CLH report

Proposal for Harmonised Classification and Labelling

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

Chemical name:

N-1,3-dimethylbutyl-*N*'-phenyl-*p*-phenylenediamine

EC Number: 212-344-0

CAS Number: 793-24-8

Index Number: -

Contact details for dossier submitter:

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CONTENTS

ABBREVIATIONS

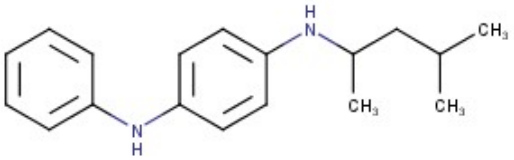
AC	Article Category
AGD	Anogenital distance
ATE	Acute Toxicity Estimate
bw	Body weight
BCF	Bioconcentration factor
BPS	Balanopreputial separation
B / vB	Bioaccumulative / very bioaccumulative
CAS	Chemical Abstract Service
CL	Confidence Limit
d	Day
DS	Dossier submitter
DPM	Disintegration per minute
Drg	Danger
FCA	Freud's Complete Adjuvant
GD	Gestation Day
GLP	Good Laboratory Practice
HCD	Historical control data
4-HDPA	4-Hydroxypropylamine
HRIPT	Human Repeat Insult Patch Test
ICDRG	International Contact Dermatitis Research Group
IPPD	*N*-Isopropyl-*N*'-phenyl-*p*-phenylenediamine
Kow	Partition coefficient octanol/water
LD50	Lethal dose, 50%
LC50	Lethal concentration, 50%
m/f	Male/female
MITI	Ministry of International Trade and Industry, Japan
4-NDPA	4-Nitrodiphenylamin
NONS	Notification of new substances
OECD	Organisation for Economic Co-operation and Development
P / vP	Persistent / very persistent
PC	Product Category
44PD	*N,N'*-di-sec-butyl-*p*-phenylenediamine
QSAR	Quantitative structure activity relationship

RAR	Risk Assessment Report
SU	Sector of Use
TG	Test Guideline
SI	Stimulation Index

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)	4-*N*-(4-methylpentan-2-yl)-1-*N*-phenylbenzene-1,4-diamine
Other names (usual name, trade name, abbreviation)	6PPD 1,4-Benzenediamine, *N*1-(1,3-dimethylbutyl)-*N*4-phenyl- *N*1-(4-Methylpentan-2-yl)-*N*4-phenylbenzene-1,4-diamine *N*-(1,3-Dimethylbutyl)-*N*'-phenyl-1,4-phenylenediamine
ISO common name (if available and appropriate)	-
EC number (if available and appropriate)	212-344-0
EC name (if available and appropriate)	*N*-1,3-dimethylbutyl-*N*'-phenyl-*p*-phenylenediamine
CAS number (if available)	793-24-8
Other identity code (if available)	-
Molecular formula	C18H24N2
Structural formula	 (**source:** European Chemicals Agency, http://echa.europa.eu/)
SMILES notation (if available)	CC(C)CC(C)NC1=CC=C(C=C1)NC2=CC=CC=C2
Molecular weight or molecular weight range	268.4
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)	Not relevant

1.2 Composition of the substance

Table 2: Constituents

Constituent (Name and numerical identifier)	**Concentration range (% w/w minimum and maximum in multi-constituent substances)**	**Current CLH in Annex VI Table 3 (CLP)**	**Current self-classification and labelling (CLP)**
N-1,3-dimethylbutyl-*N*'-phenyl-*p*-phenylenediamine EC 212-344-0	Conf.	-	Summary: Acute Tox. 4, H302; Skin Irrit. 2, H315; Eye Irrit. 2, H319; STOT SE 3, H335; STOT RE 2, H373; Skin Sens 1, H317; Repr. 1B, H360; Aquatic Acute 1; H400; Aquatic Chronic 1; H410

Based on the boundary composition given in the registration impurities do not contribute to the classification and labelling. Confidential information on concentration ranges of the constituent and impurities is given in a separate Annex to this document.

Information on the purity of the substance used for testing is given in the study descriptions if available.

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 3: For substance with no current entry in Annex VI of CLP

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATEs	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	No current Annex VI entry										
Dossier submitter's proposal	TBD	*N*-1,3-dimethylbutyl-*N*'-phenyl-*p*-phenylenediamine	212-344-0	793-24-8	Repr. 1B Acute Tox. 4 Skin Sens. 1A Aquatic Acute 1 Aquatic Chronic 1	H360FD H302 H317 H400 H410	GHS08 GHS07 GHS09 Dgr	H360FD H302 H317 H410		Oral: ATE = 890 mg/kg bw M = 10000 M = 10	

Table 4: Reason for not proposing harmonised classification and status under consultation

Hazard class	Reason for no classification	Within the scope of 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	**Acute Tox. 4, H302**	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	*data lacking*	No
Skin sensitisation	**Skin Sens. 1A, H317**	Yes
Germ cell mutagenicity	*hazard class not assessed in this dossier*	No
Carcinogenicity	*hazard class not assessed in this dossier*	No
Reproductive toxicity	**Repr. 1B, H360FD**	Yes
Specific target organ toxicity-single exposure	*hazard class not assessed in this dossier*	No
Specific target organ toxicity-repeated exposure	*data conclusive but not sufficient for classification*	Yes
Aspiration hazard	*hazard class not assessed in this dossier*	No
Hazardous to the aquatic environment	**Aquatic Acute 1, H400** **Aquatic Chronic 1, H410**	Yes
Hazardous to the ozone layer	*hazard class not assessed in this dossier*	No

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

The substance was not discussed previously by the TC C&L (Dir. 67/548/EEC). The substance has no harmonized classification so far.

The substance has 265 C&L notifications with self-classifications (summary) as Acute Tox. 4, H302; Skin Irrit. 2, H315; Eye Irrit. 2, H319; STOT SE 3, H335; STOT RE 2, H373; Skin Sens 1, H317; Repr. 1B, H360; Aquatic Acute 1; H400; Aquatic Chronic 1; H410 [status 03/2022].

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

There is no requirement for justification that action is needed at Community level.

Further detail on need of action at Community level
Beside reproductive toxicity the substance shows several hazards (acute toxicity, skin sensitisation, STOT RE, Aquatic toxicity) which also have been evaluated in this CLH Dossier as there are differences in self-classification, especially concerning the M-factor for Aquatic Toxicity.

5 IDENTIFIED USES

6PPD is registered in the EU in the tonnage band of ≥ 10 000 to < 100 000 tonnes per year. The substance is an antidegradant (antioxidant and antiozonant) added to tire rubber to protect the rubber polymer from reaction with oxygen (O_2) and ozone (O_3) (Cal DTSC, 2022).

Table 5: The following uses are indicated at ECHA dissemination site [accessed 03/2022]:

Categories	Use(s)	Technical function
Manufacture	Manufacture of the substance	-
Formulation	Production of tyre and general rubber goods (PC 32: Polymer preparations and compounds) Industrial manufacture and formulation of additised acrylate Antioxidant use in SBR polymer (PC 32: Polymer preparations and compounds) Industrial formulation, repacking and distribution of antioxidant cocktails and fuel blends (PC 13: Fuels)	-
Uses at industrial sites	Production of tyre and general rubber goods (PC 32: Polymer preparations and compounds; SU 10: Formulation [mixing] of preparations and/or re-packaging (excluding alloys); SU11: Manufacture of rubber products; SU 12: Manufacture of plastics products, including compounding and conversion) Industrial use if additised acrylate Industrial use of antioxidant in fuel blends (PC 13: Fuels)	-
Uses by professional workers	Tyre mounting and dismounting and handling of technical rubber goods (PC 32: Polymer preparations and compounds; SU 22: Public domain)	-

	Retreading and recycling (PC 32: Polymer preparations and compounds; SU 11: Manufacture of rubber products; SU 3: Industrial Manufacturing; SU 22: Public domain Profesional use of antioxidant in fuel blends (PC 13: Fuels)	
Consumer Uses	Use of tyres or technical rubber goods Consumer use if antioxidant in fuel blends (PC 13: Fuels)	-
Article service life	Article service life of technical rubber goods (AC 2: Machinery, mechanical appliances, electrical/electronic articles; AC 3: Electrical batteries and accumulators; AC 10: Rubber articles) Article service life of tyres (AC 1: Vehicles; AC 2: Machinery, mechanical appliances, electrical/ electronic articles; AC 3: Electrical batteries and accumulators; AC 10: Rubber articles) Retreading and recycling Tyre mounting and dismounting and handling of technical rubber goods (AC 10: Rubber articles)	-

6 DATA SOURCES

ECHA dissemination site: Registration Dossier - ECHA (europa.eu)

Original study reports on 6PPD were provided by registrant(s) for most of the studies; for some studies only parts of the results or only the information from ECHA dissemination site or IUCLID was available.

In addition actual scientific literature has been considered till January 2022 (with the exception of Brinkmann et al., 03/2022).

Structurally similar substances to 6PPD like *N,N'*-di-sec-butyl-*p*-phenylenediamine (44PD) were used in the registration dossier of 6PPD, but as no proper read-across justification could be found, the data for these potential read-across substances were not included in the CLH-dossier.

Several studies included in the registration for 6PPD were conducted with tyre wear particles. These studies were not used for the CLH-Dossier as no substance specific information could be retrieved from these studies.

Please see Chapter 14 for details on references.

Information concerning oxidation/hydrolysis products:

Data on the oxidation product 6QDI and the hydrolysis products 4-hydroxypropylamine, 1,3 dimethylbutylamine and *N*-phenyl-*p*-benzoquinone imine, *p*-hydroquinone and benzoquinone were gained from the IUCLID dossier and ECHA dissemination site of 6PPD. For some of these studies the original studies or parts of the studies were made available by the registrants of 6PPD.

For the oxidation product 6PPD quinone several studies were retrieved from open literature, no ecotoxicity data are available in the 6PPD registration dossier.

For the substance 1,3-dimethyl-butylamine, not registered under REACH, read-across data on sec. butylamine and octylamine were included in the 6PPD registration dossier. As no proper read-across

justification could be found the data for these potential read-across substances were not used for classification purposes.

For the following registered oxidation and hydrolysis products the substance specific ECHA dissemination website and partly also the corresponding IUCLID dossiers were consulted: 6QDI, 4HDPA, *p*-hydroquinone and *p*-benzoquinone.

For the hydrolysis product aniline much data is available and the substance has a harmonised classification as Aquatic Acute 1. As it is neither deemed proportional nor essential, the acute aquatic studies on aniline are not included in this CLH-Dossier. Due to efficiency reasons only the most sensitive relevant study per trophic level is included in the dossier (see Risk Assessment Report by European Union, 2004 and ECHA dissemination website on aniline).

7 PHYSICOCHEMICAL PROPERTIES

Table 6: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Solid; dark, brown powder	ECHA dissemination site [03/2022]	-
Melting/freezing point	49.4°C	ECHA dissemination site [03/2022]	EU Method A.1
Boiling point	163°C	ECHA dissemination site [03/2022]	Handbook
Relative density	0.995 g/ml (50°C)	ECHA dissemination site [03/2022]	Not specified
Vapour pressure	0.004 Pa (25°C)	ECHA dissemination site [03/2022]	QSAR, EPI Suite v4.11
Surface tension	-	ECHA dissemination site [03/2022]	waiving
Water solubility	1 mg/L (50°C)	ECHA dissemination site [03/2022]	Modified OECD Guideline 105, hydrolyses rapidly
	1.879 mg/L (25°C)	WSKOW v.1.41	Modelling by AT
Partition coefficient n-octanol/water (logarithmic scale)	4.68 (20°C)	ECHA dissemination site [03/2022]	QSAR, EPI Suite v4.11
Flash point	202.5°C (1013 hPa)	ECHA dissemination site [03/2022]	EU Method A.9 (Flash-Point)
Flammability	Not flammable	ECHA dissemination site [03/2022]	waiving
Explosive properties	Non explosive	ECHA dissemination site [03/2022]	waiving
Self-ignition temperature	-	ECHA dissemination site [03/2022]	waiving
Oxidising properties	No	ECHA dissemination site [03/2022]	waiving
Granulometry	-	ECHA dissemination site [03/2022]	waiving

Property	Value	Reference	Comment (e.g. measured or estimated)
Stability in organic solvents and identity of relevant degradation products	-	ECHA dissemination site [03/2022]	waiving
Dissociation constant	6.7 (20°C)	ECHA dissemination site [03/2022]	QSAR, ACD 7.0 software program
Viscosity	-	ECHA dissemination site [03/2022]	Substance is a solid.

8 EVALUATION OF PHYSICAL HAZARDS

Not addressed in this dossier.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 7: Summary table of toxicokinetic studies

Method	Results	Remarks	Reference
Hydrolysis study in simulated gastric juice GLP 500 µg/100 ml of simulated gastric juice Samples taken at 0, 4, 11.75, 24 and 48h Gas chromatography Mass spectrometry	hydrolysis rate: -0.0188 half-life: 36.9 hours solubility in gastric juice: 173 mg/ml major hydrolysis product: aniline minor intermediate hydrolyse products: benzoquinoneimine-*N*-phenyl, *N*-1,3 dimethyl-butylamine *p*-phenol	Test substance 6PPD, purity unknown Solvent: nanograde acetone	Anonymous (1986)

In a 48h hydrolysis study in simulated gastric juice approximately 60% of 6PPD was hydrolysed (Anonymous, 1986). Simulated gastric juice was prepared by dissolving 2.0 g sodium chloride, 3.2 g pepsine and 7.0 ml concentrated hydrochloric acid in a total volume of 1000 ml deionized water. 6PPD (500 µg/100 ml) was added, stirred and incubated at 37°C for 48h. At 0, 4, 11.75, 24 and 48h samples (in duplicate) were taken and quantified by gas chromatography (detection limit for 6PPD was 20 µg). Based on these data a hydrolysis rate constant of -0.0188 and a half-life of 36.9 hours were calculated. The approximate solubility of 6PPD in gastric juice was determined to be 173 mg/l. Mass spectrometry was performed for the purpose to identify hydrolysis products. The major observed hydrolysis product was aniline. Traces of two unstable intermediate hydrolysis products, benzoquinoneimine-*N*-phenyl and *N*-1,3 dimethyl-butylamine *p*-phenol were detected. Three final hydrolysis products are proposed for 6PPD: aniline, quinone and 2 amino-4 methyl pentane. However, attempts to identify the last two products were not successful in this study.

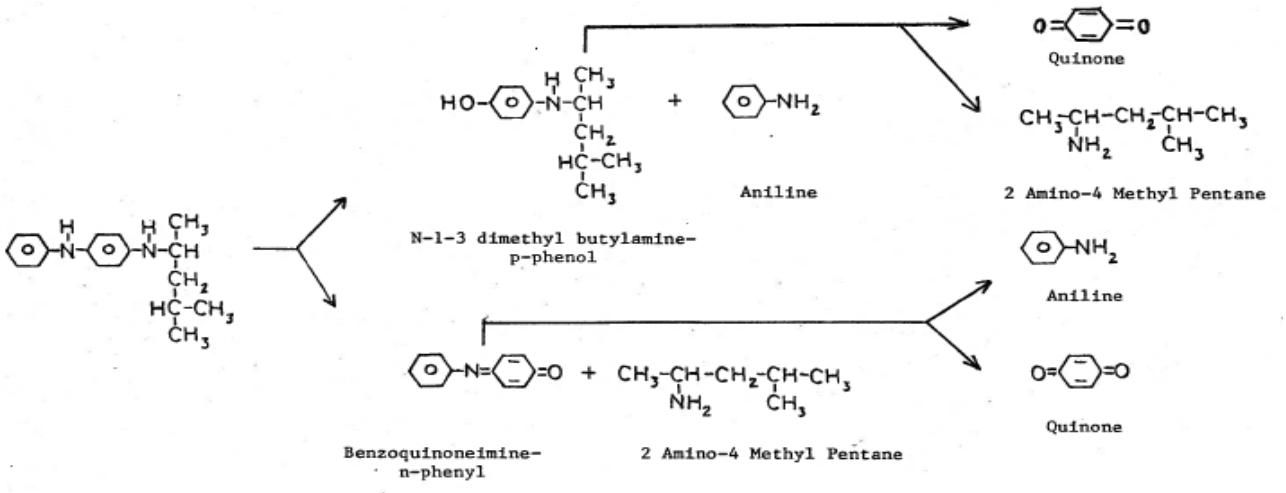


Figure 1: Proposed hydrolysis pathway of 6PPD in simulates gastric juice (Anonymous, 1986).

6PPD is absorbed by all routes of exposure. This is demonstrated by systemic effects after oral/dermal acute and repeated exposure in animal studies (see following Chapters) and biomonitoring of workers has shown that 6PPD can be resorbed after exposure by inhalation and possibly after skin contact. Carlucci et al. (1984) found 6PPD in about 15 % of the urine samples in a cohort of 21 workers exposed via inhalation or skin contact. The maximum amount found was 1.3 µg/l urine (detection limit of 0.1 µg/l). Rimatori and Castellino (1989) documented the concentration of 6PPD in 341 urine samples of rubber industry workers ranging from < 1 to 300 µg/g creatinine (with a peak value of 580 µg/g) from 1982 to 1987. The concentration was depending on the 6PPD concentration in respiratory air at the workplace which reached up to 6.6 mg/m^3 [cited from OECD, 2004].

Pavan (1987) reported that 6PPD in rubber industry was detected in the urine of exposed workers (sample collection 1982 – 1987). Air levels in the range of < 0.01 - 260 ug/mc (peak 6600) correspond to urine levels in the range of < 1 - 300 ug/g creatinine (peak 580). However, some uncertainty concerning substance identity has been identified as in the publication the abbreviation 6PPD is used, however, the substance is called *N*-(2,3-dimethylpropyl)-*N*-phenyl-1,4-benzenediamine with the CAS-No. 739-24-8 [cited from OECD, 2004].

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

Available human and animal data demonstrate that 6PPD is absorbed by all routes of exposure. Systemic toxicity after oral and dermal exposure in animals shows the principal bioavailability of 6PPD via these routes.

10 EVALUATION OF HEALTH HAZARDS

Acute toxicity

10.1 Acute toxicity - oral route

For evaluation of acute toxicity via oral exposure two well reported studies in rats (OECD 401, Limit test) are available. Limited information on five additional studies is available and reported below.

Table 8: 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}	Reference
OECD TG 401 GLP	Rat, Crj:CD n=5/sex/dose	6PPD (purity 99%)	0, 250, 500, 1000 and 2000 mg/ kg bw Vehicle: corn oil Gavage Observation periode: 15d	LD_{50} males = 1005 mg/kg bw (CI 711 – 1604 mg/kg bw) LD_{50} females = 893 mg/kg bw (CI 634 – 1346 mg/kg bw) Deaths at 1000 mg/kg bw: 2/5 m, 3/5 f Deaths at 2000 mg/kg bw: 10/10 (m+f) At 1000 mg/kg bw or higher: hypoactivity, diarrhea, bradypnea, hypothermia, prone position at 2000 mg/kg bw: abnormal gait (m), lacrimation and weakness of limbs (f) Pathological lesions in digestive organs and the respiratory system	Anonymous (1999a) [study in Japanese] Klimisch 1
Limit test (EPA OTS 798.1175) GLP	Rat, Sprague-Dawley n=5/sex/dose <u>Range finding test</u> n=1/sex/dose	6PPD (purity 97.6%)	5000 mg/kg bw (warmed) Gavage, no vehicle Observation periode: 15d	LD_{50} > 5000 mg/kg bw Deaths: m 2/5; f 1/5 Clinical signs: decreased fecal output, fecal/urine stains, rough coat, piloerection, diarrhea/soft stools, arks material around facial area. Body weigt loss in one surviving male and three females. Necropsy findings: (1) black, hard material in the stomach of dead and surviving animals (2) black/yellow-green mucoid content throughout the digestive tract, eroded areas with perforations of the stomach, reddened mucosa/dark red foci of the stomach <u>Range finding test:</u> 500, 1000, 3000, 4000 and 5000 mg/kg bw High dose male died on day 5.	Anonymous (1991) Klimisch 1

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels, duration of exposure	Value LD_{50}	Reference
-	Rat, Sprague-Dawley Albino n=5/dose	6PPD (purity unknown)	2510, 3160, 3980, 5010 and 6310 mg/kg bw (warmed) Single exposure via gavage Observation periode: 12d	LD_{50} = 3580 mg/kg bw (CI: 3400 – 3760 mg/kg bw) Number of deaths in the different dose groups: 1/5, 2/5, 3/5, 3/5, 4/5 Signs of intoxication: reduced appetite, reduced activity, increased weakness, diarrhea, ocular discharge, collaps Decedents: hemorrhagic lungs; liver discoloration (jaundice), acute gastrointestinal inflammation	Anonymous (1973) Klimisch 2
-	Rat, Sprague-Dawley	6PPD (purity unknown), undiluted	2510, 3160, 3980 and 5010 mg/kg bw n=5/dose (3f and 2m each), (high dose: 4m and 1f). stomach tube	LD_{50} = 3340 mg/kg bw (CI: 2890-3875 mg/kg bw) Number of deaths in the different dose groups: 1/5, 2/5, 3/5, 5/5 Collapse; in some cases severe diarrhea, loss of appetite, salivation, dyspnea	Anonymous (1962) Klimisch 2
-	White rats White mice	6PPD in sunflower	No information on dosing Induction into the stomach	LD_{50} white rat = 2500 mg/kg bw LD_{50} white mice = 3200 ± 310 mg/kg bw Deaths occurred day 1-3. Clinical picture: narcotic action	Stasenkova (1970) Klimisch 3
-	-	-	-	LD_{50} = 1120 mg/kg bw	Goodyear (1973) [cited from OECD, 2004]
OECD TG 423 GLP	Rat, Wistar	6PPD 300 mg/kg bw: n=3/sex/dose 2000 mg/kg bw: n=1/sex/dose	300, 2000 mg/kw bw Gavage Olive oil	LD_{50} > 300 < 2000 mg/kg bw	Anonymous (2007) [cited from ECHA dissemination site, study report not available] Klimisch 3

In an OECD guideline study (TG 401) (Anonymous, 1999a) 5 male and 5 female rats were dosed with 0, 250, 500, 1000 and 2000 mg 6PPD/kg bw in corn oil by gavage. Deaths, presented in Table 9, resulted in LD_{50} values between 1000 - 2000 mg/kg for male rats and 500 - 1000 mg/kg for female rats. Registrants calculated LD_{50} values in male rats of 1005 mg/kg bw and female rats of 893 mg/kg bw.

Table 9: Mortality of rats (m, f) after single oral exposure of 6PPD (Anonymous, 1999a).

Sex	Dose [mg/kg bw]	Total number of animals	Number of deaths					
			Day 1	Day 2	Day 3	Day 4	Day 5-9	Day 10-14
males	0	5	0	0	0	0	0	0
	250	5	0	0	0	0	0	0
	500	5	0	0	0	0	0	0
	1000	5	0	1	0	1	0	0
	2000	5	0	4	1	-	-	-
females	0	5	0	0	0	0	0	0
	250	5	0	0	0	0	0	0
	500	5	0	0	0	0	0	0
	1000	5	0	0	1	2	0	0
	2000	5	0	2	3	-	-	-

In another acute oral toxicity study (Anonymous, 1991) 5 male and 5 female rats were exposed to a limit dose of 5000 mg/kg bw (based on a range finding study). The solid test substance was warmed to 55°C for dosing to produce a liquid and then allowed to cool to 50°C for dosing. Animals were observed frequently on the day of dosing and once daily thereafter for the following 15 days. Mortality checks were performed twice daily (morning and afternoon). Individual body weights were determined and recorded on days 1, 8 and 15 or at the time of death. At the time of death or scheduled sacrifice all animals were subjected to a gross necropsy examination. Two males were found dead on day 6 and 9, one female on day 6. Necropsy observations for these animals were documented as black/yellow-green mucoid contents throughout the digestive tract, eroded areas with perforations of the stomach and reddened mucosa/dark red foci of the stomach. Many animals showed black, hard (rock-like) material contents in the stomach. Clinical signs were decreased fecal output, fecal/urine stains, rough coat, piloerection, diarrhea/soft stools and dark material around the facial area. Body weight loss was documented for some animals. An LD_{50} of >5000mg/kg bw was determined by study authors.

Anonymous (1973) applied single oral doses of 6PPD (undiluted and warmed to ~46°C to liquefy) in doses of 2510, 3160, 3980, 5010 and 6310 mg/kg bw to rats. Mortalities, documented in Table 10, occured from day two to day eleven with most deaths within seven days. An LD_{50} of 3580 mg/kg bw was determined (calculation method of De Beer). Reduced appetite and activity (days 2-5 in survivors), increasing weakness, diarrhea, ocular discharge and collapse are documented. Gross authopsy showed hemorrhagic lungs, liver discoloration (jaundiced) and acute gastrointestinal inflammation. Survivors showed slight liver discoloration in some cases.

Table 10: Mortalities after single oral exposure (Anonymous, 1973).

Dose [mg/kg bw]	Deaths of males	Deaths of females	Combined deaths
2510	0/2	1/3	1/5
3160	1/3	1/2	2/5
3980	0/2	3/3	3/5
5010	2/3	1/2	3/5
6310	2/2	2/3	4/5

In an older oral toxicity study (Anonymous, 1962) rats (3f and 2m/dose) were fed undiluted 6PPD by stomach tube in doses of 2510, 3160, 3980 and 5010 mg/kg bw (high dose: 4 males and 1 female). Survival time was 2-5 days. Toxic symptoms included collapse within 15 - 30 minutes after application, followed by recovery after several hours in some cases. Severe diarrhea, loss of appetite, salivation and dyspnea were also noted. Necropsy revealed inflammation of the gastric mucosa as well as renal and liver congestion. An LD_{50} of 3340 mg/kg bw was determined.

Stasenkova (1970) reported in a short review some LD_{50} values without further study details. The aim of this study was to compare three well-known stabilisers (derivatives of p-phenylenediamine), including 6PPD. White rats and white mice were dosed once (into the stomach) with 6PPD in sunflower oil. Determined LD_{50} values were 2500 mg/kg bw for white rats and 3200 ± 310 mg/kg bw for white mice. Animals showed marked narcotic effects and deaths occurred in the first three days.

In a recent OECD TG 423 study Wistar rats were exposed to 300 or 2000 mg/kg bw 6PPD in olive oil. No report and study details are available. An LD_{50} > 300 and < 2000 mg/kg bw was documented (Anonymous, 2007 cited from ECHA dissemination site).

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

Seven studies, reporting LD_{50} values from 893 to > 5000 mg/kg bw, are available. This range of values may be explained by the different ways of applying the lipophilic test substance (undiluted, diluted in oil, warmed) and therefore influencing the bioavailability.

In one well documented OECD TG 401 study (Anonymous, 1999a), rats were dosed with 0, 250, 500, 1000 and 2000 mg 6PPD/kg bw in corn oil. At the highest dose all animals died. Females appeared to be most sensitive with a LD_{50} = 893 mg/kg bw, compared to males with a LD_{50} = 1005 mg/kg bw.

For another guideline study (OECD TG 423) only limited information is available (Anonymous, 2007). However, the substance was dissolved in olive oil and resulted in LD_{50} > 300 < 2000 mg/kg bw in Wistar rats.

Two studies using warmed 6PPD (Anonymous, 1991 and 1973) for dosing (via gavage) in rats resulted in high values of LD_{50} = 3580 mg/kg bw and LD_{50} > 5000 mg/kg bw. For the remaining studies only limited information is available. Stasenkova (1970) applied 6PPD dissolved in sunflower oil and reported a LD_{50} = 2500 mg/kg bw in rats. Anonymous (1962) applied undiluted 6PPD, the resulting LD_{50} was 3340 mg/kg bw.

10.1.2 Comparison with the CLP criteria

According to Table 3.1.1 of Regulation (EC) No. 1272/2008 a substance shall be classified as

- Acute Tox 4 (oral) if the LD_{50}/ATE values are > 300 and ≤ 2000 mg/kg bw.

- Acute Tox 3 (oral) if the LD_{50}/ATE values are > 50 and ≤ 300 mg/kg bw.

Available studies, including three guideline and four non-guideline studies, gave a range of oral LD_{50} values from 893 to > 5000 mg/kg bw.

Inconsistency may be due to differences in the application of the test substance (undiluted, warmed, diluted in oil) influencing the bioavailability of 6PPD. In the OECD 401 guideline study the lipophilic substance 6PPD was diluted in corn oil and resulted in the lowest LD_{50} value of 893 mg/kg bw (females rats). This study was considered therefore as most relevant for classification and is supported by the OECD 423 study with LD_{50} > 300 < 2000 mg/kg bw.

10.1.3 Conclusion on classification and labelling for acute oral toxicity

Based on the lowest available LD_{50} of 893 mg/kg bw, derived for females rats in a well documented guideline study, a classification as Acute Tox 4, H302 is proposed.

An ATE value of 890 mg/kg bw has to be assigned.

10.2 Acute toxicity - dermal route

Not assessed in this dossier.

10.3 Acute toxicity - inhalation route

No data available.

10.4 Skin corrosion/irritation

Not assessed in this dossier.

10.5 Serious eye damage/eye irritation

Not assessed in this dossier.

10.6 Respiratory sensitisation

No data available.

10.7 Skin sensitisation

For evaluation of a potential skin sensitizing property of 6PPD two LLNAs, three GPMTs and two additional animal studies are available. Human data on skin sensitisation include thirteen patch tests, one case report and a field report.

Table 11: Summary table of animal studies on skin sensitisation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
LLNA OECD TG 429 GLP	Mouse, CBA/J (f), (8-12 weeks old) n=4/dose	6PPD Vehicle: acetone/olive oil (4:1 v/v) Solvent control: acetone/ olive oil (4:1) Positive control: alpha-hexylcinnamaldehyde 25%	10%, 25%, 50% 25 µl volume to the dorsum of each ear on days 1 – 3.	sensitizing SI (pooled radioactive incorporation treatment group / incorporation of pooled control): 10%: 1.55 25%: 12.37 50%: 12.38 Pos control: 3.16 EC3 = 12.06 %.	Anonymous (2007a) [according to REACH registration, study report not available]
LLNA: BrdU-ELISA (Method according to Yamano et al., 2003, similar to OECD 442B)	Mouse, Balb/c (f), (6-8 weeks old) n=4/group	6PPD (no info on vehicle)	0, 0.1 %, 0.3 %, 1%, 3 %	sensitizing not irritating BrdU incorporation index: 0.3%: 1.5 ± 0.2 1 %: 1.8 ± 0.3 3%: 2.3 ± 0.3 SI [cellularity index x BrdU incorporation index]:	Yamano et al. (2009) Klimisch 2

Method, guideline, deviations if any	**Species, strain, sex, no/group**	**Test substance**	**Dose levels duration of exposure**	**Results**	**Reference**
				0.3%: 1.35 1 %: 2.34 3%: 5.06 EC3 not given	
GPMT (Magnusson & Kligman)	Guinea pig, Hartley (f) n=20/dose	6PPD Pos control: p-phenylenediamine	Induction: intradermal: 0.5 % in olive oil + complete FCA one week later epicutaneous: 1% Vaseline Challenge: 0.05 % and 0.5% vaseline open epicutaneous Reading 48h after challenge	sensitizing 0.05 % 6PPD 10/20 positive reactions (50%) 0.5 % 6PPD 18/20 positive reactions (90%) Neg control group: 0.05%: 0/20 positive reactions 0.5%: 4/20 positive reaction Grading according Magnusson&Kligman Cross sensitisation	Herve-Bazin et al. (1977) Klimisch 2
GPMT (according to Nakamura et al., 1994; method described as almost in accordance with Magnusson & Kligman)	Guinea pig, Hartley (f), 5 to 6 weeks old n= 4/dose	6PPD and other p-Phenylenediamine (PPD)-related chemicals Vehicle: acetone	Induction: Intradermal 500 ppm in acteone Epicutaneous (occluded) 500 ppm in acteone Challenge: Epicutaneous occlusive 50 ppm (0.005 %) and 5000 ppm (0.5 %) in acetone Reading 48h after challenge	sensitizing 0.005 % (50 ppm): 4/4 positive reactions (100%), mean skin reaction score 3.5 0.5 % (5000 ppm): 4/4 positive reactions (100%), mean skin reaction score 6.25 Corss reactivity with other other p-Phenylenediamine (PPD)-related chemicals	Yamano et al. (2009) Klimisch 2
GPMT (similar to OECD TG 406) GLP	Guinea pigs n=15/dose	6PPD Vehicle: polyethylene glycol 400	Induction: intradermal 1 % + FCA, epicutaneous, occlusive (48h):	sensitizing 12,5% - 1st reading (24h after patch removal):	Anonymous (1980a) Klimisch 2

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
			2% Challenge (after 2 weeks): epicutaneous, occlusive (24h) 12.5% and 25% Scoring 24h and 48h after patch removal	Neg control: 0/15 [average grading score: 0.0] Test group: 6/15 (40%) [average grading score: 0.4] 12,5% - 2nd reading (48h) Neg control: 0/15 [average grading score: 0.0] Test group: 3/15 (20%) [average grading score: 0.2] 25% - 1st reading (24h): Neg control: 2/15 [average grading score: 0.1] Test group: 15/15 (100%) [average grading score: 1.4] 25% - 2nd reading (48h) Neg control: 2/15 [average grading score: 0.1] Test group: 14/15 (93%) [average grading score: 1.3]	
-	Guinea pigs n=45/dose	6PPD No data on vehicle	Daily application of a 50 % paste over 20 days (clipped skin on the back) Challenge: concentrations of 10, 20, 30, 50 and 100 % were applied to new	No sensitizing reaction Remark: IPPD (CAS 101-72-4) and 77PD (CAS 3081-14-9), known sensitisers[1], were also negative/slightly positive in this study	Stasenkova (1970) Klimisch 3

[1] IPPD: Harmonized classification as Skin Sens 1, H317; 77PD: Self-classified as Skin Sens 1, H317

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
			areas of the back		
TINA-Test	Guinea pigs	6PPD	Induction I (5 times): 0.5% 6PPD in olive oil + FCA (5 injections in neck and femur) Followed by Induction II: (4 times) 0.5% 6PPD in olive oil (injection, neck region) and 1% 6PPD in petrolatum (flank, occlusive, 24h) Challenge (on day 44): 0.5% 6PPD in petrolatum	sensitizing 6/20 positive	Ziegler (1987) [cited from OECD, 2004]

In a recent LLNA (Anonymous, 2007a), female mice (4/dose group) were exposed to 10%, 25%, 50% 6PPD (in 25 µl volume, vehicle acetone/olive oil (4:1 v/v)), which was applied to the dorsum of each ear. Application was done on day one, two and three. On day four and five no treatment was done. On day six the body weight was recorded and 250 µl of phosphate-buffered saline containing 20 µCi of 3H-methyl thymidine was injected into all test and control mice via the tail vein. Five hours later, the animals were killed. The draining auricular lymph nodes from each ear were excised and pooled in PBS for each experimental group (pooled treatment group approach). Clinical observations were documented daily (0: no effects, + weak effect, ++ moderate effect, +++ strong effect). For the determination of cell proliferation incorporation of 3H-methyl thymidine into the lymph node cells was measured by ß-scintillation counting on liquid scintillation analyzer as disintegration per minute (DPM). The incorporation was expressed as DPM/treatment group. The SI values were obtained by dividing the pooled radioactive incorporation for each treatment group by the incorporation of the pooled vehicle control group. The concentration eliciting a SI of 3 is identified as the EC3 value and is calculated by linear interpolation of point on the dose-response curve. Clinical observations documented no effects and during the study no decrease in body weights were noted (see Table 12). The SIs were determined to be 1.55 for 10 % 6PPD, 12.37 for 25 % and 12.38 for 50 %. The calculated EC_3 value was 12.06 %. After 6PPD-application, lymph node weight in comparison to control was increased (see Table 12). The greatest increase was seen at the 25% group. DPM were 359.9 for control and 556.8 for 10% 6PPD. For the positive control an SI of 3.16 and a DPM of 1136.7 was reported[2]. Full study report is not available, therefore registrants did not rank this study for reliability. However, as the study was done according to OECD TG 429, GLP and the results are well documented the study was rated for this dossier as reliable.

[2] For 25% and 50% an error in the reported DPM data has been identified. The values are therefore not presented in this report but were estimated (SI x DPM of control) to be around 4450.

Table 12: Mean body weights and lymph node weights after treatment with 6PPD (Anonymous, 2007a).

Treatment group	Initial weight (mean) [g]	Terminal weight (mean) [g]	Lymph node weight (mean) [g]
Vehicle control	22.52 ±0.048	23.74 ±0.056	0.0280
10 % 6PPD	22.44 ± 0.346	24.02 ± 0.784	0.0369
25% 6PPD	21.11 ±1.243	21.56 ± 1.117	0.0525
50% 6PPD	22.81 ± 1.146	23.36 ± 1.418	0.0498
Positive control	22.99 ± 2.019	24.08 ± 1.695	0.0498

In another LLNA: BrdU-ELISA (according to the method described in Yamano et al., 2003, similar to OECD TG 442B) six- to eight-week-old female BALB/c mice (4 per group) were investigated (Yamano et al, 2009). Irritation was judged by measuring ear thickness at the time of auricular lymph node excision. Total lymph node cell count in a treated animal divided by mean lymph node cell count in the control group was designated as the cellularity index (parameter not defined in OECD 442B). BrdU incorporation index (treated animal divided by control group) was determined. A stimulation index (SI) was calculated by multiplying the cellularity index by the BrdU incorporation index. Statistical analyses were done via Dunnett's or Steel's multiple comparison method. Results are presented in Table 13. A chemical was designated positive when a statistically significant increase in the SI value was obtained using a non-irritant dose (= minimum induction dose). 1% 6PPD resulted in a SI of 2.34 and 3 % in a SI of 5.06; study authors concluded that 6PPD produced a significant increase in the SI at a non-irritant dose with a minimum induction dose of 1%.

According to the TG OECD 442B the SI has to be derived by dividing the mean BrdU labelling index/mouse within each test chemical group and the positive control group by the mean BrdU labelling index for the solvent group. Based on the available documentation by Yamano et al (2009) this seems to be equal to the BrdU incorporation index as displayed in Table 13. Based on the criteria defined in OECD 442B result can be regarded as positive when SI ≥ 1.6. Therefore reaction at 1% 6PPD can be considered as positive, strengthened by a clear dose-response relationship. An EC_3 value was not calculated but may be >3% 6PPD based on the BrdU incorporation index.

Table 13: Results of LLNA with 6PPD (Yamano et al., 2009).

% 6PPD	Ear thickness index	Cellularity index [1]	BrdU incorporation index [2]	Cellularity index x BrdU incorporation index (here called stimulation index)
0	1.00 ± 0.03	1.0 ± 0.1	1.0 ± 0.2	1.0
0.1	0.99 ± 0.06	0.9 ± 0.1	1.1 ± 0.1	0.99
0.3	0.97 ± 0.03	0.9 ± 0.1	1.5 ± 0.2*	1.35
1	1.05 ± 0.02	1.3 ± 0.1*	1.8 ± 0.3**	**2.34**
3	1.02 ± 005	2.2 ± 0.2**	2.3 ± 0.3**	**5.06**

*, ** Significantly different from control, p<0.05 and 0.01, respectively.

Bold: values described as significant, but not defined further

[1] =Total lymph node cell count in a chemical-treated animal divided by mean lymph node cell count in the control group

[2] = BrdU incorporation (expressed as optical density in enzyme-linked immunosorbent assay) per well of cells from a chemical-treated animal divided by mean BrdU incorporation per well of cells from the control group

The sensitizing potential of 6PPD was also tested in a non-guideline GPMT (Herve-Bazin et al., 1977). Induction was done intradermal with 0.5% of the test substance in olive oil with complete FCA followed one week later by cutaneous application of 1% in vaseline. Challenge with 0.05% or 0.5% 6PPD in vaseline and grading according to Magnusson and Kligman resulted in positive reactions in 50% or 90% of guinea pigs,

respectively. 30% of animals sensitized to 6PPD showed cross-sensitization to 0.05% N-phenyl-N'-cyclohexyl-p-phenylenediamine (CPPD) in vaseline. Cross-sensitization to 6PPD was also documented for p-phenylenediamine (PPD) or to N-isopropyl-N‘-phenyl-p-phenylenediamine (IPPD) (see Table 14).

Table 14: Cross sensitization in guinea-pigs (Herve-Bazin et al., 1977).

% Animals cross sensitized	CPPD (0.05% in vaseline)	6PPD (0.05% in vaseline)	PPD (0.05% in vaseline)	IPPD (0.05% in vaseline)
CPPD	50	5	15	nd
6PPD	30	50	nd	nd
PPD	100	95	80	95
IPPD	90	85	100	10

In a second non-guideline GPMT (according to Nakamura, 1994 - test protocol not reported in detail but described as almost in accordance with the design of Mangusson & Kligman) the sensitizing potential of 6PPD and other p-phenylenediamine (PPD)-related chemicals[3] were investigated (Yamano et al., 2009). Guinea pigs (4 females/dose) were exposed intradermal to 500 ppm of the test substance in acetone followed by epicutaneous exposure to 500 ppm in acetone (induction). Two weeks after the second induction, 12 circles (16 mm in diameter) were marked on the dorsum of each animal, and the animals were challenged all at once with 50 ppm and 5000 ppm of the six sensitizers in 50 ml acetone (for 24h). 48h after application each site was scored for erythema (0 - 4) and oedema (0 - 3) based on the criteria of Sato (1981)[4]. Total group scores (erythema plus oedema) based on the same challenge concentration and chemical in each group were added up and divided by the number of animals in the group to give the mean response value as an index of skin reaction to challenge. 100% of guinea pigs showed positive skin reactions with scores of 3.5 and 6.25 after challenge with 50 ppm and 5000 ppm 6PPD, respectively. 6PPD was evaluated as strong sensitizer.

In another GPMT (similar to OECD TG 406) three substances were tested in parallel, including 6PPD (Anonymous, 1980a). For the first intracutaneous induction 1% of the test substance in polyethyleneglycol (+FCA) was used (in deviation to OECD 406). Second cutaneous induction one week later was done with 2% test substance in polyethyleneglycol, occlusive for 48h. Control groups were treated with vehicle (+FCA) only. Two weeks later each animal was treated (cutaneous, occlusive for 24h) with 6PPD in polyethyleneglycol (12.5%, 25%). Incidences and intensity of cutaneous reactions (grading from 0 to 3) were documented 24h and 48h after patch removal. Numbers are presented in Table 11. The authors concluded that the test substance is a sensitizer and stated that the finding was supported by histological examinations of the treated skin areas (no further information given).

A short description of a non-LLNA study is presented by Stasenkova (1970). The test substance in the form of a 50% paste was applied daily for twenty days to the clipped skin of the back of guinea pigs. No marked irritation was observed during repeated applications and general conditions of test animals did not differ from control. After twenty applications the test substance was applied in different concentrations (100, 50, 30, 20, and 10 %) to new areas of the back. No increase of sensitivity to 6PPD was observed. The similar substances IPPD (CAS 101-72-4) and 77PD (CAS 3081-14-9), known sensitisers[5], were also negative/slightly positive in this study; the study is therefore rated as Klimisch 3. No further details available.

[3] Investigated chemicals: 6PPD, N,N9-diphenyl-p-phenylenediamine (DPPD); N-isopropyl- N9-phenyl-p-phenylenediamine (IPPD); N-(1-methylheptyl)-N9-phenyl-p-phenylenediamine (MHPPD); p-phenylenediamine (PPD); p-aminodiphenylamine.

[4] Erythema formation: 0 – no erythema, 1 – very slight erythema, 2 – well defined erythema, 3 – moderate to severe erythema, 4 – severe erythema; Edema formation: 0 - no edema, 1 – slight edema, 2 – moderate edema, 3 – severe edema

[5] IPPD: Hamonized classification as Skin Sens 1, H317; 77PD: Self-classified as Skin Sens 1, H317

Positive results were obtained also in a study, not conducted according to a guideline, using the TINA test (Tierexperimenteller Nachweistest): induction took place with 0.5% 6PPD in olive oil (a mixture of 0.1 ml allergen and 0.1 ml Freund's complete adjuvant) by means of 5 intramuscular injections in the neck and femur regions of guinea pigs. On day 15, one flank was treated with 10% sodium dodecylsulfate in petrolatum, and on day 16 0.1 ml of a 0.5% 6PPD preparation in olive oil was injected into the neck region and 1% 6PPD in petrolatum was applied occlusively for 24 hours to the same region that had received the application of sodium dodecylsulfate. This treatment was repeated on days 22/23, 29/30 and 36/37. The challenge took place on day 44 by means of an occlusive application of 0.5% 6PPD in petrolatum, to which 6 of 20 animals reacted (cited from OECD, 2004).

Table 15: Summary table of human data on skin sensitisation

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
Human patch test	6PPD 2% in lanolin	Reading within 48h	Cross-sensitisation 15/15 IPPD-allergic patients tested positive with 6PPD	Herve-Bazin (1977)
Human patch test	6PPD	Investigation of 135 patients (from 1991-1995) with suspicion of contact dermatitis to rubber chemicals	6PPD: positive in 6/135 (4.4%) contact dermatitis patients Cross-sensitisation: 23/28 patients sensitized to IPPD showed also positive reactions to 6PPD	Heise (1997) [cited from REACH registration and OECD, 2004; limited data, original study not available]
Human patch test	6PPD 0.1% in pet	Readings after 30 min and 24h after patch removal (according to ICDRG criteria)	5/9 contact dermatitis patients showed a positive reaction to 6PPD cross-sensitization to N-isopropyl-N'-phenyl-p-phenylenediamine, p-phenylenediamine and p-aminoazobenzene is reported	Nishioka (1996) [cited from REACH registration and OECD, 2004]
Repeat Insult Patch Test	6PPD 50 % w/v in dimethylphthalate (for induction and challenge)	Patch 3 x 3 cm on upper arm Serie of 15 applications: exposure 24h – rest 24h Reading after patch removal and 24h later Grading on a scale of 0 – 4 Challenge after 2 weeks for 24h, readings after removal and 24h and 48h afterwards.	5/50 volunteers reacted positive No cross sensitisation to IPPD	Anonymous (1976a)
Repeat Insult Patch Test, Draize test	6PPD (with impurity 0.05% amino diphenyl anime) or 6PPD (with	0.2 ml on patch; occlusive for 48-72h Upper arms or back Application 3 times a week (for 3 weeks)	Negative for both grades	Anonymous (1978)

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
	impurity 2.5% amino diphenyl anime) 1% in petrolatum	Approx. 200 volunteers tested Challenge: two weeks later, for 48-72h Scoring 96h after application		
Repeat Insult Patch Test	6PPD	-	5/50 volunteers showed positive skin reactions	Anonymous (1974) [cited from ECHA dissemination site; original study not available]
Repeat Insult Patch Test	6PPD	-	0/50 volunteer subjects (not previously exposed to p-phenylenediamine-derivatives) reacted positive.	Anonymous (1973a) [cited from ECHA dissemination site; original study not available]
Repeat Insult Patch Test	6PPD, 0.1% w/v solution in dimethylphthalate	-	0/50 volunteers reacted positive	Anonymous (1972) [cited from ECHA dissemination site and OECD, 2004; original study not available]
Repeat Insult Patch Test (Shelanski method)	Rubber masterbatch + 6PPD (2 parts per hundred parts of rubber) (also several other anitdegradants were tested in this series)	Application: squares of the test material, 24h, to arms of 50 volunteers 15 applications to the same site Two week rest Challenge: for 24h	20/50 reacted positive to rubber formulation including 6PPD (1st experiment) 19/50 reacted positive to to rubber formulation including 6PPD (2nd experiment) 18/50 reacted positive to rubber formulation including 6PPD (from crude 4-NDPA)	Anonymous (1964a)
Repeat Insult Patch Test (Shelanski	Rubber masterbatch + 6PPD (2 parts per hundred parts of	Application: squares of the test material, 24h, to arms of 50 volunteers 15 applications to the	17/50 reacted positive to rubber formulation including 6PPD 16/50 reacted positive to rubber formulation including 6PPD (from	Anonymous (1964b)

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
method)	rubber) (also several other anitdegradants were tested in this series)	same site Two week rest Challenge: for 24h	crude 4-NDPA), unvucanized	
Repeat Insult Patch Test (Shelanski method)	Rubber masterbatch + 6PPD (2 parts per hundred parts of rubber) Vulcanised/non vulcanized	Application: squares of the test material, 24h, to arms of 50 volunteers 15 applications to the same site Two week rest Challenge: for 24h	0/50 reacted positive to rubber formulation including 6PPD, vulcanized 0/50 reacted positive to rubber formulation including 6PPD, unvulcanized	Anonymous (1964c)
Repeat Insult Patch Test (Shelanski method)	Rubber master batch + combination of antidegradants (including 6PPD)	Application: squares of the test material, 24h, to back of 50 volunteers 15 applications to the same site Two week rest Challenge: for 24h	negative	Anonymous (1963a)
Repeat Insult Patch Test (Shelanski method)	Rubber master batch + 6PPD (from crude 4-NDPA) (1, 2 or 3 parts per hundred parts of rubber)	Application: squares of the test material, 24h, to back of 50 volunteers 15 applications to the same site Two week rest Challenge: for 24h	0/50 positive reactions to rubber formulation including 6PPD (1 part per hundred) 3/50 positive reactions to rubber formulation including 6PPD (2 parts per hundred) 0/50 positive reactions to rubber formulation including 6PPD (3 parts per hundred)	Anonymous (1963b)
Case report	-	48-year old woman 16 years in rubber industry	Pruritus, erythema, vesicles contact allergies to 6PPD (2%), polymerized TMQ and IPPD	Hansson (1994)
Field report	-	Factory, exposure up to 32 years About 70 workers (with personal protection, safety instructions, technical ventilation)	No sensitization documented	Anonymous (2012)

Like for animals cross-sensitisation is also documented for humans by e.g. Herve-Bazin (1977). 15/15 IPPD-allergic patients were tested positive with 6PPD (concentration 2 % in lanolin; readings taken during the 48th hours). Heise (1997) presents a compilation of data from 135 patients (from 1991-1995) patch-tested with rubber additives. A positive reaction to 6PPD was documented for 6/135 contact dermatitis patients; partly

with cross-sensitizations (23 of 28 patients sensitized to IPPD showed also positive reactions to 6PPD). In the publication by Nishioka (1996) the results of patch testing (occlusive, Finn chambers for 48h) of 9 farmers with contact allergy due to rubber boots are reported. 19 rubber additives, including 6PPD (0.1% in pet.) were investigated. 5/9 contact dermatitis patients showed a positive reaction to 6PPD. In addition cross-sensitization to N-isopropyl-N'-phenyl-p-phenylenediamine, p-phenylenediamine and p-aminoazobenzene is reported.

In a human repeat insult patch test (HRIPT) (Anonymous, 1976a) 50 human volunteers were exposed to 6PPD (50 % w/v in dimethylphthalate). The patch (3 x 3 cm) was placed on the upper arm, covered and sealed with overlapping strips of Blenderm tape. After 24h of exposure the patch was removed and the site was examined and graded (scale 0 – 4). After a rest of 24h the site was re-examined and the test material was reapplied to the same site. In total 15 exposures (every Monday, Wednesday and Friday) were done with each volunteer. If significant irritation (≥2) was observed additional resting or application of the test substance to a new site was done. After these 15 applications and a resting period of two weeks a challenge with 6PPD (50 % w/v in dimethylphthalate for 24h, occlusive) was done. After patch removal sites were examined immediately, 24h and 48h later. The results are presented in Table 16. 5/50 individuals showed positive results after challenge. All individuals who had shown substantial reactions to 6PPD during the induction period or the challenge underwent a second challenge, this time using N-isopropyl-N'-phenyl-p-phenylenediamine (IPPD). The purpose of this second challenge was to ascertain if the individuals who had reacted to 6PPD were also allergic to IPPD. However, none of the tested individuals responded to this challenge.

Table 16: Number of reactions after repeated patch testing (Anonymous, 1976a).

No. of applications	Grading [number of reactions]				
	0	1	2	3	4
1 - 9	50	0	0	0	0
10	49	1	0	0	0
11	48	1	1	0	0
12	47	0	3	0	0
13	49	1	0	0	0
14	50	0	0	0	0
15	50	0	0	0	0
Challenge	45	0	1	4	0

Anonymous (1978) investigated the sensitizing potential of 6PPD in about 200 volunteers. 1% 6PPD in petrolatum was tested in two grades (impurity: 0.05 or 2.5% amino diphenyl anime). Applications (patch, occluded) were done three times a week (for 48-72h) and always on the same site on the upper arms or nacks. After 10 application and a rest periode of approximately two weeks a challenge application was made (occlusive, 48-72h). Grading (scoring from 0 – 4) was done 96 hours after application. No positive reactions are documented.

A 0.1% w/v solution of 6PPD in dimethylphthalate was investigated in a repeated insult patch test (Anonymous, 1972). 0/50 volunteers reacted positive.

In the REACH registration two studies (Anonymous, 1974 and 1973a) are cited reporting positive results after patch exposure to 6PPD in 5/50 and 0/50 volunteers, respectively. No further details available.

In 1964 several Repeated Insult Patch Tests to determine the effect of rubber formulations were conducted (Anonymous, 1964a and b). A rubber masterbatch was milled in different experiments with different antidegradants (e.g. 6PPD) in a proportion of 2 parts per hundred parts of rubber. The composition of the masterbatch was the same in each experiment. After vulcanization a square of the test material was applied to the arm of each subject and held in place with an adhesive tape for 24h. 50 volunteers were exposed and for each single experiment the same subjects were exposed. After removal the site was examined for reactions and afterwards the test material was reapplied to the same site. In events of great reactions a new site was chosen or the application was determined. After 15 applications a two weeks rest period followed. Then challenge exposure was applied as before. The results after challenge are presented in Table 17. Typically, the reactions extended beyond the area of contact.

Table 17: Human patch test results of rubber material after challenge exposure (Anonymous 1964a, b, c and 1963b).

Material tested	No. of subjects tested	Grading [number of reactions]				
		0	1+ (mild erythema)	2+ (well-defined erythema)	3+ (well-defined erythema + edema)	4+
Experiment series 1 (Anonymous 1964a) [on arms of volunteers]						
Blank (assumption: masterbatch without antidegradant) [sample #16-81-1]	50	46	4	0	0	0
Masterbatch + 6PPD (reference sample) (1st experiment) [sample #16-81-3]	50	30	4	14	2	0
Masterbatch + 6PPD (reference sample) (2nd experiment) [sample #16-81-9]	50	31	5	11	3	0
Masterbatch + 6PPD (from crude 4-NDPA) [sample #16-81-4]	50	32	5	9	4	0
Experiment series 2 (Anonymous 1964b) [on arms of volunteers]						
Masterbatch + 6PPD [sample #16-86-1]	50	33	5	6	6	0
Masterbatch + 6PPD (from crude 4-NDPA), non vulcanized [sample #16-86-6}	50	34	3	8	5	0
Experiment series 3 (Anonymous 1964c) [on arms of volunteers]						
Blank, unvulcanized (assumption: masterbatch without antidegradant) [sample #30-11-1]	100	100	0	0	0	0
Masterbatch + 6PPD, unvulcanised [sample #30-11-2]	50	50	0	0	0	0
Masterbatch + 6PPD, vulcanised [sample #30-11-3]	50	50	0	0	0	0
Experiment series 3 (Anonymous 1963b) [on back of volunteers]						
Masterbatch + 6PPD (1 part per hundred)	50	50	0	0	0	0
Masterbatch + 6PPD (2 parts per hundred)	50	47	3	0	0	0
Masterbatch + 6PPD (3 parts per hundred)	50	50	0	0	0	0

In a follow up experiment series (Anonymous, 1964c) 100 volunteers were tested using the same procedure and masterbatch as described for Anonymous (1964a and b). This experiment was set up to compare the effect of vulcanisation. Therefore, 50 subjects were exposed to blank and the unvulcanised 6PPD and 50 were exposed to blank and the vulcanized form of 6PPD. In contrast to the pervious experiments no reactions to any of the three rubber stocks could be detected (see Table 17).

In 1963 a Repeated Insult Patch Test Series with application of squares of test material to the back of volunteers was done. The test material consisted of the same masterbatches as for Anonymous (1964). While in Anonymous (1963a) added antidegradants were mixtures (no application of 6PPD only) in Anonymous (1963b) 6PPD only was added. Exposure procedure was as described for the series in 1964 (15 applications, 2 weeks rest, and challenge). All experiments with mixtures gave negative results (not presented in Table 17), no sensitisation was documented. Results with 6PPD were equivocal (see Table 17).

In the REACH registration some more results are displayed for the Patch Test Serie in 1963 and 1964, however, as no further description is available and further study details have not been provided, the reliability is questionable and the data has not been taken up into the CLH-Dossier.

To conclude, from these experiments it can be concluded that 6PPD increases the allergic reaction towards rubber formulations, however, due to several limitations (limited reporting, co-exposure to rubber ingredients, possibility of cross-sensitisation with other antidegradants in the course of the study) the results of these studies can only be used as supportive evidence. OECD (2004) concuded that in healthy volunteers not previously exposed to test rubber formulations, no sensitization or only a low sensitization rate to 6PPD was noted, while the sensitization rate was much higher in persons who had been previously sensitized to rubber samples.

The case of a 48 year old woman who had worked for 16 years in a rubber factory is described by Hansson (1994). Her new work included the weighing of rubber chemicals (powdered form or as pellets) and she wore leather gloves and a face mask. However, after a short period of time pruritus, erythema and vesicles developed on the uncovered skin of the hands and neck. Away from work symptoms recovered but relapsed when returning to work. Sensitisation was noted to 6PPD (1+ reaction to 2%), 2,2,4-trimethyl-1,2-dihydroquinoline and also to IPPD.

A field report from a manufacturing site in the EU documents experiences with the substance 6PPD over a period of 34 years (Anonymous, 2012; unpublished internal data). Airborne dust of 6PPD may arise from the manufacturing process and workers wear protective gloves, glasses, helmet, shoes and clothing. This is supported with regular safety instructions. Local exhaust ventilation at the filling station and efficient exhaust ventilation in the working area are also described. Medical checkups were made since 1982 for the about 70 workers. No sensitization was documented so far under the described conditions.

10.7.1 Short summary and overall relevance of the provided information on skin sensitisation

A LLNA according OECD TG 429 resulted in positive reactions in female mice and an EC_3 value of 12.06 % (Anonymous, 2007a). In a LLNA (Yamano, 2009) with a non-radioactive method (BrdU) 1% and 3 % 6PPD resulted in a BrdU incorporation index of 1.8 and 2.3, respectively. An EC_3 was not calculated but can assumed to be >3% 6PPD. Both experiments show a dose response.

Three well documented GPMTs with different vehicles and different test concentrations are available for 6PPD. In an older study (Herve-Bazin, 1977) challenge with 0.05% 6PPD resulted in 50% (10/20) positive reactions, while 0.5% resulted in 90% (18/20) positive. Yamano et al. (2009) documents in a publication 100% positive reactions (4/4) after exposure to either 0.005% or 0.5% 6PPD. However, in this study also several derivatives have been tested in parallel on the same animals, therefore cross-reactions cannot be excluded. Anonymous (1980a) challenged guinea pigs with 12.5% or 25% 6PPD resulting in 40% or 100% positive reactions after 24h, respectively.

A positive result was also obtained in a non-standard TINA-test in guinea pigs. Inductions via intramuscular injections followed by cutaneous exposure challenge with 0.5% 6PPD resulted in 30% (6/20) positive reactions.

Stasenkova (1970) reports a guinea pig test where daily application of a 50% paste followed by a challenge with different concentrations did not reveal any response. This test is not considered reliable as also the known sensitisers IPPD and 77PD were negative (or only slightly positive).

Several human patch tests are documented for 6PPD showing positive reactions to 6PPD (15/15 and 5/9 dermatitis patients reacted positive to 2% and 0.1% 6PPD, respectively) and cross-sensitisation (Herve-Bazin, 1977; Heise, 1997; Nishioka, 1996).

Repeat insult patch tests with 50% 6PPD (w/v in dimethylphthalate) gave positive results (5/50 volunteers), while 1% (in petrolatum) as well as 0.1% (w/v in dimethylphthalate) were negative (Anonymous, 1976a, 1978 and 1972). For several reported patch tests no exposure concentrations are documented (Anonymous, 1974, 1973a).

Several tests of rubber master batches including 6PPD (most of the time in concentrations of 2 parts per hundred parts rubber) gave positive as well as negative results (Anonymous, 1964 a, b, c, 1963 a, b). However, these data cannot be used for classification purposes as co-exposure and/or cross-sensitisation to several other chemicals has to be assumed.

In a case report, where a 48-year old women was exposed to rubber chemicals, the development of pruritus, erythema and vesicles was described, with a recovery of symptoms away from work. Patch testing with 2% 6PPD gave (beside other chemicals) a positive result.

10.7.2 Comparison with the CLP criteria

Category	Criteria
Category 1	Substances shall be classified as skin sensitisers (Category 1) where data are not sufficient for sub- categorisation in accordance with the following criteria: (a) if there is evidence in humans that the substance can lead to sensitisation by skin contact in a substantial number of persons; or (b) if there are positive results from an appropriate animal test
Subcategory 1A:	Substances showing a high frequency of occurrence in humans and/or a high potency in animals can be presumed to have the potential to produce significant sensitisation in humans. Severity of reaction may also be considered.
Subcategory 1B:	Substances showing a low to moderate frequency of occurrence in humans and/or a low to moderate potency in animals can be presumed to have the potential to produce sensitisation in humans. Severity of reaction may also be considered.

Further details on sub-categorisation based on human data are given in CLP, Annex I, 3.4.2.2.2.1. and 3.4.2.2.2.2.:

Human evidence for sub-category 1A can include	(a) positive responses at ≤ 500 μg/cm^2 (HRIPT, HMT — induction threshold); (b) diagnostic patch test data where there is a relatively high and substantial incidence of reactions in a defined population in relation to relatively low exposure; (c) other epidemiological evidence where there is a relatively high and substantial incidence of allergic contact dermatitis in relation to relatively low exposure.
Human evidence for sub-category 1B can include	a) positive responses at > 500 μg/cm^2 (HRIPT, HMT — induction threshold); (b) diagnostic patch test data where there is a relatively low but substantial incidence of reactions in a defined population in relation to relatively high exposure; (c) other epidemiological evidence where there is a relatively low but substantial incidence of allergic contact dermatitis in relation to relatively high exposure.

Sub-categorisation based on animal data according CLP, Annex I, 3.4.2.2.3.:

Sub-Category	Assay	Criteria
Sub-category 1A	Local lymph node assay	EC3 value ≤ 2 %
	Guinea pig maximisation test	≥ 30 % responding at ≤ 0,1 % intradermal induction dose or ≥ 60 % responding at > 0,1 % to ≤ 1 % intradermal induction dose
	Buehler assay	≥ 15 % responding at ≤ 0,2 % topical induction dose or ≥ 60 % responding at > 0,2 % to ≤ 20 % topical induction dose
Sub-category 1B	Local lymph node assay	EC3 value > 2 %
	Guinea pig maximisation test	≥ 30 % to < 60 % responding at > 0,1 % to ≤ 1 % intradermal induction dose or ≥ 30 % responding at > 1 % intradermal induction dose
	Buehler assay	≥ 15 % to < 60 % responding at > 0,2 % to ≤ 20 % topical induction dose or ≥ 15 % responding at > 20 % topical induction dose

6PPD shows clear positive results in animal studies. One LLNA resulted in an EC3 values of 12.06% (stimulation index at 25% 6PPD was 12.37) and another LLNA (BrdU-ELISA) resulted in an EC3 value >3% 6PPD. While the first test corresponds with subcategory 1B no guidance for sub-categorization based on LLNA (BrdU-ELISA) exists (ECHA, 2017).

In the three available GPMTs several concentrations in different vehicles were tested. All gave positive results and two of them indicate classification in subcategory 1A:

Test concentration (challenge)	Vehicle	Positive reactions	Reference
0.05 % 0.5 %	olive oil or vaseline	10/20 (**50%)** 18/20 (90%)	Herve-Bazin et al (1977)
0.005 % 0.5 %	acetone	4/4 (**100%**) 4/4 (100%)	Yamano et al, (2009)
12,5% 25%	polyethylene glycol	6/15 (40%) after 24h 3/15 (20%) after 48h 15/15 (100%) after 24h 14/15 (93%) after 48h	Anonymous (1980a)

A positive non guideline TINA-test, with induction and challenge in guinea pigs, supports the positive GPMT results.

In human studies sensitisation to 6PPD was documented several times in patch tests with dermatitis patients as well as in HRIPTs with volunteers. The documentation sometimes is very limited and only rather high or rather low concentrations have been tested. However, for HRIPTs it can be summarized that 50% 6PPD (w/v in dimethylphthalate) gave positive results in 5/50 (10%) volunteers, while 1% (in petrolatum) as well as

0.1% (w/v in dimethylphthalate) were negative. For human patch tests 15/15 and 5/9 dermatitis patients reacted positive to 2% and 0.1% 6PPD, respectively. Cross-sensitisation to other similar substances (e.g. IPPD, p-phenylenediamine and p-aminoazobenzene) was also documented. A case report describes symptoms like pruritus, erythema and vesicles after exposure to rubber chemicals (including 6PPD) supported by positive patch testing with 2% 6PPD.

Based on the GLP guidance (ECHA, 2017) for HRIPTs it can be summarize that sensitization is documented at relatively high exposure at high frequency in humans. Sub-categorisation is not possible based on these data.

Overall human and animal data clearly support that 6PPD is a sensitizer. Regarding potency one LLNA indicates lower potency while another LLNA, which cannot be directly compared to the criteria as it used BrdU for labelling, indicates high potency. Two of three GMPT tests also support high potency. For human patch tests in dermatitis patients positive reactions are documented with high frequency at relatively low exposure indicating classification in sub-category 1A.

Overall, it can be concluded that based on a weight of evidence analysis, the majority of the available human and animals results points towards high potency and classification in sub-category 1A.

10.7.3 Conclusion on classification and labelling for skin sensitisation

Available human and animal data clearly demonstrate the sensitizing property of 6PPD. Based on positive results in GPMTs at low doses supported by human evidence a classification as Skin Sens. 1A is proposed.

10.8 Germ cell mutagenicity

Not addressed in this dossier.

10.9 Carcinogenicity

Not addressed in this dossier.

10.10 Reproductive toxicity

10.10.1 Adverse effects on sexual function and fertility

For evaluation of adverse effects on sexual function and fertility an EOGRTS and an associated dose range finding study with rats is available. In addition two reproduction/developmental toxicity screening studies are reported. Additional information comes from a three generation study and an OECD 452 study. All studies have been done with rats (Sprague Dawley, Wistar). Study summaries are presented below and information relevant for repeated dose toxicity is presented in detail in Chapter 10.12.

Table 18: 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	Results	Reference
OECD 443 extended one-generation reproductive toxicity - with developmental neurotoxicity (Cohorts 1A, 1B	6PPD (purity 96.9%) 0, 7, 20, 60 mg/kg bw/d Oral, gavage Vehicle: corn oil 7d/week	NOAEL (parental tox) = 20 mg/kg bw/d NOAEL fertility (females) = 7 mg/kg bw/d NOAEL fertility (males) = 60 mg/kg bw/d **F0:** 60 mg/kg bw/d	Anonymous (2019b) Klimisch 1

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
without extension, 2A and 2B) GLP Rat, Crl:CD(SD) F0: N=25/sex/group High dose group: n= 30/sex/dose	F0 females: 70d+mating+gestation+lactation F0 males: like females F1: day of weaning – day prior to euthanasia (PND 21 [Cohort 2B], PND 91 [Cohort 1A], PND 78 [Cohort 2A], and PND 98 [Cohort 1B])	Mortality due to dystocia/prolonged labour (f: 5/30), total litter loss in 5 f abs. kidney weights ↑ (m +9.9% p<0.01; f + 8.6% not signif.) abs. liver weights ↑ (m +23.7%, p<0.01; f +27.5%, p<0.01) liver vacuolation (m, minimal to moderate), pigment deposition in kidneys (m, f; minimal to mild) <u>20 mg/kg bw/d</u> Mortality due to dystocia/prolonged labour (f: 2/25) rel. liver weights ↑ (m; +8.2%, p<0.05), liver vacuolation (m, minimal), pigment deposition in the kidneys (m, minimal) total litter loss in 1 f <u>7 mg/kg bw/d</u> liver vacuolation (m, minimal), pigment deposition in the kidneys (m, minimal) <u>control</u> total litter loss in 3 f **F1:** <u>60 mg/kg bw/d</u> Postnatal survival ↓ (PND 1-7) (not stat. signif. but above HCD), 111(20) pups (litters) found dead or were euthanized in extremis; 21/111 with no milk in stomach; mean bw on PND 21↓ (m -8.1% and f -7.4%, not stat. signif.) and on PND 28 mean bw remained lower (m-5.9%, f -8.6%, not stat. signif.), no effect on bw on PND 98 Cohort 1A: mean albumin↑ (m +11.9%, p<0.01; f +16.3%, p<0.01), total protein↑ (m +12.5%, p<0.01; f +15.4%, p<0.01), globulin↑ (m +13.6%, p<0.01; f +13.6%, p<0.05), cholesterol level ↑ (m +36.6%, p<0.01; f +32.5%, p<0.01) adrenal gland weights ↑ (rel to final bw: m +15.4%, p<0.01, f +20.8%, p<0.01), abs. and rel. kidney weight ↑ (rel. to final bw: m +10.6%, p<0.01; f +15.4%, p<0.01), abs. and rel. liver weight ↑ (rel to final bw: m +18.9%; f +30.5%, p<0.01) Cohort 2B (PND 21): brain weight ↓ (m, -7.3%, p<0.01) Cohort 2A (PND 78): brain weight↓ (m, -5.2%, not stat. signif.) <u>20 mg/kg bw/d</u> Postnatal survival ↓ (not signif) Cohort 1A: Mean cholesterol level ↑ (m +20.7%, p<0.05)	

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
		adrenal gland weights ↑ (f +14.8%, p <0.05), liver weight↑ (f + 15.3%, p<0.01)	
Dose-range finding study similar to OECD 421 GLP Rat, Crl:CD(SD) N= 15/sex/dose	6PPD (purity 96.9%) 0, 50, 75 and 100 mg/kg bw/day vehicle: corn oil m:28d f: 14 days prior to mating, continuing through mating, gestation, and lactation until lactation day 21 F1 pups: PND 21 through 49.	LOAEL (parental tox) = 50 mg/kg bw/d LOAEL fertility (females) = 50 mg/kg bw/d NOAEL fertility (males) = 100 mg/kg bw/d NOAEL (dev tox) = 75 mg/kg bw **F0:** <u>100 mg/kg bw</u> Dystocia/total litter loss (5), Gestation length↑ (p<0.05) Abs. liver weight↑ (m +26.1%, p<0.01; f +28.1%, p<0.01), abs. thyroid gland weights↑ (m + 31.2%, p<0.01) <u>75 mg/kg bw</u> Dystocia (1) Abs. liver weight↑ (m +25.0%, p<0.01; f +19.8%, p<0.01) <u>50 mg/kg bw</u> Dystocia (1), Gestation length↑ (p<0.05) Abs. liver weight↑ (m +16.2%, p<0.01) **F1**: <u>100 mg/kg bw</u> postnatal survival↓ (2 total litter losses due to poor maternal care), live litter size ↓ (-14.7%, not stat.signif.), bad clinical conditions of pups (thin or cold body), abs. liver weight PND50 ↑ (m +29.9%, p<0.01; f +41.8%, p<0.01), hepatocellular vacuolation (f, minimal/mild) <u>75 mg/kg bw</u> abs. liver weight PND50 ↑ (m +25.3%, p<0.01; f +32.4%, p<0.01), hepatocellular vacuolation (f, minimal) <u>50 mg/kg bw</u> anogenital distance↑ (f) abs. liver weight PND50 ↑ (m +18.6%, p<0.01; f +16.7%, p<0.01), hepatocellular vacuolation (f,	Anonymous (2019a) Klimisch 1

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
		minimal)	
OECD 421 (Reproduction/developmental toxicity screening test) GLP Rat, Crj:CD(SD) m/f n=12/sex/dose	6PPD (purity 99.4%) 0, 6, 25, 100 mg/kg bw/d Oral (gavage) Vehicle: corn oil M: 48d F: 14d before mating – day 3 of lactation No recovery group	NOAEL (parental tox) = 6 mg/kg bw/d NOAEL fertility = 25 mg/kg bw/d **F0:** <u>100 mg/kg bw/d</u>: duration of gestation↑ (22.7 ± 0.5; p ≤ 0.05) **F1:** <u>100 mg/kg bw/d:</u> body weights PND 0↑ (m +11.9%, p≤0.01, f +10.7%, p ≤ 0.05) body weights PND 4↑ (m +19.2%, not stat. signif, f +22.2%, p ≤ 0.05) External anomalies (in 1/131 pups; imperforated anus, pes varus and filamentous tail) No. of total (live) pups born ↓ (-28.4%, not statist signif.) <u>25 mg/kg bw/d:</u> body weights PND 0↑ (m +11.9%, p≤0.01, f +10.7%, p ≤ 0.05) body weights PND 4↑ (m +24.4%, p ≤ 0.05, f +27.8%, p ≤ 0.01) No. of total (live) pups born ↓(-19%, p≤0.05)	Tanaka (2001) Klimisch 2 [Japanese study, English summary]
Three generation study non GLP limited quality assurance documented (final comparison of the raw data with the final report missing; several limitations rats, Charles River CD N = 8m + 16 f (per group and	6PPD (Santoflex 13) 0, 100, 300 or 1000 ppm (approx. 0, 8, 23, 75 mg/kg bw) Oral (diet, ad libitum) (6PPD premixed in acetone)	NOAEL = 100 ppm (75 mg/kg bw/d) High mortality in control and dose groups <u>1000ppm</u> Relative liver weight (to body weight) was statistically significant increased in F1 (+34%, p<0.01) and F2 (+41%, p<0.05) generation	Anonymous (1980b) Klimisch 2 (by reg.) [short summary published by Stevens et al., 1981]

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
generation)			
OECD 452 Rat, Sprague-Dawley N= 70 rats/sex/group; at 12 months 20 rats/sex/group were sacrificed; after 24 months all survivors sacrificed	6PPD (Santoflex 13, 100% active ingredient) 0, 50, 250 or 1500 ppm (nominal in diet: males 2.6, 13.5 or 84.8 mg/kg bw, females 3.2, 16.5 or 109.5 mg/kg bw) Oral (feed) Duration of treatment ~730 days	NOEL (general toxicity) = 50 ppm (m: 2.6 mg/kg bw/d, f: 3.2 mg/kg bw/d) NOAEL (fertility) = 1500 ppm No changes in reproductive organs (weight, histology) reported	Anonymous (1993) Klimisch 2 [cited from ECHA disseminatio n site and a summary report, detailed data not available]
OECD 421 Reproduction/dev elopmental toxiciy screening study Rat, Wistar N=12/sex/dose Exposure duration males 28d, females 41-56d Evaluation by registrants: study has deficits in retrieval, presentation and interpretation of data which render reliability of data debatable	6PPD (Dusantox, purity: 97-98.7%) 0, 2.5, 12.5, 25 mg/kg bw/d Oral (gavage) Vehicle: olive oil	NOAEL (maternal tox) = 2.5 mg/kg bw/d <u>25 mg/kg bw/d</u> 1f died during delivery Trend of gestation length increase (2 f with 24 d) Total litter loss of 1 female <u>2.5 mg/kg bw/d</u> 1f died during delivery <u>control</u> 1f died during delivery	Anonymous (2009) Klimisch 4 (by reg.) [cited from ECHA disseminatio n site]

Remark: the study cited as No 006 in the registration (and referred as Anonymous 1981 in the IUCLID file) is, based on the data presented, the same as the three generation study Anonymous (1980b).

