Health Council of the Netherlands

N-fluoren-2-ylacetamide

Evaluation of the effects on reproduction, recommendation for classification

Gezondheidsraad
Health Council of the Netherlands

Aan de staatssecretaris van Sociale Zaken en Werkgelegenheid

Onderwerp : toezending advies *N-fluoreen-2-ylaceetamide*
Uw kenmerk : DGV/MBO/U-932542
Ons kenmerk : U-6770/HS/fs/543-E12
Bijlagen : 1
Datum : 19 oktober 2011

Geachte staatssecretaris,

Graag bied ik u hierbij het advies aan over de effecten van N-fluoreen-2-ylaceetamide op de vruchtbaarheid en het nageslacht; het betreft ook effecten die optreden na blootstelling via de borstvoeding. Dit advies maakt deel uit van een uitgebreide reeks waarin voor de voortplanting giftige stoffen worden geclassificeerd volgens richtlijnen van de Europese Unie. Het gaat om stoffen waaraan mensen tijdens de beroepsuitoefening kunnen worden blootgesteld.

Dit advies is opgesteld door een vaste commissie van de Gezondheidsraad, de Subcommissie Classificatie reproductietoxische stoffen. Het is vervolgens getoetst door de Beraadsgroep Gezondheid en omgeving van de raad.

Er zijn onvoldoende gegevens over de effecten van 2-AAF op de voortplanting. De subcommissie kan daarom geen aanbevelingen doen ten aanzien van classificatie voor zowel effecten op de vruchtbaarheid als op het nageslacht. 2-AAF wordt gebruikt als positieve controle in carcinogeniteits- en genotoxiciteitsproeven. De subcommissie constateert echter dat 2-AAF niet is geclassificeerd wat betreft zijn kankerverwekkende eigenschappen. Ik adviseer u dan ook de classificatie van 2-AAF op te nemen in het werkprogramma Classificatie van kankerverwekkende stoffen.

Ik heb dit advies vandaag ter kennisname toegezonden aan de staatssecretaris van Infrastructuur en Milieu en aan de minister van Volksgezondheid, Welzijn en Sport.

Met vriendelijke groet,

prof. dr. L.J. Gunning-Schepers,
voorzitter

Bezoekadres
Parnassusplein 5
2511 VX Den Haag
Telefoon (070) 340 70 04
E-mail: h.stouten@gr.nl

Postadres
Postbus 16052
2500 BB Den Haag
Telefax (070) 340 75 23
www.gr.nl

N-fluoren-2-ylacetamide

Evaluation of the effects on reproduction, recommendation for classification

Subcommittee on the Classification of Reproduction Toxic Substances,
a Committee of the Health Council of the Netherlands

to:

the State Secretary of Social Affairs and Employment

No. 2011/22, The Hague, October 19, 2011

The Health Council of the Netherlands, established in 1902, is an independent scientific advisory body. Its remit is "to advise the government and Parliament on the current level of knowledge with respect to public health issues and health (services) research..." (Section 22, Health Act).

The Health Council receives most requests for advice from the Ministers of Health, Welfare & Sport, Infrastructure & the Environment, Social Affairs & Employment, Economic Affairs, Agriculture & Innovation, and Education, Culture & Science. The Council can publish advisory reports on its own initiative. It usually does this in order to ask attention for developments or trends that are thought to be relevant to government policy.

Most Health Council reports are prepared by multidisciplinary committees of Dutch or, sometimes, foreign experts, appointed in a personal capacity. The reports are available to the public.

The Health Council of the Netherlands is a member of the European Science Advisory Network for Health (EuSANH), a network of science advisory bodies in Europe.

The Health Council of the Netherlands is a member of the International Network of Agencies for Health Technology Assessment (INAHTA), an international collaboration of organisations engaged with *health technology assessment*.

INAHTA

This report can be downloaded from www.healthcouncil.nl.

Preferred citation:
Health Council of the Netherlands. N-fluoren-2-ylacetamide; Evaluation of the effects on reproduction, recommendation for classification. The Hague: Health Council of the Netherlands, 2011; publication no. 2011/22.

all rights reserved

ISBN: 978-90-5549-855-0

Contents

Samenvatting *7*

Executive summary *8*

1 Scope *9*
1.1 Background *9*
1.2 Committee and procedure *9*
1.3 Effects on or via lactation *10*
1.4 Data *11*
1.5 Presentation of conclusions *11*
1.6 Final remark *11*

2 N-fluoren-2-ylacetamide *12*
2.1 Introduction *12*
2.2 Human studies *13*
2.3 Animal studies *13*
2.4 In vitro studies *18*
2.5 Conclusion *19*

References *21*

Annexes *25*
A The Committee *26*
B Regulation (EC) 1272/2008 of the European Community *28*
C Additional considerations to Regulation (EC) 1272/2008 *40*
D Fertility and developmental toxicity studies *42*

Samenvatting

In het voorliggende advies heeft de Gezondheidsraad N-fluoreen-2-ylaceetamide onder de loep genomen. N-fluoreen-2-ylaceetamide wordt vooral gebruikt in laboratoria als positieve controle in genotoxiciteits- en carcinogeniteitsstudies. Dit advies past in een reeks adviezen waarin de Gezondheidsraad op verzoek van de minister van Sociale Zaken en Werkgelegenheid de effecten van stoffen op de voortplanting beoordeelt. Het gaat vooral om stoffen waaraan mensen tijdens de beroepsuitoefening kunnen worden blootgesteld. De Subcommissie Classificatie reproductietoxische stoffen van de Commissie Gezondheid en beroepsmatige blootstelling aan stoffen (GBBS) van de raad, hierna aangeduid als de commissie, kijkt zowel naar effecten op de vruchtbaarheid van mannen en vrouwen als naar effecten op de ontwikkeling van het nageslacht. Bovendien worden effecten van blootstelling van de zuigeling via de moedermelk beoordeeld.

Op basis van Verordening (EG) 1272/2008 van de Europese Unie doet de commissie een voorstel voor classificatie. Voor N-fluoreen-2-ylaceetamide komt de commissie tot de volgende aanbevelingen:

- voor effecten op de fertiliteit adviseert de commissie om N-fluoreen-2-ylaceetamide niet te classificeren wegens onvoldoende geschikte gegevens
- voor effecten op ontwikkeling adviseert de commissie om N-fluoreen-2-ylaceetamide niet te classificeren wegens onvoldoende geschikte gegevens
- voor effecten tijdens de lactatie adviseert de commissie om N-fluoreen-2-ylaceetamide te kenmerken met H362 (*kan schadelijk zijn via de borstvoeding*).

Executive summary

In the present report, the Health Council of the Netherlands reviewed N-fluoren-2-ylacetamide. N-fluoren-2-ylacetamide is predominantly used in laboratories as a positive control in mutagenicity and carcinogenicity studies. This report is part of a series, in which the Health Council evaluates the effects of substances on reproduction, at the request of the Minister of Social Affairs and Employment. It mainly concerns substances to which man can be occupationally exposed. The Subcommittee on the Classification of Reproduction Toxic Substances of the Dutch Expert Committee on Occupational Safety (DECOS) of the Health Council, hereafter called the Committee, evaluates the effects on male and female fertility and on the development of the progeny. Moreover, the Committee considers the effects of a substance on lactation and on the progeny via lactation.

The Committee recommends classification according to Regulation (EC) 1272/2008 of the European Union. For N-fluoren-2-ylacetamide, these recommendations are:

- for effects on fertility, the Committee recommends not classifying N-fluoren-2-ylacetamide due to a lack of appropriate data
- for developmental toxicity, the Committee recommends not classifying N-fluoren-2-ylacetamide due to a lack of appropriate data
- for effects during lactation, the Committee recommends labelling N-fluoren-2-ylacetamide with H362 (*may cause harm to breast-fed children*).

Chapter 1

Scope

1.1 Background

As a result of the Dutch regulation on registration of compounds toxic to reproduction that came into force on 1 April 1995, the Minister of Social Affairs and Employment requested the Health Council of the Netherlands to classify compounds toxic to reproduction. This classification is performed by the Health Council's Subcommittee on the Classification of Reproduction Toxic Substances of the Dutch Expert Committee on Occupational Safety (DECOS). The classification is performed according to European Union Regulation (EC) 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures. The CLP guideline is based on the Globally Harmonised System of Classification and Labelling of Chemicals (GHS). The subcommittee's advice on the classification will be applied by the Ministry of Social Affairs and Employment to extend the existing list of compounds classified as reproductive toxicant (category 1A and 1B and 2) or compounds with effects on or via lactation.

1.2 Committee and procedure

This document contains the classification of N-fluoren-2-ylacetamide by the Health Council's Subcommittee on the Classification of Reproduction Toxic Substances, hereafter called the Committee. The members of the Committee are listed in Annex A. The classification is based on the evaluation of published

human and animal studies concerning adverse effects with respect to fertility and development as well as lactation of the above mentioned compound.

