Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

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

International Chemical Identification:

Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene

EC Number: 270-128-1

CAS Number: 68411-46-1

Index Number: Not listed in Annex VI of CLP [2]

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The annex I was completed as follows:

For reproductive toxicity, all studies have been included except the range finding studies.

For environmental hazards, only studies considered reliable and in which toxicity was observed were included.

1 PHYSICAL HAZARDS

Evaluation not performed for these substances.

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

/

3 HEALTH HAZARDS

3.1 Acute toxicity - oral route

Evaluation not performed for these substances.

3.2 Acute toxicity - dermal route

Evaluation not performed for these substances.

3.3 Acute toxicity - inhalation route

Evaluation not performed for these substances.

3.4 Skin corrosion/irritation

Evaluation not performed for these substances.

3.5 Serious eye damage/eye irritation

Evaluation not performed for these substances.

3.6 Respiratory sensitisation

Evaluation not performed for these substances.

3.7 Skin sensitisation

Evaluation not performed for these substances.

3.8 Germ cell mutagenicity

Evaluation not performed for these substances.

3.9 Carcinogenicity

Evaluation not performed for these substances.

3.10 Reproductive toxicity

3.10.1 Animal data

Studies 1, 2 and 3 were performed in the same laboratory.

When DS assessment of the results deviates from the study author's assessment it is clearly indicated as DS point of view.

3.10.1.1 Study 1

Study reference:

Unpublished Study Report (2020a) Reaction products of benzeneamine, N-phenyl with nonene (branched), Reproduction/Developmental Toxicity Screening Test in Wistar Rats Administration via the Diet and recovery period of 2 weeks.

Test type

- GLP-study
- OECD TG 421 (2016)
- Deviations

Additional investigations were implemented in the study: sperm and spermatid examinations, determination of organ weights of brain, heart, kidneys, liver, spleen and thymus, several organ or tissue fixations, and histopathology of liver.

Test substance

- Test material used in the study is equivalent to **Reaction products of diphenylamine with nonene, branched** identified in the CLH dossier
- Name of test substance: Reaction products of benzeneamine, N-phenyl with nonene (branched)
- Batch identification.: 0016046440

Test animals

- Wistar Rat; Crl:WI(Han) Male/Female
- 10 per sex per dose
- Age and weight at the study initiation: 28 ±1

Administration/exposure and description of test design

- Administration via diet to groups of 10 male and 10 female Wistar rats (F0 animals) at concentrations of 0 ppm (test group 0), 500 ppm (test group 1), 1500 ppm (test group 2) and 5000 ppm (test group 3).
- The duration of treatment covered a 10-week premating period and a 2-week mating period in both sexes (mating pairs were from the same test group) as well as entire gestation period as well as 13 days of lactation period in females up to one day prior to the day of scheduled sacrifice of the animals.
- Additional treated but not mated animals (recovery animals) to groups of 10 male and 10 female animals at nominal doses of 0 (test group 10) and 5000 ppm (test group 13) was maintained for a subsequent period of at least 14 days of no test substance administration in order to observe reversibility.
- Actual doses (mg/kg bw/day):
  Controls (group 0/10): 0
  Test group 1: 500 ppm (40 mg/kg bw/d in males, 44 mg/kg bw/d in females)
  Test group 2: 1500 ppm (122 mg/kg bw/d in males, 133 mg/kg bw/d in females)
  Test group 3 / 13: 5000 ppm (397 mg/kg bw/d in males, 419 mg/kg bw/d in females)

During the lactation period reduced to 50% to maintain the dams at the desired target doses during this period of increased food intake.

- Historical control of the laboratory from 44 OECD TG 422 studies 2015-2018 (43 by gavage while the current study is by diet). HCD from the same laboratory also available in the study report of the EORGTS.
- F0 animals were mated for a maximum of two weeks after the beginning of treatment to produce a litter (F1 generation pups). As soon as sperm was detected in the vaginal smear, mating was discontinued. F0 animals were examined for their reproductive performance including determinations of the number of implantations and the calculation of the postimplantation loss in all F0 females.
- Food consumption of the F0 parents was determined regularly once weekly before and after the mating period, as well as in dams during gestation (days 0-7, 7-14, 14-20) and lactation (days 1-4, 4-7, 7-10, 10-13).
- Estrous cycle data were evaluated for all females of the pool and F0 generation females over a 10-week period prior to premating and mating until evidence of mating occurred. Moreover, the estrous stage of each female was determined on the day of scheduled sacrifice.
- The pups were sexed and examined for macroscopically evident changes on PND 0. They were weighed on PNDs 1, 4, 7 and on PND 13 and their viability was recorded. On day, 1 after birth the anogenital distance (AGD) was determined on all live male, female and uncertain pups. On PND 4, the individual litters were standardized in such a way that, whenever possible, each litter contains 4 male and 4 female pups (as a rule, the first 4 surviving pups/sex in each litter were taken for further rearing). On PND 13, all male F1 pups were examined for retention of nipples/areolae. The number of nipples/areolae anlagen were counted.
- At necropsy on PND 4, all pups were sacrificed under isoflurane anaesthesia by decapitation. Blood was sampled for determination of thyroid hormone concentrations. After sacrifice, the pups were examined externally and eviscerated, and their organs were assessed macroscopically.
- At necropsy on PND 13, one selected male and one female pup per litter were sacrificed under isoflurane anaesthesia by decapitation. Blood was sampled for determination of thyroid hormone concentrations. Thyroid glands/parathyroid glands were fixed in neutral buffered 4% formaldehyde solution and were transferred to the Pathology Laboratory for possible further processing. The remaining pups were sacrificed under isoflurane anaesthesia with CO2. After sacrifice, all pups were examined externally and eviscerated, and their organs were assessed macroscopically.
- Additionally, blood samples from all dams at PND 14 and all males at termination were taken by puncturing the retrobulbar venous plexus under isoflurane anaesthesia.

- Clinicochemical and hematological examinations were performed in 5 animals per sex and group towards the end of the administration period as well as in all animals of the recovery groups at the end of the administration period and at the end of the recovery period.
- All F0 parental animals were sacrificed by decapitation, under isoflurane anaesthesia, and were assessed by gross pathology. Weights of selected organs were recorded and a histopathological examination was performed.
- All animals of test groups 10 and 13 (recovery animals) were maintained for a 2-week recovery period after the administration period without test substance exposure. Further examinations in these animals were depend on the findings observed in the animals of the main groups. No estrous cycle was determined during the recovery period.

Results

- No animal died prematurely in the present study.
- *Food consumption:*

During in-life period (premating) decreased food consumption in female animals of group 3 during premating mating (-11.6%), gestation (-9%) and lactation (-23.5).

- *Body weight*

During the in-life period, including premating, a decreased body weight was observed in male animals of test group 3 (5000 ppm) from study days 21 to 91 (-12.0% on study day 91).
In the recovery group, no significant body weight deviations from control were determined in male animals
During premating, a decreased body weight was observed in female animals of test group 3 (5000 ppm) -10.8% on study day 70.
During the in-life phase of female animals of the recovery group 13 (5000 mg/kg bw/d), slightly to significantly reduced body weight was observed from study days 21 to 119 (up to -16.8% on study day 119).
During the whole gestation period a decreased body weight in female animals of test group 3 (5000 ppm) was observed from study days 0 to 20 with increasing deviation from control (-18.5% on gestation day 20).
A decreased body weight in female animals of test group 2 (1500 ppm) was observed on study day 20 (-8.8%).
During lactation period a decreased body weight in female animals of test group 3 (5000°ppm) was observed from lactation days 1 to 13 (-16.9% on lactation day 13) and in test group 2 (1500 ppm) only on lactation day 13 (-7.1%).
No relevant changes at lower dose levels were noted.

- *Body weight changes*

In the main groups, the body weight change was decreased in in-life period in male animals on different days of test group 3 (5000 ppm) ending up in a deviation of -16.5% from control between study days 0 and 91. A

decreased body weight change was observed in female animals of test group 3 (5000 ppm) between study days 21 to 28 as well as 0 to 70 (-20%).

In the recovery male animals of test group 13 (5000 mg/kg bw/d) body weight gain was significantly decreased only between study days 56 to 63 (-27%) resulting in a non-statistically significant decrease from study day 0 to 91 (-7%) during the entire in-life phase (administration). The recovery of the animals from the treatment is indicated by the increased body weight gain in males during the entire recovery phase (+82%).

Mean body weight gain was significantly decreased in recovery female animals of test group 13 (5000 mg/kg bw/d) on different study days 7-14, 28-35, and 49-56 of the in-life phase as well as over the entire administration period between study days 0 and 119 (-26%).

The recovery is indicated by the increased body weight gains during the recovery phase on study days 121 to 128 and the entire recovery period 121 to 134 (+155%).

During gestation period body weight change was decreased in female animals in test group 3 (5000 ppm) at all time points as well as between gestation days 0 to 20 (-31%) and in test group 2 (1500 ppm) between gestation days 14 to 20 as well as between gestation days 0 to 20 (-19%).

During lactation period body weight change was decreased in female animals in test group 2 (750 ppm) between lactation days 10 to 13 (-105%).

- *Estrous cycle*

Data, generated during the premating phase, revealed regular cycles in the females of all test groups including the control. The mean number of estrous cycle was 4.2
/ 4.3 / 4.5 and 3.9 in test groups 0 - 3. The estrous cycle length was increased in high dose group without reaching statistical significant difference to the control.

Table 1: Summary of estrous cycles report

		Test Group 0/ F 0 ppm	Test Group 1/ F 500 ppm	Test Group 2/ F 1500 ppm	Test Group 3/ F 5000 ppm
Number of Cycles	Mean	4.2 k	4.3	4.5	3.9
	S.d.	0.4	0.5	0.5	0.9
	N	10	10	10	10
Cycles Length (days)	Mean	4.0 v	4.0	4.0	4.3
	S.d.	0.0	0.0	0.1	0.5
	N	10	10	10	10
Cycling Normally (3-6 Days)	N	10	10	10	10
	%	100.0	100.0	100.0	100.0
Long Estrous (3 Days)	N	0	0	0	0
	%	0.0	0.0	0.0	0.0
Long Diestrous (4 Days)	N	0	0	0	1
	%	0.0	0.0	0.0	10.0

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
k=KRUSKALL-WALLIS; v=KRUSKALL-WALLIS-WILCOX

- *Males reproductive data*

The male mating indices calculated after the mating period to produce F1 litter were 100% in all test groups.

One control male (No. 8) and one mid-dose male (No. 23; 1500 ppm) did not generate F1 pups.

Thus, the male fertility index was 90% in test groups 0 and 2 and 100% in test groups 1 and 3. This reflects the normal range of biological variation inherent in the strain of rats used for this study and all respective values were within the range of the HCD.

Regarding the incidence of abnormal sperms in the cauda epididymidis, sperm head counts in the testis and in the cauda epididymidis no treatment-related effects were observed.

After the recovery period, in males of test group 13 (5000 ppm) motility of the sperms and sperm head counts in the testis were significantly decreased. However, the values were within historical control ranges (males, motility 79-93 %, sperm head counts in the testis 87- 126 Mio/g testis). Therefore, these alterations were regarded as incidental and not treatment-related. Sperm head counts in the cauda epididymidis and incidences of abnormal sperms were not changed.

Table 2: Spermanalysis main groups

		Test Group 0/M 0 ppm	Test Group 1/M 500 ppm	Test Group 2/M 1500 ppm	Test Group 3/M 5000 ppm
MOTILE_C	Mean	90 x-	84 *	86	89
[%]	S.d.	5	6	8	6
day 94	N	10	10	10	10
	Median	92	83	87	90
	Deviation Vs Control [%]		-7	-4	-2
TS/gT	Mean	115 x-	X	X	127
[Mio/g]	S.d.	11			21
day 94	N	10	0	0	10
	Median	114			125
	Deviation Vs Control [%]	0			10
TS/gC	Mean	571 x-	X	X	544
[Mio/g]	S.d.	110			59
day 94	N	10	0	0	10
	Median	544			565
	Deviation Vs Control [%]	0			-5
ABNORMAL5_C	Mean	5.2 x+	X	X	5.0
[%; Cut off 5%]	S.d.	0.5			0.0
day 94	N	10	0	0	10
	Median	5.0			5.0
	Deviation Vs Control [%]	0.0			-2.9

Statistic Profile = Wilcoxon with Bonferroni-Holm (one-sided+), Wilcoxon with Bonferroni-Holm (one-sided-), Wilcoxon test (one-sided-), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
x=WILCOX

Table 3: Table Spermanalysis recovery groups

		G 10 / M 0 ppm	G13 / M 5000 ppm
MOTILE_C	Mean	91 x-	87 *
[%]	S.d.	2	5
day 111	N	10	10
	Median	92	88
	Deviation Vs Control [%]		-5
TS/gT	Mean	138 x-	125 *
[Mio/g]	S.d.	18	16
day 111	N	10	10
	Median	136	126
	Deviation Vs Control [%]		-10
TS/gC	Mean	603 x-	574
[Mio/g]	S.d.	68	79
day 111	N	10	10
	Median	609	564
	Deviation Vs Control [%]		-5
ABNORMAL5_C	Mean	5.0 x+	5.3
[%; Cut off 5%]	S.d.	0.2	0.6
day 111	N	10	10
	Median	5.0	5.0
	Deviation Vs Control [%]		5.0

Statistic Profile = Wilcoxon with Bonferroni-Holm (one-sided+), Wilcoxon with Bonferroni-Holm (one-sided-), Wilcoxon test (one-sided-), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
x=WILCOX

- *Female reproduction and delivery data*

The female mating index calculated after the mating period for F1 litter was 100% in all test groups.

The mean duration until sperm was detected (GD 0) was 2.4 / 4.0 / 2.4 and 2.6 days in test groups 0 – 3, respectively.

The fertility index ranged between 90% and 100% without showing any relation to dosing.

