mean measured concentrations were 87-100% of nominal and the LC50 based on mean measured concentrations was determined to be 100 µg/L. The NOEC based on mean measured concentrations was 9.4 µg/L. The LC50 will be used for classification purposes.

Anonymous (1998b) tested acute toxicity on *Mysidopsis bahia* according to the FIFRA 72-3 test guideline. The animals were exposed for 96 hours in a flow through test set-up to the test compound which had a purity of 99.9%. Two replicates with each 10 mysids were performed with nominal concentrations of 0, 13, 22, 36, 60 and 100 µg/L. The concentration of the test substance was analytically verified at the start of the exposure and at termination. The measured concentrations ranged from 98-110%. Only in the lowest test concentration no mortality was observed. The EC50 was determined to be 66 µg/L, based on the mean measured concentrations. The NOEC is determined to be 13 µg/L this value is based on mean measured concentrations which is for this concentration identical to the nominal concentration. The EC50 will be used for classification purposes.

In addition to the studies in the DAR and in the renewal dossier, one study was retrieved from public literature (Stevens et al., 2005) that has tested an acetamiprid formulation (225 g AI/L) on *Chironomus tepperi* and reported an LC50 of 2.22 µg/L for the active ingredient. The publication does however contain only limited information of the test set-up, e.g. test concentration are not reported, because of which the reliability of the study cannot be assessed. Also from the publication, it is concluded that the test concentrations are not verified by analysis. Therefore, the endpoint is considered unreliable and will not be used for classification purposes.

11.5.3 Acute (short-term) toxicity to algae or other aquatic plants

One additional study on toxicity to algae was included in the renewal dossier for acetamiprid since the previous evaluation (EC, 2004). The descriptions below are based on the summaries in the RAR.

Aquatic toxicity to algae was tested by Suteau (1996) on *Scenedesmus subspicatus* in a 72 hours exposure according to OECD test guideline 201. The acetamiprid with a purity of 100% was test in nominal concentrations of 1.9, 4.3, 9.4, 20.7, 45.5 and 100 mg/L in three replicates. At test initiation and test termination the exposure concentration were analysed and the concentrations were within 95 to 106% of nominal. The mean measured concentrations are 1.9, 4.3, 9.2, 20.6, 44.7 and 98.3 mg/L. Over the exposure period no inhibition of growth was observed and the EC50 was determined to be >98.3 mg/L. The test is considered reliable.

Hoberg (1997a) tested the toxicity to the blue-green algae *Anabaena flos-aquae* in a 5 day exposure according to the FIFRA test guideline 122-2 and 123-2. Acetamiprid with a purity of 99.9% was tested in only one nominal concentration of 1.0 mg/L in three replicates. The test concentration was analytically verified at day 0 and day 5 and was 1.3 mg/L (130% of nominal). The cell density was not significantly influenced and there for the EC50 was determined to be > 1.3 mg/L. The test is considered reliable and the EC50 will be used for classification purposes.

The effect on *Lemna gibba* was tested in a 14 day test by Hoberg (1997b) according to the FIFRA test guideline 122-2 and 132-2 briefly described in the DAR. The exposure concentration was a mean measured concentration of 1.0 mg/L and the EC50 was reported as > 1.0 mg/L. Given the fact that the endpoint is based on mean measured concentrations the study is considered reliable and the EC50 will be used for classification purposes.

11.5.4 Acute (short-term) toxicity to other aquatic organisms

In the RAR a publication describing the drift-initiating action by acetamiprid among other pesticides in a microcosm was summarised (Beketov and Liess, 2008). In addition to the summary in the DAR the publication of the study was also assessed. In this test, acute toxicity was determined in 100 ml glass beakers with 60 ml test solution with exposure for 96 hours. The microcosms used for chronic toxicity consisted of glass channels 1.2 m long, 10.5 cm high and 4.5 cm wide with a current of 0.06 m/s in a closed circulation system with 5 L water. The test organisms were *Gammarus pulex*, *Simulium latigonium* and *Baetis rhodani*. The test concentrations for the acute test are not reported and also not confirmed by analysis. The 96 h EC50 for *Gammarus pulex* and *Simulium latigonium* were reported to be 50 µg/L and 3.73 µg/L respectively. For *Baetis rhodani* the LC50 was not determined. The drift responses in the microcosms were reported to be 7-22 times lower than the respective LC50 values. Since the exposure concentrations and other details of the test set-up were not reported the study is considered not assignable for the reliability assessment of the LC50 values (Ri4) and will not be used for classification purposes. It should be noted that in the DAR it was also stated that the study is of limited reliability.

11.6 Long-term aquatic hazard

Table 54: Summary of relevant information on chronic aquatic toxicity

Method	Species	Test material	Results	Remarks	Reference
Fish					
GLP Static Early Life Stages test according to OECD guideline 210	*Pimephales promelas*	Acetamiprid Purity: 100%	NOEC = 19.2 mg/L (mean measured)	The study is considered reliable (RI = 2)*	Anonymous (1997g)
Invertebrates					
Static renewal GLP test according to EPA/FIFRA guideline 72-4	*Daphnia magna*	Acetamiprid Purity: 99.9%	NOEC = 5 mg/L (mean measured)	The study is considered reliable (RI = 2)*	Suteau (1997)
GLP static sediment-water	*Chironomus riparius*	Acetamiprid Purity: 99.9%	**NOEC = 0.96 µg/L EC10 = 0.235 µg/L**	The study is considered	Mc Elligott (1999)