Classification for reproduction (fertility (F) and development (D)):	
Category 1	Known or presumed human reproductive toxicant (H360(F/D))
Category 1A	Known human reproductive toxicant
Category 1B	Presumed human reproductive toxicant
Category 2	Suspected human reproductive toxicant (H361(f/d))
No classification for effects on fertility or development	
Classification for lactation:	
	Effects on or via lactation (H362)
	No labelling for lactation

The classification and labelling of substances is performed according to the guidelines of the European Union (Regulation (EC)1272/2008) presented in Annex B. The classification of compounds is ultimately dependent on an integrated assessment of the nature of all parental and developmental effects observed, their specificity and adversity, and the dosages at which the various effects occur. The guideline necessarily leaves room for interpretation, dependent on the specific data set under consideration. In the process of using the regulation, the Committee has agreed upon a number of additional considerations (see Annex C).

In 2011, the President of the Health Council released a draft of the report for public review. No comments were received.

1.3 Effects on or via lactation

The recommendation for classifying substances for effects on or via lactation is also based on Regulation (EC) 1272/2008. The guideline defines that substances which are absorbed by women and have been shown to interfere with lactation or which may be present (including metabolites) in breast milk in amounts sufficient to cause concern for the health of a breastfed child, shall be classified and labelled. Unlike the classification of substances for fertility and developmental effects, which is based on hazard identification only (largely independent of dosage), the labelling for effects during lactation is based on risk characterization and therefore, it also includes consideration of the level of exposure of the breastfed child.

Consequently, a substance should be labelled for effects during lactation when it is likely that the substance would be present in breast milk at potentially toxic levels. The Committee considers a concentration of a compound as

potentially toxic to the breastfed child when this concentration leads to exceeding the exposure limit for the general population, e.g. the acceptable daily intake (ADI).

1.4 Data

Literature searches were conducted in the on-line databases XTOXLINE, MEDLINE, and CAPLUS, up to June 2009 without a starting date. An updating search in PubMed in January 2011 did not result in relevant additional information. Literature was selected primarily on the basis of the text of the abstracts. Publications cited in the selected articles, but not selected during the primary search, were reviewed if considered appropriate. In addition, handbooks and a collection of most recent reviews were consulted. References are divided in literature cited and literature consulted but not cited.

The Committee describes both human and animal studies in the text. The animal data are described in more detail in Annex D as well. Of each study the quality of the study design (performed according to internationally acknowledged guidelines) and the quality of documentation are considered.

1.5 Presentation of conclusions

The classification is given with key effects, species, and references specified. In case a substance is not classified as toxic to reproduction, one of two reasons is given:

Lack of appropriate data preclude assessment of the compound for reproductive toxicity

Sufficient data show that no classification for toxic to reproduction is indicated.

1.6 Final remark

The classification of compounds is based on hazard evaluation only (Niesink *et al*., 1995)[27], which is one of a series of elements guiding the risk evaluation process. The Committee emphasizes that for derivation of health-based occupational exposure limits these classifications should be placed in a wider context. For a comprehensive risk evaluation, hazard evaluation should be combined with dose-response assessment, human risk characterization, human exposure assessment, and recommendations of other organizations.

Chapter 2

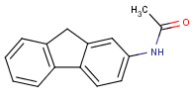
N-fluoren-2-ylacetamide

2.1 Introduction

N-fluoren-2-ylacetamide (2-AAF) is predominantly used in laboratories, since 2-AAF is used as a positive control in mutagenicity and carcinogenicity studies. Occupational exposure may occur in its production and use in laboratories. Chemists, chemical stockroom workers, and biomedical researchers have the greatest possibility of occupational exposure to 2-AAF. The primary routes of potential exposure to 2-AAF are inhalation and dermal contact. 2-AAF was intended for use as a pesticide, but was never marketed because of its carcinogenicity in experimental animals.[2,26] 2-AAF is not classified with respect to its carcinogenic properties in the EU (see Table 3.2. of Regulation (EC) 1272/2008) or the Netherlands. 2-AAF is considered a human carcinogen by the US National Toxicology Program (NTP).[2,26]

The identity and some of the physicochemical properties of 2-AAF are given below.

chemical name	:	N-fluoren-2-ylacetamide
CAS registry number	:	53-96-3
EC/EINECS number	:	200-188-6
synonyms	:	acetamide, N-9H-fluoren-2-yl-; 2-acetamidofluorene; N-2-fluorenylacetamide; 2-acetylaminofluorene; 2-AAF
colour and physical state	:	tan, crystalline powder

molecular weight	:	223.26
molecular formula	:	$C_{15}H_{13}NO$
structure	:	
melting point	:	193 °C
boiling point	:	303 °C
Log $P_{(octanol\text{-}water)}$	:	3.21 (estimated)
vapour pressure	:	1.26 x 10^{-5} Pa at 25°C (estimated)
solubility	:	slightly soluble in water (5.5 mg/L) (estimated)

Data from [1]

2.2 Human studies

Fertility studies

No data are available regarding the effects of exposure to 2-AAF on human fertility.

Developmental toxicity studies

No data are available regarding the effects of exposure to 2-AAF on development in humans.

Lactation

No data are available regarding the excretion of 2-AAF in breast milk or the effects of exposure to 2-AAF on infants during the lactation period.

2.3 Animal studies

Fertility and developmental toxicity studies with 2-AAF in laboratory animals are summarized in Annex D.

Fertility studies

Lafaurie investigated the hormonal status of rats during 2-AAF-induced hepatocarcinogenesis. Male rats (n=60) were administered 0.06% 2-AAF in a semi-synthetic diet deprived of protein and riboflavin to enhance the occurrence of liver cancer for 30 days (0.06% is equivalent to approximately 30 mg/kg bw/day, assuming 1 mg/kg food is equivalent to 20 mg/kg bw/day). Thereafter, treatment was continued by six periods of four weeks in which the animals received 0.06% 2-AAF in the semi-synthetic diet for two weeks followed by two weeks of semi-synthetic diet only. Control groups (n=60/group) receiving either a normal diet or the semi-synthetic diet were included. Four animals of each group were sacrificed 2, 4, 6, 9, 16, 30, 45, 60, 90, 120, 150, 180, and 210 days after the start of treatment. In the 2-AAF-treated groups, decreased body weight, which was attributed to the semi-synthetic diet, decreased absolute testis weight, reduced spermatogenic activity*, histological testicular lesions (e.g. near total loss of germinal epithelium, interstitial oedema, tubules containing only Sertoli cells, thickened basement membrane of tubules and tunica albuginea), and decreased testosterone levels were observed.[23]

The Committee remarks that the aim of the study was not to investigate the effects of 2-AAF on male fertility but to explore the relation between hormonal status and hepatocarcinogenesis. The Committee notes that the study did not include a group that was exposed to 2-AAF and fed a normal diet. Furthermore, the results of the end points measured varied considerably in the dosed group as well as in both control groups. Based on especially the unusual pattern found in serum testosterone levels in the group receiving a normal diet only, the Committee questions the reliability of the results. Overall, the Committee is of the opinion that due to the design and the variable results in all experimental groups no firm conclusions on the effects of 2-AAF on male fertility parameters can be drawn from this study.

In a subchronic study in rats, testicular effects were noted after exposure to 2-AAF. A group of 25 male rats was given a diet containing 0.06% 2-AAF for three weeks, followed by one week control diet (0.06% is equivalent to approximately 30 mg/kg bw/day, assuming 1 mg/kg food is equivalent to 20 mg/kg bw/day). This schedule was repeated three times. One group of five male rats was treated with 30 mg/kg bw/day 2-AAF for one period of three weeks only; a con-

* as evaluated by determining the percentage of stages VII and VIII of the cycle of the seminiferous epithelium by the greatest diameter of frontal sections of the testes

trol group of 27 males was included. At 55 weeks after start of the study, all surviving animals were sacrificed and subjected to complete necropsy and histopathology. In the group treated for 12 weeks, marked body weight depression and mortality (in 22/25 animals) were observed as well as testicular mesotheliomas (in 9/25), liver carcinomas (in 24/25; with extensive lung metastases in 8/25), cholangiosarcomas (in 5/25), and Zymbal gland carcinomas (no incidence presented). No tumours, mortality, or effects on body weight were seen in the other two groups.[6]

Administration (gavage) of doses of 2-AAF (in corn oil or olive oil) ranging from 110 to 1010 mg/kg bw/day, for three to five days, did not increase the proportion of morphological abnormalities in sperm obtained from five to nine CD-1, five HA/ICR, or seven or ten $B6C3F_1$ mice approximately five weeks after the last treatment.[16,30,31]

Treatment (gavage) with doses of 2-AAF (in trioctanoin) of 5 or 10 mg/kg bw/day, five days/week, for eight weeks, did not affect sperm morphology in 16 F344 rats (sampling time: 2.5 weeks after the last treatment).[5,30]