The mean duration of gestation varied between 22.3 (test group 0), 21.9 (test group 1), 21.7** (test group 2) [**p<=0.01] and 22.1 (test group 3) days. In the absence of dose-dependency, this finding was assessed as incidental and not related to treatment-related.

The gestation index was 100% in all test groups 0-3.

The number of implantation sides was significantly decreased in test group 2 (1500 ppm, 10.9) and in test group 3 (5000 ppm, 9.9).

The values of all tested groups were in the range of the provided historical control data HCD (9.8-14.2) while the value of the concurrent control was slightly higher. However, the range of HCD for the number of implantation sites from 2008 to 2018 period provided in the study report of the OECD TG 443 (Unpublished study report, 2021) performed with Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene, Reproduction/Developmental Toxicity Screening Test by the same laboratory, was 11.1 - 15.3 sites and 11.2-15.3 when considering a more appropriate timeframe (2015-2018). When considering the later HCD, the value of the concurrent control is well within the HCD range while the values of the mid-dose and high-dose groups are outside (Table 15). HCD were largely based on studies conducted by gavage while the present study was performed in diet (only 2/40 in HCD of OECD 421 or 422).

The postimplantation loss not affected by treatment.

No treatment-related effect was observed on the live birth index (number of liveborn pups at birth/ total number of pups born x 100).

Table 4: Summary Litter Report

		Test Group 0/ F 0 ppm		Test Group 1/ F 500 ppm	Test Group 2/ F 1500 ppm	Test Group 3/ F 5000 ppm
Total Number of Pregnant Females	N	9		10	9	10
Total number of litters	N	9		10	9	10
With liveborn pups	N	9	f-	10	9	10
	%	100.0		100.0	100.0	100.0
With stillborn pups	N	0	f+	2	0	2
	%	0.0		20.0	0.0	20.0
With all pups stillborn	N	0	f+	0	0	0
	%	0.0		0.0	0.0	0.0
Implantation Sites	N	130		131	98	99
	Mean	14.4	x-	13.1	10.9 **	9.9 **
	S.d.	1.9		2.1	1.6	2.3
	N	9		10	9	10
Pups delivered	N	113		129	92	87
	Mean	12.6	x-	12.9	10.2 *	8.7 **
	S.d.	2.2		2.1	1.7	2.1
	N	9		10	9	10
Postimplantation Loss	Mean%	12.1	x+	1.6	5.7	10.4
	S.d.	15.7		3.5	11.1	16.1
	N	9		10	9	10

Statistic Profile = Wilcoxon with Bonferroni-Holm (one-sided-), Wilcoxon with Bonferroni-Holm (one-sided+), Wilcoxon test (two-sided), Fisher's exact test (one-sided-), Fisher's exact test (one-sided+), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
f=FISHER-EXACT; x=WILCOX

- *F1 generation litter/pups*

The mean number of delivered F1 pups per dam were significantly decreased in test group 2 (1500 ppm) with 10.2 pups per litter and in test group 3 (5000 ppm) with 8.7 pups per litter. The historical control range

provided in the FSR is 9.0-13.2 pups delivered per litter, while the HCD range from the EOGRTS is 10.3-14.9 and 10.9-14.9 when considering a more appropriate timeframe (2015-2018).

The viability index on DND4 and the survival index on PND13 were unaffected by treatment.

The sex distribution and sex ratios of live F1 pups on the day of birth and PND 13 was not affected.

There was decreased body weight in male and female pups as well as both sexes combined on PND 7 (-15.7% combined) and PND 13 (-18.8% combined) and decreased body weight changes in male and female pups during the postnatal period (PND 1-13 both sexes combined (-22.5%) starting on PND 4.

The anogenital distances and anogenital indexes for male and female pups of test group 3 (5000 ppm) were comparable to the current control and within the historical control range

The apparent number and percentage of male pups having areolae was not influenced by the test substance when examined on PND 13.

- *Clinical pathology*

Haematology: changes after the administration period were regarded as incidental and not treatment-related, because the values were within historical control ranges.

Clinical chemistry

The following parameters were affected at the high dose level (5000 ppm): increased alkaline phosphatase (ALP) activities in males and dams females, increased γ-glutamyl transferase (GGT) activities triglycerides and cholesterol values in dams, decreased prothrombin time (HQT), total protein and albumin values in dams and decreased glucose and cholesterol values in males.

At the mid dose level (1500 ppm) : increased alkaline phosphatase (ALP) activities and triglyceride values in and decreased total protein and albumin values in dams.

At the low dose level (500 ppm): decreased total protein and albumin values in dams.

In the recovery group increased alkaline phosphatase (ALP) activities in males and dams females while less pronounced.

Thyroid hormones

In parental males (test groups 1, 2 and 3; 500, 1500 and 5000 ppm) a non-statistically significant increase of thyroid stimulating hormone (TSH) levels was observed in both mid and high-dose male.

In male and female pups at PND13 (test groups 11, 12 and 13; 500, 1500 and 5000 ppm), no treatment-related alterations of T4 and TSH levels were observed.

Table 5: Hormones levels

		Test Group 0/M 0 ppm	Test Group 1/M 500 ppm	Test Group 2/M 1500 ppm	Test Group 3/M 5000 ppm
T4	Mean	62.84 k	63.11	63.39	57.75
[nmol/L]	S.d.	9.44	6.72	10.77	8.75
day 94	N	10	10	10	10
	Median	67.60	62.18	63.14	60.31
	Deviation Vs Control [%]		0.43	0.88	-8.09
TSH	Mean	5.94 k	6.51	8.58	8.64
[µg/L]	S.d.	1.74	2.57	3.23	2.94
day 94	N	10	10	10	10
	Median	6.12	6.01	7.93	8.78
	Deviation Vs Control [%]		9.50	44.41	45.37

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
k=KRUSKALL-WALLIS

- *Pathology*

Organ weigts

Increased liver weights in males and females and decreased ovary weight in high dose group were considered adverse. The study author did not considered the ovary weight as treatment related since it was inside the HCD range. Considering the statistical significance (despite the low number of animal per group in a screening study) and the dose-response relationship, the decrease of ovary weight is considered treatment-related and adverse from the mid dose by the DS.

Other stastistiscal difference were considered secondary to the final body weight decreased or accidental.

Effects on organ weight in the recovery group were considered secondary to the final body weight

Table 6: Summary of organ absolute weights

Absolute weights	Males			Females		
Test group (ppm)	1 (500)	2 (1500)	3 (5000)	1 (500)	2 (1500)	3 (5000)
Final body weight	97%	96%	**87%****	102%	97%	**87%****
Epididymides	98%	94%	**91%***			
Heart	97%	93%	**87%***	103%	**93%***	**87%***
Kidneys	**111%***	101%	**87%***	103%	**89%***	97%
Ovaries				94%	**82%***	**60%****

* : p <= 0.05, **: p <= 0.01

Table 7: Summary of organs relative weights

Relative weights	Males			Females		
Test group (ppm)	1 (500)	2 (1500)	3 (5000)	1 (500)	2 (1500)	3 (5000)
Brain	102%	107%	**116%****	99%	101%	**114%***
Kidneys	**116%****	**110%***	104%	102%	93%	**114%****
Liver	103%	**112%****	**127%****	**105%***	**112%****	**125%****
Ovaries				93%	85%	**69%****
Thyroid glands	101%	100%	**119%***			

* : p <= 0.05, **: p <= 0.01

Histopathology

Treatment-related findings were observed in the liver of male and females with incidences and grading according reported in the Table below

Table 8: Selected histopathological findings.

	Male animals		Female animals	
Test group (ppm)	10 (0)	13 (5000)	10 (0)	13 (5000)
No. of animals	10	10	10	10
Fatty change, midzonal	0	3	0	0
• Grade 1		3		
Fatty change, periportal	0	0	0	3
• Grade 1				3

No histopathological findings were noted in the ovaries.

Thyroid was not investigated.

Conclusion

The study author proposed the following NOAELs

The NOAEL for general systemic toxicity was 500 ppm for male (40 mg/kg bw/d) and female rats (44 mg/kg bw/d) based on altered liver parameters in clinical pathology and pathology.

The NOAEL for reproductive performance and fertility was 1500 ppm for male (122 mg/kg bw/d) and female (133 mg/kg bw/d) rats.

The NOAEL for developmental toxicity in the offspring was 1500 ppm (133 mg/kg bw/d in parental females).

- The DS considers that effects relevant for classification for fertility are observed (decreased number of implantation and sonsequent decreased litter size as well as decreased ovary weights are adverse from the mid dose level.

3.10.1.2 Study 2

Study reference

Unpublished Study Report (2020b) Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene, Reproduction/Developmental Toxicity Screening Test in Wistar Rats Administration via the Diet.

Test type

- GLP-study
- OECD TG 421 (2016)
- Deviations

Only 6 F pregnant in the low dose group while 8 per group is the minimum acceptable number according to the TG.

Additional investigations were implemented: Sperm and spermatid examinations, determination of organ weights of brain, heart, kidneys, liver, spleen and thymus, several organ or tissue fixations, and histopathology of liver. Histopathology of the liver in males and females and hormone measurement (TSH T4) in females

Test substance

- Test material used in the study is equivalent **to Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene** identified in the CLH dossier
- Name of test substance: Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene
- Batch No.: 50116118D

Test animals

- Wistar Rat; Crl:WI(Han) Male/Female
- 10 per sex per dose
- Age and weight at the study initiation: 27 ±1

Administration/exposure and description of test design

- Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene was administered via diet to groups of 10 male and 10 female Wistar rats (F0 animals) at concentrations of 0 ppm (test group 0), 300 ppm (test group 1), 1000 ppm (test group 2) and 3000 ppm (test group 3).
- The duration of treatment covered a 10-week premating period and a 2-week mating period in both sexes (mating pairs were from the same test group) as well as entire gestation period as well as 13 days of lactation period in females up to one day prior to the day of scheduled sacrifice of the animals.
- Actual doses (mg/kg bw/day):
  Test group 0 (control): 0
  Test group 1: 300 ppm (26 mg/kg bw/d in parental males, 28 mg/kg bw/d in parental females)
  Test group 2: 1000 ppm (87 mg/kg bw/d in parental males, 95 mg/kg bw/d in parental females)
  Test group 3: 3000 ppm (260 mg/kg bw/d in parental males, 271 mg/kg bw/d in parental females)
  During the lactation period reduced to 50% to maintain the dams at the desired target doses during this period of increased food intake.
- Historical control of the laboratory from 44 OECD TG 422 studies 2015-2018 (43 by gavage while the current study is by diet). HCD from the same laboratory also available in the study report of the EORGTS.

Description of test design:

- F0 animals were mated for a maximum of two weeks after the beginning of treatment to produce a litter (F1 generation pups). As soon as sperm was detected in the vaginal smear, mating was discontinued. F0 animals were examined for their reproductive performance including

determinations of the number of implantations and the calculation of the postimplantation loss in all F0 females.

- Food consumption of the F0 parents was determined regularly once weekly before and after the mating period, as well as in dams during gestation (days 0-7, 7-14, 14-20) and lactation (days 1-4, 4-7, 7-10, 10-13).
- In general, the body weights of F0 animals were determined once respectively twice a week. However, during gestation and lactation, F0 females were weighed on gestation days (GD) 0, 7, 14 and 20, and on postnatal days (PND) 1, 4, 7, 10 and 13.
- Estrous cycle data were evaluated for all females of the pool and F0 generation females over a 3-week period prior to premating and mating until evidence of mating occurred. Moreover, the estrous stage of each female was determined on the day of scheduled sacrifice.
- The pups were sexed and examined for macroscopically evident changes on PND 0. They were weighed on PNDs 1, 4, 7 and on PND 13 and their viability was recorded. One day after birth, the anogenital distance (AGD) was determined on all live male, female and uncertain pups. On PND 4, the individual litters were standardized in such a way that, whenever possible, each litter contains 4 male and 4 female pups (as a rule, the first 4 surviving pups/sex in each litter were taken for further rearing). On PND 13, all male F1 pups were examined for retention of nipples/areolae. The number of nipples/areolae anlagen were counted.
- At necropsy on PNDs 4 and 13, all pups were sacrificed with CO2 under isoflurane anaesthesia and examined macroscopically for external and visceral findings. Blood samples were taken from all surplus pups at PND 4 as well as one male and one female pup per litter at PND 13 by decapitation under isoflurane anesthesia. Additionally, blood samples from all dams at PND 14 and all males at termination were taken by puncturing the retrobulbar venous plexus under isoflurane anesthesia. Thyroid glands/parathyroid glands were fixed in neutral buffered 4% formaldehyde solution and transferred to the Laboratory Pathology for possible further processing.
- All F0 parental animals were sacrificed by decapitation under isoflurane anaesthesia and were assessed by gross pathology. Weights of selected organs were recorded, and a histopathological examination was performed.

Results

- No treatment-related death or clinical findings were observed in male and female *animals*
- *Food consumption*

During In-life period food consumption in male animals of test group 3 (3000 ppm) was significantly decreased on study days 0 to 14 (up to -8.8%) and on study days 42 to 49 (-22.5%).

During gestation mean food consumption was significantly decreased in female animals of test group 3 (3000 ppm) on gestational days 0 to 20 (-22.9%; up to -25.1% on gestational days 0 to 7) and in test group 2 (1000 ppm) on gestational days 0 to 20 (-11.8%; up to -12.9% on gestational days 14 to 20).
During lactation mean food consumption was significantly decreased in female animals of test group 3 (1500 ppm) on lactation days 1 to 13 (-19.7%; up to -23.5% on lactation day 1 to 4).