test according to BBA guideline			(mean measured)	reliable (RI = 2)*	
Algae/Aquatic Plants					
Static GLP study according to OECD guideline 201	*Scenedesmus subspicatus*	Acetamiprid Purity: 100%	NOEC ≥ 98.3 mg/L (mean measured)	Study is considered reliable (Ri=2)*	Suteau (1996)
Static GLP study according to FIFRA guideline 122-2 and 123-3	*Anabaena flos-aquae*	Acetamiprid (N-25) Purity: 99.9%	NOEC ≥ 1.3 mg/L (mean measured)	Study is considered reliable (Ri=2)*	Hoberg (1997a)
Static GLP study according to FIFRA guideline 122-2 and 132-3	*Lemna gibba*	Acetamiprid Purity: 99.9%	NOEC ≥ 1.0 mg/L (mean measured)	Study is considered reliable (Ri=2)*	Hoberg (1997b)
Other aquatic organisms					
Flow-through GLP study according to OECD guideline 231	*Xenopus laevis*	Acetamiprid Purity: 99.9%	NOEC = 2.6 mg/L (mean measured)	Study is considered reliable (Ri=2)*	Anonimous (2013)

* Reliability according to Klimisch et al. (1997), since assessment is based on summaries in the DAR, Ri=1 is not given.

11.6.1 Chronic toxicity to fish

No new studies on chronic toxicity to fish were included in the renewal dossier for acetamiprid since the previous evaluation (EC, 2004). The descriptions below are based on the summaries in the RAR.

Chronic toxicity in fish was determined in *Pimephales promelas* according to the OECD test guideline 210 and US-EPA guideline 72-4 (Anonymous, 1997g). The test compound with a purity of 100% was tested in the five nominal concentrations of 9.4, 18.8, 37.5, 75 and 150 mg/L, and mean measured concentrations were 89 to 117% of nominal (9.9, 19.2, 38.4, 76.0 and 147.5 mg/L). Exposure lasted for 35 days, inclusive 4 days pre hatching. At completion of hatching, 96.3% survival was observed for the control which decreased to 87.5 for the treatments but this was only significant different for the 76.0 exposure. At test termination, 100% survival was observed in the two lowest concentrations and in the control, in these groups larval weight and length were also not affected. Therefore, the NOEC, based on mean measured concentrations, was determined to be 19.2 mg/L. The study is considered reliable and the NOEC can be used for classification purposes.

11.6.2 Chronic toxicity to aquatic invertebrates

In addition to the existing two studies, no new studies on chronic toxicity to invertebrates were included in the renewal dossier for acetamiprid since the previous evaluation (EC, 2004). The descriptions below are based on the summaries in the RAR.

A 21 day static renewal test on *Daphnia magna* was performed by Suteau (1997). The test was performed according to the EPA/FIFRA guideline 72-4 from 1987. The nominal exposure concentrations were 2, 5, 9, 19, 38 and 75 mg/L. 22 animals were tested at each exposure concentration. The test concentrations were analytically determined and the mean measured concentrations were 2, 5, 9, 18, 37 and 74 mg/L. Survival of the first generation was 100% for most of the exposure groups, only for the 5 and 74 mg/L survival was 86 and 57% respectively but these were not statistically significant different from the other exposures. Length and weight were significantly different from the control for the 9 mg/L exposure and the higher exposure concentrations. For these concentrations, the number of offspring was also significantly lower than the control. For the concentrations of 18 mg/L and higher, the first brood release was two days later than the

control and the lower exposure groups. Because of these observations, the NOEC, based on mean measured concentrations was determined to be 5 mg/L. This value is considered reliable and can be used for classification purposes.

Mc Elligott (1999) performed a 28 day exposure test on *Chironomus riparius* in a static sediment water system. The following summaries are taken over from the original DAR and RAR and adapted for classification purposes:

reference	:	Mc Elligott (1999)	species	:	*Chironomus riparius* (first instar, 2-3 d old at test inititiation)
type of study	:	Chronic toxicity	exposure duration	:	28 days
year of execution	:	1999	nominal dose	:	0, 1.3, 2.5, 5, 10 µg/L
GLP statement	:	yes	dosing method	:	Static sediment water system
guideline	:	BBA guideline (1995)	acceptability	:	Acceptable
test substance	:	acetamiprid , lot/batch no. NHE-19	Endpoint	:	NOEC = 0.96 µg/L (mean measured) EC10 = 0.235 µg/L (mean measured)
a.s. content	:	100 %			

This study was already included in the DAR, but the endpoint has been recalculated because it was expressed as nominal concentration while concentrations drop <LOQ at the end of the study.

Summary as presented in the DAR (2001):
Mc Elligott "Acetamiprid: Toxicity to the sediment dwelling chironomid larvae (Chironomus riparius) – 28 days" 1999 (not published). Guidelines : BBA guideline proposal (1995) GLP : yes

Materials & methods
A total of 500 organisms (4 replicates of 25 organisms per test group) were exposed to 4 concentrations of the test substance acetamiprid (lot NHE-19, certified purity 100%) and a dilution water-sediment control for an exposure period of 28 days or until emergence of adult insects, under static conditions. The test system included 3 l glass beakers, 2 cm sediment layer (artificial sediment prepared according to OECD 207), and 2.5 l reconstituted overlaying water. One day after the introduction of the test organisms, appropriate volumes of the respective stock solutions were added to the water column of the test systems. The definitive test concentrations, which were chosen following preliminary testing are 1.3, 2.5, 5, and 10 microg/l. The chemical analysis confirmed the nominal exposure concentrations at test initiation. The concentrations of the test substance in the water column decreased during the test period. Samples from the water column were collected for analysis one hour, 7, 13 and 28 days after test initiation.