In a dominant lethal assay, male F344 rats were given doses of 2-AAF (in trioctanoin) of 0, 25, or 50 mg/kg bw/day by gavage for five days, followed by 0, 12.5, or 25 mg/kg bw/day, five days/week, for 18 days. The day following the last treatment, 15 males per group were each caged with two nulliparous females for two consecutive five-day post-treatment mating intervals. Nineteen days after the first day of the mating intervals, all females were sacrificed and their uterine contents were examined. No effects were observed on the mean numbers of implantations and non-viable and viable implantations per female, on the frequencies of females with one or more or with two or more non-viable implantations, nor on the mean foetal death rate and the percentage foetal lethality. However, decreases in the percentages of females with implantations after the first mating trials were seen in both dose groups (low dose: 7/30; high dose 0/8; controls: 17/30). Because of severe body weight loss after the first treatment week, doses were lowered by one half, while after the fourth treatment week, high-dose animals began to die from apparent liver toxicity. During the two-week mating trials, 4/18 and 14/18 males of the low- and high-dose group, respectively, died. At necropsy on the 31th day after the last treatment of 10 low-dose animals and the four surviving high-dose animals, especially histological lesions in the bladder were reported. In an additional experiment, rats were given 40 daily doses of 5 mg/kg bw/day and 32-35 and 36-40 daily doses of 10 mg/kg bw/day (five per

week). Treatment did not affect the mean numbers of implantations and non-viable and viable implantations per female, the frequencies of females with one or more or with two or more non-viable implantations, nor the mean foetal death rate and the percentage foetal lethality. However, decreased percentages of females with implantations were observed when they were mated with males immediately after they had received 32 doses or more of 10 mg/kg bw.[4]

Male B6D2F_1 mice (n=30/group) were given 350 or 700 mg 2-AAF/kg bw by gavage (in olive oil) and were caged immediately thereafter with non-treated virgin females according to the above described schedule. Females were sacrificed around the 13th day of pregnancy and the number of corpora lutea and dead and living implantations were counted. Treatment did not cause differences in the frequencies of fertile matings, females with one or more dead implantations and dominant lethal mutations, nor in the numbers of corpora lutea, implantations, and living implantations per female. Mortality was reported for 6/30 males given 700 mg 2-AAF/kg bw.[33]

Intraperitoneal

In an *in vivo* sperm morphology assay, male (C3H x C57BL6)F_1 mice (n=6) were given intraperitoneal injections of 2-AAF (in DMSO/water) at 0, 125, 250, or 500 mg/kg bw/day for five days. Absolute testis weights were decreased at the high dose and a dose-related increase in sperm abnormalities (not further specified) was noted at all dose levels.[29]

No increases in sperm head abnormalities were seen in male (CBA x BALB/c)F_1 mice following five daily intraperitoneal injections of 2-AAF (in 0.5% Tween 80 in water) of 0, 100, 250 500, 750, 1000, or 1500 mg/kg bw/day. Mortality was observed at the three highest doses.[35]

Following intraperitoneal injections of doses of 2-AAF (in DMSO) of 220 to 1320 mg/kg bw for three or five days, dose-related increases were found in the percentage of abnormal sperm obtained from two to ten B6C3F_1 or B6D2F_1 mice (sampling time: five weeks after last treatment).[3,30,32]

In a dominant lethal assay, male Swiss mice (n=60-78/group) were treated with a single intraperitoneal injection (in DMSO) of 56, 112, or 167 mg 2-AAF/kg bw. An untreated control and a solvent control group were included. Approximately 24 hours after treatment, each male was paired with one untreated virgin female.

The females were replaced with new ones at four-day intervals for subsequent matings. Females were sacrificed 17 to 18 days from their initial caging with the males, and their uterine contents were evaluated for live foetuses and dead implantations (early and late death). No differences were found in the frequencies of dead implantations, females with dead implantations and dominant lethal mutations, nor in the mean numbers of implantations and dead and living implantations.[7]

When (101/E1 x C3H/E1)F_1 mice (n=45/group) were similarly treated with 300 or 600 mg 2-AAF/kg bw (in DMSO), no significant differences were seen for the frequencies of fertile matings, dead implants, and dominant lethal mutations and for the numbers of corpora lutea, implantations, and living implantations per female in the two dose groups. Slight toxicity (not specified) was seen at 300 mg/kg bw, but injection of 600 mg/kg caused mortality in 47% of the animals. The low mating frequency during the first interval was considered the result of the toxicity of 2-AAF.[8]

In a dominant lethal test with females, T-stock and (C3H x C57BL)F_1 animals (n=41-48/group) were treated with single intraperitoneal injections (in DMSO) of 1500 mg 2-AAF/kg bw and mated during post-treatment days 0.5-4.5 or 5.5-9.5. Females were sacrificed for uterine examination 17 days after mating. Treatment did not affect any of the end points examined (frequencies of dead implants and of females with one or more dead implants; numbers of implantations, living and dead implantations per female).[15]

Developmental studies

Izumi treated groups of 10-12 pregnant mice with single intraperitoneal injections of 100 mg/kg bw of 2-AAF in propylene glycol on single days during gestational day 8 to 15. Two control groups receiving no injection or propylene glycol, respectively, were included. The animals were killed on gestational day 18. No information was provided on maternal toxicity. Treatment may have induced decreased foetal body weights, increased resorptions, and skeletal malformations and variations.[20,21]

Due to poor reporting – articles were in Japanese with only summary and tables in English – and design, however, the Committee could not evaluate this study properly.

Lactation

When rats were intraperitoneally injected with doses of 2-AAF of 0.2 mmol/kg bw/day (45 mg/kg bw/day) on five or six days between lactational days five to 17, 0.05 to 0.13% of the dose administered was excreted in 1 mL of milk sampled four to five hours after the third, fourth, fifth, and sixth injection. The milk contained both parent compounds and metabolites.[25] Malejka-Giganti *et al*. very briefly reported that treatment of lactating rats with ten intraperitoneal 2-AAF injections at total doses of 0.2 or 0.3 mmol/kg bw (45 or 67 mg/kg bw) induced tumour incidences in the suckling young of 12/29 (total tumour number: 16) and 11/36 (total tumour number: 26), respectively, with mean latency periods of 15.7 and 12.1 month, respectively. In female offspring, there were mainly mammary tumours, 28% of which were malignant; in male offspring, ear duct tumours and occasionally osteogenic sarcomas and lung adenocarcinomas. In the controls, 5/24 rats had benign mammary tumours with a latency period of 17 months.[24,25]

2.4 *In vitro* studies

The capacity of 2-AAF and its metabolites to produce malformations was investigated in a series of *in vitro* experiments using cultured whole rat embryos. The results indicated that metabolic, cytochrome P450-dependent activation was required to induce malformations which included defects of the anterior neural tube (incomplete or lack of closure; misalignment) and of the prosencephalon (hypoplasia). None of the metabolites, tested without metabolic activation, produced this same spectrum of malformations. Neural tube defects were caused only by 7-hydroxy-AAF, but 3-, 5-, and 9-hydroxy-AAF, which were not or hardly active, may contribute to these defects. Prosencephalon defects (hypoplasia; ventrolateral protrusion) were induced by N-hydroxy-AF and N-acetoxy-AAF. N-hydroxyaminofluorene, N-O-sulfonoxy-2-AAF, and nitrosofluorene caused axial rotation (flexure) defects, which were rarely induced by bioactivated 2-AAF.[9,10-12,17-19,22,34]

When tested in the Frog Embryo Teratogenesis Assay – *Xenopus Iaevis* (FETAX), 2-AAF induced malformations such as pericardial oedema, craniofacial maldevelopment, skeletal kinking, and microencephaly. Generally, effects occurred at lower concentrations and were more severe when a metabolic system had been added.[13,14,28]

The Committee notes that the relevance of these *in vitro* tests for the *in vivo* situation is limited.

2.5 Conclusion

Fertility

No studies are available regarding the effects of exposure to 2-AAF on human fertility.

No studies are available regarding the effects of exposure to 2-AAF on functional fertility in laboratory animals. In a study designed to explore the relation between hormonal status and hepatocarcinogenesis, effects on testes, spermatogenesis, and serum testosterone levels were found in rats given 2-AAF in a synthetic diet deprived of protein and riboflavin to enhance liver cancer daily for 30 days and intermittently for another 24 weeks.[23] Due to the design and the variable results in all experimental groups, the Committee could not evaluate the results of this study properly. There were no effects on sperm morphology in mice[16,30,31] or rats[5,30] given 2-AAF by gavage for three to five days or eight weeks, respectively. Intraperitoneal sperm morphology assays were positive in mice[3,30,32] and conflicting in rats[29,35]. Oral dominant lethal assays in rats[4] and mice[4,33] were negative.

Overall, the Committee proposes not to classify 2-AAF for effects on fertility due to a lack of appropriate human and animal data

Developmental toxicity

No studies are available regarding the effects of exposure to 2-AAF on development in humans.

No valid studies are available regarding the effects of 2-AAF on development in laboratory animals.

Overall, the Committee proposes not to classify 2-AAF for effects on development due to a lack of appropriate human and animal data.