- *Body weight*

In test group 3 (3000 ppm) a decreased body weight in male animals was observed during the in-life period from study days 28 to 84 (up to -9.3% on study day 49; -9.0% on study day 84 towards end of administration) and in female animals during pre-mating from study days 35 to 70 (up to -9.4% on study day 70). In test group 2 (1000 ppm) a decreased body weight was observed only in females and only on study day 70 during pre-mating (-6.9%).
During gestation period a decreased body weight in female animals of test group 3 (3000 ppm) was observed from study days 0 to 20 (up to -16.8% on gestational day 14; -16.7% on gestational day 20) and in test group 2 (1000 ppm) from study days 7 to 20 (up to -10.0% on study day 14; -8.2% on gestation day 20).
During lactation period a decreased body weight in female animals of test group 3 (1500 ppm) was observed from lactation days 1 to 13 (up to -15.8% on lactation day 10; -14.9% on lactation day 13) and in test group 2 (500 ppm) from lactation days 1 to 13 (up to -9.2% on lactation day 1; -8.3% on lactation day 13).
In test group 1 (300 ppm) no treatment-related effect on body weight was observed for males and females at any time period of the study.

- *Body weight changes*

The body weight change was decreased in in-life period in male animals of test group 3 (3000 ppm) between study days 0-7, 14-28, and 35-49, as well as over the in-life period from study day 0 to 84 (-11.7%) and decreased in female animals in test group 3 (3000 ppm) between pre-mating days 21-28 as well as over the entire pre-mating period (-14.3%), and in females of test group 2 (1000 ppm) between study days 0 and 70 (-10.9%).
In test group 1 (300 ppm) no treatment-related effect on body weight change was observed for males and females at any time period of the study. The statistically significant decrease of body weight change of females between pre-mating days 28-35 was considered as incidental and not related to treatment. The corresponding overall body weight change in females of test group 1 (300 ppm) from pre-mating day 0 to 70 was comparable to control.
In test group 3 (3000 ppm) and test group 2 (1000 ppm), during gestation period body weight changes were decreased in female animals between gestation days 0-14 resulting in decreased body weight changes from gestation days 0 to 20 (-31.7% and -14.9%, respectively).
During gestation the body weight change in test group 1 (300 ppm) was comparable to control. During lactation, the body weight changes in all test groups were comparable to control.

- *Estrous cycle*

In test group 3 (3000 ppm) the estrous cycle length was prolonged significantly (4.7 days).
The number of estrous cycles in the observation period was lower (3.8 vs. 4.3 in control) but not statistically significant. The reduced number of estrous cycles might be related to the longer estrous cycle length but was in the historical control range for the estrous cycle number 2.1-4.6 cycles.

Table 9: Summary of estrous cycles report

		Test Group 0/ F 0 ppm	Test Group 1/ F 300 ppm	Test Group 2/ F 1000 ppm	Test Group 3/ F 3000 ppm
Number of Cycles	Mean	4.3 v	4.7	4.8	3.8
	S.d.	0.5	0.5	0.4	0.6
	N	10	10	10	10
Cycles Length (days)	Mean	4.0 v	4.0	3.9	4.7 **
	S.d.	0.3	0.3	0.1	0.7
	N	10	10	10	10
Cycling Normally (3-6 Days)	N	10	10	10	10
	%	100.0	100.0	100.0	100.0
Long Estrous (3 Days)	N	0	0	0	0
	%	0.0	0.0	0.0	0.0
Long Diestrous (4 Days)	N	0	1	0	1
	%	0.0	10.0	0.0	10.0

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
v=KRUSKALL-WALLIS-WILCOX

- *Males reproductive data*

The male mating indices calculated after the mating period to produce F1 litter were 100% in all test groups.
No treatment-related finding in fertility was observed for the F0 parental males. However, not all parental males produced F1 litters. The male fertility index was in test group 0 and 3 (3000 ppm) 90% and in test group 2 (1000 ppm) 80%. These values are within the historical control range of the male fertility index (80-100%). In test group 1 (300 ppm) the male fertility index was only 60%. Since this decrease in male fertility index was not observed in dose dependency, it was assessed as incidental and not related to treatment.

Table 10: Male fertility indices for F0 males

	Test group 0 (0 ppm)	Test group 1 (300 ppm)	Test group 2 (1000 pm)	Test group 3 (3000 ppm)
Male fertility index [%]	90.0	60.0	80.0	90.0

* p ≤ 0.05; ** p ≤ 0.01

Concerning motility of the sperms and the incidence of abnormal sperms in the cauda epididymidis as well as sperm head counts in the testis, no treatment-related effects were observed. Sperm head counts in the cauda epididymidis of males in test group 3 (3000 ppm) were significantly decreased, but the values were within the historical control range (males, sperm head counts in the cauda epididymidis 520-890 Mio/g) and similar to that of controls in Study 1. No histologic alteration in the epididymis was observed. Therefore, this unique change in the sperm analysis was regarded as non-adverse if at all treatment-related.
Regarding the incidence of abnormal sperms in the cauda epididymidis, sperm head counts in the testis and in the cauda epididymidis no treatment-related effects were observed.

Table 11: Spermanalysis

		Test Group 0/M 0 ppm	Test Group 1/M 300 ppm	Test Group 2/M 1000 ppm	Test Group 3/M 3000 ppm
MOTILE_C	Mean	89 x-	89	86	87
[%]	S.d.	4	4	3	5
day 87	N	10	10	10	10
	Median	90	88	87	88
	Deviation Vs Control [%]		0	-3	-2
TS/gT	Mean	103 x-	X	X	102
[Mio/g]	S.d.	25			15
day 87	N	10	0	0	10
	Median	99			98
	Deviation Vs Control [%]	0			-2
TS/gC	Mean	801 x-	X	X	619 **
[Mio/g]	S.d.	157			123
day 87	N	10	0	0	10
	Median	806			639
	Deviation Vs Control [%]	0			-23
ABNORMAL5_C	Mean	5.0 x+	X	X	5.2
[%; Cut off 5%]	S.d.	0.0			0.3
day 87	N	10	0	0	10
	Median	5.0			5.0
	Deviation Vs Control [%]	0.0			3.0

Statistic Profile = Wilcoxon with Bonferroni-Holm (one-sided+), Wilcoxon with Bonferroni-Holm (one-sided-), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
x=WILCOX

- *Female reproduction and delivery data*

The female mating index calculated after the mating period for F1 litter was 100% in all test groups.

The mean duration until sperm was detected (GD 0) was 2.8 days for test group 0 (0 ppm), 1.6 days for test group 1 (300 ppm), 2.2 days for test group 2 (1000 ppm) and 2.6 days for test group 3 (3000 ppm).

No treatment-related finding in fertility was observed for the F0 parental females. However, not all parental females produced F1 litters. The female fertility index was in test group 0 (control) and 3 (3000 ppm) 90% and in test group 2 (1000 ppm) 80%. These values are within the historical control range of the male fertility index (80-100%). In test group 1 (300 ppm) the female fertility index was only 60%. Since this decrease in female fertility index was not observed in dose dependency, it was assessed as incidental and not related to treatment.

The mean duration of gestation was comparable in all groups and the gestation index was 100% in all test groups.

The mean number of implantation sites was significantly lower (-36%) in the high-dose group compared to the concurrent control group (8.8 vs 13.8 respectively). In the mid-dose group, while non-statistically significant, a decrease of 14% as compared to control group was already observed (Table 17).

Considering the statistical significance at the high-dose (despite the low number of animal per group in a screening study) and the dose-response relationship, these changes are considered by the DS to be treatment-related and adverse in view of their magnitude.

The postimplantation loss not affected by treatment.

No treatment-related effect was observed on the live birth index (number of liveborn pups at birth/ total number of pups born x 100).

Table 12: Litter report

		Test Group 0/ F 0 ppm	Test Group 1/ F 300 ppm	Test Group 2/ F 1000 ppm	Test Group 3/ F 3000 ppm
Total Number of Pregnant Females	N	9	6	8	9
Total number of litters	N	9	6	8	9
With liveborn pups	N	9 f-	6	8	9
	%	100.0	100.0	100.0	100.0
With stillborn pups	N	0 f+	0	1	0
	%	0.0	0.0	12.5	0.0
With all pups stillborn	N	0 f+	0	0	0
	%	0.0	0.0	0.0	0.0
Implantation Sites	N	124	84	95	79
	Mean	13.8 x-	14.0	11.9	8.8 **
	S.d.	2.0	1.1	1.4	1.6
	N	9	6	8	9
Pups delivered	N	110	84	92	72
	Mean	12.2 x-	14.0	11.5	8.0 **
	S.d.	3.2	1.1	1.6	2.0
	N	9	6	8	9
Postimplantation Loss	Mean%	11.5 x+	0.0	4.3	9.7
	S.d.	20.7	0.0	4.6	8.4
	N	9	6	8	9

Statistic Profile = Wilcoxon with Bonferroni-Holm (one-sided-), Wilcoxon with Bonferroni-Holm (one-sided+), Wilcoxon test (two-sided), Fisher's exact test (one-sided-), Fisher's exact test (one-sided+), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
f=FISHER-EXACT; x=WILCOX

- *F1 generation litter/pups*

Consequently to the decreased number of implantation sites, the mean number of delivered F1 pups per dam was significantly decreased (-34%) in the high-dose group with 8.0 pups per litter. The mean number of delivered F1 pups per dam were non-significantly decreased in test group 2 (1000 ppm) with 11.5 pups per litter. The historical control range provided in the FSR is 9.0-13.2 pups delivered per litter, while the HCD range from the EOGRTS is 10.3-14.9 and 10.9-14.9 when considering a more appropriate timeframe (2015-2018).

Considering the dose-response relationship, these changes are considered treatment-related. Considering their nature and magnitude, the DS consider them adverse.

The viability index on DND4 and the survival index on PND13 were unaffected by treatment.

The sex distribution and sex ratios of live F1 pups on the day of birth and PND 13 was not affected.

At both mid and high-dose levels, a significant decrease of mean pup body weight was observed from PND 7 to termination (-8% and -26% both sexes combined on PND13 at 1000 ppm and 3000 ppm respectively) as well as a significant decrease of mean pup body weight changes.

The anogenital distances and anogenital indexes for male and female pups were not affected by treatment.

There was an increased incidence of nipple development (100% vs. 79.6% in control) and number of nipples per animal (5.2 to control 2.5) on postnatal day 13. This observation is at least partially related to the delay of general development in male pups. This assessment is supported by looking at the individual data were the male pups with the highest number of nipples (n=8) represent the male pups with the lowest body weights in this test group (19.9- 22.2 g).

Table 13: Nipple retention on PND13

		Test Group 0/ F 0 ppm	Test Group 1/ F 300 ppm	Test Group 2/ F 1000 ppm	Test Group 3/ F 3000 ppm
Nipple development	Passed				
[Incidence]	-N	28	21	26	29
day 13 Males	-%	80	88	79	100
	Failed				
	-N	7	3	7	0
	-%	20	12	21	
Nipple development [%]	Mean	79.6 x+	87.5	80.6	100.0 *
[%]	S.d.	17.24	20.92	30.52	0.00
day 13 Males	N	9	6	8	9
Nipple Number	Mean	2.5 x+	2.9	3.2	5.2 **
[#]	S.d.	1.43	1.20	1.89	1.14
day 13 Males	N	9	6	8	9

Statistic Profile = Wilcoxon with Bonferroni-Holm (one-sided+), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
x=WILCOX

- *Clinical pathology*

Haemathology: No treatment-related changes among hematological parameters were observed.

Clinical chemistry

In male and female rats of test groups 2 and 3 (1000 and 3000 ppm) alkaline phosphatase activities were significantly increased. The same was true for ALP activities in females of test group 1 (300 ppm). Additionally, in females of test group 3 (3000 ppm) γ-glutamyl transferase (GGT) activities were significantly increased. In males and females of test groups 2 and 3 total bile acid (TBA) levels were significantly decreased. Additionally, in both sexes of test group 3 triglyceride levels were significantly increased. In dams of test groups 1 and 2 triglyceride values were already higher compared to controls (in test group 1 not statistically significantly). In dams of test group 3, albumin levels were significantly decreased whereas cholesterol levels were significantly increased. Total bilirubin values were significantly decreased in males of test group 3, but they were increased in females (not statistically significantly). These mentioned alterations were regarded as treatment-related and adverse.

Thyroid hormones

In parental males of test group 3 (3000 ppm) T4 values were significantly decreased and TSH values were significantly increased.

In parental males of test group 2 (1000 ppm) T4 values were already significantly decreased. However, T4 and TSH mean were within historical control ranges (F0 males, T4 44.65-73.22 nmol/L; TSH 4.32-9.80 µg/L). In male PND13 pups of test group 12 (1000 ppm) T4 values were significantly increased, but the T4 mean as well as the TSH mean in among these individuals were within historical control ranges (PND13 males, T4 46.18-76.60 nmol/L; TSH 3.00-5.34 µg/L). In female PND13 pups of test groups 11, 12 and 13 (300, 1000 and 3000 ppm) as well as in F0 dams at PND14 of test groups 1, 2 and 3 (300, 1000 and 3000 ppm) no T4 and TSH changes were observed.

Table 14: Hormones levels in adult males

		Test Group 0/M 0 ppm	Test Group 1/M 300 ppm	Test Group 2/M 1000 ppm	Test Group 3/M 3000 ppm
T4	Mean	63.39 v	55.80	56.31 *	45.80 **
[nmol/L]	S.d.	7.92	6.82	5.84	7.74
day 87	N	10	10	10	10
	Median	63.41	56.50	56.03	43.30
	Deviation Vs Control [%]		-11.98	-11.16	-27.75
TSH	Mean	9.36 v	9.51	9.67	22.10 **
[µg/L]	S.d.	3.31	4.95	2.67	9.68
day 87	N	10	10	10	10
	Median	9.20	8.07	9.60	19.87
	Deviation Vs Control [%]		1.60	3.23	136.01

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
v=KRUSKALL-WALLIS-WILCOX

- *Pathology*

Organ weights

When compared to control group 0 (=100%), the mean absolute liver weights was significantly increased in males. The mean ovary absolute weight was significantly increase in high dose females, which was considered by the study author as secondary to the decreased body weight. The DS considers that a treatment-related effect cannot be excluded.