Findings & conclusions
One hour after test initiation analytical verification of the test concentrations in the overlaying dilution water showed the measured values were close to the nominal concentrations (92 – 100% recovery). Further on, the test concentrations on day 7 showed a reduction of [*added by RMS for AIR:* the nominal test concentrations] 1.3 and 2.5 microg/l below the limit of quantification (1 microg/l) and recoveries of 30 and 45% of the initial measured values observed at the higher nominal concentrations of 5 and 10 microg/l, respectively. Following 13 days exposure, 17% recovery of initial measured value was observed at the highest nominal concentration of 10 microg/l and recoveries at the three lower test concentrations were all below the limit of quantification. A final analytical verification at test termination (day 28) showed the recoveries from all of the [*added by RMS for AIR:* nominal] test concentrations were below the limit of quantification of 1 microg/l for the test substance under the conditions of the test. The results are expressed in nominal concentrations in microg/l. Following the 28 day exposure period, the mean rate of emergence (ER) was 0.830, 0.860, 0.620, 0.690 and 0.04 in the control group and at the nominal concentrations of 1.3, 2.5, 5 and 10 microg/l, respectively. The mean development rate (DR) for larvae was 0.089, 0.089, 0.090, 0.087 and 0.03 microg/l in the control and at the nominal concentrations of 1.3, 2.5, 5 and 10 microg/l, respectively. Statistical analysis of the mean emergence rate (ER) and the mean development rate (DR) data showed a significant difference (α=0.01) in both mean emergence rate and mean development rate between the control group and the highest nominal concentration of 10 microg/l at the end of the test period. No statistically significant differences were observed for either variable between the control group and the three lower concentrations of the test substance. Therefore it can be concluded that the 28 day NOEC of acetamiprid to the sediment dwelling life

stage of Chironomus riparius in a static sediment water system, is estimated to be 5 microg/l, and the 28 day LOEC was observed to be 10 microg/l.

Additional evaluation presented in the RAR for renewal:
In this study, the results are expressed in nominal concentrations in µg/L. Since the measured concentrations in the water column drop below 80 % of the nominal concentration and no measurements were performed in the sediment, the endpoint should be recalculated based on the geomean of the measured concentration. The metabolite (IM-1-4 is formed for 55%) is less toxic than the parent.
The course of the sediment -water concentration of acetamiprid is as follows:
One hour after test initiation analytical verification of the test concentrations in the overlaying dilution water showed the measured values were close to the nominal concentrations (92 – 100% recovery). Further on, the test concentrations on day 7 showed a reduction of the nominal test concentrations 1.3 and 2.5 microg/l below the limit of quantification (1 microg/l) and recoveries of 30 and 45% of the initial measured values observed at the higher nominal concentrations of 5 and 10 microg/l, respectively. Following 13 days exposure, 17% recovery of initial measured value was observed at the highest nominal concentration of 10 microg/l and recoveries at the three lower test concentrations were all below the limit of quantification. A final analytical verification at test termination (day 28) showed the recoveries from all of the nominal test concentrations were below the limit of quantification of 1 microg/l for the test substance under the conditions of the test. The Geomean is calculated based on measured concentrations :

Table 55: Measured concentrations of acetamiprid during the test

	measured test concentration (µg a.s./L)				
nominal test concentration (µg a.s./L)	1 h	7 days	13 days	28 days	
0	0	0	0	0	
1.3	1.3	<1	<1	<1	
2.5	2.5	<1	<1	<1	
5	4.6	1.4	<1	<1	
10	9.4	4.2	1.6	<1	

Calculation of the geometric mean measured concentration is not straightforward since several measured concentrations were below the LOQ of the method, which was 1 µg/L. Calculation of the DT50 for decline of the concentration of acetamiprid in the water phase is possible for the two highest concentrations, where 2 data points with concentrations >LOQ are available at a nominal concentration of 5 µg/L, and 3 data points with concentrations >LOQ at a nominal concentration of 10 µg/L. This was done by the RMS using Microsoft Excel, the rate constant k was derived as the slope of the regression curve when the natural logarithm of the concentration was plotted against time. The DT50, derived as ln(2)/k, was 97.6 and 122 hours at nominal concentrations of 5 and 10 µg/L, respectively. The corresponding regression curves were:
Nominal 10 µg/L: y = -0.0057X + 2.2911 (R2=0.9911)
Nominal 5 µg/L: y = -0.0071X + 1.5332 (R2=1, only 2 data points),
where Y = ln(concentration) and X is the time in hours

Using the above regression equations, estimations can be made for the concentrations which were below the LOQ of 1 µg/L, and for the concentration at t=0 hour. The resulting extrapolated concentrations are shown in the Table below (yellow shaded values).

Extrapolations at nominal concentrations of 2.5 and 1.3 µg/L will be based on the worst case DT50 of 97.6 hours. Extrapolation of this DT50 to lower concentrations of 1.3 and 2.5 µg/L is considered to be acceptable since there was no remarkable difference between the rate of dissipation at 5 and 10 µg/L, suggesting no clear concentration dependence within this range, and all nominal test concentrations were within a fairly narrow range (less than one order of magnitude). The corresponding regression curves are:
Nominal 2.5 µg/L: y = -0.0071X + 0.9234

Nominal 1.3 µg/L: y = -0.0071X + 0.2695,
where Y = ln(concentration) and X is the time in hours
The above intercepts b (0.9234 and 0.2695) were calculated by implementing the t=1 measured concentration in the regression equation y = -0.0071X + b.

The Table below presents the measured values combined with the extrapolated values calculated using the above procedure. The Table also presents the reported results for mean development rate and emergence rate. The overall geometric mean concentration was calculated according to the procedures outlined in Annex 6 of the OECD 211 guideline.