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

A recent **OECD 443 study (Anonymous, 2019b)** is available including cohorts 1A, 1B and 2A, 2B. Rats were exposed (oral, gavage) to 0, 7, 20 or 60 mg/kg bw/d (vehicle: corn oil, dose volume 5ml/kg) according to the dosing scheme for the relevant cohort (see Table 18). Dose levels were determined based on a dose-range-finding study (Anonymous, 2019a; described below). Five extra females (and corresponding males) were included in the high-dose group to accommodate potential higher losses in this group, based on observations of dystocia, moribundity, mortality, and total litter losses in the dose range-finding study. Concentration, homogeneity and stability of the test preparations has been analysed on a regular basis.

Viability of animals was checked twice daily, clinical observations prior to dosing and 3h post dosing. Body weights were determined weekly, for females during pregnancy and lactation every 3 or 4 days. Vaginal lavages of F0 females were performed daily (14d prior to cohabitation until mating; and on day of necropsy) to determine the stage of the estrus cycle. Sperm parameters (sperm motility, morphology) of each male were recorded. Litter weights were recorded on PND 1. The allocation of offspring is summarized in Table 19. Pups were sexed on PND 0, 4, 14, 21 and anogenital distance (AGD) was measured on PND 1. Thoracic nipples/areola of male pups were evaluated on PND 13. All parental animals were subjected to a complete necropsy examination at end of lactation. Offspring were examined (organ weights, tissue) according to the requirements of the cohorts. Culled pups were euthanized on PND 4. On PND 21, nonselected pups were subjected to a complete necropsy examination. For further details on termination procedures see Table 20.

Table 19: Study design - allocation of offspring (Anonymous 2019b).

Cohort	Number of pups selected	Parameters evaluated
1A	1/sex/litter/group → up to a total of 20/sex/group	Reproductive/developmental toxicity assessment
1B	1/sex/litter/group → up to a total of 25/sex/group	Follow-up reproductive assessment (not required)
2A	1/sex/litter/group → up to a total of 12/sex/group (representing as many litters as possible)	Neurobehavioral testing (startle response, motor activity, and FOB) PND 78 neuropathology
2B	1/litter → up to a total of 12/sex/group; representing ≥19 litters (representing as many litters as possible)	PND 21 neuropathology

Table 20: Terminal procedures (Anonymous 2019b).

Dose level [mg/kg bw/d]	No. of males	No. of females	Euthanasia day	Necropsy	Tissue collection	Organ weights	Histology	Histopathology
F0 – parental animals								
0	25	25	Day 129 - 132	x	x	x	Full tissues	Full tissues
7	24	25		x	x	x	Gross lesions, targeted/selected tissues[a]	Gross lesions, targeted/selected tissues
20	25	22		x	x	x	Gross lesions, targeted/selected tissues	Gross lesions, targeted/selected tissues
60	29	25		x	x	x	Full tissues	Full tissues
Unscheduled deaths				x	x	-	Full tissues	Full tissues
F1 – culled and nonselected pups								
0	84		PND 4 Culled pups	x	x[b]	-	-	-
7	87			x	x[b]	-	-	-
20	91			x	x[b]	-	-	-
60	77			x	x[b]	-	-	-
0	11		PND 21 Nonselected pups	x	x[c]	x[d]	-	-
7	20			x	x[c]	x[d]	-	-
20	12			x	x[c]	x[d]	-	-
60	12			x	x[c]	x[d]	-	-
Unscheduled deaths				x	x	-	-	-
F1 – Cohort 1								

0	18	19	PND 91 – cohort 1A	x	x	x	Full tissues	Full tissues
7	19	17		x	x	x	Gross lesions	Gross lesions
20	18	19		x	x	x	Gross lesions	Gross lesions
60	19	18		x	x	x	Full tissues	Full tissues
0	18	18	PND 98 – cohort 1B	x	x	x	-	-
7	19	18		x	x	x	-	-
20	18	18		x	x	x	-	-
60	19	19		x	x	x	-	-
Unscheduled deaths				x	x	-	Full tissues	Full tissues
F1 - Cohort 2A and 2B								
0	12	12	PND 21 – cohort 2B	x	x	x	Brain	Brain
7	12	12		x	x	x	-	-
20	12	12		x	x	x	-	-
60	12	11		x	x	x	Brain	Brain
0	10	12	PND 78 – cohort 2A	x	x	x	Nervous system tissues	Nervous system tissues
7	11	12		x	x	x	-	-
20	12	12		x	x	x	-	-
60	12	12		x	x	x	Nervous system tissues	Nervous system tissues

[a] reproductive organs from all animals suspected of reduced fertility
[b] thyroid gland with parathyroid, all gross lesions
[c] brain, mammary gland, spleen, thymus, thyroid, all gross lesions
[d] up to ten pups/sex/group; for brain, spleen, thymus and thyroid only

In F1 males balanopreputial separation was observed beginning on PND 35. F1 females were observed for vaginal perforation beginning on PND 25. Subsequently vaginal lavages were performed daily (cohort 1A) to determine estrous cycle of each female until first sign of estrus. For F1 animals (cohort 1A) hematology, serum chemistry and urinalysis were done on PND 91.

On animals assigned to cohort 2A an auditory startle response test was performed on PND 23, FOB findings were recorded on PND 65 and motor activity on PND 65. For animals perfused on PND 78 (cohort 2A) a broad range of tissues (brain, spinal cord, trigeminal ganglion/nerves, root fibres/ganglions, pituitary gland, caudal/sciatic/sural/tibial/peroneal nerves, nasal tissue, optic nerves, eyes, skeletal muscle) were examined to determine possible neurotoxicity of 6PPD. In addition, morphometric analysis on section of brain were performed.

Neuropathological evaluation of all major brain regions was performed for all animals of control and high dose perfused on PND 21 (cohort 2B).

For thyroid hormone evaluation (total T4, TSH), hematology, clinical chemistry und urinalysis samples were collected from ten F0 animals/sex/dose in week 19. For thyroid hormone evaluation in pups samples were collected on PND 4 from culled pups and PND 21 from non-selected pups as well as on PND 90 for other F1 animals.

Results of F0 generation:

Mortality was observed in males and females. One F0 male each in the 7 and 60 mg/kg bw/day groups died. The death of the males were considered not to be treatment related (low dose male without remarkable observations, high dose male killed due to swallowing of the dosing cannula). 3 and 5 F0 females in the 20 and 60 mg/kg bw/day groups, respectively, were found dead or were euthanized in extremis during the time of parturition or early lactation (gestation day 21 through lactation day 2). Dystocia and/or prolonged labor was identified as cause of moribundity; for information on relevant findings in these animals see Table 21. One additional female of the 20 mg/kg bw/d group died due to a gavage error. All others survived to the scheduled necropsy.

Table 21: Unscheduled deaths in F0 females (Anonymous, 2019b).

Female No.	Type and day of death	Clinical observations	Relevant macroscopic and microscopic§ findings
20 mg/kg bw			
9532	Found dead, GD 24	GD 23: decreased activity*, pale/cool body GD 24: yellow diarrhea around rectum*, delay in skin turgor*, unstable to walk*, dilated pupils*	Enlarged adrenal glands (adrenal cortical hypertrophy§), suppurative inflammation of lungs and uterus§, myeloid hyperplasia of bone marrow§, 14 dead fetuses in utero **cause of death: dystocia, septicemia**
9538	Found dead, GD 21	GD 20: pale body, pale ears and extremities*, hunched in corner at rest*	11 dead fetuses and 1 early resorption in utero **cause of death: dystocia**
60 mg/kg bw			
9548	Euthanized in extremis, LD 0	Postcohabitation Day 24 to LD 0: partially closed eyes Postcohabitation Day 25: hunched posture*, piloerection*, dermal atonia*, yellowish green diarrhea*, LD 0: unstable gait* Delivered 13 dead pups and 4 live pups	Dark red areas in stomach (erosion§), small thymus (lymphoid depletion§) **cause of moribundity: dystocia§**
9550	Euthanized in extremis, GD 22	GD 22: decreased activity*, severe delay in skin turgor/dehydration*, eyes partially closed*, yellow diarrhea*, pale*, cool to touch*	Enlarged adrenal glands (adrenal cortical hypertrophy§), pale kidneys (mineralization/cytoplasmic vacuolation§), white areas on the liver (vacuolation§), dark red areas in the stomach, small and pale spleen (red and white pulp atrophy§), small thymus (lymphoid depletion§), 16 live fetuses and 2 early resorptions in utero **cause of moribundity: dystocia§**
9555	Euthanized in extremis, LD 0	LD 0: cool body, eyes partially closed, red vaginal discharge, pale*, cool*, piloerection*, Delivered 1 dead pup and 2 live pups	Pale kidneys (mineralization/cytoplasmic vacuolation§), dark red areas in the stomach (erosion§), dark red uterine contents (dilation§), lymphoid depletion of thymus§, white pulp atrophy of spleen, 11 dead fetuses and 1 early resorption in utero **cause of moribundity: dystocia§**
9603	Euthanized in extremis, LD 2	LD 1: piloerection, severe delay in skin turgor*, Delivered 5 dead pups and 12 live pups (that subsequently died or were missing)	Pale and enlarged adrenal glands (adrenal cortical hypertrophy§), pale liver (vacuolation), small thymus (lymphoid depletion§), white pulp atrophy of spleen§, erosion of stomach§ **cause of moribundity: dystocia§**
9624	Euthanized in extremis, GD 24	GD 23 and/or 24: cool/pale body, piloerection, partially closed eyes*, yellowish diarrhea*, delay in skin turgor*, hunched*, pale*, cool to touch*	Enlarged adrenal glands (adrenal cortical hypertrophy§), pale kidneys (cytoplasmic vacuolation§), kidneys- rough surface (mineralization§), dark red contents in stomach, pale spleen (red and white pulp atrophy§), 14 live fetuses and 2 late resorptions in utero **cause of moribundity: dystocia§**

GD= Gestation day. LD= Lactation day. §= Microscopic findings. * Observations recorded by veterinary staff.

For survivers no test-substance related **clinical observation** were recorded. No test-substance related effects on mean **body weights, weight gains and food consumption** were observed. **Hematology** showed some statistically significant changes, however, findings were not considered test substance related. No correlating microscopic findings are documented. For further details see Chapter 10.12.

Mean **T4 levels** in the 20 and 60 mg/kg bw/day group males and females were statistically significantly higher than in the control group, but within the historical control data range (limitation is the limited number of animals for HCD). No significant changes in the TSH levels were observed. No effects on thyroid weights and no microscopic findings were documented.

Table 22: Total T4 and TSH levels in F0 animals, week 19 (mean±SD, %), n=10 per group (Anonymous, 2019b).

	Control	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d	HCD# for Crl:CD(SD) Rats
Males					
Total T4 [pg/ml]	44060.0 ± 7130.1	43760.0 ± 8874.3 -0.7 %	55560.0* ± 12115.8 26.1 %	60640.0** ± 9060.4 37.6 %	59218.0 ± 22344.3 [4 studies, 42 animals]
TSH [ng/ml]	7.8 ± 3.62	7.2 ± 3.34 -7.7 %	11.4 ± 6.65 46.2 %	10.4 ± 4.89 33.3 %	11.7 ± 3.7 [5 studies, 55 animals]
Females					
Total T4 [pg/ml]	36810.0 ± 8368.8	36230.0 ± 5652.7 -1.6 %	47500.0* ± 9808.2 29 %	54530.0** ± 11846.7 48.1 %	31306.7 ± 4637.6 [3 studies, 28 animals]
TSH [ng/ml]	5.2 ± 0.79§	5.2 ± 2.13 0.0 %	6.1 ± 2.72 17.3 %	7.1 ± 3.14 36.5 %	6.1 ± 1.9 [4 studies, 40 animals]

* p<0.05; ** p<0.01 (Dunnett´s test); § n=8
# HCD study date range 05/14 – 05/18 (OECD 421/422/443; CTA); males: from 4 studies with total 42 animals, females: from 3 studies with total 28 animals

Clinical chemistry, urinanalysis, gross pathology, organ weights and **histology** of F0 generation are discussed in Chapter 10.12 (specific target organ toxicity).

F0 sperm parameters (mean testicular and epididymal sperm numbers and sperm production rate, motility, progressive motility, and morphology) were not affected by treatment. Differences from control were slight and not statistically significant.

No treatment-related effects on the **reproductive performance** of F0 were observed (Table 23). 3, 3, and 2 males in the control, 7 and 20 mg/kg bw/day groups, respectively, did not sire a litter. 3, 4, and 2 females in these same respective groups were determined to be nongravid. The mean number of days between pairing and coitus as well as the mean lengths of estrous cycles in the test substance treated groups were similar to the control group values; there were no statistically significant differences. Gestation lengths for all substance treated groups were between 22.0 and 22.2 days and were similar to the control group mean of 22.0 days and the historical control data (21.8 days).

Dystocia and/or prolonged labour was identified as cause of death for 2 and 5 females in the 20 and 60 mg/kg bw/d groups, respectively. No signs of dystocia were noted at 7 mg/kg bw/d (see also Table 21).

Table 23: Reproductive performance of F0 generation (Anonymous, 2019b).

	0 mg/kg bw/d	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d	Historic. Control [mean and range]
Male mating index (%) [No of m with evidence of mating / total No of m x 100]	96.0	100.0	91.7	100.0	98.0 (83.3 – 100.0)
Female mating index (%) [No of f with conf. mating / total No of f x 100]	96.0	100.0	91.7	100.0	98.0 (83.3 – 100.0)
Male fertility index (%) [No of m siring a litter / total No of m x 100]	88.0	87.5	91.7	100.0	93.9 (80.0 – 100.0)
Female fertility index (%) [No of f with conf. pregnancy / total No of f x 100]	88.0	84.0	91.7	100.0	93.9 (80.0 – 100.0)
Male copulation index (%) [No of m siring a litter / No of m with evidence of mating x 100]	91.7	87.5	100.0	100.0	95.7 (80.0 – 100.0)
Female conception index (%) [No of f with conf. pregnancy / No of f with evidence of mating x 100]	91.7	84.0	100.0	100.0	95.7 (80.0 – 100.0)
Estrus Cycle Length (d)	4.2	4.2	4.4	4.3	4.2 (3.9 – 5.2)
Pre-coital Interval (d)	2.4	3.1	2.5	3.2	2.7 (1.4 - 4.5)

Results of F1 generation:

Mean live litter size, number of pups born and sex at birth was unaffected by treatment. Lower mean **postnatal survival** was reported at 60 mg/kg bw/d from PND 1 till PND 7 (see Table 24). The reduction was not statistically significant different from control, but the values were outside the historical control range of the laboratory and therefore considered relevant. Postnatal survival seems to be already lower at 20 mg/kg bw considering that in the control group the postnatal survival was lower than the HCD. **Total litter loss** (100% pup death) was reported for 3 (PND 0, 2, 9), 0, 1 (PND 2) and 5 (PND 2, 2, 4, 7, 9) females between LD 0 and 9 in the control, 7, 20, and 60 mg/kg bw/day group, respectively. The effect at the highest dose is considered test-substance related.

Table 24: PND 0 litter data and postnatal survival [mean ±SD, n] (Anonymous, 2019b).

	0 mg/kg bw/d	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d	Historic. Control * [mean and range]
PND 0 litter data (1)					
Number born	12.9 ± 4.14 22	13.6 ± 2.78 19	14.4 ± 2.37 20	13.3 ± 3.01 27	-
Sex at birth (% males per litter)	54.4 ± 12.70 21	50.1 ± 14.80 19	49.5 ± 12.89 20	52.3 ± 16.56 27	-
Live litter size PND 0	12.5 ± 4.34	13.5 ± 2.80	14.1 ± 2.39	12.4 ± 3.42	-

	22	19	20	27	
Postnatal survival [% per litter] (2)					
PND 0	92.4 ± 22.94 22	99.6 ± 1.76 19	97.6 ± 5.43 20	97.4 ± 7.16 25	-
PND 0 – PND 1	97.3 ± 4.69 21	98.6 ± 2.84 19	93.9 ± 16.32 20	90.1 ± 22.95 25	98.9 (89.1–100.0)
PND 1 – PND 4 (pre-selection)	91.1 ± 22.35 21	94.3 ± 15.43 19	89.9 ± 23.03 20	79.2 ± 33.31 25	98.3 (87.5–100.0)
PND 4 (post-selection) – PND 7	91.3 ± 16.27 20	94.9 ± 14.68 19	93.4 ± 12.75 19	83.1 ± 28.36 22	99.5 (96.7–100.0)
PND 7 – PND 14	91.9 ± 22.56 20	99.1 ± 3.82 19	91.9 ± 17.84 19	90.9 ± 22.96 21	-
PND 14 – PND 21	100.0 ± 0.00 19	100.0 ± 0.00 19	100.0 ± 0.00 19	98.0 ± 6.16 20	-
Birth – PND 4 (pre-selection)	84.2 ± 28.69 22	92.6 ± 15.16 19	85.2 ± 22.29 20	76.0 ± 32.64 25	95.7 (84.5–100.0)
PND 4 (post-selection) – PND 21	86.9 ± 24.49 20	94.2 ± 15.86 19	87.5 ± 20.83 19	77.9 ± 33.11 22	99.6 (95.8–100.0)

Statistics: (1) Dunn´s test for sex at birth; others: Dunnett´s modified statistics, (2) Dunn´s test
* HCD data: Crl:CD(SD), OECD 421/422/443; CTA, 06/2006 – 05/2018, in total 77 studies

General clinical condition of F1 pups was unaffected. 42 (13), 19(6), 45(14), and 111(20) pups (litters) were found dead or euthanized in extremis in the control, 7, 20, and 60 mg/kg bw/day groups, respectively. 18 (8), 9(5), 17(10), and 29(13) pups (litters) in the same respective groups were missing and presumed to have been cannibalized. Evaluation of this unscheduled deaths revealed no malformations in the 60 mg/kg bw/day group; the malformations and variations noted in a single pup in the 20 mg/kg bw/day group were not considered test-substance related. 21 of 111 unscheduled deaths in 60 mg/kg bw/d group had no milk present in the stomach (compared to 7/42 in control).

Mean male and female pup birth weights on PND 1 were comparable across all groups. Mean pup body weight gains in the 60 mg/kg bw/day group were slightly lower than the control group throughout the preweaning period (PND 1–21), resulting in mean **body weights** on PND 21 that were 8.1% and 7.4% lower for males and females, respectively. Bw and bw changes in the 20 mg/kg bw/d group were slightly lower than control, but the differences remain within 5%.

Anogenital distance was not statistically significant from control. No effects on **nipples/areolae** are documented (PND 13). No test-substance related effects on T4 (PND 4, 21) and TSH levels (PND 21) are reported.

No **internal findings** were documented for culled pups euthanized on PND 4 and non-selected pups euthanized on PND 21. At the necropsy of pups euthanized due to the death of dam, internal findings (including milk not present in the stomach, renal papillae not developed and distended ureter and distended urinary bladder) were noted for 5 pups in 2 litters in the 60 mg/kg bw/day group.

No test substance related effects on **organ weights** of F1 pups on PND 21 could be identified. The mean ages of attainment of **balanopreputial separation** in male were 47.7, 45.7, 47.0, and 47.1 days in the control, 7, 20, and 60 mg/kg bw/day groups, respectively. The mean ages of attainment of **vaginal patency** in females were 36.3, 35.0, 35.9, and 36.4 days in the control, 7, 20, and 60 mg/kg bw/day groups, respectively. The mean ages at the first occurrence of **estrus** were 38.3, 37.3, 37.8 and 39.5 days in the same respective groups and the duration from vaginal opening to first estrus were 2.7, 3.2, 2.4, and 2.8 days. None of these differences were statistically significant compared to control.

Evaluation of cohort 2A revealed no test substance related effects on **auditory startle responsiveness** (PND 23), **functional observational battery** (PND 65) and on **locomotor activity** (PND 65).

Results of F1 after weaning:

Survival of F1 generation following weaning was not affected by the test substance at any dose level. 3 males in the control group and 1 male and 1 female in the 7 mg/kg bw/day group were found dead. One male and 1 female in each of the control and 60 mg/kg bw/day groups were euthanized in extremis.

No test substance-related **clinical observations** were noted during the F1 generation at the daily examinations or approximately 3 hours postdosing. Findings in the treated groups included red/yellow/clear material, hair loss, and/or scabbing on various body surfaces. These effects occurred infrequently and/or at similar frequencies in the control group.

No test substance-related effects on mean **body weights** and body weight gains were noted in the 7 and 20 mg/kg bw/day groups. In the 60 mg/kg bw/d group lower mean absolute body weights on PND 21 were found (see above), due to the test substance related decrements in mean body weight gain at the end of the preweaning period. On PND 28 mean body weight remained lower in males (-5.9%) and females (-8.6%), however, differences were not statistically significant. At PND 98 no significant differences in mean body weights (m, f) compared to control are reported. Food consumption was not affected.

The mean lengths of **estrous cycles** for F1 females in treated groups during PND 75–91 were similar to the control group value.

Sperm parameters (mean testicular and epididymal sperm numbers and sperm production rate, motility, progressive motility, and morphology) of F1 males revealed no test substance related effects at any dose level. Differences from the control group were slight and were not statistically significant.

On PND 91 no substance-related significant effects on **thyroid hormones** in males and females could be detected. Relevant impaired parameters of **hematology** in males are presented in Table 25. No test substance related findings for females were reported. **Clinical chemistry** resulted in statistically signif. (mostly $p<0.01$) higher mean albumin (+11.9%, +16.3%), total protein (+12.5%, 15.4%), and globulin (+13.6%, 13.6%) concentrations in the 60 mg/kg bw/day group males and females, respectively. Mean cholesterol levels in the 20 and 60 mg/kg bw/day group males (+20.7%, +36.6%) and the 60 mg/kg bw/day group females (+32.5%) were higher than the control group. There were no test substance-related effects on **urinalysis** parameters.

Table 25: Hematology (selected parameters) of F1 generation, males, PND 91 [mean ± SD, %, n] (Anonymous, 2019b).

Mean ±SD	Control	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d
Males				
WBC [thous/µl]	10.98 ± 2.643 10	13.00 ± 3.510 18.4% 10	14.46 ± 2.674* 31.7% 10	14.75 ± 3.264* 34.3% 9
MCH [pg]	18.6 ± 0.58 10	18.1 ± 0.64 -2,7% 10	18.4 ± 0.68 -1.1% 10	17.6 ± 0.96 -5.4% 9
Retic [%]	3.2 ± 0.53 10	3.3 ± 0.94 3.1% 10	4.2 ± 0.75 * 31.3% 10	3.9 ± 0.72 21.9% 9
Retic. absolute [thous/µL]	248.3 ± 31.05 10	264.1 ± 56.47 6.4% 10	332.2 ± 57.76 ** 33.8% 10	322.2 ± 56.67 * 29.8% 9
Lymph. absolute [thous/µl]	8.31 ± 2.517 10	10.25 ± 3.254 23.3% 10	11.25 ± 2.827 35.4% 10	12.21 ± 3.115* 46.9% 9

* $p<0.05$, ** $p<0.01$ (Dunnett´s test)

Gross pathology revealed only dark red areas / dark discoloration in the lungs of some treated males (0/18, 3/19, 2/18, 3/19 and 0/18, 0/19, 1/18, 1/19, respectively) and in one female of the 20 mg/kg bw/d group.

Detailed examination of **organ weights** of the Cohort 1A revealed effects in adrenal gland weights, kidney and liver weight. For details see Table 26. Higher adrenal gland weights had no microscopic correlation and were within the historical control range. Higher kidney weights were also within the historical control range. For higher liver weights no microscopic correlation was found. Changes not presented in the table: (1) higher left cauda epididymis weights (relative to bw (+9.8%) and relative to brain weight (+10.1%); statistically significant in 60 mg/kg bw/day males; (2) higher (statistically significant) LABC muscle weight relative to final body weight in low dose males; and (3) higher (statistically significant) right testis weight relative to brain weight (+8.1%) in 60 mg/kg bw/day males. For left testis a relative to brain weight increase of 6.8% was seen (not significant).

No treatment related effects were reported for Cohort 1B with limited tissues (reproductive organs, pituitary gland) investigated.

Table 26: Final body and relevant organ weights of F1 generation, Cohort 1A (mean ±SD, %, n) (Anonymous, 2019b).

Mean ±SD	Control	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d
Males				
Final body weight [g]	485. ± 61.5 17	463. ± 55.1 -4.5% 19	470. ± 37.3 -3.1% 17	467. ± 48.9 -3.7% 19
Adrenal glands [g]	0.0623 ± 0.01119 18	0.0597 ± 0.01034 -4.2% 19	0.0586 ± 0.00816 -5.9% 18	0.0683 ± 0.01153 9.6% 19
Adrenal glands [g/100g final bw]	0.013 ± 0.0015 17	0.013 ± 0.0020 19	0.013 ± 0.0016 17	0.015 ± 0.0019**
Adrenal glands [g/100g brain]	2.975 ± 0.4360 18	2.876 ± 0.4862 19	2.840 ± 0.3828 18	3.386 ± 0.4909* 19
Kidney [g]	3.22 ± 0.439 18	3.15 ± 0.418 -2.2% 19	3.24 ± 0.341 0.6% 18	3.45 ± 0.429 7.1% 19
Kidney [g/100g final bw]	0.670 ± 0.0524 17	0.680 ± 0.0459 19	0.691 ± 0.0449 17	0.741 ± 0.0717** 19
Kidney [g/100g brain]	154.324 ± 18.3073 18	151.475 ± 17.3629 19	156.876 ± 17.8615 18	171.219 ± 14.8602* 19
Liver [g]	16.56 ± 2.465 18	16.33 ± 2.482 -1.4% 19	16.73 ± 2.386 1.0% 18	19.10 ± 2.086** 15.3% 19
Liver [g/100g final bw]	3.441 ± 0.2765 17	3.519 ± 0.2841 19	3.586 ± 0.3702 17	4.091 ± 0.2165** 19
Liver [g/100g brain]	793.458 ± 104.7698 18	784.096 ± 101.1956 19	810.512 ± 119.1736 18	948.965 ± 86.8768** 19
Females				
Final body weight [g]	265. ± 25.1 18	270. ± 23.6 1.9% 17	272. ± 30.3 2.6% 18	264. ± 24.1 -0.4% 18
Adrenal glands [g]	0.0636 ± 0.01190 19	0.0691 ± 0.01146 8.6% 17	0.0730 ± 0.00973* 14.8% 19	0.0775 ± 0.01306** 21.9% 18
Adrenal glands [g/100g final bw]	0.024 ± 0.0038 18	0.026 ± 0.0035 17	0.027 ± 0.0044 18	0.029 ± 0.0039** 18

Adrenal glands [g/100g brain]	3.294 ± 0.5556 19	3.532 ± 0.5329 17	3.839 ± 0.5629* 19	4.095 ± 0.6181** 18
Kidney [g]	1.88 ± 0.243 19	2.06 ± 0.295 9.6% 17	2.05 ± 0.290 9.0% 19	2.16 ± 0.369* 14.9% 18
Kidney [g/100g final bw]	0.709 ±0.0618 18	0.764 ± 0.0717 17	0.751 ± 0.0617 18	0.818 ± 0.1111** 18
Kidney [g/100g brain]	97.462 ± 10.5447 19	105.315 ± 11.7515 17	107.351 ± 9.6646 19	113.973 ± 17.5973** 18
Liver [g]	8.84 ± 1.080 19	9.65 ± 1.176 9.2% 17	10.19 ± 1.195** 15.3% 19	11.55 ± 1.480** 30.7% 18
Liver [g/100g final bw]	3.350 ± 0.2742 18	3.579 ± 0.3298 17	3.761 ± 0.3139** 18	4.371 ± 0.2705** 18
Liver [g/100g brain]	458.108 ± 43.4444 19	494.049 ± 58.8987 17	534.482 ± 59.7466** 19	611.171 ± 73.7302** 18

* p<0.05; ** p<0.01 (Dunnett´s test)

Histopathology revealed no treatment related findings in F1 males and females. Minimal to marked mineralization of the outer strip of the kidney medulla and variable degrees of basophilic tubules in the outer cortex were described for control and high dose females; these were considered secondary to the soy free 5K96 diet (Parker, 2018). There were no treatment-related effects on ovarian follicle counts in F1 females from Cohort 1A.

There was no clear evidence of developmental neurotoxicity. **Macroscopic examination** of Cohorts 2A and 2B did not reveal findings in brain and spinal cord at any dose level. In 60 mg/kg bw/d males (Cohort 2B, PND 21) a lower group mean absolute brain weight (-7.3%, p<0.01) was seen, while the relative brain weight to bw was similar to control. The final body weight in the high dose group was reduced -9.8% compared to control (see Table 27). According to the study authors the results were within the neurotoxic historical control range for PND 21 male Sprague Dawley rats (data not presented). In 60 mg/kg bw/d males of cohort 2A (PND 78) brain weight was also reduced (-5.2%, compared to final bw -3.5%), but not statistically significant (see Table 28). Females showed only slight effects (cohort 2B -1.9%, cohort 2A -2.8%).

Table 27: Mean brain weights of F1 Cohort 2B (PND 21) [mean ±SD] (Anonymous, 2019b).

Mean ±SD	Control	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d
Males				
No of male animals	12	12	12	12
Final body weight [g]	51.0	54.0	49.0	46.0*
Brain weight [g]	1.6565 ± 0.07639	1.6737 ± 0.08154	1.6301 ± 0.06797	1.5352** ± 0.09174
Females				
No of male animals	12	12	12	11
Final body weight [g]	49.0	52.0	49.0	47.0

Brain weight [g]	1.5769 ± 0.07087	1.5869 ± 0.11265	1.5726 ± 0.07990	1.5477 ± 0.09329

*p<0.05; **p<0.01

Table 28: Mean brain weights of F1 Cohort 2A (PND 78) [mean ±SD] (Anonymous, 2019b).

Mean ±SD	Control	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d
Males				
No of male animals	10	11	12	12
Final body weight [g]	457.0	452.0	474.0	441.0
Brain weight [g]	2.33 ± 0.097	2.33 ± 0.139	2.32 ± 0.124	2.21 ± 0.137
Females				
No of male animals	12	12	12	12
Final body weight [g]	259.0	258.0	277.0	268.0
Brain weight [g]	2.11 ± 0.159	2.13 ± 0.112	2.12 ± 0.080	2.05 ± 0.128

*p<0.05; **p<0.01

There were no **microscopic findings** in Cohort 2A and 2B animals. **Morphometric analysis** (only in control and high dose) revealed statistically significant lower width of the caudate-putamen (S3) (-6.2%, p<0.01) and lower thickness of the hippocampus (S5) (-5.1%, p<0.05) in the 60 mg/kg bw/day males at PND 78 (Cohort 2A). In general, for males and females a trend for lower values in the high dose group is seen (see Table 29), however, without statistical significance.

Table 29: Brain morphometry F1 Cohort 2A (PND 78), males and females [mean ±SD] (Anonymous, 2019b).

Mean [µm] ±SD	Control	60 mg/kg bw/d	Control	60 mg/kg bw/d
	Males		Females	
Number of animals	10	12	12	12
S1 – Thickness Frontal Cortex	2217 ± 158	2123 ± 148	2230 ± 113	2198 ± 109
S2 Thickness Parietal Cortex	2391 ± 149	2281 ± 137	2245 ± 138	2199 ± 109
S3 – Width Caudate Putamen	4293 ± 210	4028** ± 199	4203 ± 215	4120 ± 223
S4 – Thickness Corpus Callosum	355 ± 35	327 ± 66	335 ± 31	336 ± 52
S5 – Thickness Hippocampus	1712 ± 90	1624* ± 69	1558 ± 93	1550 ± 76
S6 – Height Cerebellum	6129 ± 356	5983 ± 294	5770 ± 350	5559 ± 345

*p<0.05; **p<0.01

For systemic toxicity in F0 a NOAEL of 20 mg/kg bw/d can be derived based on effects on organ weights (liver, kidney, adrenal glands) seen at 60 mg/kg bw/d. For female reproductive toxicity a NOAEL of 7 mg/kg bw can be derived based on dystocia seen at 20 and 60 mg/kg bw. For male reproductive toxicity a NOAEL of 60 mg/kg bw/d (highest dose tested) can be derived.

For discussion of effects relevant for developmental toxicity see Chapter 10.10.5.

In a **dose range finding study according to OECD 421** (Anonymous, 2019a) 10 week old Crl:CD(SD) rats were exposed to 0, 50, 75 or 100 mg/kg bw/d (oral, gavage) (homogeneity and concentration analyzed). On lactation day 13 exposure via milk was analysed. Mean concentrations of 6PPD in rat milk were 43.9, 53.2 and 59.5 ng/mL in the 50, 75, and 100 mg/kg bw/day groups, respectively.

Table 30: Terminal procedures (Anonymous, 2019a).

Dose level [mg/kg bw/d]	No. of males	No. of females	Euthanasia day	Necropsy	Tissue collection	Organ weights	Histology	Histopathology
F0 – parental animals								
0	15	15	Males: day 28 Females: lactation day 21*	x	x	x	Gross lesions, selected tissues	
50	14	13						
75	15	14						
100	15	12						
Unscheduled deaths				x	x	-	Full tissues	Full tissues
F1								
0	70		PND 4 (culled pups)	x	x	x	Gross lesions, selected tissues (liver, traches with thyroid gland/parathyroid)	
50	59							
75	45							
100	39							
0	46		PND 13	x	x	x	Gross lesions, selected tissues (liver, traches with thyroid gland/parathyroid)	
50	44							
75	39							
100	32							
0	54		PND 21 – non selected pups	x	x	x	Gross lesions, selected tissues (liver, thyroid gland with parathyroids)	
50	47							
75	41							
100	18							
Unscheduled deaths				x	x	-	-	-
0	15	15	PND 50	x	x	x	Gross lesions, selected tissues (liver, thyroid gland with parathyroids)	Selected Tissues (liver thyroid gland, all gross lesions)
50	15	15						
75	15	15						
100	15	13						

* Females with no evidence of mating or that failed to deliver were euthanized on Postcohabitation Day 25 or Postmating Day 25, respectively, and females with total litter loss were euthanized within 24 hours of litter loss.

Survival of males was unaffected. One female each in the 50 and 75 mg/kg bw/day groups was found dead and 1 and 3 females in the 50 and 100 mg/kg bw/day groups, respectively, were euthanized in extremis. One and 2 females in the 75 and 100 mg/kg bw/day groups, respectively, were euthanized due to total litter loss after the pups in these litters were euthanized in extremis due to poor maternal care. As cause of death/euthanasia several times dystocia is reported (see Table 31).

Table 31: Unscheduled deaths of F0 female rats (Anonymous, 2019a).

Animal number	Day		Bw changes/food consumption	Macroscopic and microscopic findings
50 mg/kg bw/d				
2994	LD 0, euthanized in extremis	Pale extremities, cool body, labored respiration, decreased defecation, unkempt appearance, red material around mouth, anogenital and urogenital areas; red vaginal discharge	11.2 % BW loss GD 20–22 0–1 g feed/day GD 20–22	Pale kidneys; white areas on the liver; 14 late resorptions in utero Mineralization in kidney; hepatocellular Centrilobular necrosis **dystocia**
2999	LD 10, found dead	-	-	Dark red contents in stomach; dark Red discoloration in lungs Pulmonary haemorrhage (cause of death) [gavage error]
75 mg/kg bw/d				
3038	LD 1, total litter loss	Red material on forelimbs, ventral trunk anogenital and urogenital areas, around mouth	-	-
3001	GD 23, found dead	-	-	12 dead foetuses in utero (cause of death:**dystocia**)
100 mg/kg bw/d				
3037	GD 23, euthanized in extremis	Pale extremities; pale body during parturition; cool body and extremities (no pups delivered)	10.8% BW loss GD 20–23 0-2 g feed/day GD 20–23	16 dead foetuses in utero (cause of euthanasia: **dystocia**)
3021	GD 24, euthanized in extremis	Red material on forelimbs, anogenital area, mouth; cool and pale body during parturition; vaginal discharge/ material (no pups delivered)	9.6% BW loss GD 21–24 / 0–1 g feed/day GD 22–24	15 late resorptions in utero (cause of euthanasia: **dystocia**)
3014	LD 0, euthanized in extremis	Prostrate; cool, pale body and extremities at daily or parturition examinations; red vaginal discharge; red material around mouth; diminished nursing behavior; delivered 2 live and 1 dead pup on GD 23	13.5% BW loss GD 21–23 1–9 g feed/day GD 21–23	11 dead foetuses in utero/vagina (cause of euthanasia: **dystocia**)
2969	LD 2, total litter loss	Pale, cool extremities, pale body , red materialarou nd nose and mouth, forelimbs, anogenital and urogenital areas	Body weights – no effect 0-7 g feed/day GD 20–LD 2	Pale adrenal glands
3005	LD 0, total litter loss	Pale, cool extremities, bale body, unkempt appearance, red material around nose and mouth forelimbs, anogenital and urogenital areas	-4.9% BW loss GD 21–22 4-11 g feed/day GD 20-22	-

FD = found dead. EE = euthanized in extremis. TLL = total litter loss. SD = Study Day; GD = Gestation Day; LD = Lactation Day.

No adverse effects on F0 **body weight or body weight gains** were reported in any dose group. Some statistically significant differences occurred but there was no dose-response and effects were transient. During gestation and lactation female bw and bw gain were generally similar to the control group with the exemption of dams that were euthanized in extremis or due to total litter loss. No adverse effects on food consumption are documented.

Reproductive performance is presented in Table 32. 2, 0, 0, and 2 males did not sire a litter and 2, 0, 0, and 2 females in the control 50, 75 and 200 mg/kg bw/d group, respectively, were determined to be nongravid.

Table 32: Reproductive performance of F0 generation (Anonymous, 2019a).

	0 mg/kg bw/d	50 mg/kg bw/d	75 mg/kg bw/d	100 mg/kg bw/d	Historic. Control [mean and range]
Male mating index (%) [No of m with evidence of mating / total No of m x 100]	100.0	100.0	100.0	93.3	97.9 (83.3 – 100.0)
Female mating index (%) [No of f with conf. mating / total No of f x 100]	100.0	100.0	100.0	93.3	97.9 (83.3 – 100.0)
Male fertility index (%) [No of m siring a litter / total No of m x 100]	86.7	100.0	100.0	86.7	94.0 (80.0 – 100.0)
Female fertility index (%) [No of f with conf. pregnancy / total No of f x 100]	86.7	100.0	100.0	86.7	94.0 (80.0 – 100.0)
Male copulation index (%) [No of m siring a litter / No of m with evidence of mating x 100]	86.7	100.0	100.0	92.9	95.9 (80.0 – 100.0)
Female conception index (%) [No of f with conf. pregnancy / No of f with evidence of mating x 100]	86.7	100.0	100.0	92.9	95.9 (80.0 – 100.0)
Estrus Cycle Length (d)	4.1	3.7	4.3	4.1	4.2 (3.9 – 5.2)
Pre-coital Interval (d)	1.9	2.3	2.8	1.9	2.7 (1.4 - 4.5)
Gestation length (d)	21.9 ± 0.28	22.5* ± 0.52	22.2 ± 0.60	22.5* ± 0.52	21.8

*Dunnett´s test, $p<0.05$

Gestation length was statistically significantly increased at 50 and 100 mg/kg bw/d. During the period of expected parturition, females were observed twice daily for initiation and completion of parturition and for dystocia or other difficulties. Delayed, difficult, and/or prolonged parturition was noted at all dosage levels. In summary distress-related observations of **dystocia** and pale/cool body during parturition were noted for 1, 1, and 5 females in the 50, 75, and 100 mg/kg bw/day groups, respectively, leading to the death, euthanasia, or total litter loss of these females (Table 31). These findings were noted in conjunction with pale and/or cool extremities, pale and/or cool body, unkempt appearance, prostration, red material on various body surfaces, and/or labored respiration or abnormal nesting behavior (scattered nest, diminished nursing) at the daily examinations for 1, 2, and 4 females in the 50, 75, and 100 mg/kg bw/day groups, respectively. The litters of

1 and 2 females in the 75 and 100 mg/kg bw/day groups, respectively, were euthanized due to maternal neglect.

Thyroid hormone analysis were done for males on study day 28 and females on lactation day 21. No significant differences were noted.

Investigation of **organ weights** showed higher mean liver weights (absolute and relative to final body weight and brain weight) in all treated dose groups of males and in the 75 and 100 mg/kg bw/day females. There were also test substance-related higher thyroid gland weights (absolute and relative to final body weight and brain weight) in 100 mg/kg bw/day males and higher group mean kidney weight relative to final body weight in 75 mg/kg bw/day males. For further details see Chapter 10.12. (STOT RE).

No test substance- related **microscopic findings** in males and females were found. Thyroid glands from each F0 animal were given a severity score for the degree of hypertrophy/hyperplasia of follicular cells, however, any variation in the degree of change in this parameter between dose groups was within the range of expected biological variability. According to study authors the reported slightly higher degree of hypertrophy/hyperplasia in 100 mg/kg bw males does not explain the higher group mean thyroid gland weights and there was no dose response for severity. For females there was higher grade of hypertrophy/hyperplasia of follicular cell epithelium of the thyroid glands in 50, 75 and 100 mg/kg bw/day dose groups, however, like in the males there was no dose response for severity (see Table 33).

There was a slight dose-response increased incidence of mononuclear cell infiltration (minimal/mild) in the prostate gland without disruption of the glandular architecture and without correlation with prostate weights; the incidences were within the HCDs. Liver was not examined microscopically. There were no effects on male and female reproductive tissues. Remaining findings were considered incidential.

Table 33: Incidence of histopathologic findings in thyroid gland and prostate gland of F0 animals [(number of tissues examined), incidence] (Anonymous, 2019a).

Finding	0 mg/kg bw/d	50 mg/kg bw/d	75 mg/kg bw/d	100 mg/kg bw/d
Males				
Thyroid gland, hypertrophy/hyperplasia, follicular cell	(15)	(14)	(15)	(15)
Minimal	12	6	4	7
Mild	3	8	9	7
Moderate	0	0	2	1
Prostate gland Infiltrate, mononuclear cell	(1)	(1)	(3)	(5)
Minimal	1	2	2	3
Mild	0	0	1	2
Females				
Thyroid gland, hypertrophy/hyperplasia, follicular cell	(12)	(11)	(12)	(8)
Minimal	6	0	0	2
Mild	6	10	7	4
Moderate	0	1	5	2

Results of F1 – generation

The mean number of pups born and percentage of males per litter at birth were similar to the control (see Table 34). F1 survival was affected by poor maternal care for litters from dams with dystocia or adverse clinical signs around parturition or expected parturition. On PND 0 the mean live litter size was lower in all

test substance-treated groups, however, the mean live litter size at 50 and 75 mg/kg bw/d were similar to the number of pups born. On PND 0 and on PND 0-4 postnatal survival was reduced, not statistically significant, in the high dose group. However, the value for PND 0 – 4 was lower than the minimum mean value in the Charles River Ashland historical control data. This decrease in postnatal survival in the 100 mg/kg bw/day group was due to 2 total litter losses on PND 0 or 2; these total litter losses were due to the euthanasia of pups following adverse clinical signs of thin and cool bodies resulting from poor maternal care.

Table 34: PND 0 litter data and postnatal survival [mean ±SD, n] (Anonymous, 2019a).

	0 mg/kg bw/d	50 mg/kg bw/d	70 mg/kg bw/d	100 mg/kg bw/d
PND 0 litter data				
Number born	15.6 ± 1.61 13	13.5 ± 5.01 15	13.8 ± 3.65 13	15.3 ± 2.45 # 10
Sex at birth (% males per litter)	46.3 ± 13.15 13	55.6 ± 17.65 15	48.0 ± 12.57 13	52.4 ± 11.73 11
Live litter size PND 0	15.6 ± 1.61 13	13.1 ± 5.08 15	13.5 ± 3.62 13	13.3 ± 5.12 # 10
Postnatal survival [% per litter]				
PND 0	100.0 ± 0.00 13	91.2 ± 25.68 15	98.1 ± 6.93 13	89.3 ± 31.46 10
PND 0 – PND 1	99.5 ± 1.85 13	99.2 ± 2.08 14	88.3 ± 29.41 13	100.0 ± 0.00 9
PND 1 – PND 4 (pre-selection)	99.1 ± 2.22 13	99.6 ± 1.67 14	99.5 ± 1.70 12	88.1 ± 33.12 9
PND 4 (post-selection) – PND 7	100.0 ± 0.00 13	98.6 ± 3.63 14	100.0 ± 0.00 12	97.5 ± 4.63 8
PND 7 – PND 14	100.0 ± 0.00 13	98.9 ± 3.96 13	100.0 ± 0.00 12	100.0 ± 0.00 8
PND 14 – PND 21	100.0 ± 0.00 13	100.0 ± 0.00 13	100.0 ± 0.00 12	100.0 ± 0.00 8
Birth – PND 4 (pre-selection)	98.6 ± 2.71 13	90.0 ± 25.46 15	87.8 ± 29.26 13	78.7 ± 41.67 10
PND 4 (post-selection) – PND 21	100.0 ± 0.00 13	96.8 ± 8.01 13	100.0 ± 0.00 12	95.8 ± 7.72 8

Statistics: Dunnetts test;
one female was euthanized in extremis on LD 0 with 10 dead fetuses in utero, not included in calculation of mean

The **clinical findings** are summarized in Table 35. In the high dose group, 11 (of 2 litters) and 14 pups (of 2 litters) were noted as thin or with a cool body, respectively. The general physical condition of F1 pups in the 50 and 75 mg/kg bw/day groups was similar to the control group.

Table 35: Clinical findings F1 pups (Anonymous, 2019a).

Total occurance/no. of pups (litters)	0 mg/kg bw/d	50 mg/kg bw/d	70 mg/kg bw/d	100 mg/kg bw/d
No of litters	(13)	(14)	(13)	(8)
Found dead, partially cannibalized	0 (0)	0 (0)	2 (1)	0 (0)
Found dead	1 (1)	6 (4)	16 (3)	16 (5)
Ethanized in extremis	0 (0)	1 (1)	5 (1)	18 (2)
Euthanized, death of dam	0 (0)	10 (1)	0 (0)	2 (1)
Missing	2 (2)	5 (3)	1 (1)	3 (2)
Scheduled euthanasia	100 (13)	91 (13)	80 (12)	50 (8)

Gross pathology of unscheduled deaths (found dead or euthanized in extremis) showed a dose-related increase in the number of pups with no milk in the stomach in all test substance-treated groups (see also Chapter 10.10.7). With the exception of 2 other pups from separate litters that were found dead on PND 0, this finding corresponded to poor maternal care. No other relevant internal findings in other pups examined.

The **anogenital distance** of all F1 pups was measured on PND 1. 50 mg/kg bw/d females showed a signif. difference (1.82mm*± 0.217) from control (1.64mm± 0 075) but no dose-response relationship.

On PND 13, all male pups were evaluated for the presence of thoracic **nipples/areola**. No retention was noted.

Pups were weighed individually on PND 1, 4, 7, 10, 13, 17, and 21 and on a regular basis in the post-weaning period. **Body weights** in dosed groups were generally similar to the control group throughout the postnatal period. Mean body weight gain was generally lower in the first 7 days but the only statistically significant differences were in the 75 mg/kg bw/day group males during PND 4–7 (-13%), in the 100 mg/kg bw/day group males and females during PND 10–13 (-11.3%, -11.4%), and in the 50 mg/kg bw/day group females during PND 10–13 (-8.8%). These changes were transient and did not result in statistically significantly lower mean absolute pup weights. In the post-weaning period mean F1 body weights and body weight gains in the test groups were generally similar to the control group throughout the generation (with the exception of some differences on some points in time which were transient).

In F1 pups **thyroid hormones** were investigated on PND 4, PND 13 and PND 21 as well as PND 50. The results are displayed in the following table. No clear trend can be seen, however, also taking into account the results from Anonymous (2019b), a disturbance of the thyroid axis by 6PPD can be assumed (and maybe relevant for a future ED classification).

Table 36: Thyroid hormone values of F1 pups [mean ± SD, % difference, n] (Anonymous, 2019a).

	0 mg/kg bw/d	50 mg/kg bw/d	75 mg/kg bw/d	100 mg/kg bw/d
PND 4 – culled pups				
Total T3 [pg/ml]	241. ± 40.4 - 10	198.* ± 43.8 -17.8% 10	159.* ± 18.1 -34.0% 9	177.** ± 25.6 -26.6% 8
Total T4 [pg/ml]	21180. ± 2996.2 - 10	21120. ± 4217.9 -0.3% 10	19689. ± 4930.4 -7.0% 9	18950. ± 4421.1 -10.5% 8
TSH [ng/ml]	5.3 ± 1.94 - 9	4.0 ± 0.92 -24.5% 10	4.4 ± 1.64 -17.0% 9	4.6 ± 0.84 -13.2% 8
PND 13 pups - males				
Total T3 [pg/ml)	573. ± 106.6 - 10	477. ± 101.9 -16.8% 10	632. ± 83.8 10.3% 9	677. ± 111.2 18.2% 8
Total T4 [pg/ml)	120340. ± 26611.6 - 10	110180 ± 37125.0 -8.4% 10	120433. ± 26797.2 0.1% 9	103550. ± 43337.6 -14.0% 8
TSH [ng/ml]	4.7 ± 1.38 - 10	5.0 ± 0.89 6.4% 10	6.2 ± 1.99 31.9% 10	4.8 ± 2.13 2.1% 8
PND 13 pups - females				
Total T3 [pg/ml)	538. ± 135.2 - 10	478. ± 51.2 -11.2% 10	637. ± 97.6 18.4% 10	655. ± 161.1 21.7% 8
Total T4 [pg/ml)	91680. ± 50536.9 - 10	110800. ± 37444.1 20.9% 10	116820. ± 34681.7 27.4% 10	95463. ± 49082.6 4.1% 8
TSH [ng/ml]	5.0 ± 1.45 -	5.2 ± 1.63 4.0%	6.0 ± 1.76 20.0%	5.2 ± 3.30 4.0%

	10	10	10	8
PND 21 pups -males				
Total T3 [pg/ml)	842. ± 114.8 - 13	782. ± 109.7 -7.1% 13	880. ± 136.0 4.5% 12	887. ± 87.0 5.3% 8
Total T4 [pg/ml)	29792. ± 6199.1 - 13	29023. ± 6146.2 -2.6% 13	39583.** ± 9852.8 32.9% 12	35438. ± 6766.5 19.0% 8
TSH [ng/ml]	4.1 ± 0.99 - 13	4.4 ± 1.84 7.3% 12	5.1 ± 1.30 24.4% 11	4.2 ± 0.48 2.4% 8
PND 21 pups -females				
Total T3 [pg/ml)	796. ± 57.0 - 13	776. ± 89.2 -2.5% 13	841. ± 108.1 5.7% 12	907.* ± 130.8 13.9% 8
Total T4 [pg/ml)	35338. ± 10903.9 - 13	29400. ± 5825.5 -16.8% 13	35042. ± 7659.6 -0.8% 12	30800. ± 6601.3 -12.8% 8
TSH [ng/ml]	4.3 ± 1.05 - 13	3.9 ± 1.67 -9.3% 13	4.6 ± 1.45 7.0% 12	4.3 ± 1.13 0.0% 8
PND 50 - males				
Total T3 [pg/ml)	660. ±73.9 - 10	642. ± 80.3 -2.7% 10	542.* ±85.5 -17.9% 10	531.** ± 104.6 -19,5% 10
Total T4 [pg/ml)	42200. ± 6192.6 - 10	47030. ± 7873.5, 11.4% 10	54760.* ± 14474.1, 29.8% 10	44370. ± 7986.2 5.1% 10
TSH [ng/ml]	6.4 ± 3.06 - 10	9.5 ± 5.04 48.4% 9	8.3 ± 3.67 29.7% 8	6.8 ± 4.34 6.3% 10
PND 50 - females				
Total T3 [pg/ml)	686. ± 130.4 - 10	681. ± 176.7 -0.7% 10	659. ± 172.8 -3,9% 10	571. ± 127.5 -16.8% 10
Total T4 [pg/ml)	35310. ± 9034.2 - 10	36980. ± 13225.7, 4.7% 10	46560. ± 28729.3, 31.9% 10	35580. ± 11146.6, 0.8% 10
TSH [ng/ml]	4.6 ± 2.63 - 9	4.9 ± 1.66 6.5% 10	4.7 ± 1.44 2.2% 10	4.6 ± 1.94 0.0% 10

Test substance related effects on **liver weights** of pups were documented for all investigated timepoints. In general there were no corresponding microscopic changes in the liver.

- At PND 4 statistically significantly higher liver weight relative to final body weight was seen in 75 mg/kg bw/day males and females, and 100 mg/kg bw/day females. Changes were considered test substance-related even though some of the change in relative weights may have been attributed to 9.1% lower group mean final body weight in these same dose groups.
- At PND 13 there was statistically significantly higher liver weight relative to body weight in the 100 mg/kg bw/day males and 75 and 100 mg/kg bw/day females, with no change in the group mean absolute liver weights.
- At PND 21 statistically significantly higher liver weights relative to final body weight was reported in 50, 75 and 100 mg/kg bw/day males and females, with no statistically significant change in group mean absolute liver weights.
- Pups at PND 50 showed higher liver weights (absolute and relative to final body weight) in the 50, 75 and 100 mg/kg bw/day males and females. For further details see Table 37. This correlates to hepatocellular vacuolation in the liver of females (Table 38), an effect not seen in males.

Table 37: Final bodyweights and liver weights of F1 pups at PND 50 [mean ±SD, %, n] (Anonymous, 2019a).

Mean ±SD	0 mg/kg bw/d	50 mg/kg bw/d	75 mg/kg bw/d	100 mg/kg bw/d
F1 males – PND 50				
Final bodyweight [g]	296. ± 20.0 - 15	298. ± 22.0 0.7% 15	301. ± 29.5 29.5% 15	303. ± 19.8 19.8% 15
Liver [g]	15.02 ± 1.203 - 15	17.82** ± 1.395 18.6% 15	18.82** ± 2.436 25.3% 15	19.51** ± 2.078 29.9% 15
Liver [g/100g final bw]	5.076 ± 1.203 - 15	5.991** ± 0.3066 18.0% 15	6.249**±0.4855 23.1% 15	6.433**±0.4150 26.7% 15
F1 females – PND 50				
Final bodyweight [g]	206. ± 17.0 - 15]	198.0 ± 21.8 -3.9% 15	208. ± 13.0 1.0% 15	214. ± 22.1 3.9% 13
Liver [g]	9.96 ± 1.313 - 15	11.62** ± 1.451 16.7% 15	13.19** ± 0.993 32.4% 15	14.12** ± 1.784 41.8% 13
Liver [g/100g final bw]	4.826 ± 0.3430 - 15	5.863** ± 0.4305 21.5% 15	6.340** ± 0.2888 31.4% 15	6.587** ± 0.2837 36.5% 13

* p<0.05; ** p<0.01 (Dunnett´s test)

There were no test substance-related effects on the **microscopic appearance** of the thyroid glands, parathyroid glands or liver of male or female pups at PND 4, 13 and 21. Microscopic findings in thyroid glands of PND 50 males and females (see Table 38) were not considered test substance related. Test-substance related hepatocellular vacuolation in the liver was also documented; the content of the vacuoles was consistent with lipid.

Table 38: Incidence of histopathologic findings in thyroid gland and liver of F1 animals, PND 50 [(number of tissues examined), incidence] (Anonymous, 2019a).

Finding	0 mg/kg bw/d	50 mg/kg bw/d	75 mg/kg bw/d	100 mg/kg bw/d
F1 PND 50 - Males				
Thyroid gland, hypertrophy/hyperplasia, follicular cell	(15)	(15)	(15)	(15)
Minimal	0	1	1	1
Mild	11	9	8	8
Moderate	4	5	6	6
Liver, vacuolation, hepatocellular, periportal	(0)	(0)	(1)	(1)
Mild	0	0	1	1
F1 PND 50 - Females				
Thyroid gland, hypertrophy/hyperplasia, follicular cell	(15)	(15)	(15)	(13)
Minimal	9	9	3	3
Mild	6	5	11	10
Moderate	0	1	1	0

Liver, vacuolation, hepatocellular, periportal	(0)	(3)	(9)	(6)
Minimal	0	2	8	3
Mild	0	1	1	3

Each male was observed for **balanopreputial separation** beginning on PND 35. No statistically significant differences were observed. The mean ages of attainment of balanopreputial separation were 43.4, 42.7, 43.6, and 42.1 days in the control, 50, 75, and 100 mg/kg bw/day groups, respectively. The mean body weights were 232.8 g, 231.5 g, 240.2 g, and 225.1 g in the same respective groups.

Each female was observed for **vaginal perforation** beginning on PND 25. The mean ages of attainment of vaginal patency were 33.4, 33.3, 32.5, and 33.2 days in the control 50, 75, and 100 mg/kg bw/day groups. Mean body weights at the age of attainment were 124.5 g, 121.5g, 118.4 g, and 123.4 g in the same respective groups.

For the dose range-finding study it can be concluded that dystocia/adverse clinical signs during parturition in 1, 1, and 5 females in the 50, 75 and 100 mg/kg bw group lead to death, euthanasia, or total litter loss of these females. Gestation length was statistically increased at 50 and 100 mg/kg bw/d. F1 survival was also affected due to poor maternal care or adverse clinical signs around parturition. Absence of milk was documented in a dose dependant manner. No effects on development of F1 pups was reported. Higher liver weight in 50, 75 and 100 mg/kg bw/day F0 males and 75 and 100 mg/kg bw/day F0 females were considered test substance related. Increased liver weight was also seen in the F1 generation (PND 50), in F1 females accompanied with microscopic findings. Based on these results a LOAEL (parental tox) of 50 mg/kg bw/d can be derived. For effects on female fertility (dystocia) a LOAEL of 50 mg/kg bw/d can be established.

In a **reproduction/developmental toxicity screening test** according to OECD TG 421 (Tanaka, 2001) ten week old rats were exposed to 0, 6, 25 or 100 mg 6PPD/kg bw/d. Males were exposed for 48 days, females 14 days before mating until day 3 of lactation. Body weight and food consumption was examined on a regular basis. Organ weights of liver, adrenals, testes, epididymides were determined. Macroscopical examination of liver and reproductive organs and microscopical examination of liver, kidney, skin, and reproductive organs are documented.

No effects on body weights of males and females are reported. Food consumption was increased in high-dosed males at some points in time and in all high dosed females during lactation. At the end of exposure absolute and relative liver and adrenal weights were elevated in high dose males. In females absolute and relative liver weights were increased in the mid and high dose groups. No effects on testes and epididymides weights are documented. For further details on repeated dose toxicity see Chapter 10.12.

For evaluation of the reproductive performance copulation index, fertility index, estrus cycle length, duration of gestation, number of corpora lutea, of implantations, of pups born, of live pups born, and of live pups on day 4, sex ratio, gestation index, implantation index, delivery index, live birth index, viability index on day 4, body weight change of pups were examined. Reproductive parameters are presented in Table 39. The **duration of gestation** was significantly increased ($p \leq 0.05$) at the highest dose. The number of total (live) pups born was reduced in the 25 mg/kg bw/d group ($p \leq 0.05$). There were no effects on estrus cycle and no effects on sex ratio.

Body weight increases of F1 pups were statistically significant at 25 and 100 mg/kg bw/d (see Table 40). All live pubs were examined for abnormalities. External anomalies were found in 1/131 pups of the highest dose, showing imperforated anus, pes varus and filamentous tail.

The NOEL for parenal toxicity was considered to be 6 mg/kg bw/d based on liver effects at 25 mg/kg bw/d in both sexes. Considering the prolonged gestation length the NOAEL for fertility was 25 mg/kg bw/d, for development 100 mg/kg bw/d, based on the lack of evidence.

Table 39: Reproductive parameters and delivery findings (Tanaka, 2001).

	0 mg/kg bw/d	6 mg/kg bw/d	25 mg/kg bw/d	100 mg/kg bw/d
Fertility				
No. of pairs mated	12	12	12	12
No .of pairs copulated	12	12	12	12
No. of pregnant females	12	11	12	11
Copulation index (%) [1)]	100.0	100.0	100.0	100.0
Fertility index (%) [2)]	100.0	91.7	100.0	91.7
Findings of delivery				
No of dams observed	12	11	12	10
No of dams delivered live pups	12	11	12	10
Duration of gestation [d]	22.2 ± 0.4	22.3 ± 0.5	22.4 ± 0.5	22.7 ± 0.5*
No. of total corpora lutea (mean ± SD)	241 (20.1 ± 4.4)	198 (18.0 ± 2.3)	203 (16.9 ± 3.0)	181 (18.1 ± 3.2)
No. of total implants(mean ± SD)	195 (16.3 ± 1.9)	167 (15.2 ± 1.0)	166 (13.8 ± 2.8)	150 (15.0 ± 1.6)
No. of total pups born (mean ± SD)	184 (15.3 ± 2.5)	160 (14.5 ± 1.0)	149 (12.4 ± 2.4)*	131 (13.1 ± 2.9)
No. of total live pups born (mean ± SD)	183 (15.3 ± 2.5)	158 (14.4 ± 0.9)	148 (12.3 ± 2.3)*	131 (13.1 ± 2.9)
Gestation index [%] [3)]	100.0	100.0	100.0	90.0
Implantation index [%] [4)]	82.9 ± 12.3	85.2 ± 9.0	82.4 ± 13.9	84.2 ± 10.8
Delivery index [%] [5)]	93.9 ± 7.8	95.9 ± 4.2	90.3 ± 7.4	87.8 ± 18.0
Live birth index [%] [6)]	99.5 ± 1.7	89.8 ± 2.6	99.4 ± 2.1	100.0 ± 0.0

** significantly different from control, p≤0.01; , * significantly different from control, p≤0.05
[1)] (No. of animals with successful copulation / No. of animals mated) x 100
[2)] (No. of pregnant animals / No. of animals with successful copulation) x 100
[3)] (No. of females with live pups / No. of pregnant females) x 100
[4)] (No. of implants / No. of corpora lutea) x 100
[5)] (No. of pups born / No. of implants) x 100
[6)] (No. of live pups born / No. of pups born) x 100

Table 40: Body weight changes [g] of F1 pups after subchronic exposure to 6PPD (Tanaka, 2001).

[mean ± SD, %]	Days after birth	0 mg/kg bw/d	6 mg/kg bw/d	25 mg/kg bw/d	100 mg/kg bw/d
Males	0	5.9 ± 0.4 -	6.1 ± 0.5 3.4%	6.6 ± 0.7** 11.9%	6.6 ± 0.5** 11.9%
	4	7.8 ± 1.6 -	8.6 ± 1.4 10.3%	9.7 ± 1.4* 24.4%	9.3 ± 1.5 19.2%
Females	0	5.6 ± 0.4 -	5.8 ± 0.5 3.6%	6.2 ± 0.6* 10.7%	6.2 ± 0.5* 10.7%
	4	7.2 ± 1.8 -	8.3 ± 1.2 15.3%	9.2 ± 1.4** 27.8%	8.8 ± 1.5* 22.2%

** significantly different from control, p≤0.01; , * significantly different from control, p≤0.05

In a **three generation reproductive study** (Anonymous, 1980b) Charles River CD rats (groups of 8 males and 16 females) were exposed to 6PPD concentrations of 0, 100, 300 or 1000 ppm (approx. 0, 8, 23, 75 mg/kg bw/d) via diet (ad libitum). Animals were allowed to reach maturity, mate, and produce two litters (F1a, F1b). 8 males and 16 females were retained at weaning from the second litters (F1b) of each group to serve as parental animals of the next generation (F2a, F2b). Again 8 males and 16 females were retained at weaning from the second litters (F2b) of each group to serve as parental animals of the next generation (F3a, F3b). The first litters (a) in each generation as well as excess pups of the second litter were weaned 21 days

postpartum, sacrificed and discarded in the absence of untoward development. Pups with abnormal findings were subject to gross pathology. Complete gross pathology and histologic examination was done only for 10 male and 10 female pups of the F3b litters (control and high dose).

Parental animals were weighed weekly in the pre-mating period and final bw were determined. Food intake measurements were conducted at 3 intervals (week 4, 5, 9) during the F0 generation's pre-mating period for several animals of each dietary group. Daily observations of each generation for mortality and behavioral reactions were made. Animals were also observed for fertility, gestation time, and lactation performance. For unscheduled deaths a complete necropsy was performed. After the second litter all parental males and females were sacrified and subject to gross pathology. Next to final body weight also weight of brain, gonads, heart, kidneys, liver and spleen were recorded. Microscopic investigations of a full list of organs/tissues were done for 5 males and 5 females from control and high dose group as well as selected animals.

Progeny were examined for anomalies at birth, viability, number of stillborn/cannibalized at the day of parturition. Survival on lactation days 1, 4, 12 and 21 were maintained. Litters with more than 10 pups were reduced to 10 on lactation day 4. Pup development during lactation was recorded. Body weight and sex was determined of each pup surviving to weaning.

In the study protocol several shortcomings are documented: (1) no quality assurance review, (2) general high mortality data in control and dose groups, (3) no evidence that all pups with abnormal development were subject to gross pathology, (4) only partly randomized procedures for selection of animals, (5) documentation of replacement animals inconsistent. (6) acetone has been used as solvent for 6PPD, the diet of the control group contained acetone from the fifth week on. (7) A large number of treated and control F0 animals were reported as possibly having respiratory infection. In addition, the number of females was lower than for a study according to OECD 416 (recommended not less than 20), however, this may be compensated by the two litters (a and b) per female.

Mortality was documented for test and control animals during each generation, the numbers being unusually high in some instances. A correlation with the administration of the test substance cannot be made.

Table 41: Mortality data – males (Anonymous, 1980b).

Dose [ppm]	Pre-mating	Mating	Post-mating	Mating	Post-mating	Total incidence (total number of males per group)
	First mating			Second mating		
F0 males						
0	0	0	1	0	1	2 (8)
100	0	2	0	0	0	2 (8)
300	1	3	1	1	1	7 (8)
1000	0	4	0	1	0	5 (8)
F1 males						
0	2	2	0	1	1	6 (10)
100	1	0	1	0	1	3 (9)
300	0	1	0	0	0	1 (8)
1000	1	0	1	0	2	4 (9)
F2 males						
0	1	1	0	0	0	2 (9)
100	1	0	1	2	0	4 (9)
300	0	0	0	0	0	0 (8)
1000	0	1	0	0	0	1 (8)

Table 42: Mortality data – females (Anonymous, 1980b).

Dose [ppm]	Pre-M	M	G	P	L	R	M	G	P	L	C	Total incidence (total number of males per group)
	First mating						Second mating					
F0 females												
0	0	1	0	0	0	2	0	0	0	0	1	4 (16)
100	1	0	1	0	1	0	0	1	0	0	0	4 (16)
300	0	0	0	0	0	0	0	0	0	0	2	2 (16)
1000	0	0	1	0	0	1	0	0	0	0	0	2 (16)
F1 females												
0	1	1	0	1	0	0	0	0	0	0	1	4 (17)
100	4	1	0	0	1	0	0	1	0	0	0	7 (17)
300	0	3	0	0	0	0	0	0	0	0	0	3 (16)
1000	0	2	0	0	0	0	0	0	0	0	0	2 (16)
F2 females												
0	0	0	0	0	0	0	0	0	0	1	0	1 (16)
100	0	0	0	0	0	0	2	0	0	0	0	2 (16)
300	0	0	1	0	0	1	0	0	0	0	0	2 (16)
1000	2	1	0	0	0	0	0	0	0	0	0	3 (18)

Pre-M = premating, M = mating period, G = gestation period, P = parturition, L = Lactation, R = rest period between litters (10 days), C = completed (ready for sacrifice)

Body weight (gains) were affected on some points in time with no clear dose dependency. Reduced body weight gains (without statistical significance) were reported for F0 parental males in the high dose group (-12.6%) and for F2 parental males in mid (-16.9%) and high dose groups (-12.5%). Final body weights were lower (without statistical significance) for F1 parental males in the mid (-9.9 %) and high dose groups (-20.4%), for F2 males in the mid (-10.2%) and high dose group (-7.6%). For F2 females statistically significant reduction of final bw (-11.9%) in the mid dose group is documented. For **food intake** no marked differences between test and control groups are documented.