When compared to control group 0 (=100%), the mean relative liver weights were significantly increased from the low dose in males and from the mid dose in females.

The significant relative weight increases of the brain in males and females of test group 3 were secondary to their respective final body weight.

The significant relative weight increase of the thyroid glands in males of test group 3 correlated with histopathological and hormonal changes and was assessed as treatment-related.

Table 15: Summary of organs absolute weights

Absolute weights	Males			Females		
Test group (ppm)	1 (300)	2 (1000)	3 (3000)	1 (300)	2 (1000)	3 (3000)
Final body weight				95%	**91%****	**85%****
Liver	99%	**112%***	**128%****			
Ovaries				103%	98%	**75%****

* : p <= 0.05, **: p <= 0.01

Table 16: Summary of organs relative weights

Relative weights	Males			Females		
Test group (ppm)	1 (300)	2 (1000)	3 (3000)	1 (300)	2 (1000)	3 (3000)
Brain	103%	100%	**109%***	103%	107%	**114%***
Liver	**104%***	**114%****	**143%****	99%	**116%****	**130%****
Thyroid glands	110%	116%	**132%****	103%	**124%****	111%

* : p <= 0.05, **: p <= 0.01

Histopathology

Treatment-related findings were observed in the liver and the thyroid glands.

A dose-dependent hepatocellular hypertrophy in the liver was seen starting from test group 1 in males and from test group 2 in females. In test group 1 and 2, a centrilobular hypertrophy predominated, whereas in test group 3 the pattern was mainly diffuse with a mild centrilobular accentuation. Similarly, the fatty change was dose dependent and showed different patterns. In male animals, the fatty change in test group 2 was predominantly of macrovesicular type with a midzonal localization. In males and females of test group 3, the fatty change was periportal and mainly microvesicular with some macrovesicular type. Minimal single cell necrosis/apoptosis was noted only in males of test 2 scattered in the centrilobular to midzonal areas. Focal necrosis in males of test groups 2 and 3 was also noted.

Table 17: Liver histopathological findings

Liver	Male animals				Female animals			
Test group (ppm)	0 (0)	1 (300)	2 (1000)	3 (3000)	0 (0)	1 (300)	2 (1000)	3 (3000)
No. of animals	10	10	10	10	10	10	10	10
Hypertrophy, diffuse, centrilobular accentuated	0	0	0	10	0	0	0	10
• Grade 1				2				3
• Grade 2				3				5
• Grade 3				5				2
Hypertrophy, centrilobular	0	2	10	0	0	0	8	0
• Grade 1		1	1				8	
• Grade 2		1	9					
Fatty change, periportal	0	0	0	6	0	0	0	5
• Grade 1				6				3
• Grade 2								2
Fatty change, midzonal	0	0	4	0	0	0	0	0
• Grade 1			4					
Single cell necrosis/ apoptosis	0	2	6	0	0	0	0	0
• Grade 1		2	6					
Necrosis, focal	0	0	1	1	0	0	0	0
• Grade 1			1	1				

Thyroid effects were observed from the low dose levels (minimal hypertrophy/hyperplasia of follicular cells of 3/10 males and 3/10 females in combination with altered colloid). In high-dose animals, thyroid effects consisted in a significantly increased relative weight (17%) in males, corroborated by hypertrophy/hyperplasia of follicular cells of 9/10 males (minimal to moderate) and of 6/10 females (minimal to mild).

Table 18: Thyroid histopathological findings

Thyroid glands	Male animals				Female animals			
Test group (ppm)	0 (0)	1 (300)	2 (1000)	3 (3000)	0 (0)	1 (300)	2 (1000)	3 (3000)
No. of animals	10	10	10	10	10	10	10	10
Hypertrophy/hyper-plasia, follicular cell	2	3	4	9	0	3	2	6
• Grade 1	2	3	4	2		2	2	5
• Grade 2				4		1		1
• Grade 3				3				
Altered colloid	2	2	3	7		3	2	6
• Grade 1	2	2	2	3		3	2	6
• Grade 2			1	4				

Conclusion

The study author proposed the following NOAELs

The LOAEL for general systemic toxicity was 300 ppm for male (26 mg/kg bw/d) and female Wistar rats (28 mg/kg bw/d) based on liver effects in males and thyroid effects in both males and females.

The NOAEL for reproductive performance and fertility was 1000 ppm for males (87 mg/kg bw/d) and females (95 mg/kg bw/d) based on an in increased estrous cycle length as well as decreased number of implantations sites and pups delivered.

The NOAEL for developmental toxicity in the offspring was 1000 ppm for males (87 mg/kg bw/d) and females (95 mg/kg bw/d) based on decreased body weight from postnatal day (PND) 7.

- The DS considers that effects relevant for classification for fertility are observed (decreased number of implantation and consequent decreased litter size) from the mid dose level.

3.10.1.3 Study 3

Study reference

Anonymous, Report Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene Extended One-Generation Reproduction Toxicity Study in Wistar Rats Administration via the Diet , 2021

Test type

- OECD TG 443 (2018) including cohort 1B extension to produce F2 generation, cohorts 2A and 2B dedicated to developmental neurotoxicity screening.
- GLP compliant.
- Deviations:

No histopathology performed on F1C1B (since suspected to be ED, histopathology of cohort 1B should have been performed. Since liver and thyroid are identified as target organs they should also have been analysed)

Thyroid hormones not measured in F2 pups.

DNT: No historical control data (HCD) no positive control, statistical analysis not appropriate for motor activity, auditory startle response and morphometrics.

Test substance

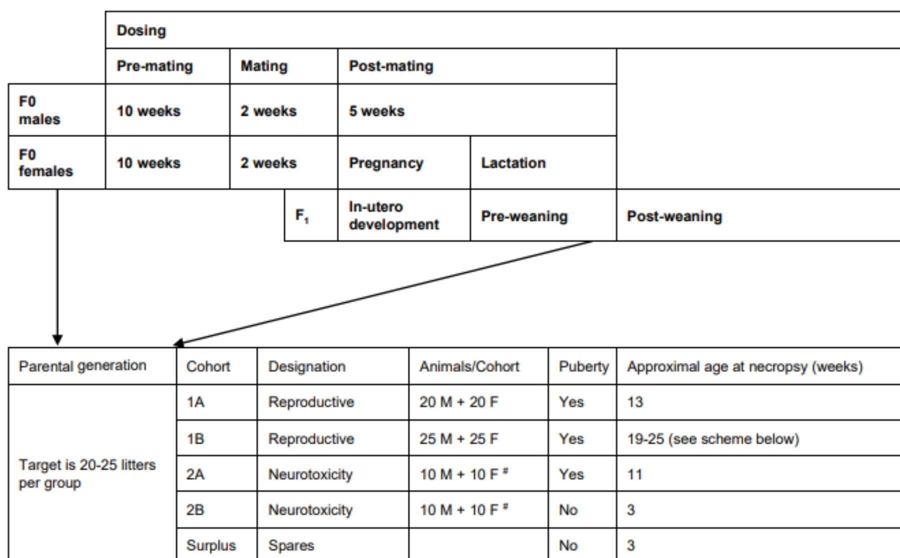
- Test material used in the study is equivalent **to Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene** identified in the CLH dossier
- Reaction products of diphenylamine with 2,4,4-trimethylpentene
- Batch: 501161180D

Test animals

- Wistar rats, strain Crl:WI(Han), males anf females
- P0: 25/sex/dose, F1C1A: 20/sex/dose, F1C1B (= P1): 25/sex/dose, F1C2A: 10/sex/dose and F1C2B: 10/sex/dose
- 28 (±1) days, acclimatization 9 days, 37 (±1) days old at the beginning of treatment, males: 118g, females 111g

Administration/exposure

- Oral, feed
- duration and frequency of test/exposure period



	Dosing				
	Pre-mating	Mating	Post-mating		
F0 males	10 weeks	2 weeks	5 weeks		
F0 females	10 weeks	2 weeks	Pregnancy	Lactation	
		F1	In-utero development	Pre-weaning	Post-weaning

Parental generation	Cohort	Designation	Animals/Cohort	Puberty	Approximal age at necropsy (weeks)
Target is 20-25 litters per group	1A	Reproductive	20 M + 20 F	Yes	13
	1B	Reproductive	25 M + 25 F	Yes	19-25 (see scheme below)
	2A	Neurotoxicity	10 M + 10 F #	Yes	11
	2B	Neurotoxicity	10 M + 10 F #	No	3
	Surplus	Spares		No	3

one per litter and representative of 20 litters in total where possible

- 0, 200, 600 and 1800 ppm. During the lactation period, concentrations in the diet of the P0 and P1 females were reduced to 50%.
- Dose level selection based on a dose range finding study (OECD TG 421, 0, 300, 1000 and 3000 ppm). According to advice on dose-level selection (ECHA January 2022), the same high dose levels could have been tested in the EOGRTS since no mortality and no severe suffering were observed at 3000 ppm).
- Historical control data: HCD have been provided, however several shortcomings limit their reliability. While data collected within 5 years of the study being evaluated is recommended, the collection period exceeds 12 years (e.g. from 2008 to 2018 while the current study was performed in 2020). The protocol of the studies is not clearly indicated (e.g. whether OECD TG 416 or TG 443 where followed, the route of administration not indicated for all studies). The HCD studies are different according to the parameters considered, which further limits the transparency and

readability of those data. In view of the limitations of the provided HCD, they will not be given much weight compared to the concurrent control group, which is still the most relevant comparator for determining treatment-related effects if the concurrent control is not aberrant.

HCD for the DNT part: No HCD nor positive control for (auditory startle response, motor activity, morphometrics)

- Various analyses confirmed the achieved concentration, stability and homogeneity of the preparation
- Actual doses: 0, 18, 54 and 167 mg/kg bw/day for the males and 0, 18, 54 and 166 mg/kg bw/day for the females

Description of test design: in accordance with OECD TG 443 (2018)

- Each of the male and female animals was mated overnight at a 1 : 1 ratio for a maximum of 2 weeks. Vaginal smear after each mating and examined for the presence of sperm. If sperm was detected, pairing of the animals was discontinued. The day on which sperm were detected was denoted " gestation day (GD) 0" and the following day "gestation day (GD) 1"
- **10-week** premating exposure period for males and females (P0 and P1)
- Standardization of litters: yes on PND 4 where possible, each litter contained 5 male and 5 female pups
- State of health checked each day, parental animals examined for their mating and reproductive performances. A detailed clinical observation (DCO) was performed in all P0 parents and F1 animals in cohorts 1A, 1B and 2A at weekly intervals.
- Food consumption determined once weekly, In general, body weights of P0 parents and F1 rearing animals were determined once weekly. However, during gestation and lactation P0 and F1B females were weighed on GD 0, 7, 14 and 20 and on PND 1, 4, 7, 10, 14, 18 and 21.
- Estrous cycle data were evaluated for P0 and P1 (F1C1B) females over a three-week period prior to mating until evidence of mating occurred. In all cohort 1A females, vaginal smears were collected after vaginal opening until the first cornified smear (estrous) was recorded. The estrous cycle also was evaluated in cohort 1A females for 2 weeks around PND 75. Moreover, the estrous stage of each P0, F1C1A and F1C1B female was determined on the day of scheduled sacrifice.
- Various sperm parameters (motility, sperm head count, morphology) were assessed in the P0 generation males and cohort 1A males at scheduled sacrifice.
- Blood and urine samples were withdrawn from 10 selected P0 and cohort 1A animals per sex and group for clinical pathological investigations (haematology, clinical chemistry, thyroid hormones and urinalysis.
- All P0 and P1 (F1C1B) parental animals were assessed by gross pathology (including weight determinations of several organs) and subjected to an extensive histopathological examination; special attention being paid to the organs of the reproductive system. A quantitative assessment of primordial and growing follicles in the ovaries was performed for all control and high-dose F1 rearing females of cohort 1A.
- The F1 and F2 pups were sexed on the day of birth (PND 0) and on PND 21. They were weighed on the first day after birth (PND 1) as well as on PND 4, 7, 14 and 21. Their viability was recorded. At necropsy, all pups were examined macroscopically (including weight determinations of brain, spleen and thymus in one pup/sex/litter of F2 pups).
  Anogenital distance measurements were conducted in a blind randomized fashion, using a measuring ocular on all live male and female pups on PND 1.
  All surviving pups were examined for the presence or absence of nipple/areola anlagen on PND 13 and were re-examined on PND 20.

Date of sexual maturation, i.e. day of vaginal opening (females) or balanopreputial separation (males), of all F1 pups selected to become F1 rearing animals (except F1C2B rearing animals sacrificed at PND22).
Further blood samples were taken from all surplus (culled) PND 4 pups per sex and group as well as from 10 surplus PND 22 pups per sex and group to investigate thyroid hormone levels.

- DNT: Beside DCO, Auditory Startle Response on PND 24, FOB and motor activity on PND 69 were carried out in all F1C2A animals. On PND 22 (F1C2B) and on PND 77 (F1C2A) animals were weighed, subjected to deep anesthesia (pentobarbital) and sacrificed by perfusion fixation for detailed neuropathology investigations including morphometry for F1C2A animals.

Results and discussion

CLINICAL EXAMINATIONS AND EXAMINATION OF REPRODUCTIVE PERFORMANCE

P0 and F1 adults

- There were no test substance-related or spontaneous mortalities in any of the groups of P0, F1C1A, F1C1B (P1) or F1C2A.
- No clinical signs or changes of general behaviour, which may be attributed to the test substance, were detected in any of the male and female animals in any of the groups of P0, F1C1A, F1C1B (P1) or F1C2A.
- Food consumption of all treated males of P0, P1(F1C1B) F1C1A or F1C2A was comparable to the concurrent control values throughout the entire study.