Table 56: Measured and extrapolated concentrations of acetamiprid during the test, and overall geomean concentration

nominal test concentration (µg a.s./L)	measured test concentration (µg a.s./L)						mean emergence rate	mean development rate
	0 h	1 h	7 days (168 h)	13 days (312 h)	28 days (672 h)	Geo-mean		
control	0	0	0	0	0	0	0.830	0.089
1.3	1.31	1.3	0.40	0.14	0.011	0.27	0.860	0.089
2.5	2.52	2.5	0.76	0.27	0.021	0.52	0.620	0.090
5	4.63	4.6	1.4	0.51	0.039	0.96	0.690	0.087
10	9.9	9.4	4.2	1.6	0.21	2.56	0.040*	0.030*

Yellow marked concentrations are extrapolated values (see text)
* Statistically significant (α = 0.01).

In reponse to data requirement 5.9, the notifier submitted a document in which the L(E)C10, L(E)C20 and NOEC values from 14 chronic/long-term/reproductive studies were calculated. For the current study the following was derived:

Table 57: Endpoints calculated by notifier based on nominal concentrations

Study #	**Author (year)**	**Organism**	**Test material**	**Units**	**Endpoint (duration)**	**Nominal/ measured**	**Stats method**	**LC/EC20**	**LC/EC10**	**NOEC**
RDII02191	McElligott (1999)	Midge (*Chironomus riparius*)	Acetamiprid	µg/L	Emergence (28 day)	nominal	Non-linear regr.	2.37	1.8	5
RDII02191	McElligott (1999)	Midge (*Chironomus riparius*)	Acetamiprid	µg/L	development (28 day)	nominal	Non-linear regr.	6.39	5.62	5

The endpoints derived by the notifier are based on the nominal concentrations. However, for classification purposes the endpoint based on geometric mean measured concentrations will be used. The NOEC is 0.96 µg/L for emergence rate and development rate. The EC10 and EC20 values based on the geometric mean measured concentrations were calculated by the RMS using Toxrat version 3.2. The results are shown in the table below. A statistically significant fit could not be obtained for emergence rate of males and females combined when using the data for all exposure groups. Therefore, the analysis was repeated after omission of the data for the second or third exposure group (0.52 or 0.96 µg a.s./L). A statistically significant fit was obtained for the reduced data set of control and 0.27, 0.52 and 2.56 µg a.s./L, but not for the reduced data set of control and 0.27, 0.96 and 2.56 µg a.s./L. Judging from the normalized width (NW) of the confidence intervals, the estimated EC10 and EC20 values for emergence rate (obtained for the reduced data set containing data for the control and 0.27, 0.52 and 2.56 µg a.s./L) are reliable (i.e. NW <0.5). The EC10 and EC20 values estimated for development rate for all larvae and for males are less reliable (NW ≥0.8).

A statistically significant fit could not be obtained for the development rate of females; using a reduced dataset did not provide a fit either. This is not crucial since emergence rate was the more sensitive parameter.

Table 58: Endpoints (µg a.s./L) calculated by RMS based on geometric mean measured concentrations

	EC10 (95% confidence interval)	**EC20 (95% confidence interval)**	**Statistical method**
Emergence rate			
Males + Females (All data)	0.257 -	0.391 -	Probit (fit not statistically significant)
	0.231 -	0.408 -	Weibull (fit not statistically significant)
Males + Females (reduced data set: control, 0.27, 0.52 and 2.56 µg a.s./L)	0.235 (0.183-0.283)	0.333 (0.275-0.389)	Probit (fit statistically significant)
Males + Females (reduced data set: control, 0.27, 0.96 and 2.56 µg a.s./L)	0.310 -	0.457 -	Probit (fit not statistically significant)
	0.331 -	0.533 -	Weibull (fit not statistically significant)
Development rate			
Males + Females	1.40 (0.80-2.45)	1.90 (1.26-2.86)	3-CDF (fit statistically significant)
Males	1.56 (0.37-6.89)	2.36 (0.74-7.92)	3-CDF (fit statistically significant)
Females	-	-	No fit could be obtained

Conclusions

In the RAR it is concluded that the lowest statistically significant EC10 and EC20 values are 0.235 and 0.333 µg a.s./L, and the NOEC 0.96 µg/L. Where EC10 values are available, they are preferred over NOEC values for the same endpoint (ECHA, 2015, OECD, 2006). The Dossier Submitter notes that the study setup contains sediment. Actual concentrations were determined in the water column, but not in the sediment. The measured concentrations in the water column dropped below 80% of the nominal concentrations during testing. It is therefore necessary to determine via which route exposure mainly occurred. For sediment no sorption constants were reported for acetamiprid. However, as discussed under section 11.3, acetamiprid does not strongly adsorb to soil with adsorption constants (K_{oc}) ranging 71 to 138 L/kg, i.e. log K_{oc} of 2.02 . Thus, while exposure via the sediment and/or food can occur, it is expected that it mainly occurred via overlaying/pore water. Overall, the results are considered reliable and will be used for classification purposes.Chronic toxicity to algae or other aquatic plants

One additional study on toxicity to algae was included in the renewal dossier for acetamiprid since the previous evaluation (EC, 2004). The descriptions below are based on the summaries in the RAR.

Aquatic toxicity to algae was tested by Suteau (1996) on *Scenedesmus subspicatus* in a 72 hours exposure according to OECD test guideline 201. The acetamiprid with a purity of 100% was tested with nominal concentrations of 1.9, 4.3, 9.4, 20.7, 45.5 and 100 mg/L in three replicates. At test initiation and test termination the exposure concentration were analysed and the concentrations were within 95 to 106% of nominal. The mean measured concentrations are 1.9, 4.3, 9.2, 20.6, 44.7 and 98.3 mg/L. Over the exposure period no inhibition of growth was observed and the NOEC, based on mean measured concentrations, is determined to be ≥98.3 mg/L. The test is considered reliable.