Lactation

The Committee notes that 2-AAF is used in laboratories as a positive control in carcinogenicity and mutagenicity studies. Oral (diet) administration of 2-AAF for 12 weeks to rats induced liver tumours as well as testicular mesotheliomas, extensive lung metastases, cholangiosarcomas, and Zymbal gland carcinomas.[6] Intraperitoneal administration to lactating rats resulted in increased incidences of mammary tumours in female offspring and ear duct tumours and occasionally osteogenic sarcomas and lung adenocarcinomas in male offspring later in life.[24] Experiments in which 2-AAF was intraperitoneally injected into lactating rats showed the presence of 2-AAF and its metabolites in breast milk.[25] The amounts excreted may have been relatively small and the intraperitoneal route may be considered less relevant. The Committee assumes that occupational exposure to 2-AAF leads to internal exposure and to the presence of 2-AAF and its metabolites in breast milk. Since the Committee is of the opinion that the excretion of these genotoxic carcinogenic compounds in breast milk should be avoided, it, therefore, proposes to label 2-AAF for effects during lactation.

Proposed classification for fertility

Lack of appropriate data precludes the assessment of N-fluoren-2-ylacetamide (2-AAF) for effects on fertility.

Proposed classification for developmental toxicity

Lack of appropriate data precludes the assessment of N-fluoren-2-ylacetamide (2-AAF) for effects on development.

Proposed labelling for effects during lactation

H362.

References

1 2-Acetylaminofluorene. ChemIDplus Advanced, http://chem.sis.nlm.nih.gov/chemidplus/jsp/common/PhysicalProperties.jsp

2 2-acetylaminofluorene, CASNR: 53-96-3. HSDB online: http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?./temp/~eUW4mV:1

3 Arany J, Erna J. Murine sperm-shape abnormality assay. In: Ashby J, editor. Evaluation of short-term tests for carcinogens. Report of the IPCS Collaborative Study on in vivo Assays. New York: Press Syndicate of the University of Cambridge 1988; 2.251-2.253.

4 Bishop JB, Teaf CM, Campbell VE. Assessment of foetal death among in utero progeny of Fischer 344 rats after gavage treatment of males with 2-acetylaminofluorene or 4-acetylaminofluorene. In: Ashby J, editor. Evaluation of short-term tests for carcinogens. Report of the IPCS Collaborative Study on in vivo Assays. New York: Press Syndicate of the University of Cambridge 1988; 2.215-2.224.

5 Bishop JB, Teaf CM, Salamone MF. Assessment for abnormality in head shape of epidiymal sperm from Fischer 344 male rats treated by gavage with 2-acetylaminofluorene and 4-acetylaminofluorene. In: Ashby J, editor. Evaluation of short-term tests for carcinogens. Report of the IPCS Collaborative Study on in vivo Assays. New York: Press Syndicate of the University of Cambridge 1988; 2.258-2.260.

6 Cabral JRP, Neal GE. Testicular Mesotheliomas in rats exposed to N-2-fluorenylacetamide (FAA). Tumori 1983; 69: 195-199.

7 Chauhan PS, Aravindakshan M, Sundaram K. Dominant lethal test in Swiss male mice. In: Ashby J, editor. Evaluation of short-term tests for carcinogens. Report of the IPCS Collaborative Study on in vivo Assays. New York: Press Syndicate of the University of Cambridge 1988; 2.194-2.201.

8 Ehling UH. Dominant lethal studies in male mice. In: Ashby J, editor. Evaluation of short-term tests for carcinogens. Report of the IPCS Collaborative Study on in vivo Assays. New York: Press Syndicate of the University of Cambridge; 1988: 2.202-2.207.

9 Faustman-Watts E, Greenaway JC, Namkung MJ, Fantel AG, Juchau MR. Teratogenicity in vitro of 2-acetylaminofluorene: role of biotransformation in the rat. Teratology 1983; 27(1): 19-28.

10 Faustman-Watts EM, Giachelli CM, Juchau MR. Carbon monoxide inhibits monooxygenation by the conceptus and embryotoxic effects of proteratogens in vitro. Toxicol Appl Pharmacol 1986; 83(3): 590-595.

11 Faustman-Watts EM, Namkung MJ, Greenaway JC, Juchau MR. Analysis of metabolites of 2-acetylaminofluorene generated in an embryo culture system. Relationship of biotransformation to teratogenicity in vitro. Biochem Pharmacol 1985; 34(16): 2953-2959.

12 Faustman-Watts EM, Namkung MJ, Juchau MR. Modulation of the embryotoxicity in vitro of reactive metabolites of 2-acetylaminofluorene by reduced glutathione and ascorbate and via sulfation. Toxicol Appl Pharmacol 1986; 86(3): 400-410.

13 Fort DJ, James BL, Bantle JA. Evaluation of the developmental toxicity of five compounds with the frog embryo teratogenesis assay: Xenopus (FETAX) and a metabolic activation system. J Appl Toxicol 1989; 9(6): 377-388.

14 Fort DJ, Rogers RL, Stover EL, Finch RA. Optimization of an exogenous metabolic activation system for FETAX. I. Post-isolation rat liver microsome mixtures. Drug Chem Toxicol 2001; 24(2): 103-115.

15 Generoso WM, Cain KT, Hughes LA, Braden PW. Test for induction of presumed dominant-lethal effects in female mice. In: Ashby J, editor. Evaluation of short-term tests for carcinogens. Report of the IPCS Collaborative Study on in vivo Assays. New York: Press Syndicate of the University of Cambridge 1988; 2.189-2.193.

16 Harper BL, Legator MS. Sperm morphology test results for benzo(a)pyrene, pyrene, 2-acetylaminofluorene, and 4-acetylaminofluorene in ICR Swiss mice. In: Ashby J, editor. Evaluation of short-term tests for carcinogens. Report of the IPCS Collaborative Study on in vivo Assays. New York: Press Syndicate of the University of Cambridge 1988; 2.254-2.257.

17 Harris C, Hiranruengchok R, Lee E, Berberian RM, Eurich GE. Glutathione status in chemical embryotoxicity: synthesis, turnover and adduct formation. Toxicology In Vitro 1995; 623-631.

18 Harris C, Stark KL, Juchau MR. Glutathione status and the incidence of neural tube defects elicited by direct acting teratogens in vitro. Teratology 1988; 37(6): 577-590.

19 Harris C, Stark KL, Luchtel DL, Juchau MR. Abnormal neurulation induced by 7-hydroxy-2-acetylaminofluorene and acetaminophen: evidence for catechol metabolites as proximate dysmorphogens. Toxicol Appl Pharmacol 1989; 10(3): 432-446.

20 Izumi T. Congenital skeletal abnormalities in offspring of mice induced by administration of 2-acetylaminofluorene during pregnancy. Acta Anat Nippon 1962; 37: 250-259.

21 Izumi T. Developmental anomalies in offspring of mice induced by administration of 2-acetylaminofluorene during pregnancy. Acta Anat Nippon 1962; 37: 239-249.

22 Juchau MR. Bioactivation in chemical teratogenesis. Annu Rev Pharmacol Toxicol 1989; 29: 165-187.

23 Lafaurie CA, Castelli D, Krebs B, Stora C. Sex steroid hormones levels and testicular activity during hepatocarcinogenesis by N-2-fluorenylacetamide. Res Commun Chem Pathol Pharmacol 1982; 37: 49-63.

24 Malejka-Giganti D, Decker RW, Rydell RE. Metabolites of N-2-fluorenylacetamide (2-AAF) excreted in milk of lactating rats and the carcinogenicity for suckling young. Proc Am Assoc Cancer Res 1983; 24: 76.

25 Malejka-Giganti D, Magat WJ, Adelmann AM, Decker RW. Metabolite profile in milk of lactating rats after treatment with a carcinogen, N-2-fluorenylacetamide. Drug Metab Dispos 1987; 15: 760-766.

26 National Toxicology Program. 2-Acetylaminofluorene, CAS No. 53-96-3. Report of Carcinogens, Eleventh Edition, http://ntp.niehs.nih.gov/ntp/roc/eleventh/profiles/s002acet.pdf

27 Niessink R, de Vries J, Hoolinger M. Toxicology principles and applications. Boca Raton, FL, USA: CRC Press 1995.

28 Propst TL, Fort DJ, Stover EL, Schrock B, Bantle JA. Evaluation of the developmental toxicity of benzo[alpha]pyrene and 2-acetylaminofluorene using Xenopus: modes of biotransformation. Drug Chem Toxicol 1997; 20(1&2): 45-61.

29 Quinto I, De ME. Evaluation of Propineb, a dithiocarbamate pesticide, in the mouse-sperm morphology assay. Mutat Res 1983; 124(3-4): 235-240.

30 Salamone MF. Summary report on the performance of the sperm assays. In: Ashby J, editor. Evaluation of short-term tests for carcinogens. Report of the IPCS Collaborative Study on in vivo Assays. New York: Press Syndicate of the University of Cambridge 1988; 2.229-2.234.