  Food consumption of the high-dose P0 females was statistically significantly below the concurrent control values during the entire gestation and major parts (PND 1 - 18 and 1 - 21) of the lactation period (up to 14%, 16% and 13%, respectively). Food consumption of the high-dose P1females was statistically significantly below the concurrent control values during premating days 0 - 7, 42 - 49, 56 - 70 and 0 - 70 (up to 10%) and during the entire gestation and lactation periods (up to 16% and 22%, respectively). Food consumption in other treated females was not affected.
- Mean body weights of all treated P0 male animals were comparable to the concurrent control values throughout the entire study. Mean body weights were statistically significantly below the concurrent control values for the high-dose P0 females during premating days 28 - 70 and during the entire gestation and lactation period (-8.2%, -12.1% and -9.4% at the end of the premating period, gestation and lactation respectively).

  Mean body weight was affected in high dose F1 animals as follows:

  - Decreased body weights in high dose P1 males during major parts of the study (6.2% below control at termination) and in females during major parts of the premating period and the entire gestation and lactation period (9%, 13% and 12% below control at the end of the premating period, gestation and lactation respectively).
  - Decreased body weights in F1C1A males and females during the entire study (at termination 8.4% and 6.4% below control, respectively)
  - Decreased body weights in high dose F1C2A males on study day 21 and during study days 7 - 21 (about 10% below control and -7.9% at termination)

Table 19: P0 body weight (grams [g])

	P0 males				P0 females			
Dose levels (ppm)	**0**	**200**	**600**	**1800**	**0**	**200**	**600**	**1800**
Start premating	118.9	118.7	119.1	118.5	111.4	111.2	111.9	111.6
Start mating	394.9	386.3	382.0	381.4	223.0	221.3	225.7	**204.6****
Difference from controls								**↓8.2%**
Start gestation	–	–	–	–	226.4	225.6	228.7	**207.3****
Difference from controls								**↓8.5%**
End gestation	–	–	–	–	337.8	326.0	333.4	**297.0****
Difference from controls								**↓12.1%**
Start lactation	–	–	–	–	255.2	250.3	252.1	**226.6****
Difference from controls								**↓11.2%**
Termination/End lactation	450.9	437.8	435.0	435.0	279.8	274.4	282.4	**253.6****
Difference from controls								**↓9.4 %**

Statistical significance: **: p<0.01 - **In bold: considered adverse**

Table 20: F1 body weight (grams [g])

		F1 males				F1 females			
	Dose levels (ppm)	**0**	**200**	**600**	**1800**	**0**	**200**	**600**	**1800**
F1	**weaning (3 w)**	51.4	52.1	49.0	**45.0****	50.1	50.7	**48.1***	**44.5****
	Difference from controls				**↓12.4**			**↓5.4%**	**↓11.7%**
F1C1A	**Termination (13 w)**	361.0	351.5	349.9	**330.8****	214.1	217.7	213.3	**200.3****
	Difference from controls				**↓8.4%**				**↓6.4%**
F1C2A	**Termination (11 w)**	324.0	331.6	321.9	**298.3**	197.1	201.9	197.2	187.2
	Difference from controls				***↓7.9%***				*↓5%*
F1C1B = P1	**Start premating (5 w)**	87.2	88.3	87.2	81.7	81.1	82.5	80.6	76.7
	Difference from controls				*↓6.3%*				*↓5.4%*
	Start mating (15 w)	370.4	371.6	379.4	**346.2***	224.9	229.1	223.0	**203.6****
	Difference from controls				**↓6.5%**				**↓9.4%**
	Start gestation	–	–	–	–	229.1	232.4	227.6	**208.0****
	Difference from controls								**↓9.2%**
	End gestation	–	–	–	–	336.4	339.5	329.4	**298.6****
	Difference from controls								**↓13%**
	Start lactation	–	–	–	–	257.4	261.9	253.7	**220.9****
	Difference from controls								**↓14.2%**
	End lactation/Termination	413	413	422	**387.3***	287.8	287.4	283.0	**253.2****
	Difference from controls				**↓6.2%**				**↓12%**

Statistical significance: *: p<0.05 **: p<0.01 - **In bold: considered adverse**

- **Estrous cycle**

While estrous cycle data from P0 females and F1C1A females did not revealed any treatment-related effect, in estrous cycle data from P1 female, the mean estrous cycle duration was: 4.0 / 4.0 / 4.0 and 4.3** (**:p<=0.01) days in control, LD, MD and HD groups respectively. The slightly prolonged average in the HD group is mainly driven by an increased number of days in diestrous stage.

The apparent prolongation was considered as a spurious finding by the study author since the average cycle length is within the historical control range (HCD = 3.9 - 4.6) and a comparable increase was not observed in the corresponding F1C1A females.

However, it is noteworthy that F1C1A females were exposed for a shorter period and increased estrous cycles length (4.7 days vs. control 4.0 days) and increased number of days in diestrous stage were observed in the OECD TG 421 studies in females exposed to 3000 ppm.

- **Reproduction and delivery data**

For all P0 and P1 males, which were placed with females to generate pups, copulation was confirmed. Thus, the male and the female mating index was 100% in all test groups for both generations. The male and female fertility index ranged between 96% and 100% in P0 generation and between 92% and 100% in P1 generation without any relation to the dose.

There was no treatment-related effect on gestation lenth and gestation index.

In P0 females the mean number of implantation sites was statistically significantly below the concurrent control values in the high-dose group (12.1 / 11.0 / 11.9 and 10.7** implants/dam in control, LD, MD and HD groups respectively) and below the provided historical control range (HCD = 11.1 - 15.3 implants/dam).

As a consequence of the lower number of implants the mean number of F1 pups delivered per dam (average litter size) was statistically significantly below the concurrent control values in the high-dose group (12.0 / 11.3 / 11.5 and 9.6** pups/dam, respectively in control, LD, MD and HD group, and outside the historical control range (HCD = 10.3 - 14.9 pups/dam).

In P1, the mean number of implantation sites was also statistically significantly below the concurrent control values in the high-dose group (12.3 / 11.8 / 11.2 and 10.2** (**:p<=0.01) implants/dam in test groups 10 - 13, respectively) and below the historical control range (HCD = 11.1 - 15.3 implants/dam in control, LD, MD and HD groups respectively). It was just inside the historical control range in the mid-dose group. However as already mentioned, the HCD covered a too long timeframe.

The mean number of F2 pups delivered per dam (average litter size) was statistically significantly below the concurrent control values in the mid- and high-dose group (12.0 / 11.2 / 10.8* (*:p<=0.05) and 9.8** (**:p<=0.01) pups/dam, respectively in test groups 10 - 13). The lower average litter size is for both groups considered a consequence of a lower number of implants. While the litter size of the high-dose group was outside the historical control range (HCD = 10.3 - 14.9 pups/dam), it is still within range in the mid-dose group.

Considering the observed dose-response relationship treatment related and Evaluation firstly based on concurrent controls. Dose-related effect and concurrent control not aberrant.

Decreased number of implantation sites and correlated decreased litter size was also observed in the OECD TG 421 carried out with the substance and observed in OECD TG 421 and with a structural analogue.

Based on the above-mentioned considerations, the effect is considered adverse from the mid-dose level in P1 females.

Postimplantation loss was not affected by treatment in either generation.

Table 21: Summary Delivery and Litter Report

		P0				P1				
Dose levels (ppm)		**0**	**200**	**600**	**1800**	**0**	**200**	**600**	**1800**	***HCD#***
Number of females at start	N	25	25	25	25	25	25	25	25	
Total Number of Pregnant Females	N	24	24	25	25	23	24	24	25	
Total Number of Delivering Females	N	24	23**a**	25	25	23	24	24	25	
With liveborn pups	N	24	23	25	25	23	24	24	25	
	%	100	100	100	100	100	100	100	100	
With stillborn pups	N	2	1	0	2	1	1	1	3	
	%	8.3	4.3	0	8	4.3	4.2	4.2	12	
With all pups stillborn	N	0	0	0	0	0	0	0	0	
	%	0	0	0	0	0	0	0	0	
Implantation Sites	N	302	276	298	267	283	282	270	255	
	Mean	12.6	11.5	11.9	**10.7****	12.3	11.8	**11.2**	**10.2****	*11.1 - 15.3*
	↓%control		8.7	5.6	**15.1**		4.1	**8.9**	**17.1**	
	SD	1.6	2.6	2.1	1.5	2.3	2.2	1.9	1.9	
	N	24	24	25	25	23	24	24	25	
Pups delivered	N	288	261	288	239	277	270	260	244	
	Mean	12	11.3	11.5	**9.6****	12	11.2	**10.8***	**9.8****	*10.3 - 14.9*
	↓%control		5.8	4.2	**20.0**		6.7	**10.0**	**18.3**	
	SD	1.9	1.3	2.1	2.2	2.2	2.3	1.8	1.9	
	N	24	23	25	25	23	24	24	25	
Postimplantation Loss	Mean%	4.8	9	3.4	10.2	2	4.5	3.3	4	
	SD	8.7	19.9	5.9	19	4	6.2	6.9	7.5	
	N	24	24	25	25	23	24	24	25	

Statistical significance: *: $p<0.05$ **: $p<0.01$. **In bold considered adverse**

a: One sperm positive low-dose female did not deliver F1 pups but had implants in the utero.

 # HCD not considered fully reliable 2008-2018 (covering a too long period) 10.3 from a 2008 study while the current study was performed in 2020

- **F1 and F2 generation pups/litters**

As a consequence of the lower number of implants, the mean number of F1 pups delivered per dam (average litter size) was statistically significantly below the concurrent control values in the HD group (12.0 / 11.3 / 11.5 and 9.6** pups/dam, respectively in control, LD, MD and HD groups). The mean number of F2 pups delivered per dam (average litter size) was statistically significantly below the concurrent control values from the MD group (12.0 / 11.2 / 10.8* (*:p<=0.05) and 9.8** (**:p<=0.01) pups/dam respectively in control, LD, MD and HD groups) in test groups 10 - 13).

There were no test substance-related adverse effects on clinical signs, sex ratio or on live birth, viability (PND 0-4), lactation (PND 4-24) indices.

Mean body weights were affected from the MD in F1 generation and limited to HD group in F2 generation.

Table 22: Pups bodyweight (in grams)

		Males				Females			
	Dose levels (ppm)	**0**	**200**	**600**	**1800**	**0**	**200**	**600**	**1800**
F1 pups	**PND 1**	7.0	7.1	6.7	6.8	6.7	6.8	6.4	6.5
	PND 7	17.1	17.2	**16.0***	**15.3****	16.60	16.70	**15.4***	**15.0****
	Difference from controls			**↓6.5%**	**↓10.5%**			**↓7.1%**	**↓9.7%**
	PND 21	51.4	52.1	49.0	**45****	50.1	50.7	**47.3***	**44.2****
	Difference from controls				**↓12.4%**			**↓5.5%**	**↓11.7%**
F2 pups	**PND 1**	6.8	7.1	6.8	6.4	6.5	6.7	6.6	6.1
	PND 7	16.7	17.7	16.7	**14.7****	16.2	17.1	16.2	**14.2****
	Difference from controls				**↓12.3%**				**↓12.4%**
	PND 21	51.1	53.2	49.7	**42.7****	49.6	51.2	48.1	**41.9****
	Difference from controls				**↓16.3%**				**↓15.5%**

Statistical significance: *: p<0.05 **: p<0.01 - **In bold: considered adverse**

The anogenital distance and anogenital index of all test substance treated male and female pups was comparable to the concurrent control values in both generation.

Nipple/ areola anlagen

F1 pups: in HD group at PND20, 2 pups from the same litter had 2 nipple/areola anlagen. This was considered to be a spontaneous event by the study author.

F2 pups: at PND13 mean nipple number in HD males was statistically significantly above the concurrent control and was considered by the study author to be the consequence of a general delay of pup development, rather than a specific effect on hormonal homeostasis.

However, the very high background incidence at PND13 compromises a reliable assessment of the potential effect of the test substance. No persistence of nipple/areola anlagen on PND20 was reported in historical control data (range 0-0). In the OECD TG 421 at HD level (3000 ppm), a statistically significant increased incidence of nipple development (100% vs. 79.6% in control) and a statistically significant increased number of nipples per animal (5.2 to control 2.5) were also observed.

Table 23: Nipple retention

		F1 males pups				F2 males pups				*HCD range 2007-2018*
	Dose levels (ppm)	**0**	**200**	**600**	**1800**	**0**	**200**	**600**	**1800**	
PND 13	**% males with nipple**	67	57	79	78	79	63	76	86	*8.6-95*
	Affected per litter mean%	66.8	57.2	76.1	78.5	77.1	59.1	74.2	86.4	*8.7-84*
	Mean nipple number	2	1.5	2.7	2.4	2.6	1.8	2.9	**4.1***	*not available*
PND 20	**% males with nipple**	0	0	0	**2**	0	0	0	0	*0-0*
	Affected per litter mean%	0	0	0	1.4	0	0	0	0	*0-0*
	Mean nipple number	0	0	0	0	0	0	0	0	*not available*

* p ≤0.05

Sexual maturation data

The mean number of days to reach vaginal opening in the control, LD, MD and HD groups was 31.0; 31.1; 31.3 and 31.8* (* = p≤0.05) days, respectively.

The mean number of days to reach preputial separation in the control, LD, MD and HD groups was 42.1, 41.5, 42.3 and 43.5** (** = p≤0.01) days, respectively.

A delay in puberty onset was observed in both sexes of the HD group, which is not supportive of an endocrine mode of action. Furtheremore, a decreased body weight was noted at weaning in HD animals while the weight at puberty onset is similar in all groups (Table XXX). The delay in sexual maturation observed at the high dose level is therefore considered as a consequence of the delayed general development (lower pup weights).