Reproductive performance was not affected by 6PPD treatment. Mating indices for males and females, male and female fertility indices and incidence of parturition were mostly comparable between control and treated group across the different generations. In the 300 ppm group, F1b male fertility (66.7%) and F2a female fertility indices (56.3%) were lower than controls, but these differences were believed to be related to the poor health of the parental animals in this test group and high mortality of parental animals during the mating period for these mid dose litters. The mean number of live or dead pups at birth, and the number of pups weaned, were comparable between control and treated groups. **Survival indices** were also comparable between most dose groups. Pups in the mid dose group had reduced survival indices in some litters (see Table 43). Mean **body weights of pups** measured at day 21 of lactation were considered comparable for all groups; only some minor statistical variations were noted. No unusual behavioral reactions related to treatment were noted for offspring during the study.

Table 43: Survival indices (Anonymous, 1980b).

Dose [ppm]	Litter	Live birth index [%]	Survival index [%]			
			24h *	4d *	12d #	21d #
0	F1A	97.3	96.7	90.0	87.5	76.5
	F1B	99.2	97.6	91.9	97.2	81.1
100	F1A	98.8	96.7	88.0	100.0	99.1
	F1B	99.3	98.5	94.8	92.6	71.3
300	F1A	98.8	98.8	95.2	81.7	71.1
	F1B	95.0	99.3	75.7	93.2	80.6
1000	F1A	97.6	99.4	98.8	90.2	77.6
	F1B	99.5	98.2	84.6	75.7	69.6
0	F2A	93.2	98.4	95.9	89.4	86.7
	F2B	96.5	100.0	78.0	76.5	61.7
100	F2A	95.2	100.0	99.0	82.3	72.9
	F2B	97.8	98.9	98.9	97.6	91.5
300	F2A	100.0	96.4	77.4	72.6	54.8
	F2B	94.3	97.0	83.3	86.5	82.7
1000	F2A	96.6	98.8	97.6	96.3	79.3
	F2B	99.0	99.0	97.1	93.7	76.8
0	F3A	91.7	100.0	87.0	91.8	89.4
	F3B	96.8	96.7	73.8	95.4	83.9
100	F3A	91.5	97.5	89.9	85.6	76.0
	F3B	95.2	100.0	97.0	94.7	82.1
300	F3A	94.4	90.6	74.1	62.3	57.4
	F3B	92.8	89.1	76.6	58.7	56.5
1000	F3A	96.3	98.1	94.2	83.3	67.7
	F3B	93.5	94.8	85.3	91.2	80.2

*Number of pups viable at LD 1 (or 4) / number of viable pups born x 100
number of pups viable at LD 12 (or 21) / number of pups retained at LD 4 x 100

Gross necropsy observations of F0, F1, and F2 adults sacrificed after weaning of their second litters did not reveal any adverse effects related to test material administration. No treatment related statistical differences in **organ weights** were noted for F0, F1, or F2 parental animals in any treatment group with the exception of liver weight. Female total liver weight was (not statistically significant) increased in a dose dependent manner in F0, F1 and F2. Female relative liver weight (to body weight) was statistically significantly increased in 1000ppm F1 (+34%, $p<0.01$) and F2 (+41%, $p<0.05$) generation. Effects on male liver weights were not significant. **Microscopic examination** of tissues from selected F0, F1 and F2 parental animals and F3b pups from control and high dose groups, and from selected animals from low and mid dose groups, showed no test-substance related abnormalities.

In summary in this three generation study with administration of 6PPD via diet (despite the high mortality in all groups) no adverse effects on parental animals was seen. Fetal and pup survival were not affected. No changes in fertility indices were reported. 100 ppm can be considered a NOAEL in this study.

In a **chronic feeding study according to OECD 452** (Anonymous, 1993, cited from REACH registration) rats were dosed with 0, 50, 250 or 1500 ppm (nominal in diet for males: 0, 2.6, 13.5, 84.8 mg/kg bw/d; for females: 0, 3.2, 16.5, 109.5 mg/kg bw/d). For further details on study design and results on general repeated dose toxicity see Chapter 10.12. In this study, beside others, also weight of reproductive organs as well as histopathological changes of reproductive organs (mammary glands, uterus, ovaries, testes, accessory genital organs as prostate, seminal vesicles, epididymides) were investigated. No changes up to the highest dose tested were documented.

In a **reproductive / developmental toxicity screening study** according to OECD 421 (Anonymous, 2009; cited from ECHA dissemination site) Wistar rats (12/sex/dose) were exposed to 6PPD concentrations of 0, 2.5, 12.5, 25 mg/kg bw/d (vehicle olive oil). Males were exposed for 28 days (14d pre-mating and 14d mating) and females for 41 to 56 days (14d premating, ~14d mating, ~21-24d gestation, 4d lactation). Body weights were recorded weekly, food consumption daily. Clinical observations were done once a day (1h after application). After necropsy organ weights of liver, kidneys, testes, epididymides, uterus and ovaries were determined from parental animals. All animals underwent macroscopic evaluation and histological examination including uterus, ovaries, epididymides, prostate, kidney of control and high dose group, as well as livers of all dose group animals.

Study authors concluded that no test-substance related **death** occurred: one male of the mid dose group died on day 16 immediately after application (probably by inspiration of the test substance, animal was replaced), one control, one female of the low and one female of the high dose group died during delivery of pups. **Clinical signs** are described as sporadic occurrence of smooth stool in males of the low (2/12), mid (2/12) and high dose group (2/12) and one female of the mid dose group. These signs appeared temporary and were considered as not test substance related. Administration of 6PPD did not affect **body weight** and food consumption of males and females. No statistically significant differences in relative **organ weights** were reported in treated animals compared to control. Male liver weight shows a tendency of weight reduction while females show a weight increase with increasing test concentration (control 4.58g ± 0.06, high dose 5.23g ± 0.067; not statistically significant). **Histopathological findings** are documented for liver and reproductive organs (see Table 44).

Table 44: Relevant histopathological findings (Anonymous, 2009).

Organ	Observation	Test group [no. of lesions / no. of animals]			
		0 mg/kg bw/d	2.5 mg/kg bw/d	12.5 mg/kg bw/d	25 mg/kg bw/d
Males					
Liver	Focal necrosis	0/12	0/12	2/11	7/12
	Mononuclear nodules in parenchyma or occurrence of inflammatory cells	0/12	3/12	8/11	8/12
	Small-or medium droplet vacuolization of hepatocytes	1/12	7/12	9/11	4/12
Testes	Hypoplasia of seminiferous tubules and absence of spermatogenic cells	0/12	-	1/1[#]	0/12
Females					
Liver	Focal necrosis	2/11	-	-	2/12
	Mononuclear nodules in parenchyma	4/11	-	-	5/12
	Small- or medium droplet vacuolization of hepatocytes	4/11	-	-	9/12
Uterus	Focal inflammatory lesion in myometrium	0/11	-	-	1/12

microscopic evaluation performed because of macroscopic finding in one male

Mating was done 1:1 until copulation (from day 1 to day 14). One male died on day 16 and was replaced. Three non-successful males in the mid dose were replaced by other males of the same dose group, these males were successful and females became pregnant, except for one female which became not pregnant. One non-successful high dose male was replaced by another high dose male; this male was successful and the female became pregnant.

One female of the mid dose group (12.5 mg/kg bw/d) did not become pregnant, no sperm or vaginal plugs were observed. One female of the low dose group became not pregnant, although sperms were detected. Three pregnant females died (1 from control, 1 from low dose, 1 from high dose). 1 female of the mid dose group was pregnant for 21 d, the majority of females had **pregnancy durations** of 22 days (10 control, 8 low, 8 mid and 7 high dose). A duration of 23 days was noted in 2 females of control, 3 of low dose, 2 of mid dose and 3 females of the high dose group. For 2 females of high dose group a length of 24 days was observed (see Table 47)[6]. No HCD data are available. The indices of **reproduction parameters** (%) were calculated; no test substance-related effects were noted.

Table 45: Reproduction parameter (Anonymous, 2009; cited from ECHA dissemination site). [remark: missing definition of calculated parameters]

	Sperm positive %	Pregnant %	With live pups %	Fertility %	Gestation %	Live birth viability %	Viability %
Control	100	100	91.67	100	83.33	89.69#	98.86#
2.5mg/kg bw	100	91.67	83.33	91.67	81.82	88.89	100
12.5 mg/kg bw	91.67	91.67	91.67	91.67	100	97.54	100
25 mg/kg bw	100	100	91.67	100	91.67	98.17	99.07

remark registrant: different data documented in study report

No treatment related differences were noted in dams with live pups. **Alive and death pups** were determined on post-natal day 1 and 4. No treatment related effects were observed in number of live and dead pups (postnatal day 1 and 4) (see Table 46). No treatment-related effects were noted in **sex-distribution**. The **litter weights** at birth (mean) and at day 4 (mean) were slightly increased in treated dams compared to control, but no dose-response relationship was indicated. A summary of observations is presented in Table 47. Based on the presented data total litter loss till PND 4 of one high dose female can be assumed (only 10 dams with live young at day 4 pp). No dose response for loss of offspring is documented. No further information is available.

Table 46: Offspring survival (Anonymous, 2009; cited from ECHA dissemination site).

	Number of pups				Live pups %	Death pups %
	total	mean per dam	Live	Death		
Control	105#	8	96	9	91.4#	8.6#
2.5 mg/kg bw	108	10	96	12	88.89	11.11

[6] Registrants comment that the mode of paring is not exact described so that a difference in the exact insemination time point of several hours can not be excluded.

12.5 mg/kg bw	122	11	119	3	97.54	2.46
25 mg/kg bw	109	9	108	1	99.08	0.92

remark registrant: new calculated by registrants from report data

Table 47: Observations – reproduction parameters (Anonymous, 2009; cited from ECHA dissemination site).

	0 mg/kg bw/d	2.5mg/kg bw/d	12.5 mg/kg bw/d	25 mg/kg bw/d
Pairs started (n)	12	12	12	12
Females evidence of copulation (n)	12	12	11	12
Females became pregant (n)	12	11	11	12
Conceiving days 1-5 (n)	12	12	8	11
Conceiving days 6-14 (n)	0	0	3	1
Pregnancy <21 days (n)	0	0	1	0
Pregnancy = 22 days (n)	10	8	8	7
Pregnancy > 23 days (n)	2	3	2	5
Dams with live young born (n)	10	9	11	11
Dams with live young at day 4 pp (n)	10	9	11	10
Corpora lutea/dam (mean)	12	13	13	12
Implants/dam (mean)	10	11	11	11
Live pups/dam at birth (mean)	8	10	11	9
Live pups/dam at day 4 (mean)	9	10	11	9
Sex ratio (m/f) at day 4 (mean)	5/4	5/6	5/5	5/5
Litter weight at birth (mean)	51.36	58.50	65.91	62.27
Litter weight at day 4 (mean)	69.55	80.50	89.09	80.91
Loss of offsprings				
Preimplantation loss (corpora lutea minus implantations)				
Females with 0	3	1	4	3
Females with 1	0	0	4	2
Females with 2	4	8	0	3
Females with ≥3	5	1	3	4
Pre-natal/post-implantations (implantations minus live birth)				
Females with 0	4	6	8	7
Females with 1	3	3	1	3
Females with 2	1	0	1	0
Females with ≥3	4	2	1	2
Post-natal (live births minus alive at postnatal day 4)				
Females with 0	9	9	11	9
Females with 1	1	0	0	2
Females with 2	0	0	0	0
Females with ≥3	0	0	0	0

Original report was not available but the study was assessed by registrants: The overall assessment of the study was that it has deficits in retrieval, presentation and interpretation of data which render reliability of data debatable and it was concluded that the report is not assignable for regular purposes before thorough revision and therefore categorized with a reliability of 4. An evaluation in the course of the dossier preparation was not possible. In general, the study was done according to OECD 421, GLP and detailed data are presented, however, some details are missing (e.g. individual litter data, standard deviations, missing definitions, observations during parturition, cause of deaths in dams) and hinder a sound conclusion from the study.

10.10.3 Comparison with the CLP criteria

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 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). • The classification of a substance in Category 1A is largely based on evidence from humans. • 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 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.
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 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.

No human data is available to justify classification in Category 1A.

Dystocia has been observed in an OECD 443 and OECD 421 study in multiple dose groups after treatment with 6PPD and considered as key effect in rats. According to the GLP guidance (ECHA, 2017) for classification any effect that has the potential to interfere with sexual function and fertility, including e.g. parturition and pregnancy outcomes, has to be considered.

In the OECD 443 study (Anonymous 2019b) mortality due to dystocia/prolonged labour was seen at 20 and 60 mg/kg bw in 2/25 and 5/30 females, respectively. A NOAEL of 7 mg/kg bw/d can be derived for female fertility. No effects on male fertility are reported. For systemic toxicity in F0 a NOAEL of 20 mg/kg bw/d can be derived based on effects on organ weights (liver, kidney, adrenal glands) seen at 60 mg/kg bw/d. Pigment deposition in the kidney was already seen at 7 mg/kg bw/d.

In the OECD 421 dose-range-finding study (Anonymous, 2019a) dystocia/adverse clinical signs during parturition in 1, 1, and 5 females in the 50, 75 and 100 mg/kg bw group lead to death, euthanasia, or total litter loss of these females. Gestation length was statistically increased at 50 and 100 mg/kg bw/d. For effects on female fertility (dystocia) a LOAEL of 50 mg/kg bw/d can be established. Systemic toxicity (F0) is manifested in higher liver weight in 50, 75 and 100 mg/kg bw/day F0 males and 75 and 100 mg/kg bw/day females.

In another OECD 421 study (Tanaka, 2001) the duration of gestation was significantly increased at the highest dose of 100 mg/kg bw/d resulting in a NOAEL for female fertility of 25 mg/kg bw/d. Systemic toxicity (liver effects) were seen at 25 and 100 mg/kg bw/d in both sexes.

In a third OECD 421 study (Anonymous, 2009) an increase of gestation duration is documented for the highest dose (25 mg/kg bw/d). Mortalities of dams around parturition cannot be evaluated in detail due to missing information in the report and therefore the presented data cannot be considered for classification.

In a three generation reproductive study (Anonymous, 1980b) the test substance was administered via diet up to 100 ppm and no adverse effects were observed on fertility of parental animals and viability of pups.

A chronic feeding study according to OECD 452 (Anonymous, 1993) showed no changes in reproductive organs up to the highest dose of 1500 ppm.

10.10.4 Adverse effects on development

For evaluation of this endpoint two studies in rabbits (OECD TG 414 and a teratogenicity study) are reported. One OECD 414 study, a reproduction/developmental toxicity screening test, an OECD 443 study and a dose range finding study with rats are also available. Additional information is presented from a three generation study in rats, a range finding teratology study in rats, three older teratology studies with limited reporting in rats and rabbits and a pre-screening test with chicken embryos.

Also, three pubertal studies have been conducted with 6PPD, two in female and one in male rats, to assess a potential effect on the endocrine system.

Table 48: 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	Reference
OECD 414, PNDT study GLP Rat, Charles River, CD (~90 days old) GD 6-15 (termination on GD 20) N= 25f/group	6PPD (Sanotflex 13, 100%) 0, 50, 100, 250 mg/kg bw/d Oral, gavage Vehicle: corn oil	NOAEL (dev) = 250 mg/kg bw/d No substance related adverse effects on pups No substance related malformations	Anonymous (1987) Klimisch 1
OECD 414, PNDT study GLP Rabbit, New Zealand white N=24f (28 at top dose) GD 7-28	6PPD (purity 96.9%) 0, 25, 50 or 100 mg/kg bw/d Oral, gavage Vehicle: 1% methyl cellulose 400cP	NOAEL maternal tox = 25 mg/kg bw/d NOAEL dev tox = 25 mg/kg bw/d <u>100 mg/kg bw/d</u> decreased defecation, brown material on the facial area abort (3/24), mean terminal bw ↓ (-6.1%, p<0.01), food consumption↓, body weight gains↓, gravid uterine weight↓ (-18.3%, p<0.01), liver weights ↑ (abs +28.3%, p<0.01) postimplantation loss (11.6 % per litter, not stat signif. but exceeding HCD), viable fetuses ↓ (88.4% per litter, not stat signif but exceeding HCD) mean fetal weights ↓(-18.0%, p<0.01) <u>50 mg/kg bw/d</u> mean body weights ↓ (-5.0% to 5.5% during GD 24-27), food consumption↓, body weight gains↓ liver weights ↑ (rel +15.5%, p<0.01) mean fetal weights ↓(-8.7%,<0.05)	Anonymous (2018c) Klimisch 1

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Teratogenicity study Rabbit, New Zealand Albino GD 6-18, sacrif. on GD 29 N= 17 for control and low dose, 23 for high dose (after artificial insemination 15, 16 and 17 were pregnant)	6PPD (Santoflex 13) Oral (gelous capsules)	NOAEL (dev) = 30 mg/kg bw/d <u>30 mg/kg bw/d</u> Body weight loss during gestation Mortality: 6/17 (GD 17, GD 19 abortion and death; GD 13, GD 28 with abortion on GD 22; GD 19, GD 26) No. of early resorptions ↑ (33 compared to 10 in control), No. of live young↓ (38.6 per 100 implantation sites compared to 68.6 in control) spina bifida 1/28 fetuses <u>10 mg/kg bw/d</u> Body weight loss during gestation Mortality: 3/16 (GD 6; GD 20 with abortion on GD 17; GD 12) live young↓ (48.3 per 100 implantation sites compared to 68.6 in control) <u>control</u> Body weight loss during gestation Mortality: 5/15 (GD 15, GD1, GD21, GD22, GD23) Pilot study with 0, 30, 100 or 300 mg/kg bw/d: general high mortality	Anonymous (1976b) Klimisch 2
OECD 421 (Reproduction/developmental toxicity screening test) GLP Rat, Crj:CD(SD) m/f n=12/sex/dose	6PPD (purity 99.4%) 0, 6, 25, 100 mg/kg bw/d Oral (gavage) Vehicle: corn oil M: 48d F: 14d before mating – day 3 of lactation No recovery group	NOAEL (parental tox) = 6 mg/kg bw/d NOAEL fertility = 25 mg/kg bw/d NOAEL (dev) = 6 mg/kg bw/d **F0:** <u>100 mg/kg bw/d</u>: duration of gestation↑ (22.7 ± 0.5; p ≤ 0.05) **F1:** <u>100 mg/kg bw/d:</u> body weights PND 0↑ (m +11.9%, p≤0.01, f +10.7%, p ≤ 0.05) body weights PND 4↑ (m +19.2%, not stat. signif, f +22.2%, p ≤ 0.05) External anomalies (in 1/131 pups; imperforated anus, pes varus and	Tanaka (2001) Klimisch 2 [Japanese study, English summary]

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
		filamentous tail) No. of total (live) pups born ↓ (-28.4%, not statist signif.). <u>25 mg/kg bw/d:</u> body weights PND 0↑ (m +11.9%, p≤0.01, f +10.7%, p ≤ 0.05) body weights PND 4↑ (m +24.4%, p ≤ 0.05, f +27.8%, p ≤ 0.01) No. of total (live) pups born ↓ (-19%, p≤0.05).	
OECD 443 extended one-generation reproductive toxicity - with developmental neurotoxicity (Cohorts 1A, 1B without extension, 2A and 2B) GLP Rat, Crl:CD(SD) F0: N=25/sex/group High dose group: n= 30/sex/dose	6PPD (purity 96.9%) 0, 7, 20, 60 mg/kg bw/d Oral, gavage Vehicle: corn oil 7d/week F0 females: 70d+mating+gestation+lactation F0 males: like females F1: day of weaning – day prior to euthanasia (PND 21 [Cohort 2B], PND 91 [Cohort 1A], PND 78 [Cohort 2A], and PND 98 [Cohort 1B])	NOAEL (parental tox) = 20 mg/kg bw/d NOAEL (dev tox) = 7 mg/kg bw/d **F0:** <u>60 mg/kg bw/d</u> total litter loss in 5 f <u>20 mg/kg bw/d</u> total litter loss in 1 f <u>control</u> total litter loss in 3 f **F1:** <u>60 mg/kg bw/d</u> Postnatal survival ↓ (PND 1-7) (not stat. signif. but above HCD), 111(20) pups (litters) found dead or were euthanized in extremis; 21/111 with no milk in stomach; mean bw on PND 21↓ (m -8.1% and f -7.4%, not stat. signif.) and on PND 28 mean bw remained lower (m-5.9%, f -8.6%, not stat. signif.), no effect on bw on PND 98 Cohort 2B (PND 21): brain weight ↓ (m -7.3%, p<0.01) Cohort 2A (PND 78): brain weight↓ (m -5.2%, not statistically significant. <u>20 mg/kg bw/d</u> Postnatal survival ↓ (not signif)	Anonymous (2019b) Klimisch 1
Dose-range finding study similar to OECD 421 GLP Rat, Crl:CD(SD)	6PPD (purity 96.9%) 0, 50, 75 and 100 mg/kg bw/day vehicle: corn oil	LOAEL (parental tox) = 50 mg/kg bw/d NOAEL (dev tox) = 75 mg/kg bw/d **F1**: <u>100 mg/kg bw</u>	Anonymous (2019a) Klimisch 1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
N= 15/sex/dose	m:28d f: 14 days prior to mating, continuing through mating, gestation, and lactation until lactation day 21 F1 pups: PND 21 through 49.	postnatal survival↓ (2 total litter losses due to poor maternal care), live litter size ↓ (-14.7%, not stat.signif.), bad clinical conditions of pups (thin or cold body), abs. liver weight PND50 ↑ (m +29.9%, p<0.01; f +41.8%, p<0.01), hepatocellular vacuolation (f, minimal/mild) <u>75 mg/kg bw</u> abs. liver weight PND50 ↑ (m +25.3%, p<0.01; f +32.4%, p<0.01), hepatocellular vacuolation (f, minimal) <u>50 mg/kg bw</u> anogenital distance↑ (f) abs. liver weight PND50 ↑ (m +18.6%, p<0.01; f +16.7%, p<0.01), hepatocellular vacuolation (f, minimal)	
Three generation study non GLP limited quality assurance documented (final comparison of the raw data with the final report missing; several limitations rats, Charles River CD N = 8m + 16 f (per group and generation)	6PPD (Santoflex 13) 0, 100, 300 or 1000 ppm (approx. 0, 8, 23, 75 mg/kg bw) Oral (diet, ad libitum) (6PPD premixed in acetone)	NOAEL (dev) = 100 ppm (75 mg/kg bw/d) High mortality in control and dose groups <u>1000ppm</u> Relative liver weight (to body weight) was statistically significant increased in F1 (+34%, p<0.01) and F2 (+41%, p<0.05) generation	Anonymous (1980b) Klimisch 2 (by reg.) [short summary published by Stevens et al., 1981]
Range finding teratology study GLP Rat, Sprague-Dawley GD 6-15 N=5/dose	6PPD (Sanotflex 13) 0, 100, 300, 600, 1000 and 2000 mg/kg bw/day oral: gavage vehicle corn oil	NOAEL (dev) = 300 mg/kg bw/d <u>600, 1000 and 2000 mg/kg bw/d:</u> Mortality of all dams Intrauterine survival was not affected by treatment at ≤ 300 mg/kg bw/d	Anonymous (1986a) Klimisch 2 [study report not available, cited from ECHA dissemination site]
Pilot study teratology Rat	6PPD 30, 100, 300 mg/kg bw (not	<u>300 mg/kg bw/d</u> Mortality of dams (80%), resorptions↑ <u>100 mg/kg bw/d</u>	Anonymous (1976c) Klimisch 4 [study report

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
-	fully specified)	absorptions↑, resorptions↑ <u>30 mg/kg bw/d</u> resorptions↑, reduced pup survival	not available, cited from ECHA dissemination site]
Teratology study Rat -	6PPD	<u>30 mg/kg bw/d:</u> maternal toxicity (abortions and body weight changes) slight increases in resorptions and fetal death (not signif) <u>10 mg/kg bw/d:</u> slight increases in resorptions and fetal death (not signif)	Anonymous (1976d) Klimisch 4 [study report not available, cited from ECHA dissemination site]
Pilot teratology study Rabbit GD 6-18	6PPD (Santoflex 13) 0, 30, 100 or 300 mg/kg bw/d	<u>100 and 300 mg/kg bw/d:</u> Maternal toxicity <u>30 mg/kg:</u> mild body weight losses along with a marginal increase in fetal resorption	Anonymous (1976e) Klimisch 4 [study report not available, cited from ECHA dissemination site]
Prescreening test with chicken embryos	6PPD (technical grade)	ED_{50} = 1.5µmol (dose/egg) Dose/egg [µmol] / Affected embryos% (early/late deaths, malformations) 0.55 / 0 (n=10) 1.1. / 20 (n=30) 2.2 / 43 (n=30) 4.4 / 33 (n=30)	Korhonen (1983) Klimisch 3
- Rat	-	Negative [no further details available]	Schardein (1993) Klimisch 4 [study report not available, cited from ECHA dissemination site]

Table 49: Summary table of animal studies investigating possible endocrine effects

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Female pubertal study EPA OPPTS Guideline 890.1450 GLP Rat, Crl:CD(SD), f n=15f/group PND 22-42/43	6PPD (purity 98.2%) 0, 250, 500 mg/kg bw/d Oral, gavage Vehicle: corn oil	LOAEL = 250 mg/kg bw/d <u>500 mg/kg bw/d</u> Earlier age at vaginal opening (33.2 d compared to control with 35.2 d; p<0.05) Higher age at first oestrus (39.2 d compared to control 6.3 d, p<0.05) Bw at vaginal opening ↓ (-18.8%, p<0.05) Pale liver in 2/15 correlates with hepatocellular vacuolation Bilirubin ↑, (+700%, p<0.05), GGT ↑ (+100%, not signif), AST ↓ (-47.3%, p<0.05), triglycerides ↓ (-51.2%, p<0.05), serum cholesterol ↑ (+24.7%) T4 ↓ (-22.2%, p<0.05) , TSH ↑ (+72.3%, p<0.05) ovary weight ↓ (-21.5%, p<0.05), uterus weight ↓ (-39.3%, p<0.05), kidney weight↑ (+10.6%, p<0.05), liver weight↑ (52.4%, p<0.05) , thyroid gland weight↑ (11.7%, not signif), pituitary ↓ (-22.3%, p<0.05) immature uterus (2/12) thyroid gland: follicular cell height (p<0.05), lower colloid area (p<0.05) <u>250 mg/g bw/d</u> Bw at vaginal opening ↓ (-11.5%, p<0.05) AST↓ (-39.8%, p<0.05), triglycerides ↓ (-33.9%, p<0.05), serum cholesterol↑ (+23.7%, p<0.05) TSH ↑ (+92.3%, p<0.05) uterus weight ↓ (-18.7%, not signif), liver weight ↑ (+42.2%, p<0.05, kidney weight↑ (+9.8%, p<0.05), thyroid gland: follicular cell height ↑, lower colloid area;	Anonymous (2016a) Klimisch 1
Pubertal study EPA OPPTS Guideline 890.1450 GLP Rat, Crl:CD(SD), f N=15f/dose PND 22-42/43	6PPD (98.2%) 0, 10, 100, 300 mg/kg bw/d Oral, gavage Vehicle: corn oil	<u>300 mg/kg bw/d</u> lower mean body weight at attainment of vaginal opening (-11.9%, not signif) irregular estrous cycles / not cycling (not signif. but above HCD) liver weights ↑ (+47.8%, p<0.05), TSH ↑ (+80.5%, p<0.05), T4 ↓(-8.3%, not signif) ovarian weight ↓ (-16.9%, p<0.05), uterine weight ↓ (blotted and wet: -33.8% and -30.5%, p<0.05) follicular cell heights↑ (p<0.05), lower thyroid colloid area (p<0.05) <u>100 mg/kg bw/d</u> liver weights ↑ (+17.9%, p<0.05), TSH ↑ (+15%, not signif) follicular cell heights↑ (p<0.05), lower thyroid colloid area	Anonymous (2017) Klimisch 1

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
		(p<0.05)	
Male pubertal study EPA OPPTS Guideline 890.1500 GLP Rat, Crl:CD(SD), f N=15m/dose PND 23-53/54	6PPD (98.2%) 0, 250, 500 mg/kg bw/d Oral, gavage Vehicle: corn oil	<u>500 mg/kg bw/d</u> Salivation, clear and/or red material around the mouth body weight gain ↓ (-27.5%, p<0.05) delayed mean age of attainment of balanopreputial separation (49.3 d compared to control at 46.2d), body weight at BPS (-14.5%, p<0.05)↓ ALT↑ (+54.9%, p<0.05), GGT↑ (0.8 U/l compared to control 0.0 u/l), serum triglyceride ↓ (-75.8%, p<0.05), T4↓ (-32-6%, p<0.05), testosterone↓ (-82.5%, p<0.05), TSH ↑ (+79.2%, not signif) prostate weight ↓ (dors.-39.8, ventr.-48%, p<0.05), epididymides weight ↓(rt: -15.3%, lt -16.5%, p<0.05), pituitary gland ↓ (-26.0%, p<0.05), testis ↓ (-11.0%, p<0.05), thyroid gland ↓ (-22.%, p<0.05), liver ↑ (+32.6%, p<0.05), LABC muscle ↓ (-40.9%, p<0.05) thyroid: higher follicular cell height (not stat. signif) and lower colloid area (signif) <u>250 mg/kg bw/d</u> Salivation, clear and/or red material around the mouth body weight gain ↓ (-9.6%, p<0.05) body weight at BPS (-11.2%, p<0.05) ↓ serum triglyceride ↓ (-62.0%) adrenal glands↑ (+11.5%, p<0.05), prostate weight ↓ (dors. -17.3%, ventr. -18.3%, p<0,05), liver ↑ (31.0%, p<0,05), LABC muscle ↓ (-14.3%, p<0.05) thyroid: higher follicular cell height (not stat. signif) and lower colloid area (signif)	Anonymous (2016b) Klimisch 1

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

In a **prenatal developmental toxicity study** (OECD 414, Anonymous, 1987) female rats were mated (1:1, confirmed by presence of copulatory plug) and subsequently exposed to 6PPD concentrations of 0, 50, 100, 250 mg/kg bw/d (oral, gavage; in corn ail prepaired daily) from GD 6-15. Analysis of the -dosage preparations for concentration were conducted three times (1st day, midway through dosing, last day of dosing). The average concentrations of 6PPD analyzed in the three preparations was 98.2%, 92.7% and 99.0%, respectively, of the targeted test concentration.

Throughout gestation females were observed twice daily. Body weights as well as food consumption were recorded on gestation days 0, 6, 9, 12, 16 and 20. Study was terminated on GD 20 and a gross postmortem examination was performed on all animals. Organ weights of liver, kidney and spleen were determined. Live and dead fetuses, early and late resorptions, implantation sites and corpora lutea were recorded. Fetuses were

weighed, sexed and examined for gross and skeletal malformations. The crown-rump length of late resorptions was recorded. Half of the fetuses were sectioned and examined for soft tissue malformations (fetal kidneys were examined and graded for renal papillae development). The remaining half were processed for subsequent skeletal examination.

100% survival in all study groups is documented. Abortions or premature deliveries are not reported. Clinical signs (salivation prior to dosing, soft stool, diarrhea, green fecal discoloration and green staining of the anogenital fur) were seen at the two highest test concentrations (dose-related). Some single findings were also made in the low dose.

In general, maternal **body weights** were comparable between treated groups and control. A slight increase in mean bw was seen for the 100 mg/kg bw/d dose group on GD 12, 16 and 20; bodyweight gains were also increased during this time. The mean values were statistically significant when compared with the control group. Study authors assume that these differences were probably due to an unusually large litter size in this group, a condition not related to 6PPD treatment. This was also accompanied by an increased **food consumption** (GD 9-12 and GD 16-20). In the high dose group, a slight reduced food intake during the first days of treatment was seen, during the remainder of the treatment period (GD 9-12 and 12-16) food consumption was slightly increased. GD16-20 food intake was significantly increased in this group.

Pathology showed no relevant substance related findings.

Termination on GD 20 revealed no differences between control and test substance groups with respect to numbers of viable fetuses, early and late resorptions, fetal sex ratio and fetal weights. A significant increase ($p < 0.05$) in the mean numbers of viable fetuses, implantation sites and corpora lutea occurred in the 100 mg/kg bw/day group when compared with the control group, however, due to the study design (dosing on GD 6-15) this finding is not test substance related.

Investigation of **fetal malformations** showed one fetus in the control group with a diaphragmatic hernia. In addition, two fetuses from two litters in the 100 mg/kg bw/day group were malformed (one fetus with unilateral microphthalmia, one with multiple anomalies). No malformations were observed in the 250 mg/kg bw/day dose group.

Investigation of **skeletal variations** showed a small numerical increase (not statistically significant) in the incidence of a number of skeletal variations in treated groups. These included an increase in the percentage of fetuses with sternebrae no. 5 and/or 6 unossified and malaligned sternebrae (100 and 250 mg/kg bw/day groups), sternebrae no. 1, 2, 3 and/or 4 unossified and 27 presacral vertebrae (50, 100 and 250 mg/kg bw/day groups) and 7th cervical ribs (250 mg/kg bw/day group) (Table 50). Study authors concluded that these are common developmental variations in this species and all have been observed to occur with similar incidence in the historical data. The remaining developmental variations occurred with similar frequency between the control group and the treated groups

Based on the available data a NOAEL of 250 mg/kg bw/d can be derived for developmental toxicity of 6PPD. No marked maternal toxicity is reported.

Table 50: Skeletal variations[#] (Anonymous, 1987).

Finding	Fetuses				Litters			
	control	50 mg/kg bw/d	100 mg/kg bw/d	250 mg/kg bw/d	Control	50 mg/kg bw/d	100 mg/kg bw/d	250 mg/kg bw/d
Number examined viscerally	144	168	185	173	20	23	23	23
Renal papillae not developed and/or distended ureter(s)	5	1	0	2	4	1	0 *	2
Number examined skeletally	148	173	186	167	20	23	23	23
Sternebrae no. 5	14	16	26	20	7	10	13	11

and/or 6 unossified								
Sternebrae malaligned	10	6	15	16	9	6	10	12
Reduced ossification of 13th rib(s)	0	1	3	1	0	1	2	1
14th rudimentary rib(s)	7	13	14	10	4	6	10	4
Sternebrae no. 1, 2, 3 and/or 4 unossified	0	1	1	2	0	1	1	1
27 presacral vertebrae	0	1	1	4	0	1	1	1
Bent rib(s)	0	0	1	0	0	0	1	0
Hyoid unossified	0	0	1	0	0	0	1	0
7th cervical rib(s)	0	0	0	1	0	0	0	1

* signif. different from control (0.05), Fisher´s Exact test
# presented HCD incidences: sternebrae no. 5 and/or 6 unossified in 1962/10040; sternebrae malaligned in 239/10040; 14th rudimentary rib(s) in 449/10040; sternebrae no. 1, 2, 3 and/or 4 unossified in 55/10040; 27 presacral vertebrae in 12/10040.

In a recent **OECD 414 study** (Anonymous, 2018c) rabbits were dosed (oral, gavage) daily from GD 7-28 with concentrations of 0, 25, 50 or 100 mg 6PPD/kg bw/d. The dosing formulations were prepared approximately weekly (stability for up to 12 days when stored refriged was demonstrated) and were analyzed for verification of concentration. Animals were observed for general health and moribundity twice daily.

For detailed clinical observations the animals were removed from the cage once daily. During the dosing period, these observations were performed prior to dosing. On dosing days, clinical observations were also recorded 3 h after dosing. Individual animals were weighed on GD 0 (by supplier), 4, and 7–29. Food consumption was quantitatively measured on GD 4–29. Gravid uterine weight was collected and net body weight (weight on GD 29 minus weight of the uterus and contents) as well as net body weight change (body weight change GD 0-29 minus weight of the uterus and contents) were calculated for each gravid female at the scheduled laparohysterectomy. Number and distribution of corpora lutea, implantation sites, live and dead fetuses, and early and late resorptions as well as early implantation loss (10% ammonium sulphide solution) was investigated. External, internal, and skeletal findings were recorded (number of foetuses, number of litters). Each viable fetus was examined in detail, sexed, weighed, tagged, and euthanized. Nonviable fetuses were examined, crown-rump length measured, weighed, sexed and tagged individually. The crown-rump length of late resorptions was measured, the degree of autolysis recorded, a gross external examination performed (if possible), and the tissue discarded. All fetuses were examined for visceral anomalies by dissection in the fresh (non-fixed) state. A skeletal examination was made following fixation in alcohol and staining.

Clinical signs included decreased defecation (correlation to reduced food consumption) and brown material on the facial area in the mid and high dosed groups and mucoid feces (GD 21, 23) at high dose. Other clinical observations occurring infrequently and similar to control were rales, soft feces, green staining of the fur on the dorsal trunk, and brown material, scabbing, or hair loss on various body surfaces.

Three females in the 100 mg/kg bw/day group aborted during on GD 22 or 24 following marked body weight losses (12.7% to 18.3% from GD 7 to the day of abortion) and reduced food consumption (≤ 47 g/day):

Table 51: Rabbits with abortions at 100 mg/kg bw/d (Anonymous, 2018c).

Animal number	Abortion	Bw/food consumption	Clinical observation	Necropsy findings

4169	1 dead fetus on GD 22	marked body weight loss (-13.4%) limited food consumption (0–32 g/day) from GD 7–22	thin body during GD 18-22 decreased defecation sporadically primarily during GD 14-22 red material on the cage floor on the day of abortion	dam internally normal 5 dead fetuses and 2 late resorptions with no apparent malformation in utero
4195	1 dead fetus on GD 24	body weight losses (-18.3%) from GD 7–24 reduced food consumption (1-20 g/day) from GD 14–24	Decreased defecation and brown material on various body surfaces sporadically during GD 16-24	dam was internally normal 4 viable fetuses with no apparent malformations in utero
4249	2 dead fetuses on GD 24	body weight losses (-12.7%) reduced food consumption (0-47 g/day) from GD 7–24	sporadic decreased defecation from GD 12–23 mucoid feces on GD 21 brown material on the facial area on GD 22.	dam internally normal 13 viable fetuses with no apparent malformations in utero

In the 25 mg/kg bw/day group, female No. 4225 was found dead on GD 28. This animal was noted with gasping and brown material around the nose and mouth on the day of death, but there were no effects on body weight gain and food consumption noted. At necropsy the female was noted with an accessory spleen, brown matting of the skin, lungs that were not fully collapsed, and dark red areas in the lungs and the rabbit had 8 dead fetuses with no apparent malformations in utero. In the absence of other signs of toxicity at this dosage level, this mortality was not considered test substance-related. All other females survived to the scheduled necropsy.

At 25 mg/kg bw/d body weight parameters and gravid uterine weight were unaffected. **Mean body weights** were significantly ($p<0.05$ or $p<0.01$) lower in the 50 mg/kg bw/day group (5.0% to 5.5% during GD 24-27) and 100 mg/kg bw/day group (ranging from 6.1 % to 10.0% during GD 13-29) compared to the control. Mean body weight losses and lower mean body weight gains were noted in the 50 and 100 mg/kg bw/day groups during GD 7–10, 10–13, and 13–20; differences from the control group were generally significant ($p<0.01$). Mean body weight gains in these groups were comparable to or slightly higher than the control group during GD 20–29. The decrements in body weight gain in the 50 and 100 mg/kg bw/day groups resulted in significantly ($p<0.01$) lower mean body weight gains when the entire treatment period (GD 7-29) was evaluated (347g, 324g, 197g and 158g in control, low, mid and high dose, respectively)**.** Significantly ($p<0.05$ or $p<0.01$) lower mean **gravid uterine weight** at 100 mg/kg bw/day (-18.3%) and net body weight change at 50 and 100 mg/kg bw/day were noted compared to the control group (see Table 52). While the reduction of the corrected maternal body weight in the high dose group was -4.6% and in the mid dose group -3.8% the effect on gravid uterus weight was higher with -18.3 and -10%, respectively. According to CLP regulation (Section 3.7.2.4.4) in rabbits, the body weight gain may not be a useful indicator of maternal toxicity because of normal fluctuations in body weight during pregnancy. Therefore, effects on gravid uterus weight were considered relevant for classification.

Table 52: Summary of body weight, gravid uterine weight parameters and liver weight [g] [mean ± SD, n, %] (Anonymous, 2018c).

	Control	25 mg/kg bw/d	50 mg/kg bw/d	100 mg/kg bw/d
Initial body weight	3246. ± 226.8 23	3260. ± 241.8 23	3248. ± 226.6 24	3251. ± 212.7 24
Terminal body weight	3699. ± 226.8 23	3656. ± 264.4 23	3529. ± 243.0 24	3472.** ± 281.7 24

		-1.2%	-4.6%	-6.1%
Gravid uterine weight	485.6 ± 71.05 23	498.6 ± 86.50 23 +2.7%	437.2 ± 85.57 24 -10.0%	396.6** ± 134.62 23 -18.3%
Net body weight	3213.3 ± 215.90 23	3156.9 ± 250.28 23	3091.3 ± 210.70 24	3064.6 ± 247.29 23
Net body weight change	-32.9 ± 113.96 23	-102.6 ± 163.60 23	-156.7* ± 142.76 24	-180.8** ± 212.33 23
Liver	82.17 ± 12.986 23	87.79 ± 14.306 23 +6.8%	91.35 ± 11.936 24 +11.2%	105.44** ± 13.841 24 +28.3%
Liver (g/100 g net body weight)	2.556 ± 0.3517 23	2.771 ± 0.3151 23	2.952** ± 0.2948 24	3.394** ± 0.2621 23

** = Significantly different from the control group at 0.01 using Dunnett's test

Food consumption in the 25 mg/kg bw/day group was unaffected by test substance administration. Mean food consumption (g/animal/day and g/kg/day) in the 50 and 100 mg/kg bw/day groups was generally lower than in the control group beginning on GD 7, and continuing through GD 24 or 25. Thereafter, mean food consumption in these groups were comparable to the control group. Investigating cumulative intervals the mean food consumption in the 50 and 100 mg/kg bw/day groups was significantly ($p<0.05$ or $p<0.01$) lower in periods GD 7–10, 10–13, 13–20, and 7–29. These reductions correspond to the reduced mean body weight gains or losses, were generally dose-responsive and were considered test substance related. Generally individual food consumption of rabbits varied over days, especially in the 50 and 100 mg/kg bw/day dose groups. E.g. female No 4232 consumed 20 g/day from GD 7–10 and 1 g/day from GD 10-13 and had 9 early resorptions with no viable fetuses at necropsy (control group: with 156 and 159 g/d respectively). But on the other hand female No 4211 consumed about 2.5g/day from GD 8- 16 and showed no resorptions at all but had 9 viable fetuses at necropsy. Several females in the 50 and 100 mg/kg bw/day groups experienced periods of 3–14 days of markedly reduced food consumption during the implantation period (GD 7–20). These females tended to have increased food consumption during the fetal period (GD 21–29; with the exception of the 3 females that aborted on either GD 22 or 24 at 100 mg/kg bw/day), although their food consumption levels did not recover completely. However, beside a general lower food consumption in the 50 and 100 mg/kg bw/day groups no clear correlation between reduced food consumption, reduced net female body weight and resorptions can be established based on individual animal data.

Gross pathology showed at necropsy on GD 29 no test substance related internal findings in any dose group. However, increased incidences of green discoloration of the fur were noted in the 50 and 100 mg/kg bw/day groups compared to the control group and was not considered toxicologically relevant by study authors. One female each in the control and 100 mg/kg bw/day group were determined to be non gravid.

Higher mean absolute and relative (to net body weight) **liver weights** were noted in the 50 and 100 mg/kg bw/day groups; differences from the control group were generally significant ($p < 0.01$) (see Table 52).

Fetal data at necropsy are presented in Table 53. The mean litter proportion of postimplantation loss (see Table 54) in the 100 mg/kg bw/day group, primarily consisting of early resorptions (11.6 % per litter) was higher when compared to the concurrent control group (3.5% per litter). The difference was not statistically significant, however, the value exceeded the maximum mean value in the Charles River Ashland HCD version 2018.01 (10.15% per litter). A corresponding lower (not statistically significant) mean litter proportion of viable fetuses was noted in this group (88.4% per litter) when compared to the concurrent control group (96.5% per litter) and the minimum mean value in the Charles River Ashland HCD (89.85% per litter). Test substance-related lower mean fetal body weights (male, female, and combined) were noted in the 50 and 100 mg/kg bw/day groups (correlating with the effects on uterine weights) when compared to the

concurrent control group and the mean values in the Charles River Ashland HCD (42.370 g, 41.124 g, and 41.792 g, respectively).

Table 53: Fetal data (rabbits) at scheduled necropsy (Anonymous, 2018c).

	Control	25 mg/kg bw/d	50 mg/kg bw/d	100 mg/kg bw/d
No of gravid females	23	23	24	24
Viable fetuses	192	209	196	196
Sex [m/f]	93/99	114/95	118/78	88/108
Dead fetuses	0	0	0	0
Early resorptions	6	3	5	19
Late resorptions	1	0	0	8
Post implantation loss	7	3	5	27
Implantation sites	199	212	201	223
Corpora lutea	214	225	215	238
Pre-implantation loss	15	13	14	15
Fetal weight [mean ±SD in g]	42.7 ± 3.40	40.5 ± 4.24 -5.2%	39.0* ± 4.64 -8.7%	35.0** ± 6.09 -18.0%

* = Significantly different from the control group at 0.05; ** = significantly different from the control group at 0.01

Table 54: Fetal data (rabbits) at scheduled necropsy displayed in % per litter (Anonymous, 2018c).

	Control	25 mg/kg bw/d	50 mg/kg bw/d	100 mg/kg bw/d
Corpora lutea	9.3 ±1.49 23	9.8 ± 1.91 23	9.0 ± 1.52 24	9.9 ± 2.43 24
Implantation sites	8.7 ± 1.50 23	9.2 ± 2.07 23	8.4 ± 1.69 25	9.3 ± 2.48 24
Viable fetuses	96.5 ± 5.51 23	98.3 ± 4.54 23	97.7 ± 5.68 24	88.4 ± 21.08 24
Dead fetuses	0.0 ± 0.00 23	0.0 ± 0.00 23	0.0 ± 0.00 24	0.0 ± 0.00 24
Early resorptions	3.0 ± 5.29 23	1.7 ± 4.54 23	2.3 ± 5.68 24	8.6 ± 21.33 24
Late resorptions	0.5 ± 2.31 23	0.0 ± 0.00 1.0 23	0.0 ± 0.00 1.0 24	2.0 ± 6.13 3.0 24
Total resorption	3.5 ± 5.51 23	1.7 ± 4.54 23	2.3 ± 5.68 24	11.6 ± 21.08 24
Pre-implantation loss	6.5 ± 10.87 23	6.0 ± 7.67 23	6.6 ± 9.04 24	7.3 ± 16.12 24
Post-implantation loss	3.5 ± 5.51 23	1.7 ± 4.54 23	2.3 ± 5.68 24	11.6 ± 21.08 24
Males	47.5 ± 22.14	53.8 ± 18.37	61.6 ± 17.14	44.2 ± 20.12

	23	23	24	23
Females	52.5 ± 22.14 23	46.2 ± 18.37 23	38.4 ± 17.14 24	55.8 ± 20.12 23
Male fetal weights [g]	43.6 ± 4.38 23	41.7 ± 4.64 -4.4% 23	39.3** ± 4.70 -9.9% 24	33.9** ± 4.48 -22-2% 22
Female fetal weights [g]	41.8 ± 3.74 22	39.3 ± 4.54 -6.0% 23	38.6 ± 5.31 -7.7% 22	34.6** ± 5.94 -17.2% 23
Combined fetal weights [g]	42.7 ± 3.20 23	40.5 ± 4.24 -5.2% 23	39.0* ± 4.64 -8.7% 24	35.0** ± 6.09 -18.0% 23

* = Significantly different from the control group at 0.05 (Dunnett´s test)
** = Significantly different from the control group at 0.01 (Dunnett´s test)

External malformation was noted in 1 fetus in the 25 mg/kg bw/day groups (polydactyly, one extra digit on a hindpaw), and was not considered substance related. One control fetus had anophthalmia (unilateral), exencephaly with an open eyelid (unilateral), cheilognathoschisis (involving right naris), and microtia (unilateral; bent). **Visceral malformations** (see Table 55) occurred in single fetuses, were not observed in a dose-related manner and were not statistically significant, therefore, they were not attributed to the test substance. In addition, no test substance-related visceral developmental variations were noted. **Skeletal malformations** were also not considered test substance related. Skeletal developmental variations were infrequently observed in the test substance-treated groups, were similar to the control group, and were not observed in a dose-related manner, the differences in the mean litter proportions were not statistically significant and/or the values were within the ranges of the Charles River Ashland HCD.

Table 55: Malformations in rabbits, total numbers (Anonymous, 2018c).

Finding	Fetuses				Litters			
	control	25 mg/kg bw/d	50 mg/kg bw/d	100 mg/kg bw/d	control	25 mg/kg bw/d	50 mg/kg bw/d	100 mg/kg bw/d
No. examined	192	209	196	196	23	23	24	23
External - total	1	1	0	0	1	1	0	0
Polydactyly	0	11	0	0	0	1	0	0
Microphthalmia and/or anophthalmia	1	0	0	0	1	0	0	0
Exencephaly with or without open eyelid	1	0	0	0	1	0	0	0
Cheilognathoschisis	1	0	0	0	1	0	0	0
Microtia	1	0	0	0	1	0	0	0
Soft tissue - total	5	8	11	5	4	5	5	5
Lungs – lobular agenesis	5	7	10	4	4	4	4	4
Hydrocephaly	0	0	0	1	0	0	0	1
Interrupted aortic arch	0	1	0	0	0	1	0	0

Teratology of fallot	0	0	1	0	0	0	1	0
Skeletal - total	5	2	4	1	4	2	4	1
Vertebral anomaly with or without associated rib anomaly	0	0	3	1	0	0	3	1
Costal cartilage anomaly	4	1	2	0	3	1	2	0
Sternebra(e) malaligned (severe)	1	0	0	0	1	0	0	0
Skull anomaly	0	1	0	0	0	1	0	0

For this OECD 414 study in rabbit it can be summarized that at 100 mg/kg bw/day a higher mean litter proportion of postimplantation loss resulted in a lower mean litter proportion of viable fetuses. In addition, significant lower mean fetal body weight at 50 and 100 mg/kg bw/d (up to 8.7% and 18.0%, respectively) were reported. Three females in the high dose group (3/24) aborted (reported HCD: 8 of 2065 rabbits aborted). Test-substance related effects on maternal body weight and food consumption are reported for 50 and 100 mg/kg bw/d. As no clear correlation of food consumption with resorptions in these dose groups could be established these effects on progeny are considered relevant for classification. Lower mean gravid uterine weight at 100 mg/kg bw/day correlated with a higher mean litter proportion of postimplantation loss and lower mean fetal body weights in this group.

In another **teratogenicity study** (Anonymous, 1976b) with New Zealand Albino rabbits 6PPD was administered orally in gelatin capsules on GD 6-18 once daily. Sacrifice was done on GD 29. In a pilot study, concentrations of 0, 30, 100 or 300 mg/kg bw/d were administered (n=5/group). Based on these results, showing high mortality in rabbits (see Table 56), the test concentrations for the main study were determined to be 0, 10 and 30 mg/kg bw/d. Body weights were determined on GD 0, 6, 9, 12, 15, 18 and 29. Animals were observed daily for mortality and abnormal reactions. After sacrifice on GD 29 the body weight of foetuses was determined and an external examination was conducted. Immediately afterwards the viable young were placed in an incubator and viability was checked after 7 and 24h. All young rabbits were examined by dissection (size, shape, organs, blood vessels, skeletal tissue).

Table 56: Result of pilot study with rabbits (Anonymous, 1976b).

	Maternal bw change [g]		No of implantation sites	No of resorption sited	No of fetuses	Bw [g]	Comments
	GD 6-18	GD 0-29					
control	-750	-1000	Not pregnant	-	-	-	
	+100	+350	7	1	6	46	1 young died between 7 and 24h incubation
	-	-50	Not pregnant	-	-	-	
	-100	-150	3	0	3	43	
	+50	died	-	-	-	-	Found dead on GD 27
30 mg/kg bw	-300	-100	9	2	7	40	
	-	-	2	0	2	48	
	+100	+450	9	2	7	39	5 young died during incubation
	-200	-50	9	1	8	40	1 young died during incubation
	-	-	5	3	2	50	

100 mg/kg bw	-200	-200	Not pregnant	-	-	-	
	-800	-600	Not pregnant	-	-	-	
	-850	-1000	Aborted, GD 18	-	-	-	
	-250	-200	7	7	0	-	
	-400	-600	Aborted, GD 23	-	-	-	
300 mg/kg bw	-950	-1350	10	10	0	-	
	-	-	Died, GD 17	-	-	-	
	-1050	-	Died, GD 22	-	-	-	
	-	-	Died, GD 9	-	-	-	
	-1050	-	Died GD 19	-	-		

Rabbits in the three groups of the main study showed mean **body weight loss** during the dosing period (see Table 57), the control and the high dose group showed mean body weight loss overall.

Mortality: 5, 3 and 6 animals died in the control, 10 or 30 mg/kg bw/d group, respectively. In 10/14 animals found dead, respiratory insufficiency or failure was diagnosed. 5 of these 10 animals were control animals, therefore this effect was determined as not substance-related by study authors. The number of pregnant survivors at the end of the study was 10, 13 and 11 in the three dose groups, respectively.

Table 57: Mortalities and mean body weights during gestation [kg/rabbit] (Anonymous, 1976b).

Group	Number of dead rabbits (day of gestations found dead)	No. of does surviving	Gestation day [mean bw in kg/rabbit]						
			0	6	9	12	15	18	29
Control	5 (GD 15*, GD16*, GD21*, GD22*, GD23*)	10	4.19	4.15	4.12	4.11	4.13	4.07	4.15
10 mg/kg bw/d	3 (GD 6; GD 20 with abortion on GD 17; GD 12*)	13	4.16	4.14	4.10	4.11	4.11	4.07	4.24
30 mg/kg bw/d	6 (GD 17, GD 19 abortion and death*; GD 13, GD 28 with abortion on GD 22*; GD 19, GD 26*)	11	3.93	3.93	3.92	3.84	3.74	3.68	3.83

* cause of death: respiratory insufficiency

Reproductive data are presented in Table 58. A slight increase of resorption sites per 100 implantation sites is documented for the high dose (38.6%) compared to control (31.4%). Values for control and low dose group were at the high end of the range for control New Zealand albino rabbits used in similar studies by the same laboratory. A moderate decrease of live young per 100 implantation sites is documented for the low (48.3%) and the high dose (38.6%) compared to control (68.6%). This reduction may be due to the number of foetuses aborted.

Table 58: Summary of reproductive data, main study (Anonymous, 1976b).

Group	No. of pregnant animals	No. of implantation sites (IS)	No. of early resorptions	No. of late resorptions	No. of does showing resorptions	No. of live young	No. of resorption sites per 100 IS	No. of live young per 100 IS	No. of foetuses aborted	No. of does showing abortion
Control	10	70	10	12	7	48	31.4	68.6	0	0
10 mg/kg bw/d	14	118	28	8	8	57	30.5	48.3	25	2
30 mg/kg	11	85	33	1	8	34	38.6	38.6	20	2

bw/d										

IS…implantation sites

Examination of foetuses for external abnormalities revealed one in the high dose group with spina bifida (1 of 34 fetuses total), due to the low incidence in the litter (1of 6 pups) it was not considered indicative of a teratogenic response by the study authors. The mean body weights of progeny were 42.5g, 41.0g and 44.0g in the control, mid and high dose group, respectively. The 24h survival (incubation period) of the high dose group (viability index 94.1%) was comparable to control (93.8%). In the 10 mg/kg bw/d group a reduction was seen (80.7%) due to 2 litters having 50% or less survival. In the following dissection no internal abnormalities were seen. Skeletal findings are shown in Table 59. Incomplete ossified sternum sections, non-ossified sternum sections and supernumerary ribs were classified as incidental findings. The incidence of non-ossified sternum sections was at the high end of the range normally observed for foetuses from control in New Zealand albino rabbits in similar studies by this laboratory.

Table 59: Skeletal abnormalities in young rabbits (Anonymous, 1976b).

Group	No of foetuses examined		Incidence	% of total examined
Control	48	• Incomplete ossified sternum sections	32	66.7
		• Non-ossified sternum sections	0	0.0
		• Supernumerary ribs	24	50.0
		• Dual ossification sternum sections	1	2.1
		No. of foetuses with skeletal abnorm.	1	2.1
		No. of foetuses with findings	43	89.6
		No. of foetuses with no findings	5	10.4
10 mg/kg bw/d	57	• Incomplete ossified sternum sections	39	68.4
		• Non-ossified sternum section(s)	3	5.3
		• Supernumerary ribs	14	24.6
		No. of foetuses with skeletal abnorm.	0	0.0
		No. of foetuses with findings	44	77.2
		No. of foetuses with no findings	13	22.8
30 mg/kg bw/d	26*	• Incomplete ossified sternum sections	17	65.4
		• Non-ossified sternum section(s)	3	11.5
		• Supernumerary ribs	11	42.3
		• Spina bifida	1	3.9
		No. of foetuses with skeletal abnorm.	1	3.9
		No. of foetuses with findings	23	88.5
		No. of foetuses with no findings	3	11.5

*Eight foetuses were destroyed during processing

For this teratogenicity study in rabbits it can be concluded that the quality is limited due to high mortality and body weight loss in all dose groups, however, developmental toxicity was only seen in dosed animals. Therefore, abortions in the mid and high dose group as well as increased resorptions at high dose and resulting reduced number of viable young give some cause for concern.

In a **reproduction/developmental toxicity screening test** according to OECD TG 421 (Tanaka, 2001) ten week old rats were exposed to 0, 6, 25 or 100 mg 6PPD/kg bw/d. Males were exposed for 48 days, females 14 days before mating until day 3 of lactation. Body weight and food consumption was examined on a regular basis. Organ weights of liver, adrenals, testes, epididymides were determined. Macroscopical examination of liver and reproductive organs and microscopical examination of liver, kidney, skin, and reproductive organs are documented.

No effects on body weights of males and females are reported. Food consumption was increased in high-dosed males at some points in time and in all high dosed females during lactation. At the end of exposure absolute and relative liver and adrenal weights were elevated in high dose males. In females absolute and relative liver weights were increased in the mid and high dose groups. No effects on testes and epididymides weights are documented. For further details on repeated dose toxicity see Chapter 10.12. For evaluation of the reproductive performance see Chapter 10.10.1. Reproductive parameters are presented in Table 60. The duration of gestation was significantly increased ($p \leq 0.05$) at the highest dose. The number of total (live) pups born was significantly reduced in the 25 mg/kg bw/d group ($p \leq 0.05$) and also reduced (not statistically signif) at 100 mg/kg bw/d. There were no effects on estrus cycle and no effects on sex ratio.

Body weights of F1 pups were statistically significantly increased at 25 and 100 mg/kg bw/d (see Table 61), this may be related to a lower (dose related) number of implants/pups born especially in mid and high dose dams. All live pups were examined for abnormalities. External anomalies were found in 1/131 pups of the highest dose, showing imperforated anus, pes varus and filamentous tail.

The NOEL for parental toxicity was considered to be 6 mg/kg bw/d based on liver effects at 25 mg/kg bw/d in both sexes. Considering the prolonged gestation length the NOAEL for fertility was 25 mg/kg bw/d, for development effects on number of total live pups are described at 25 and 100 mg/kg bw/d. A NOAEL of 6 mg/kg bw/d can be set.

Table 60: Reproductive parameters and delivery findings (Tanaka, 2001).

	0 mg/kg bw/d	6 mg/kg bw/d	25 mg/kg bw/d	100 mg/kg bw/d
Fertility				
No. of pairs mated	12	12	12	12
No. of pairs copulated	12	12	12	12
No. of pregnant females	12	11	12	11
Copulation index (%) [1)]	100.0	100.0	100.0	100.0
Fertility index (%) [2)]	100.0	91.7	100.0	91.7
Findings of delivery				
No. of dams observed	12	11	12	10
No. of dams delivered live pups	12	11	12	10
Duration of gestation [d]	22.2 ± 0.4	22.3 ± 0.5	22.4 ± 0.5	22.7 ± 0.5*
No. of total corpora lutea (mean ± SD)	241 (20.1 ± 4.4)	198 (18.0 ± 2.3)	203 (16.9 ± 3.0)	181 (18.1 ± 3.2)
No. of total implants(mean ± SD)	195 (16.3 ± 1.9)	167 (15.2 ± 1.0)	166 (13.8 ± 2.8)	150 (15.0 ± 1.6)
No. of total pups born (mean ± SD)	184 (15.3 ± 2.5)	160 (14.5 ± 1.0)	149 (12.4 ± 2.4)*	131 (13.1 ± 2.9)
No. of total live pups born (mean ± SD)	183 (15.3 ± 2.5)	158 (14.4 ± 0.9)	148 (12.3 ± 2.3)*	131 (13.1 ± 2.9)
Gestation index [%] [3)]	100.0	100.0	100.0	90.0
Implantation index [%] [4)]	82.9 ± 12.3	85.2 ± 9.0	82.4 ± 13.9	84.2 ± 10.8
Delivery index [%] [5)]	93.9 ± 7.8	95.9 ± 4.2	90.3 ± 7.4	87.8 ± 18.0
Live birth index [%] [6)]	99.5 ± 1.7	89.8 ± 2.6	99.4 ± 2.1	100.0 ± 0.0

** significantly different from control, $p \leq 0.01$; , * significantly different from control, $p \leq 0.05$

[1)] (No. of animals with successful copulation / No. of animals mated) x 100

[2)] (No. of pregnant animals / No. of animals with successful copulation) x 100

[3)] (No. of females with live pups / No. of pregnant females) x 100

[4)] (No. of implants / No. of corpora lutea) x 100
[5)] (No. of pups born / No. of implants) x 100
[6)] (No. of live pups born / No. of pups born) x 100

Table 61: Body weight changes [g] of F1 pups after subchronic exposure to 6PPD (Tanaka, 2001).

[mean ± SD]	Days after birth	0 mg/kg bw/d	6 mg/kg bw/d	25 mg/kg bw/d	100 mg/kg bw/d
Males	0	5.9 ± 0.4	6.1 ± 0.5	6.6 ± 0.7**	6.6 ± 0.5**
	4	7.8 ± 1.6	8.6 ± 1.4	9.7 ± 1.4*	9.3 ± 1.5
Females	0	5.6 ± 0.4	5.8 ± 0.5	6.2 ± 0.6*	6.2 ± 0.5*
	4	7.2 ± 1.8	8.3 ± 1.2	9.2 ± 1.4**	8.8 ± 1.5*

** significantly different from control, p≤0.01; , * significantly different from control, p≤0.05

A recent **OECD 443 study (Anonymous, 2019b)** is available including cohorts 1A, 1B and 2A, 2B. Rats were exposed (oral, gavage) to 0, 7, 20 or 60 mg/kg bw/d (vehicle: corn oil, dose volume 5ml/kg). The study is described in detail in Chapter 10.10.2.

For F1 pups a NOAEL (developmental toxicity) of 7 mg/kg bw/d can be derived based on a dose dependent impairment of pup survival already seen at lower extent at 20 mg/kg bw/d (PND 1-4), which increased in the 60 mg/kg bw group (PND 1-7) - still not statistically significant but outside the historical control range. Total litter loss between LD 0 and 9 was reported for 3 (PND 0, 2, 9), 0, 1 (PND 2) and 5 (PND 2, 2, 4, 7, 9) females in the control, 7, 20, and 60 mg/kg bw/day group, respectively. Mean male and female pup birth weights on PND 1 were comparable across all groups. In the 60 mg/kg bw/d group lower mean absolute body weights on PND 21 (m -8.1% and f -7.4%) were found which remained low on PND 28 (m -5.9%, f -8.6%), but were similar to control on PND 98.

No clear evidence for developmental neurotoxicity was documented. Only lower absolute brain weight (in correlation with lower final body weight) at 60 mg/kg bw/d males (LOEL) and a trend seen in females is described.

In a previously conducted **dose range finding study according to OECD 421** (Anonymous, 2019a) 10 week old Crl:CD(SD) rats were exposed to 0, 50, 75 or 100 mg/kg bw/d (oral, gavage). The study results of F0 and F1 generation are also described in detail in Chapter 10.10.2. On PND 0 and on PND 0-4 postnatal survival was reduced in the high dose group, not statistically significant but below the HCD. This decrease in postnatal survival in the 100 mg/kg bw/day group was due to 2 total litter losses on PND 0 or 2 due to the euthanasia of pups following adverse clinical signs of thin and cool bodies resulting from poor maternal care. F1 body weights in dosed groups were generally similar to the control group throughout the postnatal period while mean body weight gain was generally lower in the first 7 days - but the only statistically significant differences were in the 75 mg/kg bw/day group males during PND 4–7 (-13%), in the 100 mg/kg bw/day group males and females during PND 10–13 (-11,3%, -11.4%), and in the 50 mg/kg bw/day group females during PND 10–13 (-8.8%). Based on the presented thyroid hormone data a disturbance of the thyroid axis by 6PPD can be assumed, which might be relevant for future with regard to a potential ED classification.

In a **three generation reproductive study** (Anonymous, 1980b) Charles River CD rats (groups of 8 males and 16 females) were exposed to 6PPD concentrations of 0, 100, 300 or 1000 ppm (approx. 0, 8, 23, 75 mg/kg bw/d) via diet (ad libitum). For further details on study design see Chapter 10.10.2.

In the study protocol several shortcomings are documented: (1) no quality assurance review, (2) general high mortality data in control and dose groups, (3) no evidence that all pups with abnormal development were subject to gross pathology, (4) only partly randomized procedures for selection of animals, (5) documentation of replacement animals inconsistent. (6) acetone has been used as solvent for 6PPD, the diet of the control group contained acetone from the fifth week on. (7) A large number of treated and control F0 animals were reported as possibly having respiratory infection.

Mortality was documented for test and control animals during each generation, the numbers being unusually high in some instances. A correlation with the administration of the test substance cannot be made. **Body weight (gains)** were affected on some points in time with no clear dose dependency. For further details see Chapter 10.10.2

Reproductive performance was not affected by 6PPD treatment. For information on fertility see Chapter 10.10.2. The mean number of live or dead pups at birth, and the number of pups weaned, were comparable between control and treated groups. **Survival indices** were also comparable between most dose groups. Pups in the mid dose group had reduced survival indices in some litters (see Table 62). Mean **body weights of pups** measured at day 21 of lactation were considered comparable for all groups; only some minor statistical variations were noted. No unusual behavioral reactions related to treatment were noted for offspring during the study.

Table 62: Survival indices (Anonymous, 1980b).

Dose [ppm]	Litter	Live birth index [%]	Survival index [%]			
			24h *	4d *	12d #	21d #
0	F1A	97.3	96.7	90.0	87.5	76.5
	F1B	99.2	97.6	91.9	97.2	81.1
100	F1A	98.8	96.7	88.0	100.0	99.1
	F1B	99.3	98.5	94.8	92.6	71.3
300	F1A	98.8	98.8	95.2	81.7	71.1
	F1B	95.0	99.3	75.7	93.2	80.6
1000	F1A	97.6	99.4	98.8	90.2	77.6
	F1B	99.5	98.2	84.6	75.7	69.6
0	F2A	93.2	98.4	95.9	89.4	86.7
	F2B	96.5	100.0	78.0	76.5	61.7
100	F2A	95.2	100.0	99.0	82.3	72.9
	F2B	97.8	98.9	98.9	97.6	91.5
300	F2A	100.0	96.4	77.4	72.6	54.8
	F2B	94.3	97.0	83.3	86.5	82.7
1000	F2A	96.6	98.8	97.6	96.3	79.3
	F2B	99.0	99.0	97.1	93.7	76.8
0	F3A	91.7	100.0	87.0	91.8	89.4
	F3B	96.8	96.7	73.8	95.4	83.9
100	F3A	91.5	97.5	89.9	85.6	76.0
	F3B	95.2	100.0	97.0	94.7	82.1
300	F3A	94.4	90.6	74.1	62.3	57.4
	F3B	92.8	89.1	76.6	58.7	56.5
1000	F3A	96.3	98.1	94.2	83.3	67.7
	F3B	93.5	94.8	85.3	91.2	80.2

*Number of pups viable at LD 1 (or 4) / number of viable pups born x 100
Number of pups viable at LD 12 (or 21) / number of pups retained at LD 4 x 100

Gross necropsy observations of F0, F1, and F2 adults sacrificed after weaning of their second litters did not reveal any adverse effects related to test material administration. No treatment related statistical differences in **organ weights** were noted for F0, F1, or F2 parental animals in any treatment group with the exception of liver weight. Female total liver weight was (not statistically significant) increased in a dose dependent manner in F0, F1 and F2. Females relative liver weight (to body weight) was statistically significantly

increased in 1000ppm F1 (+34%, $p<0.01$) and F2 (+41%, $p<0.05$) generation. Effects on male liver weights were not significant. **Microscopic examination** of tissues from selected F0, F1 and F2 parental animals and F3b pups from control and high dose groups, and from selected animals from low and mid dose groups, showed no test-substance related abnormalities.

In summary in this three generation study with administration of 6PPD via diet, despite the high mortality in all groups, no adverse effects of 6PPD on parental animals was seen. Fetal and pup survival were not affected. 100 ppm can be considered as NOAEL in this study.

In a **range-finding teratology study** (Anonymous, 1986a; cited from ECHA dissemination site) mated Sprague-Dawley rats were treated by oral gavage at dosage levels of 0, 100, 300, 600, 1000 and 2000 mg/kg bw/day (n=5/group) on GD 6 through 15. Animals were observed daily for mortality or signs of toxicity. Maternal body weights were recorded at various intervals during the study. All surviving dams were sacrificed on GD 20. Uterus and ovaries were examined and the number and location of viable and nonviable fetuses, early and late resorptions and number of total implantations and corpora lutea were recorded. All animals in the 600, 1000 and 2000 mg/kg bw/d groups died from GD 8 to 12. At gross necropsy, the most common lesions for early death animals were congested kidneys, enlarged adrenals, intestinal and gastric epithelial loss, hyeremic lungs and meningeal congestion or hemorrhage. No treatment related gross lesions were observed in the 100 and 300 mg/kg bw/day group, all of whom survived to termination. Lethargic behaviour, pale tail, body cool to the touch, dried red material around mouth and nose and diarrhea were associated with treatment at 300 mg/kg bw/day and higher dose levels. Excessive toxicity (death) was reported for concentrations ≥ 600 mg/kg bw/d. Intrauterine survival was not affected by treatment at ≤ 300 mg/kg bw/d. No further data available.

In a **pilot rat teratology study** (Anonymous, 1976a; cited from ECHA dissemination site) rats were exposed at least to concentrations of 30, 100 and 300 mg/kg bw/d. Significant maternal toxicity in terms of mortality (80%) occurred at a dosage of 300 mg/kg while absorptions were increased at 100 mg/kg bw/d. Signs of developmental toxicity included increased resorptions at 30, 100 and 300 mg/kg bw/d with reduced pup survival for 24 hours in an incubator after maternal C-section at the low-dose of 30 mg/kg bw/d only. No further data available.

In a **teratology study** (Anonymous, 1976b; cited from ECHA dissemination site) signs of maternal toxicity (abortions and body weight changes) were noted in 30 mg/kg bw/d treated dams. Signs of developmental toxicity (slight increases in resorptions and fetal death) were observed at 10 and 30 mg/kg bw/d. Because there were no statistically significant differences between control and treated groups for these responses and no trend was evident, the study authors considered these responses as questionable biological relevant. However, results have been considered as supportive evidence.