31 Salamone MF, Chiu S, Logan DM. Abnormal sperm test results for benzo(a)pyrene, pyrene, 2-acteylaminofluorene, and 4-acetylaminofluorene using both hybrid and outbred mice II. Gavage treatment. In: Ashby J, editor. Evaluation of short-term tests for carcinogens. Report of the IPCS Collaborative Study on in vivo Assays. New York: Press Syndicate of the University of Cambridge 1988; 2.243-2.250.

32 Salamone MF, Logan DM. Abnormal sperm test results for benzo(a)pyrene, pyrene, 2-acteylaminofluorene, and 4-acetylaminofluorene using hybrid mice I. Intraperitoneal treatment. In: Ashby J, editor. Evaluation of short-term tests for carcinogens. Report of the IPCS Collaborative Study on in vivo Assays. New York: Press Syndicate of the University of Cambridge 1988; 2.235-2.242.

33 Shibuya T, Miyamae Y, Murota T. Mouse dominant lethal test on four chemicals. In: Ashby J, editor. Evaluation of short-term tests for carcinogens. Report of the IPCS Collaborative Study on in vivo Assays. New York: Press Syndicate of the University of Cambridge 1988; 2.208-2.214.

34 Stark KL, Harris C, Juchau MR. Modulation of the embryotoxicity and cytotoxicity elicited by 7-hydroxy-2-acetylaminofluorene and acetaminophen via deacetylation. Toxicol Appl Pharmacol 1989; 97(3): 548-560.

35 Topham JC. Do induced sperm-head abnormalities in mice specifically identify mammalian mutagens rather than carcinogens? Mutat Res 1980; 74(5): 379-387.

Literature consulted but not cited

Beckman DA, Fawcett LB, Brent RL. The effects of maternal drugs on the developing fetus. In: Avery GB, Fletcher MA, MacDonald MG, editors. Neonatology: pathophysiology and management of the newborn, 5th edition. Philadelphia PA, USA: Lippincott Willimas & Wilkins; 1999. p 209-36.

Berry DL, Zachariah PK, Namkung MJ, Juchau MR. Transplacental induction of carcinogen-hydroxylating systems with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Appl Pharmacol 1976; 36: 569-84.

Derewlany LO, Koren G. Biotransformation of carcinogenic arylamines and arylamides by human placenta. J Lab Clin Med 1994; 124: 134-41.

Macina OT, Sussman NB, Grant SG, Thomas CA, Mattison DR, Woodruff T, *et al*. Computational evaluation of hazardous air pollutants for developmental toxicity using a structure activity approach. SOT 2000 Annual Meeting 2000; 298.

A The Committee

B Regulation (EC) 1272/2008 of the European Community

C Additional considerations to Regulation (EC) 1272/2008

D Fertility and developmental toxicity studies

Annexes

Annex **A**

The Committee

- A.H. Piersma, *chairman*
  Professor of Reproductive and Developmental Toxicology, Utrecht University, Utrecht; National Institute of Public Health and the Environment, Bilthoven
- D. Lindhout
  Professor of Medical Genetics, Paediatrician (not practizing), Clinical Geneticist, University Medical Centre, Utrecht
- N. Roeleveld
  Reproductive Epidemiologist, Radboud University Nijmegen Medical Centre, Nijmegen
- J.G. Theuns-van Vliet
  Reproductive Toxicologist, TNO Triskelion BV, Zeist
- D.H. Waalkens-Berendsen
  Reproductive Toxicologist, Zeist
- P.J.J.M. Weterings
  Toxicologist, Weterings Consultancy BV, Rosmalen
- A.S.A.M. van der Burght, *scientific secretary*
  Health Council of the Netherlands, Den Haag
- J.T.J. Stouten, *scientific secretary*
  Health Council of the Netherlands, Den Haag

The first draft of this report was prepared by drs. I.A. van de Gevel, from the Regulatory Affairs Department of NOTOX BV (Den Bosch, the Netherlands), by contract with the Ministry of Social Affairs and Employment

The Health Council and interests

Members of Health Council Committees are appointed in a personal capacity because of their special expertise in the matters to be addressed. Nonetheless, it is precisely because of this expertise that they may also have interests. This in itself does not necessarily present an obstacle for membership of a Health Council Committee. Transparency regarding possible conflicts of interest is nonetheless important, both for the President and members of a Committee and for the President of the Health Council. On being invited to join a Committee, members are asked to submit a form detailing the functions they hold and any other material and immaterial interests which could be relevant for the Committee's work. It is the responsibility of the President of the Health Council to assess whether the interests indicated constitute grounds for non-appointment. An advisorship will then sometimes make it possible to exploit the expertise of the specialist involved. During the inaugural meeting the declarations issued are discussed, so that all members of the Committee are aware of each other's possible interests.

Annex **B**

Regulation (EC) 1272/2008 of the European Community

3.7 Reproductive toxicity

3.7.1 Definitions and general considerations

3.7.1.1 Reproductive toxicity includes adverse effects on sexual function and fertility in adult males and females, as well as developmental toxicity in the offspring. The definitions presented below are adapted from those agreed as working definitions in IPCS/EHC Document No 225, Principles for Evaluating Health Risks to Reproduction Associated with Exposure to Chemicals. For classification purposes, the known induction of genetically based heritable effects in the offspring is addressed in Germ Cell Mutagenicity (section 3.5), since in the present classification system it is considered more appropriate to address such effects under the separate hazard class of germ cell mutagenicity.

In this classification system, reproductive toxicity is subdivided under two main headings:

(a) adverse effects on sexual function and fertility;

(b) adverse effects on development of the offspring.

Some reproductive toxic effects cannot be clearly assigned to either impairment of sexual function and fertility or to developmental toxicity. Nonetheless, substances with these effects, or mixtures containing them, shall be classified as reproductive toxicants.

3.7.1.2 For the purpose of classification the hazard class Reproductive Toxicity is differentiated into:

- adverse effects
  - on sexual function and fertility, or
  - on development;
- effects on or via lactation.

3.7.1.3 Adverse effects on sexual function and fertility

Any effect of substances that has the potential to interfere with sexual function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive cycle normality, sexual behaviour, fertility, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems.

3.7.1.4 Adverse effects on development of the offspring

Developmental toxicity includes, in its widest sense, any effect which interferes with normal development of the conceptus, either before or after birth, and resulting from exposure of either parent prior to conception, or exposure of the developing offspring during prenatal development, or postnatally, to the time of sexual maturation. However, it is considered that classification under the heading of developmental toxicity is primarily intended to provide a hazard warning for pregnant women, and for men and women of reproductive capacity. Therefore, for pragmatic purposes of classification, developmental toxicity essentially means adverse effects induced during pregnancy, or as a result of parental exposure. These effects can be manifested at any point in the life span of the organism. The major manifestations of developmental toxicity include (1) death of the developing organism, (2) structural abnormality, (3) altered growth, and (4) functional deficiency.

3.7.1.5 Adverse effects on or via lactation are also included in reproductive toxicity, but for classification purposes, such effects are treated separately (see Table 3.7.1 (b)). This is because it is desirable to be able to classify substances specifically for an adverse effect on lactation so that a specific hazard warning about this effect can be provided for lactating mothers.

3.7.2 Classification criteria for substances

3.7.2.1 Hazard categories

3.7.2.1.1 For the purpose of classification for reproductive toxicity, substances are allocated to one of two categories. Within each category, effects on sexual function and fertility, and on development, are considered separately. In addition, effects on lactation are allocated to a separate hazard category.

Table 3.7.1(a) Hazard categories for reproductive toxicants.

Categories		Criteria
CATEGORY 1		Known or presumed human reproductive toxicant Substances are classified in Category 1 for reproductive toxicity when they are known to have produced an adverse effect on sexual function and fertility, or on development in humans or when there is evidence from animal studies, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with reproduction in humans. The classification of a substance is further distinguished on the basis of whether the evidence for classification is primarily from human data (Category 1A) or from animal data (Category 1B).
	Category 1A	Known human reproductive toxicant The classification of a substance in Category 1A is largely based on evidence from humans.
	Category 1B	Presumed human reproductive toxicant The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate.
CATEGORY 2		Suspected human reproductive toxicant Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects.

Table 3.7.1(b) Hazard category for lactation effects.

EFFECTS ON OR VIA LACTATION
Effects on or via lactation are allocated to a separate single category. It is recognised that for many substances there is no information on the potential to cause adverse effects on the offspring via lactation. However, substances which are absorbed by women and have been shown to interfere with lactation, or which may be present (including metabolites) in breast milk in amounts sufficient to cause concern for the health of a breastfed child, shall be classified and labelled to indicate this property hazardous to breastfed babies. This classification can be assigned on the: (a) human evidence indicating a hazard to babies during the lactation period; and/or (b) 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; and/or (c) absorption, metabolism, distribution and excretion studies that indicate the likelihood that the substance is present in potentially toxic levels in breast milk.