Table 24: Age and weight at vaginal opening

Dose levels (ppm)		0 ppm	200 ppm	600 ppm	1800 ppm	*HCD range 2008-2018*
Days at vaginal opening	Mean	31.0	31.1	31.3	**31.8***	*29.5- 38.8*
	S.d.	1.1	1.6	1.4	2.0	
	N	55	55	55	54	
Weight at vaginal opening	Mean	96.0	97.1	94.3	92.2	*84.7-105.8*
	S.d.	8.1	10.4	10.1	9.7	
	N	55	55	55	54	

* p≤0.05, ** p≤0.01

Table 25: Age and weight at preputial separation

Dose levels (ppm)		0 ppm	200 ppm	600 ppm	1800 ppm	*HCD range 2008-2018*
Days at preputial separation	Mean	42.1	41.5	42.3	**43.5****	*40.1-45.2*
	S.d.	2.1	1.7	1.5	1.9	
	N	55	55	55	55	
Weight at preputial separation	Mean	181.2	176.7	180.9	175.3	*158.2-221.1*
	S.d.	14.5	13.3	13.8	15.1	
	N	55	55	55	55	

** p≤0.01

CLINICAL PATHOLOGY and PATHOLOGY

P0 and F1 adults

Reproductive system:

Females:

Decreased absolute ovary weight was observed in both HD P0 and HD P1 females. No correlated histopathological findings were noted in P0 females (while histopathology was not performed in P1 females).

Table 26: Ovary weights

	P0 females			**P1 (F1C1B) females**		
Test group (ppm)	200	600	1800	200	600	1800
Terminal body weight	98%	99%	**90%****	101%	100%	**90%****
Absolute ovaries weights	100%	99%	**87%****	100%	103%	**88%****
Relative ovaries weights	102%	100%	97%	99%	103%	97%

Statistical significance: *: $p<0.05$ **: $p<0.01$

In cohort F1C1A females (terminated at 11-week old), there was no test substance-related effect on ovary weight and histopathology. The results of the differential ovarian follicle count (DOFC) – comprising the numbers of primordial and growing follicles, as well as the combined incidence of primordial plus growing follicles – did not reveal significant differences between the F1C1A control group and the F1C1A HD group. There were no test substance-related effects on uterus in any generations.

Males

There were no test substance-related effects on tested or any secondary sex organs in any generations.

Sperm analysis: no treatment-related effects were observed concerning motility of the sperms and the incidence of abnormal sperms in the cauda epididymidis as well as sperm head counts in the testis and in the cauda epididymidis of P0 and F1C1A males.

Other organs/systems

Regarding clinical pathology and pathology, except slight hemathological findings observed in the group (1800 ppm), all the other treatment-related changes were related to the liver and the thyroid in P0 and F1 males and females therefore the different findings (biochemistry and histopathology) are reported according to the these two target organs.

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered incidental or spontaneous in origin and without any relation to treatment.

Haematology

Slight haematological findings were observed in the HD groups (1800 ppm) of P0 and F1C1A.In both P0 and F1C1A males and females of the HD groups (1800 ppm) haemoglobin and haematocrit values were marginally but significantly decreased (less than 5% decrease). Additionally, in HD P0 and F1C1A males absolute reticulocyte counts were significantly increased (regenerative anaemia). Decreased platelet counts

was also noted at this dose level P0 females and F1C1A males and females while prothrombin time (HQT) was reduced in both HP0 and F1C1A females.

Liver effects

A consistent pattern of pathological effects was observed in both generation that is also consistent with the liver effects observed in the other available studies (i.e. OECD TG 421, OECDE TG 422).

Liver weights

In P0 generation, males and females of the MD and HD groups (600 and 1800 ppm) showed an increase in liver weight. In both F1C1A and F1C1B (P1) cohorts, the effects on liver weight were limited to the HD groups.

Table 27: Liver weights

		Males			Females		
	Dose levels (ppm)	**200**	**600**	**1800**	**200**	**600**	**1800**
P0	Terminal BW	98%	97%	97%	98%	99%	**90%****
	Liver abs weight	101%	105%	**115%****	100%	**107%****	**123%****
	rel weight	103%*	**108%****	**119%****	102%	**108%****	**136%****
F1C1A	Terminal BW	97%	97%	**91%****	101%	99%	**93%****
	Liver abs weight	94%	97%	99%	100%	105%	**121%****
	rel weight	96%	100%	**109%****	99%	106%*	**131%****
F1C1B	Terminal BW	99%	101%	**94%****	101%	100%	**90%****
	Liver abs weight	98%	104%	106%	101%	107%	**119****
	rel weight	99%	103%	**114%****	100%	107%	**132%****

Statistical significance: *: p<0.05 **: p<0.01

Histopathology

P0: Male animals of MD and HD groups (600 and 1800 ppm) revealed a minimal to moderate centrilobular liver cell hypertrophy. In addition, most of these animals showed a microvesicular vacuolation in the transition region between centrilobular and midzonal area. The livers of 2 HD males showed a positive reaction when stained with Oil Red O while those from control males were negative. Therefore, the microvesicular vacuolation was shown to be neutral lipids (fatty change).

Females of all test groups showed a centrilobular liver cell hypertrophy. Additionally, six HD females revealed a diffuse hepatocellular hypertrophy.

2/20 males of the LD group (200 ppm) revealed fatty change and 4/20 minimal centrilobular hypertrophy.

F1C1A: Males of MD and HD groups (600 and 1800 ppm) revealed all a minimal to moderate centrilobular liver cell hypertrophy. In addition, some of them showed a microvesicular vacuolation (fatty change). Females of MD and HD groups showed also centrilobular liver cell hypertrophy.

2/20 males of the LD group (200 ppm) revealed minimal centrilobular hypertrophy.

Table 28: Liver histopathological findings in P0

	P0 males				P0 females			
dose level (ppm)	**0**	**200**	**600**	**1800**	**0**	**200**	**600**	**1800**
N	20	21	21	20	20	20	20	20
Hypertrophy, diffuse	0	0	0	0	0	0	0	**6**
• Grade 2								**5**
• Grade 3								**1**
Hypertrophy, centrilob.	0	4	**20**	**20**	0	3	**5**	**12**
• Grade 1		4	**9**			3	**4**	**3**
• Grade 2			**11**	**4**			**1**	**5**
• Grade 3				**16**				**4**
Fatty change, centrilob.	0	**2**	**19**	**14**	0	0	0	0
• Grade 1		**1**	**13**	**5**				
• Grade 2		**1**	**5**	**5**				
• Grade 3			**1**	**3**				
• Grade 5				**1**				

In bold: considered adverse

Table 29: Liver histopathological findings in F1C1A

	F1C1A males				F1C1A females			
dose level (ppm)	**0**	**200**	**600**	**1800**	**0**	**200**	**600**	**1800**
N	20	20	20	20	20	20	20	20
Hypertrophy, centrilob.	0	2	**19**	**20**	0	0	4	**18**
• Grade 1		2	**6**				4	**9**
• Grade 2			**13**	**16**				**6**
• Grade 3				**4**				**3**
Fatty change, centrilob.	0	0	**7**	**10**	0	0	0	**0**
• Grade 1			**4**	**8**				
• Grade 2			**2**	**2**				
• Grade 3			**1**					

In bold: considered adverse

Biochemistry

P0: In male and female rats of MD and HD groups, (600 and 1800 ppm), alkaline phosphatase (ALP) activities and triglyceride (TRI) values were significantly increased. Additionally, in both sexes of the HD group albumin (ALB) levels were significantly decreased. In males of this test group, total protein values were also significantly decreased, whereas in females globulin levels were significantly increased. In

addition, in MD and HD males of alanine aminotransferase (ALT) activities were marginally, but significantly increased, and in HD females γ-glutamyl transferase (GGT) activities and cholesterol levels were significantly higher compared to controls.
In male and female rats of the LD group (200 ppm) ALP activities were significantly increased. In addition, (TRI) values were significantly increased and TBIL in males but without reaching statistical significance in females. ALB levels were also significantly decreased in females.
F1C1A: in both sexes of the MD and HD groups 1 (600 and 1800 ppm), ALP activities were significantly increased whereas ALB values were significantly decreased. Additionally, in males of these test groups total protein and globulin values were significantly decreased. In HD male and female rats of test group 13 total bilirubin values were significantly decreased. Additionally, in HD females of this test group (GGT) activities, triglyceride, cholesterol and globulin values were significantly increased.
In male and female rats of the LD group (200 ppm) ALP activities were significantly increased. In addition, ALB levels were also significantly decreased in females.

Table 30: Biochemistry P0 Males

		Group 00/M 0 ppm	Group 01/M 200 ppm	Group 02/M 600 ppm	Group 03/M 1800 ppm
ALT	Mean	0.63 v	0.71	0.76 **	0.95 **
[µkat/L]	S.d.	0.05	0.15	0.15	0.49
day 123	N	10	10	10	10
	Median	0.60	0.66	0.70	0.75
	Deviation Vs Control [%]		13.42	21.25	51.60
AST	Mean	1.67 k	1.65	1.71	1.83
[µkat/L]	S.d.	0.27	0.28	0.22	0.27
day 123	N	10	10	10	10
	Median	1.65	1.58	1.67	1.81
	Deviation Vs Control [%]		-1.44	2.51	9.58
ALP	Mean	1.00 v	1.26 *	1.94 **	2.66 **
[µkat/L]	S.d.	0.16	0.22	0.15	0.36
day 123	N	10	10	10	10
	Median	0.97	1.32	1.94	2.68
	Deviation Vs Control [%]		25.60	94.50	166.40
GGT_C	Mean	25 x+	25	25	25
[nkat/L]	S.d.	0	0	0	1
day 123	N	10	10	10	10
	Median	25	25	25	25
	Deviation Vs Control [%]		0	0	1
UREA	Mean	4.60 k	5.08	4.34	4.77
[mmol/L]	S.d.	0.64	1.40	0.41	0.83
day 123	N	10	10	10	10
	Median	4.66	4.80	4.30	4.43
	Deviation Vs Control [%]		10.43	-5.65	3.56
CREA	Mean	30.2 k	29.9	28.1	28.1
[µmol/L]	S.d.	5.8	6.7	2.5	2.5
day 123	N	10	10	10	10
	Median	30.6	28.8	28.0	27.9
	Deviation Vs Control [%]		-1.1	-7.1	-6.9
GLUC	Mean	6.42 k	6.48	6.64	6.51
[mmol/L]	S.d.	0.63	0.83	0.68	0.47
day 123	N	10	10	10	10
	Median	6.36	6.69	6.82	6.38
	Deviation Vs Control [%]		0.84	3.39	1.42
TBIL_C	Mean	1.60 v	1.36 *	1.36	1.15 **
[µmol/L]	S.d.	0.23	0.34	0.27	0.25
day 123	N	10	10	10	10
	Median	1.48	1.26	1.35	1.17
	Deviation Vs Control [%]		-14.97	-14.65	-27.85
TPROT	Mean	62.96 v	62.82	61.90	60.87 *
[g/L]	S.d.	1.74	1.37	1.52	2.52
day 123	N	10	10	10	10
	Median	63.40	62.56	61.58	60.86
	Deviation Vs Control [%]		-0.22	-1.68	-3.31
ALB	Mean	37.11 v	37.20	36.74	35.09 **
[g/L]	S.d.	0.98	0.67	0.59	1.00
day 123	N	10	10	10	10
	Median	37.08	37.22	36.58	35.14
	Deviation Vs Control [%]		0.24	-0.99	-5.44
GLOB	Mean	25.85 k	25.63	25.16	25.78
[g/L]	S.d.	1.00	0.95	1.26	2.01
day 123	N	10	10	10	10
	Median	26.18	25.40	24.93	25.30
	Deviation Vs Control [%]		-0.87	-2.67	-0.26
CHOL	Mean	1.62 k	1.56	1.44	1.52
[mmol/L]	S.d.	0.34	0.24	0.28	0.29
day 123	N	10	10	10	10
	Median	1.54	1.50	1.41	1.46
	Deviation Vs Control [%]		-3.71	-11.00	-6.00
TRIG	Mean	0.88 v	1.34 **	1.49 **	1.77 *
[mmol/L]	S.d.	0.25	0.38	0.46	0.86
day 123	N	10	10	10	10
	Median	0.75	1.38	1.38	1.92
	Deviation Vs Control [%]		53.60	70.74	101.94