A **pilot rabbit teratology study** (Anonymous, 1976c; cited from ECHA dissemination site) was conducted with groups of pregnant rabbits orally administered 0, 30, 100 or 300 mg/kg bw/d of 6PPD (GD 6-18). Maternal toxicity was encountered at dose levels of 100 and 300 mg/kg bw/d. The group treated with 30 mg/kg bw/d showed mild body weight losses along with a marginal increase in fetal resorption.

6PPD was tested for embryotoxicity and induction of malformations in three-day **chicken embryos** (Korhonen, 1983). Substance was dosed by dropping the chemical into the air chamber of the egg. Two days later dead embryos were scored and discarded. Remaining embryos were checked for deaths and malformations up to day 14 of incubation (11d after treatment). The effective dose ED_{50} (dose/egg) of 6PPD was determined to be 1.5 µmol (maximum malformed: 40 %). For details see Table 48. Study authors tested about 80 chemicals with this setting and mention that an interesting aspect was the universal occurrence of malformations probably as a result of the use of doses close to the LD_{50}. The study was not considered relevant for classification purpose.

In a publication by **Schardein** (1993, cited from ECHA dissemination site) a negative result in rats is reported. No further details are available.

Three **pubertal studies** were done with 6PPD to assess the potential effect on the endocrine system, by investigating effects on pubertal development and thyroid function.

Anonymous (2016a) administered 6PPD (oral, gavage) to juvenile/peripubertal female rats (n=15/group) in concentrations of 0, 250 or 500 mg/kg bw/d from PND 22 – 42/43. The dose levels were based on the results of a previous dose range finding study where female juveniles (5/group) were dosed at 125, 250, and 500 mg/kg bw/day for 3 consecutive days. The females were observed twice daily for mortality and moribundity from weaning through study termination. Clinical observations and body weights were recorded daily. All females were observed daily for vaginal opening. Vaginal lavages were performed from that time on to determine the stage of the estrous cycle. Necropsy was done for animals found dead and survivors on scheduled euthanasia PND 42/43. Selected organs were weighted (uterus, adrenal glands, kidney, liver ovaries, pituitary gland, thyroid) and histopathological evaluation of the thyroid, kidney, ovary, and uterus was performed. T4 and TSH as well as serum chemistry were done on PND 42/43.

Two females were found dead in the high dose group (PND 25, 40) and one in the control (PND 40) with no significant clinical findings. One additional female in the high dose group died (PND 25) as result of an intubation error. Clinical findings included salivation prior to dosing, clear material around the mouth or yellow material around the urogenital area in the treated groups.

Lower initial mean body weight gains were noted for females in the two dosed groups following the first 2 (250 mg/kg bw/day) or 3 days (500 mg/kg bw/day) of 6PPD administration; this effects was considered test substance related. As a result, mean body weights in these groups were up to 8.37% and 14.83% lower during the treatment period, respectively, compared to control. Throughout the remainder of the treatment period mean body weight gains in these groups were generally similar to the control group and when the entire treatment period (PND 22-42) was evaluated.

Vaginal opening (complete and incomplete) was 35.2 d in the control and 33.2 d at 500 mg/kg bw/d (see Table 63). At this time lower mean body weights were noted for females in the 250 (-11.48%) and 500 (-18.8%) mg/kg bw/day groups. In the high dose group first estrous was observed after 39.2 d, compared to the control group with 36.3 days. Due to the low number of females that were cycling in the dosed groups (6 and 2 in the mid and high dose, respectively), estrous cycle lengths could not be evaluated (see Table 64). The lower number of females that were cycling in these groups was partially due to the numbers of females with insufficient data[7] in the 250 and 500 mg/kg bw/day group (3 and 4 females, respectively).

Table 63: Vaginal opening and general growth (unadjusted) [mean ±SD, n] (Anonymous, 2016a).

Dose group	Age at opening [PND]	Age at opening (incomplete) [PND]	Body weight at opening [g]	Final body weight [% of control]
Control (n=15)	35.2 ± 1.9	35.2 ± 1.9	128.1 ± 8.73	-
250 mg/kg bw/d (n=15)	34.3 ± 2.32	34.3 ± 2.32	113.4 ± 16.69*	-2.72
500 mg/kg bw/d (n=13)	33.2 ± 3.03	32.3 ± 1.93*	104.0 ± 21.1*	-5.9

*p<0.05 using Dunnett's test

[7] Insufficient data: the animal does not display at least 1 complete cycle but has at least 1 E and/or P and a partial cycle of 5 days or fewer, no E present on any days of estrous cycle determination and 4 or fewer days of data collected, or at least 1 E or P present and only 1-4 days of data collected.

Table 64: Estrus cycle data and T4, TSH levels [mean ±SD, n] (Anonymous, 2016a).

Dose group	Age at first estrus [days ± SD, n]	Estrus cycle length [days ± SD, n]	Cycling [%]	Regularly cycling [%]	Total T4 [µg/dL]	TSH [ng/m]
Control	36.3 ± 2.25 15	4.8 ± 0.42 10	100	100	4.5 ± 0.700 14	6.5 ± 2.8 14
250 mg/kg bw/d	36.8 ± 2.68 15	5.0 ± 0.63 6	93.3	45.5 +	4.3 ± 0.458 15	12.5 ± 6.40* 15
500 mg/kg bw/d	39.2 ± 3.70* 13	4.8 ± 0.35 2	83.3	14.3 ++	3.50 ± 0.525* 12	11.2 ± 5.25* 12

* Significantly different from the control group at 0.05 using Dunnett's test
\+ Significantly different from the control group at 0.05 using Chi-Square test
++ Significantly different from the control group at 0.01 using Chi-Square test

Clinical chemistry revealed higher mean total bilirubin (+700%, $p<0.05$) and GGT (+100%, not signif) in the high dose group and higher serum cholesterol in the low (23.7%, $p<0.05$) and high dose groups (24.7%, $p<0.05$); all exceeding the historical control reference range. Lower mean AST (-39.8% and -47.3%) and lower serum triglycerides (-33.9% and -51.2%) (all $p<0.05$) are documented for low and high dose groups; values were within the historical control range. Lower serum T4 and higher TSH in the 250 and 500 mg/kg bw/day group females (see Table 64), were considered test substance related but non-adverse by the study authors.

Pale liver was found in 2/15 females in the 500 mg/kg bw/day group; this correlated to a microscopic finding of hepatocellular vacuolation. Lower uterus weights (blotted and unblotted) and higher kidney, liver, and thyroid gland weights in the 250 and 500 mg/kg bw/day group females and lower ovary weights in the 500 mg/kg bw/day group were recorded ($p<0.05$) (see Table 65). Microscopic investigations revealed higher follicular cell height and lower colloid area in the thyroid glands of 250 and 500 mg/kg bw/day group females, vacuolation of the liver in high dose females, an absence of corpora lutea with increased tertiary follicles (i.e., non-cycling) in the ovaries at 500 mg/kg bw/day, and immature uterus of 500 mg/kg bw/day group females (see Table 66).

Table 65: Selected organ weights, females [mean ±SD, unadjusted] (Anonymous, 2016a).

Dose group	Adrenal glands [mg]	Kidney [g]	Liver [g]	Ovaries [mg]	Pituitary [mg]	Thyroid glands [mg]	Uterus – blotted [mg]	Uterus wet [mg]
Control N=14	35.9 ± 6.75	1.32 ± 0.100	7.67 ± 0.678	80.1 ± 13.26	10.3 ± 2.32	11.51 ± 1.822	259.8 ± 74.60	334.3 ±185.2
250 mg/kg bw/d N=15	41.0 ± 6.22 14.2%	1.45 ± 0.140* 9.8%	10.91 ± 1.839* 42.2%	73.6 ± 8.62 -8.1%	9.2 ± 1.30 -10.7%	12.28 ± 1.969 6.7%	211.1 ± 68.40 -18.7%	247.7 ± 102.4 -25.9%
500 mg/kg bw/d N=12	38.7 ± 5.61 7.8%	1.46 ± 0.191* 10.6%	11.69 ± 1.258* 52.4%	62.9 ± 19.59* -21.5%	8.0 ± 1.46* -22.3%	12.86 ± 2.201 11.7%	157.6 ± 73.96* -39.3%	187.6* ± 108.96 -43.9%

*p<0.05

Table 66: Microscopic findings, statistic analysis (Anonymous, 2016a).

Finding	Grade	Control	250 mg/kg bw/d	500 mgkg bw/d
Total examined		14	15	12

Thyroids – colloid area	Grade 3	0	9	8
	Grade 4	2	6	4
	Grade 5	12	0	0
Thyroids - follicular cell height	Grade 1	12	1	0
	Grade 2	2	6	5
	Grade 3	0	8	7
Ovaries – corpora lutea, growth phase	Grade 1	0	0	4
	Grade 3	14	15	8
Ovaries – corpora lutea, mature phase	Grade 1	0	0	4
	Grade 3	14	15	8
Ovaries – corpora lutea, regressive phase	Grade 1	0	0	4
	Grade 3	14	15	8
Ovaries - follicles tertiary	Grade 3	14	15	8
	Grade 4	0	0	4
Uterus	Examined unremarkable	14	15	10
	immature	0	0	2

Shaded values p<0.05 (Mann-Whitney U test)

To summarize, changes in the ovaries and the uterus as well as changes in reproductive endpoints including age at attainment of vaginal opening, body weight at attainment of vaginal opening, and age at first estrus give some evidence of possible test substance-related endocrine-mediated effects. Effects on thyroid hormones are inconsistent, however microscopic investigations revealed higher follicular cell height and lower colloid area in the thyroid glands of 250 and 500 mg/kg bw/day group females. This could be mediated by liver enzyme induction, for which the observed hepatomegaly could be an indication, however, no clear evidence for this mode of action is available.

In a second study (**Anonymous, 2017**) juvenile/peripubertal female rats were exposed to 0, 10, 100 or 300 mg/kg bw/d (n=15/group) from PND 22 to 42/43. The study design was the same as for Anonymous (2016a).

One female in the 100 mg/kg bw/day group was found dead (PND 40) following body weight loss (9.1% from PND 28-39). Clear material around the mouth and/or nose were noted following administration (2h) in the 100 and 300 mg/kg bw/d groups. Mean body weights and body weight gains were unaffected in all dose groups.

A lower mean body weight (-11.9%, not signif) at attainment of vaginal opening was noted in the high dose group compared to the control group, however, due to the absence of a test substance-related effect on the day of attainment of vaginal patency, the difference was attributed to biological variability in this group (see Table 67). No effects were seen at 10 and 100 mg/kg bw/day.

Table 67: Vaginal opening [mean ±SD, n] (Anonymous, 2017).

Dose group	Age at opening [PND]	Age at opening (incomplete) [PND]	Body weight at opening [g]	Final body weight (PND 42) [% of control]
Control (n= 15)	34.7 ± 3.04	34.2 ± 2.23	127.1 ± 19.00	168.1 ± 14.11
10 mg/kg bw/d	33.8 ± 3.36	32.8 ± 2.11	117.7 ± 20.97	162.3 ± 14.76

(n= 15)				
100 mg/kg bw/d (n=15)	34.3 ± 3.33	33.7 ± 2.32	122.0 ± 19.72	165.6 ± 16.36
300 mg/kg bw/d (n= 15)	33.3 ± 2.37	33.3 ± 2.37	112.0 ± 16.61	166.9 ± 16.68

*p<0.05

Ten females in the 300 mg/kg bw/day group were noted with irregular estrous cycles and 2 females not cycling compared to the control group. Consequently, a lower number (2f) and percentage of females (16.7%) in this group were noted as cycling normally compared to the control group. Differences were not statistically significant, the proportion of females in the 300 mg/kg bw/day group that were cycling regularly was below the minimum mean value in the Charles River Ashland historical control data (42.9%). This was considered test substance related. Cyclicity in other groups was similar to control. Mean ages at the first occurrence of estrous and estrous cycle length at 10, 100, and 300 mg/kg bw/day were similar to the control group (HCD 36.8 days).

Table 68: Estrus cycle data and T4, TSH levels [mean ±SD, n] (Anonymous, 2017).

Dose group	Age at first estrus [days ± SD, n]	Estrus cycle length [days ± SD, n] #	Cycling [%]	Regularly cycling [%]	Total T4 [µg/dL]	TSH [ng/m]
Control	36.6 ± 3.10 14	4.5 ± 0.50 7	100.0	63.5	5.4 ± 0.831 15	9.4 ± 4.686 15
10 mg/kg bw/d	35.4 ± 3.26 12	4.8 ± 0.27 5	85.7	50.0	5.96 ± 0.923 14	9.52 ± 4.002 15
100 mg/kg bw/d	36.5 ± 3.53 11	4.9 ± 0.66 6	84.6	36.4	6.1 ± 0.96 14	10.81 ± 4.576 14
300 mg/kg bw/d	36.8 ± 2.17 13	4.0 ± 0.00 4	86.7	16.7	4.95 ± 0.627	16.97 ± 8.681*

* Significantly different from the control group at 0.05
# Historical control data: 4.0 – 5.0 days

Test substance-related higher liver weights (+47.8%), increased TSH (+15% and +80.5%, p<0.05) and follicular cell height as well as lower thyroid colloid area in the 100 and 300 mg/kg bw/day groups, and lower T4 (-8.3%) in the 300 mg/kg bw/day group were noted. Additionally, test substance-related findings were noted in serum chemistry and organ weights. Specifically, higher albumin, globulin, total protein, calcium, and cholesterol, and lower chloride, triglyceride, albumin to globulin ratio, and AST were considered test substance-related.

Table 69: Microscopic findings – statistical analysis (Anonymous, 2017).

Finding	Grade	Control	10 mg/kg bw/d	100 mg/kg bw/d	300 mg/kg bw/d
Total examined		15	15	14	15
Thyroids – colloid area	Grade 2	0	0	1	5
	Grade 3	0	1	4	7
	Grade 4	15	14	9	3
Thyroids -	Grade 2	15	13	5	0

follicular cell height	Grade 3	0	2	9	13
	Grade 4	0	0	0	2

Shades values p<0.05 (Mann-Whitney U test)

Study authors considered hepatomegaly as cause for microscopic changes in the thyroid, higher liver weights, lower levels of T4, triglycerides and AST, and higher TSH and cholesterol.

Lower ovarian (-16.9%) and uterine weights (-33.8%) were noted in the 300 mg/kg bw/day group and correlated with a lower percentage of regularly cycling females in this dose group. Taken together this study supports the effects seen in the first study (Anonymous, 2016a).

Potential effects of 6PPD on the endocrine system in the juvenile/peripubertal male rat were investigated by **Anonymous (2016b)**. Male rats were exposed to 0, 250 or 500 mg/kg bw/d (oral, gavage) from PND 23 – 53/54. The dose levels were based on the results of a previous dose range finding study where male juvenils (5/group) were dosed at 125, 250, and 500 mg/kg bw/day for 7 consecutive days. Main study design was the same as described by Anonymous (2016a), with the modification that all males were observed daily (from PND 30 on) for balanopreputial separation. Hormone (T4, TSH, and testosterone) and clinical pathology evaluations (serum chemistry) were conducted on all surviving animals on PND 53/54. Histopathological evaluation of the thyroid, kidney, testes, and epididymis was performed.

One male from the high dose group was euthanized in extremis on PND 25 following 2 days of severe body weight loss (18.9%). Cool and pale body, soft faeces and yellow material on the urogenital/anogenital areas were reported on the day of death. No mortality was seen in the control and low dose group. Clinical signs of all dosed animals included increased incidences of salivation prior to dosing and clear and/or red material around the mouth following dose administration in a dose-responsive manner.

Lower mean **body weight** gains were noted in the 250 and 500 mg/kg bw/day groups throughout the dosing period from PND 23-53 (see Table 70). The severity of this effect was dose-responsive. As a result, mean body weights in the high dose group were 12.39% lower than the control group on PND 25 and continued to be lower (14.51% to 22.33%) through the end of the dosing period (PND 53). In the low dose group mean body weights were lower (5.62% to 8.69%) than the control group during PND 25-53. Differences were generally significant.

The mean age of attainment of **balanopreputial separation** (BPS) was delayed in the 500 mg/kg bw/day group (49.3 d) compared to control (46.2 d) and lower mean body weights on the day of attainment of balanopreputial separation were noted in both dosed groups (see Table 70). The reduction was -11.2% in the low and -14.5% in the high dose group, respectively.

Table 70: Selected male parameters [mean ±SD, n, %] (Anonymous, 2016b).

Dose group	Body weight gain (PND 23-53) [g]	Age at balanopreputial separation (BPS) [days]	Body weight at BPS [g]	Testosterone [ng/ml]	Total T4 [mg/dl]	TSH [ng/ml]
Control	232.6 ± 26.34 15	46.2 ± 2.68 15	236.9 ± 29.79 15	3.48 ± 1.933 15	5.33 ± 0.622 15	10.6 ± 3.84 15
250 mg/kg bw/d	210.3 ± 13.71* 15 -9.6%	45.3 ± 2.55 15	210.4 ± 16.61* 15 -11.2%	0.91 ± 0.802* 13 -73,9%	4.75 ± 0.651* 15 -10.9%	13.1 ± 8.03 15 +23.6%
500 mg/kg bw/d	168.5 ± 27.72* 14 -27.5%	49.3 ± 3.77* 14	202.6 ± 19.58* 14 -14.5%	0.61 ± 0.664* 11 -82.5%	3.59 ± 0.605* 14 -32.6%	19.0 ± 13.97 14 +79.2%

* Significantly different from the control group at 0.05

Administration of 6PPD was associated with higher ALT (+54.9%) and GGT (0.8 U/l compared to control 0.0 u/l) in the 500 mg/kg bw/day males, lower mean serum triglyceride levels in the 250 (-62.0%) and 500 (-74.8%) mg/kg/day group males, lower total T4 and testosterone and higher serum TSH levels in both dosed groups (see Table 70), higher liver weights as well as lower epididymides, testes, prostate, and seminal vesicle/coagulating gland weights in the 500 and/or 250 mg/kg bw/day group males (see Table 71) and higher follicular cell height and lower colloid area in both groups (see Table 72). These changes were not considered to be a direct toxic effect of 6PPD by study authors. Microscopic examination showed no test substance related changes in the testes or epididymides (low incidence of mild decreased sperm was noted in 1, 2 and 1 male of control low and mid dose, respectively). The microscopic changes in the thyroid gland (follicular cell height increase and decreased colloid area), higher liver weights, lower serum T4, higher serum TSH, and higher ALT and GGT were, like for females, considered secondary to hepatomegaly related to metabolic enzyme induction by the study authors. The lower testosterone levels and the lower male reproductive organ weights were considered secondary to stress and/or lower body weights by study authors.

Table 71: Selected organ weights, males [mean ±SD, %] (Anonymous, 2016b).

Dose group	Adrenal glands [mg]	Dorso prostate [mg]	Ventral prostate [mg]	Epididymis, lt [mg]	Epididymis, rt [mg]	Pituitary [mg]	Testis, left [mg] #	Thyroid glands [mg]	Liver [g]	LABC muscle [mg]
Control N=15	39.2 ± 7.43	105.4 ± 23.31	233.4 ± 69.07	201.7 ± 23.42	204.7 ± 24.31	10.0 ± 1.44	1428.1 ± 109.05	12.68 ± 2.672	13.50 ± 1.879	534.4 ± 81.92
250 mg/kg bw/d N=15	43.7 ± 5.83* 11.5%	87.2 ± 22.94* -17.3%	190.6 ± 52.46* -18.3%	198.4 ± 21.86 -1.6%	204.7 ± 20.92 0.0%	9.1 ± 0.88 -9.0%	1424.9 ± 120.60 -0.2%	11.76 ± 2.499 -7.2%	17.68 ± 1.950* +31.0%	458.2 ± 60.61* -14.3%
500 mg/kg bw/d N=14	38.4 ± 5.68 -2.0%	63.4 ± 18.40* -39.8%	135.3 ± 43.64* -48.0%	168.5 ± 25.12* -16,5%	173.4 ± 25.01* -15.3%	7.4 ± 0.98* -26.0%	1270.9 ± 108.68* -11.0%	9.87 ± 1.997* -22.2%	17.90 ± 4.181* 32.6%	315.6 ± 85.42* -40.9%

*Statistically significant at the 0.05 level (Dunnett)
Values similar to testis right

Table 72: Microscopic findings – statistical analysis (Anonymous, 2016b).

Finding	Grade	Control	250 mg/kg bw/d	500 mg/kg bw/d
Total examined		15	15	14
Thyroids – colloid area	Grade 2	0	4	5
	Grade 3	9	10	8
	Grade 4	5	1	1
	Grade 5	1	0	0
Thyroids - follicular cell height	Grade 1	1	0	0
	Grade 2	7	4	3
	Grade 3	7	8	10
	Grade 4	0	3	1

Shades values p<0.05 (Mann-Whitney U test)

A delay in the onset of puberty can sometimes be explained by lower body weight or body weight gain and a general delay in development. However, it is also reported that puberty onset is insensitive to changes in growth (Stump et al., 2014). The study authors of the present study concluded that changes in the mean age when BPS was attained were considered to be due to the lower mean body weights in these groups. In addition it is cited in the study report, that in a **Hershberger assay** 6PPD showed no androgenic or antiandrogenic effects when administered to peripubertal orchidoepididymectomized rats (no further details available). In the present study a considerable and dose dependent decrease in testosterone levels was seen in both treatment groups (-74% and -83% in low and high dose group, respectively). A potential relation to the observed delay in BPS cannot be excluded. The fact that puberty onset was earlier in females but delayed in males does not necessarily invalidate the findings, as especially for endocrine mediated effects different responses in males and females are not uncommon. Overall, it is not clear whether the lower body weight and / or the lowered testosterone levels or any other effect was the underlying cause of the observed delay in BPS.

10.10.6 Comparison with the CLP criteria

Substances are classified in Category 1 for reproductive toxicity when they are known to have produced an adverse effect 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). Adverse effects on development • The classification of a substance in Category 1A is largely based on evidence from humans. • 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 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.
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 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.

No human data is available to justify classification in Category 1A.

In an OECD 414 study (Anonymous, 2018c) after administration of 100 mg 6PPD/kg bw/d to rabbits from GD 7-28 postimplantation loss (exceeding HCD), including early and late resorptions, as well as reduced mean fetal body weight (-18.0%) on GD 29 is documented. Reduced fetal weight is also reported at 50 mg/kg bw/d (-8.7%). Gravid uterine weight was reduced significantly at 100 mg/kg bw/day (-18.3%) but also at 50 mg/kg bw/d (-10.0%, not statist. signif.) compared to a minimal decrease of corrected maternal bw of -4.6% in the high dose and -3.8% in the mid dose. Reduced gravid uterine weight correlates with reduced fetal weight. Fluctuations in individual food consumption are documented but no clear correlation with resorptions can be made, on the basis of individual animal data. A NOAEL of 25 mg/kg bw/d can be derived for developmental toxicity as well as maternal toxicity (reduced body weight, increased liver weight).

Rabbits were also investigated by Anonymous (1976b) (GD 6-18). High mortality was seen in all dose and control groups and impairs the quality of the study, however, developmental toxicity (increased resorptions, reduced number of viable young) was seen only in dosed groups and is supported by similar effects seen in an OECD 414 study. At a concentration of 30 mg/kg bw/d an increased number of early resorptions and

reduced number of live young was documented. Abortions were seen at 10 mg/kg bw/d and 30 mg/kg bw/d but not in the control group. A LOAEL of 10 mg/kg bw/d can be established.

In an OECD 414 study in rats (Anonymous, 1987) investigating doses up to 250 mg/kg bw/d in rats (GD 6-15, termination on GD 20) no substance related adverse effects on pups are documented.

Tanaka (2001) reports an OECD 421 screening study in rats. The number of total (live) pups born was significantly reduced in the 25 mg/kg bw/d group ($p \leq 0.05$) and also reduced (not statistically signif) at 100 mg/kg bw/d with no marked maternal toxicity. These results support findings from rabbit studies. A NOAEL of 6 mg/kg bw/d can be derived.

In an OECD 443 study (Anonymous, 2019b) for F1 pups a NOAEL (developmental toxicity) of 7 mg/kg bw/d can be derived based on a dose dependent impairment of pup survival already seen at lower extend at 20 mg/kg bw/d (PND 1-4), which increased in the 60 mg/kg bw/d group (PND 1-7) (outside HCD). Substance related total litter loss was seen at 60 mg/kg bw/day in 5 females. In the 60 mg/kg bw/d group lower mean absolute body weights on PND 21 (m -8.1% and f -7.4%) were found which remained low on PND 28 (m -5.9%, f -8.6%), but were similar to control on PND 98. The effect on male F1 brain weight seen at 60 mg/kg bw/day (-7.3%) was not considered relevant for classification as this finding was in correlation with reduced final body weight of F1 pups and effect on brain weight was only seen in this study. For F0 no effects on thyroid weights and no microscopic findings were documented, T4 levels were dose-dependent increased but TSH also showed a trend of increase. For F1 no test-substance related effects on T4 (PND 4, 21, 91) and TSH levels (PND 21, 91) are reported.

In a dose range finding study according to OECD 421 (Anonymous, 2019a) on PND 0 and on PND 0-4 postnatal survival was reduced at 100 mg/kg bw/d (below HCD) due to 2 total litter losses on PND 0 or 2 (poor maternal care). F1 body weights in dosed groups were generally similar to the control group throughout the postnatal period while mean body weight gain was generally lower in the first 7 days. Based on the presented thyroid hormone data of F1 a disturbance of the thyroid axis by 6PPD can be assumed, which might be relevant for future with regard to a potential ED classification.

In a three generation study (Anonymous, 1980b) high mortality in control and high dose rats was seen. Respiratory infection is reported. No developmental toxicity was observed up to the highest dose tested (75 mg/kg bw/d).

A range finding teratology study (Anonymous, 1986a) showed mortality of all dams (rats) at concentrations ≥ 600 mg/kg bw/d. Intrauterine survival was not affected by treatment at ≤ 300 mg/kg bw/d.

For three older studies only a short description with very limited information is available; registrants indicate Klimisch 4 score. Anonymous (1976c) describes a pilot teratology study in rats with high mortality of dams (80%) and increased resorptions at 300 mg/kg bw/d. Increased resorptions were also seen at 100 and 30 mg/kg bw/d. In the following teratology study (Anonymous, 1976d) a slight increase of resorptions and fetal death was seen down to a concentration of 10 mg/kg bw/d. A pilot study in rabbits (Anonymous, 1976e) with exposure from GD 6-18 showed maternal toxicity at 100 and 300 mg/kg bw/d and some mild effects (mild body weight losses, marginal increase in fetal resorption) at 30 mg/kg bw/d. Generally the information from these studies is limited but resorptions seen in rats in all three studies can be used as supportive evidence for an adverse effect on development.

Korhonen (1983) describes a prescreening test with chicken embryos with a resulting ED_{50} of 1.5µmol (dose/egg), the read out was early / late death, malformations.

Two pubertal studies in female rats give some cause for concern for a potential interference with the endocrine system due to effects on hormone dependent tissues reported. An early onset of puberty is indicated by a dose dependent earlier onset of vaginal opening, despite lower body weights. Lower ovaries and uterus weights were reported at 500 and 200 mg/kg bw/d and microscopic investigations revealed an absence of corpora lutea with increased tertiary follicles (i.e., non-cycling) in the ovaries, and immature uterus in females of the 500 mg/kg bw/d group. It can be summarised that irregular cycling females (at 300 mg/kg bw/d), earlier age at vaginal opening (at 500 mg/kg bw/d), lower body weight at attainment of vaginal opening (at 250 mg/kg bw/d and 500 mg/kg bw/d), and higher age at first oestrus (at 500 mg/kg bw/d) were observed.

In a pubertal study with male rats higher mean age of attainment of BPS (at 500 mg/kg bw/d), lower mean testes weight (at 500 mg/kg bw/d) and a decrease in testosterone levels (-74% and -83% at 250 and 500 mg/kg bw/d, respectively) is reported. However, no sound conclusion can be drawn on the relevance of the delay in puberty onset, due to a general impact on body weight gain which may confound the results on puberty onset. The lowered testosterone levels and the lowered testis weight is not considered sufficiently severe in order to demonstrate an adverse effect and no link to the delay in puberty onset can be made (lack of mechanistic information).

6PPD seems to have an impact on the thyroid axis which is evidenced by some histopathological changes and changes in thyroid hormone levels. Some studies indicate that an increased catabolism by induced liver enzymes could be involved in these changes. However, although thyroid hormones were affected in several studies the changes were no consistent between or even within studies.

In the available studies no relevant malformations were observed in neither rats nor rabbits, the main effect was on foetal / pup body weights and pre- and post-natal survival. It is noted that low birth weight has been linked to adverse outcome later in life in animals as well as humans. All these effects are clearly adverse and considered supportive for a classification for developmental toxicity.

10.10.7 Adverse effects on or via lactation

Table 73: 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	Reference
OECD 443 extended one-generation reproductive toxicity - with developmental neurotoxicity (Cohorts 1A, 1B without extension, 2A and 2B) GLP Rat, Crl:CD(SD) F0: N=25/sex/group High dose group: n= 30/sex/dose	6PPD (purity 96.9%) 0, 7, 20, 60 mg/kg bw/d Oral, gavage Vehicle: corn oil 7d/week F0 females: 70d+mating+gestation+lactation F0 males: like females F1: day of weaning – day prior to euthanasia (PND 21 [Cohort 2B], PND 91 [Cohort 1A], PND 78 [Cohort 2A], and PND 98 [Cohort 1B])	NOAEL (parental tox) = 20 mg/kg bw/d NOAEL fertility (females) = 7 mg/kg bw/d NOAEL fertility (males) = 60 mg/kg bw/d NOAEL (dev tox) = 7 mg/kg bw **F1:** <u>60 mg/kg bw/d</u> Postnatal survival ↓ (PND 1-7) (not stat. signif. but above HCD), 111(20) pups (litters) found dead or were euthanized in extremis; 21/111 with no milk in stomach; mean bw on PND 21 ↓ (m -8.1% and f -7.4%, not stat. signif.) and on PND 28 mean bw remained lower (m -5.9%, f -8.6%, not stat. signif.), no effect on bw on PND 98 Cohort 1A: mean albumin↑ (m +11.9%, p<0.01; f +16.3%, p<0.01), total protein↑ (m +12.5%, p<0.01; f +15.4%, p<0.01), globulin↑ (m +13.6%, p<0.01; f +13.6%, p<0.05), cholesterol level ↑ (m +36.6%, p<0.01; f +32.5%, p<0.01) adrenal gland weights ↑ (rel to final bw: m +15.4%,	Anonymous (2019b) Klimisch 1

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
		p<0.01, f +20.8%, p<0.01), abs. and rel. kidney weight ↑ (rel. to final bw: m +10.6%, p<0.01; f +15.4%, p<0.01), abs. and rel. liver weight ↑ (rel to final bw: m +18.9%; f +30.5%, p<0.01) Cohort 2B (PND 21): brain weight ↓ (-7.3%, p<0.01) Cohort 2A (PND 78): brain weight↓ (-5.2%, not stat. signif.) <u>20 mg/kg bw/d</u> Postnatal survival ↓ (not signif) Cohort 1A: Mean cholesterol level ↑ (m +20.7, %, p<0.05) adrenal gland weights ↑ (f +14.8%, p <0.05), liver weight↑ (f +15.3%, p<0.01)	
Dose-range finding study similar to OECD 421 GLP Crl:CD(SD) rats N= 15/sex/dose	6PPD (purity 96.9%) 0, 50, 75 and 100 mg/kg/day vehicle: corn oil m:28d f: 14 days prior to mating, continuing through mating, gestation, and lactation until lactation day 21 F1 pups: PND 21 through 49.	LOAEL (parental tox) = 50 mg/kg bw/d LOAEL fertility (females) = 50 mg/kg bw/d NOAEL fertility (males) = 100 mg/kg bw/d NOAEL (dev tox) = 75 mg/kg bw **F1**: <u>100 mg/kg bw</u> postnatal survival↓ (2 total litter losses due to poor maternal care), live litter size ↓ (-14.7%, not stat.signif.), bad clinical conditions of pups (thin or cold body), abs. liver weight PND50 ↑ (m +29.9%, p<0.01; f +41.8%, p<0.01), hepatocellular vacuolation (f, minimal/mild) <u>75 mg/kg bw</u> abs. liver weight PND50 ↑ (m +25.3%, p<0.01; f +32.4%, p<0.01), hepatocellular vacuolation (f, minimal) <u>50 mg/kg bw</u> anogenital distance↑ (f) abs. liver weight PND50 ↑ (m +18.6%, p<0.01; f +16.7%, p<0.01), hepatocellular vacuolation (f, minimal)	Anonymous (2019a) Klimisch 1

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

In an OECD 443 study (Anonymous, 2019b), described in detail in Chapter 10.10.1, 21 of 111 unscheduled deaths in 60 mg/kg bw/d group had no milk present in the stomach (compared to 7/42 in control). For further details see Table 74. Postnatal survival of F1 generation (PND 1 – PND 7), including total litter loss (from PND 0 – PND 9), was impaired at the highest dose but may be contributed to poor maternal care. Mean pup body weight gains in the 60 mg/kg bw/day group were slightly lower than the control group throughout the preweaning period (PND 1–21), resulting in mean body weights on PND 21 that were 8.1% and 7.4% lower for males and females, respectively. No test substance related effects on organ weight of F1 pups on PND 21 could be identified.

Table 74: Necropsy findings, selected parameter: stomach (Anonymous, 2019b).

	Pups				Litters			
Dose group	control	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d	control	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d
F1 – unscheduled deaths								
Number examined	42	19	45	111	13	6	14	20
Stomach – milk absent	7	2	8	21	3	2	5	7
Stomach – milk present	1	0	0	0	1	0	0	0
F1 – PND 21 non selected pups and pups euthanized due to death of dam								
Number examined	0	1	0	6	0	1	0	2
Stomach – milk absent	0	0	0	5	0	0	0	2

In the dose range finding study (Anonymous, 2019a) gross pathology of unscheduled deaths (found dead or euthanized in extremis) showed a dose-related increase in the number of pups (litters) with no milk in the stomach (0 (0), 6 (4), 15 (2), 20(3)) in control 50, 75 and 100 mg/kg bw/d group, respectively. With the exception of 2 other pups from separate litters that were found dead on PND 0, this finding corresponded to poor maternal care. On lactation day 13 exposure via milk was analysed. Mean concentrations of 6PPD in rat milk were 43.9, 53.2 and 59.5 ng/mL in the 50, 75, and 100 mg/kg bw/day groups, respectively. F1 survival was affected by poor maternal care for litters from dams with dystocia or adverse clinical signs around parturition or expected parturition. On PND 0 and on PND 0-4 postnatal survival was reduced, not statistically significant, in the high dose group. Body weights were generally similar to the control group throughout the postnatal period; only some transient changes were reported. Increased liver weights were documented for F1 pups during lactation (details see chapter 10.10.2).

Overall, it can be concluded that the observations in the offspring cannot be clearly allocated to either in utero exposure or exposure during lactation, as exposure took place over both life phases.

10.10.9 Comparison with the CLP criteria

According to CLP regulation 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 basis of: (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.

In an OECD 443 study with 6PPD the absence of milk in pups with unscheduled death was reported for 21/111 pups at 60 mg/kg bw/d. In the corresponding dose range finding study (OECD 421) also a dose-related increase in the number of pups (litters) with no milk in the stomach (0 (0), 6 (4), 15 (2), 20(3)) in control 50, 75 and 100 mg/kg bw/d group, respectively, is documented. A correlation with poor maternal care was assumed.

The transfer of 6PPD via milk to the progeny is demonstrated by Anonymous (2019a). Effects in the F1 generation that may be contributed to the transfer of 6PPD via milk are reduced bodyweight (OECD 443) or increased liver weight (OECD 421).

10.10.10 Conclusion on classification and labelling for reproductive toxicity

Fertility

The key effect dystoca is documented in two recent studies with rats down to a concentration of 20 mg/kg bw/d. A NOAEL of 7 mg/kg bw/d can be derived for this effect. Increase of gestation duration was also seen in three OECD 421 studies at 100 mg/kg bw/d (two times) or 25 mg/kg bw/d, a finding which is often linked to dystocia, thereby supporting the biological relevance of this effect.

For exposure via diet lower bioavailability can be assumed compared to administration via gavage in oily vehicles. This is confirmed by two studies with administration of 6PPD via diet showing no adverse effects on fertility, viability of pups and/or reproductive organs, in contrast to the gavage studies.

Based on these effects in female rats, leading to reduced pregnancy outcomes, a classification as **Repr. 1B for fertility** is proposed.

Developmental toxicity

Developmental toxicity in rabbits is documented in two PNDT studies. Postimplantation loss/early resorptions, a reduced number of viable fetuses as well as reduced mean fetal body weights are described down to concentrations of 30 mg/kg bw/d. Test substance related abortions were seen even at 10 mg/kg bw/d. A direct correlation with reduced maternal food intake could not be established.

Rats have been investigated in several studies (PNDT, screening study, three generation study, EOGRTS). Increased number of resorptions, reduced number of live pups, impairment of pup survival, lower fetal body weight, with some effects already seen at 10 mg/kg bw/d are reported. These findings clearly support effects seen in the guideline conform rabbits studies.

Three pubertal studies show effects on the onset of puberty, especially in females (earlier age at vaginal opening despite lower body weights, higher age at first oestrus, irregular cycling). Interference with the endocrine system can be assumed based on effects on hormone dependent tissues/organs (ovaries, uterus, testes). However, no clear link can be derived between the clearly adverse effects on the offspring and the observed endocrine changes. According to the CLP Regulation (Annex I, 3.7.1.3) any effect on the onset of puberty should be covered under sexual function and fertility. On the other hand, as stated in CLP, Annex I, section 3.7.1.4, 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. […] These effects can be manifested at any point in the life span of the organism. Therefore, the effects on the onset of puberty described in the pubertal studies are considered relevant for classification for developmental toxicity. Further support for this conclusion comes from a recently agreed

RAC note on developmental neurotoxicity, which also refers to the above referenced CLP paragraph (Annex I, section 3.7.1.4) and which is published on the ECHA website[8].

Based on the presented data a classification for **Repr. 1B for development** is proposed.

Effects on or via lactation

Some indications of a possible adverse effect on or via lactation were identified in an OECD 443 study and an OECD 421 study with 6PPD. Among other findings, reduced F1 body weights and increased liver weights were reported for F1 pups which may be a direct adverse effect of 6PPD transferred via milk to the progeny. Transfer of the substance to milk has been proven. Absence of milk has been reported (in a dose dependant manner) in pups with unscheduled death, however, also poor maternal care is reported.

As the observed effects in the offspring could have been induced either during in utero exposure or during lactation it is not possible to clearly identify whether the effects are supportive for a classification for effects on or via lactation. However, the effects are covered under the classification for developmental toxicity.

10.11 Specific target organ toxicity-single exposure

Not addressed in this dossier.

10.12 Specific target organ toxicity-repeated exposure

For evaluation of this endpoint all relevant (sub-)chronic animal data is presented below. It includes two 28-day studies in rats, one 90-day study, two 24-month feeding studies, one EOGRTS (with developmental neurotoxicity) and a corresponding dose range findings study (similar to OECD TG 421), two reproduction/developmental toxicity screening studies, one chronic and one subchronic feeding study, one 24-day study and a three generation study. Beside these oral studies in rats two oral studies in rabbits are also included – one PNDT study with 21 days of exposure and one teratogenicity study with 12 days of exposure. In addition, one study with exposure of rats via inhalation is available, but with limited reporting.

Table 75: Summary table of animal studies on STOT RE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference

[8] https://echa.europa.eu/documents/10162/17090/rac_clh_guidance_note_neurotoxicity_en.pdf/96717ed9-55d3-10e0-785b-093d07e267f3?t=1665034511575).

28 day study (according: Guidelines for 28-day repeat Dose Toxicity Testing of Chemicals, Japan) GLP Rat, Sprague-Dawley (m/f) 4, 20 mg/kg bw/d: N= 5/sex/dose 0, 100 mg/kg bw/d: N=10/sex/dose	6PPD (99% purity) Oral, gavage (5 ml/kg) 0, 4, 20, 100 mg/kg bw/d Recovery groups (14d): 0, 100 mg/kg bw/d Vehicle: corn oil	NOAEL (female) = 4 mg/kg bw/d NOAEL (male) = 20 mg/kg bw/d <u>100 mg/kg bw/d</u> Males: rel. liver weight ↑ (+28%, p<0.01) reversible liver enlargement 2/5 periportal fatty change (slight to moderate) 5/5 total protein↑ (+9.8%, p<0.01), total cholesterol↑ (+82%, p<0.01), creatinine↑ (+20%, p<0.01), Ca↑ (+6.6%, p<0.01), albumin/globuline↓ (-14.2%, p<0.01) hematokrit↓ (-8.9%, p<0.01) (after rec. +1.9%, p<0.01), MCV↓ (-6.8%, p<0.01) (also after rec. -9.1%, p<0.05), MCHC↑ (+4.2%, p<0.01), platelets↑ (+16.1%, p<0.05) (also after rec. +7.2%, p<0.05) Females: rel. liver weight ↑ (+35.4%, p<0.01), slightly (+4%) also after recovery (p<0.05) liver: reversible liver enlargement 1/5; reversible accentuated lobular pattern (2/5), yellowish colour (1/5), dark colour (3/5) periportal fatty change (very slight to moderate) 5/5 total protein↑ (+16%, p<0.01), inorganic phosphate↓ (-11%, p<0.05), albumin↑ (+12.9%, p<0.05) haemoglobin ↓ (-8.3%, p<0.01) (also after rec.) hematocrit ↓ (-10.5%, p<0.01) (also after recovery -5.3%, p<0.05), MCV↓ (-5.4%, p<0.01) (also after recovery -8%, p<0.05), platelets↑ (+16.4%, p<0.01), prothrombin time↓ (-10.1%, p<0.05), APTT↓ (-10.7%, p<0.01) <u>20 mg/kg bw/d:</u> Females: periportal fatty change (very slight to moderate) 5/5 total protein↑ (+8%, p<0.05), inorganic phosphate↓ (-13.6%, p<0.01) <u>4 mg/kg bw/d:</u> Females: absolute brain weight ↑ (+7.4%, p<0.01)	Anonymous (1999b) [Japanese study, Englisch summary] Klimisch 1

OECD TG 452 Rat, Sprague-Dawley n=70/sex/dose at 12 months 20 rats/sex/group were sacrificed; after 24 months all survivors sacrificed	6PPD 0, 50, 250, 1500 ppm (nominal in diet for males: 0, 2.6, 13.5, 84.8 mg/kg bw/d; for females: 0, 3.2, 16.5, 109.5 mg/kg bw/d) Oral (feed) Duration of treatment ~730 days	NOEL = 50 ppm (m: 2.6 mg/kg bw/d, f: 3.2 mg/kg bw/d) <u>1500 ppm:</u> Mean body weight (m) ↓ (-9.9%), (f) ↓ (-18.4%) Mean food consumption (m) ↑ (+5.5%), (f) ↑ (+17.3%) cholesterol↑ slight anemia; hemoglobin (m, f)↓, hematocrit (m, f) ↓, erythrocyte counts(f) ↓, platelet counts (m, f) ↑, MCV↓, MCH↓ cholesterol levels (m, f) ↑ absolute and relative kidney weights (m, f)↑ (at 12 month), chronic nephropathy absolute and relative liver weights (m, f)↑ pigment in the hepatocytes and reticuloendothelial cells (f) and cytoplasmic vacuolization (f) absolute and relative spleen weights (m, at 12 month only) ↑ follicular cell carcinoma and adenoma slightly↑ (m) <u>250 ppm:</u> Mean body weight (f) ↓ (-5.4%). Mean food consumption (f) ↑ (+4.1%) Mean absolute and relative liver weights (m, f) ↑	Anonymous (1993) Klimisch 2 [cited from ECHA dissemination site and a summary report, detailed data e.g absolute numbers or significance values are not available]

OECD TG 443 extended one-generation reproductive toxicity - with developmental neurotoxicity (Cohorts 1A, 1B without extension, 2A and 2B) GLP Rat, Crl:CD(SD) F0: N=25/sex/group High dose group: n= 30/sex/dose	6PPD (purity 96.9%) 0, 7, 20, 60 mg/kg bw/d Oral, gavage Vehicle: corn oil 7d/week F0 females: 70d+mating+gestation+lactation F0 males: like females F1: day of weaning – day prior to euthanasia (PND 21 [Cohort 2B], PND 91 [Cohort 1A], PND 78 [Cohort 2A], and PND 98 [Cohort 1B])	NOAEL (parental tox) = 20 mg/kg bw/d **F0:** <u>60 mg/kg bw/d</u> Mortality due to dystocia/prolonged labour (f: 5/30), total litter loss in 5 f abs. kidney weights ↑ (m +9.9% p<0.01; f + 8.6% not signif.) abs. liver weights ↑ (m +23.7%, p<0.01; f +27.5%, p<0.01) liver vacuolation (m, minimal to moderate), pigment deposition in the kidneys (minimal to mild; m 22/29, f 15/25; control 0/25) <u>20 mg/kg bw/d</u> Mortality due to dystocia/prolonged labour (f: 2/25) rel. liver weights ↑ (m +8.2%, p<0.05), liver vacuolation (m, minimal), pigment deposition in the kidneys (m, minimal) total litter loss in 1 f <u>7 mg/kg bw/d</u> liver vacuolation (m, minimal), pigment deposition in the kidneys (m, minimal) <u>control</u> total litter loss in 3 f **F1:** <u>60 mg/kg bw/d</u> Postnatal survival ↓ (PND 1-7) (not stat. signif. but above HCD), 111(20) pups (litters) found dead or were euthanized in extremis; 21/111 with no milk in stomach; mean bw on PND 21↓ (m -8.1% and f -7.4%, not stat. signif.) and on PND 28 mean bw remained lower (m-5.9%, f -8.6%, not stat. signif.), no effect on bw on PND 98 Cohort 1A: mean albumin↑ (m +11.9%, p<0.01; f +16.3%, p<0.01), total protein↑ (m +12.5%, p<0.01; f +15.4%, p<0.01), globulin↑ (m +13.6%, p<0.01; f +13.6%, p<0.05), cholesterol level ↑ (m +36.6%, p<0.01; f +32.5%, p<0.01) adrenal gland weights ↑ (rel to final bw: m +15.4%, p<0.01, f +20.8%, p<0.01), abs. and rel. kidney weight ↑ (rel. to final bw: m +10.6%, p<0.01; f +15.4%, p<0.01), abs. and rel. liver weight ↑ (rel to final bw: m +18.9%; f +30.5%, p<0.01) Cohort 2B (PND 21): brain weight ↓ (-7.3%, p<0.01) Cohort 2A (PND 78): brain weight↓ (-5.2%, not stat. signif.) <u>20 mg/kg bw/d</u> Postnatal survival ↓ (not signif) Cohort 1A: Mean cholesterol level ↑ (m +20.7, %, p<0.05) adrenal gland weights ↑ (f +14.8%, p <0.05), liver weight↑ (f + 15.3%, p<0.01)	Anonymous (2019b) Klimisch 1

Dose-range finding study similar to OECD TG 421 GLP Crl:CD(SD) rats N= 15/sex/dose	6PPD (purity 96.9%) 0, 50, 75 and 100 mg/kg bw/day vehicle: corn oil m:28d f: 14 days prior to mating, continuing through mating, gestation, and lactation until lactation day 21 F1 pups: PND 21 through 49	LOAEL (parental tox) = 50 mg/kg bw/d **F0:** <u>100 mg/kg bw/d</u> Dystocia/total litter loss (5), Gestation length↑ ($p<0.05$) Abs. liver weight↑ (m +26.1%, $p<0.01$; f +28.1%, $p<0.01$), Abs. thyroid gland weights↑ (m +31.2%, $p<0.01$) <u>75 mg/kg bw/d</u> Dystocia (1) Abs. liver weight↑ (m +25.0%, $p<0.01$; f +19.8%, $p<0.01$) <u>50 mg/kg bw/d</u> Dystocia (1), Gestation length↑ ($p<0.05$) Abs. liver weight↑ (m +16.2%, $p<0.01$)	Anonymous (2019a) Klimisch 1
OECD 421 (Reproduction/developmental toxicity screening test) GLP Rat, Crj:CD(SD) m/f n=12/sex/dose	6PPD (purity 99.4%) 0, 6, 25, 100 mg/kg bw/d Oral (gavage) Vehicle: corn oil M: 48d F: 14d before mating – day 3 of lactation No recovery group	NOAEL (m/f) = 6 mg/kg bw/d <u>100 mg/kg bw/d</u>: salivation (m, f) mortality: 1f on day 23 of gestation food consumption ↑ (m: intermittendly, f during lactation) absolute (and relative) liver weight ↑ (m +38.2% $p<0.01$; f +36.8% $p<0.01$) absolute (and relative) adrenal weight ↑ (m +17.6%, $p<0.01$) liver enlargement (m: 9/12, f :7/10) vacuolar degeneration of the liver (m: 9/11; 8 slight and 1 severe) <u>25 mg/kg bw/d:</u> salivation (m) absolute (and relative) liver weight ↑ (f +17.8%, $p<0.01$) relative liver weight ↑ (m +10.1%, $p<0.05$) liver enlargement (m: 2/12); vacuolar degeneration of the liver (m: 2/12; 1 slight and 1 moderate) <u>6 mg/kg bw/d:</u> Liver: cellular infiltration (slight) of lymphocytes (m: 4/11)	Tanaka (2001) Klimisch 1 [Japanese study, English summary]

OECD TG 421 Reproduction/developmental toxicity screening study Rat, Wistar N=12/sex/dose Exposure duration males 28d, females 41-56d Evaluation by registrants: study has deficits in retrieval, presentation and interpretation of data which render reliability of data debatable	6PPD (Dusantox, purity: 97-98.7%) 0, 2.5, 12.5, 25 mg/kg bw/d Oral (gavage) Vehicle: olive oil	NOAEL (maternal tox) = 2.5 mg/kg bw/d <u>25 mg/kg bw/d</u> smooth stool, males (2/12) liver, males: focal necrosis (7/12), mononuclear nodules (8/12), vacuolization (4/12) liver, females: focal necrosis (2/12), mononuclear nodules (5/12), vacuolization (9/12) uterus, females: focal inflammation lesion (1/12) liver weight ↓ in males (-6.0%, not stat. signif) liver weight ↑ in females (+14.2%, not stat. signif) <u>12.5 mg/kg bw/d</u> smooth stool, males (2/12) , females 1/12 liver, males: focal necrosis (2/12), mononuclear nodules (8/12), vacuolization (9/11) testes: hypoplasia of seminiferous tubules, absence of spermatogenic cells (1/1 investigated due to macroscopic finding) <u>2.5 mg/kg bw/d</u> smooth stool, males (2/12) liver, males: mononuclear nodules (3/12), vacuolization (7/12)	Anonymous (2009) Klimisch 4 (by registrant) [cited from ECHA dissemination site]
Chronic feeding study (2 years) Rat, Charles River (CD outbred), m+f N= 50/sex/dose	6PPD 0, 100, 300, 1000 ppm (ca. 0, 8, 23 or 75 mg/kg bw/d) Oral (feed) 24 months	NOAEL = 300 ppm (~ 23 mg/kg bw/d) <u>1000 ppm:</u> body weight and bw gain ↓ (m, f; p<0.01) food consumption ↓(m, f) (during first weeks). absolute and relative kidney weights ↓ (f, p<0.05) absolute and relative liver weights ↑ (m, p<0.05) absolute and relative spleen weights ↓ (f) Erythrocyte counts ↓ (m at 3 months, p<0.01) (f at 3, 6 and 12 months, p<0.01) Hemoglobin concentrations ↓ (m at 3, p<0.01 and 12 and 18 months, p<0.05) (f at 6, p<0.01 and 12/18 months, p<0.05) Hematocrit values ↓ (m, f at 3 and 6, p<0.01; 12 months, p<0.05)	Anonymous (1978a) Klimisch 2 [cited from ECHA dissemination site, study and detailed data not available; short summary report published by Stevens et al., 1981]

Subchronic study, 3 months GLP Rat, Sprague-Dawley, m+f N= 25/sex/dose	6PPD (purity 97.1%) 0, 250, 1000 or 2500 ppm (equal to - m: 15.7, 62.3, 153.8 mg/kg bw/d; f: 18.5, 75.0, 172.1 mg/kg bw/d) Oral (feed) 13 weeks	NOAEL = 250 ppm (m: 15.7 mg/kg bw/d; f: 18.5 mg/kg bw/d) <u>2500 ppm:</u> Body weight ↓ (m -16.1, f -12.7; both p≤0.01); body weight gain ↓ (m -25.3%, f -28.1%; both p≤0.01) Absolute liver weight ↑ (m +17.6%, f +25.3%, both p≤0.01) Absolute testis weight ↓ (m -10.2%, p≤0.05); absolute spleen weight ↓ (f -20.8%, p≤0.01) Anemia, mild (m, f) <u>1000 ppm:</u> Body weight ↓ (m -9.9%, p≤0.01); body weight gain ↓ (m -15.2%, p≤0.01, f -14.7%, p≤0.01) Absolute liver weight ↑ (f +14.4, p≤0.05) Anemia, mild (m, f)	Anonymous (1987a) Klimisch 1
Subchronic study rat	6PPD Dose: 250 mg/kg bw/d for the first 4 d, thereafter being increased 50 % every 5 d Oral, gavage 24 d	NOAEL not determinable Clear signs of intoxication (increased oxygen consumption, suppression of the central nervous system decreased synthezising function of the liver, decreased ascorbic acid content in the liver)	Stasenkova (1970) Klimisch 3
Chronic toxicity and reproduction study Rat, Charles River CD N= 50 m + 50 f/group	6PPD 0, 100, 300 or 1000 ppm (~ 0, 8, 23 or 75 mg/kg bw/d) Oral, feed 24 months	<u>1000 ppm:</u> reduced body weight gain (with decreased food consumption during the first week of the study) decrease in erythrocyte counts, hemoglobin concentration and hematocrit values (at some interim intervals, but not at the end) increased kidney and spleen weights at terminal sacrifice (f)	Anonymous (1981)[9] Klimisch 2 [cited from ECHA dissemination site, original report not available; short summary published by Stevens et al, 1981]

[9] High similarity with Anonymous (1978a); only difference in kidney and spleen weight, however, different original study reports are cited

OECD TG 408 Rat, Wistar (m, f) GLP N=15/sex/dose recovery group (control and high dose) n=9/sex/group	6PPD 0, 2.5, 12.5, 25 mg/kg bw/day Oral:gavage Vehicle: olive oil 90 days 28 days recovery periode	LOAEL = 2.5 mg/kg bw/d <u>25 mg/kg bw/d (90d):</u> Hematocrit ↓ (m, -3.8%, p<0.05), hemoglobin ↓ (m -4.0%, f -5.5%; both p<0.05), MCV ↓ (m -4.2%, p<0.05), MCH ↓ (m -4.5%, f -4.2%, both p<0.05), MCHC↓ (m -0.3% , f -4.0%, both p<0.05), total cholesterol ↑ (m, p<0.05), total protein ↑ (m, p<0.05), albumin ↑ (m, p<0.05); kidney - moderate degenerative changes in proximal tubules (14/15 m); sporadic glomerular atrophy and hyaline deposition (14/14 f) Heart - Small focal muscular dystrophy in left myocardium (12/15 m) vacuolization of hepatocytes (f 14/15) + necrosis (f 12/14) not statistically significant increase of relative liver and kidney weights (m + 26.9% and +9.7%, f +25.2% and +10.5%) <u>12.5 mg/kg bw/d (90d):</u> hemoglobin ↓ (m -4.8%, p<0.05); MCV ↓ (m -3.6%, p<0.05), MCH ↓ (m -4%, p<0.05), total protein ↑ (m, p<0.05) vacuolization of hepatocytes (f 8/15) + monocellular necrosis (f 4/13), sporadic glomerular atrophy (f 13/13) statistically significant increase of relative kidney weights (f +8.9%, p<0.05) <u>2.5 mg/kg bw/d (90d):</u> Monocellular necrosis (f 2/15), sporadic glomerular atrophy (14/15 f))	Anonymous (2008a) Klimisch 2 [cited from ECHA dissemination site, study not available]
28 day study GLP Rat, Wistar (m,f) N=12/sex/dose Satellite group 8/sex/dose	6PPD No info on dosing Oral, gavage 28 days	No results reported	Anonymous (2008b) Klimisch 4 [cited from ECHA dissemination site, study not available]

Three generation study non GLP limited quality assurance documented (final comparison of the raw data with the final report missing; several limitations rats, Charles River CD N = 8m + 16 f (per group and generation)	6PPD (Santoflex 13) 0, 100, 300 or 1000 ppm (approx. 0, 8, 23, 75 mg/kg bw/d) Oral (diet, ad libitum) (6PPD premixed in acetone)	NOAEL = 100 ppm (75 mg/kg bw/d) High mortality in control and dose groups <u>1000ppm</u> Relative liver weight (to body weight) was statistically significant increased in F1 (+34%, $p<0.01$) and F2 (+41%, $p<0.05$) generation	Anonymous (1980b) Klimisch 2 (by reg.) [short summary published by Stevens et al., 1981]
OECD TG 414, PNDT GLP Rabbit, New Zealand white N=24f (28 at top dose) GD 7-28	6PPD (purity 96.9%) 0, 25, 50 or 100 mg/kg bw/d Oral, gavage Vehicle: 1% methyl cellulose 400cP	NOAEL maternal tox = 25 mg/kg bw/d <u>100 mg/kg bw/d</u> decreased defecation, brown material on the facial area abort (3/24), mean terminal bw ↓ (-6.1%, $p<0.01$), food consumption↓, body weight gains↓, gravid uterine weight↓ (-18.3%, $p<0.01$), liver weights ↑ (abs +28.3%, $p<0.01$) <u>50 mg/kg bw/d</u> Mean body weights ↓ (-5.0% to 5.5% during GD 24-27), food consumption↓, body weight gains↓ liver weights ↑ (rel +15.5%, $p<0.01$)	Anonymous (2018c) Klimisch 1
Teratogenicity study Rabbit, New Zealand Albino GD 6-18, sacrif. on GD 29 N= 17 for control and low dose, 23 for high dose (after artificial insemination 15, 16 and 17 were pregnant)	6PPD (Sanotflex 13) Oral (gelous capsules)	LOAEL = 10 mg/kg bw/d <u>30 mg/kg bw/d</u> Body weight loss during gestation, mortality (6/17) <u>10 mg/kg bw/d</u> Body weight loss during gestation, mortality (3/16) <u>control</u> Body weight loss during gestation, mortality (5/15) Pilot study with 0, 30, 100 or 300 mg/kg bw/d: general high mortality	Anonymous (1976b) Klimisch 2

Non guideline subacute dust inhalation study GLP Rat, Sprague-Dawley (m,f) N=5/sex/dose	6PPD 51, 247, 498 mg/m^3 Inhalation, whole body exposure 4 weeks, 6h/d, 5d/weeks	NOAEL not determinable due to limited reporting <u>498 mg/m^3:</u> Hypoactivity, swollen snouts, scratching Abs. liver weight ↑ (m, p<0.05) Abs. + rel liver weight ↑ (f, p<0.01) Abs. + rel lung weight ↓ (m, p<0.01/p<0.05) Hemoglobin dose dependant decrease (f) dose-dependent increase in leukocytes (f) dose dependent decrease in MCV and MCH (m) <u>247 mg/m^3:</u> Hypoactivity, swollen snouts, scratching Abs. + rel. kidney weight ↑ (f, p<0.05) Abs. + rel liver weight ↑ (m p<0.01/p<0.05, f, p<0.01) Abs. + rel lung weight ↓ (f, p<0.05) Abs. + rel. spleen weight ↑ (m, p<0.05) Hemoglobin dose dependant decrease (f) dose-dependent increase in leukocytes (f) dose dependent decrease in MCV and MCH (m) <u>51 mg/m^3:</u> Hypoactivity Rel. kidney weight ↑(f, p<0.05) Abs. liver weight ↑ (m, p<0.01; f, p<0.05)	Anonymous (1979) Klimisch 3 [cited from ECHA dissemination site, study not available]

In a **28-day study according to Japanese guideline** (Anonymous, 1999b) rats were exposed to 0, 4, 20 or 100 mg/kg bw/d (oral, gavage). For control and high dose group a recovery of 14 days was included. Body weights were examined on days 1, 4, 8, 11, 15, 18, 22, 25, 28 and on recovery days 1, 4, 8, 11 and 14. Haematology, clinical chemistry, urinalysis as well as organ weights (brain, thymus, heart, liver, kidneys, spleen, adrenal glands, testes, epididymides, ovaries) with macroscopic and microscopic examinations are documented for all animals. No animal died during exposure or recovery. No effects on body weight gain were found. Food consumption was affected in the high dose female group on day 1 (decrease –13%, p<0.01) and in the high dose male group on day 1 of the recovery period (increase +16%, p<0.01). In females significant increase (p<0.01) of absolute brain weight at 4 mg/kg bw/d and absolute liver weight at 100 mg/kg bw/d is documented on day 28 of exposure but not after recovery. In males and females relative liver weight was increased at 100 mg/kg bw/d, in females also after end of recovery (see Table 76). Gross pathology documented a reversible liver enlargement in two males and one female at 100 mg/kg bw/d. Livers of high dose females also showed reversible accentuated lobular pattern (2/5), yellowish colour (1/5) or dark colour (3/5). Histopathology revealed periportal fatty change at end of administration at 20 mg/kg bw/d for females (very slight to moderate, 5/5), in males also (very slight to slight, 3/5) but not statistically significant) and at 100 mg/kg bw/d for both sexes (m: slight to moderate, 5/5; f: very slight to moderate, 5/5) (p<0.05). At the end of recovery these changes were reported as very slight (f: 3/5, m: 3/5) but not statistically significant.

Table 76: Relative and absolute body/organ weights (m/f) (selected) after oral administration of 6PPD for 28 days (Anonymous, 1999b).

n=5/sex/dose	Males		Females		
	Body weight, absolute [g±SD]	Liver, relative weight [mg/g±SD]	Body weight, absolute [g±SD]	Brain, absolute weight [mg±SD]	Liver, relative weight [mg/g±SD]
Control	351.9 ± 25.9 -	34.948 ± 2.562 -	210.7 ± 16.9 -	1747.2 ± 44.2 -	30.271 ± 2.120 -
4 mg/kg bw/d	351.9 ± 15.0 0.0%	36.430 ±1.247 +4.2%	221.6 ± 19.8 +5.2%	1877.1 ** ± 49.4 +7.4%	30.838 ± 0.925 +1.9%
20 mg/kg bw/d	354.5 ± 24.0 -0.7%	37.056 ± 1.761 +6.0%	206.9 ± 11.4 -1.8%	1753.0 ± 73.1 +0.3%	32.686 ± 1.142 +8.0%
100 mg/kg bw/d	332.7 ± 34.5 -5.5%	44.718** ± 2.422 +28%	214.5 ± 16.9 +1.8%	1744.4 ± 51.1 -0.2%	40.991 ** ± 2.469 +35.4%
Control after recovery	398.8 ± 27.3 -	30.478 ± 2.115 -	259.4 ± 12.8 -	1773.9 ± 54.9 -	29.488 ± 0.945 -
100 mg/kg bw/d after recovery	418.9 ± 36.5 +5.0%	34.119 ± 4.045 +12%	248.9 ± 18.1 -4.0%	1844.9 ± 98.3 +4.0%	31.599 * ± 1.138 +7.2%

** significantly different from control, p<0.01;, * significantly different from control, p<0.05

In clinical chemistry males showed no effects in lower doses; at 100 mg/kg bw/d a significant but reversible increase in total protein, total cholesterol, creatinine and Ca and a decrease in albumin/globuline is documented. Triglyceride were increased at end of recovery (see Table 77). Females showed an increase of total protein at 20 and 100 mg/kg bw/d, a decrease of inorganic phosphate at 20 and 100 mg/kg bw/d and an increase of albumin at 100 mg/kg bw/d (see Table 78). Some effects on haematology parameters are reported for males and females at high dose and after recovery. Details are presented in Table 79. Urinanalysis showed a reversible increase of protein in males and females at 100 mg/kg bw/d.

Table 77: Clinical chemistry of male rats after 28 days of exposure and recovery, selected values (Anonymous, 1999b).

n=5/sex/dose	Total protein [g/dL±SD]	Albumin/globuline	Total cholesterol [mg/dL]	Creatinine [mg/dL]	Ca [mg/dL]	Triglyceride [mg/dL]
Control	5.1 ± 0.1	1.34 ± 0.08	45 ± 11	0.5 ± 0.0	9.0 ± 0.1	74 ± 24
4 mg/kg bw/d	5.0 ± 0.3	1.23 ± 0.06	52 ± 5	0.5 ± 0.1	9.2 ± 0.3	85 ± 19
20 mg/kg bw/d	5.2 ±0.2	1.28 ± 0.14	59 ± 19	0.6 ± 0.1	9.3 ± 0.2	76 ± 28
100 mg/kg bw/d	5.6 ± 0.1**	1.15 ± 0.02**	82 ± 18**	0.6 ± 0.0**	9.6 ± 0.2**	40 ± 21
Control after recovery	5.4 ± 0.2	1.22 ± 0.10	43 ± 8	0.6 ± 0.0	8.8 ± 0.3	47 ± 7
100 mg/kg bw/d after recovery	5.7 ± 0.3	1.08 ± 0.11	59 ± 16	0.6 ± 0.1	8.9 ± 0.2	69 ± 17*

** significantly different from control, p<0.01; , * significantly different from control, p<0.05

Table 78: Clinical chemistry of female rats after 28 days of exposure and recovery, selected values (Anonymous, 1999b).

n=5/sex/dose	Total protein [g/dL±SD]	Albumin [g/dL]	Total cholesterol [mg/dL]	Creatinine [mg/dL]	Ca [mg/dL]	Triglyceride [mg/dL]	Inorg. Phos. [mg/dL]
Control	5.0 ± 0.2	3.1 ± 0.2	56 ± 14	0.6 ± 0.0	8.0 ± 0.3	44 ± 20	8.1 ± 0.5
4 mg/kg bw/d	5.2 ± 0.1	3.2 ± 0.2	47 ± 8	0.6 ± 0.0	7.7 ± 2.5	34 ± 7	7.6 ± 0.6
20 mg/kg bw/d	5.4 ± 0.1*	3.2 ± 0.2	56 ± 11	0.7 ± 0.1	9.0 ± 0.2	31 ± 4	7.0 ± 0.4**
100 mg/kg bw/d	5.8 ± 0.3**	3.5 ± 0.3*	76 ± 19	0.6 ± 0.1	8.8 ± 1.1	22 ± 10	7.2 ± 0.3*
Control after recovery	5.7 ± 0.3	3.3 ± 0.2	55 ± 5	0.7 ± 0.1	9.0 ± 0.3	45 ± 15	7.1 ± 0.3
100 mg/kg bw/d after recovery	5.6 ± 0.3	3.1 ± 0.3	63 ± 18	0.7 ± 0.0	9.0 ± 0.3	38 ± 8	7.4 ± 0.6

** significantly different from control, p<0.01; * significantly different from control, p<0.05

Table 79: Selected haematology parameters of males and females after 28 days of exposure and recovery (Anonymous, 1999b).

n=5/sex/ dose	Haemoglobin [g/dL]	Hematocrit [%]	MCV [µm³]	MCH [pg]	MCHC [%]	Reticulocyte [%]	Platelet [x10⁴/mm³]	Prothrombin time [sec]	APTT [10][sec]
Males									
Control	14.0 ± 0.5	41.8 ± 1.1	63.7 ± 1.7	21.4 ± 0.6	33.6 ± 0.6	5.7 ± 1.1	100.4 ± 8.0	19.5 ± 3.0	22.9 ± 1.6
4 mg/kg bw/d	14.2 ± 0.3	41.5 ± 0.7	63.1 ± 2.0	21.6 ± 0.8	34.2 ± 0.8	5.9 ± 0.4	103.3 ± 6.9	20.3 ± 3.7	24.0 ± 2.9
20 mg/kg bw/d	14.1 ± 0.6	41.0 ± 1.6	62.6 ± 2.2	21.6 ± 0.8	34.4 ± 0.5	5.1 ± 0.8	106.4 ± 9.2	19.6 ± 3.8	24.2 ± 2.0
100 mg/kg bw/d	13.3 ± 0.5	38.1 ± 1.4**	59.4 ± 0.8**	20.8 ± 0.4	35.0 ± 0.4**	6.1 ± 1.2	116.6 ± 10.3*	17.5 ± 2.7	24.2 ± 2.4
Control after recovery	15.0 ± 0.3	45.2 ± 0.9	60.8 ± 0.4	20.2 ± 0.3	33.3 ± 0.4	4.0 ± 0.8	100.2 ± 5.1	26.3 ± 4.9	24.8 ± 1.8
100 mg/kg bw/ d after recovery	14.3 ± 0.5*	42.6 ± 1.0**	57.9 ± 2.3*	19.4 ± 0.7*	33.5 ± 0.5	5.6 ± 1.3*	107.6 ± 5.0*	23.9 ± 2.8	25.9 ± 1.3
Females									
Control	14.4 ±	41.9 ±	62.5 ±	21.6 ±	34.5 ±	3.0 ± 1.0	99.6 ±	14.9 ±	20.6 ± 0.8

[10] APTT = Activated partial thromboplastin time

	0.7	1.8	1.2	0.9	1.0		6.1	1.0	
4 mg/kg bw/d	14.1 ± 0.6	41.1 ± 25.	61.5 ± 1.9	21.2 ± 0.4	34.4 ± 0.6	3.2 ± 0.8	98.8 ± 3.8	14.8 ± 0.5	20.9 ± 0.6
20 mg/kg bw/d	13.7 ± 0.4	39.8 ± 1.6	61.2 ± 0.4	21.1 ± 0.3	34.5 ± 0.6	4.1 ± 1.1	102.8 ± 3.8	14.6 ± 0.7	20.6 ± 0.8
100 mg/kg bw/d	13.2 ± 0.5**	37.5 ± 1.4**	59.1 ± 1.5**	20.8 ± 0.5	35.2 ± 0.3	4.8 ± 1.7	115.9 ± 8.0**	13.4 ± 0.5*	18.4 ± 1.0**
Control after recovery	15.1 ± 0.7	44.1 ± 1.9	60.2 ± 0.5	20.7 ± 0.2	34.3 ± 0.3	3.6 ± 1.0	99.8 ± 4.5	16.8 ± 0.4	21.7 ± 0.9
100 mg/kg bw/d after recovery	13.5 ± 0.8**	39.7 ± 2.5*	57.5 ± 2.0*	19.6 ± 0.6**	34.1 ± 0.4	6.2 ± 2.2	107.4 ± 9.8	15.9 ± 1.4	20.5 ± 1.2

** significantly different from control, $p<0.01$; * significantly different from control, $p<0.05$

Females were identified as more sensitive than males. The study authors derived a NOEL of 4 mg/kg bw/d based on liver effects (reversible periportal fatty change of the liver; increased total serum protein) seen in female rats. For males a NOAEL of 20 mg/kg bw/d can be derived.