3.7.2.2 Basis of classification

3.7.2.2.1 Classification is made on the basis of the appropriate criteria, outlined above, and an assessment of the total weight of evidence (see 1.1.1). Classification as a reproductive toxicant is intended to be used for substances which have an intrinsic, specific property to produce an adverse effect on reproduction and substances shall not be so classified if such an effect is produced solely as a non-specific secondary consequence of other toxic effects.

The classification of a substance is derived from the hazard categories in the following order of precedence: Category 1A, Category 1B, Category 2 and the additional Category for effects on or via lactation. If a substance meets the criteria for classification into both of the main categories (for example Category 1B for effects on sexual function and fertility and also Category 2 for development) then both hazard differentiations shall be communicated by the respective hazard statements. Classification in the additional category for effects on or via lactation will be considered irrespective of a classification into Category 1A, Category 1B or Category 2.

3.7.2.2.2 In the evaluation of toxic effects on the developing offspring, it is important to consider the possible influence of maternal toxicity (see section 3.7.2.4).

3.7.2.2.3 For human evidence to provide the primary basis for a Category 1A classification there must be reliable evidence of an adverse effect on reproduction in humans. Evidence used for classification shall ideally be from well conducted epidemiological studies which include the use of appropriate controls, balanced assessment, and due consideration of bias or confounding factors. Less rigorous data from studies in humans shall be supplemented with adequate data from studies in experimental animals and classification in Category 1B shall be considered.

3.7.2.3 Weight of evidence

3.7.2.3.1 Classification as a reproductive toxicant is made on the basis of an assessment of the total weight of evidence, see section 1.1.1. This means that all available information that bears on the determination of reproductive toxicity is considered together, such as epidemiological studies and case reports in humans and specific reproduction studies along with sub-chronic, chronic and special study results in animals that provide relevant information regarding toxicity to reproductive and related endocrine organs. Evaluation of substances chemically related to the substance under study may also be included, particularly when information on the substance is scarce. The weight given to the available evidence will be influenced by factors such as the quality of the studies, consistency of results, nature and severity of effects, the presence of maternal toxicity in experimental animal studies, level of statistical significance for inter-group differences, number of endpoints affected, relevance of route of administration to humans and freedom from bias. Both positive and negative results are assembled together into a weight of evidence determination. A single, positive study performed according to good scientific principles and with statistically or biologically significant positive results may justify classification (see also 3.7.2.2.3).

3.7.2.3.2 Toxicokinetic studies in animals and humans, site of action and mechanism or mode of action study results may provide relevant information which reduces or increases concerns about the hazard to human health. If it is conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expressed in humans then a substance which produces an adverse effect on reproduction in experimental animals should not be classified.

3.7.2.3.3 If, in some reproductive toxicity studies in experimental animals the only effects recorded are considered to be of low or minimal toxicological significance, classification may not necessarily be the outcome. These effects include small changes in semen parameters or in the incidence of spontaneous defects in the foetus, small changes in the proportions of common foetal variants such as are observed in skeletal examinations, or in foetal weights, or small differences in postnatal developmental assessments.

3.7.2.3.4 Data from animal studies ideally shall provide clear evidence of specific reproductive toxicity in the absence of other systemic toxic effects. However, if developmental toxicity occurs together with other toxic effects in the dam, the potential influence of the generalised adverse effects shall be assessed to the extent possible. The preferred approach is to consider adverse effects in the embryo/foetus first, and then evaluate maternal toxicity, along with any other factors which are likely to have influenced these effects, as part of the weight of evidence. In general, developmental effects that are observed at maternally toxic doses shall not be automatically discounted. Discounting devel-

opmental effects that are observed at maternally toxic doses can only be done on a case-by-case basis when a causal relationship is established or refuted.

3.7.2.3.5 If appropriate information is available it is important to try to determine whether developmental toxicity is due to a specific maternally mediated mechanism or to a non-specific secondary mechanism, like maternal stress and the disruption of homeostasis. Generally, the presence of maternal toxicity shall not be used to negate findings of embryo/foetal effects, unless it can be clearly demonstrated that the effects are secondary non-specific effects. This is especially the case when the effects in the offspring are significant, e.g. irreversible effects such as structural malformations. In some situations it can be assumed that reproductive toxicity is due to a secondary consequence of maternal toxicity and discount the effects, if the substance is so toxic that dams fail to thrive and there is severe inanition, they are incapable of nursing pups; or they are prostrate or dying.

3.7.2.4 Maternal toxicity

3.7.2.4.1 Development of the offspring throughout gestation and during the early postnatal stages can be influenced by toxic effects in the mother either through non-specific mechanisms related to stress and the disruption of maternal homeostasis, or by specific maternally-mediated mechanisms. In the interpretation of the developmental outcome to decide classification for developmental effects it is important to consider the possible influence of maternal toxicity. This is a complex issue because of uncertainties surrounding the relationship between maternal toxicity and developmental outcome. Expert judgement and a weight of evidence approach, using all available studies, shall be used to determine the degree of influence that shall be attributed to maternal toxicity when interpreting the criteria for classification for developmental effects. The adverse effects in the embryo/foetus shall be first considered, and then maternal toxicity, along with any other factors which are likely to have influenced these effects, as weight of evidence, to help reach a conclusion about classification.

3.7.2.4.2 Based on pragmatic observation, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed foetal weight, retarded ossification, and possibly resorptions and certain malformations in some strains of certain species. However, the limited number of studies which have investigated the relationship between developmental effects and general maternal toxicity have failed to demonstrate a consistent, reproducible relationship across species. Developmental effects which occur even in the presence of maternal toxicity are considered to be evidence of developmental toxicity, unless it can be unequivocally demonstrated on a case-by-case basis that the developmental effects are secondary to maternal toxicity. Moreover, classification shall be considered where there is a significant toxic effect in the offspring, e.g. irreversible effects such as structural malformations, embryo/foetal lethality, significant post-natal functional deficiencies.

3.7.2.4.3 Classification shall not automatically be discounted for substances that produce developmental toxicity only in association with maternal toxicity, even if a specific maternally-mediated mechanism has been demonstrated. In such a case, classification in Category 2 may be considered more appropriate than Category 1. However, when a substance is so toxic that maternal death or severe inanition results, or the dams are prostrate and incapable of nursing the pups, it is reasonable to assume that developmental toxicity is produced solely as a secondary consequence of maternal toxicity and discount the developmental effects. Classification is not necessarily the outcome in the case of minor developmental changes, when there is only a small reduction in foetal/pup body weight or retardation of ossification when seen in association with maternal toxicity.

3.7.2.4.4 Some of the end points used to assess maternal effects are provided below. Data on these end points, if available, need to be evaluated in light of their statistical or biological significance and dose response relationship.

Maternal mortality:

an increased incidence of mortality among the treated dams over the controls shall be considered evidence of maternal toxicity if the increase occurs in a dose-related manner and can be attributed to the systemic toxicity of the test material. Maternal mortality greater than 10 % is considered excessive and the data for that dose level shall not normally be considered for further evaluation.

Mating index

(no. animals with seminal plugs or sperm/no. mated × 100) (*)

Fertility index

(no. animals with implants/no. of matings × 100)

Gestation length

(if allowed to deliver)

Body weight and body weight change:

Consideration of the maternal body weight change and/or adjusted (corrected) maternal body weight shall be included in the evaluation of maternal toxicity whenever such data are available. The calcula-

* () It is recognised that the Mating index and the Fertility index can also be affected by the male.

tion of an adjusted (corrected) mean maternal body weight change, which is the difference between the initial and terminal body weight minus the gravid uterine weight (or alternatively, the sum of the weights of the foetuses), may indicate whether the effect is maternal or intrauterine. In rabbits, the body weight gain may not be useful indicators of maternal toxicity because of normal fluctuations in body weight during pregnancy.

Food and water consumption (if relevant):

The observation of a significant decrease in the average food or water consumption in treated dams compared to the control group is useful in evaluating maternal toxicity, particularly when the test material is administered in the diet or drinking water. Changes in food or water consumption need to be evaluated in conjunction with maternal body weights when determining if the effects noted are reflective of maternal toxicity or more simply, unpalatability of the test material in feed or water.

Clinical evaluations (including clinical signs, markers, haematology and clinical chemistry studies):

The observation of increased incidence of significant clinical signs of toxicity in treated dams relative to the control group is useful in evaluating maternal toxicity. If this is to be used as the basis for the assessment of maternal toxicity, the types, incidence, degree and duration of clinical signs shall be reported in the study. Clinical signs of maternal intoxication include: coma, prostration, hyperactivity, loss of righting reflex, ataxia, or laboured breathing.

Post-mortem data:

Increased incidence and/or severity of post-mortem findings may be indicative of maternal toxicity. This can include gross or microscopic pathological findings or organ weight data, including absolute organ weight, organ-to-body weight ratio, or organ-to-brain weight ratio. When supported by findings of adverse histopathological effects in the affected organ(s), the observation of a significant change in the average weight of suspected target organ(s) of treated dams, compared to those in the control group, may be considered evidence of maternal toxicity.