Hoberg (1997a) tested the toxicity to the blue-green algae *Anabaena flos-aquae* in a 5 day exposure according to the FIFRA test guideline 122-2 and 123-2. Acetamiprid with a purity of 99.9% was tested in only one nominal concentration of 1.0 mg/L in three replicates. The test concentration was analytically verified at day 0 and day 5 and was 1.3 mg/L (130% of nominal). The cell density was not significantly influenced and therefore the NOEC, based on mean measured concentrations, is determined to be ≥1.3 mg/L. The test is considered reliable and the NOEC will be used for classification purposes.

The effect on *Lemna gibba* was tested in a 14 day test by Hoberg (1997b) according to the FIFRA test guideline 122-2 and 132-2. The exposure concentration was only one mean measured concentration of 1.0 mg/L. The NOEC for frond density and biomass is determined to be ≥ 1.0 mg/L. Given the fact that the

endpoint is based on mean measured concentrations, the study is considered reliable and the NOEC will be used for classification purposes.

11.6.3 Chronic toxicity to other aquatic organisms

For the renewal dossier of acetamiprid an additional study on chronic aquatic toxicity to frogs was included (Anonimous, 2013). In this test, performed according to OECD test guideline 231, *Xenopus laevis* was exposed under flow-through conditions to three mean measured acetamiprid (purity 99.9%) concentrations of 0.24, 2.6 and 26 mg/L. The exposure lasted for 21 days, with four replicates each containing 20 tadpoles of 16 days post fertilization. The test concentrations were confirmed analytically after conditioning of the diluter and after 0, 7, 14 and 21 days. The measured concentration ranged from 59 to 109% of nominal. The mean measured concentrations are based on the samples from days 0, 7, 14 and 21. Snout-to-vent length was reduced in the highest exposure group, other parameters were not affected. On the basis of this a NOEC for non-thyroid specific toxicity of 2.6 mg/L was determined. This value can be considered for classification purposes.

11.7 Comparison with the CLP criteria

11.7.1 Acute aquatic hazard

For acetamiprid there are reliable acute data for all three trophic levels. The lowest endpoint for fish is 92 mg/L (*Cyprinodon variegatus)*, for invertebrates this is 20.7 µg/L (*Chironomus riparius)* and for algae and aquatic plants this is >1.0 mg/L (*Lemna gibba)*. The lowest value of 20.7 µg/L is below 1 mg/L (Table 4.1.0 (a) of the CLP guidance) therefore acetamiprid should be classified as Aquatic acute 1. This classification should be accompanied with an M-factor of 10 since the LC50 of 20.7 µg/L falls in the toxicity range of $0.01 < L(E)C50 \leq 0.1$.

11.7.2 Long-term aquatic hazard (including bioaccumulation potential and degradation)

The substance is not readily biodegradable based on a 28-day test for ready biodegradability (Anonymous, 1999c). According to the decision scheme on rapid degradability of the CLP guidance section II.4 (a) acetamiprid can be considered as not rapidly degradable.

Rapid degradation was observed in a surface water simulation test with half-lives of 3.9 and 8.5 days (Möndel, 2014). However, according to the Guidance on the Application of the CLP Criteria version 4.1, a substance can be considered rapidly degradable when it "is demonstrated to be ultimately degraded in a surface water simulation test with a half-life of < 16 days". Since a major metabolite is detected with increasing concentration up to 81.5% and 70.8%, ultimate degradation is not demonstrated. Furthermore, an aquatic sediment simulation study is available (McMillan-Staff and Austin, 2001, Jarvis and Montesano, 2014) but also in this case it is required that ultimate degradation should be demonstrated and the half-life should be <16 days, this requirements is not fulfilled by this test. The available data on abiotic processes, hydrolysis (Gomyo and Kobayashi, 1993) and photochemical degradation (Hausmann and Class, 1998), does not support a conclusion on rapid degradability.

Therefore, acetamiprid is considered not rapidly degradable and the chronic classification of acetamiprid will be based on the criteria for non-rapidly degradable substances. Acetamiprid is considered to have a low potential for bioaccumulation based on a log Pow value of 0.80 (measured).

Experimental chronic toxicity endpoints are available for all three trophic levels. The lowest endpoint for fish is a NOEC of 19.2 mg/L (*Pimephales promelas)*, for invertebrates this is an EC10 of 0.235 µg/L (*Chironomus riparius)* and for algae and aquatic plants this is ≥1.0 mg/L (*Lemna gibba)*. The lowest chronic endpoint for aquatic toxicity of 0.235 µg/L is lower than 0.1 mg/L (Table 4.1.0 (b) (ii) of the CLP guidance) therefore acetamiprid should be classified as Aquatic chronic 1. This classification should be accompanied

with an M-factor of 100 since the EC10 of 0.235 µg/L falls in the toxicity range of 0.0001 < ECx ≤ 0.001 for non-rapidly degradable substances.

11.8 CONCLUSION ON CLASSIFICATION AND LABELLING FOR ENVIRONMENTAL HAZARDS

The proposed classification for aquatic acute toxicity is Aquatic acute 1 with an M-factor of 10.

The proposed classification for aquatic chronic toxicity is Aquatic chronic 1 with an M-factor of 100.

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Acetamiprid, which belongs to the neonicotinoid insecticides, is currently classified as Aquatic Chronic 3 in Annex VI to the CLP Regulation. The DS proposed to classify the substance as Aquatic Acute Category 1 based on the 48h LC_{50} of 20.7 µg/L for *Chironomus riparius* and in Aquatic Chronic Category 1 based on the 28 day EC_{10} of 0.235 µg/L for *Chironomus riparius* and the substance being not rapidly degradable. The proposed M-factors were 10 (0.0207 mg/L ≤ 1 mg/L) for acute toxicity and 100 (0.0001 mg/L < 0.000235 mg/L ≤ 0.001 mg/L) for chronic toxicity.