Table 31: Biochemistry P0 Females

		Group 00/F 0 ppm	Group 01/F 200 ppm	Group 02/F 600 ppm	Group 03/F 1800 ppm
ALT	Mean	0.55 k	0.59	0.60	0.64
[µkat/L]	S.d.	0.06	0.12	0.20	0.18
day 132	N	10	10	10	10
	Median	0.55	0.55	0.56	0.62
	Deviation Vs Control [%]		7.80	9.26	15.97
AST	Mean	1.56 k	1.47	1.67	1.64
[µkat/L]	S.d.	0.35	0.27	0.28	0.33
day 132	N	10	10	10	10
	Median	1.47	1.50	1.68	1.64
	Deviation Vs Control [%]		-5.59	7.45	5.39
ALP	Mean	0.73 v	1.21 **	1.62 **	2.98 **
[µkat/L]	S.d.	0.25	0.28	0.36	0.99
day 132	N	10	10	10	10
	Median	0.70	1.22	1.71	2.78
	Deviation Vs Control [%]		66.85	123.38	309.22
GGT_C	Mean	25 x+	25	25	40 **
[nkat/L]	S.d.	0	0	0	21
day 132	N	10	10	10	10
	Median	25	25	25	32
	Deviation Vs Control [%]		0	0	62
UREA	Mean	6.32 k	6.00	6.77	6.43
[mmol/L]	S.d.	0.60	0.82	1.03	0.90
day 132	N	10	10	10	10
	Median	6.23	5.75	7.08	6.70
	Deviation Vs Control [%]		-5.08	7.18	1.72
CREA	Mean	30.9 v	34.9 **	35.0 **	31.6
[µmol/L]	S.d.	2.1	5.3	3.8	3.8
day 132	N	10	10	10	10
	Median	30.5	34.7	35.4	31.2
	Deviation Vs Control [%]		13.1	13.3	2.3
GLUC	Mean	5.28 k	5.25	5.38	5.59
[mmol/L]	S.d.	0.43	0.54	0.55	0.43
day 132	N	10	10	10	10
	Median	5.22	5.30	5.34	5.48
	Deviation Vs Control [%]		-0.72	1.87	5.75
TBIL_C	Mean	1.93 k	1.62	1.74	1.73
[µmol/L]	S.d.	0.53	0.38	0.26	1.07
day 132	N	10	10	10	10
	Median	1.86	1.53	1.72	1.49
	Deviation Vs Control [%]		-16.19	-9.94	-10.34
TPROT	Mean	63.84 k	61.89	60.46	63.22
[g/L]	S.d.	3.59	1.68	1.88	2.89
day 132	N	10	10	10	10
	Median	62.82	62.20	60.71	63.17
	Deviation Vs Control [%]		-3.06	-5.29	-0.98
ALB	Mean	39.04 v	36.44 **	35.62 **	35.06 **
[g/L]	S.d.	1.85	1.19	1.07	1.16
day 132	N	10	10	10	10
	Median	38.84	36.46	35.47	34.67
	Deviation Vs Control [%]		-6.66	-8.77	-10.19
GLOB	Mean	24.80 v	25.45	24.85	28.16 **
[g/L]	S.d.	2.03	1.04	1.22	2.05
day 132	N	10	10	10	10
	Median	24.14	24.88	24.63	27.84
	Deviation Vs Control [%]		2.60	0.18	13.51
CHOL	Mean	1.40 v	1.28	1.62	2.66 **
[mmol/L]	S.d.	0.36	0.32	0.22	0.63
day 132	N	10	10	10	10
	Median	1.40	1.14	1.67	2.48
	Deviation Vs Control [%]		-8.29	15.73	90.49
TRIG	Mean	0.77 v	1.04	1.24 *	3.48 **
[mmol/L]	S.d.	0.26	0.57	0.45	1.56
day 132	N	10	10	10	10
	Median	0.70	0.86	1.31	3.58
	Deviation Vs Control [%]		34.59	61.77	352.41

Table 32: Biochemistry F1C1A Males

		Group 00/M 0 ppm	Group 01/M 200 ppm	Group 02/M 600 ppm	Group 03/M 1800 ppm
ALT	Mean	0.63 v	0.71	0.76 **	0.95 **
[µkat/L]	S.d.	0.05	0.15	0.15	0.49
day 123	N	10	10	10	10
	Median	0.60	0.66	0.70	0.75
	Deviation Vs Control [%]		13.42	21.25	51.60
AST	Mean	1.67 k	1.65	1.71	1.83
[µkat/L]	S.d.	0.27	0.28	0.22	0.27
day 123	N	10	10	10	10
	Median	1.65	1.58	1.67	1.81
	Deviation Vs Control [%]		-1.44	2.51	9.58
ALP	Mean	1.00 v	1.26 *	1.94 **	2.66 **
[µkat/L]	S.d.	0.16	0.22	0.15	0.36
day 123	N	10	10	10	10
	Median	0.97	1.32	1.94	2.68
	Deviation Vs Control [%]		25.60	94.50	166.40
GGT_C	Mean	25 x+	25	25	25
[nkat/L]	S.d.	0	0	0	1
day 123	N	10	10	10	10
	Median	25	25	25	25
	Deviation Vs Control [%]		0	0	1
UREA	Mean	4.60 k	5.08	4.34	4.77
[mmol/L]	S.d.	0.64	1.40	0.41	0.83
day 123	N	10	10	10	10
	Median	4.66	4.80	4.30	4.43
	Deviation Vs Control [%]		10.43	-5.65	3.56
CREA	Mean	30.2 k	29.9	28.1	28.1
[µmol/L]	S.d.	5.8	6.7	2.5	2.5
day 123	N	10	10	10	10
	Median	30.6	28.8	28.0	27.9
	Deviation Vs Control [%]		-1.1	-7.1	-6.9
GLUC	Mean	6.42 k	6.48	6.64	6.51
[mmol/L]	S.d.	0.63	0.83	0.68	0.47
day 123	N	10	10	10	10
	Median	6.36	6.69	6.82	6.38
	Deviation Vs Control [%]		0.84	3.39	1.42
TBIL_C	Mean	1.60 v	1.36 *	1.36	1.15 **
[µmol/L]	S.d.	0.23	0.34	0.27	0.25
day 123	N	10	10	10	10
	Median	1.48	1.26	1.35	1.17
	Deviation Vs Control [%]		-14.97	-14.65	-27.85
TPROT	Mean	62.96 v	62.82	61.90	60.87 *
[g/L]	S.d.	1.74	1.37	1.52	2.52
day 123	N	10	10	10	10
	Median	63.40	62.56	61.58	60.86
	Deviation Vs Control [%]		-0.22	-1.68	-3.31
ALB	Mean	37.11 v	37.20	36.74	35.09 **
[g/L]	S.d.	0.98	0.67	0.59	1.00
day 123	N	10	10	10	10
	Median	37.08	37.22	36.58	35.14
	Deviation Vs Control [%]		0.24	-0.99	-5.44
GLOB	Mean	25.85 k	25.63	25.16	25.78
[g/L]	S.d.	1.00	0.95	1.26	2.01
day 123	N	10	10	10	10
	Median	26.18	25.40	24.93	25.30
	Deviation Vs Control [%]		-0.87	-2.67	-0.26
CHOL	Mean	1.62 k	1.56	1.44	1.52
[mmol/L]	S.d.	0.34	0.24	0.28	0.29
day 123	N	10	10	10	10
	Median	1.54	1.50	1.41	1.46
	Deviation Vs Control [%]		-3.71	-11.00	-6.00
TRIG	Mean	0.88 v	1.34 **	1.49 **	1.77 *
[mmol/L]	S.d.	0.25	0.38	0.46	0.86
day 123	N	10	10	10	10
	Median	0.75	1.38	1.38	1.92
	Deviation Vs Control [%]		53.60	70.74	101.94

Table 33: Biochemistry F1C1A Females

		Group 00/F 0 ppm	Group 01/F 200 ppm	Group 02/F 600 ppm	Group 03/F 1800 ppm
ALT	Mean	0.55 k	0.59	0.60	0.64
[µkat/L]	S.d.	0.06	0.12	0.20	0.18
day 132	N	10	10	10	10
	Median	0.55	0.55	0.56	0.62
	Deviation Vs Control [%]		7.80	9.26	15.97
AST	Mean	1.56 k	1.47	1.67	1.64
[µkat/L]	S.d.	0.35	0.27	0.28	0.33
day 132	N	10	10	10	10
	Median	1.47	1.50	1.68	1.64
	Deviation Vs Control [%]		-5.59	7.45	5.39
ALP	Mean	0.73 v	1.21 **	1.62 **	2.98 **
[µkat/L]	S.d.	0.25	0.28	0.36	0.99
day 132	N	10	10	10	10
	Median	0.70	1.22	1.71	2.78
	Deviation Vs Control [%]		66.85	123.38	309.22
GGT_C	Mean	25 x+	25	25	40 **
[nkat/L]	S.d.	0	0	0	21
day 132	N	10	10	10	10
	Median	25	25	25	32
	Deviation Vs Control [%]		0	0	62
UREA	Mean	6.32 k	6.00	6.77	6.43
[mmol/L]	S.d.	0.60	0.82	1.03	0.90
day 132	N	10	10	10	10
	Median	6.23	5.75	7.08	6.70
	Deviation Vs Control [%]		-5.08	7.18	1.72
CREA	Mean	30.9 v	34.9 **	35.0 **	31.6
[µmol/L]	S.d.	2.1	5.3	3.8	3.8
day 132	N	10	10	10	10
	Median	30.5	34.7	35.4	31.2
	Deviation Vs Control [%]		13.1	13.3	2.3
GLUC	Mean	5.28 k	5.25	5.38	5.59
[mmol/L]	S.d.	0.43	0.54	0.55	0.43
day 132	N	10	10	10	10
	Median	5.22	5.30	5.34	5.48
	Deviation Vs Control [%]		-0.72	1.87	5.75
TBIL_C	Mean	1.93 k	1.62	1.74	1.73
[µmol/L]	S.d.	0.53	0.38	0.26	1.07
day 132	N	10	10	10	10
	Median	1.86	1.53	1.72	1.49
	Deviation Vs Control [%]		-16.19	-9.94	-10.34
TPROT	Mean	63.84 k	61.89	60.46	63.22
[g/L]	S.d.	3.59	1.68	1.88	2.89
day 132	N	10	10	10	10
	Median	62.82	62.20	60.71	63.17
	Deviation Vs Control [%]		-3.06	-5.29	-0.98
ALB	Mean	39.04 v	36.44 **	35.62 **	35.06 **
[g/L]	S.d.	1.85	1.19	1.07	1.16
day 132	N	10	10	10	10
	Median	38.84	36.46	35.47	34.67
	Deviation Vs Control [%]		-6.66	-8.77	-10.19
GLOB	Mean	24.80 v	25.45	24.85	28.16 **
[g/L]	S.d.	2.03	1.04	1.22	2.05
day 132	N	10	10	10	10
	Median	24.14	24.88	24.63	27.84
	Deviation Vs Control [%]		2.60	0.18	13.51
CHOL	Mean	1.40 v	1.28	1.62	2.66 **
[mmol/L]	S.d.	0.36	0.32	0.22	0.63
day 132	N	10	10	10	10
	Median	1.40	1.14	1.67	2.48
	Deviation Vs Control [%]		-8.29	15.73	90.49
TRIG	Mean	0.77 v	1.04	1.24 *	3.48 **
[mmol/L]	S.d.	0.26	0.57	0.45	1.56
day 132	N	10	10	10	10
	Median	0.70	0.86	1.31	3.58
	Deviation Vs Control [%]		34.59	61.77	352.41

Thyroid effects

Thyroid weights:

P0: in both sexes of the HD groups (1800 ppm), the thyroid weights were significantly increased (absolute and relative weights in males and relative weight in females). While not statistically significant an increase of the absolute and relative thyroid weights were also observed in females of the MD group.

F1C1A: in females of the MD and HD groups (600 and 1800 ppm), the absolute and relative thyroid weights were significantly increased. While not statistically significant an increase of the relative thyroid weights were also observed in males of the HD group.

F1C1B (P1): thyroids were not weighted.

Table 34: Thyroid weights

			Males			Females		
	Dose levels (ppm)		**200**	**600**	**1800**	**200**	**600**	**1800**
P0	**Terminal BW**		98%	97%	97%	98%	99%	**90%****
	Thyroid	**abs weight**	105%	106%	**124%****	96%	**111%**	108%
		rel weight	108%	109%	**127%****	99%	**112%**	**121%****
F1C1A	**Terminal BW**		97%	97%	**91%****	101%	99%	**93%****
	Thyroid	**abs weight**	101%	105%	102%	101%	**113%***	**113%***
		rel weight	103%	108%	113%	100%	**114%***	**122%****

Statistical significance: *: p<0.05 **: p<0.01

Histopathology

P0: Males and females of all test groups revealed a higher incidence of thyroid follicular cell hypertrophy/hyperplasia. Animals of the LD group showed only minimally increased incidences compared to control. In addition, there was an increase of more floccular, basophilic colloid (altered colloid) in HD males and females of all test groups.

F1C1A: Males and females of the HD group (1800 ppm) revealed a higher incidence of thyroid follicular cell hypertrophy/hyperplasia and a higher incidence of altered colloid.

Increased incidence of thyroid follicular cell hypertrophy/hyperplasia was also observed in female of the MD group.

F1C1B (P1): thyroid histopathology was not performed.

Table 35: Thyroid histopathological findings in P0

	P0 males				P0 females			
Dose level (ppm)	**0**	**200**	**600**	**1800**	**0**	**200**	**600**	**1800**
N	20	20	20	20	20	20	20	20
Hypertrophy/hyperplasia, follicular	5	7	**10**	**15**	0	**2**	**7**	**16**
• Grade 1	5	7	**10**	**9**		**2**	**7**	**12**

• Grade 2				**5**				**4**
• Grade 3				**1**				
Altered colloid	12	5	**10**	**17**	3	**7**	**10**	**19**
• Grade 1	10	4	**7**	**3**	3	**7**	**8**	**1**
• Grade 2	2	1	**3**	**8**			**2**	**12**
• Grade 3				**5**				**6**
• Grade 4				**1**				

Table 36: Thyroid histopathological findings in F1C1A

	F1C1A males				**F1C1A females**			
Dose level (ppm)	**0**	**200**	**600**	**1800**	**0**	**200**	**600**	**1800**
N	20	20	20	20	20	20	20	20
Hypertrophy/hyperpl., follicular	1	4	2	**7**	0	0	**3**	**8**
• Grade 1	1	4	2	**6**			**3**	**6**
• Grade 2				**1**				**2**
Altered colloid	0	0	0	**2**	0	0	0	**3**
• Grade 1								**2**
• Grade 2				**2**				
• Grade 3								**1**

Thyroid hormones

P0: In P0 males of all test groups (200, 600 and 1800 ppm), T4 values were significantly decreased, although not dose dependent, while a dose-dependent but not statistically significant increase of TSH values was observed.

In females of all test groups (200, 600 and 1800 ppm) TSH values were significantly increased. T4 values were not changed.