In a **chronic feeding study according to OECD TG 452** (Anonymous, 1993, cited from REACH registration) rats were dosed with 0, 50, 250 or 1500 ppm (nominal in diet for males: 0, 2.6, 13.5, 84.8 mg/kg bw/d; for females: 0, 3.2, 16.5, 109.5 mg/kg bw/d). Analytical verification was reported and within a range of ±15%. At the beginning of the experiment 70 rats/sex/group were dosed with 6PPD. After 12 months about 20 rats/sex/group were sacrificed. After 24 months of treatment (between 729 and 737 days) all survivors were sacrificed. Body weight and food consumption were recorded weekly throughout the first 13 weeks and monthly thereafter. Ophthalmoscopic examination was done after 12 months and at study termination. Hematology (20 animals per time point/sex/group), clinical chemistry (10 animals per time point/sex/group), and urine analysis (10 animals per time point/sex/group) were performed prior to study initiation, during months 3, 6, 12, 18, and at termination. Postmortem gross examination was reported for all animals. Organ weights (adrenals, brain, kidneys, testes with epididymides, liver, ovaries, and spleen) were recorded for ≥ 10 animals/sacrifice timepoint/sex/dose. For the following organs in the control and high dose group a histopathologic evaluation was done: adrenals, aorta (abdominal), bone (including the articular surface, sternum and femur), bone marrow (sternum), brain (medulla/pons, cerebellar cortex and cerebral cortex), esophagus, eyes, heart, intestine, cecum, colon, duodenum, ileum, jejunum, rectum, kidneys, liver, lungs (including trachea) – inflated with fixative, lymph nodes (mesenteric, mediastinal), mammary gland (right inguinal), ovaries, pancreas, peripheral nerve – sciatic, taken with biceps femoris, pituitary, accessory genital organs (prostate, seminal vesicles, epididymides), salivary glands (submandibular), skeletal muscle (right biceps femoris), skin, spinal cord (midthoracic, lumbar, cervical), spleen, stomach, testes, thymus, thyroid (including the parathyroids), urinary bladder – inflated with fixative, uterus, all gross lesions and tumors.
No mortalities or clinical signs were observed. Ophthalmic examination was negative. Mean body weight was decreased at 1500 ppm in m (-9.9%) and f (-18.4%) and at 250 ppm in females only (-5.4%). In contrast mean food consumption was increased in m (+5.5%) and f (+17.3%) at 1500 ppm and f at 250 ppm (+4.1%). In high dose groups signs of slight anemia are reported. Hemoglobin was slightly reduced in males compared to control animals by 7, 8, and 14% at the high dose at 3, 6, and 12 months, respectively. Slight reduction was observed in females of the high dose group at all time points (12, 17, 14, 10 and 17% at 3, 6, 12, 18, and 24 months, respectively). Hematocrit was slightly reduced in males compared to control animals by 5, 7, and 13% at the high dose at 3, 6, and 12 months, respectively. Slight reduction was observed in females in the

high dose group at all time points (12, 11, 11, 8, and 17% at 3, 6, 12, 18, and 24 months, respectively). Erythrocyte counts were not affected in males but slightly reduced (between 7 and 13%) in females in the high dose group at 3, 6, 12, and 24 months. Platelet counts were slightly increased at the high dose group in males at a single point in time (14% at 3 months). In females a slight increase between 8 and 30% was observed at 3, 6, 12, and 24 months. MCV was slightly reduced (between 3 and 8% compared to control) in the high dose group in males and females at 3, 6, 12 and 18 months, but not at termination. MCH was slightly reduced (between 5 and 9% compared to control) in the high dose group in males and females at 3, 6, 12, and 18 months, but not at termination. MCHC was slightly reduced in the high-dose group in males at termination (3%) and in females at 3 and 6 months (3 and 3%, respectively). Total and differential leucocyte counts were not affected at any dose group or at any time point.

Clinical chemistry revealed consistent statistically significant increased cholesterol levels in males and females in the high dose groups at 6, 12, 18, and 24 months. In the high dosed group also effects like increased total protein, globulin and calcium are reported, but the effects were generally slight and not consistent over time.

Mean absolute and relative kidney weights were increased in the high-dose groups in males and females at 12 months, but not at termination. The incidence of chronic nephropathy was similar between the control groups and the dose groups, but the severity increased. Chronic nephropathy correlated with an increased incidence of irregularities of the kidney surface observed macroscopically in males and females at the high dose groups.

Mean absolute and relative liver weights were increased in males and females after 12 and 24 months in the high dose groups and in the mid dose groups at termination. Microscopic observations indicate increased incidence in pigment in the hepatocytes and reticuloendothelial cells and cytoplasmic vacuolization of the liver in females at the high dose. No effect was observed in males or females in the mid dose or males in the high dose. Mean absolute and relative spleen weights were increased in the high dose males only at 12 months, but not at termination. Detailed values are not available.

Examination of the thyroid showed slight follicular cell hyperplasia in treated males. Slight increased follicular carcinoma were observed in males of mid and high dose groups. The incidences are presented in Table 80. Fisher Exact Test showed that the increased incidence of follicular cell carcinoma seen in males at the mid and high dose groups was not statistically significantly different from controls. However, the incidences are slightly above the historical control incidences observed in that laboratory (4/501; 0.8%). Increased liver activity is known to induce thyroid follicular hypertrophy and hyperplasia and might lead to increased thyroid tumors as a secondary effect. To set the two effects in relation registrants report detailed liver weight data. Unfortunately, liver weight has not been investigated for all animals with thyroid follicular tumors. Liver weight data are available for the high dose group for one male with follicular carcinoma and two males with follicular adenoma. The absolute and relative organ weights of these three males were not only elevated compared to controls but also at the higher end within the high dose group. The three males in question were the animals with the highest absolute or relative liver weights in the entire study (liver/brain weight for these males: 10.67, 11.58, 11.82; for comparison: mean liver/brain weight in the high dose group: 9.85; mean liver/brain weight in the control group: 7.00).

Table 80: Follicular adenoma and carcinoma after chronic exposure to 6PPD (Anonymous, 1993).

	Follicular carcinoma		Follicular adenoma	
	female	male	female	male
0 ppm	1/69 (1.4%)	0/70 (0%)	0/69 (0%)	3/70 (4.3%)
50 ppm	2/70 (2.8%)	0/69 (0%)	1*/70 (1.4%)	2/69 (2.9%)
250 ppm	1/69 (1.4%)	2/70 (2.8%)	0/69 (0%)	3/70 (4.3%)
1500 ppm	1/69 (1.4%)	3/69 (4.4%)	1/69 (1.4%)	3/69 (2.9%)

*animal with follicular adenoma and carcinoma

No changes in weight of reproductive organs and no histopathological changes of reproductive organs (mammary glands, uterus, ovaries, testes, accessory genital organs as prostate, seminal vesicles, epididymides) up to the highest dose tested.

Based on elevated absolute and relative liver weights in males and females as well as reduced body weight in females at 250 ppm (at termination) a NOAEL of 50 ppm can be derived.

A recent **OECD 443 study** (Anonymous, 2019b) is available including cohorts 1A, 1B and 2A, 2B. Rats were exposed (oral, gavage) to 0, 7, 20 or 60 mg/kg bw/d (vehicle: corn oil). Details on study design and results relevant for reproductive toxicity are presented in Chapter 10.10.2.

Mortality was observed in males and females; the death of the males were considered not to be treatment related. Dystocia and/or prolonged labor was identified as cause of moribundity for females (see Table 21).

For survivors no test-substance related clinical observations were recorded. Findings noted in treated survivors were hair loss, scrabbing and red material around the nose/mouth. These findings occurred infrequent, at similar frequencies in control and/or not dose-related.

No test substance-related effects on mean body weights, weight gains and food consumption were observed. Some statistically significant differences on some days in time occurred in all groups, but these changes were transient, not of sufficient magnitude and/or not in a dose related manner. Mean maternal body weights, body weight gains and food consumptions were unaffected by test substance administration during gestation and lactation (differences recorded were slight and not statistically significant).

Hematology showed statistically significantly lower mean corpuscular hemoglobin concentration and statistically significantly higher reticulocyte counts in the 60 mg/kg bw/day group males compared to the control group (Table 81). Higher mean red blood cell distribution width (RDW) was noted in 60 mg/kg bw/day females. Coagulation parameters were not affected. Findings were not considered test substance related. No correlating microscopic findings are documented.

Table 81: Haematology of F0 – selected parameters, week 19, n=10 (Anonymous, 2019b).

Mean ±SD	Control	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d
Males				
MCHC [g/dl]	32.8 ± 0.43	38.8 ± 0.56	32.4 ± 0.74	31.7 ± 0.73**
Retic. [%]	2.4 ± 0.29	2.3 ± 0.28	2.6 ± 0.43	3.0 ± 0.49 ** n=9
Retic. absolute (thous/µL]	219.7 ± 30.46	200.1 ± 20.24	226.3 ± 27.13	262.1 ± 29.46** n=9
Females				
RDW (%)	11.9 ± 0.49	12.1 ± 0.66	12.5 ± 0.81	12.9 ± 0.88**

* p<0.05; ** p<0.01 (Dunnett´s test)

Mean T4 levels in the 20 and 60 mg/kg bw/day group males and females were statistically significant higher than in the control group, but within the historical control data range. No significant changes in the TSH levels were observed. No effects on thyroid weights and no microscopic finding were documented.

Table 82: Total T4 and TSH levels in F0 animals, week 19, n=10 per group (Anonymous, 2019b).

Mean ±SD, %	Control	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d	HCD# for Crl:CD(SD) Rats
Males					
Total T4 [pg/ml]	44060.0 ± 7130.1	43760.0 ± 8874.3 -0.7 %	55560.0* ± 12115.8 26.1 %	60640.0** ± 9060.4 37.6 %	59218.0 ± 22344.3 [4 studies, 42 animals]
TSH [ng/ml]	7.8 ± 3.62	7.2 ± 3.34 -7.7 %	11.4 ± 6.65 46.2 %	10.4 ± 4.89 33.3 %	11.7 ± 3.7 [5 studies, 55 animals]

Females					
Total T4 [pg/ml]	36810.0 ± 8368.8	36230.0 ± 5652.7 -1.6 %	47500.0* ± 9808.2 29 %	54530.0** ± 11846.7 48.1 %	31306.7 ± 4637.6 [3 studies, 28 animals]
TSH [ng/ml]	5.2 ± 0.79§	5.2 ± 2.13 0.0 %	6.1 ± 2.72 17.3 %	7.1 ± 3.14 36.5 %	6.1 ± 1.9 [4 studies, 40 animals]

* p<0.05; ** p<0.01 (Dunnett´s test); § n=8
# HCD study date range 05/14 – 05/18 (OECD 421 / 422 / 443; CTA)

Clinical chemistry revealed significantly higher (p<0.01) mean albumin (+9.5%, +13.0%), total protein (+10.4%, +12.7%) and globulin concentrations (+12.0%, +12.0%) in the 60 mg/kg bw/day group males and females, respectively. Also mean cholesterol levels in the 20 and 60 mg/kg bw/day group males (+36.2%, +53.6%) and the 60 mg/kg bw/day group females (+33.0%) were significantly higher than in the control group. Other changes were not considered treatment related (small magnitude, no dose response).

Urinanalysis showed a statistically significant (p<0.05) higher mean urobilinogen level in high dose females (0.6 ± 0.61 versus control of 0.2 ± 0.00 mg/dl). This finding was not considered test substance-related (no other effects, no correlating microscopic changes, only in females).

Gross pathology showed pale liver in one male of 20 mg/kg bw/d and one male of 60 mg/kg bw/d correlating with minimal and marked vacuolation of the liver, respectively.

In high dose males and females higher absolute kidney weights (m +9.9% p<0.01; f +8.6% not signif.) and kidney weights relative to final body weight and relative to brain weight were noted. However, weights did not exceed historical control range. In high dose males and females statistically significant (p<0.01) higher absolute liver weights (m +23.7%, f +27.5%) and liver weights relative to final body weight and relative to brain weight were noted. Higher liver weight relative to final body weight (p<0.05) was also present in the 20 mg/kg bw/day males. Higher weights were exceeding the historical control. See also Table 83.

Table 83: Final body and organ weights of F0 (mean ±SD, %, n) (Anonymous, 2019b).

Mean ±SD	Control	7 mg/kg bw/d	20 mg/kg bw/d	60 mg/kg bw/d
Males				
Final body weight [g]	641. ± 61.6 25	638. ± 62.9 -0.5% 24	652. ± 66.6 1.7% 25	625. ± 65.6 -2.5% 29
Kidney [g]	3.74 ± 0.325 25	3.75 ± 0.395 0.3% 24	3.85 ± 0.379 2.9% 25	4.11 ± 0.430** 9.9% 29
Liver [g]	19.39 ± 3.071 25	19.55 ± 2.288 0.8% 24	21.39 ± 3.359 10.3% 25	23.99 ± 3.537** 23.7% 29
Liver [g/100g final bw]	3.023 ± 0.3476 25	3.073 ± 0.2926 1.7% 24	3.271* ± 0.2994 8.2% 25	3.835** ± 0.3254 26.9% 29
Females				
Final body weight [g]	319. ± 30.2 25	329. ± 38.0 3.1% 25	328. ± 36.1 2.8% 22	226 ± 38.0 5.3% 25
Kidney [g]	2.21 ± 0.271 25	2.26 ± 0.176 2.3% 25	2.28 ± 0.269 3.2% 22	2.40 ± 0.281 8.6% 25
Liver [g]	11.04 ± 1.548 25	11.56 ± 1.534 4.7% 25	11.87 ± 1.373 7.5% 22	14.08 ± 2.141** 27.5% 25

Liver [g/100g final bw]	3.464 ± 0.3729 25	3.524 ± 0.3059 1.7% 25	3.618 ± 0.2506 4.4% 22	4.185** ± 0.3629 20.8% 25

* p<0.05; ** p<0.01 (Dunnett´s test)

Histopathology revealed treatment-related findings in liver and kidney of 7, 20, and 60 mg/kg bw/d males and 60 mg/kg bw/d females. In males a dose related increase in incidence and severity of vacuolation of the liver was seen. In females this was limited to the high dose group. Vacuolation was characterized by multiple clear vacuoles expanding the cytoplasm of the hepatocytes especially in the midzonal region of the hepatic lobules. Study authors concluded that the vacuolation may have contributed in part to higher liver weights but was not the primary cause for elevation in liver weights. Pigment deposition in the kidneys was increased in high dose females (15/25) and dose-related in males (0/25, 7/23, 12/25, 22/29 in control, 7, 20 and 60 mg/kg bw/d, respectively). Pigment deposition was characterized as yellow to brown pigment in the cytoplasm of proximal convoluted tubular epithelium and within the lumen of these proximal convoluted tubules. A special staining of selected male kidneys revealed that the pigment was negative for bile and/or iron (with exception of one male with minimal positive iron staining) but it could have been a metabolite or the test substance itself. Other remaining histologic changes were considered to be incidental findings.

Table 84: Incidence of histopathologic findings in liver and kidney (Anonymous, 2019b).

	Males				Females			
Dose groups [mg/kg bw/d]	0	7	20	60	0	7	20	60
Liver – No. of tissues examined	25	24	25	39	25	25	22	25
Vacuolation	0	12	16	25	0	0	0	3
Minimal	-	11	15	18	-	-	-	3
Mild	-	1	1	3	-	-	-	0
Moderate	-	0	0	3	-	-	-	0
Marked	-	0	0	1	-	-	-	0
Liver – No. of tissues examined	25	23	25	29	25	25	22	25
Pigment	0	7	12	22	0	0	0	15
Minimal	-	7	12	17	-	-	-	15
Mild	-	0	0	5	-	-	-	0

Other histologic changes were: (1) moderate dilatation of all chambers of the heart in one high dose female, (2) focal minimal necrosis of skeletal muscle in one high dose female, (3) a minimal to moderate mineralization of the outer stripe of the kidney medulla was described for all groups of females (including control) and it was considered secondary to the diet, (4) variations in the incidence of chronic progressive nephropathy and/or basophilic tubules in all animals were considered secondary to the diet. (5) In ovaries of F0 animals frequently reduced numbers of corpora lutea and retained antral follicles were detected. The incidence was 6/25, 6/25, 4/22, and 5/25 in the 0, 7, 20 and 60 mg/kg bw/day groups, respectively. Many of these animals were identified as animals with reduced fertility. (6) The incidence of follicular cysts (focal or multifocal) was 2/25, 0/25, 1/22, and 4/25, respectively. The cysts were slightly larger than a tertiary ovulatory follicle but were not noted on gross examination. There was no difference in the appearance of the cysts between the various dose groups. The cysts were considered incidental by the study authors, within an expected incidence rate for adult fertile rats.

F1 generation (after weaning) also showed higher liver and kidney weights as well as adrenal gland weights in high dose males and high/mid dose females. For details see Table 26. Histopathology revealed no treatment related findings in F1 males and females.

In a **dose range finding study according to OECD 421** (Anonymous, 2019a) 10 week old Crl:CD(SD) rats were exposed to 0, 50, 75 or 100 mg/kg bw/d (oral, gavage). Details on study design and results relevant for reproductive toxicity are presented in Chapter 10.10.2.

Survival of males was unaffected. As cause of death/euthanasia in extremis of females several times dystocia is reported. For details see Table 31. No adverse effects on F0 body weight or body weight gains were reported in any dose group. Some statistically significant differences occurred but there was no dose-response and effects were transient. During gestation and lactation female bw and bw gain were generally similar to the control group with the exemption of dams that were euthanized in extremis or due to total litter loss. No adverse effects on food consumption are documented.

Investigation of organ weights showed higher mean liver weights (absolute and relative to final body weight and brain weight) in all treated dose groups of males and in the 75 and 100 mg/kg bw/day females (see Table 85). The data show a dose-response and HCDs were exceeded. There was also test substance-related higher thyroid gland weight (absolute and relative to final body weight and brain weight) in 100 mg/kg bw/day males. Only the thyroid gland weight relative to body weight exceeded the Charles River Ashland historical control data base (+/- 2 SD). There was higher group mean kidney weight relative to final body weight in 75 mg/kg bw/day males, however, absolute weight was not affected.

Table 85: Organ weights (selected values) of F0 males and females (mean ±SD, % diff, n) (Anonymous, 2019a).

Mean ±SD, %, n	Control	50 mg/kg bw/d	75 mg/kg bw/d	100 mg/kg bw/d
Males				
Liver [g]	17.53 ± 1.563 - 15	20.37** ± 2.339 16.2% 14	21.92** ± 1.867 25.0% 15	22.11** ± 2.225 26.1% 15
Liver [g/100g final bw]	3.780 ± 0.2012 - 15	4.377** ± 0.2974 15.8% 14	4.696** ± 0.1612 24.2% 15	4.854** ± 0.2131 28.4% 15
Thyroid/Para [g]	0.0202 ± 0.00430 - 15	0.0236 ± 0.00359 16.8% 14	0.0217 ± 0.00332 7.4% 15	0.0265** ± 0.00773 31.2% 15
Thyroid/Para [g/100g final bw]	0.004 ± 0.0011 - 15	0.005 ± 0.0010 25% 14	0.005 ± 0.0007 25% 15	0.006** ± 0.0018 50% 15
Females				
Liver [g]	17.14 ± 1.397 - 13	18.59 ± 1.790 8.5% 13	20.53** ± 2.149 19.8% 12	21.96** ± 2.311 28.1% 8
Liver [g/100g final bw]	5.172 ± 0.2551 - 13	5.502 ± 0.5577 6.4% 13	5.956** ± 0.5260 15.2% 12	6.252** ± 0.3907 20.9% 8

* $p<0.05$; ** $p<0.01$ (Dunnett´s test)

No test substance related microscopic findings in males and females were found. Thyroid glands from each F0 animal were given a severity score for the degree of hypertrophy/hyperplasia of follicular cells, however, any variation in the degree of change in this parameter between dose groups was within the range of expected biological variability. According to study authors the reported slightly higher degree of hypertrophy/hyperplasia in 100 mg/kg bw/d males does not explain the higher group mean thyroid gland weights and there was no dose response for severity. For females there was higher grade of hypertrophy/hyperplasia of follicular cell epithelium of the thyroid glands in 50, 75 and 100 mg/kg bw/day dose groups, however, like in the males there was no dose response for severity (see Table 86).

There was a slight dose-response increased incidence of mononuclear cell infiltration (minimal/mild) in the prostate gland without disruption of the glandular architecture and without correlation with prostate weights; the incidences were within the HCDs. Liver was not examined microscopically. There were no effects on male and female reproductive tissues. Remaining findings were considered incidental.

Table 86: Incidence of histopathologic findings in thyroid gland and prostate gland of F0 animals [(number of tissues examined), incidence] (Anonymous, 2019a).

Finding	Control	50 mg/kg bw/d	75 mg/kg bw/d	100 mg/kg bw/d
Males				
Thyroid gland, hypertrophy/hyperplasia, follicular cell	(15)	(14)	(15)	(15)
Minimal	12	6	4	7
Mild	3	8	9	7
Moderate	0	0	2	1
Prostate gland Infiltrate, mononuclear cell	(1)	(1)	(3)	(5)
Minimal	1	2	2	3
Mild	0	0	1	2
Females				
Thyroid gland, hypertrophy/hyperplasia, follicular cell	(12)	(11)	(12)	(8)
Minimal	6	0	0	2
Mild	6	10	7	4
Moderate	0	1	5	2

F1 data is presented in Chapter 10.10. Test substance related effects on **liver weights** of pups were documented for all investigated timepoints. In general, there were no corresponding microscopic changes in the liver.

- At PND 4 statistically significantly higher liver weight relative to final body weight was seen in 75 mg/kg bw/day males and females, and 100 mg/kg bw/day females. Changes were considered test substance related even though some of the changes in relative weights may have been attributed to 9.1% lower group mean final body weight in these same dose groups.
- At PND 13 there was statistically significant higher liver weight relative to body weight in the 100 mg/kg bw/day males and 75 and 100 mg/kg bw/day females, with no change in the group mean absolute liver weights.
- At PND 21 statistically significant higher liver weights relative to final body weight was reported in 50, 75 and 100 mg/kg bw/day males and females, with no statistically significant change in group mean absolute liver weights.
- Pups at PND 50 showed higher liver weights (absolute and relative to final body weight) in the 50, 75 and 100 mg/kg bw/day males and females. For further details see Table 87. This correlates to hepatocellular vacuolation in the liver of females (Table 88), an effect not seen in males.

Table 87: Liver weights of F1 pups at PND 50 [Mean ±SD, %, n] (Anonymous, 2019a).

Mean ±SD, %, n	Control	50 mg/kg bw/d	75 mg/kg bw/d	100 mg/kg bw/d
F1 males – PND 50				
Final bodyweight [g]	296. ± 20.0 - 15	298. ± 22.0 0.7% 15	301. ± 29.5 29.5% 15	303. ± 19.8 19.8% 15
Liver [g]	15.02 ± 1.203 - 15	17.82** ± 1.395 18.6% 15	18.82** ± 2.436 25.3% 15	19.51** ± 2.078 29.9% 15
Liver [g/100g final bw]	5.076 ± 1.203 - 15	5.991** ± 0.3066 18.0% 15	6.249**± 0.4855 23.1% 15	6.433**± 0.4150 26.7% 15
F1 females – PND 50				
Final bodyweight [g]	206. ± 17.0 - 15]	198.0 ± 21.8 -3.9% 15	208. ± 13.0 1.0% 15	214. ± 22.1 3.9% 13
Liver [g]	9.96 ± 1.313 - 15	11.62** ± 1.451 16.7% 15	13.19** ± 0.993 32.4% 15	14.12** ± 1.784 41.8% 13
Liver [g/100g final bw]	4.826 ± 0.3430 - 15	5.863** ± 0.4305 21.5% 15	6.340** ± 0.2888 31.4% 15	6.587** ± 0.2837 36.5% 13

* p<0.05; ** p<0.01 (Dunnett´s test)

There were no test substance-related effects on the **microscopic appearance** of the thyroid glands, parathyroid glands or liver of male or female pups at PND 4, 13 and 21. Microscopic findings in thyroid glands of PND 50 males and females (see Table 88Table 38) were not considered test substance related. Test-substance related hepatocellular vacuolation in the liver was also documented; the vacuoles were consistent with lipid.

Table 88: Incidence of histopathologic findings in thyroid gland and liver of F1 animals, PND 50 [(number of tissues examined), incidence] (Anonymous, 2019a).

Finding	Control	50 mg/kg bw/d	75 mg/kg bw/d	100 mg/kg bw/d
F1 PND 50 - Males				
Thyroid gland, hypertrophy/hyperplasia, follicular cell	(15)	(15)	(15)	(15)
Minimal	0	1	1	1
Mild	11	9	8	8
Moderate	4	5	6	6
Liver, vacuolation, hepatocellular, periportal	(0)	(0)	(1)	(1)
Mild	0	0	1	1
F1 PND 50 - Females				
Thyroid gland, hypertrophy/hyperplasia, follicular cell	(15)	(15)	(15)	(13)
Minimal	9	9	3	3
Mild	6	5	11	10
Moderate	0	1	1	0

Liver, vacuolation, hepatocellular, periportal	(0)	(3)	(9)	(6)
Minimal	0	2	8	3
Mild	0	1	1	3

In a **Reproduction/developmental toxicity screening test** according to OECD TG 421 (Tanaka, 2001) ten week old rats were exposed to 0, 6, 25 or 100 mg 6PPD/kg bw/d. Males were exposed for 48 days, females 14 days before mating until day 3 of lactation. Body weight and food consumption was examined on a regular basis. Organ weights of liver, adrenals, testes, epididymides were determined. Macroscopical examination of liver and reproductive organs and microscopical examination of liver, kidney, skin, and reproductive organs are documented. Reproductive performance was also examined, for further details see Chapter 10.10.

Salivation was seen for some males and females in the 100 mg/kg bw/d group and some males in the 25 mg/kg bw/d group at some point in time. One female died on day 23 of gestation showing thymus atrophy. No effects on body weights of males and females are reported. Food consumption was increased in high dosed males at some points in time and in all high dosed females during lactation.

At the end of exposure absolute and relative liver and adrenal weights were elevated in high dose males. In females absolute and relative liver weights were increased in the mid and high dose groups (see Table 89). No effects on testes and epididymides weights are documented. 9/12 males and 7/10 females at high dose group showed liver enlargement ($p \leq 0.01$). Histology documented vacuolar degeneration of the liver in high dose males (9/11) ($p \leq 0.01$) and mid dose males (2/12), in females this effects was not observed. The effect was generally described as slight, with the exception of one high dose male with marked degeneration and one mid dose male with moderate effect. In males of the 6 mg/kg bw/day group a slight cellular infiltration of lymphocytes in the liver is documented (m: 4/11; $p \leq 0.05$).

Based on liver weight increase seen at the mid dose in males and females and vacuolar degeneration a NOAEL of 6 mg/kg bw/d can be derived. Results for fertility and developmental toxicity are shown in Chapter 10.10.

Table 89: Body and organ weights after oral subchronic exposure to 6PPD (Tanaka, 2001).

[mean ± SD, %]	0 mg/kg bw/d	6 mg/kg bw/d	25 mg/kg bw/d	100 mg/kg bw/d
Males (day 49 of experiment)				
No of animals examined	12	12	12	12
Body weight [g]	477 ± 29 -	492 ± 30 3%	482 ± 29 1%	490 ± 37 2.7%
Absolute liver weight [g]	16.03 ± 2.15 -	17.70 ± 2.25 10.4%	17.84 ± 1.80 11.3%	22.15 ± 2.92** 38.2%
Absolute adrenal weight [mg]	51 ± 7 -	51 ± 6 0%	53 ± 7 3.9%	60 ± 8** 17.6%
Absolute testes weight [g]	3.32 ± 0.24 -	3.42 ± 0.19 3%	3.47 ± 0.16 4,5%	3.45 ± 0.26 3.9%
Absolute epididymides [mg]	1282 ± 80 -	1237 ± 56 -3,5%	1278 ± 78 -0.3%	1295 ± 98 1%
Relative liver weight [g%]	3.35 ± 0.34 -	3.59 ± 0.26 7.1%	3.69 ± 0.20* 10.1%	4.51 ± 0.36** 34.6%
Relative adrenal weight [mg%]	10.64 ± 1.67 -	10.37 ± 1.22 -2.5%	11.05 ± 1.14 3.9%	12.23 ± 1.52* 14.9%
Relative testes weight [g%]	0.70 ± 0.07	0.70 ± 0.07	0.72 ± 0.04	0.71 ± 0.07
Relative epididymides [mg%]	269.54 ± 23.24 -	252.12 ± 19.66 -6.5%	265.65 ± 20.19 -1.4%	265.78 ± 28.03 -1.4%
Females (day 4 of lactation)				

No of animals examined	12	11	12	210
Body weight [g]	280 ± 26 -	290 ± 22 3.6%	296 ± 20 5.7%	296 ± 17 5.7%
Absolute liver weight [g]	11.14 ± 1.05 -	12.11 ± 1.21 8.7%	13.12 ± 0.97** 17.8%	15.24 ± 1.09** 36.8%
Absolute adrenal weight [mg]	73 ± 12 -	67 ± 7 -8.2%	71 ± 9 -2.7%	79 ± 12 8.2%
Relative liver weight [g%]	3.98 ± 0.21 -	4.19 ± 0.34 5.3%	4.43 ± 0.10** 11.3%	5.16 ± 0.34** 29.6%
Relative adrenal weight [mg%]	26.48 ± 5.49 -	23.12 ± 2.06 -12.7%	24.06 ± 3.39 -9.1%	26.67 ± 3.51 0.7%

** significantly different from control, p≤0.01; * significantly different from control, p≤0.05

In a **reproductive / developmental toxicity screening study** according to OECD 421 (Anonymous, 2009; cited from ECHA dissemination site) Wistar rats (12/sex/dose) were exposed to 6PPD concentrations of 0, 2.5, 12.5 or 25 mg/kg bw/d (vehicle olive oil). Males were exposed for 28 days (14d pre-mating and 14d mating) and females for 41 to 56 days (14d premating, ~14d mating, ~21-24d gestation, 4d lactation). Body weights were recorded weekly, food consumption daily. Clinical observations were done once a day (1h after application). After necropsy organ weights of liver, kidneys, testes, epididymides, uterus and ovaries were determined from parental animals. All animals underwent macroscopic evaluation and histological examination included uterus, ovaries, epididymides, prostate, kidney of control and high dose group, livers of low and mid dose group animals.

Study authors concluded that no test-substance related death occurred: one male of the mid dose group died on day 16 immediately after application (probably by inspiration of the test substance, animal was replaced), one control, one female of the low and one female of the high dose group died during delivery of pups. Clinical signs are described as sporadic occurrence of smooth stool in males of the low (2/12), mid (2/12) and high dose group (2/12) and one female of the mid dose group. These signs appeared temporary and were considered as not test substance related. Administration of 6PPD did not affect body weight and food consumption of males and females. No statistically significant differences in relative organ weights were reported in treated animals compared to control. Male liver weights showed a tendency of weight reduction (control 4.46g ± 0.33, high dose 4.19g ± 0.31, not signif) while females showed liver weight increase with increasing test concentration (control 4.58g ± 0.06, high dose 5.23g ± 0.067; not statistically significant). Histopathological findings are documented for liver and reproductive organs (see Table 90).

Table 90: Relevant histopathological findings (Anonymous, 2009).

Organ	Observation	Test group [no of lesions / no of animals]			
		control	2.5 mg/kg bw/d	12.5 mg/kg bw/d	25 mg/kg bw/d
Males					
Liver	Focal necrosis	0/12	0/12	2/11	7/12
	Mononuclear nodules in parenchyma or occurrence of inflammatory cells	0/12	3/12	8/11	8/12
	Small-or medium droplet vacuolization of hepatocytes	1/12	7/12	9/11	4/12
Testes	Hypoplasia of seminiferous tubules and absence spermatogenic cells	0/12	-	1/1[#]	0/12
Females					
Liver	Focal necrosis	2/11	-	-	2/12
	Mononuclear nodules in parenchyma	4/11	-	-	5/12
	Small- or medium droplet vacuolization of hepatocytes	4/11	-	-	9/12
Uterus	Focal inflammatory lesion in	0/11	-	-	1/12

	myometrium				

microscopic evaluation performed because of macroscopic finding in one male

Further details on reproductive parameters or offsprings are presented in Chapter 10.10.

In a **chronic feeding study** (Anonymous, 1978a; cited from ECHA dissemination site) 6PPD was administered in doses of 0, 100, 300 or 1000 ppm (equal to ~ 0, 8, 23 or 75 mg/kg bw/d) for 24 months. Animals were observed daily (mortality, toxic signs). Body weights were measured weekly for 13 weeks and monthly thereafter. Food consumption was measured for 5 rats per sex from dietary level weekly for the first 13 weeks and for one week in each month of the study thereafter. Ten animals per sex from control and high dose groups were examined at 3, 6, 12, and 18 months for variations in hematological values (hemoglobin, hematocrit, erythrocyte and leukocyte counts, mean corpuscular volume and hemoglobin concentration), clinical chemistry values (blood urea, nitrogen, glutamic pyruvic transaminase, glutamic oxalacetic transaminase, alkaline phosphatase, glucose), and urinalyses (specific gravity, pH, albumin, glucose, microscopic elements). Ten animals per sex from each group were also examined for variations in these parameters at 12 and 24 months.

Complete gross necropsies were conducted on animals found dead, on all animals sacrificed in extremis and on all animals surviving at 24 months. From all animals sacrificed at 24 months organ weights and organ/body weight or organ/brain weight were recorded (brain, gonads, heart, kidneys, liver, spleen). Histopathological examinations were conducted on adrenal, bone marrow, brain, esophagus, eye, optic nerve, ovary/testis, epididymis, heart, cecum, colon, duodenum, ileum, jejunum, kidney, liver, lung, pancreas, pituitary, skeletal muscle, spinal cord, spleen, stomach, thyroid parathyroid, urinary bladder, uterus, prostate, mammary gland, and additional grossly observed alterations or tissue masses from some animals found dead, selected animals sacrificed in extremis, and all surviving animals sacrificed at 24 months.

The survival rates for 0, 100, 300 and 1000 ppm were 24/28/26/28 in males and 54/52/44/52 in females. No treatment related clinical sign were noted. At 1000 ppm body weight and bw gain was reduced in males and females; while males showed a reduced bw ($p<0.01$) only during the initial 5 weeks of testing females exhibit lower body weights ($p<0.01$) throughout most of the study. Food consumption was reduced in high dose animals during the first few weeks. Gross pathology revealed no test substance related deaths or lesions.

Examination of organ weights showed a decrease in absolute and relative kidney weights in high dose females (abs. weight $p<0.05$), increase in absolute and relative liver weights in high dose males (rel weight $p<0.05$), and decrease in absolute and relative spleen weights in high dose females. Histopathologic examination could not identify treatment related lesions. Urinalyses and clinical biochemistry showed no effects (or within the historic control data range). Hematology showed some variations: Erythrocyte counts were lower for high dose males (at 3 months) and females (at 3, 6 and 12 months) ($p<0.01$), however, not in subsequent blood collections. Hemoglobin concentration was reduced for high dose males (at 3, 12 and 18 months) ($p<0.01$ or 0.05) and females (at 6, 12 and 18 months). Although reduced, most hemoglobin concentration results were considered within normal range. Hematocrit values among high dose animals were significantly lower than controls and were at the lower limits at 3, 6 (females only, $p<0.01$) and 12 months testing ($p<0.05$). Hematocrit values increased slightly for these animals at 18 and 24 months. The 12 -month additional bleeding (conducted 5 days later) on control and all test groups revealed no statistical differences in any of the parameters evaluated.

For male animals of the high dose group the incidence of neoplastic lesions was slightly but not significantly elevated compared to control (see Table 91). The greatest differences between males of control and treated groups were noted in lymph nodes and in the pituitary and parathyroids. However, the number of tumors noted was considered to be within the historical range for this strain of animal. For females the incidence of neoplastic lesions was reduced for treated groups versus controls. Individual types of neoplastic lesions were comparable to controls or within the historical range for animals of this strain and age.

Based on this study description a NOAEL of 300 ppm can be derived.

Table 91: Neoplastic lesions after chronic exposure to 6PPD (Anonymous, 1978a).

		0 ppm	100 ppm	300 ppm	1000 ppm
Males	Numbers examined	19	15	18	25
	Neoplastic lesions	0.94	0.80	1.11	1.12
Females	Numbers examined	37	28	38	37
	Neoplastic lesions	1.51	1.14	1.29	1.32

In a **three month feeding study** (Anonymous, 1987a) Sprague Dawley rats (6 weeks old) were exposed via diet to 0, 250, 1000 or 2500 ppm 6PPD (equal to males: 15.7, 62.3, 153.8 mg/kg bw/d; females: 18.5, 75.0, 172.1 mg/kg bw/d). Analyses to verify stability and concentration of the test material in diet were performed. Body weight and food consumption were investigated weekly, mortality twice daily and detailed observations for signs of toxicity once weekly. Ophthalmoscopic examination was done at the beginning and the end of the study. Clinical pathology (hematology, clinical chemistry) was done for 10/sex/group in study week 6-7 and at final sacrifice. Organ weights of brain, kidney, liver, spleen, testes with epididymides were determined. Macroscopic and microscopic investigations have been documented.

No mortality occurred and clinical signs showed no treatment related effects. There were no ophthalmic findings. Body weight and body weight gain was reduced in males and females at 2500 ppm and in males at 1000 ppm (Table 92). Food consumption was reduced for animals of the two highest dose groups for the first week of testing (possibly due to unpalatability of the test material/diet mixture). Then food consumption by males and females at the high dose level and males at the middle level remained consistently below control levels. Absolute liver weight was increased in males at high dose and females at mid and high dose. Spleen weight was decreased in high dose females and testes weight was decreased in high dose males. Histology revealed no test related effects.

Table 92: Body weights and relevant absolute organ weights at study termination (Anonymous, 1987a).

n=15/sex/dose	Males				Females			
	Body weight, absolute, mean [g±SD] (%)	Body weight gain, mean [g], (%)	Absolute liver weight, mean [g], (%)	Absolute testis weight, mean [g], (%)	Body weight, absolute, mean [g±SD]	Body weight gain, mean [g], (%)	Absolute liver weight, mean [g], (%)	Absolute spleen weight, mean [g], (%)
Control	558.0 ± 49.05	341.9 ± 44.92 (+161.6%)	15.263 ± 0.491	5.634 ± 0.133	312.3 ± 33.60	130.2 ± 26.55 (+72.5%)	8.370 ± 0.239	0.596 ± 0.029
250 ppm	534.5 ± 21.94 (-4.2%)	321.5 ± 17.91 (+150.5%)	15.347 ± 0.299 (0.6%)	5.459 ± 0.170 (-3.1%)	312.2 ± 24.00 (0%)	129.3 ± 18.40 (+72.4%)	9.262 ± 0.303 (+10.6)	0.594 ± 0.027 (-0.3%)
1000 ppm	502.9 ± 26.27** (-9.9%)	290.0 ± 25.41** (+136.0%)	16.873 ± 0.557 (+10.5%)	5.290 ± 0.129 (-6.1%)	290.6 ± 28.08 (-6.9%)	111.1 ± 20.54* (+60.7%)	9.577 ± 0.297* (+14.4%)	0.581 ± 0.033 (-2.5%)
2500 ppm	468.4 ± 31.46** (-16.1%)	255.3 ± 24.92** (+119.6%)	17.942 ± 0.599** (+17.6%)	5.058 ± 0.155* (-10.2%)	272.5 ± 21.25** (-12.7%)	93.6 ± 13.50** (+51.7%)	10.485 ± 0.355** (+25.3%)	0.472 ± 0.016** (-20.8%)

*Dunnett´s test p≤0.05; ** p≤0.01

Hematology showed signs of a mild anemia in mid and high dose males and females as reflected by mild but significant (p<0.01) decrease in RBC (m -12.3%, f -16.3%), HGB (m -9.0%, f -14.5%), and MCV (m -5.5%, f -6.3%) and corresponding deviations in the calculated values for HCT, MCH and MCHC. Platelet counts were significantly elevated (>20%) in mid and high dose animals (thrombocytosis). Reticulocyte values were

significantly elevated ($p<0.01$) for high dose males at the interim and the terminal sampling and high dose females at the end of the study. Decreased WBC counts (due to reduced numbers of lymphocytes) were observed in females from all test levels at the terminal sampling.

Elevations of total bilirubin in males, and total protein, albumin, globulin, calcium, and/or cholesterol in both sexes were present in high, and often mid dose animals at interim and terminal sampling. Decreases of SCOT, SGPT, creatinine, and BUN were also observed sporadically in either or both sexes, usually at the higher dietary levels.

A NOAEL of 250 ppm can be derived based on reduced body weight gain, mild anemia and increased liver weights seen at 1000 ppm.

Stasenkova (1970) gives a rough description of an **oral short-term repeated dose toxicity study**. The documentation is rather limited, however, the following information can be given: 6PPD was introduced in the stomach of white rats once a day for 24 days. The starting dose was 250 mg/kg bw/d (1/10 of a determined LD_{50} value of 2500 mg/kg bw/d) and was increased by 50% every 5 days. No effect on body weight was documented, however, suppression of the CNS, a tendency to increased oxygen consumption, a clear suppression of the synthesizing function of the liver (measured via the content of hippuric acid in 24h urine samples) and a statistically reliable fall in the content of ascorbic acid in the liver were observed.

In **a chronic feeding study** (24 months) (Anonymous, 1981) rats (50m+50f per dose) were exposed to 0, 100, 300 or 1000 ppm 6PPD (~ 0, 8, 23 or 75 mg/kg bw/d). Body weight and food consumption were investigated on a regular basis (weekly, monthly). Haematology as well as urinalysis of control and high dose group (n=10/sex) was done at 3, 6 and 18 months. All groups were investigated only at 12 and 24 months. Microscopic examination was done on selected tissues from the chest, the abdominal regions and the CNS of all high dose and control animals.

The survival rate was comparable to control (m: 24, 28, 26, 28; f: 54, 52, 44, 51 for 0, 100, 300 or 1000 ppm, respectively). No changes in organ weights and no gross or microscopic tissue changes are documented. At 1000 ppm reduced body weight gain (with decreased food consumption during the first week of the study), decrease in erythrocyte counts, hemoglobin concentration and hematocrit values at some interim intervals, but not at the end of the study are reported. Increased kidney and spleen weights at terminal sacrifice were only described in females. For males the incidence of non-neoplastic lesions was comparable between control and high dose groups, for females the incidence was reduced in treated animals compared to controls.

In an **OECD 408 study** (Anonymous, 2008a) Wistar rats were treated orally for 90 days with 0, 2.5, 12.5, 25 mg 6PPD/kg bw/day in olive oil. A recovery group of 28 days was included for high dose animals and control. Haematology and clinical chemistry was done on day 0, 30, 60 and 90 as well as 28d after last application for the satellite group. Urinalysis was done at the end of study and clinical observations were made once a day one hour after application of 6PPD. Organ weights of liver, kidneys, adrenals, heart, brain, spleen, thymus, seminal glands and ovaries were determined. Microscopic investigation (control and high dose animals) of all weighted organs as well as lungs, lymph nodes, bone marrow, stomach small and large intestine, trachea, thyroid, esophagus, aorta, salivary gland, urinary bladder, peripheral nerve, brain, pituitary, males and females reproduction organs and all big additional findings was done. In addition livers and hearts of mid dose group males and kidneys and livers of mid and low dose group females were microscopically evaluated due to findings in high dose group. According to registrants the study has limitations in study design and documentation (e.g. no analytic for homogeneity and stability of test substance solution performed, no historical control data given).

Five unscheduled deaths were observed evenly distributed among all dose and control groups (1/24 control f, 2/15 medium dose f, 1/24 high dose f, 1/15 low dose m) and therefore considered accidental. Clinical observation reported some cases of piloerection, alopecia and smooth stool. Body weights showed some slight but not statistically significant decrease in the high dose group (see Table 93). The food consumption in males and females of all dose levels was similar to the control group (with exception of some cases in some weeks).

Table 93: Mean body weights of males and females after treatment (90d) with 6PPD (Anonymous, 2008a).

Body weight (mean + SD)	Day 1	Day 30	Day 60	Day 90	Recovery day 28
Males					
Control (n=24)	168.3 ± 17.6	263.8 ± 22.4	329.6 ± 26.0	362.9 ± 29.1	361.1 ± 27.6 (n=9)
2.5 mg/kg bw/d (n=15)	172.0 ± 17.8	262.7 ± 22.2	335.3 ± 33.6	376.4 ± 41.6	-
12.5 mg/kg bw /d(n=15)	185.3 ± 16.4*	268.7 ± 23.6	340.7 ± 28.1	368.7 ± 32.0	-
25 mg/kg bw/d (n=24)	176.7 ± 14.0	260.0 ± 18.2	319.2 ± 26.4	343.3 ± 28.2	337.8 ± 15.6* (n=9)
Females					
Control (n=24)	140.4 ± 13.0	176.3 ± 21.2	211.7 ± 22.8	220.4 ± 23.4	220.0 ± 21.5 (n=9)
2.5 mg/kg bw/d (n=15)	144.0 ± 15.5	180.0 ± 22.4	205.3 ± 24.5	217.3 ± 26.9	-
12.5 mg/kg bw/d (n=15)	139.3 ± 8.8	175.0 ± 16.1	203.6 ± 16.5	213.8 ± 15.0	-
25 mg/kg bw/d (n=24)	145.4 ± 15.0	187.5 ± 20.3	215.4 ± 25.4	219.6 ± 25.5	217.8 ± 27.5 (n=9)

* $p<0.05$

In urinalysis no significant changes against normal physiological conditions were detected. The urine of some animals contained small amounts of protein, ketones and presence of blood was observed. The authors stated that these findings are considered as physiological. Changes in RBC, PLT, lymphocytes and neutrophiles were noted in control and treated animals, more often in males than in females; the authors conclude that these changes are not treatment related. Further changes are reported in Table 94.

Table 94: Hematology (Anonymous, 2008a).

Value [unit]	Day 30	Day 60	Day 90	Recovery day 28
Males				
Hematocrit	-	-	control: 48.81 ± 2.97 25 mg/kg: 46.95 ± 1.43* (-3.8%)	-
Hemoglobin	-	control: 16.14 ± 0.92 25 mg/kg: 15.18 ± 0.85* (-5.9%)	control: 15.89 ± 0.95 12.5 mg/kg: 15.12 ± 0.57* (-4.8%) 25 mg/kg: 15.25 ± 0.48* (-4.0%)	-
MCV [µm3]	-	-	control: 55.58± 1.98 12.5 mg/kg: 53.60 ± 2.44* (-3.6%) 25 mg/kg: 53.25 ± 1.78* (-4.2%)	-
MCH [pg]	control 19.89 ± 0.67 25 mg/kg: 19.09 ± 0.66* (-4.0%)	control: 18.20 ± 0.82 25 mg/kg: 17.23 ± 0.61* (-5.3%)	control: 18.07 ± 0.54 12.5 mg/kg: 17.35 ± 0.54* (-4%) 25 mg/kg: 17.26 ± 1.78* (-4.5%)	-
MCHC [g/dL)] (1)	control: 35.35 ± 1.28	control:33.19 ± 1.59 25 mg/kg: 31.21 ±	control: 32.58 ± 1.05 25 mg/kg: 32.47 ± 0.53* (-0.3%)	-

	25 mg/kg: 33.86 ± 1.16* (-4.2%)	1.39* (-6%)		
Quick test (s)	-	-	control: 12.64 ± 0.52 2.5 mg/kg: 12.24 ± 0.59* (-3.2%) 12.5 mg/kg: 12.13 ± 0.43* (-4%) 25 mg/kg: 12.06 ± 0.92* (-4.6%)	control: 11.54 ± 0.97, 12.70 ± 0.42*
Females				
Hemoglobin	-	-	control: 15.84 ± 0.89 25 mg/kg: 14.97 ± 1.04* (-5.5%)	-
MCH [pg]	-	control: 19.26 ± 1.00 25 mg/kg: 18.54 ± 0.45* (-3.7%)	control: 19.76 ± 0.95 25 mg/kg: 18.93 ± 0.68* (-4.2%)	-
MCHC [g/dL)]	-	-	control: 34.80 ± 1.78 25 mg/kg: 33.40 ± 0.81* (-4.0%)	-
Quick test (s)	-	-	control: 12.75 ± 0.71 12.5 mg/kg: 11.79 ± 0.55* (-7.5%) 25 mg/kg: 12.00 ± 0.55* (-5.9%)	-

* $p<0.05$
(1) for this parameter also on day 0 signif. differences are reported: control: 34.19 ± 1.45, 25 mg/kg: 33.06 ± 1.08*

In clinical chemistry some effects were concluded as effects of biological variability (eg. increases in enzyme activity of ALP in males with lack of dose response; sporadic changes of glucose, creatinine, urea). A significant increase ($p<0.05$) of total cholesterol [mmol] was noted in males of the high dose group after 30, 60 and 90 days of application compared to control (but not after recovery). In females of the mid and high dose groups an increase in cholesterol values were noted after 30, 60 and 90 days of treatment (significant after 30 and 60 d). High dose females of the satellite group showed an increase, but not significant, in cholesterol. Total protein was significantly increased in mid and high dose males after 90 d (but not after recovery). Females of the high dose group had increased values after 30, 60, 90 d and 28d post-treatment (statistically signif. only on day 30 and 60). For albumin a significant increase ($p<0.05$) was noted in high dose males throughout the study (day 0, 30, 60, 90, 28d post-treatment); an increase was noted in high dose females after 30, 60 and 90 days (on day 60 signif, $p<0.05$); no increase was noted in high dose females of the satellite group compared to control. No values for clinical chemistry are reported.

Increases (not statistically significant) of relative liver weights as well as kidney weights are documented in males and females. Also a slight increase in relative testes weights were observed in treated males compared to control. For further details see Table 95. Absolute organ weights are not reported.

Table 95: Relative organ weights, males and females [mean ± SD, %] (Anonymous, 2008a).

%	Liver, relative	Kidney, right, relative	Kidney, left, relative	Testis, right, relative	Testis, left, relative
Males					
Control (n=15)	25.35 ± 1.35	2.96 ± 0.28	2.89 ± 0.25	6.16 ± 0.71	6.20 ± 0.80
2.5 mg/kg bw/d (n=14)	27.59 ± 2.17 8.8%	2.98 ± 0.24 0.7%	2.95 ± 0.27 2.1%	5.50 ± 0.95 -10.7%	5.68 ± 0.88 -8.4%
12.5 mg/kg bw/d (n=15)	29.54 ± 1.48 16.5%	3.14 ± 0.21 6.1%	3.11 ± 0.30 7.6%	6.32 ± 0.91 2.6%	6.14 ± 0.73 -1%
25 mg/kg bw/d (n=15)	32.18 ± 5.31 26.9%	3.18 ± 0.21 7.4%	3.17 ± 0.25 9.7%	6.39 ± 0.62 3.7%	6.52 ± 0.63 5.1%
Control recovery	26.59 ± 1.29	3.05 ± 0.24	3.05 ± 0.25	5.63 ± 1.75	5.79 ± 1.60

(n=9)	-	-	-	-	-
High dose recovery (n=9)	28.96 ± 1.24* 8.9%	3.21 ± 0.21 5.2%	3.18 ± 0.22 4.3%	5.63 ± 1.00 0.0%	5.75 ± 1.14 -0.7%
Females					
Control (n=15)	25.33 ± 2.21	3.14 ± 0.26	3.02 ± 0.24	-	-
2.5 mg/kg bw/d (n=15)	27.11 ± 2.52 7.0%	3.18 ± 0.22 1.3%	3.14 ± 0.27 4.0%	-	-
12.5 mg/kg bw/d (n=13)	28.74 ± 2.33 13.5%	3.32 ± 0.24 * 5.7%	3.29 ± 0.29* 8.9%	-	-
25 mg/kg bw/d (n=14)	31.71 ± 2.20 25.2%	3.47 ± 0.35 10.5%	3.37 ± 0.29 11.6%	-	-
Control recovery (n=8)	25.91 ± 2.40 -	3.26 ± 0.21 -	3.15 ± 0.16 -	-	-
High dose recovery (n=9)	27.93 ± 2.62 7.8%	3.22 ± 0.23 -1,2%	3.16 ± 0.31 0.3%	-	-

* p<0.05

Macroscopic evaluation revealed no treatment related gross lesions. Atrophy of both testes was found in 1/24 males from control group and in 1/24 males from high dose level. Results of histological examination are shown in Table 96. In addition, in testes atrophy of tubules and absence of spermatogenic cells were observed in 1/9 males from control satellite group and 1/9 males from high dose satellite group. In 1/15 high dose males droplet vacuolization of most cells in adrenals was observed.

Table 96: Number of relevant histological findings (Anonymous 2008a).

Organ	Lesion	Males				Females			
		control	2.5 mg/kg bw/d	12.5 mg/kg bw/d	25 mg/kg bw/d	control	2.5 mg/kg bw/d	12.5 mg/kg bw/d	25 mg/kg bw/d
Liver	Diffuse smalldroplet vacuolization of hepatocytes	7/15	-	-	6/15	4/15	3/15	8/15	14/15
	Monocellular necrosis	5/15	-	-	1/15	0/15	2/15	4/13	12/14
	Small focal inflammatory lesions	7/15	-	-	5/15	-	-	-	-
Kidneys	Moderate degenerative changes in proximal tubules	0/15	-	0/15	14/15	-	-	-	-
	Inclusions in epithelial cells of proximal tubules	4/15	-	4/15	1/15	-	-	-	-
	Interstitial lymphocytic infiltration	2/15	-	1/15	1/15	-	-	-	-
	Sporadic glomerular atrophy	0/15	-	2/15	0/15	2/15	14/15	13/13	14/14
	Hyaline deposition in lumen of Proximal tubules	-	-	-	-	4/15	0/15	0/13	14/14
Heart	Small focal muscular dystrophy in left myocardium; morphology of mucle cell was changed	0/15	-	2/15	12/15	-	-	-	-
Ovary	Cyst formation	-	-	-	-	0/15	-	-	1/14
Spleen	White pulp hyperplasia	-	-	-	-	0/15	-	-	1/14

Pancreas	Chronic pancreatitis and cyst formation	-	-	-	-	0/15	-	-	1/14

Remark: Because of microscopic findings in high dose animals kidneys and hearts of mid dose group males and kidneys and livers of mid and low dose group females were microscopically evaluated.

The **28-day study** by Anonymous (2008b) is reported for completeness, but no dosing and no results are reported for this study in the registration and the orginal report is not available. No conclusion can be based on this information.

In a **three generation reproductive study** (Anonymous, 1980b) Charles River CD rats (groups of 8 males and 16 females) were exposed to 6PPD concentrations of 0, 100, 300 or 1000 ppm (approx. 0, 8, 23, 75 mg/kg bw/d) via diet (ad libitum). Details on study design are presented in Chapter 10.10. Mortality was documented for test and control animals during each generation, the numbers being unusually high in some instances. A correlation with the administration of the test substance cannot be made.

Body weight (gains) were affected on some points in time with no clear dose dependency. Reduced body weight gains (without statistical significance) were reported for F0 parental males in the high dose group (-12.6%) and for F2 parental males in mid (-16.9%) and high dose groups (-12.5%). Final body weights were lower for F1 parental males in the mid (-9.9 %) and high dose groups (-20.4%), for F2 males in the mid (-10.2%) and high dose group (-7.6%), and for F2 females statistically significant (-11.9%) in the mid dose group. For food intake no marked differences between test and control groups are documented.

Gross necropsy observations of F0, F1 and F2 adults sacrificed after weaning of their second litters did not reveal any adverse effects related to test material administration. No treatment related statistical differences in organ weights were noted for F0, F1, or F2 parental animals in any treatment group with the exception of liver weight. Female total liver weight was (not statistically significant) increased in a dose dependent manner in F0, F1 and F2. Female relative liver weight (to body weight) was statistically significant increased in 1000 ppm F1 (+34%, $p<0.01$) and F2 (+41%, $p<0.05$) generation. Effects on male liver weights were not significant. Microscopic examination of tissues from selected F0, F1 and F2 parental animals and F3b pups from control and high dose groups, and from selected animals from low and mid dose groups, showed no test-substance related abnormalities.

Two studies with rabbits (oral exposure) are available with repeated exposure to 6PPD. In an **OECD 414 study** (Anonymous, 2018c) pregnant rabbits were dosed for 21 days (GD 7-28) with concentrations of 0, 25, 50 or 100 mg 6PPD/kg bw/d. For detailed study description see Chapter 10.10.4. At 25 mg/kg bw/d body weight parameters were unaffected. Mean body weights were significantly ($p<0.05$ or $p<0.01$) lower in the 50 mg/kg bw/day group (5.0% to 5.5% during GD 24-27) and 100 mg/kg bw/day group (ranging from 6.1 % to 10.0% during GD 13-29) compared to the control. Mean body weight losses and lower mean body weight gains were noted in the 50 and 100 mg/kg bw/day groups during GD 7–10, 10–13, and 13–20; differences from the control group were generally significant ($p<0.01$). Mean body weight gains in these groups were comparable to or slightly higher than the control group during GD 20–29. The decrements in body weight gain in the 50 and 100 mg/kg bw/day groups resulted in significantly ($p<0.01$) lower mean body weight gains when the entire treatment period (Gestation Days 7-29) was evaluated (347g, 324g, 197g and 158g in control, low, mid and high dose, respectively)**.** Significantly ($p<0.05$ or $p<0.01$) lower mean gravid uterine weight at 100 mg/kg bw/day (-18.3%) and net body weight change at 50 and 100 mg/kg bw/day were noted compared to the control group (see Table 52). The aforementioned effects on mean body weight, body weight gains, and gravid uterine weight were considered test substance-related and adverse. Food consumption in the 25 mg/kg bw/day group was unaffected by test substance administration. Mean food consumption (g/animal/day and g/kg/day) in the 50 and 100 mg/kg bw/day groups was generally lower than the control group beginning on GD 7, and continuing through GD 24 or 25. Thereafter, mean food consumption in these groups were comparable to the control group.

Gross pathology at necropsy on GD 29 showed no test substance related internal findings in any dose group. Higher mean absolute and relative (to net body weight) liver weights were noted in the 50 and 100 mg/kg

bw/day groups; differences from the control group were generally significant ($p<0.01$) (see Table 97). A NOAEL of 25 mg/kg bw/d can be derived.

Table 97: Body and liver weights of rabbits [mean ± SD, n, %] (Anonymous, 2018c).

Parameter	Control	25 mg/kg bw/d	50 mg/kg bw/d	100 mg/kg bw/d
Initial body weight	3246. ± 226.8 23	3260. ± 241.8 23 +0.4%	3248. ± 226.6 24 0.0%	3251. ± 212.7 24 +0.2%
Terminal body weight	3699. ± 226.8 23	3656. ± 264.4 23 -1.2%	3529. ± 243.0 24 -4.6%	3472.** ± 281.7 24 -6.1%
Liver	82.17 ± 12.986 23	87.79 ± 14.306 23 +6.8%	91.35 ± 11.936 24 +11.2%	105.44** ± 13.841 24 +28.3%
Liver (g/100 g net body weight)	2.556 ± 0.3517 23	2.771 ± 0.3151 23 +8.4%	2.952** ± 0.2948 24 +15.5%	3.394** ± 0.2621 23 +32.7%

In a **teratogenicity study** (Anonymous, 1976b) with New Zealand Albino rabbits 6PPD was administered in doses of 0, 10 and 30 mg/kg bw/d orally in gelatin capsules on GD 6-18 once daily (13 days in total). Sacrifice was done on GD 29. Rabbits in the two test groups and the control group showed mean **body weight loss** during the dosing period, the control and the high dose group showed mean body weight loss overall. 5, 3 and 6 animals died in the control, 10 or 30 mg/kg bw/d group, respectively. In 10/14 animals found dead, respiratory insufficiency or failure was diagnosed. 5 of these 10 animals were control animals. The limited quality of the study with high mortality in the control group hinder the derivation of an effect level.

In a non-guideline **dust inhalation toxicity study** (Anonymous, 1979) rats were exposed (whole body, 6h/d, 5d/week for 4 weeks) to concentrations of 51, 247, 498 mg/m³. The doses were analytically verified but registrants report that only <2% of the particles was within the recommended particle-size range of 1.5 to 3.0 µg. The exposure of relevant regions of the respiratory tract was therefore questionable. Animals were exposed in an inhalation chamber (capacity of 500L). Dust was generated by passing streams of clean, dry air (-40°C dewpoint) through the test material powder. The resulting air/dust mixtures were then introduced into the exposure chambers at the top center and exhausted at the bottom of the chambers. Particle size distribution was determined weekly (see Table 98).

Table 98: Particle size distribution in percent [%] (Anonymous 1979).

Particle size range	Week 1	Week 2	Week 3	Week 4
Low dose, 51 mg/m³ (1)				
1-5 µm	1.9	0.9	2.3	0.9
6-10 µm	12.1	9.5	10.9	4.3
11-25 µm	31.5	28.5	29.8	20.4
>25 µm	54.5	61.1	57.0	74.3
Mid dose, 247 mg/m³				
1-5 µm	1.8	1.1	1.0	1.3
6-10 µm	12.3	10.0	7.5	8.9

11-25 µm	36.2	25.0	20.0	37.8
High dose, 498 mg/m³				
1-5 µm	7.7	12.6	11.4	11.4
6-10 µm	17.3	31.2	36.8	37.6
11-25 µm	73.1	55.0	50.9	50.2

(1) Total number of particle count for week 1 through 4 were (low dose): 257, 221, 265 and 230, respectively.

Mortality, reactions displayed and body weight were recorded. Hematologic and clinical chemistry studies and urinalyses were conducted on all test and control animals on day 23. All surviving animals were sacrificed at the end of the 28d period and subjected to gross necropsy. Organ weights were recorded from liver, kidneys, spleen, lungs, gonads, heart and brain. Histopathologic evaluations (control and high dose group) were conducted on selected tissues and organs (heart, kidney, liver, lung, lymph nodes, bronchi, pituitary gland, spleen, trachea, urinary bladder, gonads, and brain).

One mid dose male died in the study period. Hypoactivity was noted in all test groups; swollen snouts and scratching were documented for mid and high dose animals. Mean body weight gains of treated animals were comparable to control.

For organ weights only limited data is available on ECHA dissemination site. Absolute kidney weight was higher in mid dose females (no kidney effects in males). Liver was affected in both sexes: signif. higher ($p<0.01$) liver weights were seen in low and mid dose males as well as in high dose males ($p<0.05$). Mid dose males also had a higher ($p<0.05$) organ to bw ratio. Higher liver weights were also documented for females of the low dose ($p<0.05$) was well as the mid and high dose ($p<0.01$). Mid and high dose females also had a higher organ to bw and organ to brain weight ratio. For the lung lower organ weights ($p<0.01$) and organ to brain weight ratios ($p<0,05$) were reported for high dose males and mid dose females ($p<0.05$). Spleen weight and spleen to bw ratio was significantly higher in mid dose males ($p<0.05$).

No histopathologic alterations were reported. A dose-dependent decrease in hemoglobin and dose-dependent increase in leukocytes were noted in treated females. A decrease in the erythrocyte count and hematocrit value were noted in treated males and females (not dose dependent). A dose dependent decrease in MCV and MCH was noted in treated males. No further details available. Clinical chemistry and urinalysis showed no effects attributable to the test substance.

10.12.1 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure

In an oral 28-day study (Anonymous, 1999b) rats show increased liver weights at 100 mg/kg bw/d (m + 27.9%, f +35.4%), accompanied with periportal fatty change. The latter were also seen at 20 mg/kg bw/d in females only. Increased absolute brain weight was seen in females at 4 mg/kg bw/d only (+7.4%) and not considered test substance related. Based on this study a NOAEL of 4 mg/kg bw/d for females and 20 mg/kg bw/d for males can be derived.

Anonymous (1993) conducted a chronic feeding study (OECD 452). Increased liver weight is documented in males and females of the 250 and 1500 ppm groups as well as reduced mean bw and increased food consumption. Slight anemia was seen at the highest dose. Kidney weights (m, f) and spleen weights (m) were also increased at 1500 ppm resulting in a NOAEL of 50 ppm.

In an EOGRTS (Anonymous, 2019b) liver weight increase (m +23.7%, f +27.5%) is documented for the high dose (60 mg/kg bw/d) and correlates with liver vacuolation in males. In the mid dose (20 mg/kg bw/d) liver weight was only increased in males (+10.3%, not statist signif). Kidney weights were also increased at 60 mg/kg bw/d (+9.95%). A NOAEL of 20 mg/g bw/d can be determined. However, pigment deposition in the kidney was seen down to concentrations of 7 mg/kg bw/d. In the corresponding dose range finding study (Anonymous, 2019a) increased liver weights were seen at 100 mg/kg bw/d (m +26.1%, f +28.1%), 75 mg/kg bw/d (m +25.0%, f +19.8%) and 50 mg/kg bw/d (m +16.2%). No NOAEL can be determined.

In a screening study (Tanaka, 2001) rats showed at 100 mg/kg bw/d increased liver weights (m +34%, f +29.6%), liver enlargement in males (9/12) and females (7/10), vacuolar degeneration in males (9/11) and increased adrenal weights (m +14.9%). At 25 mg/kg bw/d absolute or relative liver weight was increased in females (+17.8%) and males (+10.1%), respectively, and accompanied with liver enlargement in males (2/12) and vacuolar degeneration in males (2/12). A NOAL of 6 mg/kg bw/d can be derived.

In a second oral screening study in rats (Anonymous, 2009) at 25 mg/kg bw/d focal necrosis were seen in males and mononuclear nodules in parenchyma/inflammatory cells as well as vacuolization were seen in the liver of males and females. In males of the lower dose groups (12.5 and 2.5 mg/kg bw/d groups) the same histological parameters were affected but with lower incidence. Male mean liver weight shows a tendency of weight reduction (4.46g, 4.33g, 4,27g and 4,19g in control, 2.5, 12.5 and 25 mg/kg bw/d groups respectively) while females show a weight increase with increasing test concentration (control 4.58g ± 0.06, high dose 5.23g ± 0.067; not statistically significant).

In a 24 months chronic feeding study (Anonymous, 1978a) effects at 1000 ppm were reduced bw gain and food consumption, increased liver weight in males, decreased kidney and spleen weights in females. Histopathologic examination could not identify treatment related lesions. A NOAEL of 300 ppm (23 mg/kg bw/d) can be derived. In a similar study cited as Anonymous (1981) reduced bw gain is described for the highest dose (1000 ppm), increased kidney and spleen weights at terminal sacrifice were only described in females. The reporting is limited but a NOAEL of 300 mg/kg bw/d can be assumed.

In a subchronic three month feeding study (Anonymous, 1987a) reduced bw (m -9.8%) and body weight gain (m -15.2%, f -14.7%), increased liver weight (f +14.4) and mild anemia (m, f) at 1000 ppm is reported. Same effects were seen at 2500 ppm at higher intense. A NOAEL of 250 ppm (m: 15.7 mg/kg bw/d; f: 18.5 mg/kg bw/d) can be set.

Anonymous (2008a) reports an oral 90 day study in rats. At 25 mg/kg bw/d some effects on hematology are reported as well as effects on kidney (degenerative changes in males, glomerular atrophy in females) and heart in males (small focal muscular dystrophy in left myocardium). Glomerular atrophy in females was seen down to the lowest concentration tested (2.5 mg/kg bw/d). Monocellular necrosis are seen in a dose dependent manner in females (0/15, 2/15, 4/13, 12/14), but not males.

In a three generation study (Anonymous, 1980b) high mortality in control and dose groups was seen. At 1000 ppm relative liver weight (to body weight) was statistically significant increased in F1 (+34%, $p<0.01$) and F2 (+41%, $p<0.05$) generation. Due to study limitations the study was not used for classification purpose.

Two oral studies with pregnant rabbits are available. While one cannot be used due to limited reliability (Anonymous 1976b), the second study (Anonymous, 2018c) showed effects on body weight, body weight gain and food consumption down to concentrations of 50 mg/kg bw/d. Liver weights were increased with +11.2% and 28.3% at 50 and 100 mg/kg bw/d, respectively. A NOAEL of 25 mg/kg bw/d can be set.

Stasenkova (1970), a 28day study by Anonymous (2008b) and an inhalation study (Anonymous, 1979) cannot be used for classification purpose as reporting is limited.

A clear target organ of 6PPD toxicity after repated oral exposure is the liver, which was affected in rats and rabbits. Other organs less frequently affected were the kidneys and the blood system (anemia). The key findings of the available repeated dose toxicity studies are presented in Table 99 and directly compared to the cut-off levels relevant for classification as STOT RE category 1 or 2.