Degradation

There was one ready biodegradability test available on acetamiprid (OECD TG 301B, GLP) showing 27% degradation after 28 days, which is below the CLP criteria of 70% after 28 days. Consequently, the DS considered the substance not readily biodegradable.

Hydrolysis of radiolabelled acetamiprid was tested in a GLP study in three buffer solutions of pH 4, 5, 7 and 9 and at temperatures of 22, 35 and 45 °C. The hydrolysis was performed in the dark. At pH 4, 5 and 7 the substance was stable at all test temperatures. At pH 9 the substance became instable at higher temperatures with half-lives of 52.9 days and 13 days for temperatures of 35 and 45 °C. The DS concluded that the substance is stable at environmentally relevant conditions.

In a water/sediment simulation study, radiolabelled acetamiprid was examined in a water-sediment system with sediment from two different locations (Manningtree and Ongar, Essex, UK) for 115 days mainly in the dark at a temperature of 20 °C. The overall recovery was 97.3% of the applied dose. According to the RAR (Volume 3, B8, November 2015), up to 14 days acetamiprid remained the main component of all chromatographic samples from both water and sediment. After this time, both IM-1-4 and IC-0 were the major metabolites. The reassessed (Focus Kinetics) geometric mean half-lives of 23.1 and 31.6 days were calculated for the Manningtree and Ongar whole system, respectively.

The aerobic degradation of radiolabelled acetamiprid in surface water from a natural pond was investigated (OECD TG 309, GLP). The degradation of two concentrations was tested. The total mass balance was between 97.5 and 101.6% of the applied radioactivity.

Acetamiprid declined to 6.5% and 11.5% of the applied dose in the low and high dosed systems, respectively. In all systems, one metabolite (IM-1-4) was detected with a concentration > 10% of the applied dose and the concentration of this metabolite increased to 81.5 and 70.8% in the low and high dose respectively over the 59 days of the incubation. Volatiles did not exceed 1% (≤ 0.57%). Re-evaluation of the half-lives resulted in half-lives of 2.4 and 6.8 days for the low and high dose respectively. The acute toxicity values in RAR (Volume I, November 2015) for the metabolite IM-1-4 were in the range of 10 to 100 mg/L for fish, Daphnia, Chironomus and *Mysidopsis bahia*. No algae data was available. According to the CAR, (Acetamiprid, Product type 18, August 2018) IM-1-4 was hydrolytically stable and there were not enough data available in the water/sediment study and the water degradation study to derive information about the biodegradation of IM-1-4. Based on this information the fulfilment of the criteria for classification as hazardous to the aquatic environment cannot be excluded.

Direct photochemical degradation was investigated with radiolabelled acetamiprid at 25 °C for 30 days with 12-hour light per day. The light intensity was 690 W/m^2 and wavelengths < 290 nm were filtered out. Recovery of the radiolabels was 99.5%. After 30 days, only 0.1-0.2% of the applied dose was retrieved from the volatile traps and 54% of the parent compound was still present in the test solutions. The half-live of the parent was calculated to be 34 days. Degradation products were detected.

Bioaccumulation

There was no fish bioconcentration study available. The measured log K_{ow} was 0.80 (shake flask method). Based on the available Log K_{ow} being below the CLP criterion of ≥ 4, the DS considered the substance to have a low potential for bioaccumulation.

Aquatic toxicity

Table 14: *Reliable aquatic toxicity tests on acetamiprid*

Method	Species	Test material	Results	Remarks	Reference
Fish					
OECD TG 203, GLP Static	*Oncorhynchus mykiss*	Acetamiprid 99.57%	96h LC_{50} > 100 mg/L (nominal)	Measured concentrations 101-104% of nominal (RI = 2)*	Anonymous (1997h)
Flow through toxicity study	*Lepomis macrochirus*	Acetamiprid > 99%	96h LC_{50} > 119.3 mg/L (mean measured)	Measured concentrations 93-99% of nominal (RI = 2)*	Anonymous (1997b)
FIFRA guideline 72-3, GLP Flow-through	*Cyprinodon variegatus*	Acetamiprid Purity: > 99.9%	96h LC_{50} 92 mg/L (mean measured)	Measured concentrations 88-102% of nominal (RI = 2)*	Anonymous (1998c)
OECD TG 210 Static, GLP	*Pimephales promelas*	Acetamiprid Purity: 100%	35d NOEC 19.2 mg/L (mean measured)	Measured concentrations 89-117% of nominal (RI = 2)*	Anonymous (1997g)