3.7.2.5 Animal and experimental data

3.7.2.5.1 A number of internationally accepted test methods are available; these include methods for developmental toxicity testing (e.g. OECD Test Guideline 414), and methods for one or two-generation toxicity testing (e.g. OECD Test Guidelines 415, 416).

3.7.2.5.2 Results obtained from Screening Tests (e.g. OECD Guidelines 421 — Reproduction/ Developmental Toxicity Screening Test, and 422 — Combined Repeated Dose Toxicity Study with

Reproduction/Development Toxicity Screening Test) can also be used to justify classification, although it is recognised that the quality of this evidence is less reliable than that obtained through full studies.

3.7.2.5.3 Adverse effects or changes, seen in short- or long-term repeated dose toxicity studies, which are judged likely to impair reproductive function and which occur in the absence of significant generalised toxicity, may be used as a basis for classification, e.g. histopathological changes in the gonads.

3.7.2.5.4 Evidence from in vitro assays, or non-mammalian tests, and from analogous substances using structure-activity relationship (SAR), can contribute to the procedure for classification. In all cases of this nature, expert judgement must be used to assess the adequacy of the data. Inadequate data shall not be used as a primary support for classification.

3.7.2.5.5 It is preferable that animal studies are conducted using appropriate routes of administration which relate to the potential route of human exposure. However, in practice, reproductive toxicity studies are commonly conducted using the oral route, and such studies will normally be suitable for evaluating the hazardous properties of the substance with respect to reproductive toxicity. However, if it can be conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expressed in humans then a substance which produces an adverse effect on reproduction in experimental animals shall not be classified.

3.7.2.5.6 Studies involving routes of administration such as intravenous or intraperitoneal injection, which result in exposure of the reproductive organs to unrealistically high levels of the test substance, or elicit local damage to the reproductive organs, including irritation, must be interpreted with extreme caution and on their own are not normally the basis for classification.

3.7.2.5.7 There is general agreement about the concept of a limit dose, above which the production of an adverse effect is considered to be outside the criteria which lead to classification, but not regarding the inclusion within the criteria of a specific dose as a limit dose. However, some guidelines for test methods, specify a limit dose, others qualify the limit dose with a statement that higher doses may be necessary if anticipated human exposure is sufficiently high that an adequate margin of exposure is not achieved. Also, due to species differences in toxicokinetics, establishing a specific limit dose may not be adequate for situations where humans are more sensitive than the animal model.

3.7.2.5.8 In principle, adverse effects on reproduction seen only at very high dose levels in animal studies (for example doses that induce prostration, severe inappetence, excessive mortality) would

not normally lead to classification, unless other information is available, e.g. toxicokinetics information indicating that humans may be more susceptible than animals, to suggest that classification is appropriate. Please also refer to the section on maternal toxicity (3.7.2.4) for further guidance in this area.

3.7.2.5.9 However, specification of the actual 'limit dose' will depend upon the test method that has been employed to provide the test results, e.g. in the OECD Test Guideline for repeated dose toxicity studies by the oral route, an upper dose of 1 000 mg/kg has been recommended as a limit dose, unless expected human response indicates the need for a higher dose level.

3.7.3 Classification criteria for mixtures

3.7.3.1 Classification of mixtures when data are available for all ingredients or only for some ingredients of the mixture

3.7.3.1.1 The mixture shall be classified as a reproductive toxicant when at least one ingredient has been classified as a Category 1A, Category 1B or Category 2 reproductive toxicant and is present at or above the appropriate generic concentration limit as shown in Table 3.7.2 for Category 1A, Category 1B and Category 2 respectively.

3.7.3.1.2 The mixture shall be classified for effects on or via lactation when at least one ingredient has been classified for effects on or via lactation and is present at or above the appropriate generic concentration limit as shown in Table 3.7.2 for the additional category for effects on or via lactation.

Table 3.7.2 Generic concentration limits of ingredients of a mixture classified as reproduction toxicants or foreffects on or via lactation that trigger classification of the mixture.

Ingredient classified as:	Generic concentration limits triggering classification of a mixture as:			
	Category 1A reproductive toxicant	Category 1B reproductive toxicant	Category 2 reproductive toxicant	Additional category for effects on or via l actation
Category 1A reproductive toxicant	≥ 0,3 % [Note 1]			
Category 1B reproductive toxicant		≥ 0,3 % [Note 1]		
Category 2 reproductive toxicant			≥ 3,0 % [Note 1]	
Additional category for effects on or via lactation				≥ 0,3 % [Note 1]

Note The concentration limits in the table above apply to solids and liquids (w/w units) as well as gases (v/v units).
Note 1 If a Category 1 or Category 2 reproductive toxicant or a substance classified for effects on or via lactation is present in the mixture as an ingredient at a concentration above 0,1 %, a SDS shall be available for the mixture upon request.

3.7.3.2 Classification of mixtures when data are available for the complete mixture

3.7.3.2.1 Classification of mixtures will be based on the available test data for the individual ingredients of the mixture using concentration limits for the ingredients of the mixture. On a case-by-case basis, test data on mixtures may be used for classification when demonstrating effects that have not been established from the evaluation based on the individual components. In such cases, the test results for the mixture as a whole must be shown to be conclusive taking into account dose and other factors such as duration, observations, sensitivity and statistical analysis of reproduction test systems. Adequate documentation supporting the classification shall be retained and made available for review upon request.

3.7.3.3 Classification of mixtures when data are not available for the complete mixture: bridging principles

3.7.3.3.1 Subject to paragraph 3.7.3.2.1, where the mixture itself has not been tested to determine its reproductive toxicity, but there are sufficient data on the individual ingredients and similar tested mixtures to adequately characterise the hazards of the mixture, these data shall be used in accordance with the applicable bridging rules set out in section 1.1.3.

3.7.4 Hazard Communication

3.7.4.1 Label elements shall be used for substances or mixtures meeting the criteria for classification in this hazard class in accordance with Table 3.7.3

Table 3.7.3 Label elements for reproductive toxicity.

Classification	Category 1A or Category 1B	Category 2	Additional category foreffects on or via lactation
GHS Pictograms			No pictogram
Signal Word	Danger	Warning	No signal word
Hazard Statement	H360: May damage fertility or the unborn child (state specific effect if known)(state route of exposure if it is conclusively proven that no other routes of exposure cause the hazard)	H361: Suspected of damaging fertility or the unborn child (state specific effect if known) (state route of exposure if it is conclusively proven that no other routes of exposure cause the hazard)	H362: May cause harm to breast-fed children.
Precautionary Statement Prevention	P201 P202 P281	P201 P202 P281	P201 P260 P263 P264 P270
Precautionary Statement Response	P308 + P313	P308 + P313	P308 + P313
Precautionary Statement Storage	P405	P405	
Precautionary Statement Disposal	P501	P501	

Annex **C**

Additional considerations to Regulation (EC) 1272/2008

The classification and labelling of substances is performed according to the guidelines of the European Union (Regulation (EC)1272/2008) presented in Annex B. The classification of compounds is ultimately dependent on an integrated assessment of the nature of all parental and developmental effects observed, their specificity and adversity, and the dosages at which the various effects occur. The guideline necessarily leaves room for interpretation, dependent on the specific data set under consideration. In the process of using the regulation, the committee has agreed upon a number of additional considerations:

- If there is sufficient evidence to establish a causal relationship between human exposure to the substance and impaired fertility or subsequent developmental toxic effects in the offspring, the compound will be classified in category 1A, irrespective of the general toxic effects (see Annex B, 3.7.2.2.1.).
- Adverse effects in a reproductive study, reported without information on the parental or maternal toxicity, may lead to a classification other than category 1B, when the effects occur at dose levels which cause severe toxicity in *general* toxicity studies.
- Clear adverse reproductive effects will not be disregarded on the basis of reversibility per se.

- The committee does not only use guideline studies (studies performed according to OECD* standard protocols) for the classification of compounds, but non-guideline studies are taken into consideration as well.