Table 99: Extrapolation of equivalent effective dose for toxicity studies of greater or lesser duration than 90 days

Study reference Kind of exposure	Effective dose (mg/kg/d) – lowest dose inducing signif/severe effects	Effects reported	Length of exposure	Extrapolated effective dose when extrapolated to 90-day exposure	Classification supported by the study
Rat, oral					
28-day study Anonymous (1999b) Oral (gavage)	100 mg/kg bw/d	$NOEL_{female}$ = 4 mg/kg bw/d $NOAEL_{male}$ = 20 mg/kg bw/d <u>100 mg/kg bw/d:</u> rel. **liver weight** ↑ (m + 28% p<0.01; f +35.4%, p<0.01) , **periportal fatty change** (m: slight to moderate in 5/5; f: very slight to moderate in 5/5) <u>20 mg/kg bw/d:</u> periportal **fatty change** in f (very slight to moderate in 5/5)	28 days	33 mg/kg bw/d	Category 2
OECD 452 Anonymous (1993) Oral (feed)	250 ppm (m: 13.5, f: 16.5 mg/kg bw/d)	NOAEL = 50 ppm (m: 2.6 mg/kg bw/d, f: 3.2 mg/kg bw/d) <u>250 ppm:</u> Mean body weight (f)↓ (-5.4%). Mean food consumption (f)↑ (+4.1%) Mean absolute and relative **liver weights** (m, f) ↑ [no values available] <u>1500 ppm:</u> **slight anemia:** hemoglobin (m, f)↓, hematocrit (m, f) ↓, erythrocyte counts(f) ↓, platelet counts (m, f) ↑, MCV↓, MCH↓ [no values available]	370 days	55.4 – 67.7 mg/kg bw/d	Category 2
OECD 443 Anonymous (2019b) Oral (gavage)	7 mg/kg bw/d	<u>60 mg/kg bw/d:</u> abs. kidney weights ↑ (m +9.9% p<0.01; f + 8.6% not signif.) abs. liver weights ↑ (m +23.7%, p<0.01; f +27.5%, p<0.01) rel. liver weights ↑ (m +26.9%, p<0.01; f +20.8%, p<0.01) liver vacuolation (m, minimal to moderate), pigment deposition in the kidneys (minimal to mild; m 22/29, f 15/25; control 0/25) <u>20 mg/kg bw/d</u> rel. liver weights ↑ (m +8.2% p<0.05), **pigment deposition** in the kidneys (minimal in m 12/25) <u>7 mg/kg bw/d</u> **pigment deposition in the kidneys** (minimal in m 7/23)	Day 129 – 132	10 mg/kg bw/d	Category 2

Study reference **Kind of exposure**	**Effective dose (mg/kg/d) – lowest dose inducing signif/severe effects**	**Effects reported**	**Length of exposure**	**Extrapolated effective dose when extrapolated to 90-day exposure**	**Classification supported by the study**
Dose-range finding study similar to OECD 421 Anonymous (2019a) Oral (gavage)	50 mg/kg bw/d	LOAEL = 50 mg/kg bw/d <u>100 mg/kg bw:</u> Abs. liver weight↑ (m +26.1%, p<0.01; f +28.1%, p<0.01), Abs. thyroid gland weights↑ (m +31.2%, p<0.01) <u>75 mg/kg bw:</u> Abs. liver weight↑ (m +25.0%, p<0.01; f +19.8%, p<0.01) <u>50 mg/kg bw:</u> Abs. **liver weight**↑ (m +16.2%, p<0.01)	m:28d f: 14 days prior to mating, continuing through mating, gestation, and lactation until lactation day 21	16.6 mg/kg bw/d adaptive response	-
OECD 421 Tanaka (2001) Oral (gavage)	25 mg/kg bw/d	NOEL = 6 mg/kg bw/d <u>100 mg/kg bw/d:</u> mortality (f): 1/12, absolute (and relative) liver weight ↑ (m +38.2% p<0.01; f +36.8% p<0.01) absolute (and relative) adrenal weight ↑ (m +17.6%, p<0.01) liver enlargement (m: 9/12, f: 7/10) vacuolar degeneration of the liver (m: 9/11; 8 slight and 1 severe) <u>25 mg/kg bw/d:</u> absolute (and relative) liver weight ↑ (f +17.8%, p<0.01) relative liver weight ↑ (m 10.1%, p<0.05) liver enlargement (m: 2/12), **vacuolar degeneration** of the liver (m: 2/12; 1 slight and 1 moderate) <u>6 mg/kg bw/d:</u> Liver: cellular infiltration (slight) of lymphocytes (m: 4/11)	m: 48d f: 14d before mating – day 3 of lactation	~12,5 mg/kg bw/d	Category 2
OECD 421 Anonymous (2009) Oral (gavage)	12.5 mg/kg bw/d	<u>25 mg/kg bw/d:</u> liver: focal necrosis (m 7/12, f 2/12), mononuclear nodules (m 8/12, f 5/12), vacuolization (m 4/12, f 9/12) uterus, females: focal inflammation lesion (1/12) <u>12.5 mg/kg bw/d:</u> liver (males only): **focal necrosis** (2/12), mononuclear nodules (8/12), vacuolization (9/11)	m: 28d f: 41-56d	4.2 mg/kg bw/d	- Klimisch 4

Study reference **Kind of exposure**	**Effective dose (mg/kg/d) – lowest dose inducing signif/severe effects**	**Effects reported**	**Length of exposure**	**Extrapolated effective dose when extrapolated to 90-day exposure**	**Classification supported by the study**
		<u>2.5 mg/kg bw/d:</u> liver (males only): mononuclear nodules (3/12), vacuolization (7/12)			
Chronic feeding study (2 years) Anonymous (1978a) Oral (feed)	1000 ppm (75 mg/kg bw/d)	NOAEL = 300 ppm (~23 mg/kg bw) <u>1000 ppm:</u> body weight and bw gain ↓ (m, f; p<0.01) food consumption ↓(m, f) (during first weeks) abs. and rel. kidney weights ↓ (f, p<0.05) abs. and rel. **liver weights** ↑ (m, p<0.05) abs. and rel. spleen weights ↓ (f)	24 months	600 mg/kg bw/d adaptive response	-
Subchronic study, 3 months Anonymous (1987a) Oral (feed)	1000 ppm (m: 62.3, f: 75.0 mg/kg bw/d)	NOAEL = 250 ppm (m: 15.7 mg/kg bw/d; f: 18.5 mg/kg bw/d) <u>2500 ppm:</u> Body weight ↓ (m -16.1, f -12.7; both p≤0.01); body weight gain ↓ (m -25.3%, f -28.1%; both p≤0.01) Absolute liver weight ↑ (m +17.6%, f +25.3%, both p≤0.01) Absolute testis weight ↓ (m -10.2%, p≤0.05); absolute spleen weight ↓ (f -20.8%, p≤0.01) Anemia, mild (m, f) <u>1000 ppm:</u> Body weight ↓ (m -9.9%, p≤0.01); body weight gain ↓ (m -15.2%, p≤0.01, f -14.7%, p≤0.01) Abs. **liver weight** ↑ (f +14.4, p≤0.05) **Anemia, mild** (m, f)	13 weeks	62.3 - 75.0 mg/kg bw/d	Category 2
Subchronic study Stasenkova (1970)	-	No NOAEL available Clear signs of intoxication (increased oxygen consumption, suppression of the central nervous	24 days	-	- Klimisch 3

Study reference **Kind of exposure**	**Effective dose (mg/kg/d) – lowest dose inducing signif/severe effects**	**Effects reported**	**Length of exposure**	**Extrapolated effective dose when extrapolated to 90-day exposure**	**Classification supported by the study**
Oral (gavage)		system decreased synthezising function of the liver, decreased ascorbic acid content in the liver)			
Chronic toxicity and reproduction study Anonymous (1981) Oral (feed)	1000 ppm (75 mg/kg bw/d)	NOAEL = 300 ppm (23 mg/kg bw) <u>1000 ppm:</u> Body weight gain ↓ Kidney and spleen weights ↑ (f) [limited reporting]	24 months	600 mg/kg bw/d adaptive response	-
OECD 408 Anonymous (2008a) Oral (gavage)	2.5 mg/kg bw/d	LOAEL = 2.5 mg/kg bw/d <u>25 mg/kg bw/d:</u> kidney - moderate degenerative changes in proximal tubules (14/15 m); sporadic glomerular atrophy and hyaline deposition (14/14 f) heart - small focal muscular dystrophy in left myocardium (12/15 m); vacuolization of hepatocytes (f 14/15) + monocellular necrosis (f 12/14) not statistically significant increase of relative liver and kidney weights (m + 26.9% and +9.7%, f +25.2% and +10.5%) <u>12.5 mg/kg bw/d:</u> vacuolization of hepatocytes (f 8/15) + monocellular necrosis (f 4/13), sporadic glomerular atrophy (f 13/13) relative liver weight ↑ (m +16.5%, f +13.5%; not signif), relative kidney weights↑ (f +8.9%, p<0.05) <u>2.5 mg/kg bw/d:</u> sporadic **glomerular atrophy** in the kidneys (f 14/15) **monocellular necrosis** (f 2/15)	90 days	2.5 mg/kg bw/d	Category 1
28days study Anonymous (2008b) Oral (gavage)	-	No results reported	28 days	-	- Klimisch 4

Study reference **Kind of exposure**	**Effective dose (mg/kg/d) – lowest dose inducing signif/severe effects**	**Effects reported**	**Length of exposure**	**Extrapolated effective dose when extrapolated to 90-day exposure**	**Classification supported by the study**
Three generation study Anonymous (1980b) Oral (feed)	1000 ppm (75 mg/kg bw/d)	High mortality in control and dose groups <u>1000ppm</u> Relative liver weight (to body weight) was statistically significant increased in F1 (+34%, p<0.01) and F2 (+41%, p<0.05) generation	three generation study	-	-
Rabbit (pregnant), oral					
OECD 414 Anonymous (2018c) Oral (gavage)	100 mg/kg bw/d	NOAEL = 25 mg/kg bw/d <u>100 mg/kg bw/d</u> mean terminal bw ↓ (-6.1%, p<0.01), food consumption↓, body weight gains↓, gravid uterine weight↓ (-18.3%, p<0.01), abs liver weights ↑ (+28.3%, p<0.01) <u>50 mg/kg bw/d</u> Mean body weights ↓ (-5.0% to 5.5% during GD 24-27), food consumption↓, body weight gains↓ Rel. liver weights ↑ (+15.5%, p<0.01)	21 days	33 mg/kg bw/d adaptive response	-
Teratogenicity study Anonymous (1976b) Oral (gelous capsules)	-	Body weight loss and mortality in control and dose animals	13 days	-	-
Pubertal studies					
Female pubertal study Anonymous (2016a) Oral, gavage	250 mg/kg bw/d	<u>500 mg/kg bw/d</u> Pale liver in 2/15 correlates with hepatocellular vacuolation bilirubin↑, (+700%, p<0.05), GGT↑ (+100%, not signif), AST↓ (-47.3%, p<0.05), triglycerides↓ (-51.2%, p<0.05), serum cholesterol↑(+24.7%), T4↓ (-22.2%, p<0.05) , TSH↑ (+72.3%, p<0.05) ovary weights↓ (-21.5%, p<0.05), uterus weight ↓ (-39.3%, p<0.05), kidney weight↑ (+10.6%, p<0.05), liver weight↑ (52.4%, p<0.05) ,	PND 22-42/43 (~20d)	83 mg/kg bw/d Adaptive response; other effects considered for developmental toxicity	-

Study reference **Kind of exposure**	**Effective dose (mg/kg/d) – lowest dose inducing signif/severe effects**	**Effects reported**	**Length of exposure**	**Extrapolated effective dose when extrapolated to 90-day exposure**	**Classification supported by the study**
		thyroid gland weight↑ (11.7%, not signif), pituitary ↓ (-22.3%, p<0.05) immature uterus (2/12) thyroid gland: follicular cell height (p<0.05), lower colloid area (p<0.05) <u>250 mg/g bw/d</u> AST↓ (-39.8%, p<0.05), triglycerides↓ (-33.9%, p<0.05), serum cholesterol↑ (+23.7%, p<0.05) TSH↑ (+92.3%, p<0.05) uterus weight ↓ (-18.7%, not signif), liver weight↑ (42.2%, p<0.05, kidney weight↑ (+9.8%, p<0.05), thyroid gland: follicular cell height↑, lower colloid area			
Female pubertal study Anonymous (2017) Oral, gavage	100 mg/kg bw/d	<u>300 mg/kg bw/d</u> liver weights ↑ (+47.8%, p<0.05), TSH ↑ (+80.5%, p<0.05), T4 ↓(-8.3%, not signif) ovarian weight ↓ (-16.9%, p<0.05), uterine weight (blotted and wet: -33.8% and -30.5%, p<0.05) follicular cell heights↑ (p<0.05), lower thyroid colloid area (p<0.05) <u>100 mg/kg bw/d</u> liver weights ↑ (+17.9%, p<0.05), TSH ↑ (+15%, not signif) follicular cell heights↑ (p<0.05), lower thyroid colloid area (p<0.05)	PND 22-42/43 (~20d)	33 mg/kg bw/d Adaptive response; other effects considered for developmental toxicity	-
Male pubertal study Anonymous (2016b) Oral, gavage	250 mg/kg bw/d	<u>500 mg/kg bw/d</u> Salivation, clear and/or red material around the mouth body weight gain ↓ (-27.5%, p<0.05) delayed mean age of attainment of balanopreputial separation (49.3 d compared to control at 46.2d), Body weight at BPS (-14.5%, p<0.05)↓ ALT↑ (+54.9%, p<0.05), GGT↑ (0.8 U/l compared to control 0.0 u/l), serum triglyceride ↓ (-75.8%, p<0.05), T4↓ (-32-6%, p<0.05), testosterone↓ (-82.5%, p<0.05), TSH ↑ (+79.2%, not signif) prostate weight ↓ (dors.-39.8, ventr.-48%, p<0.05), epididymides weight	PND 23-53/54 (~30d)	83 mg/kg bw/d Adaptive response; other effects considered for developmental toxicity	-

Study reference **Kind of exposure**	**Effective dose (mg/kg/d) – lowest dose inducing signif/severe effects**	**Effects reported**	**Length of exposure**	**Extrapolated effective dose when extrapolated to 90-day exposure**	**Classification supported by the study**
		↓(rt: -15.3%, lt -16.5%, p<0.05), pituitary gland ↓ (-26.0%, p<0.05), testis ↓ (-11.0%, p<0.05), thyroid gland ↓ (-22.%, p<0.05), Liver ↑ (+32.6%, p<0.05), LABC muscle ↓ (-40.9%, p<0.05) thyroid: higher follicular cell height (not stat. signif) and lower colloid area (signif) <u>250 mg/kg bw/d</u> Salivation, clear and/or red material around the mouth body weight gain ↓ (-9.6%, p<0.05) Body weight at BPS (-11.2%, p<0.05) ↓ serum triglyceride ↓ (-62.0%) adrenal glands↑ (+11.5%, p<0.05), prostate weight ↓ (dors. -17.3%, ventr. -18.3%, p<0,05), Liver ↑ (31.0%, p<0,05), LABC muscle ↓ (-14.3%, p<0.05) thyroid: higher follicular cell height (not stat. signif) and lower colloid area (signif)			
Rat, inhalation					
Non guideline subacute dust inhalation study Anonymous (1979) Inhalation study – whole body	-	NOAEL not determinable due to limited reporting <u>498 mg/kg bw/d:</u> Abs. liver weight ↑ (m, p<0.05) Abs. + rel liver weight ↑ (f, p<0.01) Abs. + rel lung weight ↓ (m, p<0.01/p<0.05) <u>247 mg/kg bw/d:</u> Abs. + rel. kidney weight ↑ (f, p<0.05) Abs. + rel liver weight ↑ (m p<0.01/p<0.05, f, p<0.01) Abs. + rel lung weight ↓ (f, p<0.05) Abs. + rel. spleen weight ↑ (m, p<0.05)	28 days	-	- Klimisch 3

Study reference **Kind of exposure**	**Effective dose (mg/kg/d) – lowest dose inducing signif/severe effects**	**Effects reported**	**Length of exposure**	**Extrapolated effective dose when extrapolated to 90-day exposure**	**Classification supported by the study**
		51 mg/kg bw/D: Rel. kidney weight ↑(f, p<0.05) Abs. liver weight ↑ (m p<0.01, f, p<0.05)			

10.12.2 Comparison with the CLP criteria

A substance is classified with STOT RE under CLP when it has produced or has been shown to have the potential to produce significant toxicity to humans or be harmful to human health following repeated exposure by the oral, dermal or inhalation routes. This can be on the basis of human data or evidence from studies in animals that cause such effects at or below given Guidance Values. All significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed are included under this classification.

Category 1	Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of: • reliable and good quality evidence from human cases or epidemiological studies; or • observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations. Guidance dose/concentration values are provided below (see 3.9.2.9), to be used as part of a weight-of- evidence evaluation.
Category 2	Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following repeated exposure. Substances are classified in category 2 for target organ toxicity (repeat exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations. Guidance dose/concentration values are provided in the CLP regulation in order to help in classification. In exceptional cases human evidence can also be used to place a substance in Category 2

To assist in classification for STOT RE the following guidance values (referring to a 90 day exposure period) are given in CLP:

Route of exposure	Unit	Guidance value (dose/concentration)	Resulting classification, category
Oral (rat)	mg/kg bw/d	C ≤ 10	Category 1
Oral (rat)	mg/kg bw/d	10 < C ≤ 100	Category 2

STOT RE is assigned on the basis of findings of ‘significant’ or ‘severe’ toxicity. In this context ‘significant’ means changes which clearly indicate functional disturbance or morphological changes which are

toxicologically relevant. All significant health effects that can impair function, reversible and irreversible, immediate and/or delayed are included. Changes in organ weights with no evidence of organ dysfunction or adaptive responses are not considered relevant for classification.

For 6PPD no information on repeated dose toxicity in humans is available.

The main target after repeated oral exposure in animals (rats and rabbits) is the liver (vacuolation, fatty changes, increased weight, necrosis, degeneration), sometimes accompanied with effects on hematology (anemia) and in some studies kidney effects were seen (pigment depositions, degenerative changes). In chronic oral (gavage) studies effects on liver weight are documented down to concentrations of 12.5 mg/kg bw/d, histopathological changes are even seen at 2.5 mg/kg bw/d in rats. In chronic oral studies with exposure via diet the NOAELs are between 50 and 300 ppm (2.6-23 mg/kg bw/d). The extrapolated effective doses based on a >10% change in liver weight, relevant histological findings in liver (necrosis, degeneration) or kidney and anemia are presented in Table 99.

Liver weight increase up to 30% is seen consistently in male and female rats and pregnant female rabbits in chronic studies. This effect also has been seen in pups at PND 4/13/21/50 in an oral EOGRTS study down to the lowest concentration tested (50 mg/kg bw/d). While the increases in liver weights and hepatocellular hypertrophy can be regarded as adaptive response, there were also indications of clear liver toxicity, i.e. vacuolar degeneration of the liver, focal necrosis, and changes in liver related blood-biochemical parameters (GGT, ALP).

Monocellular necrosis are seen in a 90-day study (Anonymous 2008a) in a dose dependent manner in females (0/15, 2/15, 4/13, 12/14), but not males. Focal necrosis are documented in a screening study (Anonymous, 2009) in a dose dependent manner in males (1/12, 0/12, 2/11, 7/12) but not in females. In this study the mean liver weight of males shows a tendency of weight reduction while females show a weight increase with increasing test concentration. In another screening study (Tanaka, 2001) vacuolar degeneration at 100 mg/kg bw/d and in a minimal extend at 20 mg/kg bw/d is described. In a 28-day study (Anonymous, 1999b) periportal fatty changes are reported down to a daily dose of 20 mg/kg bw/d. In this regard also the publication by Stasenkova (1970) has to be considered (although the reliability is limited), which reports clear signs of intoxication described as suppression of the synthesizing function of the liver and a decreased ascorbic acid content in the liver. This is, however, in contrast to the observed increased levels of mean albumin, total protein, globulin and cholesterol level, seen e.g. in the EOGRT study (Anonymous, 2019b).

Increased kidney weight is described in several chronic studies (Anonymous 2019ab, 2008a, 1993, 1981, 1979), decreased kidney weight was only seen in a chronic feeding study (Anonymous 1978a). In an EOGRTS and the corresponding range-finding study (Anonymous 2019a,b) pigment deposition down to a dose of 7 mg/kg bw/d is reported. Moderate degenerative changes and glomerular atrophy are documented in a 90 day-study (Anonymous 2008a) down to a dose of 2.5 mg/kg bw/d.

Mild anemia was seen in (sub-)chronic feeding studies (Anonymous, 1993 and 1987a).

In a 90-day study in males at a daily dose of 25 mg/kg bw/d small focal muscular dystrophy in left myocardium is described, however, this severe finding is not supported by other studies (microscopic evaluation of the heart has been done in several studies).

For exposure via inhalation only one study is available, but no NOAEL can be derived due to limited reporting.

Overall, it can be concluded that severe organ toxicity after oral administration of 6PPD is documented in some studies, however, the results are very inconsistent. In addition, severe effects on liver (necrosis, degeneration) are documented in subchronic but not in chronic studies. Effects on heart were a single finding in male rats. Effects around a possible kidney toxicity also give no clear picture.

10.12.3 Conclusion on classification and labelling for STOT RE

Beside adaptive responses of the liver some organ toxicity (liver, kidney, heart, blood) is reported in different (sub-)chronic studies, however, the results are inconsistent, and no clear picture can be drawn. No

classification for STOT RE is proposed.

10.13 Aspiration hazard

Not addressed in this dossier.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

11.1 Rapid degradability of organic substances

Abiotic loss of 6PPD is obtained via hydrolysis and/or oxidation and ozonation reactions. 6PPD undergoes rapid loss via hydrolysis (Anonymous, 2003; Anonymous, 1993; Anonymous 1979b; Hiki et al., 2021). Another important abiotic loss of 6PPD is the photo-oxidation of 6PPD, which occures fast (60% decrease of 6PPD within 72 hours, e.g. Klöckner et al., 2021) leading to 6PPD-quinone. An overview of the suggested abiotic (hydrolysis and oxidation) reactions is summarized in Figure 1. Information on the estimated biodegradability BIOWIN v4.11 (EPI SuiteTM) of all, 6PPD and other relevant substances including hydrolysis (e.g. 4-HDPA) and oxidation/ozonation (e.g. 6QDI, 6PPD-quinone) products, is presented in Annex IV. The experimental data on rapid degradability are included in Table 100 and further a comparison of all available information on the estimated and experimental ready biodegradability is shown in Table 101.

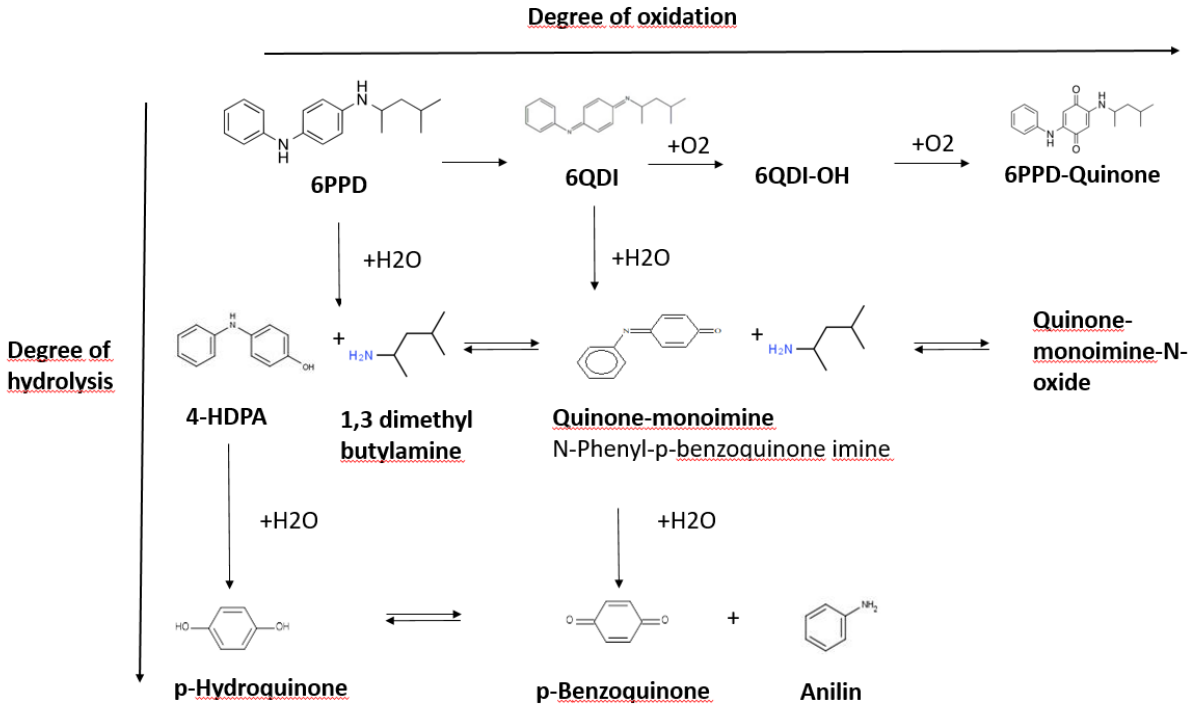


Figure 1: Suggested abiotic reactions of 6PPD (summarized from Klöckner et al., 2021; Anonymous, 2003, Hiki et al., 2021 and ECHA registration database).

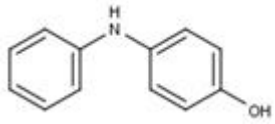
Table 100: Summary of relevant information on rapid degradability

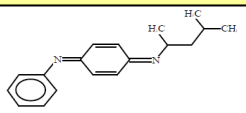
Method	Results	Remarks	Reference
Ready biodegradability			
OECD Guideline 301C Ready biodegradability: Modified MITI test (I) GLP Test item: **6PPD** Test item concentration:	2% of the initial dose was degraded within 28 days (based on BOD); ca. 92% 6PPD disappearance based on HPLC Not readily biodegradable Toxicity control: The substance had no inhibitory effect on activated sludge	Klimisch 1 (registrant) Klimisch 2 Sludge was collected from 10 sites incl. industrial locations. Adaptation cannot be	CERI (1994)

Method	Results	Remarks	Reference
100 mg/L, kept at cold temperature until use Test substance purity: 6PPD >99%, impurity: 4-Aminodiphenylamine, *N*-bis-(1,3-Dimethylbutyl)-*N*-phenyl-1,4-phenylendiamine Sludge concentration: 30 mg/L related to BOD Reference item: aniline A blank control (sterile mineral medium only), positive control (aniline as reference compound at 100 mg/L) and 6PPD in pure water (6PPD at 100 mg/L) were incubated simultaneously. % biodegradation was calculated based on BOD and HPLC analysis. Test temperature: 25°C; test performed in the dark; continuously stirred. Test duration: 28 days	microorganisms. Biodegradation rate of positive control: 64% after 7 days and 71% after 14 days. Following degradation products were determined: Phenyl benzoquinone imine, benzoquinone, 1,3-dimethylbutylamine, 4-anilinophenol (4-HDPA), aniline Although the 2-ring intermediates were degraded further, neither aniline nor p-benzoquinone were recovered in significant quantities.	ruled out.	
Method comparable to OECD 301B similar to Gledhill method listed in USEPA 40 CFR Ch1 subpart D paragraph 739.3100 Ready biodegradability Test item: **6PPD** Not GLP Acclimated inoculum used Tests substance: *N*-1,3-dimethylbutyl-*N*'-phenyl-*p*-phenylenediamine (Santoflex 13)	7.2% after 32 days (based on CO_2 evolution) Not readily biodegradable under test conditions based on CO_2 evolution.	Klimisch 2 (registrant) Klimisch 4 For this study the full study report was not available, therefore study is not assignable. Acclimated bacteria were used.	Anonymous (1979a)
OECD Guideline 301C Ready biodegradability: Modified MITI test (I) GLP: unknown	Readily biodegradable % degradation of test substance: ca. 75% (n-octylamine) and ca. 65% (n-butylamine)	Klimisch 2 (registrant) Klimisch 4 Supporting study for two read-across	Yoshimura et al. (1980)

Method	Results	Remarks	Reference
Test item: **n-octylamin** CAS 111-86-4 Test item concentration: unknown Test substance purity: unknown Sludge concentration: unknown Reference item: unknown Controls: unknown Test temperature: unknown Test duration: 12 days	after 12 days based on O_2 consumption	substances Un-adapted sludge used Read-across is not accepted by the DS.	
Ready biodegradability: Test item: **6QDI**	Biodegradation in water: 20% in 28 days according to guideline Not readily biodegradable	Klimisch 4 only information from the website available to DS	Registration Dossier - ECHA (europa.eu)
Ready biodegradability: OECD 301C (MITI I) Test item: **p-Hydroquinone** Test duration: 28 days	70% biodegradation after 14 days Under aerobic conditions: 1,4 benzoquinone, 2-hydroxy-1,4-benzoquinone, and b-ketoadipic acid identified as metabolites Positive control and blank controls were performed. ready biodegradable	Klimisch 2 (registrant) Klimisch 4 only information from the website available to DS	Registration Dossier - ECHA (europa.eu)
Ready biodegradability: OECD 301A (MITI I) Test item: **p-Benzoquinone** Test duration: 28 days	% degradation (DOC removal): 56%, 28 days not readily biodegradable	Klimisch 1 (registrant) Klimisch 4 Only information from the website available to the DS	Registration Dossier - ECHA (europa.eu)
Ready biodegradability: Test item: **Aniline**	80 – 100% in 28 days Ready biodegradable	Many tests available, Aniline is used as positive control in ready tests.	Registration Dossier - ECHA (europa.eu)
Ready biodegradability: **C1-13 primary alkylamines**	Read-across from supporting substance (chemical category: C1 – C13 primary alkylamines).	Klimisch 2 (registrant) Supporting study Klimisch 4	OECD (2011), SIDS Assessment profile: Chemical category: C1-13 Primary Amines (SIAM 32), on ECHA dissemination site
Simulation tests			
Test system: natural	pH 7.4: half-life 2.9 hours at 24 °C in entire system (active Mississippi river	Klimisch 2 (registrant)	Anonymous

Method	Results	Remarks	Reference
water Test item: **6PPD** Primary biological and chemical transformation similar to aqueous Die-Away Screening Method (in Mississippi river water)	water (glass wool filtered) pH 7.4: half-life 3.9 hours at 24 °C in water (sterile Mississippi river water (0.2µm filtered) pH 7.4: half-life 6.8 hours at 24 °C in water (purified water (Milli-Q)) Transformation products: not specified % Dissipation/Degradation of test substance: 97 after 22 h (Test mat. analysis) (active Mississippi river water (glass wool filtered) 96 after 22 h (Test mat. analysis) (sterile Mississippi river water (0.2µm filtered) 88 after 22 h (Test mat. analysis) (purified water (Milli-Q)	supporting study Klimisch 4 No OECD study GLP-study Test duration only 22 hours.	(1981)
OECD Guideline 307 4 soils under aerobic condition Test item: **14C-7PPD** GLP	Under non-flooded soil conditions with 7PPD, very high amount of non-extractable residues (NER) are formed. Based on the information it is not possible to distinguish between the different NER types. Therefore NER should be considered as non-degraded within the assessment. Further acidic reflux hampered the recovery of the parent compound, as the residues were altered. Other extraction precedures like ambient and Soxhlet did not release significant amount of radioactivity.	Klimisch 1 (registrant)	Anonymous (2015a)
OECD Guideline 307 One soil incubated under anaerobic conditions Test item: **14C-7PPD** GLP	Under aerobic conditions a fast decline of 7PPD at the beginning of the study was obtained, but from day 3 to day 56 the extractable amount of 7PPD remained more or less constant, degradation stopped completely, therefore 7PPD can be considered as persistent and very persistent under flooded soil conditions. The transformation product, 7QDI was formed only under flooded conditions during the beginning of the study, but not from day 3 onwards.	Klimisch 1 (registrant)	Anonymous (2015b)
Hydrolysis			
No guideline study No GLP study Hydrolysis Test item: **6PPD** Conditions: light, head space air saturated, algae nutrient medium used	pH 7: half-life 5 hours at 50 °C pH 7: half-life 14 hours at 26 °C (buffered solution) pH 7: half-life 8 hours at 26 °C (nutrient medium) major intermediate:	Klimisch 2 (registrant) Klimisch 3 No OECD study. No GLP study. No mass balance available.	Anonymous (2003)

Method	Results	Remarks	Reference
	4-HDPA (CAS 122-37-2, 4-aniliniophenol)  Detected with HPLC with UV detection in maximum formation: 54% in buffer, 99% in nutrient media at low temp., 75% in nutrient media at high temp.	No recoveries indicated. Algae medium used, test substance concentration used unknown. Hydrolysis not performed at pH 4 and 9, only at pH 7.	
EPA OTS 796.1860, 1990 **6PPD** Hydrolysis as a function of pH at 24 ± 1°C	pH 7: half-life 3.3 hours at 24 °C (Santoflex 13, 6PPD) Transformation products: not identified	Klimisch 2 (registrant) Klimisch 3 GLP study Tested only at one temperature, and only at pH 7.	Anonymous (1993)
Hydrolysis rate in simulated gastric juice Test item: **6PPD** Test duration: 48 hours	36.9 hours, rate constant: -0.019 Solubility of 6PPD in simulated gastric juice: 173 mg/L Hydrolysis products observed: - Major: Aniline - traces of: Benzoquinoneimine-N-phenyl, N-1,3 Dimethyl-Butylamine p-phenol	Klimisch 2 (registrant) Klimisch 4 Substance was monitored, not considered relevant for the environment by DS	Anonymous (1986b)
Transformation / degradation of **6PPD** in deionized water (stirred, aerated)	pH 7: half-life < 1 day at 24 °C total recovery of test substance in %: 100 at 24°C after 0 hour 85.5 at 24°C after 1 hour 84.6 at 24°C after 2 hours 63.6 at 24°C after 3.5 hours 40 at 24°C after 25 hours	Klimisch 2 (registrant) Klimisch 3 No Guideline study, No GLP study No analytics	Anonymous (1979b)
Stability study in aquatic toxicity study Test item: **6PPD** Tested in de-chlorinated tap water	6PPD pH 8: half-life 5 hours at 23 °C 6PPD pH 8: half-life 8 hours at 10 °C 6PPD-Quinone: half-life 33 hours at 23 °C 6PPD-Quinone was detected in low concentration in the 6PPD-solution after 4 hours. Transformation followed a first order kinetics.	Supporting study Klimisch 3 Not according to guideline. No GLP study. Hydrolysis not performed at pH 4, 7 and 9, only at pH 8.	Hiki et al. (2021)
OECD Guideline 111 Hydrolysis Test item: **6QDI** CAS 52870-46-9	under N_2 pH 4: half-life 19 hours at 25°C pH 7: half-life 4.7 hours at 25°C pH 9: half-life 4.3 hours at 25°C	Klimisch 1 (registrants) Supporting study using surrogate Klimisch 2	Anonymous (1999c)

Method	Results	Remarks	Reference
	Hydrolysis products: 4-HDPA p-benzoquinone p-Hydroquinone Aniline	GLP study Instead of two, only one temperature used Mass balance is not available to dossier submitter, recoveries are only available for the parent. The ionic form of 6QDI is formed under low pH and is assumed to be more stable than the parent.	
Phototransformation			
Phototransformation in air, **6PPD** estimated by calculation AOP Program v1.92 of EPI-Suite software	Half- life in air: 1.7 hours concentration of OH-radicals: 0.5E6 OH/cm3, 24 hours/day	Klimisch 2 (registrant)	Anonymous (2009a)
Phototransformation in air comparison of spectra, no guidance followed Test item: **6PPD**	Maximum absorbance of UV light is at 291 nm and at 350 nm.	Klimisch 4 Since 6PPD absorbs UV-B radiation it is expected that 6PPD will undergo direct photolysis due to absorbance of environmental UV light.	Müller et al. (1988), John et al. (1983) cited in OECD SIDS report (2004).
Photodegradation of a tire composite sample (Tmix) Simulation natural sunlight (0.68 W/m2 at 340 nm; 32 °C) Samples were taken after 24, 49, and 72 hours 6PPD	Increase during exposure: Compounds 3, 6, (C15H10N3O2), 7 and 10 increased during exposure, no further identification Decrease during sunlight: 6PPD, reduction in peak intensity of 60-80% Compound 15: 6QDI-OH Compound 17: 6PPD-Quinone	Klimisch 4 72 hours exposure equals to 2-7 weeks sunshine in Germany	Klöckner et al. (2021)

11.1.1 Ready biodegradability

Estimated data

The DS presents QSAR calculations performed with BIOWIN v4.10 QSAR contained within EPI Suite™ version 4.10 (US-EPA, 2011) for 6PPD (see Annex IV) Biowin consists of seven models. The substance is predicted to not biodegrade fast using linear (Biowin 1) and non-linear (Biowin 2) biodegradation models, as the values are below 0.5. The substance lies in the applicability domain of the models and is considered valid, as the substance is in the molecular range of the training set and the fragments of the training set cover

many fragments of the substances. Ultimate biodegradation, the conversion from 6PPD to CO_2 (Biowin 3), is predicted not to occur fast. Initial steps, primary biodegradation are predicted to occur fast (Biowin 4). Biowin 5 and 6 represent MITI testing: 6PPD was not considered readily biodegradable in these models. Under anaerobic conditions (Biowin 7), the test substance is predicted not to quickly biodegrade. The overall prediction of the ready biodegradability of 6PPD and the primary hydrolysis products 4-HDPA, 1,3 dimethylbutylamin and *N*-Phenyl *p*-benzoquinone imine and the oxidation /ozonation products 6PPD-Quinone, 6QDI and 6QDI-OH (Tian et al., 2021, Klöckner et al., 2021) is "No".

Experimental data

Ready biodegradability tests

6PPD was investigated for its ready biodegradability in a GLP study according to OECD Guideline 301C (CERI, 1994). The study was performed with aerobic activated sludge (30 mg suspended solids/L) from 10 different sites (sewage treatment works, industrial treatment works, rivers, lakes and sea throughout Japan) to assess biodegradation in a culture medium with 100 mg test substance/L, which is above the water solubility of the test item (~1 mg/L (50°C)). Incubation was performed in 5-litre flasks at a temperature of 25°C in a dark room over 28 days.

Results: 2% biodegradation based on biological oxygen demand (BOD) was observed for the substance after 28 days. 6PPD was also analyzed by HPLC, after 28 days, 92% degradation or dissipation was observed. Aniline was readily biodegraded by 64% after 7 days and 71% after 14 days. Following degradation products were identified: Phenylbenzoquinone imine, *p*-benzoquinone (EC 203-405-2), 1,3-dimethylbutyl amine (EC 203-549-6-1), 4-anilinophenol (EC 204-538-9), and aniline (EC 200-539-3). As the threshold for ready biodegradability is not met within 28 days, it can be concluded that the test substance is not readily biodegradable. The study is rated as Klimisch 2.

Another ready biodegradability study (Anonymous, 1979a) was performed according to an in-house method (Monsanto Shake flask) method using Santoflex 13 as test item. The method is similar to OECD 301B, but used adapted sludge.

Results: Only 7.2 % CO_2 was obtained after 32 days. As the documentation is insufficient for assessment, the study is rated as Klimisch 4.

Ready biodegradability studies are also available on the read–across substances n-octylamin and n-butylamine (Yoshimura et al.1980, OECD 2011). This information was considered as supportive information by the registrant(s), but not considered by the DS as the read-across was not substantiated by the registrant(s) besides the structural similarity. The ready biodegradability prediction for 1,3 dimethylbutylamin is "NO".

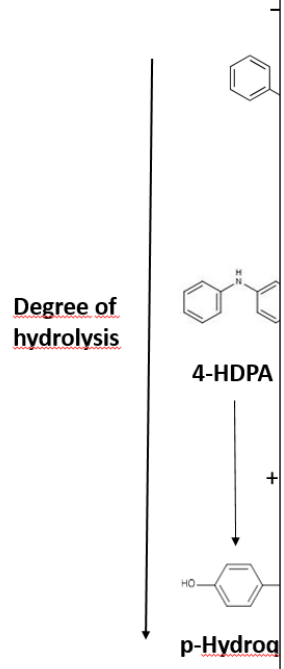
Summary on estimated and experimental data

Table 101: Summary of ready biodegradability of 6PPD and its degradation/transformation products

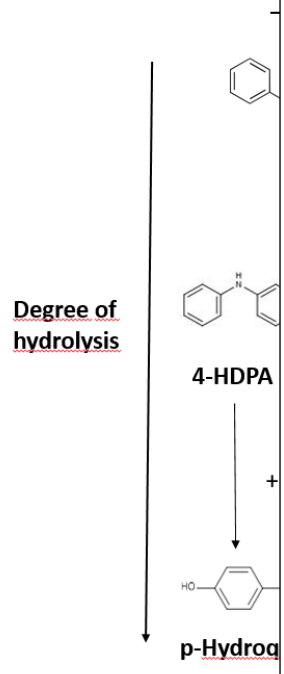
Substance	CAS or Smiles	Estimated Readily biodegradable?	Experimentally Readily biodegradable?
6PPD	793-24-8	No	No
6PPD-Quinone	C1(NC(C)CC(C)C)C(=O)C=C(Nc2ccccc2)C(=O)C=1	No	No data
6QDI	52870-46-9	No	No[11], ref. Abiotic loss of 6PPD is obtained via hydrolysis and/or oxidation

[11] Registration Dossier - ECHA (europa.eu) "Biodegradation in water: 20% in 28 days according to guideline."

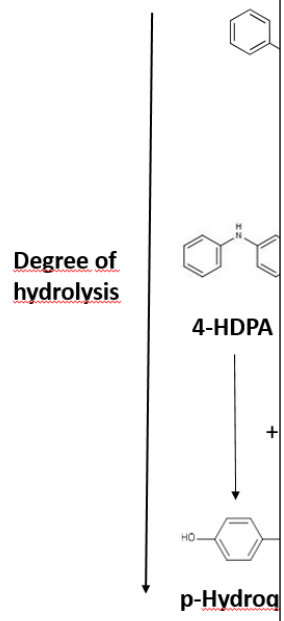
Substance	CAS or Smiles	Estimated Readily biodegradable?	Experimentally Readily biodegradable?
			and ozonation reactions. 6PPD undergoes rapid loss via hydrolysis (Anonymous, 2003; Anonymous, 1993; Anonymous 1979b; Hiki et al., 2021). Another important abiotic loss of 6PPD is the photo-oxidation of 6PPD, which occures fast (60% decrease of 6PPD within 72 hours, e.g. Klöckner et al., 2021) leading to 6PPD-quinone. An overview of the suggested abiotic (hydrolysis and oxidation) reactions is summarized in Figure 1. Information on the estimated biodegradability BIOWIN v4.11 (EPI SuiteTM) of all, 6PPD and other relevant substances including hydrolysis (e.g. 4-HDPA) and oxidation/ozonatio n (e.g. 6QDI, 6PPD-quinone) products, is presented in Annex IV. The experimental data on rapid degradability are included in Table 100 and further a

Substance	CAS or Smiles	Estimated Readily biodegradable?	Experimentally Readily biodegradable?
			comparison of all available information on the estimated and experimental ready biodegradability is shown in Table 101.  **Figure 1: Suggested abiotic reactions of 6PPD (summarized from Klöckner et al., 2021; Anonymous, 2003, Hiki et al., 2021 and ECHA registration database).** Table 100
6QDI-OH	C1(O)C(=Nc2ccccc2)C=CC(=NC(C)CC(C)C)C=1	No	No data
N-phenyl *p*-benzoquinone imine	C1(=O)C=CC(=Nc2ccccc2)C=C1	No	No data

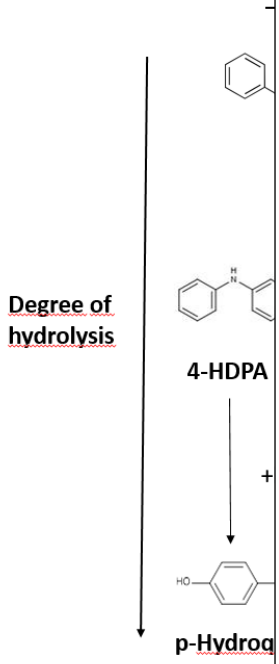
Substance	CAS or Smiles	Estimated Readily biodegradable?	Experimentally Readily biodegradable?
1,3 dimethyl-butylamine	108-09-8	No	No data
4-HDPA	122-37-2	No	No data
p-Hydroquinone	123-31-9	Yes	Yes, ref. Abiotic loss of 6PPD is obtained via hydrolysis and/or oxidation and ozonation reactions. 6PPD undergoes rapid loss via hydrolysis (Anonymous, 2003; Anonymous, 1993; Anonymous 1979b; Hiki et al., 2021). Another important abiotic loss of 6PPD is the photo-oxidation of 6PPD, which occures fast (60% decrease of 6PPD within 72 hours, e.g. Klöckner et al., 2021) leading to 6PPD-quinone. An overview of the suggested abiotic (hydrolysis and oxidation) reactions is summarized in Figure 1. Information on the estimated biodegradability BIOWIN v4.11 (EPI SuiteTM) of all, 6PPD and other relevant substances including hydrolysis (e.g. 4-HDPA) and oxidation/ozonation (e.g. 6QDI,

Substance	CAS or Smiles	Estimated Readily biodegradable?	Experimentally Readily biodegradable?
			6PPD-quinone) products, is presented in Annex IV. The experimental data on rapid degradability are included in Table 100 and further a comparison of all available information on the estimated and experimental ready biodegradability is shown in Table 101.  **Figure 1: Suggested abiotic reactions of 6PPD (summarized from Klöckner et al., 2021; Anonymous, 2003, Hiki et al., 2021 and ECHA**

Substance	CAS or Smiles	Estimated Readily biodegradable?	Experimentally Readily biodegradable?
			registration database). Table 100
p-Benzoquinone	106-51-4	Yes	No, ref. Abiotic loss of 6PPD is obtained via hydrolysis and/or oxidation and ozonation reactions. 6PPD undergoes rapid loss via hydrolysis (Anonymous, 2003; Anonymous, 1993; Anonymous 1979b; Hiki et al., 2021). Another important abiotic loss of 6PPD is the photo-oxidation of 6PPD, which occures fast (60% decrease of 6PPD within 72 hours, e.g. Klöckner et al., 2021) leading to 6PPD-quinone. An overview of the suggested abiotic (hydrolysis and oxidation) reactions is summarized in Figure 1. Information on the estimated biodegradability BIOWIN v4.11 (EPI SuiteTM) of all, 6PPD and other relevant substances including hydrolysis (e.g. 4-HDPA) and oxidation/ozonatio

Substance	CAS or Smiles	Estimated Readily biodegradable?	Experimentally Readily biodegradable?
			n (e.g. 6QDI, 6PPD-quinone) products, is presented in Annex IV. The experimental data on rapid degradability are included in Table 100 and further a comparison of all available information on the estimated and experimental ready biodegradability is shown in Table 101.  **Figure 1: Suggested abiotic reactions of 6PPD (summarized from Klöckner et al., 2021; Anonymous, 2003, Hiki et al., 2021**

Substance	CAS or Smiles	Estimated Readily biodegradable?	Experimentally Readily biodegradable?
			and ECHA registration database). Table 100
Aniline	62-53-3	Yes	Yes, ref. Abiotic loss of 6PPD is obtained via hydrolysis and/or oxidation and ozonation reactions. 6PPD undergoes rapid loss via hydrolysis (Anonymous, 2003; Anonymous, 1993; Anonymous 1979b; Hiki et al., 2021). Another important abiotic loss of 6PPD is the photo-oxidation of 6PPD, which occures fast (60% decrease of 6PPD within 72 hours, e.g. Klöckner et al., 2021) leading to 6PPD-quinone. An overview of the suggested abiotic (hydrolysis and oxidation) reactions is summarized in Figure 1. Information on the estimated biodegradability BIOWIN v4.11 (EPI SuiteTM) of all, 6PPD and other relevant substances including hydrolysis (e.g. 4-HDPA) and

Substance	CAS or Smiles	Estimated Readily biodegradable?	Experimentally Readily biodegradable?
			oxidation/ozonation (e.g. 6QDI, 6PPD-quinone) products, is presented in Annex IV. The experimental data on rapid degradability are included in Table 100 and further a comparison of all available information on the estimated and experimental ready biodegradability is shown in Table 101.  **Figure 1: Suggested abiotic reactions of 6PPD (summarized from Klöckner et al., 2021; Anonymous, 2003,**

Substance	CAS or Smiles	Estimated Readily biodegradable?	Experimentally Readily biodegradable?
			Hiki et al., 2021 and ECHA registration database). Table 100

Conclusions on ready biodegradability

6PPD is considered as not readily biodegradable (CERI, 1994), the substance showed 2% degradation based on biological oxygen demand (BOD). The ready biodegradability study revealed the presence of primary (4-HDPA, 1,3-dimethylbutylamine, phenylbenzoquinone imine) and secondary hydrolysis products (aniline, benzoquinone). The study is rated as Klimisch 2 (ref. to Abiotic loss of 6PPD is obtained via hydrolysis and/or oxidation and ozonation reactions. 6PPD undergoes rapid loss via hydrolysis (Anonymous, 2003; Anonymous, 1993; Anonymous 1979b; Hiki et al., 2021). Another important abiotic loss of 6PPD is the photo-oxidation of 6PPD, which occures fast (60% decrease of 6PPD within 72 hours, e.g. Klöckner et al., 2021) leading to 6PPD-quinone. An overview of the suggested abiotic (hydrolysis and oxidation) reactions is summarized in Figure 1. Information on the estimated biodegradability BIOWIN v4.11 (EPI SuiteTM) of all, 6PPD and other relevant substances including hydrolysis (e.g. 4-HDPA) and oxidation/ozonation (e.g. 6QDI, 6PPD-quinone) products, is presented in Annex IV. The experimental data on rapid degradability are included in Table 100 and further a comparison of all available information on the estimated and experimental ready biodegradability is shown in Table 101.

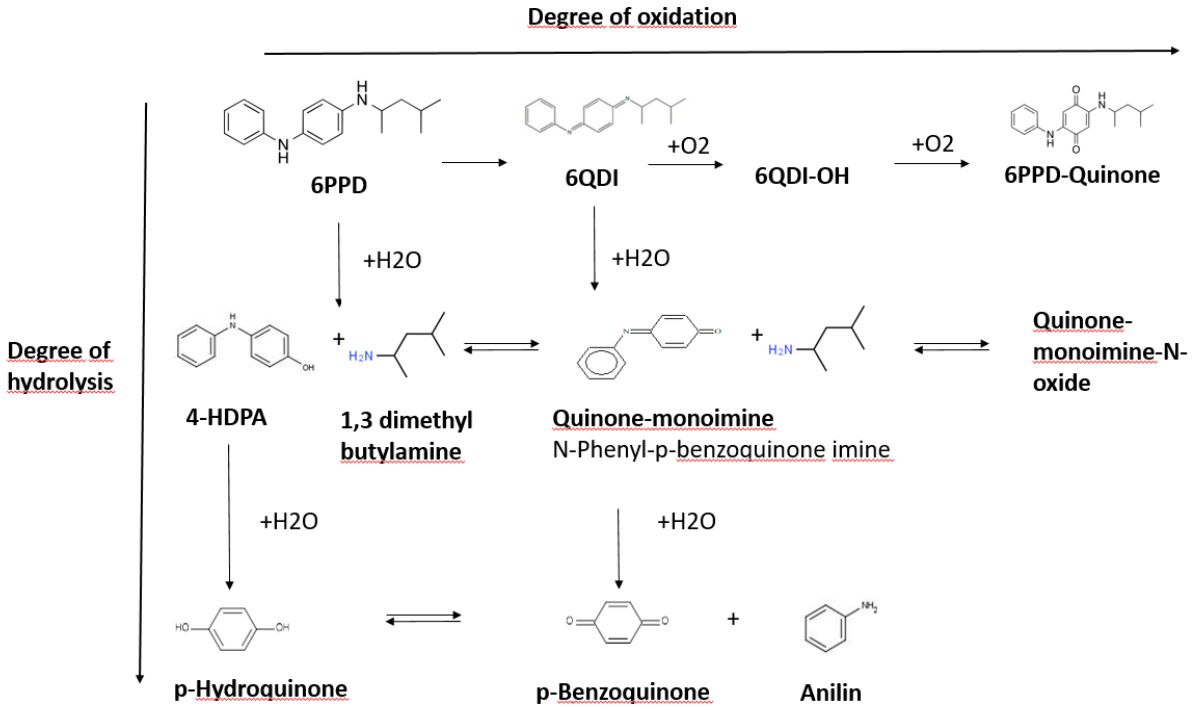


Figure 1: Suggested abiotic reactions of 6PPD (summarized from Klöckner et al., 2021; Anonymous, 2003, Hiki et al., 2021 and ECHA registration database).

Table 100).

Studies on n-octylamine (CAS 111-86-4) and n-butylamine (CAS 109-73-9) were included by the registrant(s) as a read-across for the assumed hydrolysis product 1,3-dimethylbutylamine to show that this compound is presumably ready biodegradable. Data on these substances were not taken into account in this dossier as read-across was not further justified by the registrant(s) besides the structural similarity. The primary hydrolysis products: 4-HDPA, 1,3-dimethylbutylamine and *N*-phenyl *p*-benzoquinone imine are estimated to be not readily biodegradable, experimental data are not available. It seems reasonable to assume, that the parent and and/or the hydrolysis products become to some extent unavailable for further hydrolysis or that the primary hydrolysis products are more stable in the first weeks.

During the ready biodegradability testing also oxidation might have occurred as seen in stability test (Hiki et al., 2020), leading to e.g. 6PPD-Quinone, 6QDI, 6QDI-OH, which are all estimated as not readily biodegradable. Only for 6QDI experimental data are available, which confirm the estimate of not ready biodegradability. In solution, 6PPD-Quinone was shown to be more hydrolytically stable than 6PPD (Hiki et al., 2020).

Another possible explanation for the not readily biodegradability was given by the registrant(s) as the toxicity towards bacteria of the assumed secondary hydrolysis product p-hydroquinone (EC_{50} = 71 mg/L) is high.

11.1.2 BOD_5/COD

Information of oxygen demand is not available.

11.1.3 Hydrolysis

Several studies are available showing that 6PPD undergoes rapid loss via hydrolysis (Anonymous, 2003; Anonymous, 1993; Anonymous 1979b; Hiki et al., 2021). Most of these studies investigating the hydrolysis of 6PPD are rated as Klimisch 3 or 4 (ref. to Table 100). A Klimisch 2 study performed according to OECD 111 is available on 6QDI showing a half-life at 25°C at pH 7 of 4.7 hours (Anonymous, 1999c). In Table 102 the occurrence of primary and secondary hydrolysis products is listed.

Table 102: Occurrence of primary and secondary hydrolysis products in available studies

Substance	CAS or Smiles	Study	Conditions	Remark by DS
N-phenyl p-benzoquinone imine	C1(=O)C=CC(=Nc2ccccc2)C=C1	i) 6PPD OECD 301C (CERI, 1994) ii) 6PPD hydrolysis in simulated gastric juice, 48 hours (Anonymous, 1986b)	i) Ready biodegradability test ii) Considered not relevant for environment	Not found in any hydrolysis studies acc. to OECD 111 with 6PPD or 6QDI (ref. Table 100).
1,3 dimethyl-butylamine	108-09-8	i) 6PPD OECD 301C (CERI, 1994)	i) Ready biodegradability test	Not found in any hydrolysis studies with 6PPD or 6QDI (ref. Table 100).
4-HDPA	122-37-2	i) 6PPD OECD 301C (CERI, 1994) ii) 6PPD hydrolysis study (Anonymous, 2003) iii) 6QDI hydrolysis study (Anonymous, 1999c)	i) Ready biodegradability test ii) Hydrolysis test, algae medium, light, air saturated iii) Hydrolysis test acc. OECD 111 iiii) stability test for aquatic toxicity	Found in hydrolysis studies with 6PPD and 6QDI and in ready biodegradability tests with 6PPD. Additionally found in stability test for aquatic toxicity.

Substance	CAS or Smiles	Study	Conditions	Remark by DS
		iiii) stability test for aquatic toxicity (Hiki et al., 2021)		
p-Hydroquinone	123-31-9	i) 6QDI hydrolysis study (Anonymous, 1999c)	i) Hydrolysis study	Only found in one hydrolysis study of the surrogate substance 6QDI.
p-Benzoquinone	106-51-4	i) 6QDI hydrolysis study (Anonymous, 1999c)	i) Hydrolysis study	Only found in one hydrolysis study of the surrogate substance 6QDI.
Aniline	62-53-3	i) 6PPD: OECD 301C (CERI, 1994) ii) 6PPD hydrolysis in simulated gastric juice, 48 hours, major metabolite (Anonymous, 1986b) iii) 6QDI hydrolysis study (Anonymous, 1999c)	i) Ready biodegradability test ii) Considered not relevant for environment iii) Hydrolysis test acc. OECD 111	Found in ready biodegradability tests with 6PPD and hydrolysis study with 6QDI.

The hydrolysis of 6PPD was investigated in buffered solution and algae media under aerated and light conditions (Anonymous, 2003). The obtained half-life depends on the temperature and is longer at lower temperature and in buffered solution. At 26 °C in buffer, the half-life of 6PPD is 14 hours (pH 7) and in algae medium containing traces of heavy metals like Mn, Co, Cu and Mo the half-life is 8 hours (pH 7). One major intermediate, 4-HDPA, was identified. Other transformation products were not observed and further information is not available to the dossier submitter. The study is rated as Klimisch 3, as several deviations to OECD 111 were observed. In another Klimisch 3 study, 6PPD revealed at neutral pH a half-life of 3.3 hours for 6PPD (Anonymous, 1993).

In the above-mentioned hydrolysis studies on 6PPD, half-lives at 24°C and 26°C were obtained. The temperature can be corrected by the following equation, but it should be noted that the extrapolation of hydrolysis rated from higher to lower temperature may contain remarkable uncertainties (ECHA guidance R.7b 2017, p 206): $t_{1/2}$ (X°C) = $t_{½}$ $^{e\,(0.08\,(T-X))}$.

6PPD	half-life	extrapolated pH, 12°C
Hydrolysis $t_{1/2}$ pH 7, 24°C	3.3 hours	8.62 hours
Hydrolysis $t_{1/2}$ pH 7, 26°C, algae medium	14 hours	42.91 hours

In an ecotoxicity study the stability of 6PPD was tested (Hiki et al., 2021). At pH 8 and at 10°C, the half-life of 6PPD was 8 hours compared to 5 hours at 23 °C. 6PPD-quinone was detected in the 6PPD solution used for the stability tests after 4 hours. 6PPD-Quinone is more stable (half-life 33 hours, 23°C) than the parent (half-life 5 hours, 23°C).

The hydrolysis of 6PPD was investigated in simulated gastric fluid and a half-life of 36.9 hours was obtained (Anonymous, 1986b). The study is considered not relevant for the environment by the DS. In a study conducted by Kretzschmart et al. (1992) 6PPD was stable at pH 2 for at least 4 weeks in aqueous solution in the cold; the study was not taken into account by the DS, as the pH of 2 is not considered relevant for the environment.

For 6QDI (*N*-(1,3-dimethylbutyl)-*N*'-(phenyl)-1,4-benzoquinonediimine, CAS 52870-46-9) a full OECD 111 study is available (Anonymous, 1999c), which is rated by the registrant as Klimisch 1. The half-life at the pH of 4 is the longest at 25°C with 19 hours. Following hydrolysis products were detected: 4-HDPA (4-Hydroxydiphenylamin, CAS 122-37-2), *p*-benzoquinone (CAS 106-51-4), *p*-hydroquinone (CAS 106-51-4) and aniline (CAS 62-53-4). Quinone-monoimine was not detected. The study is rated as Klimisch 2 as it is not a GLP study. Recovery and mass balance are not available.

Postulated hydrolysis of 6PPD and 6QDI consists of a primary and a secondary hydrolysis step (see also Figure 1): First, 6PPD or 6QDI are hydrolysed to 4-HDPA and *N*-phenyl-*p*-benzoquinonimine (Quinone-monoimine) and the alkyl moiety 1,3 dimethylbutylamine (CAS 108-09-8), which was not detected in hydrolysis tests. 4-HDPA and *N*-phenyl-*p*-benzoquinonimine are further hydrolysed to secondary products: *p*-Bezoquinone and p-Hydroquinone and aniline. 6QDI was not found in any of the hydrolysis studies of 6PPD. The proposed abiotic transformation pathway is included in Figure 1. QSAR calculations were performed for all primary and secondary hydrolysis products with BIOWIN v4.10 QSAR contained within EPI Suite™ version 4.10 (US-EPA, 2011) (ref to Annex IV).

Conclusion hydrolysis

6PPD undergoes rapid loss via hydrolysis, the obtained hydrolysis half-lives for 6PPD are between 3.3 hours (pH 7, 24 °C) and 14 hours (pH 7, 26°C, algae medium) (Anonymous, 2003). Further supportive evidence for the fast hydrolysis comes from a Klimisch 2 study using 6QDI (Anonymous, 1999c), a surrogate for 6PPD revealing a half–life of 4.7 hours at 25°C, which is comparable to 6PPD (Anonymous, 2003). As hydrolysis is a relevant pathway for degradation in the aquatic environment, the hydrolysis products are considered relevant for the classification of 6PPD with the following environmentally relevant hydrolysis rates: Re-calculation of the 6PPD hydrolysis at half-life from 24-26°C to 12°C at pH 7 results in values between 8.6 hours to 42 hours. Due to the rapid loss of 6PPD, ecotoxicity data on hydrolysis products can be used for classification, where information is required.

The primary hydrolysis product 4-HDPA is self-classified as Aquatic Acute 1 and Aquatic Chronic 1 and the secondary hydrolysis products aniline, p-benzoquinone and p-hydroquinone have a harmonized classification as Aquatic Acute 1, H400 (see Annex III).

6PPD undergoes fast hydrolysis, but as the hydrolysis products formed fulfil the criteria for hazardous for the aquatic environment, the substance is considered as not rapidly degradable.

In addition, in the environment and in old tyres oxidized transformation products from 6PPD were detected (Tian et al., 2021, Klöckner et al., 2021). An oxidation transformation product of 6PPD, 6PPD-Quinone was found to be present in a 6PPD-solution, which was used to test the stability of 6PPD. 6PPD-Quinone was more stable (half-life 33 hours, 23°C) than 6PPD (half-life 5 hours, 23°C) in aqueous solution.

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

6PPD is used in tyres to protect rubber from degradation by ozone and oxygen. It is clear that oxygen and ozone react with 6PPD faster than with rubber polymers. Abiotic degradation of 6PPD is one degradation pathway in the environment. Besides hydrolysis, photodegradation of 6PPD occurs when exposed to oxygen or ozone either on the tyre surface / road or in water (Klöckner et al., 2020, Tian et al., 2021, Hiki et al., 2020) and is considered as a relevant degradation pathway of 6PPD.

6PPD-quinone

Tian et al (2021) demonstrated that 6PPD can react with ozone to generate 6PPD-quinone, the responsible substance for the urban run-off mortality syndrome in the area of Washington State. The substance kills Coho salmon as they migrate up the river to spawn (Tian et al., 2021). The content of oxidation/ozonation products including 6PPD-quinone was shown to increase continuously with increasing age of tyre road wear particles (Klöckner et al., 2021).

The formation of 6PPD-quinone is not entirely clear, but it is suggested via intermediate phenols or via formation of semiquinone radicals (Tian et al, 2021). Klöckner et al., 2021 proposed a reaction pathway via formation of 6QDI (see Figure 1).

6PPD-quinone is especially relevant for many waters, which receive waters near to busy roadways. This has been already demonstrated for waterways in California, indicating that 6PPD-quinone is sufficiently persistent in aquatic system to potentially expose aquatic organisms, estimated concentrations upon retrospective analysis of 6PPD-quinone revealed mean 6PPD-quinone concentration between 1.0 – 6.1 µg/L (Tian et al., 2021). Data suggest that storm water treatment infrastructure does not remove 6PPD-quinone efficiently. 6PPD-quinone is estimated to be not readily biodegradable (ref. to Table 100) and is more stable (half-life 33 hours, 23°C) than 6PPD (half-life 5 hours, 23°C) in aqueous solution. Further it was detected in the 6PPD solution used for the stability tests after 4 hours (Hiki et al., 2021).

The high quantity of emitted tyre wear particles and steady flux of 6PPD to roads and the identification of 6PPD-quinone as the causative agent for the mortality syndrome demonstrate that 6PPD-quinone is stable enough in the aquatic environment to cause mortality towards fish (Tian et al., 2021, and 2022).

Aniline

Aniline is readily biodegradable, as the substance is also used as positive control in OECD test systems. According to information on ECHA dissemination site[12] "in sediment and soil, two competing processes are at hand, biodegradation and the formation of non-hydrolysable covalent bonds to humic substances. This binding leads to long half-lives for bound aniline of 350 and 3500 days for soil and sediment, respectively. In EU-Risk Assessment report (2004) it was assumed that approximately 80% of Aniline is covalently bound in soil."

Other transformation products

Tian et al. (2021) reported besides 6PPD-quinone also 6PPD ozonation products C18H22N2O (formula-matched).

11.1.3.2 Inherent and enhanced ready biodegradability tests

Inherent and enhanced ready biodegradability tests are not available.

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

Biodegradation in water

No simulation study is available for 6PPD.

In a non-OECD GLP study (Anonymous, 1981) the primary degradation of 6PPD was studied using Mississippi river water under aerobic conditions. No transformation products were identified. After 22 hours, which was the end of the experiment, 97% of 6PPD disappeared from the river water with a half-life between 2.9 – 6.8 hours. The study is rated as Klimisch 4 (Registrant: Klimisch 2), as the study results cannot be compared to a full OECD study. It might be possible that the substance was either transformed or became unavailable for degradation (e.g. adsorption), both cannot be evaluated based on the presented data. The pKa of 6PPD is in the range of environmentally relevant pH (6.7 at 20 °C) and therefore ionisable and adsorptive.

Biodegradation in water-sediment

No simulation study is available for 6PPD.

Biodegradation in soil

No simulation study is available for 6PPD, but an OECD 307 soil simulation test is available with 7PPD (Anonymous 2015a, 2015b). As 7PPD has a higher molecular weight the substance was not considered by the DS.

[12] Registration Dossier - ECHA (europa.eu)

11.1.3.4 Photochemical degradation

Table 103: Summary of relevant information on phototransformation

Method	Results	Remarks	Reference
Phototransformation in air 6PPD estimated by calculation AOP Programm v1.92 of EPI-Suite software	Half- life in air: 1.7 hours concentration of OH-radicals: 0.5E6 OH/cm3, 24 hours/day	Klimisch 2 (registrant)	Anonymous (2009a)
Phototransformation in air comparison of spectra, no guidance followed	Maximum absorbance of UV light is at 291 nm and at 350 nm.	Klimisch 4 Since 6PPD absorbs UV-B radiation it is expected that 6PPD will undergo direct photolysis due to absorbance of environmental UV light.	Müller et al (1988), John et al. (1983) cited in OECD SIDS report (2004).
Photodegradation of a tire composite sample (T_{mix}) Simulation of natural sunlight (0.68 W/m2 at 340 nm; 32 °C) Samples were taken after 24, 49, and 72 hours 6PPD	Increase during exposure: Compounds 3, 6, ($C_{15}H_{10}N_3O_2$), 7 and 10 increased during exposure, no further identification Decrease during sunlight: 6PPD, reduction in peak intensity of 60-80% Rel. stable after light exposure: Compound 15: 6QDI-OH Compound 17: 6PPD-Quinone	Klimisch 4 72 hours exposure equals to 2-7 weeks sunshine in Germany	Klöckner et al. (2021)

No relevant information on phototransformation in water and soil is available. Estimated data reveal a half-life in air of 1.7 hours.

In a recent publication of Klöckner et al. (2021) the peak area of 6PPD/mg tyre particle decreased (60-80%) with time after photooxidation up to 72 hours. No structure is available for increase compounds, but relative stable compounds have been identified, which resist sunlight. It is reasonable to assume that the identified oxidation/ozonation products can be used as tyre and road wear particle markers: both 6QDI-OH and 6PPD-quinone can be used for quantification in the environment.

Conclusion photodegradation

In the atmosphere, 6PPD undergoes indirect photodegradation via rapid reaction with hydroxyl radicals, resulting in a half-life in air between of 1-2 hours. Additionally, 6PPD adsorbs UV-B radiation and is expected to undergo direct photolysis. Potential tyre markers resistant to sunlight were found, e.g. ozonation/oxidation products like 6QDI-OH and 6PPD-quinone. Photooxidation of 6PPD occurred fast (60% decrease of 6PPD within 72 hours). The content of these compounds increase continuously with increasing age of tyre road wear particles. 6PPD-quinone was found in water and is highly toxic to Coho salmon.

11.2 Environmental transformation of metals or inorganic metals compounds

Not relevant for this dossier.

11.3 Environmental fate and other relevant information

Table 104: Summary of relevant information on environmental fate [EPISuite v4.11]

Method	CAS or Smiles	Estimated Log Koc	Experimentally Log koc	Reference
6PPD	793-24-8	Log Koc = 3.45	No data	Modelling, DS
6PPD-Quinone	C1(NC(C)CC(C)C)C(=O)C=C(Nc2ccccc2)C(=O)C=1	Log Koc = 3.94	No data	Modelling, DS
6QDI	52870-46-9 C1(=NC(C)CC(C)C)C=CC(=Nc2ccccc2)C=C1	Log Koc = 5.08	No data	Modelling, DS
6QDI-OH	C1(O)C(=Nc2ccccc2)C=CC(=NC(C)CC(C)C)C=1	Log Koc = 3.26	No data	Modelling, DS
N-phenyl p-benzoquinone imine	C1(=O)C=CC(=Nc2ccccc2)C=C1 2406-04-4	Log Koc = 2.40	No data	Modelling, DS
1,3 dimethyl-butylamine	108-09-8	Log Koc = 1.83	No data	Modelling, DS
4-HDPA	122-37-2	Log Koc = 2.61	No data	Modelling, DS
p-Hydroquinone	123-31-9	Log Koc = 1.58	No data	Modelling, DS
p-Benzoquinone	106-51-4	Log Koc = 1.94	No data	Modelling, DS
Aniline	62-53-3	Log Koc = 1.40	1.41 – 1.92	Modelling, DS

Log Koc values have been estimated with EPISuite v4.11 (KOCWIN, method Log Koc) and summarized in Table 104. Only for aniline experimental data are available on the ECHA dissemination platform, which are in good agreement with the estimates.

Conclusion on adsorption

4-HDPA, p-hydroquinone, p-benzoquinone, N-phenyl p-benzoquinone imine and aniline are considered as mobile based on the estimated Log Koc < 3. 6QDI with a log Koc value of 5.08 is considered as immobile in soil and sediment. 6PPD and all other transformation products exhibit log Koc values between 3.16 – 3.94.

11.4 Bioaccumulation

Table 105: Summary of relevant information on bioaccumulation

Method	Results	Remarks	Reference
QSAR model KOWWIN v1.68 (EPI SuiteTM) 6PPD	Log K_{OW} = 4.68	Indication for bioaccumulation	Modelling, DS

Method	Results	Remarks	Reference
CAS 793-24-8			
QSAR model BCFBAF v3.01 (EPI SuiteTM) 6PPD CAS 793-24-8	Estimated BCF = 569 (regression based method)		Modelling, DS
OECD 305 C *Cyprius carpio* Test item: N-phenyl p-benzoquinone imine Uptake: 42 days	BCF: 12 - 23	Klimisch 1 (registrants) Klimisch 2 No depuration phase available	CERI (1995a) MITI (1995)
OECD 305 C *Cyprius carpio* Test item: 1,3-dimethylbutylamine Uptake: 42 days	BCF: 1.7 - 17	Klimisch 1 (registrants) Klimisch 2 No depuration phase available	CERI (1995b) MITI (1995)
OECD 305 C *Cyprius carpio* Test item: 4-HDPA Uptake: 6 weeks	BCF: 3.3 – 49 Lipid content: 4.2%	Klimisch 2 (registrants) Not available to DS Klimisch 4 No depuration phase available	NITE (2002)
OECD 305 C *Cyprius carpio* Test item: N- (octane-2-yl) - N- phenyl benzene-1,4-diamine 8PPD Uptake: 6 weeks	BCFss: 1500 – 1700 Lipid content: 3.07% (start) 2.98% (end)	Klimisch 1 (registrants) Not available to DS Klimisch 4 Re-calculated to 5% Lipid content by DS: 2833 2	General Chemicals Evaluation and Research Institute (2010)

11.4.1 Estimated bioaccumulation

According to the CLP Regulation (EC) No 1272/2008 a Log $K_{OW} \geq 4$ is used to indicate a risk for bioaccumulation. Based on the estimated Log K_{OW} of 4.68 (KOWWIN v1.68), a BCF value of 569 was calculated (BCFBAF v3.01) for 6PPD.

11.4.2 Measured partition coefficient and bioaccumulation test data

Measured partition coefficient and bioaccumulation data for 6PPD are not available. Registrant(s) used a BCF value of 8PPD of 1700 in a weight of evidence approach (General Chemicals Evaluation and Research Institute, 2010). Assuming a whole fish lipid content of 3%, normalizing to 5% lipid content results in a BCF

of 2833. Hydrolysis of 6PPD is fast and yields primary hydrolysis products, 4-HDPA and N-phenyl p-benzoquinone imine and 1,3-dimethylbutylamine (CERI, 1995a,b; MITI, 1995; NITE, 2002). BCF values for these hydrolysis products are < 500.

Conclusion of aquatic bioaccumulation

The estimated derived Log K_{OW} for 6PPD is 4.68, this is higher than the trigger value of 4 given in the CLP Regulation. The estimated BCF value is 569, which is again higher than the trigger of 500. No experimental Log K_{OW} and BCF studies for 6PPD are available. Registrant(s) used the fast hydrolysis and the low BCF values from the primary hydrolysis products (4-HDPA and *N*-phenyl *p*-benzoquinone imine and 1,3-dimethylbutylamine) together with the BCF value from 8PPD to argue that the bioaccumulation potential of 6PPD is low. The lipid-normalised BCF of 8PPD is 2800, despite the similarity of 8PPD to 6PPD hydrolysis seems actually not taking place in the fish test. But overall, taking the decreasing trend in estimated Log K_{OW} and BCF values from 8PPD to 6PPD into account, is seems reasonable to assume that the bioaccumulation potential of 6PPD is low.

11.5 Acute aquatic hazard

Several studies included on the ECHA dissemination site for 6PPD were conducted with tyre wear particles. However, these studies are not included in the CLH-dossier as no substance specific information could be retrieved from these studies.

Regarding short-term aquatic toxicity several studies are available – for the parent substance as well as oxidation and hydrolysis products. For the substance 1,3-dimethyl-butylamine read-across data on sec. butylamine and octylamine were included in the 6PPD-registration dossier. For algae a study with N,N'-di-sec-butyl-p-phenylenediamine (44PD) is included in the 6PPD registration dossier with a MITI-study (2008) resulting in a 72h-EC_{50} value of 0.939 mg/L. As no proper read-across justification could be found the data for this potential read-across substances were not included to the CLH-dossier.

Another short-term daphnia toxicity study included in the 6PPD registration dossier resulting in an LC_{50} of 0.99 mg/L was not included to this CLH-dossier as there were doubts regarding the test substance identity, which was only presumably 6PPD.