Invertebrates					
OECD TG 202 Static	*Daphnia magna*	Acetamiprid (NI-25) Purity: 99.9%	48h LC_{50} 49.8 mg/L (mean measured)	Measured concentrations 94-103% of nominal (RI = 2)*	Saika (1997)
ASTM guideline E-729 and FIFRA 72-2 Static, GLP	*Chironomus riparius*	Acetamiprid Purity: 99.3%	**48h LC_{50} 20.7 µg/L** (mean measured)	Measured concentrations 92-110% of nominal (RI=2)*	Putt (2003b)
OPPTS draft guideline 850-1020 Static, GLP	*Gammarus fasciatus*	Acetamiprid Purity: 99.3%	96h LC_{50} 100 µg/L (mean measured)	Measured concentrations 87-100% of nominal (RI = 2)*	Putt (2003a)
FIFRA 72-3 Guideline Flow-through, GLP	*Mysidopsis bahia*	Acetamiprid Purity: 99.9%	96h EC_{50} 66 µg/L (mean measured)	Measured concentrations 98-110% of nominal (RI = 2)*	Anonymous (1998b)
EPA/FIFRA guideline 72-4 Static renewal, GLP	*Daphnia magna*	Acetamiprid Purity: 99.9%	21d NOEC 5 mg/L (mean measured)	Measured concentrations 95-99% of nominal (RI = 2)*	Suteau (1997)
BBA guideline Static, GLP	*Chironomus riparius*	Acetamiprid Purity: 99.9%	**Recalculated: 28d NOEC 0.96 µg/L EC_{10} = 0.235 µg/L** (mean measured)	The study is considered reliable (RI = 2)*	Mc Elligott (1999)
Algae/Aquatic Plants					
OECD TG 201 Static, GLP	*Scenedesmus subspicatus*	Acetamiprid Purity: 100%	72h EC_{50} and NOEC > 98.3 mg/L (mean measured) no effects	Measured concentrations 95-106% of nominal (RI=2)*	Suteau (1996)
FIFRA guideline 122-2 and 123-3 Static, GLP	*Anabaena flos-aquae*	Acetamiprid (N-25) Purity: 99.9%	5d EC_{50} and NOEC > 1.3 mg/L (mean measured) One concentration tested, no effects	Measured concentration 130% of nominal (RI=2)*	Hoberg (1997a)
FIFRA guideline 122-2 and 132-3 Static, GLP	*Lemna gibba*	Acetamiprid Purity: 99.9%	14-d EC_{50} and NOEC > 1.0 mg/L (mean measured) One concentration tested, no effects	(RI = 2)*	Hoberg (1997b)

* Reliability according to Klimisch et al. (1997), since assessment is based on summaries in the DAR, Ri=1 is not given.

Acute toxicity

There were three acute toxicity studies available for fish, four for invertebrates and three for algae. The lowest acute toxicity value was a 48h LC_{50} of 20.7 µg/L 8 (mean measured) for *Chironomus riparius* in a static test. The nominal test concentrations were: 6.3, 13, 25, 50 and 100 µg/L. A total of 140 organisms (5 per replicate, 4 replicates per concentration) were exposed to five concentrations of the test substance, a dilution water control and a solvent control for 48 hours. A 1.0 mg/mL stock solution was prepared by placing 0.25180 g (0.25004 g as active ingredient) of acetamiprid in a 250 mL volumetric flask and bringing to volume with acetone (0.10 mL/L). Mortality was recorded at 0, 24 and 48 hours of exposure. Biological observations and observations of the physical characteristics of each replicate test solution were also made and recorded at 0, 24 and 48 hours. At test initiation and test termination, one sample was removed from each test solution and the control for analysis of test substance concentration. Analytical results showed a mean recovery from 92 to 110% (mean measured concentrations: 6.0, 14, 26, 46 and 110 µg/L). Results were expressed in mean measured concentrations, which is acceptable. The Dossier Submitter notes that the current study setup contains no sediment, thus exposure occurs only via the water.

Chronic toxicity

There was one chronic toxicity study available for fish, two for invertebrates and three for algae.

The lowest chronic toxicity value was from a 28-day static sediment/water system test on *Chironomus riparius.* The study was included in the DAR, but the endpoint has been recalculated because it was expressed as nominal concentration while concentrations drop below the limit of quantification (LOQ) at the end of the study. The test system included 3L glass beakers, 2 cm sediment layer (artificial sediment prepared according to OECD TG 207), and 2.5L reconstituted overlaying water. Samples from the water column were collected for analysis 1 hour, 7, 13 and 28 days after test initiation. No measurements were performed on the sediment. One hour after test initiation analytical verification of the test concentrations in the overlaying dilution water showed that the measured values were close to the nominal concentrations (92-100% recovery). Further on, the test concentrations on day 7 showed that at nominal concentrations 1.3 and 2.5 µg/L the measured concentrations were below the LOQ (1 µg/L). At the higher nominal concentrations of 5 and 10 µg/L, the recoveries were 30 and 45% of the initial measured values, respectively. Following 13 days of exposure, 17% recovery of initial measured value was observed at the highest nominal concentration of 10 µg/L and recoveries at the three lower test concentrations were all below the LOQ. A final analytical verification at test termination on day 28 showed that the recoveries from all of the test concentrations were below the LOQ under the conditions of the test. The results are expressed in nominal concentrations. It was concluded that the 28-day NOEC of acetamiprid for the sediment dwelling life stage of *Chironomus riparius* in a static sediment water system, is estimated to be 5 µg/L, and the 28-day LOEC was observed to be 10 µg/L (nominal concentrations). In the RAR, a recalculation based on the geomean of the measured concentrations was presented. Using the regression equations based on the calculation of the DT_{50} for decline of the concentration of acetamiprid in the water phase for the two highest concentrations, where 2 data points with concentrations below the LOQ are available, estimations were made for the concentrations which were below the LOQ of 1 µg/L. The lowest statistically significant EC_{10} and EC_{20} values were 0.235 and 0.333 µg a.s./L, and the

NOEC 0.96 µg/L. The DS notes that the study setup contains sediment. Actual concentrations were determined in the water column, but not in the sediment. However, acetamiprid does not strongly adsorb to soil. Thus, while exposure via the sediment and/or food can occur, it is expected that it mainly occurred via overlaying/pore water. Overall, the results are considered reliable by the DS.