* Organisation for Economic Cooperation and Development

Annex **D**

Fertility and developmental toxicity studies

Table 1a Fertility studies in animals: oral administration.

author	species	experimental period/design	dose/route	general toxicity	effects on reproductive organs/ effects on reproduction
Cabral/ Neal, (1983)	male Fischer 344 rats (n=5-27/ group)	3 wk, followed by 1 wk control diet; schedule repeated 3 times; only 3 wk; control group included; sacrifice at 55 wk	0.06% in diet (30 mg/kg bw/d)	12-wk treatment: marked bw depression and mortality (in 22/25 animals); liver carcinomas (in 24/ 25; with extensive lung metastases in 8/25), cholangiosarcomas (in 5/25), Zymbal gland carcinomas (no incidence presented); other groups: no effect on bw, no mortality, no tumours	testicular mesotheliomas (in 9/ 25)
Lafaurie *et al*. (1982)	male Sprague-Dawley rats, (n=60/group)	30 d; then 6 periods of 4 wk in which animals received 2-AAF in semi-synthetic diet for 2 wk followed by 2 wk of semi-synthetic diet only	0.06% (30 mg/kg bw/d) in semi-synthetic diet deprived of protein and riboflavin (to enhance occurrence of liver cancer; 1 control group fed a normal diet; 1 control group fed the semi-synthetic diet	not reported; study designed to explore relation between hormonal status and liver cancer	decreased absolute testis weights; reduced spermatogenic activity[a]; histological testicular lesions (e.g. near total loss of germinal epithelium, interstitial oedema, tubules containing only Sertoli cells, thickened basement membrane of tubules and tunica albuginea); decreased testosterone levels generally, levels fluctuated considerably

Bishop *et al*. (1988b) Salome (1988)	male Fischer 344 rats (n=18/group)	5 d/wk, 8 wk; sacrifice 2.5 wk after final treatment	0, 5, 10 mg/kg bw/d (in trioctanoin); gavage	at 10 mg/kg: mortality after wk 6; 4/18 survived	no effect on sperm morphology
Harper/ Legator (1988) Salome (1988)	male Ha/ICR Swiss mice (n=5/group)	2 or 5 d; sacrifice 35 d after final treatment	0, 110, 222, 444, 666, 780, 888, 900 mg/kg bw/d (in olive oil); gavage	not reported; mortality in 1/5 animals at 900 mg/kg bw	no effect on sperm morphology
Salome *et al.* (1988) Salome (1988)	male CD-1 Swiss mice (n=5-9/group)	3 d; sacrifice 34-35 d after last treatment	two separate series: 0, 385, 462, 577, 728, 902 mg/kg bw/ d (in corn oil); and 0, 713, 828, 950, 1082 mg/kg bw/d (in corn oil); gavage	not reported; mortality in 3/9 at 902 mg/kg bw, and in 1/8 at both 1032, 950, 713, and 577 mg/kg bw. (also mortality in 1/5 in solvent control in 1st series)	no effect on sperm morphology
Salome *et al*. (1988) Salome (1988)	male B6C3F$_1$ mice (n=7 or 10/group)	3 or 4 d; sacrifice approx. 35 d after last treatment	0, 720, 830, 954, 1010 mg/kg bw/d (in corn oil); gavage	not reported; mortality in 1/10 at 1010 mg/kg bw and 1/7 at 830 mg/kg bw	no effect on sperm morphology
Bishop *et al*. (1988a)	male F344 rats (n=15/ group)	5 d/wk, for 23 d males mated post-treatment for 2 consecutive 5-d periods; 2 females per male; females sacrificed 19 d after mating	0, 5, 50 mg/kg bw/d for 5 d; then 0, 12.5 25 mg/kg bw/d; 5 d/ w for 18 d; gavage	severe bw loss after 1st wk (leading to lowering doses by one half); after 4th wk, mortality at high-dose from apparent liver toxicity; during 2-wk mating trials: mortality in 4/18 low-dose and 14/18 high-dose animals; bladder lesions in survivors sacrificed at post-treatment d 31	no effect on the mean numbers of implantations, non-viable and viable implantations per female; on frequencies of females with 1 or more or with 2 or more non-viable implantations; on mean foetal death rate and percentage foetal lethality; decreased percentages of females with implantations after 1st mating trials at low (7/30) and high dose (0/8) (controls: 17/30)
Bishop *et al*. (1988a)	male F344 rats (n=15/ group)	5 d/w, for 32-40 d	0, 5 mg/kg bw; 40 d 0, 10 mg/kg bw/d; 32-35 d or 36-40 d; gavage	at high dose, mortality after 6th wk of treatment	no effect on the mean numbers of implantations, non-viable and viable implantations per female; on frequencies of females with 1 or more or with 2 or more non-viable implantations; on mean foetal death rate and percentage foetal lethality; decreased percentages of females with implantations when mated with males immediately after having received 32 doses or more of 10 mg/kg bw

Shibuya *et al*. (1988)	male $B6D2F_1$ mice (n=30/ group)	5 d males mated post-treatment for 2 consecutive 5-d periods; 2 females per male; females sacrificed 13 d after mating	0, 350, 700 mg/kg bw/d; gavage	not reported; mortality in 6/30 males at 700 mg/kg bw	no effect on frequencies of fertile matings, females with one or more dead implantations and dominant lethal mutations, or on numbers of corpora lutea, implantations, and living implantations per female

[a] as evaluated by determining the percentage of stages VII and VIII of the cycle of the seminiferous epithelium by the greatest diameter of frontal sections of the testes
Abbreviations: bw: body weight; d: day(s); wk: week(s)

Table 1b Fertility studies in animals: intraperitoneal administration.

author	species	experimental period/design	dose	general toxicity	effects on reproductive organs/ effects on reproduction
Topham (1980)	male (CBA x BALB/c)F_1 mice (n=5/ group)	5 d; sacrifice 5 wk after last treatment	0, 100 250, 500, 1000, 1500 mg/kg bw/d (in Tween 80/ water)	not reported; mortality (not quantified) observed at 500, 1000, 1500 mg/kg bw	no increase in sperm head abnormalities
Salome/ Logan (1988) Salome (1988)	male $B6C3F_1$ mice (n=2-10/group)	3 or 5 d; sacrifice 35-36 d after last treatment	0, 220, 440, 660, 880, 1100, 1320 mg/kg bw/d (in DMSO)	not reported; mortality in 5-d group in 6/10 at 1320 mg/kg bw, 1/10 at 1100 mg/kg bw, 2/8 at 660 mg/kg bw, 1/8 at 220 mg/kg bw; considerable mortality in all groups treated for 3 d[a]	dose-dependent increase in percentage of abnormal sperm (stat. sign. for 4 higher doses)
Arany/Erna (1988) Salome (1988)	male $B6C2F_1$ mice (n=8-10/group)	5 d; sacrifice 5 wk after last treatment	0, 1115, 2220, 3330, 4440, 5550 mg/kg bw/d (in DMSO)	not reported; mortality in 2/8 at 1115 mg/kg bw, in 1/8 at 3330 mg/kg bw, in 2/10 at both 4440 and 5550 mg/kg bw	dose-dependent increase in percentage of abnormal sperm (stat. sign. for 3 higher doses)
Quinto/De Marinis (1983)	male ($C_{57}BL_6$ x C3H)F_1 mice	5 d	0, 125, 250, 500 mg/kg bw/d (in DMSO/water); sacrifice the 5th wk after 1st injection	not reported	at all dose levels: increase in sperm abnormalities at 500 mg/kg: reduced absolute testis weights
Cauhan (1988)	male Swiss mice (n=60-78/group)	1 d 24 h after treatment, 1 male mated with 1 female for 4 d; then female replaced with new ones 4-d intervals for at another 11 subsequent matings; females sacrificed 17-18 d after 1st mating d	0, 56, 112, 167 mg/ kg bw (in DMSO)	not reported	no effect on frequencies of dead implantations, females with dead implantations, dominant lethal mutations, on mean numbers of implantations and dead and living implantations

Ehling (1988)	male (101/E1 x C3H/E1)F_1 mice (n=45/group)	1 d see above Cauhan	0, 300, 600 mg/kg bw (in DMSO)	at 300 mg/kg bw: slight toxicity (not specified); at 600 mg/kg bw: mortality in 47%	no effect on frequencies of fertile matings, dead implants, dominant lethal mutations, on numbers of corpora lutea, implantations and living implantations per female
Generoso (1988)	female T-stock and (C3H x C57BL)F_1 mice (n=41-48/group)	1 d mated during post-treatment 0.5-4.5 and 5.5-9.5; sacrifice 17 d after mating	0, 1500 mg/kg bw (in DMSO)	not reported	no effect on frequencies of dead implants, of females with 1 or more dead implants, on numbers of implantations, living and dead implantations per female

[a] at 1320 mg/kg bw: 5/10; at 1100 mg/kg bw: 4/8; at 880 mg/kg bw: 6/9; at 660 mg/kg bw: 3/6; at 440 mg/kg bw: 2/5; at 220 mg/kg bw: 1/4

Abbreviations: bw: body weight; d: day(s); stat. sign.: statistically significant; wk: week(s)

Table 2 Developmental toxicity studies in animals.

author	species	experimental period/design	dose/route	general toxicity	developmental toxicity
Izumi (1962)	Japanese dd strain mice (n=10-12)	single dose on single gd 8, 9, 10, 11, 12, 13, 14, or 15; animals sacrificed on gestational d 18	0, 100 mg/kg bw (in propylene glycol); intraperitoneal	not reported	decreased foetal weight at gd 9, 10, 11; increased percentage of resorptions at gd 8, of number of malformed survivors at gd 10; increased number of malformations at gd 8, 9, 10, 11, 13, 14 (including adactyly and maldirection of fingers; brachydactyly, polydactyly, macrodactyly of toes; malformation of wrist or ankle joint, absence of tail, cleft palate, cleft lip, cerebral hernia); skeletal malformations after staining included: abnormalities of the ribs, retarded ossification of 13th rib, caudal vertebrae, metacarpal or metatarsal bones or phalanges of finger or toe.

Abbreviations: d: day(s); gd: gestational day