For the hydrolysis product aniline many data are available and the substance has a harmonised classification as Aquatic Acute 1. According to the ECHA dissemination site for aniline and the EU Risk Assessment Report on aniline (European Union, 2004) daphnids are the most sensitive organisms: A GLP and OECD 202 guideline conform semi-static study, performed by the Danish EPA lead to a 48h-EC_{50} of 0.16 mg/l for *Daphnia magna* based on measured concentrations (Danish Environmental Protection Agency 1996). As it is neither deemed proportional nor essential (as much data is available and the substance has a harmonised classification as Aquatic Acute 1), the aquatic acute studies on aniline are not included in this CLH-dossier. The acute aquatic toxicity of aniline is considered to be lower than for 6PPD.

For a better overview, as there are particularly many studies included in the CLH-dossier, Table 106 presents the information aggregated for the aquatic acute hazard in a substance by substance manner whereas Table 107 presents the data in a trophic level manner.

Table 106: Overview on available short-term studies on 6PPD and its oxidation and hydrolysis products in a substance by substance manner.

Short term toxicity				
Test material	**Results [mg/L]**	**Species**	**Klimisch Sore**	**Reference**
6PPD	**Fish**			
	96h-LC_{50}: 0.028 (based on measured concentrations, geometric mean)	*Oryzias latipes*	Kl. 1	MITI (2001)

	LC_0: 5 (not analytically measured) LC_{100}: 100 (not analytically measured)	*Danio rerio*	Kl. 4	Anonymous (1984a)
	96h-LC_{50}: > 0.041 in Danio rerio (based on geometric mean measured concentrations) 96h-LC_{50}: < 0.07 in *Oryzias latipes* (based on geometric mean measured concentrations)	*Danio rerio* *Oryzias latipes*	Kl. 2	Hiki et al. (2021)
	96h-LC_{50}: 0.443 (based on nominal concentrations)	*Danio rerio*	Kl. 2	Varshney et al. (2021)
	Invertebrates			
	48h-EC_{50}: 0.23 (based on mean measured concentrations)	*Daphnia magna*	Kl. 1	MITI (1999)
	48h-EC_{50}: 0.51 (nominal)	*Daphnia magna*	Kl. 2	Anonymous (1984b)
	48h-EC_{50}: 0.82 (nominal)	*Daphnia magna*	Kl. 2	Anonymous (1978b)
	48h-EC_{50}: 0.79 (nominal)	*Daphnia magna*	Kl. 4	Anonymous (1984c)
	48h-EC_{50}: < 0.342 in *Daphnia magna* (based on mean measured concentrations) 96h-LC_{50} < 0.463 in *Hyalella Azteca* (based on mean measured concentrations)	*Daphnia magna* *Hyalella azteca*	Kl. 2	Hiki et al. (2021)
	Algae and Cyanobacteria			
	96h-EC_{50}: 0.6 (nominal; based on decrease of chlorophyll a and cell numbers)	*Raphidocelis subcapitata,* formerly known as *Selenastrum capricornutum*	Kl. 3	Anonymous (1978c)
6PPD-quinone	**Fish**			
	24h-LC_{50}: 0.0007 (based on measured concentrations)	*Oncorhynchus kisutch*	Kl. 2	Tian et al. (2021)
	24h-LC_{50}: 0.000079 (based on measured concentrations)	*Oncorhynchus kisutch*	Kl. 2	Tian et al. (2022)
	24h-LC_{50}: 0.00059 in *Salvelinus fontinalis* (based on mean measured concentrations)	*Oncorhynchus mykiss* *Salvelinus fontinalis*	Kl. 2	Brinkmann et al. (2022)

	96h-LC_{50}: 0.001 in *Oncorhynchus mykiss* (based on mean measured concentrations) 96h-LC_{50}: > 0.0142 in *Salvelinus alpinus* (based on mean measured concentrations) 96h-LC_{50}: > 0.0127 in *Acipenser transmontanus* (based on mean measured concentrations)	*Salvelinus alpinus* *Acipenser transmontanus*		
	96h-LC_{50}: > 0.053 in *Danio rerio* (based on geometric mean measured concentrations) 96h-LC_{50}: > 0.030 in *Oryzias latipes* (based on geometric mean measured concentrations)	*Danio rerio* *Oryzias latipes*	Kl. 2	Hiki et al. (2021)
	96h-LC_{50}: 0.133 (based on nominal concentrations)	*Danio rerio*	Kl. 2	Varshney et al. (2021)
	Invertebrates			
	48h-EC_{50}: > 0.046 in *Daphnia magna* (based on mean measured concentrations) 96h-LC_{50}: > 0.043 in *Hyalella Azteca* (based on mean measured concentrations)	*Daphnia magna* *Hyalella azteca*	Kl. 2	Hiki et al. (2021)
6QDI	**Fish**			
	96h-LC_{50}: 0.638	*Oncorhynchus mykiss*	Kl. 4	Anonymous (a) , Registered as NONS – year not specified
	Invertebrates			
	48-hr EC_{50}: 1.41 (not specified if based on nominal or measured concentrations)	Not specified	Kl. 4	Anonymous (b) – year not specified
	Algae and Cyanobacteria			
	72-hr E_rC_{50}: 1.6 (nominal)	*Raphidocelis subcapitata,* formerly known as *Selenastrum capricornutum*	Kl. 2	Anonymous (1998)
4-	**Fish**			

Hydroxydiphenylamine (4-HDPA)	QSAR estimation: 96h-LC_{50}: 12.7 using class "Phenols"	Fish	Kl. 3	Anonymous (2012a)
	Invertebrates			
	QSAR estimation: 48-hr EC_{50}: 5.3 using class "Phenols"	Daphnid	Kl. 3	Anonymous (2012c)
	48h-LC_{50}: 0.69 (nominal)	*Daphnia magna*	Kl. 1	Anonymous (2010a)
	Algae and Cyanobacteria			
	QSAR estimation: 96h-EC_{50}: 23.4 using class "Phenols"	Green algae	Kl. 3	Anonymous (2012e)
	72-hr E_rC_{50}: 2.6 (based on geometric mean measured concentrations)	*Desmodesmus subspicatus*	Kl. 1	Anonymous (2010b)
N-Phenyl-p-Benzoquinone monoimine	**Fish**			
	QSAR estimation: 96h-LC_{50}: 1.78 for class "Schiff Base" 96h-LC_{50}: 108.1 for class "Vinyl/Allyl Ketones" 96h-LC_{50}: 76.7 for class "Neutral organics"	Fish	Kl. 3	Anonymous (2012b)
	Invertebrates			
	QSAR estimation: 48h-LC_{50}: 3.8 using class "Schiff Base" 48h-LC_{50}: 65.7 using class "Vinyl/Allyl Ketones" 48h-LC_{50}: 46.0 using class "Neutral organics"	Daphnid	Kl. 3	Anonymous (2012d)
	Algae and Cyanobacteria			
	QSAR estimation: 96h-EC_{50}: not calculated for class "Schiff Base" due to lack of data 96h-EC_{50}: 51.1 using class "Vinyl/Allyl Ketones" 96h-EC_{50}: 22.5 using	Green algae	Kl. 3	Anonymous (2012f)

	class "Neutral organics"			
p-Benzoquinone	**Fish**			
	96h-LC_{50}: 0.125 (nominal)	*Oncorhynchus mykiss*	Kl. 3	De Graeve et al. (1980)
	96h-LC_{50}: 0.045 (nominal)	*Pimephales promelas*	Kl. 3	De Graeve et al. (1980)
	Invertebrates			
	48-hr EC_{50}: 0.13 (nominal)	*Daphnia magna*	Kl. 2	Anonymous (2018a)
	Algae and Cyanobacteria			
	72-hr ErC_{50}: 1.5 (nominal)	*Desmodesmus subspicatus*	Kl. 3	Anonymous (2018b)
p-Hydroquinone	**Fish**			
	96h-LC_{50}: 0.638 (based on mean measured concentrations)	*Oncorhynchus mykiss*	Kl. 3	Hodson et al. (1984)
	96h-LC_{50}: 0.097 (nominal)	*Oncorhynchus mykiss*	Kl. 3	De Graeve et al. (1980)
	96h-LC_{50}: 0.044 (nominal)	*Pimephales promelas*	Kl. 3	De Graeve et al. (1980)
	96-hr LC_{50}: 0.17 (not specified if based on nominal or measured concentrations)	*Danio rerio*	Kl. 4	Wellens H. (1982)
	96-hr LC_{50}: > 0.4 (not specified if based on nominal or measured concentrations)	*Pimephales promelas*	Kl. 4	Terhaar et al. (1972)
	Invertebrates			
	48-hr EC_{50}: 0.061 (based on mean measured concentrations)	*Daphnia magna*	Kl. 1	Anonymous (2008a)
	48-hr EC_{50}: 0.13 (nominal)	*Daphnia magna*	Kl. 3	Crisinel et al. (1994)
	48-hr EC_{50}: 0.29 (nominal)	*Daphnia magna*	Kl. 3	Kühn et al. (1989)
	24-hr EC_{50}: 0.09 (nominal)	*Daphnia magna*	Kl. 3	Bringmann et al. (1977)
	48-hr LC_{50}: 0.05 (not specified if based on nominal or measured concentrations)	*Daphnia magna*	Kl. 2	OECD SIDS (2002)
	Algae and Cyanobacteria			
	72-hr E_rC_{50}: 0.053 (based on geometric mean measured concentrations)	*Raphidocelis subcapitata* formerly known as *Pseudokirchneriella subcapitata*	Kl. 1	Anonymous (2008b)

	72-hr E_rC_{50}: 0.335 (not specified if based on nominal or measured concentrations)	*Raphidocelis subcapitata* formerly known as *Pseudokirchneriella subcapitata*	Kl. 4	Devillers et al. (1990)

Table 107 : Summary of relevant information on acute aquatic toxicity in a trophic level manner.

Method	Species	Test material	Results	Remarks	Reference
Fish					
OECD TG 203	*Oryzias latipes*	6PPD	96-hr LC_{50}: 0.028 mg/L (based on measured concentrations, geometric mean)	Klimisch 1	MITI (2001)
OECD TG 203	*Danio rerio*	6PPD	LC_{100}: 100 mg/l, LC_0: 5 mg/L (not analytically measured)	Klimisch 4, scarce information on study description	Anonymous (1984)
OECD TG 236 OECD TG 203	*Danio rerio* *Oryzias latipes*	6PPD + 6PPD-quinone	96-hr LC_{50}: > 0.053 mg/L (mean measured based on geometric mean) in *Danio rerio* for 6PPD-quinone using OECD TG 236 96-hr LC_{50}: > 0.03 mg/L (mean measured based on geometric mean) in *Oryzias laptides* for 6PPD-quinone using OECD TG 203 96-hr LC_{50}: > 0.041 mg/L (mean measured based on geometric mean) in *Danio rerio* for 6PPD using OECD TG 236 96-hr LC_{50}: < 0.07 mg/L (mean measured based on geometric mean) in *Oryzias latipes* for 6PPD using OECD TG 203	Klimisch 2 (reliable with restrictions) Initial measured concentration varied between different experiments with no explanation	Hiki et al. (2021)
OECD TG 236	*Danio rerio*	6PPD + 6PPD-quinone	96-hr $LC_{50:}$ 0.443 mg/L for 6PPD (based on nominal concentrations) 96-hr $LC_{50:}$ 0.133 mg/L for 6PPD-quinone (based on nominal concentrations)	Klimisch 2 (reliable with restrictions) No analytical verification of test item concentration.	Varshney et al. (2021)
Testing	*Oncorhynchus*	6QDI	96-hr $LC_{50:}$ 0.638 mg/L	Klimisch 4,	Anonymous

procedure not reported	*mykiss*			Registered as NONS, no further details available. Data confidential	(a) – year not specified [cited from ECHA dissemination site]
Study similar to OECD TG 203	*Oncorhynchus kisutch*	6PPD-quinone	24-hr LC_{50}: 0.0007 mg/L (based on measured concentrations); calculation performed by DS	Klimisch 2 (reliable with restrictions) Study duration 24 hours	Tian et al. (2021)
Study similar to OECD TG 203	*Oncorhynchus kisutch*	6PPD-quinone	24-hr LC_{50}: 0.0000793 mg/L (based on measured concentrations); calculation performed by DS	Klimisch 2 (reliable with restrictions) Key study	Tian et al. (2022)
Study similar to OECD TG 203	*Oncorhynchus mykiss* *Salvelinus fontinalis* *Salvelinus alpinus* *Acipenser transmontanus*	6PPD-quinone	24-hr LC_{50}: 0.00059 mg/L (based on mean measured concentrations) in *Salvelinus fontinalis* 96-hr LC_{50}: 0.001 mg/L (based on mean measured concentrations) in *Oncorhynchus mykiss* 96-hr LC_{50}: > 0.0142 mg/L (based on mean measured concentrations) in *Salvelinus alpinus* 96-hr LC_{50}: > 0.0127 mg/L (based on mean measured concentrations) in *Acipenser transmontanus*	Klimisch 2 (reliable with restrictions)	Brinkmann et al. (2022)
OECD TG 203	*Oncorhynchus mykiss*	p-hydroquinone	96-hr $LC_{50:}$ 0.638 mg/L (based on mean measured concentrations)	Klimisch 3 (not reliable) Nominal test concentrations not specified.	Hodson et al. (1984) [cited from ECHA dissemination site]
EPA ((Fish Acute Toxicity test) 1974))	*Oncorhynchus mykiss* *Pimephales promelas*	p-hydroquinone	96-hr $LC_{50:}$ 0.097 mg/L (nominal) for *Oncorhynchus mykiss* 96-hr $LC_{50:}$ 0.044 mg/L (nominal) for *Pimephales promelas*	Klimisch 3 (not reliable) Nominal test concentrations not specified	DeGraeve et al. (1980)
EPA ((Fish Acute Toxicity test) 1974))	*Oncorhynchus mykiss*	p-benzoquinone	96-hr $LC_{50:}$ 0.125 mg/L (nominal) for *Oncorhynchus mykiss*	Klimisch 3 (not reliable)	DeGraeve et al. (1980)

	Pimephales promelas		96-hr $LC_{50:}$ 0.045 mg/L (nominal) for *Pimephales promelas*	No detailed description of measured concentrations within the report	
QSAR estimation with ECOSAR v1.00 using class "Phenols" Input parameter: Molecular weight: 185.23 g/mol logKow: 2.46 (calculated by program)	Fish	4-HDPA	96h-LC_{50}: 12.7 mg/L using class "Phenols"	Klimisch 3 (not reliable)	Anonymous (2012a)
QSAR estimation with ECOSAR v1.00 using class "Neutral organics", "Vinyl/Allyl Ketones" and "Schiff Bases" Input parameter: Molecular weight: 183.21 g/mol logKow: 2.31 (calculated by program)	Fish	N-Phenyl-p-benzoquinone monoimine	96h-LC_{50}: 1.78 mg/L for class "Schiff Base" 96h-LC_{50}: 108.1 mg/L for class "Vinyl/Allyl Ketones" 96h-LC_{50}: 76.7 mg/L for class "Neutral organics"	Klimisch 3 (not reliable)	Anonymous (2012b)
Method equivalent to OECD Guideline 203	*Danio rerio*	p-Hydroquinone	96-hr LC_{50}: 0.17 mg/L (not specified if based on nominal or measured concentrations)	Klimisch 4 (not assignable) No description of study details and testing conditions.	Wellens H. (1982) [cited in OECD SIDS report, 2002]
Method equivalent to OECD Guideline 203	*Pimephales promelas*	p-Hydroquinone	96-hr LC_{50}: > 0.4 mg/L (not specified if based on nominal or measured concentrations)	Klimisch 4 (not assignable) No description of study details and testing conditions.	Terhaar et al. (1972) [cited in OECD SIDS report, 2002]
Invertebrates					
OECD Guidline 202	*Daphnia magna*	6PPD	48-hr $EC_{50:}$ 0.23mg/L (measured)	Klimisch 1 (reliable without restrictions)	MITI (1999)

acc to GLP					
Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians (US-EPA 1975)	*Daphnia magna*	6PPD	48-hr $EC_{50:}$ 0.51 mg/L (nominal)	Klimisch 2	Anonymous (1984b)
Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians (US-EPA 1975)	*Daphnia magna*	6PPD	48-hr $EC_{50:}$ 0.82 mg/L (nominal)	Klimisch 2	Anonymous (1978b)
OECD Guidline 202	*Daphnia magna*	6PPD	48-hr $EC_{50:}$ 0.79 mg/L (nominal)	Klimisch 4, original reference is not available	Anonymous (1984c)
OECD TG 202 Method outlined by Environment and Climate Change Canada (2017)	*Daphnia magna* *Hyalella azteca*	6PPD + 6PPD-quinone	48-hr EC_{50}: < 0.342 mg/L (based on mean measured concentrations) in *Daphnia magna* for 6PPD using OECD TG 202 96-hr LC_{50}: < 0.463 mg/L (based on mean measured concentrations) in *Hyalella Azteca* for 6PPD 48-hr EC_{50}: > 0.046 mg/L (based on mean measured concentrations) in *Daphnia magna* for 6PPD-quinone using OECD TG 202 96-hr LC_{50}: > 0.043 mg/L (based on mean measured concentrations) in *Hyalella Azteca* for 6PPD-quinone	Klimisch 2 (reliable with restrictions)	Hiki et al. (2021)
Testing procedure not reported	Not specified	6QDI	48-hr EC_{50}: 1.41 mg/L (not specified if based on nominal or measured concentrations)	Registered as NONS, no further details available. Data confidential	Anonymous (b) – year not specified [cited from ECHA dissemination site]
OECD TG 202 (2004); EU method C.2 (2008) GLP	*Daphnia magna*	4-HDPA	48-hr $EC_{50:}$ 0.69 mg/L (nominal)	Klimisch 1 (reliable without restrictions)	Anonymous (2010a)
OECD TG 202	*Daphnia magna*	p-	48-hr $EC_{50:}$ 0.061 mg/L	Klimisch 1 (reliable	Anonymous

GLP		hydroquinone	(based on mean measured concentrations)	without restrictions)	(2008c) [cited from ECHA dissemination site, study report not available]
OECD TG 202	*Daphnia magna*	p-benzoquinone	48-hr $EC_{50:}$ 0.13 mg/L (nominal)	Klimisch 2 (reliable with restrictions) No analytical monitoring of test item concentration performed.	Anonymous (2018a) [cited from ECHA dissemination site, study report not available]
ISO 6341 (Water quality Determination of the Inhibition of the Mobility of Daphnia magna Straus (Cladocera, Crustacea))	*Daphnia magna*	p-hydroquinone	48-hr EC_{50}: 0.13 mg/L (nominal)	Klimisch 3 (not reliable) No detailed description of used test concentrations or if analytical verification of test item concentration was performed.	Crisinel et al. (1994)
DIN 38412, Part II 'Daphnia short-time test	*Daphnia magna*	p-hydroquinone	48-hr EC_{50}: 0.29 mg/L (nominal)	Klimisch 3 (not reliable)	Kühn et al. (1989a) [cited from ECHA dissemination site]
DIN 38412, Part II 'Daphnia short-time test'	*Daphnia magna*	p-hydroquinone	24-hr EC_{50}: 0.09 mg/L (nominal)	Klimisch 3 (not reliable)	Bringmann et al. (1977) [cited from ECHA dissemination site]
Similar to OECD Guideline 202	*Daphnia magna*	p-hydroquinone	96-hr LC_{50}: 0.05 mg/L (not specified if based on nominal or measured concentrations.	Klimisch 2 (reliable with restrictions)	OECD SIDS report (2002, Hydroquinone
QSAR estimation with ECOSAR v1.00 using class “Phenols” Input parameter: Molecular weight: 185.23 g/mol	Daphnid	4-HDPA	48h-LC_{50}: 5.3 mg/L using class “Phenols”	Klimisch 3 (not reliable)	Anonymous (2012c)

logKow: 2.46 (calculated by program)					
QSAR estimation with ECOSAR v1.00 using class "Neutral organics", "Vinyl/Allyl Ketones" and "Schiff Bases" Input parameter: Molecular weight: 183.21 g/mol logKow: 2.31 (calculated by program)	Daphnid	N-phenyl-p-benzoquinone monoimine	48h-LC_{50}: 3.8 mg/L using class "Schiff Base" 48h-LC_{50}: 65.7 mg/L using class "Vinyl/Allyl Ketones" 48h-LC_{50}: 46.0 mg/L using class "Neutral organics"	Klimisch 3 (not reliable)	Anonymous (2012d)
Algae					
Algal Assay Procedure: Bottle Test (US-EPA 1971)	*Raphidocelis subcapitata,* formerly known as *Selenastrum capricornutum*	6PPD	96-hr EC_{50}: 0.6 mg/L (nominal concentration; based on decrease of chlorophyll a and cell numbers)	Klimisch 3 due to methodological deficiencies	Anonymous (1978c)
OECD TG 201 GLP	*Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*)	6QDI	72-hr $E_rC_{50:}$ 1.6 mg/L (nominal)	Klimisch 2 (reliable with restrictions)	Anonymous (1998)
OECD Guildine 201 (2006); EU method C.3 (2009) GLP	*Desmodesmus subspicatus*	4-HDPA	72-hr $E_rC_{50:}$ 2.6 mg/L (mean measured based on geometric mean) 72-hr $E_yC_{50:}$ 0.91 mg/L (mean measured based on geometric mean)	Klimisch 1 (reliable without restrictions)	Anonymous (2010b)
Similar to OECD TG 201 GLP	*Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*)	p-Hydroquinone	72-hr $E_rC_{50:}$ 0.053 mg/L (mean measured based on geometric mean)	Klimisch 2 (reliable with restrictions)	Anonymous (2008d) [cited from ECHA dissemination site, study report not available]
OECD TG 201	*Desmodesmus subspicatus*	p-Benzoquinone	72-hr $E_rC_{50:}$ 1.5 mg/L (nominal)	Klimisch 3 (not reliable)	Anonymous (2018b) [cited from ECHA dissemination

					site, study report not available]
QSAR estimation with ECOSAR v1.00 using class "Phenols" Input parameter: Molecular weight: 185.23 g/mol logKow: 2.46 (calculated by program)	Green algae	4-HDPA	96h-EC_{50}: 23.4 mg/L using class "Phenols"	Klimisch 3 (not reliable)	Anonymous (2012e)
QSAR estimation with ECOSAR v1.00 using class "Neutral organics", "Vinyl/Allyl Ketones" and "Schiff Bases" Input parameter: Molecular weight: 183.21 g/mol logKow: 2.31 (calculated by program)	Green algae	N-Phenyl-p-benzoquinone monoimine	96h-EC_{50}: not calculated for class "Schiff Base" due to lack of data 96h-EC_{50}: 51.1 mg/L using class "Vinyl/Allyl Ketones" 96h-EC_{50}: 22.5 mg/L using class "Neutral organics"	Klimisch 3 (not reliable)	Anonymous (2012f)
Testing procedure not reported	*Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*)	p-Hydroquinone	72-hr $E_rC_{50:}$ 0.335 mg/L (not specified if based on nominal or measured concentrations)	Klimisch 4 (not assignable) No description of study details and testing conditions.	Devillers et al. (1990) [cited from ECHA dissemination site]

11.5.1 Acute (short-term) toxicity to fish

In an acute fish test according to OECD TG 203 (flow-through design) and GLP *Oryzias latipes* was exposed to **6PPD** for 96 hours (MITI, 2001). No deviations are reported and the study is rated with a Klimisch score of 1. Besides the information provided in the IUCLID dossier and on the ECHA-Website on 6PPD, only the first page of the study in Japanese and several tables including the table on mortality are available (in English).

The fish were acclimated for more than 12 days before testing with no group showing mortality for seven days, before the test started. Fish with 22.3 mm (19.4 - 24.8 mm) and 23.0 mm (20.3 - 24.4 mm) in length were chosen at random for the main test (n=10) and the additional test (n=10), respectively. Average body weight of fish was 0.1866 g and 0.1877 g for the main test (n=10 per replicate) and the additional test (n=10

per replicate), respectively. The temperature varied between 23.1 and 23.8°C, the pH between 6.7 and 7.1 and the dissolved oxygen between 7.1 - 8.6 mg/L. Water renewal was 8 times per day using tap water after dechlorination by passing through an activated carbon filter. The stock solution was prepared daily.

As solvent HCO-50 was used and a solvent control was run as well as a dilution water control and a positive control using copper (II) sulfate pentahydrate. As statistical method the LC_{50} (moving average) was calculated using TOXDATMultiMethodProgram (USEPA) and probit by EcoTox-Statics (ver 1.1, Oita University).

Nominal concentrations of the test solutions were 0, 0.15, 0.22, 0.34, 0.51, 0.76, and 1.14 mg/L in the main test and 0.07 and 0.10 mg/L in the supplemental test: These concentrations were added because the fatality observed at 0.15 mg/l in the main run was more than 50%. The mean measured concentrations based on geometric mean (at test begin and after 24 hours) were 0.08, 0.14, 0.05, 0.07, 0.11, 0.14, 0.26 and 0.44 mg/L used as the measured concentrations were less than 20% within the nominal test concentrations.

Table 108: Nominal and measured concentrations in short-term fish study with 6PPD (MITI, 2001).

Nominal concentration in mg/L	Measured concentration in mg/L and (%) measured concentration of the nominal concentration		Mean measured concentration in mg/L (geometric mean)
	0h	24h	
Control	< 0.006	< 0.006	
Solvent control	< 0.006	< 0.006	
0.07	0.009 (12.9)	0.007 (10)	0.008
0.1	0.017 (17)	0.012 (12)	0.014
Control	< 0.04	< 0.04	
Solvent control	< 0.04	< 0.04	
0.15	0.05 (33.3%)	0.05 (33.3)	0.05
0.22	0.06 (27.3)	0.08 (36.4)	0.07
0.34	0.09 (26.5)	0.14 (41.2)	0.11
0.51	0.11 (21.6)	0.17 (33.3)	0.14
0.76	0.21 (27.6)	0.32 (42.1)	0.26
1.14	0.36 (31.6)	0.53 (46.5)	0.44

An LC_{50}-value of 0.028 mg/L was gained based on measured concentrations (geometric mean). Detailed results are summarised in Table 109.

Table 109: Toxicity symptoms and mortality in short-term fish study with 6PPD (MITI, 2001).

Mean measured concentration in mg/L (geometric mean)	Symptoms of toxicity observed; in brackets number of fish affected				Cumul. number of died fish (% mortality)			
	24h	48h	72h	96h	24h	48h	72h	96h
Control	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Solvent control	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
0.008	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

0.014	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	1 (10)
Control	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Solvent control	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
0.05	0 (0)	B (1)	B (1)	C (1)	0 (0)	2 (20)	3 (30)	7 (70)
0.07	0 (0)	B (1)	B (1)	-*	0 (0)	2 (20)	5 (50)	10 (100)
0.11	0 (0)	B (1)	-*	-*	0 (0)	9 (90)	10 (100)	-*
0.14	-*	-*	-*	-*	4 (40)	10 (100)	-*	-*
0.26	C (1)	-*	-*	-*	9 (90)	10 (100)	-*	-*
0.44	-*	-*	-*	-*	10 (100)	-*	-*	-*

*No observation made, as all animals dead.
0: normal
A: abnormal respiration; B: abnormal swimming ability
C: loss of equilibrium or swimming ability; D: other symptoms

Conclusion: In an acute fish test according to OECD TG 203 (flow-through design) and GLP an LC_{50}-value of 0.028 mg/L based on measured concentrations (geometric mean) was gained for 6PPD after 96 hours. The study is rated Klimisch 1.

In a static OECD TG 203 study with *Danio rerio* with **6PPD** without analytical monitoring an LC_{100} of 100 mg/l and LC_0 of 5 mg/L were retrieved (Anonymous, 1984a).

Conclusion: In a study rated Klimisch 4 based on scarce information on study description an LC_{100} of 100 mg/l and LC_0 of 5 mg/L were gained based on nominal concentrations.

In the study by Hiki et al. (2021) the acute toxicity of **6PPD and 6PPD-quinone** to the freshwater fish *Danio rerio* and *Oryzias latipes* were assessed. Acute toxicity in fish embryo was evaluated using *Danio rerio* according to OECD Guideline 236. Twenty eggs (< than 3 hour post fertilisation) were exposed to the test items 6PPD and 6PPD-quinone at their maximum water solubility at 26 °C for 96 hours with a photoperiod of 16 hours. The pH-value was maintained at 7.7 and the dissolved oxygen concentration was 7.8 mg/L. A negative control (dechlorinated tap water), positive control (3,4-dichloroaniline) and an internal plate control (using 4 eggs) were also included. To maintain chemical concentrations, >90 % of the test solution was renewed every 48 hours. At the start and end of the renewal process water quality characteristics and test item concentrations were measured via LC/MS/MS. Mortality and any visible abnormalities were recorded every 24 hours. The acute toxicity of 6PPD and 6PPD-quinone was also assessed using *Oryzias latipes* according to OECD Guideline 203. Ten organisms were exposed to 6PPD and 6PPD-quinone at their maximum water solubility in a 5 L glass tank with one replicate for a duration of 96 hours. The test was performed at 24.5 °C with a photoperiod of 16 hours. The pH-value was maintained at 7.9 and the dissolved oxygen concentration was 7.8 mg/L. Ten additional organisms were used to measure the length at the start of exposure (17.7 mm ± 1.2 mm; mean ± SD). A negative control (dechlorinated tap water) was included. The procedure to maintain chemical concentrations was the same as for the study with *Danio rerio*. Mortality and any visible abnormalities were recorded every 24 hours.

Results: In the zebrafish embryo test no acute mortality was observed for the test item 6PPD-quinone at its maximum water solubility after study duration, revealing a 96-hr- LC_{50} of > 53 µg/L (mean measured based on geometric mean). Mortality in the negative control and positive control after 96 hours was 0 % and 60 %, respectively. The hatching rate in the negative control and internal plate control was 100 % after 96 hours. Further exposure to 6PPD-quinone did not decrease hatching rates (95 % and 100 % after 72 and 96 hours, respectively) and did not induce any visible abnormalities. Although no acute mortality was observed to zebrafish embryos exposed to 6PPD at its maximum water solubility (41 µg/L mean measured based on geometric mean) after 96 hours, 6PPD delayed hatching rates in *Danio rerio* (45 % after 72 hours) and caused abnormalities of the spine with lateral curvature in 25 % of the tested species. No acute mortality was observed in the fish acute toxicity test with *Oryzias latipes* exposed to 6PPD-quinone at its maximum water solubility, revealing a 96-hr-LC_{50} of > 30 µg/L (mean measured based on geometric mean). No mortality was

observed in the negative control. Exposure to 6PPD-quinone did not exhibit behavioural symptoms to *Oryzias latipes*. After 96 hours study duration the mortality rate of *Oryzias latipes* exposed to 6PPD at its maximum water solubility (70 µg/L mean measured based on geometric mean) was 80 % inducing a LC_{50} value < 70 µg/L (mean measured based on geometric mean). Further all fish exposed to 6PPD showed abnormal swimming behaviour within 1 hour of exposure. The validity criteria for both tests were fulfilled according to OECD Guidelines.

Conclusion: 6PPD-quinone did not exhibit acute lethal toxicity at its maximum water solubility in the limit test performed with *Oryzias latipes* and *Danio rerio*. 80 % mortality was seen for the test item 6PPD and *Oryzias latipes*, revealing a 96-hr LC_{50} of < 0.070 mg/L (mean measured based on geometric mean). The study is rated as Klimisch 2.

A fish embryo acute toxicity test was performed in the study by Varshney et al. (2021). Zebrafish embroys were exposed to **6PPD and 6PPD-quinone** over a study duration of 96 hours. Eggs were received by random mating between sexually mature individuals. The fertilization rate was > 80 % for the experiment. The ferzilized eggs were then separated from the unfertilized eggs. Prior to the main experiment a range finding test was performed (0-1500 µg/L for 6PPD and 0-1000 µg/L for 6PPD-quinone). In the main experiment the zebrafish embryos (< 16 cell stage) were exposed to nominal concentrations of 0 (control), 150, 300, 450, 600, 750, 900, 1050 and 1200 µg/L of the test item 6PPD and to nominal concentrations of 0 (control), 40, 80, 120, 160, 200, 240, 280, 320 and 360 µg/L of the test item 6PPD-quinone. A measurement of the actual test item concentration was not made as it was reported that analytical methods were not well standardized at the time of the study. The test was conducted in clear polystyrene and flat-bottomed 24 well plates which were pre-treated with the test solutions 24 hours before test start. One embryo was placed in each well with 2 mL of test solution. The test solutions were replaced every 24 hours to maintain the concentration of the test items. ISO standard fish media served as negative control. As ethanol was used for preparing the test item solutions, 0.1 % Ethanol was implemented as solvent control. 3,4-dichloroaniline served as positive control (concentration higher than recommended in OECD Guideline). Temperature (26.0 ± 1.0°C), pH (6.81 ± 0.08) and oxygen saturation (87.1 ± 2.0 %) were maintained throughout the whole study duration. Hatching rates were recorded every 24 hours, for determination of the LC_{50} values mortality was used as endpoint.

Results: The study resulted in a 96-hr LC_{50} value of 442.62 µg/L for the test item 6PPD and a 96-hr LC_{50} value of 132.92 µg/L for the test item 6PPD-quinone. The validity criteria according to OECD Guideline 236 were met and the study is rated as reliable with restrictions as there was no measurement of the actual test item contractions and the used concentration of the positive control was slightly higher than recommended in the OECD Guideline.

Conclusion: In the study rated as Klimisch 2 a 96-hr LC_{50} value of 0.133 mg/L for the test item 6PPD-quinone was gained. The study revealed also a 96-hr LC_{50} value of 0.443 mg/L for the test item 6PPD. Both values are based on nominal concentrations.

For **6QDI**, one acute study is available revealing a 96-hr LC_{50} of 0.638 mg/L for the organism *Oncorhynchus mykiss*. It is noted that the substance is registered as NONS, therefore no detailed description of the study is available, as the data are confidential. The study is assessed as not assignable.

Conclusion: In the study rated as Klimisch 4 (based on no details on study methodology) a LC_{50} of 0.638 mg/L (not specified if based on nominal or measured concentrations) were revealed.

In the study by Tian et al. (2021) coho salmon (*Oncorhynchus kisutch*) was exposed to the test item **6PPD-quinone** over a study duration of 24 hours. A ten-dilution series was used and the exposure was repeated twice. In the first batch the test organisms were exposed to nominal concentrations of 0.30, 0.39, 0.50, 0.65, 0.83, 1.08, 1.39, 1.80, 2.32 and 3.00 µg/L. In the second batch nominal concentrations of 1.0, 1.17, 1.36, 1.59, 1.85, 2.16, 2.52, 2.94, 3.43 and 4.0 µg/L were used. In addition a negative control (system water), solvent control (pure ethanol) and method blank controls (water/solvent going through fractionation) were included. Eight fish were used per concentration in 30 L of test solution. Aquaria were held in a recirculating water batch to control temperatures. Prior to fish transfer temperature (10-12 °C), pH (7.6 – 7.8), conductivity and dissolved oxygen (> 98 % saturation) were verified. The actual test item concentration was measured via HPLC and the measurement was performed prior to fish transfer. For all exposures, mortality rates were recorded after 24 hours.

Results: The following Table 110 gives an overview on the outcome of both batches including the measured concentrations and the mortality rates of the different concentration levels for *Oncorhynchus kisutch*.

Table 110: Outcome of the study by Tian et al. (2021) for *Oncorhynchus kisutch* for both individual batches.

Outcome Batch 1		Outcome Batch 2	
Measured Conc. (µg/L)	Mortality rate (%)	Measured Conc. (µg/L)	Mortality rate (%)
0.15	0	0.66	38
0.30	0	0.83	25
0.34	0	0.71	38
0.33	13	0.98	75
0.48	0	1.01	75
0.63	13	1.23	88
0.67	13	1.31	75
0.76	88	1.79	100
1.08	63	2.12	88
1.58	88	2.70	100

For the first batch the measured concentrations were in the range of 49 – 78 % of nominal concentrations. For the second batch the measured concentrations were in the range of 52 – 72 % of nominal concentrations. The study authors estimated the 24-hr LC_{50} value over both batches using the measured concentrations of each batch (using four-parameter logistic model). The authors revealed a 24-hr LC_{50} value of 0.79 µg/L (95 % CI [0.63, 0.96]). The LC_{50} value for each individual batch was estimated by DS using R statistical software. Four different models were compared including Log Logistic model 2, Log Logistic model 4, Log normal and Weibull (for details see Annex I). For Batch 1 the LC_{50} values were in the range 0.7 – 0.86 µg/L (depended of used model). For Batch 2 the LC_{50} values ranged between 0.81 – 0.96 µg/L (depended of used model). For all data pooled the LC_{50} values were in the range 0.79 – 0.84 µg/L (depended of used model). Considering the goodness-of-fit of the data points on the curve, the respective p-value, the upper and lower limit of the model and the optical determination of the curve the respective LC_{50} of 0.7 µg/L for Batch 1 (Log Logistic model 4) was viewed as most reliable and it represents the most stringent outcome. The respective outcome of other used models of the individual batches and pooled data support the outcome as they are all in the same range. Although the study duration was only 24 hours, the LC_{50} value of 0.7 µg/L (based on measured concentrations) is reliable and relevant for classification purposes, as the validity criteria according to OECD Guideline were fulfilled.

Conclusion: A 24-hr LC_{50} value of 0.0007 mg/L (0.7 µg/L) was gained based on measured concentrations calculated for batch 1. The study was rated as Klimisch 2.

In a subsequent study by Tian et al. (2022) coho salmon (*Oncorhynchus kisutch*) was again exposed to the test item **6PPD-quinone** over a test duration of 24 hours. A commercially available 6PPD-quinone standard was used instead of an in-house standard as in the previous study to re-confirm toxicity estimates in coho salmon. Prior to the definitive study a range-finding test was performed. In the range-finding study the fish were exposed to nominal concentrations of 20.0, 35.6, 63.2, 112.5, and 200.0 ng/L of the test item. In the definitive study exposures were performed in triplicates (Batch 1-3) over three weeks. For the first batch nominal concentrations of 50.0, 66.0, 87.0, 115.0, 152.0 and 200 ng/L were used. For the second and third batch the nominal concentrations were 60.0, 72.0, 86.0, 104.0, 125.0, and 150 ng/L. The study authors used a smaller range in the second and third batch to get a more accurate LC_{50}. Ethanol was used to prepare the test solutions. Negative controls were pure ethanol. In the range finding study and the definitive study six fish were placed in 70 L aquaria per test concentration. In all aquaria temperature (10 – 13 °C), pH (7.6 – 8.0), conductivity and dissolved oxygen (> 98 % saturation) were verified before fish were transferred. Analytical measurement was also performed prior to fish transfer via LC-MS/MS. Mortality rates were recorded after 24 hours.

Results: By the time of the range finding study the used quantification method was not finalized, therefore the measured concentrations were back calculated according to the median error of batch 1-3 of the definitive study (median 16 %). The measured concentrations of the range finding study were 32.1, 41.1, 73.0, 120.0 and 231.0 ng/L. In the 23.1, 41.1 and 73.0 ng/L treatment groups no mortality was observed. In the 130.0 ng/L treatment group the mortality rate was 67 %. In the 231 ng/L treatment group all fish died. All negative fish survived and did not show any symptoms at the end of the range-finding study. Table 111 gives the outcome of the definitive study with the individual batch 1-3 after the study duration of 24 hours.

Table 111: Outcome of batch 1-3 of the study by Tian et al. (2022) after 24 hour study duration

Outcome Batch 1		Outcome Batch 2		Outcome Batch 3	
Measured Conc. (ng/L)	Mortality rate (%)	Measured Conc. (ng/L)	Mortality rate (%)	Measured Conc. (ng/L)	Mortality rate (%)
53.2	0	68.1	0	70.5	50
68.9	20	83.5	33	84.6	83
97.1	67	99.3	40	105.0	100
122.0	67	115.0	67	124.0	80
162.0	83	154.0	50	151.0	67
226.0	83	194.0	83	190.0	67

The measured concentrations were systematically higher than the nominal concentrations, potentially caused by volumetric vessels (Hamilton syringes and glass pipettes) with a median of 16 % over all three batches. The measured concentrations of batch 1 were in the range 4 – 13 % of nominal concentrations. For batch 2 the measured concentrations were in the range of 10 – 29 % of nominal concentrations whereas for batch 3 the measured concentrations were in the range 10 – 29 % of nominal concentrations. To determine the LC_{50} value the study authors used the 30 fish in the range finding test and the 108 fish in the three definitive studies. As during exposure four fish jumped out of the aquaria 134 fish contributed to the LC_{50} value calculation. The study authors revealed a 24-hr LC_{50} value of 95 ng/L (95 % CI [80, 110]) which is based on measured concentrations. A two parameter logistic model was used by the study authors. The respective LC_{50} values for each individual batch was in addition calculated by DS using R statistical software. Four different models were compared including Log Logistic model 2, Log Logistic model 4, Log normal and Weibull (for details see Annex II). For batch 1 the calculation was performed with the nominal concentrations and the measured concentrations (range 4 – 13 % of nominal concentrations) and for batch 2 and 3 the calculations were only performed with the measured concentrations (range 10 – 29 % and 10 – 29 % of nominal concentrations respectively). For batch 1 the 24-hr LC_{50} value of 79.28 ng/L (based on measured concentrations) for the Log logistic model 4 was considered to be most reliable and accurate. This is based on the goodness-of-fit of the data points on the curve, the respective p-value, the upper and lower limit of the model and the optical determination of the curve as well as on the visual check of the data. For batch 1 the data of the other used models were considered supportive as they are in the same range. For batch 2 and 3 the individual LC_{50} values for the different calculation models were not considered to be reliable and robust. This due to a visual check of the data and optical determination of the curves. The validity criteria are fulfilled according to OECD Guideline, as in the control no mortalities were observed, the dissolved oxygen concentration was ≥ 60 % of the air saturation value in all test vessels throughout the exposure and an analytical measurement was performed. The 24-hr LC_{50} value of 79.28 ng/L (based on measured concentrations) calculated for batch 1 is therefore considered valid and reliable for classification purposes.

Conclusion: The study resulted in a 24-hr LC_{50} value of 0.0000793 mg/L (79.28 ng/L) based on measured concentrations calculated for batch 1. The study was rated as Klimisch 2.

In a study by Brinkmann et al. (2022) the acute toxicity of the test item **6PPD-quinone** was investigated to *Oncorhynchus mykiss*, *Salvelinus fontinalis*, *Salvelinus alpinus* and *Acipenser transmontanus*. Prior to the main experiment a range-finding study was conducted. In the main experiment *Oncorhynchus mykiss* was exposed to nominal concentrations of 0.15, 0.75, 1.5, 3 and 6 µg/L of the test item. For *Salvelinus fontinalis* nominal concentrations of 0.1, 0.5, 1.0, 2.0 and 4.0 µg/L were used. For *Salvelinus alpinus* and *Acipenser transmontanus* a limit test was performed with a nominal concentration of 20 µg/L. All experiments were conducted under static renewal conditions. For test solution preparation dimethyl sulfoxide was used. *Salvelinus fontinalis* was exposed to the test item in 150-L tanks for a period of 24 hours. Two replicates with four fish each were used per test concentration. *Oncorhynchus mykiss, Salvelinus alpinus* and *Acipenser transmontanus* were exposed to the test item in 700-L tanks over a period of 96 hours. For *Oncorhynchus mykiss* and *Salvelinus alpinus* two replicates with 5 fish each were used. For *Acipenser transmontanus* three replicates with two fish each were used. Control tanks in the respective experiments were dosed with dimethyl sulfoxide the same level as all other tanks. Test solutions were aerated, recirculated and temperature controlled during the whole study duration in all experiments. Concentrations were measured after dosing and before and after renewal of the test solution every 24 hours in experiments with *Oncorhynchus mykiss*, *Salvelinus alpinus* and *Acipenser transmontanus.* In the experiment with *Salvelinus fontinalis* the concentrations were measured after dosing and at test end. LC-HRMS was used to verify the actual test item concentration. Mortality and any visible abnormalities were recorded during the whole study duration.

Results: As over all experiments the deviation from the nominal concentrations is > 20 % the study results are based on mean measured concentrations. No mortality was observed after study duration in the experiments with *Salvelinus alpinus* and *Acipenser transmontanus,* resulting in a 96-hr LC_{50} value of > 14.2 µg/L (based on mean measured concentrations) and 96-hr LC_{50} of > 12.7 µg/L (based on mean measured concentrations) respectively. *Salvelinus fontinalis* showed the highest sensitivity with 100 % mortalities in the highest treatment group within three hours of exposure, revealing a 24-hr LC_{50} value of 0.59 µg/L (based on mean measured concentrations). A 96-hr LC_{50} value of 1.0 µg/L (based on mean measured concentrations) was recorded in the experiment with *Oncorhynchus mykiss.* In cases mortalities were observed, both *Oncorhynchus mykiss* and *Salvelinus fontinalis* showed behaviours including hovering close to the water surface, accelerated movement, gasping and spiralling motion. Although the fish were larger than recommended in the OECD Guideline the validity criteria according to OECD Guideline were fulfilled, as in the control no mortalities were observed, the dissolved oxygen concentration was ≥ 60 % of the air saturation value in all test vessels throughout the exposure and an analytical measurement was performed. The 24-hr LC_{50} value of 0.59 µg/L and 96-hr LC_{50} value of 1.0 µg/L are therefore rated as valid and reliable for classification purposes.

Conclusion: In a study rated as Klimisch 2 a 96-hr LC_{50} value of 0.001 mg/L was gained. Such value is supported with the 24-hr LC_{50} value of 0.00059 mg/L also revealed in this study. Both values are based on mean measured concentrations.

The acute toxicity of **p-hydroquinone** to the organism *Oncorhynchus mykiss* was investigated in a study according to OECD Guideline 203 under flow-through conditions (Hodson et al., 1984, cited from ECHA dissemination site). The fish were exposed to six concentrations (0 (control), 10, 18, 32, 56, 100 % of maximum test concentration – not further specified). The test substance concentration was measured daily via UV absorbance. No analytical results of the test item concentrations were available within the report. Degradation products were not analysed during this study. Three replicates per test concentration and control were used with 10 fish per test vessel (loading rate: 0.8 – 2.7 g/L). The length of the used test organism was at test end in the range of 4.6 – 6.4 cm and the weight was in the range 1.2 – 3.8 g. Temperature (14.1 – 16.5 °C), pH (7.6 ± 8.19) and dissolved oxygen concentration (5.6 – 9.4 mg/L) was determined.

Results: The study revealed a 96-hr LC_{50} of 0.638 mg/L (nominal) for the organism *Oncorhynchus mykiss*. The full study report was not available. As there are missing raw data and deviations from the OECD Guideline the study is assessed as not reliable for classification purposes.

Conclusion: A 96-hr LC_{50} of 0.638 mg/L based on nominal concentrations was revealed. The study is rated as Klimisch 3.

The short term toxicity of **p-hydroquinone** to fish (*Oncorhynchus mykiss* and *Pimephales promelas*) was investigated in a flow-through test (DeGraeve et al., 1980). The test duration was 96 hours. Both organisms were exposed to a negative control and seven test concentrations (100% well-water, highest achievable concentration and six intermediate concentrations, each 50% less toxicant than immediately higher concentration – not further specified). Each test concentration was delivered to two 28-L tanks (*Pimephales promelas*) and two 14-L tanks (*Oncorhynchus mykiss*). It is not specified within the report if ten or five fish were held in each test vessel. The loading rate for *Oncorhynchus mykiss* could therefore range from 3 g/L (assuming 5 fish/28 L tank) to 6 g/L (assuming 10 fish/28 L tank). For the organism *Pimephales promelas* the loading rate is in the range 0.18 g/L (assuming 5 fish/28 L tank) to 0.36 g/L (assuming 10 fish/28L tank). Dead fish were removed daily, weighed and measured as well the behaviour of surviving animals was recorded twice daily. During the whole study duration the fish were not fed. The concentration of the test item was monitored by high pressure liquid chromatography or gas chromatography daily. Within the report there was no detailed description of the measured concentrations. The mean length and weight of the test organisms *Pimephales promelas* was 3.5 cm and 0.5 g respectively at test end. The mean length and weight of the test organism *Oncorhynchus mykiss* was 11.3 cm and 16.8 g respectively at test end. The pH-value and dissolved oxygen concentration in the highest used test concentration was 8.1 and 6.5 mg/L, respectively. The test temperature was 14 ± 1 °C. After study duration the mortality rate was determined.

Results: The 96-hr LC_{50} for the test organism *Pimephales promelas* was 0.044 mg/L (nominal) and the LC_{50} for the test organism *Oncorhynchus mykiss* was 0.097 mg/L (nominal). The study is considered not reliable and valid as the used test concentrations are not specified, analytical results are not reported and there are uncertainties regarding the number of fish per tank. Further there are deviations from the OECD Guideline as the length of the organism *Oncorhynchus mykiss* was > than recommended in OECD Guideline and the test temperature for *Pimephales promelas* was < than recommended in OECD Guideline.

Conclusion: The study rated as Klimisch 3 exhibited a 96-hr LC_{50} of 0.044 mg/L based on nominal concentrations.

In the study by DeGraeve et al. (1980) the acute toxicity of the test item **p-benzoquinone** was also investigated to the test organisms *Oncorhynchus mykiss* and *Pimephales promelas.* For description see above. Test temperature, pH and dissolved oxygen concentration was the same as described above.

Results: The 96-hr LC_{50} for the test organism *Pimephales promelas* was 0.045 mg/L (nominal) and the LC_{50} for the test organism *Oncorhynchus mykiss* was 0.125 mg/L (nominal). Due to the mentioned reasons above the study is assessed as not reliable for classification purposes.

Conclusion: The study rated as Klimisch 3 exhibited a 96-hr LC_{50} of 0.045 mg/L based on nominal concentrations.

The acute toxicity of **4-HDPA** to fish was predicted by Anonymous (2012a) using ECOSAR v1.00. The molecular weight of 185.23 g/mol and the logKow of 2.46 (calculated by the program) were used as input parameters. ECOSAR v1.00 revealed a 96-hr LC_{50} of 12.7 mg/l using class “Phenols”. As the chemical structures of substances used for derivation of the linear regression equation are not very similar to the substance to be computed uncertainty of the revealed outcome is expected. The acute toxicity of **N-Phenyl-p-benzoquinone monoamine** to fish was also predicted by Anonymous (2012b) using ECOSAR v1.00. The molecular weight of 183.21 g/mol and the logKow of 2.31 (calculated by the program) were used as input parameter. ECOSAR v1.00 revealed a 96-hr LC_{50} of 1.78 mg/L for the class “Schiff Base”, a 96-hr LC_{50} of 108.1 mg/L for the class “Vinyl/Allyl Ketones” and a 96-hr LC_{50} of 76.7 mg/L for the class “Neutral organics”. The model for the class “Schiff Base” is based on three data points and estimates for the class “Vinyl/Allyl Ketones” are based on seven data points. As the chemical structures of substances used for derivation of the linear regression equation are not very similar to the substance to be computed uncertainty of the revealed outcome is expected. For both substances information was gained from the ECHA dissemination site as well as from the IUCLID report.

Conclusion: The predicted acute toxicity using ECOSAR v.1.00 for both substances yields results with high uncertainty and are not reliable for classification purposes.

In the OECD SIDS Document (2002) for **p-hydroquinone** two short study summaries are available. The acute toxicity of p-hydroquinone was investigated in a static test equivalent to OECD Guideline 203 with the test organism *Danio rerio*. After 96 hours a LC_{50} of 0.17 mg/L was obtained. Another short term toxicity study with the fish *Pimephales promelas* and the test item **p-hydroquinone** is reported in the document with a LC_{50} of > 0.4 mg/L. Both studies are assessed as not assignable. The detailed study description is missing. Further it is not specified if the revealed results are based on nominal or measured concentrations.

Conclusion: A LC_{50} value of 0.17 mg/L (not specified if based on nominal or measured concentrations) was reported in the OECD SIDS Document (2002). The result is rated as Klimisch 4 as there are no descriptions of the study methodology.

11.5.2 Acute (short-term) toxicity to aquatic invertebrates

A GLP-study according to OECD Guideline 202 with **6PPD** was performed under flow-through conditions with *Daphnia magna* (MITI, 1999; besides the information provided in the IUCLID dossier and the ECHA-Website on 6PPD, only the first page of the study in Japanese and the tables for the calculated EC_{50} values and NOECs are available in English). The stock solution was prepared daily and each test solution was prepared daily by diluting 10 mg/L solution containing 0.005 % HCO-50 with diluting mineral medium (Elendt M4) by using a continuous diluting apparatus.

The pH varied from 7.7 to 8.0 and the test temperature from 19.8 to 20.3°C. The hardness was 253 mg/L $CaCO_3$ and dissolved oxygen was > 60% of saturation. Two replicates with ten individuals were used. The measured concentrations were below the nominal concentrations (measurements via HPLC) and the 48h-EC_{50} for immobilisation/mortality was 0.23 mg/L. For details see Table 112.

Table 112: Nominal and measured concentrations and immobilisation/mortality with 6PPD in acute daphnia study (MITI, 1999)

Nominal concentration in mg/L	Measured concentration in mg/L and (%) measured concentration of the nominal concentration		Mean measured concentration in mg/L (geometric mean)	Cumulative number of died or immobilised and % and brackets	
	0h	24h		24h	48h
Control	< 0.01	< 0.01		0 (0)	0 (0)
Solvent control	< 0.01	< 0.01		0 (0)	1 (5)
0.11	0.02 (18.2%)	< 0.01 (-)	< 0.01	0 (0)	0 (0)
0.19	0.03 (15.8)	< 0.01 (-)	< 0.01	0 (0)	1 (5)
0.34	0.08 (23.5)	0.02 (5.9)	0.05	0 (0)	0 (0)
0.62	0.09 (14.5)	0.03 (4.8)	0.06	0 (0)	2 (10)
1.11	0.48 (43.2)	0.44 (39.6)	0.46	11 (55)	14 (70)
2	1.02 (51)	1.43 (71.5)	1.23	20 (100)	*

*No observation made, as all animals dead. # No information on data

Using a binominal statistical method the 24h-EC_{50} for immobilisation/mortality was 0.4 mg/L with a 95%-confidence limit ranging from 0.06 to 1.23 mg/L and the 48h-EC_{50} for immobilisation/mortality was 0.23 mg/L with a 95%- confidence limit ranging from 0.17 to 0.31 mg/L for 6PPD.

Conclusion: The 48h-EC_{50} for immobilisation/mortality was 0.23 mg/L based on measured concentrations (geometric mean) for 6PPD. The study is rated Klimisch 1.

Another study with **6PPD** was performed according to "Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians" (US-EPA 1975) with *Daphnia magna* (Anonymous, 1984b). The nominal concentrations in this static test were 0.25, 0.5, 1.0, 2.0 and 4.0 mg/L without analytical monitoring, resulting in a 48h-EC_{50}

of 0.51 mg/l. When the test solution was allowed to age 24 h before test, 1.0 mg/l (= solubility limit, highest tested concentration) had no effect on survival.

Conclusion: A 48h-EC_{50} of 0.51 mg/l (based on nominal concentrations) was gained in a study rated Klimisch 2.

Another static study with **6PPD** was performed according to "Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians" (US-EPA 1975) with *Daphnia magna* (Anonymous, 1978b) without analytical monitoring, resulting in a 48h-EC_{50} of 0.82 mg/l.

Conclusion: A 48h-EC_{50} of 0.82 mg/l (based on nominal concentrations) was gained in a study rated Klimisch 2.

Another static study with **6PPD** was performed according to OECD Guideline 202 with *Daphnia magna* (Anonymous, 1984c) without analytical monitoring, resulting in a 48h-EC_{50} of 0.79 mg/l.

Conclusion: A 48h-EC_{50} of 0.79 mg/l (based on nominal concentrations) was gained in study rated Klimisch 4 by the registrants as the study report is not available.

The acute toxicity of the test item **6PPD and 6PPD-quinone** to *Daphnia magna* and *Hyalella azteca* was assessed in the study by Hiki et al. (2021). The acute toxicity to *Daphnia magna* was tested according to OECD Guideline 202 over a period of 48 hours. Five neonates of daphnids (< 24 hours old) were exposed to 6PPD and 6PPD-quinone at its maximum water solubility in a 50 mL glass beaker with four replicates included (total of 20 daphnids). A photoperiod of 16 hours was used. The daphnids were fed with 0.5 mL of yeast cerophyl trout chow per beaker at the beginning of the study and after 48 hours of exposure. Mortality and immobilization were observed every 24 hours. 3,4 dichloraniline was included as a reference to check the sensitivity of the stock culture. Acute toxicity in amphipods was investigated using *Hyalella Azteca* according to the test method outlined by Environment and Climate Change Canada (2017) over a period of 96 hours. Ten neonates (3-5 days old) were exposed to 6PPD and 6PPD-quinone at its maximum water solubility in a 300 mL glass beaker with two replicates. A photoperiod of 16 hours was used. Mortality was recorded every 24 hours. Cadmium chloride was used as a reference to check the sensitivity of the stock culture. Although recommended in test guideline for *Hyalella Azteca* no substart was added to vessels.

Results: For *Daphnia magna* no acute mortality or immobility was observed after 48 hours exposed to 6PPD-quinone at its maximum water solubility, revealing a 48-hr EC_{50} of > 46 µg/L (based on mean measured concentrations). The 48-hr EC_{50} for 3,4-dichloraniline was estimated to be 569 µg/L (based on nominal concentrations). In the negative control no mortality or immobilization was observed. The mortality rate of daphnids exposed to 6PPD at its maximum water solubility (342 µg/L based on mean measured concentrations) was 100 %. For *Hyalella Azteca* no acute mortality was observed after the study duration of 96 hours and exposure to 6PPD-quinone, revealing a 96-hr LC_{50} of > 43 µg/L (based on mean measured concentrations). In the negative control 5 % of mortality was observed for *Hyalella Azteca*. For Cadmium Chloride a 96-hr LC_{50} of 6.1 µg/L was estimated. The mortality rate for *Hyalella Azteca* exposed to 6PPD at its maximum water solubility (463 µg/L based on mean measured concentrations) was 100 % after study duration. The validity criteria for both toxicity tests were fulfilled.

Conclusion: For 6PPD-quinone no acute toxicity was exhibited at its maximum water solubility neither in the test with *Daphnia magna* nor in the test with *Hyalella Azteca*. 100 % mortality was seen for the test item 6PPD at its maximum water solubility in both tests, revealing a 48-hr EC_{50} < 0.342 mg/L for *Daphnia magna* and a 96-hr LC_{50} < 0.463 mg/L for *Hyalella Azteca* (both values based on mean measured concentrations). The study is rated as Klimisch 2.

One acute study investigating the acute toxicity of **6QDI** to aquatic invertebrates (test organism not specified) revealed a 48-hr EC_{50} value of 1.41 mg/L (not specified if based on nominal or measured concentrations). As 6QDI is registered as NONS, no detailed description of the study is available, as the data are confidential.

Conclusion: The 48-hr EC_{50} value of 1.41 mg/L (not specified if based on nominal or measured concentrations) in the NONS registration is rated as Klimisch 4.

A study was conducted to evaluate the acute toxicity of **4-HDPA** to the organism *Daphnia magna* under static conditions (Anonymous, 2010a). The study was performed according to OECD Guideline 202 and

under GLP. Daphnids were exposed to nominal concentrations of 0.21, 0.47, 1, 2.3, and 5 mg/L. The actual test item concentration was measured via HPLC/UV-VIS. The test temperature was 20 ± 1 °C during the whole study and the pH-value was in the range 7.8 – 8.0. Ten daphnids were held in each test vessel. For each test concentration and control two replicates were used. Observations were made on swimming ability, immobilisation rate, water characteristics and chemical analysis after 24 and 48 hours.

Results: The results are expressed in terms of nominal concentrations, as the effective concentrations ranged from 95.4 – 100.5 % (0 hours) and from 81.9 – 90.2 % (48 hours) of nominal values. The highest used test concentration (5 mg/L) was measured after 24 hours due to early immobilisation of the daphnids. The concentration corresponds to 92.2 % of the nominal concentration. The 48-hr EC_{50} value was determined to be 0.69 mg/l based on nominal concentrations. The validity criteria of the study were met. The study is therefore valid and reliable for classification purposes.

Conclusion: A 48-hr EC_{50} of 0.69 mg/L (nominal) was gained in a study rated as Klimisch 1.

In a short-term toxicity study according to OECD Guideline 202 and GLP *Daphnia magna* was exposed to the test substance **p-hydroquinone** under semi-static conditions (Anonymous, 2008c, cited from ECHA dissemination site). The nominal test concentrations were 0.07, 0.11, 0.16, 0.24 and 0.35 mg/L. The test item concentration was measured via HPLC/UV. Immobilisation was recorded after 24 and 48 hours.

Results: The study results in a 48-hr EC_{50} of 0.061 mg/L (based on mean measured concentrations). The full study report was not available. As the study was conducted according to OECD Guideline and under GLP and the results are well documented, the study is rated as valid and reliable.

Conclusion: The study gained a 48-hr EC_{50} of 0.061 mg/L (based on mean measured concentrations). The study is rated as Klimisch 1.

The acute toxicity of **p-benzoquinone** to the test organism *Daphnia magna* was determined in a study according to OECD Guideline 202 under static conditions (Anonymous, 2018a, cited from ECHA dissemination site). The daphnids were exposed to nominal test item concentrations of 0.05, 0.10, 0.15, 0.20, 0.25 mg/L over a period of 48 hours. The actual test item concentration was not measured throughout the whole study duration. After 24 and 48 hours the daphnia mobility was checked and documented. Further temperature, pH and dissolved oxygen concentration was held within the range as recommended in the OECD Guideline.

Results: The study revealed a 48-hr EC_{50} value of 0.13 mg/L based on nominal concentrations for the test organism *Daphnia magna.* As the study was conducted according to OECD Guideline and the results are well documented, the study is rated as reliable with restrictions.

Conclusion: The study rated as Klimisch 2 gained a 48-hr EC_{50} value of 0.13 mg/L based on nominal concentrations.

The acute toxicity of **p-hydroquinone** was investigated by Crisinel et al. (1994) with the test organism *Daphnia magna* under static conditions. Within the report there was no description of used test concentrations. Further the dissolved oxygen concentration and pH was not reported within the report.

Results: The 48-hr EC_{50} was 0.13 mg/L (nominal) for the test organism *Daphnia magna*. The study is assessed as not reliable, as there is no detailed description of the study methodology.

Conclusion: The study rated as Klimisch 3 gained a 48-hr EC_{50} value of 0.13 mg/L based on nominal concentrations.

Two static acute toxicity tests were conducted with **p-hydroquinone** and the test organism *Daphnia magna* according to DIN 38412, Part II ‘Daphnia short-time test‘ (Kühn et al. (1989a) and Bringmann et al. (1977)). The full study report was not available. The reported 48-hr and 24-hr EC_{50} values are 0.29 mg/L and 0.09 mg/L (nominal) respectively. The reported values are considered as not reliable and valid for classification purposes due to missing description of the study methodology. Further the dissolved oxygen concentration and pH was not reported within the report.

Conclusion: The reported values of 48-hr EC_{50} 0.29 mg/L and 24-hr EC_{50} 0.09 mg/L (both based on nominal concentrations) are rated as Klimisch 3 due to missing description of study methodologies.

In the OECD SIDS Document (2002) a study is included testing the acute toxicity of **p-hydroquinone** to *Daphnia magna.* The test organism were exposed in a test similar to OECD 202 for 96 hours under static conditions to the test item. The study obtained a 96-hr LC_{50} of 0.05 mg/L (not specified if based on nominal or measured concentrations). The study is rated as Klimisch 2.

The acute toxicity of **4-HDPA** to daphnids was predicted by Anonymous (2012c) using ECOSAR v1.00. The molecular weight of 185.23 g/mol and the logKow of 2.46 (calculated by the program) was used as input parameter. ECOSAR v1.00 revealed a 48-hr LC_{50} of 5.3 mg/l using class "Phenols". As the chemical structures of substances used for derivation of the linear regression equation are not very similar to the substance to be computed, uncertainty of the revealed outcome is expected. The acute toxicity of **N-phenyl-p-benzoquinone monoamine** to daphnids was also predicted by Anonymous (2012d) using ECOSAR v1.00. The molecular weight of 183.21 g/mol and the logKow of 2.31 (calculated by the program) was used as input parameter. ECOSAR v1.00 revealed a 48-hr LC_{50} of 3.8 mg/L for the class "Schiff Base", a 48-hr LC_{50} of 65.7 mg/L for the class "Vinyl/Allyl Ketones" and a 48-hr LC_{50} of 46.0 mg/L for the class "Neutral organics". The model for the class "Schiff Base" is based on only one data point. As the chemical structures of substances used for derivation of the linear regression equation are not very similar to the substance to be computed, uncertainty of the revealed outcome is expected. Information for both substances was gained from the IUCLUD report as well as from the ECHA dissemination site.

Conclusion: The predicted acute toxicity using ECOSAR v1.00 for both substances yields results with high uncertainty and is therefore not reliable for classification purposes.

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

In a study with **6PPD** according to the Algal Bottle Test (US-EPA 1971) the decrease of chlorophyll a and decrease of cell numbers in exposed cultures of *Raphidocelis subcapitata*, formerly known as *Selenastrum capricornutum* was compared to control at 24, 48, 72 and 96 h (Anonymous, 1978c). The nominal concentrations tested were: 0.1, 0.3, 0.6, 1 and 3 mg/L – no analytical measurements are reported. The 96h-EC_{50} for both endpoints was 0.6 mg/L for decrease of chlorophyll a and cell numbers. The study is rated Klimisch 3 due to significant methodological deficiencies: No exponential growth was observed throughout the incubation period.

Conclusion: The 96h-EC_{50} values for decrease of chlorophyll a and cell numbers was 0.6 mg/L for 6PPD. The study is not considered reliable due to methodological deficiencies.

In a study by Anonymous (1998) the toxicity of **6QDI** was investigated to the freshwater algae *Raphidocelis subcapitata.* The study was conducted according to OECD Guideline 201 and under GLP. Based on a range-finding test the freshwater algae was exposed to nominal concentrations of 0.03, 0.06, 0.125, 0.25, 0.50, 1.0, 2.0, and 4.0 mg/L over a period of 72 hours in the definitive test. One solution containing 100% filtered alga medium served as negative control and filter alga medium containing 0.25 % acetone served as solvent control. The test was conducted in three replicates. The initial biomass concentration used was 1 x 10^4 cells/mL. Every 24 hours cells were counted. Test temperature and pH were held within the range as recommended in the OECD Guideline.

Results: The study revealed a 72-hr E_rC_{50} of 1.60 mg/L for the freshwater algae *Pseudokirchneriella subcapitata*. No chemical analysis of the test item was carried out as part of this study. The study is rated as reliable with restrictions as the test item adhered on the sides of both plastic and glass containers during testing. Further the response variable yield was not determined as part of this study and the concentration of acetone was higher than recommended in the OECD Guideline.

Conclusion: In the study rated as Klimisch 2 a 72-hr E_rC_{50} of 1.60 mg/L based on nominal concentrations was exhibited.

A study was conducted to assess the toxicity of **4-HDPA** to the freshwater algae *Desmodesmus subspicatus* (Anonymous, 2010b). The study was performed according to OECD Guideline 201 and under GLP. Cultures of *Desmodesmus subspicatus* were exposed for a period of 72 hours to nominal concentrations of 0.1, 0.32, 1.0, 3.2, 10, 32 and 100 mg/L. The maintenance of the test item concentration was proved by HPLC/UV-VIS. During the test a temperature range of 21 – 24°C was maintained in the test vessels. The pH was measured at the beginning and the end of the exposure period. An initial biomass concentration of 5000

cells/mL was used. Per test concentration and control three replicates were implemented. The cell densities were measured at 24 hour intervals.

Results: Results are expressed in terms of geometric mean measured concentrations, as the effective concentrations ranged from 34.0 – 86.5 % of nominal values at 72 hours. The study revealed a 72-hr E_rC_{50} of 2.6 mg/l (growth rate) and a 72-hr E_yC_{50} of 0.91 mg/l (yield) based on geometric mean measured concentrations. All validity criteria of the study were met. The study is rated as valid and reliable.

Conclusion: The study revealed a 72-hr E_rC_{50} of 2.6 mg/l (growth rate) based on geometric mean measured concentrations. The study was rated as Klimisch 1.

The toxicity of **p-hydroquinone** to the test organism *Raphidocelis subcapitata* was determined in a study similar to OECD Guideline 201 and under GLP (Anonymous, 2008d, cited from ECHA dissemination site). Cultures of the test organism were exposed to nominal test concentrations of 0.005, 0.01, 0.02, 0.040, 0.10, 0.25, 0.63, 1.6, and 3.9 mg/L in two separate tests under static conditions. The used initial cell density was 0.5 x 10^4. A monitoring of the test item concentration was performed via HPLC/UV. For each test concentration three replicates were used. For the negative control six replicates were used. The degradation products were not analysed as part of this study. The test temperature was in the range of 22.6 – 23.0 °C and the pH was in the range of 7.3 – 8.1 during the whole study. At study end the growth inhibition rate was determined.

Results: At test start the measured initial concentrations were between 82 and 100% of nominal and at test end in the range of 1.4 – 5.2 % of nominal. The study resulted in a 72-hr E_rC_{50} of 0.053 mg/l (growth rate) based on geometric mean measured concentrations. The full study report was not available. The information was gained from the ECHA dissemination site and the IUCLID report. As the study was conducted according to OECD Guideline and under GLP and the results are well documented, the study is rated as reliable with restrictions. Water hardness and dissolved oxygen concentration were not reported and the response variable yield was not determined as part of this study.

Conclusion: The study rated as Klimisch 2 gained a 72-hr E_rC_{50} of 0.053 mg/l (growth rate) based on geometric mean measured concentrations.

A study investigating the toxicity of **p-benzoquinone** to the freshwater algae *Desmodesmus subspicatus* is available (Anonymous, 2018b, cited from ECHA dissemination site). The algae were exposed to nominal concentrations of 0.5, 10, 2, 3 and 10 mg/L. According to a pre-test a fluorescence measurement was performed after 0, 24, 48 and 72 hours to determine the growth curve of the test organism. An analytical monitoring of the test item concentration was not performed. The tests were performed in duplicate over a 72h period of time. The test temperature was in the main test in the range 20.0 – 22.0 °C and the pH was in the range 8.1 – 9.1.

Results: The full study report was not available. The study resulted in a 72-hr E_rC_{50} of 1.5 mg/L based on nominal concentrations and the measurement of fluorescence. A NOEC was not determined as part of this study. The study is assessed as not reliable due to missing description of the study methodology. However, the revealed 72-hr E_rC_{50} is above the cut-off value triggering a harmonised classification for acute toxicity.

Conclusion: The study rated as Klimisch 3 exhibited a 72-hr E_rC_{50} of 1.5 mg/L based on nominal concentrations and the measurement of fluorescence.

The acute toxicity of **4-HDPA** to green algae was predicted by Anonymous (2012e) using ECOSAR v1.00. The molecular weight of 185.23 g/mol and the logKow of 2.46 (calculated by the program) was used as input parameter. ECOSAR v1.00 revealed a 96-hr EC_{50} of 23.4 mg/l using class “Phenols”. As the chemical structures of substances used for derivation of the linear regression equation are not very similar to the substance to be computed, uncertainty of the revealed outcome is expected. The acute toxicity of **N-Phenyl-p-benzoquinone monoamine** to green algae was also predicted by Anonymous (2012f) using ECOSAR v1.00. The molecular weight of 183.21 g/mol and the logKow of 2.31 (calculated by the program) was used as input parameter. ECOSAR v1.00 revealed a 96-hr EC_{50} of 51.1 mg/L for the class “Vinyl/Allyl Ketones” and a 96-hr EC_{50} of 22.5 mg/L for the class “Neutral organics”. For the model “Schiff Base” no result was calculated due to lack of data. The model for the class “Vinyl/Allyl Ketones” is based on only one data point. As the chemical structures of substances used for derivation of the linear regression equation are not very

similar to the substance to be computed, uncertainty of the revealed outcome is expected. Information was achieved from the ECHA dissemination site and the IUCLID report.