Comments received during consultation

Comments were received from four Member States (MS). Three of them agreed with the proposed classification. One MS had comments relating to the aquatic toxicity tests. They wanted to have more precise information on the mean measured concentrations in the 21 day semi-static *Daphnia magna* NOEC (Suteau, 1997). The issue was clarified by the DS. The MS also had concerns regarding the use of kinetic regressions in the calculation of concentrations in the chronic *Chironomus* test. They also thought it useful to present endpoints using the standard geometric mean measured calculation for analytical periods and ½ the LOQ. This information would be relevant as endpoints using this method would be in the 0.001 to 0.01 mg/L classification range indicating M = 10. They also noted that using the valid acute toxicity to *Chironomus* endpoint and the surrogate approach would result in Aquatic Chronic 1, M = 10. The DS answered that geometric mean values calculated from ½ LOQ are 0.6, 0.7, 1.1 and 2.4 µg/L. An EC_{10} based on these values is not available and it should be noted that in the opinion of the DS these values overestimate the actual exposure concentration because at multiple timepoints the measured concentrations are below the LOQ. The DS is of the opinion that the EC_{10} of 0.235 µg/L together with a NOEC of 0.96 µg/L are sufficiently reliable and the most appropriate representation of the toxicity observed in this study. Consequently, they did not consider application of the surrogate approach to be necessary.

Assessment and comparison with the classification criteria

Degradation

RAC agrees with the Dossier Submitter to consider acetamiprid as 'not rapidly degradable', based on:

- 27% degradation in 28 days in a ready biodegradability test (OECD TG 301B).
- The substance was not ultimately degraded in the aerobic degradation test (OECD TG 309) in surface water from a natural pond but the half-lives were < 16 days. However, the fulfilment of the criteria for classification as hazardous to the aquatic environment of the degradation product cannot be excluded.
- The substance was hydrolytically stable at environmentally relevant conditions.
- Half-lives of 23.1 and 31.6 days were calculated from the water/sediment simulation test, metabolites were detected but not identified.

Bioaccumulation

There was no fish bioconcentration study available. Based on the measured log K_{ow} of 0.80, RAC agrees with the DS to consider acetamiprid as having a low potential for bioaccumulation.

Acute aquatic toxicity

There were acute toxicity data available for three trophic levels. The lowest acute toxicity value was a 48-h LC_{50} of 0.0207 mg/L (mean measured) for *Chironomus riparius* and RAC agrees with the DS that this value should be used for acute hazard classification.

Chronic aquatic toxicity

There were chronic toxicity data available for all three trophic levels. The lowest chronic toxicity value was from a 28 days static sediment/water system test on *Chironomus riparius.* Originally, it was concluded that the 28 days NOEC of acetamiprid to the sediment dwelling life stage of *Chironomus riparius* in a static sediment water system is estimated to be 0.005 mg/L, based nominal concentrations.

In the RAR, a recalculation based on the geomean of the measured concentrations was presented, as described above. The lowest statistically significant EC_{10} and EC_{20} values are 0.000235 and 0.000333 mg a.s./L, and the NOEC is 0.00096 mg/L. The study setup contains sediment but actual concentrations were only determined in the water column and not in the sediment.

RAC is of the opinion that original NOEC value based on nominal concentrations is not reliable for classification purposes. The same applies to the calculations based on the kinetic regression equations. According to ECHA Guidance on IR & CSR, Chapter R7b, for static tests, where the concentrations do not remain within 80-120% of nominal, the effect concentrations should be expressed relative to the geometric mean of the measured concentrations at the start and end of the test, in static tests.

There are also concerns related to the effect of sediment in the study results. In OECD TG 219 (sediment-Water Chironomid toxicity test) it is indicated as a minimum, samples of the overlying water, the pore water and the sediment must be analysed at the start (preferably one hour after application of test substance) and at the end of the test, at the highest concentration and a lower one. It further indicates that measurements in sediment might not be necessary if the partitioning of the test substance between water and sediment has been clearly determined in a water/sediment study under comparable conditions (e.g. sediment to water ratio, type of application, organic carbon content of sediment). In the case of acetamiprid, there were no information to make such a comparison. The chromatographic samples in the water-sediment test showed that sediment concentrations during the 28-day Chironomus test were likely to be significant. In conclusion, RAC does not consider the test results reliable and they will not be used for chronic hazard classification.

As *Chironomus riparius* is the most sensitive species under acute testing, RAC is of the opinion that in the absence of reliable chronic data for this species, the chronic classification should be based on the surrogate approach.

RAC agrees to use the 48h LC_{50} of 0.0207 mg/L for *Chironomus riparius* for short-term classification. Based on this acetamiprid warrants classification as Aquatic Acute 1 with an M-factor of 10 (0.01 mg/L < $L(E)C_{50}$ ≤ 0.1 mg/L).

The surrogate approach based on the 48h LC_{50} of 0.0207 mg/L for *Chironomus riparius* for a 'not rapidly degradable' substance indicates that acetamiprid warrants classification as Aquatic Chronic 1 with an M-factor of 10 (0.01 mg/L < $L(E)C_{50}$ ≤ 0.1 mg/L).

In conclusion, RAC disagrees with the DS for chronic classification and is of the opinion that

acetamiprid warrants classification as **Aquatic Acute 1 (H400), M=10 and Aquatic Chronic 1 (H410), M=10**.

The classification might have to be revisited in case of new information on chronic toxicity on invertebrates.

12 EVALUATION OF ADDITIONAL HAZARDS

Not evaluated in this dossier

13 ADDITIONAL LABELLING

[If relevant, please justify here the reason for supplemental hazard information in accordance with Annex II of the CLP Regulation.]

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15 ANNEXES

See separate documents for Annex 1 containing the evaluated study report of the developmental neurotoxicity study and the confidential annex that contains the full references of studies with vertebrate animals in case they were not available in the public literature.