Conclusion: The predicted acute toxicity using ECOSAR v1.00 for both substances yields results with high uncertainty and is therefore not reliable for classification purposes.

In a 72 hour study, the toxicity of **p-hydroquinone** to the freshwater algae *Raphidocelis subcapitata* was determined revealing a 72-hr EC_{50} value of 0.335 mg/L (Devillers et al., 1990, cited from ECHA dissemination site). The NOEC value is not reported. The study is rated as not assignable due to insufficient documentation of study procedures and testing conditions.

Conclusion: The reported 72-hr EC_{50} of 0.335 mg/L (not specified if based on nominal or measured concentrations) is rated as Klimisch 4 as there is no description of the study methodology.

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

Not relevant.

11.6 Long-term aquatic hazard

Several studies included on ECHA dissemintion site for 6PPD were conducted with tyre wear particles. These studies are not included in the CLH-dossier as no substance specific information could be retrieved from these studies.

Regarding long-term aquatic toxicity several studies are available – for the parent substance as well as for oxidation and hydrolysis products. For algae a study with N,N'-di-sec-butyl-p-phenylenediamine (44PD) is included in the 6PPD registration dossier with a MITI-study from 2008 resulting in a NOEC value of 0.096 mg/L. As no proper read-across justification could be found, the data for this potential read-across substance was not included to the CLH-dossier.

For the hydrolysis product aniline many data are available and for efficiency reasons only the most sensitive relevant study per trophic level is included in this CLH-dossier according to the Risk Assessment Report (RAR) for aniline (European Union, 2004). For fish the most sensitive study according to the EU RAR and the ECHA dissemination site is the same study. For aquatic algae and cyanobacteria the most sensitive and relevant study is resulting in a NOEC of 2 mg/L according to the EU RAR. On ECHA dissemination site for aniline this study is rated with Klimisch 2. Nevertheless, for unknown reasons this study was not chosen as a key study, but a study resulting in a higher chronic toxicity value.

According to the ECHA dissemination site for aniline and the EU RAR on aniline daphnids are the most sensitive organisms (European Union, 2004). While in the RAR and at the dissemination website an arithmetic mean of three NOEC values is presented, in this dossier only the most sensitive and reliable long-term study with daphnids is included.

Table 113: Overview on available long-term studies with 6PPD and its oxidation and hydrolysis products

	6PPD	6QDI	4-Hydroxypropylamine (4-HDPA)	p-Hydroquinone	Aniline (most sensitive relevant study per trophic level)
long term toxicity to fish	NOEC: 0.0037 mg/L (MITI, 2002; Kl. 1))			NOEC: ≥ 66 µg/L (Anonymous, 2016c, Kl. 1)	NOEC (wet weight and total length): 0.39 mg/L (Anonymous 1991, based on measured concentrations, Kl. 2)
long term toxicity to aquatic invertebrates			NOEC (reproduction): 0.028 mg/L (Anonymous, 2010c, Kl. 2)	NOEC (reproduction): 0.0029 mg/L (Anonymous, 2008e, Kl. 1) NOEC (growth): 0.039 mg/L (Anonymous, 2008e, Kl. 1)	NOEC (reproduction): 0.004 mg/L (Kühn et al. 1989b, based on extrapolated measured concentrations; Kl. 2)
long term toxicity to aquatic algae and cyano bacteria	96-h EC_{10}: 0.2 mg/L based on decrease of chlorophyll a 96-h EC_7: 0.1 mg/L and 96-h EC_{13}: 0.3 mg/L based on decrease in cell number (Anonymous, 1978c; Kl. 3)	NOE_rC: 0.50 mg/L (Anonymous, 1998, Kl. 2)	E_rC_{10}: 0.58 mg/L (Anonymous, 2010b, Kl. 1)	NOE_rC: 0.0015 mg/L (Anonymous, 2008d, Kl. 2)	NOEC (biomass): 2 mg/L (Calamari et al., 1980, based on nominal concentrations, Kl. 2)

Table 114: Summary of relevant information on chronic aquatic toxicity

Method	Species	Test material	Results	Remarks	Reference
Fish					
OECD TG 210 GLP	*Oryzias latipes*	6PPD	30d-NOEC: 0.0037 mg/L based on effects on growth (body weight and length; arithmetic and geometric mean)	Klimisch 1 (reliable without restrictions)	MITI (2003)
OECD TG 210 GLP	*Pimephales promelas*	p-Hydroquinone	32-d NOEC: ≥ 66 µg/L (based on mean measured	Klimisch 1 (reliable without restrictions)	Anonymous (2016c) [cited from

			concentrations)		ECHA dissemination site, study report not available]
Early life stage test, not further specified	*Pimephales promelas*	Aniline	32d-NOEC for growth rate (wet weight and total length): 0.39 mg/L based on measured concentrations	Klimisch 2 (reliable with restrictions)	Anonymous (1991a)
Invertebrates					
OECD Guideline 211 GLP	*Daphnia magna*	p-Hydroquinone	21d-NOEC for reproduction: 0.0029 mg/L (based on time-weighted-mean) 21d-NOEC for growth: 0.039 mg/L (based on time-weighted-mean)	Klimisch 1 (reliable without restrictions)	Anonymous (2008e) [cited from ECHA dissemination site, study report not available]
OECD Guideline 211 (2008); EU method C.20 (2008) GLP	*Daphnia magna*	4-HDPA	21d-NOEC for reproduction: 0.028 mg/L (based on mean measured concentrations)	Klimisch 2 (reliable with restrictions)	Anonymous (2010c)
Internal method of the German Federal Environmental Agency, comparable to OECD TG 211	*Daphnia magna*	Aniline	NOEC for reproduction: 0.004 mg/L (based on extrapolated measured concentrations)	Klimisch 2 (reliable with restrictions)	Kühn et al. (1989b)
Algae					
Algal Assay Procedure: Bottle Test (US-EPA 1971)	*Raphidocelis subcapitata,* formerly known as *Selenastrum capricornutum*	6PPD	96-h EC_{10}: 2 mg/L (nominal concentration; based on decrease of chlorophyll a) 96-h EC_7: 0.1 mg/L and 96-h EC_{13}: 0.3 mg/L (nominal concentration, based on decrease in cell number)	Klimisch 3 due to methodological deficiencies	Anonymous (1978c)
OECD Guideline 201 GLP	*Raphidocelis subcapitata* (formerly	6QDI	72h-NOE_rC: 0.50 mg/L (growth rate) based on	Klimisch 2 (reliable with restrictions)	Anonymous (1998)

	known as *Pseudokirchneriella subcapitata*)		nominal concentrations)		
OECD Guideline 201 (2006); EU method C.3 (2009) GLP	*Desmodesmus subspicatus*	4-HDPA	E_rC_{10}:. 0.58 mg/L (growth rate) mean measured based on geometric mean	Klimisch 1 (reliable without restrictions)	Anonymous (2010b)
Similar to OECD TG 201 GLP	*Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*)	p-Hydroquinone	72h-NOE_rC: 0.0015 mg/L (growth rate) mean measured based on geometric mean	Klimisch 2 (reliable with restrictions)	Anonymous (2008d) [cited from ECHA dissemination site, study report not available]
US EPA (1971), algal assay test as proposed by Chiaudani G. and Vighi M. (1978)	*Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*)	Aniline	96-h NOEC: 2 mg/L (biomass) based on nominal concentrations	Klimisch 2 (reliable with restrictions)	Calamari et al. (1980)

11.6.1 Chronic toxicity to fish

In an Early-Life Stage Toxicity Test according to OECD Guideline 210 the chronic toxicity of **6PPD** towards fish was tested with *Oryzias latipes* under flow-through conditions for 41 days (MITI, 2003). The study was performed according to GLP and is rated Klimisch 1. An English translation of the Japanese study report is available. The sampling for analytical monitoring (HPLC with reversed phase column) took place on day 0, 7, 14, 22, 28 and 35. 20 embryos were exposed in triplicates.

For the preparation of the test liquid dimethylformamide solution of HCO-60 (HCO-60 concentration: 2000 mg/L) was prepared. This co-solvent was diluted with distilled water so as to form a concentration of 95 % (v/v). 380 mg test material was weighed out and dissolved in this raw liquid and stepwise diluted to reach the test concentrations. The co-solvent concentration was fixed in each concentration group, and co-solvent control groups with the same concentrations were provided (dimethylformamide: about 0.1 mL/L, HCO-60: 0.2 mg/L). The purity of the test substance was 99.8%.

The test temperature and dissolved oxygen were measured at the beginning, periodically after 1 week and at the end of the test: the temperature was maintained at 24+/-1°C and the dissolved oxygen varied between 7.0 and 8.4 mg/l. The pH and the hardness were measured at the beginning and at the end of the test at all concentrations: pH varied between 7.5 and 8.0 and hardness varied between 47 and 53 mg/l.

As the measured concentrations were below 80% of the nominal concentrations, the measured concentrations were taken (see Table 115).

Table 115: Nominal and measured concentrations in FELS with 6PPD (MITI, 2003).

Nominal concentration (mg/L)	Measured concentration (mg/L) based on arithmetic mean and SD in mg/L	Measured concentration based on arithmetic mean: coefficient of variation in %	Measured concentration in % of the nominal concentration
0.002	0.00142 (0.0002)	14.1	71
0.0053	0.00371 (0.0004)	10.8	70
0.014	0.011 (0.0006)	5.5	79
0.038	0.0385 (0.001)	2.6	101
0.1	0.0937 (0.003)	3.2	94

Results: The days to hatch were significantly prolonged and the survival rates from the start and the survival rate after hatching were significantly reduced in the highest test concentration (0.0937 mg/L, $p<0.01$ for all effects) compared to solvent control, while no effect on hatching rate was observed. Only one animal survived in the highest concentration at the end of the test.

Table 116: Time to hatch, hatching rate and survival rates at the end of the test (MITI, 2003).

Measured concentration (mg/L)	Mean days to hatch (+/- SD)	Mean hatching rate % (+/- SD)	Mean survival rate from start % (+/- SD)	Mean survival rate after hatching % (+/- SD)
Control	11.03 (0.12)	93.3 (5.8)	91.7 (2.9)	98.3 (2.9)
Solvent Control	10.89 (0.06)	98.3 (2.9)	91.7 (7.6)	93.2 (5.9)
0.00142	11.11 (0.05)	100 (0.0)	93.3 (5.8)	93.3 (5.8)
0.00371	9.61 (0.36)	95.0 (5.0)	81.7 (23.1)	85.4 (21.2)
0.011	11.25 (0.25)	95.0 (0.0)	86.7 (2.9)	91.2 (3.0)
0.0385	10.77 (0.28)	93.3 (7.6)	80.0 (13.2)	86.6 (18.9)
0.0937	12.05++ (0.58)	96.7 (2.9)	1.7 (2.9)**	1.7 (2.9)**

++: Significantly different from solvent control at $p<0.01$ (by Williams multicomparison test)
**: Significantly different from solvent control at $p<0.01$ (by Dunnett multicomparison test)

At day 6 and 8 one embryo was identified with reduced activity at 0.00371 mg/L and on day 6, 8, 11 and 13 one embryo at 0.011 mg/L. The main observed abnormality in larvae and juvenile fish was paralysis occurring occasionally at all concentrations as well as in the solvent control. A higher frequency for paralysis and also abnormal swimming were noted for the highest test concentration. Starting from day 22 to day 41 in the highest test concentrations reduced feeding activity was observed, for medium concentration (0.011 mg/L) reduced feeding activity was observed from day 25 to day 36.

Significant effects on growth were observed in the three highest test concentrations: The mean total wet body weight and the mean body length were both significantly reduced (see Table 117).

Table 117: Healthy fish rate, total mean wet body weight and body length (MITI, 2003).

Measured concentration (mg/L)	Healthy fish rate % (+/- SD)	Total mean wet body weight in mg (+/- SD)	Total mean body weight coeffecient of variation in %	Total mean body length in mm (+/- SD)	Total mean body length coeffecient of variation in %
Control	91.7 (2.9)	91 (25)	27	16.9 (1.5)	8.9
Solvent Control	91.7 (7.6)	90 (23)	26	17.0 (1.7)	10.0

0.00142	93.3 (5.8)	82 (20)	24	16.6 (1.8)	10.8
0.00371	81.7 (23.1)	82 (14)	17	16.8 (1.0)	6.0
0.011	86.7 (2.9)	66 (18)**	27	15.5 (1.9)**	12.3
0.0385	80.0 (13.2)	47 (11)**	23	13.5 (1.2)**	8.9
0.0937	1.7 (2.9) ##	15 (-)++	-	8.3 (-)++	-

##: significantly different from solvent control (p<0.01) by Dunnett multicomparison test
**: significantly different from solvent control (p<0.01) by nonparametric Dunnett multicomparison test
++: Statistical comparison test not possible as only one fish had survived at the end

Conclusion: A 30d-NOEC of 0.00371 mg/L based on growth (weight and length) based on arithmetic and also according to geometric mean (calculation by DS) was identified.

The long-term toxicity of **p-hydroquinone** to the test organism *Pimephales promelas* was investigated in a study according to OECD Guideline 210 and GLP under flow-through conditions over a period of 32 days (Anonymous, 2016c, cited from ECHA dissemination site). The nominal test item concentrations were 4.6, 10, 22, 46 and 100 µg/l, which were based on results of a range-finding test. The actual concentration was analytically verified. Per test concentration and control eighty fresh fertilised eggs were randomly distributed over four test vessels. Each test vessel contained therefore 20 eggs in 1.7 litre of test medium. During the embryonic/larvae stage the eggs/larvae were observed for survival and effects on development, appearance and swimming behaviour. At the end of the test the survived fish were measured and weighed. Test temperature was in the range 24.5 – 26.0 °C and pH- values ranges 7.3 – 7.8.

Results: Analyses showed that the measured concentrations of the test item decreased as the exposure progressed with recoveries dropping to a range of 35 – 60% of target concentrations after 21 days. The arithmetic mean measured concentrations were 2.5, 5.7, 14, 28 and 66 µg/L. It was observed that p-hydroquinone did not induce significant effects on the development of the embryos, hatching or hatching success or on survival, growth and development up to and including the highest average measured concentration of 66 µg/L. Hence the 32-d NOEC of the test item p-hydroquinone for the test organism *Pimephales promelas* was ≥ 66 µg/L under the conditions of the study. The validity criteria of the study were met. The full study report was not available. However, the study was conducted under GLP and according to OECD Guideline. Further, the methodology and results were sufficiently described.

Conclusion: A 32-d NOEC of ≥ 66 µg/L based on mean measured concentrations was identified. The study is rated as Klimisch 1.

A study by Anonymous (1991a) investigating the long term toxicity of **aniline** to the organism *Pimephales promelas* is available. Embryos less than 24 hours old were exposed in a flow-through system to the test item aniline for 32 days. The actual test item concentration was measured at least twice per week. Test endpoints included the hatching rate, survival and growth effects. After study duration of 32 days a NOEC of 0.39 mg/L (wet weight and total length) was revealed which is based on measured values. The study is included in the EU RAR of aniline (European Union, 2004).

Conclusion: A 32-d NOEC of 0.39 mg/L (wet weight and total length) which is based on measured values was gained. The study is rated as Klimisch 2.

11.6.2 Chronic toxicity to aquatic invertebrates

A chronic toxicity study to aquatic invertebrates with the test item **p-hydroquinone** was available (Anonymous, 2008e, cited from ECHA dissemination site). *Daphnia magna* was exposed to p-hydroquinone over a period of 21 days according to the OECD Guideline 211 and under GLP. Daphnids were exposed to target concentrations of 0.0081, 0.016, 0.033, 0.065 and 0.13 mg/L under semi-static conditions based on a range-finding study. The test solution was replaced every 24 hours. Test concentrations were monitored via HPLC/UV in three exposure periods. Initial concentrations of the test solution were measured at day 0, 11 and 20 and the respective aged solution at day 1, 12 and 21. Ten animals were held individually at each test concentration including the negative control series. Effect parameters observed were inhibition of

reproduction, cumulative number of surviving offspring, first day of birth, mortality, body length of parent daphnias and occurrence of aborted eggs. Degradation products were not analysed in this study.

Results: Analyses revealed mean measured initial concentrations of 0.0057, 0.012, 0.027, 0.054 and 0.106 mg/L. At the beginning of the exposure periods the measured concentrations were between 68.8 – 78.6 % (day 0), 64.2 – 90.0 % (day 11) and 72.8 – 81.8 % (day 20) of nominal. During the 24 hours exposure periods the test item concentration decreased to < 7.5 – 39.2 % (day 1), < 14.8 – 50.8 % (day 12) and < 7.5 – 40.8 % (day 21) of nominal. The results are based on time-weighted-average concentrations. The following Table 118 gives a summary of the observed effect parameters.

Table 118: Summary of observed effect parameters after 21 days on *Daphnia magna* to p-hydroquinone (Anonymous, 2008e).

Time-weighted-average (mg/L)	Mortality (%)	Average cumulative surviving offspring per surviving parent	First day of birth (day)	Body length (mm)
Control	0	95.5 ± 4.4	8.1 ± 0.74	4.3 ± 0.11
0.0029	0	94.7 ± 5.3	8.0 ± 0	4.2 ± 0.07
0.0049	20	84.1 ± 6.1*	8.5 ± 0.85	4.2 ± 0.06
0.014	20	80.1 ± 9.0*	8.4 ± 0.97	4.1 ± 0.11
0.039	20	66.9 ± 11*	8.5 ± 0.85	4.2 ± 0.13
0.076	70	46.7 ± 9*	8.7 ±1.89	4.0 ± 0.26**

* Significantly different from control (NOEC calculated using Bartlett method, ANOVA, Dunett method and Kruskal-Wallis rank sum test)
** Significantly different from control (NOEC calculated using Bartlett method, ANOVA, Dunett method and Kruskal-Wallis rank sum test)

The study revealed a 21-d NOEC of 0.0029 mg/L (based on time-weighted-average) for inhibition of reproduction and a 21-d NOEC of 0.039 mg/L (based on time-weighted-average) for inhibition of growth rate. Resting eggs or male individuals were not observed. The validity criteria of the study were met. The full study report was not available. Information was gained from the ECHA dissemination site and IUCLID report. However, the study was conducted under GLP and according to OECD Guideline. Further the methodology and results were sufficiently described.

Conclusion: A 21-d NOEC of 0.0029 mg/L based on inhibition of reproduction based on time-weighted-mean was identified. The study was rated as Klimisch 1.

A study with **4-HDPA** was performed to assess the effects on the reproductive output of *Daphnia magna* under semi static conditions (Anonymous, 2010c). In the study according to OECD Guideline 211 and under GLP the daphnids were exposed to nominal concentrations of 0.0032, 0.01, 0.032, 01 and 3.2 mg/L of the test item over a period of 21 days. The actual test item concentration was verified via HPLC/UV-VIS. For each test concentration and negative control ten replicates with one daphnid in each test vessel was used. At the end of the exposure period the total number of living offspring produced per parent animal alive at the end of the test was assessed. The test temperature was in the range of 20.5 – 21.9 °C and the pH-value was in the range of 8.0 – 8.4. Growth measurements were not performed as part of this study as recommended in OECD Guideline.

Results: Table 119 gives a summary of observed effect parameters after the exposure period.

Table 119: Observed effect parameters on *Daphnia magna* after 21 days of exposure to the test item 4-HDPA (Anonymous, 2010c).

Nominal test item concentration (mg/L)	Mean measured concentration (mg/L)	Average cumulative surviving offspring per surviving parent (absolute / %)	Mortality (%)
Control	-	104.8 /100	0
0.0032	0.0028	116.8 / 111.5	10
0.01	0.0089	113.8 / 108.6	10
0.032	0.0279	102.2 / 97.5	10
0.1	0.0869	81.5* / 77.8	0
0.32	0.2884	0.9* / 0.86	10

*statistically significant using Williams Multiple Sequential t-test Procedure (α= 0.05)

The results are expressed as mean measured concentrations as the test item concentration was outside the range of 80 – 120 % of nominal concentrations. Based on biological effects observed the study revealed a 21-d NOEC for reproduction of 0.028 mg/L. The validity criteria of the study were fullfilled. The study is rated as reliable with restrictions.

Conclusion: A 21-d NOEC of 0.028 mg/L (reproduction) based on mean measured concentrations was identified. The study is rated Klimisch 2.

In a semi-static (three renewals per week) prolonged toxicity test with **aniline** using *Daphnia magna* according to an internal method of the German Federal Environmental Agency and comparable to OECD TG 211 eight test concentrations ranging nominally from 0.1 µg/L to 316 µg/L were used (Kühn et al., 1989b). During the test conducted at 25°C the daphnids were fed on fish food and activated sludge. A 21-day NOEC for reproduction of 10 µg/L based on nominal concentration was retrieved. It was not possible to analyse the real aniline concentration in the samples (detection limit of the used method was 0.1 mg/L). In an additional test vessel without daphnids and food a nominal aniline concentration of 316 µg/L was used. The aniline concentration in this sample was only 40 to 60% of the nominal concentration after two days. Therefore, a NOEC value of 4 µg/L is extrapolated from a recovery rate of 40% as the lowest analysed concentration (minimum value) obtained during the test. This extrapolation does not take into account the possibly enhanced degradation or dissipation of aniline in the presence of daphnid food. The study is included into the EU RAR on aniline (European Union, 2004).

Conclusion: In a study rated Klimisch 2 and similar to OECD TG 211 a NOEC of 0.004 mg/L based on extrapolated measured values was gained.

11.6.3 Chronic toxicity to algae or other aquatic plants

In a study with **6PPD** according to the Algal Bottle Test (US-EPA 1971) the decrease of chlorophyll a and decrease of cell numbers in exposed cultures of *Raphidocelis subcapitata*, formerly known as *Selenastrum capricornutum* was compared to control at 24, 48, 72 and 96h (Anonymous, 1978c). The nominal concentrations tested were: 0.1, 0.3, 0.6, 1 and 3 mg/L – no analytical measurements are reported.

The 96h-EC_{10} for the decrease in chlorophyll a was 0.2 mg/L. At 0.1 mg/L the cell numbers were reduced by 7%, at 0.3 by 13% after 96h. The study is rated Klimisch 3 due to significant methodological deficiencies: No exponential growth was observed throughout the incubation period.

Conclusion: The 96h-EC_{10} for decrease of chlorophyll a was 0.2 mg/L. At 0.1 mg/L the cell numbers were reduced by 7%, at 0.3 by 13% after 96h. The study is not considered reliable due to methodological deficiencies.

Another study was conducted to determine the toxicity of **6QDI** to the test organism *Raphidocelis subcapitata* (Anonymous, 1998). The study resulted in a 72-hr NOE_rC (growth rate) of 0.50 mg/L based on

nominal concentrations. The study was rated as reliable with restrictions. For detailed test description see section 11.5.3.

Conclusion: A 72-hr NOE_rC (growth rate) of 0.50 mg/L based on nominal concentrations was identified in a study rated as Klimisch 2.

One study was conducted to assess the toxicity of **4-HDPA** to freshwater algae *Desmodesmus subspicatus* (Anonymous, 2010b). The study is described in section 11.5.3. The E_rC_{10} of 0.58 mg/L (growth rate) based on geometric mean measured concentrations is valid and reliable for classification purposes.

Conclusion: In a study rated as Klimisch 1 an E_rC_{10} of 0.58 mg/L (growth rate) based on geometric mean measured concentrations was identified.

For test description on the study of Anonymous (2008d) (cited from ECHA dissemination site) see section 11.5.3. The study revealed a 72-hr NOE_rC (growth rate) of 0.0015 mg/L based on geometric mean measured concentrations for the test organism *Raphidocelis subcapitata* with the test item **p-hydroquinone**. The full study report was not available. As the study was conducted similar to OECD Guideline and under GLP and the results are well documented, the study is rated as Klimisch 2.

Conclusion: A 72-hr NOE_rC (growth rate) of 0.0015 mg/L based on geometric mean measured concentrations was revealed in a study rated as Klimisch 2.

In a study with *Raphidocelis subcapitata* the toxicity to **aniline** was investigated in a static system (Calamari et al., 1980). Algal growth was determined by measuring fluorimetric units at 48, 72 and 96 hours. Further a measurement was performed after 7 days. An analytical monitoring for the actual test item concentration was performed using gas chromatography at the beginning of the experiment and at the end of the test. As the difference between nominal and measured concentrations was < 10 %, the NOEC was referred to nominal concentrations. The study revealed a 96-h NOEC of 2 mg/L (biomass) based on nominal concentrations. The study is included into the EU RAR of aniline (European Union, 2004).

Conclusion: A 96-h NOEC of 2 mg/L (biomass) was revealed based on nominal concentrations in a study which is rated as Klimisch 2.

11.6.4 Chronic toxicity to other aquatic organisms

Not relevant.

11.7 Comparison with the CLP criteria

11.7.1 Acute aquatic hazard

As there are acute data available on fish, invertebrates and algae, there is a need to assess the criteria given in Table 4.1.0(a) of the CLP Regulation. The classification would, subsequently, be according to the most stringent outcome.

Acute (short-term) aquatic hazard classification categories for hazardous to the aquatic environment (Table 4.1.0(a) of the CLP Regulation):

Category Acute 1: (Note 1)	
96 hr LC_{50} (for fish)	≤ 1 mg/l and/or
48 hr EC_{50} (for crustacea)	≤ 1 mg/l and/or
72 or 96 hr E_rC_{50} (for algae or other aquatic plants)	≤ 1 mg/l. (Note 2)

Note 1: When classifying substances as Acute Category 1 and/or Chronic Category 1 it is necessary at the same time to indicate the appropriate M-factor(s) (see Table 4.1.3 of CLP Regulation).

Note 2: Classification shall be based on the ErC_{50} [= EC_{50} (growth rate)]. In circumstances where the basis of the EC_{50} is not specified or no ErC_{50} is recorded, classification shall be based on the lowest EC_{50} available.

Assessing the criteria of Table 4.1.0(a), leads to classification as Aquatic Acute 1, based on the lowest acute toxicity value of 6PPD as well as its relevant oxidation and hydrolysis products, which is a 24h-LC_{50} value of 0.0000793 mg/L (based on measured concentrations, calculation performed by DS), which is derived from a fish study with the oxidation product 6PPD-quinone. A CLP notification for 2-(1,3-dimethylbutylamino)-5-(2,3,4,5,6-pentadeuterioanilino)-1,4-benzoquinone (EC 955-440-4, this substance is the deuterated form of 6PPD-quinone) was found in the C&L inventory showing that this substance would need to be classified as Aquatic Acute 1 and Aquatic Chronic 1, no M-factors were provided (status: 03/2023). An overview on the current environmental (self)classifications of the transformation products of 6PPD is given in Annex III.

As oxidation/ozonation is a relevant pathway for degradation of 6PPD, the oxidation/ozonation products are considered relevant for the classification of 6PPD: 6PPD is highly reactive toward ozone (Lattimer et al., 1983; Krüger et al., 2005). Tian et al. (2021) identified 6PPD-quinone by two-deimensional NMR spectroscopy as compound present in tyre wear leachates and further demonstrated that 6PPD-quinone was formed from the reaction of 6PPD and ozone in gas phase in the lab. Klöckner et al. (2021) investigated photo-degradation of tyre wear particles and a 60% decrease of 6PPD within 72 hours was observed. The formation of 6PPD-quinone was suggested via formation of QDI. Based on the high quantity of emitted tyre wear particles and steady flux of 6PPD, it can be assumed that high loads of oxidation/ozonation products are continuously released under environmentally relevant conditions to the environment, especially near busy roads via run-off to receiving waters. EPI Suite suggests that 6PPD-quinone (water solubility: 51.3 mg/L) is more water soluble than 6PPD. Further, 6PPD-quinone is estimated to be not readily biodegradable (ref. Annex IV) and is more stable (half-life 33 hours, 23°C) than 6PPD (half-life 5 hours, 23°C) in aqueous solution. It is acknowledged that the study duration of the key study, Tian et al. (2022) is short with 24 hours. Nevertheless, the study is considered usable and relevant for classification purposes: In the CLP guidance the following is stated: *"…….durations shorter than 96 hours are generally less sensitive. However, for classification, they could be used if no acceptable data with the smaller fish for 96 hours are available or the results of these tests with different size fish or test durations would influence classification in a more hazardous category. Tests consistent with OECD Test Guideline 203 (Fish 96 hour LC_{50}) or equivalent should be used for classification."*

The study from Tian et al. (2022) is considered to be equivalent to OECD Test Guideline 203 and the use of a 24h-test is deemed acceptable, as this study with shorter study duration influence classification in a way leading to a higher M-Factor.

According to Table 4.1.3 of the CLP Regulation an acute M-Factor of 10000 is warranted.

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

According to 4.1.2.9.5. of Annex I of Regulation (EC) No 1272/2008 (CLP Regulation) substances are considered rapidly degradable in the environment if one of the following criteria holds true:

(a) if, in 28-day ready biodegradation studies, at least the following levels of degradation are achieved:

(i) tests based on dissolved organic carbon: 70 %;

(ii) tests based on oxygen depletion or carbon dioxide generation: 60 % of theoretical maximum or

(b) if, in those cases where only BOD and COD data are available, when the ratio of BOD5/COD is ≥ 0,5; or

(c) if other convincing scientific evidence is available to demonstrate that the substance can be degraded (biotically and/or abiotically) in the aquatic environment to a level > 70 % within a 28-day period.

Based on Annex II of the guidance on the application of CLP criteria, data on hydrolysis might be considered for classification purpose only when the longest half-life is shorter than 16 days. It is further clearly stated that hydrolysis is not an ultimate degradation, as degradation products might be formed, which slowly degrade. Only when it can be satisfactorily demonstrated that the hydrolysis products formed do not fulfil the criteria for classification as hazardous for the aquatic environment, data from hydrolysis studies could be considered.

6PPD undergoes rapid loss via hydrolysis and results in re-calculated half-lives for 12°C at pH 7 between 8.62 hours in buffered solution to 42 hours in algae media. The primary hydrolysis product 4-HDPA is self-classified as Aquatic Acute 1 and Aquatic Chronic 1 and the secondary hydrolysis products aniline, p-benzoquinone and p-hydroquinone have a harmonized classification as Aquatic Acute 1, H400. As these hydrolysis products are classified, 6PPD cannot be considered to be rapidly biodegradable.

6PPD is not readily biodegradable based on a 28-day test for ready biodegradability (modified MITI test (I)). The substance showed 2% degradation (based on BOD) and had no inhibitory effect on activated sludge microorganisms (CERI, 1994).

Another abiotic process to transform 6PPD is oxidation and ozonation. Photooxidation of 6PPD occurs when exposed to oxygen or ozone either on the tyre surface / road or in water.

In the atmosphere, 6PPD undergoes indirect photodegradation via rapid reaction with hydroxyl radicals, resulting in a half-life in air of 1-2 hours. Additionally, 6PPD adsorbs UV-B radiation and is expected to undergo direct photolysis. Potential tyre markers resistant to sunlight were found, namely 6QDI-OH and 6PPD-quinone. 6PPD-quinone, 6QDI, 6QDI-OH are all estimated to be not readily biodegradable. Only for 6QDI experimental data are available, which confirm that the substance is not readily biodegradable. The content of these compounds increases continuously with increasing age of tyre road wear particles.

Based on the high quantity of emitted tyre wear particles and steady flux of 6PPD, it can be assumed that high loads of oxidation and ozonation products are continuously released under environmentally relevant conditions to the environment, especially near busy roads to receiving waters.

6PPD-quinone is more stable (half-life 33 hours, 23°C) than 6PPD (half-life 5 hours, 23°C) in aqueous solutions. Monitoring shows that 6PPD-quinone occurred in waterways e.g. in California, demonstrating the relevance of this substance for classification of 6PPD, as it needs to be assumed that tyre wear particles and the 6PPD-quinone reach the aquatic environment. 6PPD-quinone was identified as the causative agent for the mortality syndrome demonstrating that 6PPD-quinone is stable enough in the aquatic environment to cause mortality towards fish.

Photooxidation is fast as investigated with substances in tyre particles, as the peak area of 6PPD decrease within 72 hours at least 60%. 6PPD-quinone is highly toxic towards *Coho salmon* and it is necessary to consider this transformation product also for classification and labelling.

Based on all information available, 6PPD can be considered as not rapidly degradable.

Long-term aquatic hazard

As there are long-term (chronic) data available on fish, invertebrates and algae, and the substance is not rapidly degradable there is a need to assess the criteria given in Table 4.1.0(b)(i) of the CLP Regulation. The classification would, subsequently, be according to the most stringent outcome.

(i) Non-rapidly degradable substances (Note 3) for which there are adequate chronic toxicity data available (Table 4.1.0(b)(i) of the CLP Regulation)

Category Chronic 1: (Note 1)	
Chronic NOEC or EC x (for fish)	≤ 0,1 mg/l and/or
Chronic NOEC or EC x (for crustacea)	≤ 0,1 mg/l and/or
Chronic NOEC or EC x (for algae or other aquatic plants)	≤ 0,1 mg/l.
Category Chronic 2:	
Chronic NOEC or EC x (for fish)	> 0,1 to ≤ 1 mg/l and/or
Chronic NOEC or EC x (for crustacea)	> 0,1 to ≤ 1 mg/l and/or
Chronic NOEC or EC x (for algae or other aquatic plants)	> 0,1 to ≤ 1 mg/l.

Assessing the criteria of Table 4.1.0(b)(i), leads to classification as Aquatic Chronic 1, based on the lowest chronic toxicity value of 6PPD as well as its relevant oxidation and hydrolysis products, which is a 72h-

NOErC of 0.0015 mg/L (growth rate) based on geometric mean measured concentrations and derived from an algae study with the hydrolysis product p-hydrochinone.

The hydrolysis product p-hydrochinone has a harmonised classification as Aquatic Acute 1, H400 with an acute M-factor of 10. Further, p-hydrochinone is self classified as Aquatic Chronic 1 with a chronic M-factor of 1 as well as Aquatic Acute 1 with an acute M-factor of 1 or 10 by the registrants (status: 03/2023, see also Annex III).

6PPD undergoes a rapid loss via hydrolysis forming primary (e.g. 4-HDPA) and secondary hydrolysis products (e.g. p-hydroquinone). As hydrolysis is a relevant pathway for degradation in the aquatic environment, the hydrolysis products are considered relevant for the classification of 6PPD with the following environmentally relevant hydrolysis rates: Re-calculation of the 6PPD hydrolysis at half-life from 24-26°C to 12°C at pH 7 results in values between 8.6 hours to 42 hours. Due to the rapid loss of 6PPD, ecotoxicity data on hydrolysis products can be used for classification, where information is required.

This classification is supported by a 30d-NOEC of 0.0037 mg/L for 6PPD for growth (weight and length; based on measured concentrations using arithmetic or geometric mean), a 21d-NOEC for daphnia of 0.0029 mg/L (reproduction, based on time-weighted-mean) for the hydrolysis product p-hydroquinone and a 21d-NOEC of 0.004 mg/L based on extrapolated minimum measured concentrations for the hydrolysis product aniline.

According to Table 4.1.3 of the CLP-Regulation a chronic M-factor of 10 is warranted for a not rapidly degradable substance.

11.8 Conclusion on Classification and Labelling for Environmental Hazards

Based on the available data it is proposed to classify 6PPD as Aquatic Acute 1; H400 (Very toxic to aquatic life) with an acute M-factor = 10000 and Aquatic Chronic 1; H410 (Very toxic to aquatic life with long lasting effects) with a chronic M-factor = 10.

12 EVALUATION OF ADDITIONAL HAZARDS

12.1 Hazardous to the ozone layer

Not assessed in this dossier.

13 ADDITIONAL LABELLING

Not relevant for this dossier.

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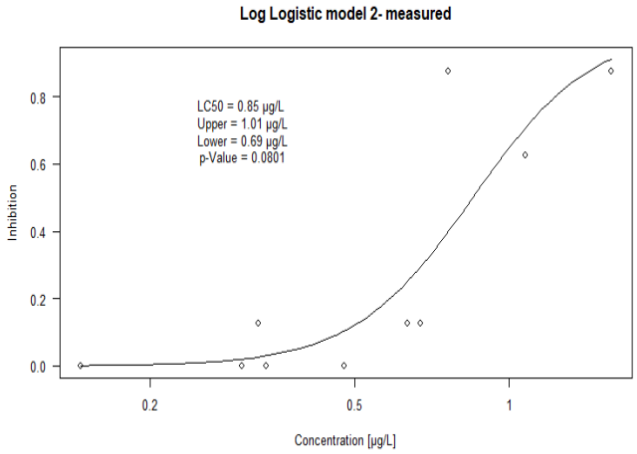
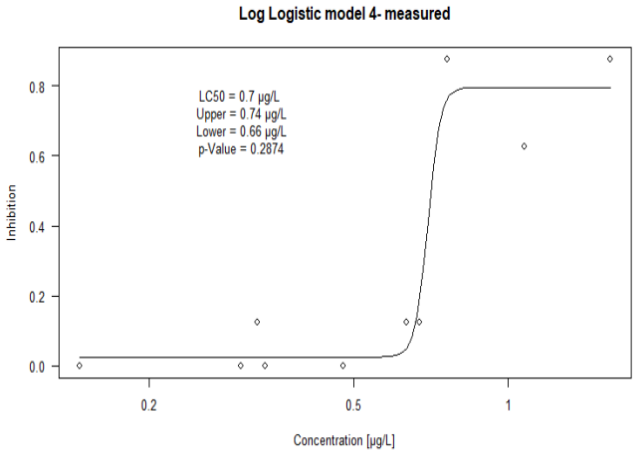
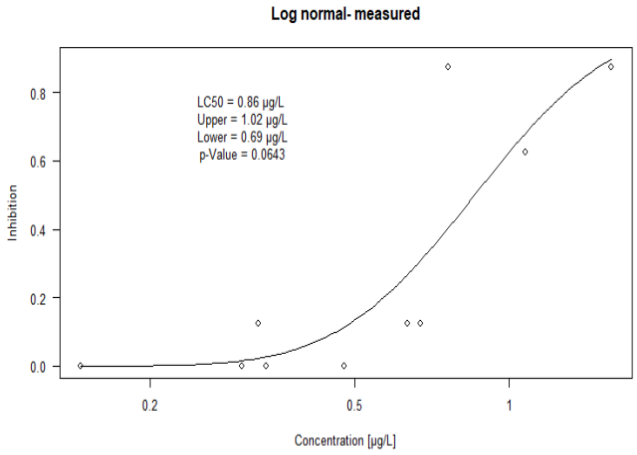
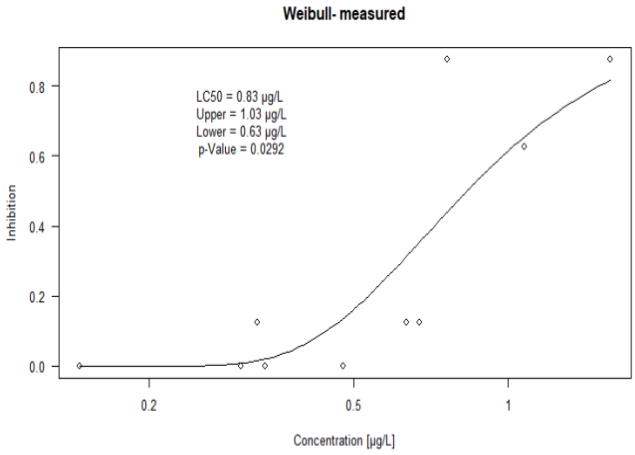
ANNEX I - ESTIMATION LC50 VALUES OF THE STUDY TIAN ET AL. (2021) USING R STATISTICAL SOFTWARE (V 1.3.1056; DRC PACKAGE V 3.0-1) BY DS

Graphs of Batch 1 of the study by Tian et al. (2021) estimated by DS using R statistical software. Measured concentrations were used for estimation of the LC_{50} values. Four different models (Log Logistic model 2, Log Logistic model 4, Log normal and Weibull) were compared.

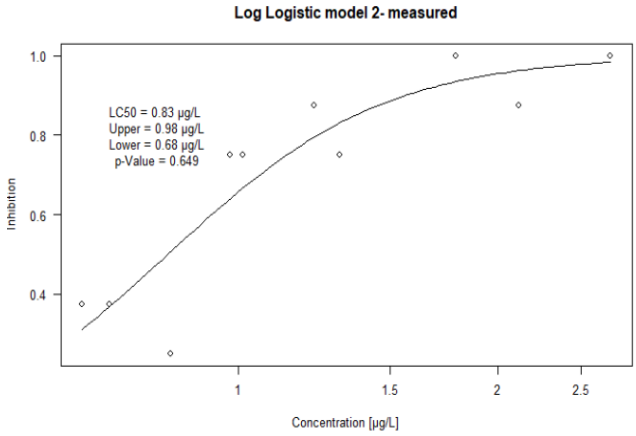


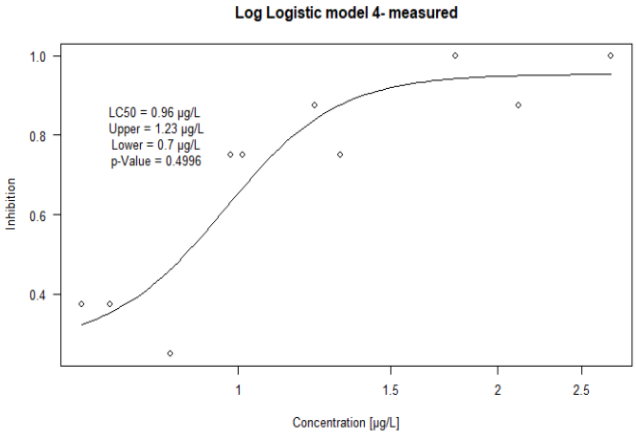


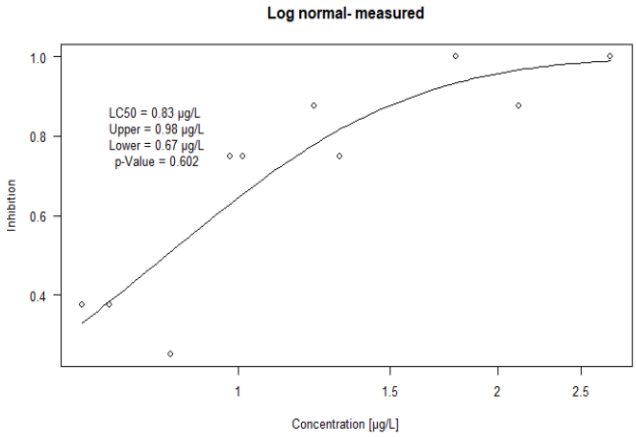


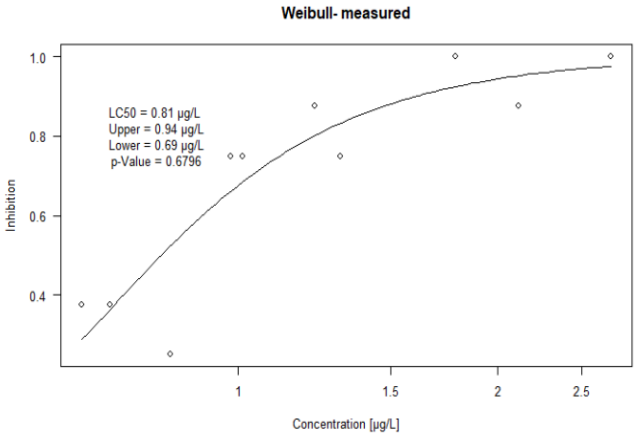


Graphs of Batch 2 of the study by Tian et al. (2021) estimated by DS using R statistical software. Measured concentrations were used for estimation of the LC_{50} values. Four different models (Log Logistic model 2, Log Logistic model 4, Log normal and Weibull) were compared.



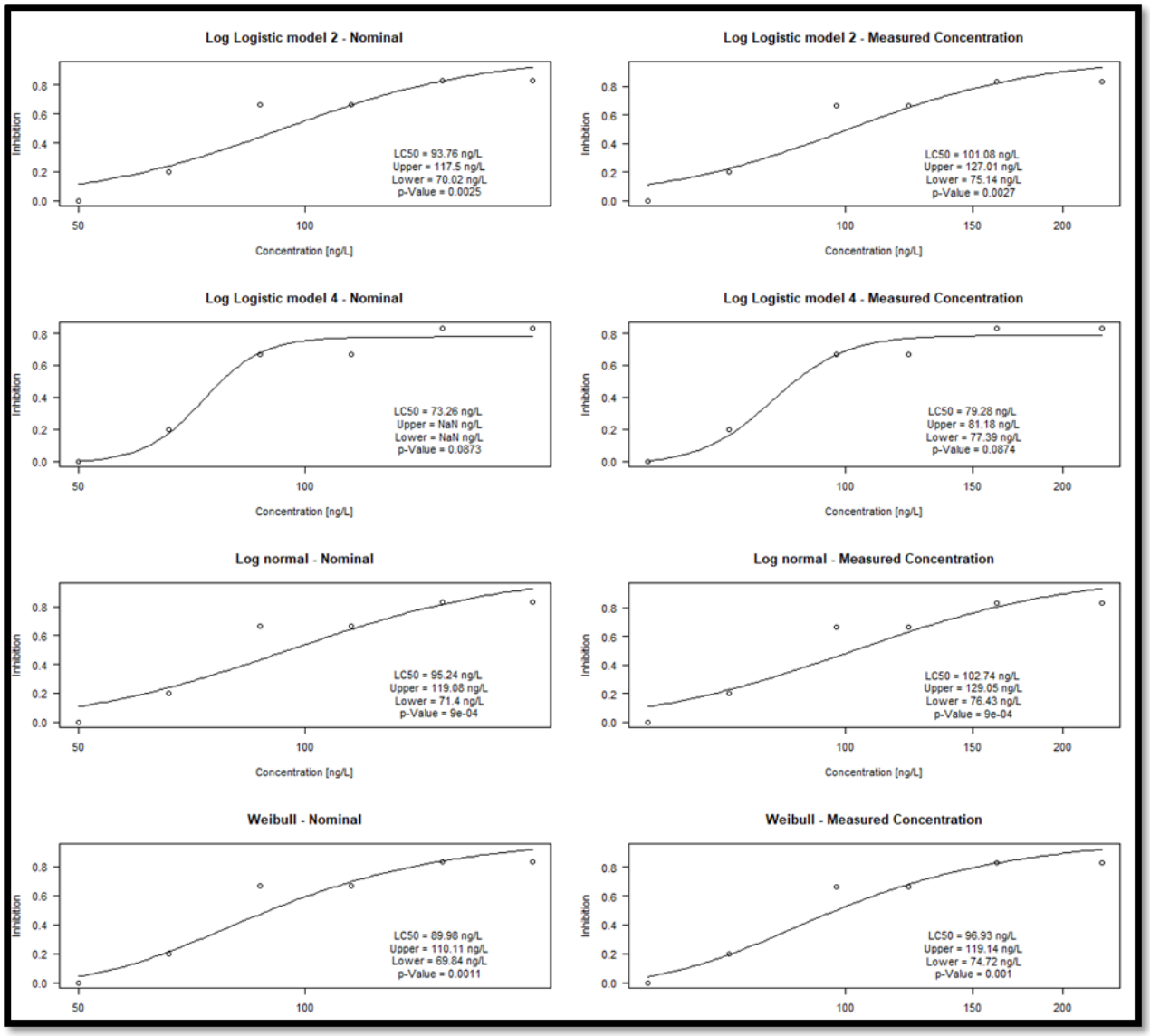




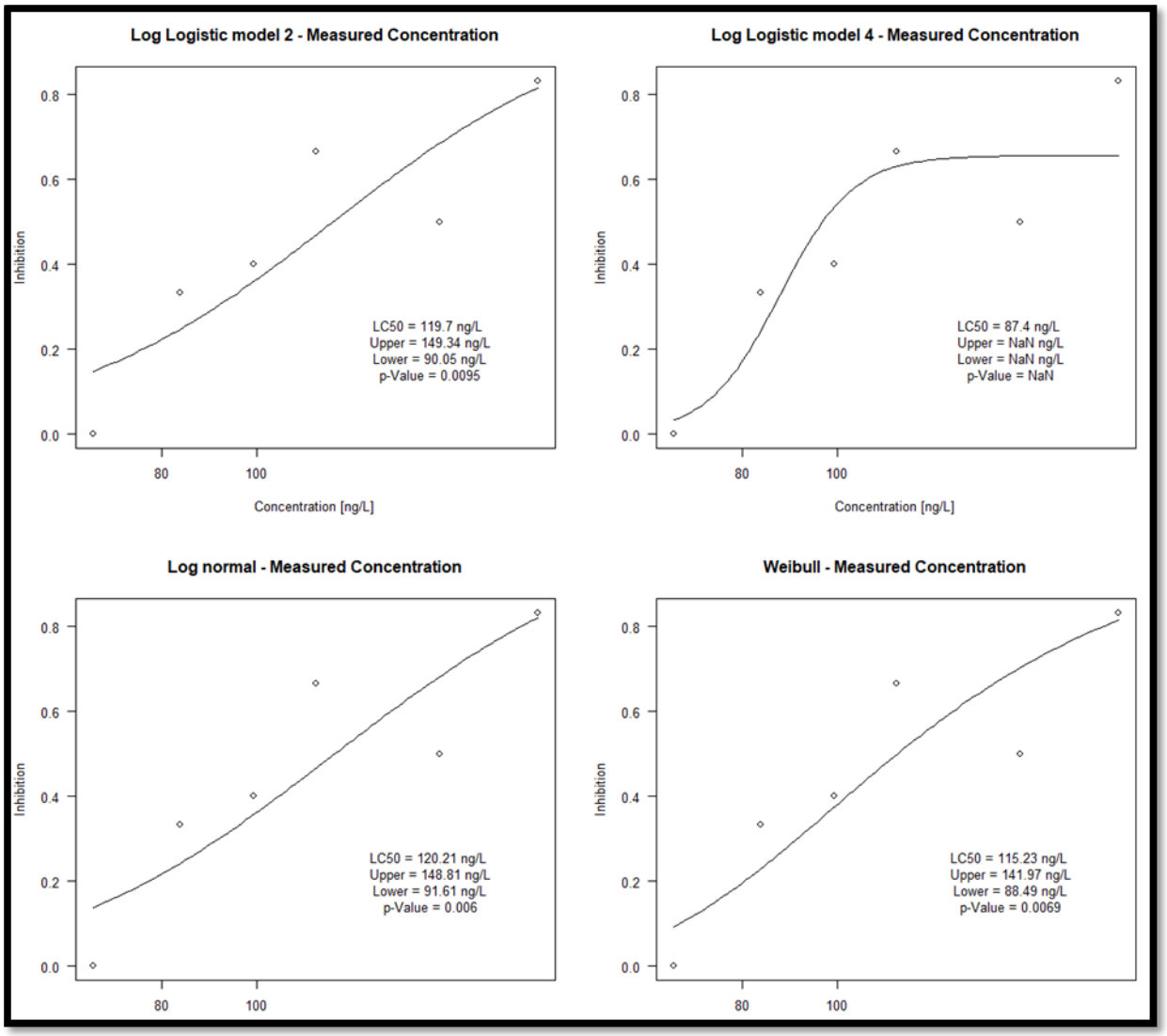


ANNEX II – ESTIMATION LC50 VALUES OF THE STUDY TIAN ET AL. (2022) USING R STATISTICAL SOFTWARE (V 1.3.1056; DRC PACKAGE V 3.0-1) BY DS

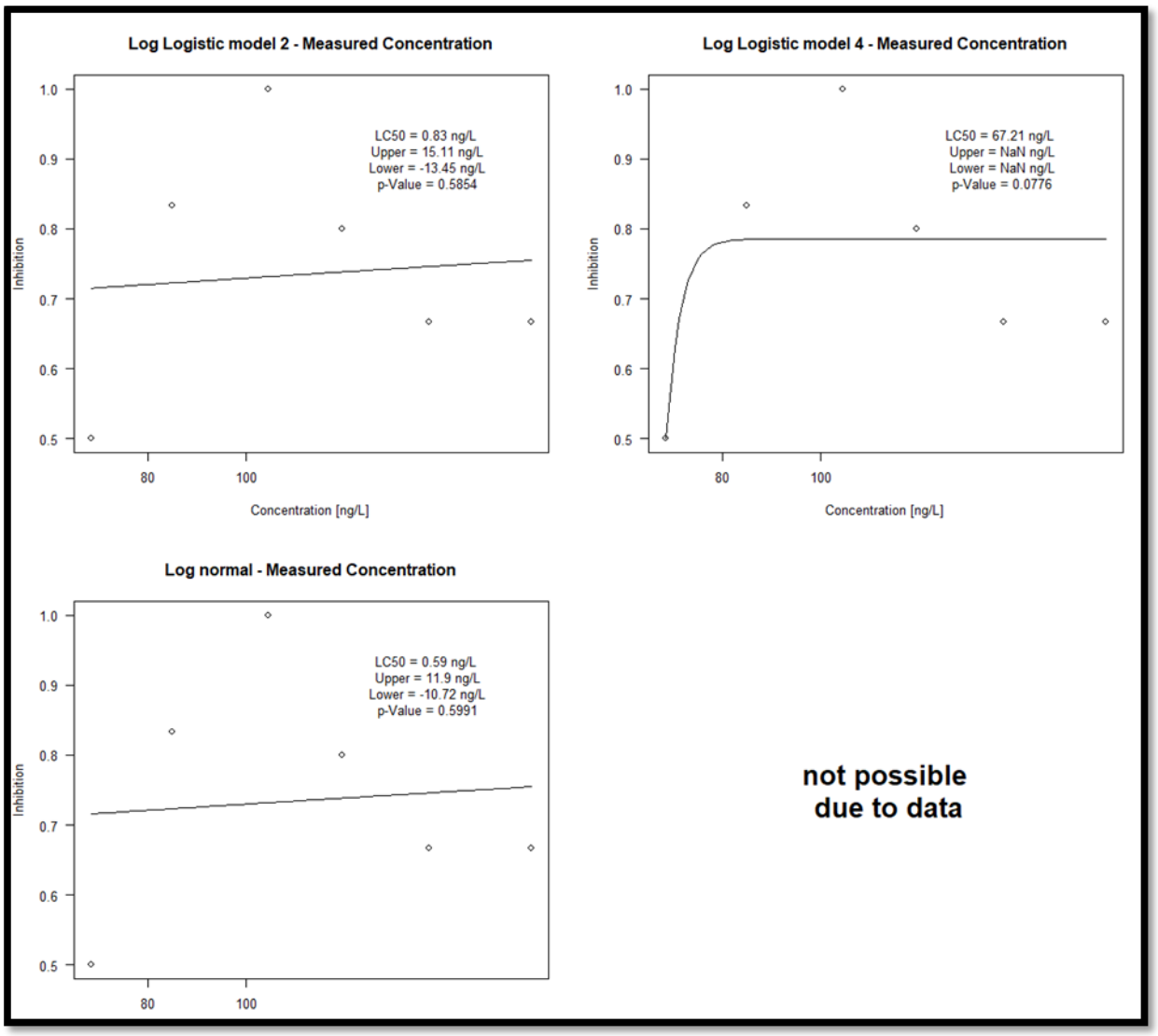
Graphs of Batch 1 of the study by Tian et al. (2022) estimated by DS using R statistical software. Measured concentrations and nominal concentration were used for estimation of the LC_{50} values. Four different models (Log Logistic model 2, Log Logistic model 4, Log normal and Weibull) were compared.



Graphs of Batch 2 of the study by Tian et al. (2022) estimated by DS using R statistical software. Measured concentrations were used for estimation of the LC_{50} values. Four different models (Log Logistic model 2, Log Logistic model 4, Log normal and Weibull) were compared.



Graphs of Batch 3 of the study by Tian et al. (2022) estimated by DS using R statistical software. Measured concentrations were used for estimation of the LC_{50} values. Four different models (Log Logistic model 2, Log Logistic model 4, Log normal and Weibull) were compared. Due to the data the estimation is not considered reliable and robust.



ANNEX III – STATUS OF CLASSIFICATION FOR AQUATIC TOXICITY OF OXIDATION AND HYDROLYSIS PRODUCTS OF 6PPD

The Annex III provides an overview on the current status of environmental classification of the hydrolysis and oxidation products of 6PPD.

For 6PPD-quinone only information on the deuterated form 2-(1,3-dimethylbutylamino)-5-(2,3,4,5,6-pentadeuterioanilino)-1,4-benzoquinone (EC No 955-440-4) is found in the C&L inventory which is presented here.

Transformation product	Current environmental CLH in Annex VI Table 3 (CLP)	Current environmental self-classification and labelling (CLP) - Summary
Oxidation products		
6QDI CAS 52870-46-9	Aquatic Acute 1 (H400); Aquatic Chronic 1 (H410) – no acute and chronic M-factor provided	Not self-classified
2-(1,3-dimethylbutylamino)-5-(2,3,4,5,6-pentadeuterioanilino)-1,4-benzoquinone – deuterated form of 6PPD-quinone EC 955-440-4	No harmonised classification	Aquatic Acute 1 (H400); Aquatic Chronic 1 (H410) – no acute and chronic M-factors provided
Hydrolysis products		
4-Hydroxydiphenylamine (4-HDPA) CAS 122-37-2	No harmonised classification	Aquatic Acute 1 (H400); Aquatic Chronic 1 (H410) – no acute and chronic M-factors provided or not self-classified;
1,3 Dimethylbutylamine CAS 108-09-8	No harmonised classification	Not self-classifiied
N-Phenyl-p-Benzoquinone monoimine -	No information available	No information available
p-Hydrochinone CAS 123-31-9	Aquatic Acute 1 (H400) with an acute M-factor of 10	Aquatic Acute 1 (H400) with an acute M-factor of 1 or 10; Aquatic Chronic 1 (H410) with a chronic M-factor of 1
p-Benzoquinone CAS 106-51-4	Aquatic Acute 1 (H400) with an acute M-factor of 10	Aquatic Acute 1 (H400) with an acute M-Factor of 10; Aquatic Chronic 1 (H410) with a chronic M-factor of 1 or 10
Aniline CAS 62-53-3	Aquatic Acute 1 (H400) – no acute M-factor provided	Aquatic Acute 1 (H400) with an acute M-factor of 1; Aquatic Chronic 1 (H410) with a chronic M-factor of 1; Aquatic Chronic 2 (H411)

ANNEX IV – ESTIMATIONS ON READY BIODEGRADABILITY

This Annex provides an overview on the rapid degradability of 6PPD and hydrolysis and oxidation/ozonation products using BIOWIN v4.11 (EPI SuiteTM).

Method	Results	Remarks	Reference
Ready biodegradability			
BIOWIN v4.11 (EPI Suite™) *N*-1,3-dimethylbutyl-*N*'-phenyl-*p*-phenylenediamine **6PPD**	Biowin 1= 0.28 (<0.5) Biowin 2 = 0.056 (<0.5) for Biowin 1 and 2, a fast degradation is defined as predicted probability >0.5 Biowin 3= 2.36 (<2.5) For Biowin 3, a fast ultimate degradation is defined as > 2.5 Biowin 4= 3.25 (<2.26 (-2.75)) A fast primary degradation is defined as > 2.5 Biowin 5= -0.38 (<0.5) Biowin 6= 0.0018 (<0.5) for Biowin 5 and 6, a fast degradation is defined as predicted probability >0.5 Biowin 7= -0.90 (<0.5) For Biowin 7 a fast degradation is defined as predicted probability > 0.5 Ready biodegradability prediction: **NO**	The substance is in the applicability domain of the used models, results are considered valid. Biowin 1 and 2: degradation is not fast, as values are below 0.5 Biowin 3: primary degradation is not fast Biowin 4: ultimate degradation is fast Biowin 5 and 6: not readily biodegradable based on linear and non-linear models Biowin 7: anaerobic degradation is not fast. The prediction for the overall degradability prediction is NO, which is based on Biowin 3 (≤2.75) and Biowin 5 results (≤0.5). According to ECHA Guidance R.11 p.49[13], Biowin 3 (value < 2.25 (to 2.75)) is considered borderline and Biowin 6 (<0.5) values are lower than the screening threshold values indicating that more degradation relevant information is warranted.	Modelling, DS
BIOWIN v4.11 (EPI SuiteTM) Transformation product after oxidation / ozonation: 2-((4-Methylpentan-2-Yl)amino)-5-(Phenylamino)cyclohexa-	Biowin 1= 0.67 (<0.5) Biowin 2= 0.24 (<0.5) Biowin 3= 2.41 (<2.5) Biowin 4= 3.31(<2.26 (-2.75)) Biowin 5= -0.06 (<0.5) Biowin 6= 0.007 (<0.5)	The prediction for the overall degradability prediction is NO, which is based on Biowin 3 (≤2.75) and Biowin 5 results (≤0.5). According to ECHA Guidance R.11 p.49[14],	Modelling, DS

[13] e56a6015-807e-46eb-b808-e5a7dc9fd572 (europa.eu)

[14] e56a6015-807e-46eb-b808-e5a7dc9fd572 (europa.eu)

Method	Results	Remarks	Reference
2,5-Diene-1,4-Dione 6PPD-Quinone (obtained in e.g. Tian et al., 2022, Hiki et al., 2021)	Biowin 7=-0.999 (<0.5) Ready biodegradability prediction: **NO**	Biowin 3 (value < 2.25 (to 2.75)) is considered borderline and Biowin 6 (<0.5) values are lower than the screening threshold values indicating that more degradation relevant information is warranted.	
BIOWIN v4.11 (EPI SuiteTM) Transformation product after oxidation / ozonation, Hydrolysis study for 6QDI available. 6QDI (mentioned in Klöckner et al., 2021)	Biowin 1= 0.75 (<0.5) Biowin 2= 0.74 (<0.5) Biowin 3= 2.63 (<2.5) Biowin 4= 3.47 (<2.26 (-2.75)) Biowin 5= -0.06 (<0.5) Biowin 6= 0.015 (<0.5) Biowin 7= -0.26 (<0.5) Ready biodegradability prediction: **NO**	Biowin 1 and 2: degradation is fast, as values are higher 0.5 Biowin 3: primary degradation is fast Biowin 4: ultimate degradation is fast Biowin 5 and 6: not readily biodegradable based on linear and non-linear models Biowin 7: anaerobic degradation is not fast. The prediction for the overall degradability prediction is NO, which is based on Biowin 3 (≤2.75) and Biowin 5 results (≤0.5). According to ECHA Guidance R.11 p.49[15], Biowin 3 (value < 2.25 (to 2.75)) is considered borderline and Biowin 6 (<0.5) values are lower than the screening threshold values indicating that more degradation relevant information is warranted.	Modelling, DS
BIOWIN v4.11 (EPI SuiteTM) Transformation product after oxidation / ozonation: 6QDI-OH obtained in (obtained in	Biowin 1= 0.90 (<0.5) Biowin 2= 0.87 (<0.5) Biowin 3= 2.757 (<2.5) Biowin 4= 3.57 (<2.26 (-2.75)) Biowin 5= 0.04 (<0.5)	Biowin 1 and 2: degradation is fast, as values are higher 0.5 Biowin 3: primary degradation is fast Biowin 4: ultimate degradation is fast	Modelling, DS

[15] e56a6015-807e-46eb-b808-e5a7dc9fd572 (europa.eu)

Method	Results	Remarks	Reference
Klöckner et al., 2021)	Biowin 6= 0.025 (<0.5) Biowin 7=-0.0551 (<0.5) Ready biodegradability prediction: **NO**	Biowin 5 and 6: not readily biodegradable based on linear and non-linear models Biowin 7: anaerobic degradation is not fast. The prediction for the overall degradability prediction is NO, which is based on Biowin 3 (≤2.75) and Biowin 5 results (≤0.5). According to ECHA Guidance R.11 p.49[16], Biowin 3 (value < 2.25 (to 2.75)) is considered borderline and Biowin 6 (<0.5) values are lower than the screening threshold values indicating that more degradation relevant information is warranted.	
BIOWIN v4.11 (EPI SuiteTM) Primary hydrolysis product of 6PPD: N-Phenyl-p-benzoquinone imine (obtained in ready biodegradability tests CERI, 1994; and Quinone-monoimine is a proposed primary hydrolysis product of PPDs)	Biowin 1= 0.79 (<0.5) Biowin 2= 0.85 (<0.5) Biowin 3= 2.79 (<2.5) Biowin 4= 3.56 (<2.26 (-2.75)) Biowin 5= 0.35 (<0.5) Biowin 6= 0.23 (<0.5) Biowin 7=-0.11 (<0.5) Ready biodegradability prediction: **NO**	Biowin 1: degradation is fast, as value is higher 0.5 Biowin 2: degradation is, as value is higher 0.5 Biowin 3: primary degradation is fast Biowin 4: ultimate degradation is fast Biowin 5 and 6: not readily biodegradable based on linear and non-linear models Biowin 7: anaerobic degradation is not fast. The prediction for the overall degradability prediction is NO, which is based on Biowin 3 (≤2.75) and Biowin 5 results (≤0.5).	Modelling, DS
BIOWIN v4.11 (EPI SuiteTM) Primary hydrolysis	Biowin 1= 0.67 (<0.5) Biowin 2= 0.76 (<0.5) Biowin 3= 2.73 (<2.5)	Biowin 1 and 2: degradation is fast, as values are higher than 0.5	Modelling, DS

[16] e56a6015-807e-46eb-b808-e5a7dc9fd572 (europa.eu)

Method	Results	Remarks	Reference
product of 6PPD: 4-anilinophenol 4-HDPA CAS 122-37-2 (obtained in several hydrolysis studies with 6PPD, 6QDI)	Biowin 4= 3.52 (<2.26 (-2.75)) Biowin 5= 0.14 (<0.5) Biowin 6= 0.077 (<0.5) Biowin 7= -0.002 (<0.5) Ready biodegradability prediction: **NO**	Biowin 3 and 4 values are higher than the QSAR based screening criteria, indicating that primary and ultimate degradation is fast. Biowin 5 and 6: not readily biodegradable based on linear and non-linear models Biowin 7: anaerobic degradation is not fast. The prediction for the overall degradability prediction is NO, which is based on Biowin 3 (≤2.75) and Biowin 5 results (≤0.5). According to ECHA Guidance R.11 p.49[17], Biowin 3 (value < 2.25 (to 2.75)) is considered borderline and Biowin 6 (<0.5) values are lower than the screening threshold values indicating that more degradation information is warranted.	
BIOWIN v4.11 (EPI SuiteTM) Primary hydrolysis product of 6PPD: 4-anilinophenol 1,3 dimethylbutylamin CAS 108-09-8 (obtained in several hydrolysis studies with 6PPD, 6QDI)	Biowin 1= 0.85 (<0.5) Biowin 2= 0.94 (<0.5) Biowin 3= 3.00 (<2.5) Biowin 4= 3.75 (<2.26 (-2.75)) Biowin 5= 0.39 (<0.5) Biowin 6= 0.40 (<0.5) Biowin 7= 0.47 (<0.5) Ready biodegradability prediction: **NO**	Biowin 1 and 2: degradation is fast, as values are higher than 0.5 Biowin 3 and 4 values are higher than the QSAR based screening criteria, indicating that primary and ultimate degradation is fast. Biowin 5 and 6: not readily biodegradable based on linear and non-linear models Biowin 7: anaerobic degradation is not fast. The prediction for the overall degradability prediction is NO, which is based on Biowin 3 (≤2.75) and Biowin 5	Modelling, DS

[17] e56a6015-807e-46eb-b808-e5a7dc9fd572 (europa.eu)

Method	Results	Remarks	Reference
		results (≤0.5).	
BIOWIN v4.11 (EPI SuiteTM) Secondary hydrolysis product of 6QDI: p-hydroquinone CAS 123-31-9 (obtained in following hydrolysis studies: 6QDI (Anonymous, 1981), and 7PPD at pH 4[18]	Biowin 1= 0.93 (<0.5) Biowin 2= 0.96 (<0.5) Biowin 3= 3.07 (<2.5) Biowin 4= 3.77 (<2.26 (-2.75)) Biowin 5= 0.55 (<0.5) Biowin 6= 0.69 (<0.5) Biowin 7= 0.62 (<0.5) Ready biodegradability prediction: **YES**	The substance is in the applicability domain of the used models, results are considered valid. Biowin 1 and 2: degradation is fast, as values are higher than 0.5 Biowin 3 and 4: values are higher than the QSAR based screening criteria, indicating that primary and ultimate degradation is fast. Biowin 5 and 6: values are higher than 0.5, indicating readily biodegradable based on linear and non-linear models. Biowin 7: anaerobic degradation is fast. The prediction for the overall degradability prediction is YES, which is based on Biowin 3 (≤2.75) and Biowin 5 results (≤0.5). Estimations confirmed by experimental ready biodegradability testing showing that the substance is readily biodegradable[19].	Modelling, DS
BIOWIN v4.11 (EPI SuiteTM) Secondary hydrolysis product of 6QDI and 7PPD: p-Benzoquinone CAS106-51-4 (obtained in following hydrolysis studies: 6QDI (Anonymous, 1981), and	Biowin 1= 0.71 (<0.5) Biowin 2= 0.64 (<0.5) Biowin 3= 2.92 (<2.5) Biowin 4= 3.65 (<2.26 (-2.75)) Biowin 5= 0.65 (<0.5) Biowin 6= 0.77 (<0.5) Biowin 7= -0.24 (<0.5) Ready biodegradability prediction: **YES**	The substance is in the applicability domain of the used models, results are considered valid. Biowin 1 and 2: degradation is fast, as values are higher than 0.5 Biowin 3 and 4: values are higher than the QSAR based screening criteria,	Modelling, DS

[18] Registration Dossier - ECHA (europa.eu)

[19] Registration Dossier - ECHA (europa.eu)

Method	Results	Remarks	Reference
7PPD at pH 4 and 7[20], and 77PD.		indicating that primary and ultimate degradation is fast. Biowin 5 and 6: values are higher than 0.5, indicating readily biodegradable based on linear and non-linear models Biowin 7: anaerobic degradation is not fast. The prediction for the overall degradability prediction is YES, which is based on Biowin 3 (≤2.75) and Biowin 5 results (≤0.5). Estimations are not confirmed, as p-Benzoquinone is considered as not readily biodegradable based on a ready biodegradability test OECD 301A[21].	
BIOWIN v4.11 (EPI SuiteTM) Secondary hydrolysis product of 6PPD, 6QDI and 7PPD: Aniline CAS 62-53-3 (obtained in several hydrolysis studies with 6PPD, 6QDI, 7PPD)	Biowin 1= 0.93 (<0.5) Biowin 2= 0.96 (<0.5) Biowin 3= 3.07 (<2.5) Biowin 4= 3.79 (<2.26 (-2.75)) Biowin 5= 0.55 (<0.5) Biowin 6= 0.69 (<0.5) Biowin 7= 0.62 (<0.5) Ready biodegradability prediction: **YES**	The substance is in the applicability domain of the used models, results are considered valid. Biowin 1 and 2: degradation is fast, as values are higher than 0.5 Biowin 3 and 4: values are higher than the QSAR based screening criteria, indicating that primary and ultimate degradation is fast. Biowin 5 and 6: values are higher than 0.5, indicating readily biodegradable based on linear and non-linear models Biowin 7: anaerobic degradation is fast.	Modelling, DS

[20] Registration Dossier - ECHA (europa.eu)

[21] Registration Dossier - ECHA (europa.eu)

Method	Results	Remarks	Reference
		The prediction for the overall degradability prediction is YES, which is based on Biowin 3 (≤2.75) and Biowin 5 results (≤0.5). Estimations are confirmed by experimental data.	