Table 37: Levels of Thyroid hormones in P0 males

		Group 00/M 0 ppm	Group 01/M 200 ppm	Group 02/M 600 ppm	Group 03/M 1800 ppm
T4	Mean	46.66 v	39.15 *	36.68 **	38.62 **
[nmol/L]	S.d.	3.04	7.72	7.51	3.66
day 123	N	10	10	10	10
	Median	46.83	40.52	35.80	37.69
	Deviation Vs Control [%]		-16.08	-21.38	-17.23
TSH	Mean	9.17 k	10.37	10.86	11.91
[µg/L]	S.d.	1.82	3.92	6.92	4.22
day 123	N	10	10	10	10
	Median	8.48	9.36	8.77	11.40
	Deviation Vs Control [%]		13.09	18.42	29.91

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
v=KRUSKALL-WALLIS-WILCOX; k=KRUSKALL-WALLIS

Table 38: Levels of Thyroid hormones in P0 females

		Group 00/F 0 ppm	Group 01/F 200 ppm	Group 02/F 600 ppm	Group 03/F 1800 ppm
T4 [nmol/L] day 132	Mean	24.28 k	23.28	23.85	27.68
	S.d.	3.86	4.10	5.81	10.22
	N	10	10	10	10
	Median	24.40	24.06	22.66	25.75
	Deviation Vs Control [%]		-4.12	-1.77	13.98
TSH [µg/L] day 132	Mean	5.19 v	8.22 **	6.55 *	9.87 **
	S.d.	1.14	3.08	1.41	3.94
	N	10	10	10	10
	Median	4.98	7.28	6.77	9.66
	Deviation Vs Control [%]		58.39	26.19	90.13

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided). * p<=0.05, ** p <=0.01, X = Group excluded from statistics
k=KRUSKALL-WALLIS; v=KRUSKALL-WALLIS-WILCOX

F1C1A: In males of MD and HD groups (600 and 1800 ppm), T4 values were significantly decreased. In HD males, TSH values was significantly increased.

In females of MD and HD groups (600 and 1800 ppm), a dose-dependent increased of TSH values was observed (statistically significant only at the HD).

Table 39: Levels of Thyroid hormones in F1C1A males

		G 10 / M 0 ppm	G 11 / M 200 ppm	G 12 / M 600 ppm	G 13 / M 1800 ppm
T4 [nmol/L] day 90	Mean	66.23 v	59.94	50.88 **	48.22 **
	S.d.	7.15	9.66	7.57	7.42
	N	10	10	10	10
	Median	63.95	61.01	50.48	50.22
	Deviation Vs Control [%]		-9.50	-23.18	-27.19
TSH [µg/L] day 90	Mean	6.96 v	6.90	7.57	9.59 *
	S.d.	1.81	1.98	3.38	2.20
	N	10	10	10	10
	Median	6.70	6.84	6.95	9.71
	Deviation Vs Control [%]		-0.88	8.68	37.79

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided). * p<=0.05, ** p <=0.01, X = Group excluded from statistics
v=KRUSKALL-WALLIS-WILCOX

Table 40: Levels of Thyroid hormones in F1C1A females

		G 10 / F 0 ppm	G 11 / F 200 ppm	G 12 / F 600 ppm	G 13 / F 1800 ppm
T4 [nmol/L] day 90	Mean	41.52 v	38.97	32.08 *	44.71
	S.d.	6.15	5.44	9.43	5.82
	N	10	10	10	10
	Median	41.56	40.34	31.26	43.44
	Deviation Vs Control [%]		-6.13	-22.73	7.70
TSH [µg/L] day 90	Mean	4.55 v	4.86	6.16	7.24 **
	S.d.	0.70	0.99	1.98	2.67
	N	10	10	10	10
	Median	4.42	4.62	5.74	6.96
	Deviation Vs Control [%]		6.99	35.56	59.18

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided). * p<=0.05, ** p <=0.01, X = Group excluded from statistics
v=KRUSKALL-WALLIS-WILCOX

PND 4 and 22 F1-offspring

PND4: In HD, group (1800 ppm) hormone values of only 2 pups of each sex could be measured due to the decreased litter sizes. T4 values in males of all test groups including the control group and in females of the MD and HD groups were below the HCD. While T4 values in male and female pups were not statistically significantly changed, it could not be ruled out that the decreased observed from the MD was related to treatment.

There was no significant effect on TSH values of males and females (all values were within the HCD range).

At PND 22: There was no significant effect on T4 values of males and females (all values were within the HCD range). TSH values in male and female PND22 pups of the MD and HD groups (600 and 1800 ppm) were significantly and dose-dependently increased. While TSH values of all test groups were within HCD

ranges, considering the dose-response relationship, the increased TSH level in PND22 pups from the MD is considered treatment related.

Thyroid hormones were not investigated in F2 pups.

Table 41: Levels of Thyroid hormones PND4 males

		Group 00/M 0 ppm	Group 01/M 200 ppm	Group 02/M 600 ppm	Group 03/M 1800 ppm
T4 [nmol/L] day 4	Mean	16.02 k	15.76	14.12	10.14 X
	S.d.	5.36	2.78	2.30	2.50
	N	9	7	9	2
	Median	16.76	14.89	13.71	10.14
	Deviation Vs Control [%]	0.00	-1.61	-11.88	-36.68
TSH [µg/L] day 4	Mean	4.43 k	4.50	4.47	3.98 X
	S.d.	0.33	0.44	0.42	0.32
	N	9	7	9	2
	Median	4.44	4.35	4.56	3.98
	Deviation Vs Control [%]	0.00	1.46	0.83	-10.29

Statistic Profile - Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
k=KRUSKALL-WALLIS; X = Group excluded from statistics

HCD range 12 studies 2015-2019	
T4 nmol/L	18.36-36.79
TSH µg/L	3.19-5.25

Table 42: Levels of Thyroid hormones PND4 females

		Group 00/F 0 ppm	Group 01/F 200 ppm	Group 02/F 600 ppm	Group 03/F 1800 ppm
T4 [nmol/L] day 4	Mean	18.74 k	17.95	14.89	15.71 X
	S.d.	4.62	5.00	2.66	5.49
	N	10	8	9	2
	Median	17.70	17.01	15.37	15.71
	Deviation Vs Control [%]	0.00	-4.19	-20.54	-16.16
TSH [µg/L] day 4	Mean	4.46 k	4.72	4.74	3.83 X
	S.d.	0.41	0.81	0.60	0.81
	N	10	8	9	2
	Median	4.52	4.66	4.70	3.83
	Deviation Vs Control [%]	0.00	5.96	6.30	-14.07

Statistic Profile - Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
k=KRUSKALL-WALLIS

HCD range 10 studies 2015-2019	
T4 nmol/L	17.88-34.51
TSH µg/L	3.05-6.36

Table 43: Levels of Thyroid hormones PND22 males

		Group 00/M 0 ppm	Group 01/M 200 ppm	Group 02/M 600 ppm	Group 03/M 1800 ppm
T4 [nmol/L] day 22	Mean	53.52 k	49.56	54.46	56.49
	S.d.	10.26	6.66	9.35	9.57
	N	10	10	10	10
	Median	53.72	47.84	52.26	57.46
	Deviation Vs Control [%]		-7.39	1.77	5.55
TSH [µg/L] day 22	Mean	3.51 v	3.78	4.19 *	4.86 **
	S.d.	0.57	0.64	0.71	0.63
	N	10	10	10	10
	Median	3.54	3.71	4.12	4.88
	Deviation Vs Control [%]		7.78	19.45	38.44

Statistic Profile - Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
k=KRUSKALL-WALLIS; v=KRUSKALL-WALLIS-WILCOX

HCD range 10 studies 2015-2019	
T4 nmol/L	50.57-71.39
TSH µg/L	3.40- 4.87

Table 44: Levels of thyroid hormones in PND22 females

		Group 00/F 0 ppm	Group 01/F 200 ppm	Group 02/F 600 ppm	Group 03/F 1800 ppm
T4 [nmol/L] day 22	Mean	52.85 k	49.21	58.31	55.84
	S.d.	7.22	5.60	13.55	9.29
	N	10	10	10	10
	Median	52.89	49.21	52.78	57.71
	Deviation Vs Control [%]		-6.89	10.34	5.67
TSH [µg/L] day 22	Mean	3.57 v	3.99	4.05 *	4.15 **
	S.d.	0.45	0.81	0.52	0.36
	N	10	10	10	10
	Median	3.49	3.92	3.91	4.18
	Deviation Vs Control [%]		11.58	13.23	16.23

Statistic Profile - Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics
k=KRUSKALL-WALLIS; v=KRUSKALL-WALLIS-WILCOX

HCD range 10 studies 2015-2019	
T4 nmol/L	44.85-73.70
TSH µg/L	2.92-5.13

DEVELOPMENTAL NEUROTOXICITY

No clinical signs or changes of general behaviour, which may be attributed to the test substance, were detected in any of the male and female animals in any of the groups.

Auditory Startle Response F1C2A animals on PND24

According to the study author, no influence of the test substance on auditory startle habituation (maximum amplitude and latency) was observed in any male or female animal in all treated groups.

The ASR test presented some limitations: poor reporting of the apparatus used, statistical analysis not in line with the NAFTA guidance (i.e. no mention, or results presented for testing for interactions of sex, trial blocks and treatment) for maximal amplitude and latency as well as a complete absence of any statistical testing for habituation (a variable required under OECD 443). Furthermore, the lack of HCD and positive control increases the possibility the risk of false negative findings taking into account the low statistical power in DNT investigations. Therefore, the effects from the mid-dose level on the mean maximal amplitude in males as well as habituation (proxy calculation mean Block1 minus mean Block 5) from the mid-dose in males and in males and females combined are considered biologically relevant from the DS point of view, considering their magnitude in the absence of appropriate statistical analysis (testing for interactions of sex, trial blocks and treatment) and positive controls

Table 45: Mean maximal amplitude in males females over time, overall mean (Blocks 1 to 5) and habituation (Block1 minus Block 5)

Males	Max. Ampl.								habituation	
		Block 1	Block 2	Block 3	Block 4	Block 5	Block 1-5	% controls	Block1 - block5	%control
0ppm	Mean	578.3	386.6	368.6	290.6	368.2	**398.5**	100	210.1	100
	SD	299.7	231.7	257.9	114.1	293.3	227.4			
	CV (%)	51.8	59.9	70.0	39.3	79.7	57.1			
	N	10	10	10	10	10	10			
200 ppm	Mean	746.6	572.7	381.3	318.5	366.2	**477.1**	120	380.4	181
	SD	262.2	171.7	165.9	125.9	170.3	176.8			
	CV (%)	35.1	30.0	43.5	39.5	46.5	37.1			
	N	10	10	10	10	10	10			
600 ppm	Mean	453.4	348.1	350.5	314.2	328	**350.8**	88	125.4	60
	SD	282.2	205.0	155.2	122.9	154.7	181.8			
	CV (%)	62.3	58.9	44.3	39.1	47.2	51.8			
	N	10	10	10	10	10	10			
1800 ppm	Mean	395.6	342.6	298.7	281.4	305.4	**324.7**	81	90.2	43
	SD	247.1	185.9	139.4	120.2	131.7	162.3			
	CV (%)	62.5	54.3	46.7	42.7	43.1	50.0			
	N	10	10	10	10	10	10			

Table 46: Mean maximal amplitude in females over time, overall mean (Blocks 1 to 5) and habituation (Block1 minus Block 5)

Females	Max. Ampl.								habituation	
		Block 1	Block 2	Block 3	Block 4	Block 5	Block 1-5	% controls	Block1 - block5	%control
0ppm	Mean	478.8	328.5	276.4	276.0	275.4	327.1	100.0	203.4	100
	SD	219.9	109.1	50.4	48.2	95.1	66.9			
	CV (%)	45.9	33.2	18.2	17.5	34.5	20.5			
	N	10	10	10	10	10	10			
200 ppm	Mean	455.7	299.7	385.2	347.7	346.8	367.0	112.2	108.9	54
	SD	161.0	65.1	224.1	147.7	126.9	120.2			
	CV (%)	35.3	21.7	58.2	42.5	36.6	32.8			
	N	10	10	10	10	10	10			
600 ppm	Mean	408.5	335.2	269.9	252.7	295.8	312.2	95.4	112.7	55
	SD	195.0	157.8	60.0	49.5	81.9	89.6			
	CV (%)	47.7	47.1	22.2	19.6	27.7	28.7			
	N	10	10	10	10	10	10			
1800 ppm	Mean	465.5	319.2	338.3	321.1	334.2	355.7	108.7	131.3	65
	SD	200.0	83.9	112.6	94.3	92.4	85.2			
	CV (%)	43.0	26.3	33.3	29.4	27.7	23.9			
	N	10	10	10	10	10	10			

Table 47: Mean maximal amplitude in males and females combined females over time, overall mean (Blocks 1 to 5) and habituation (Block1 minus Block 5)

M & F	Max. Ampl.								habituation	
		Block 1	Block 2	Block 3	Block 4	Block 5	Block 1-5	% controls	Block1 - block5	%control
0ppm	Mean	528.6	357.5	322.6	283.3	321.9	362.8	100.0	206.65	100
	SD	260.9	178.8	187.0	85.7	217.6	167.1			
	CV (%)	49.4	50.0	58.0	30.3	67.6	46.1			
	N	20	20	20	20	20	20			
200 ppm	Mean	601.1	436.2	383.3	333.1	356.5	422.1	116.4	244.7	118.4
	SD	400.1	287.4	223.5	134.0	150.2	201.4			
	CV (%)	66.6	65.9	58.3	40.2	42.1	47.7			
	N	20	20	20	20	20	20			
600 ppm	Mean	431.0	341.7	310.2	263.5	311.9	331.5	91.4	119.1	57.6
	SD	161.2	148.4	112.5	55.9	97.1	87.4			
	CV (%)	37.4	43.4	36.3	21.2	31.1	26.4			
	N	20	20	20	20	20	20			
1800 ppm	Mean	430.6	331.0	318.5	301.4	319.8	340.3	93.8	110.8	53.6
	SD	164.6	113.4	97.1	75.1	76.1	74.6			
	CV (%)	38.2	34.3	30.5	24.9	23.8	21.9			
	N	20	20	20	20	20	20			

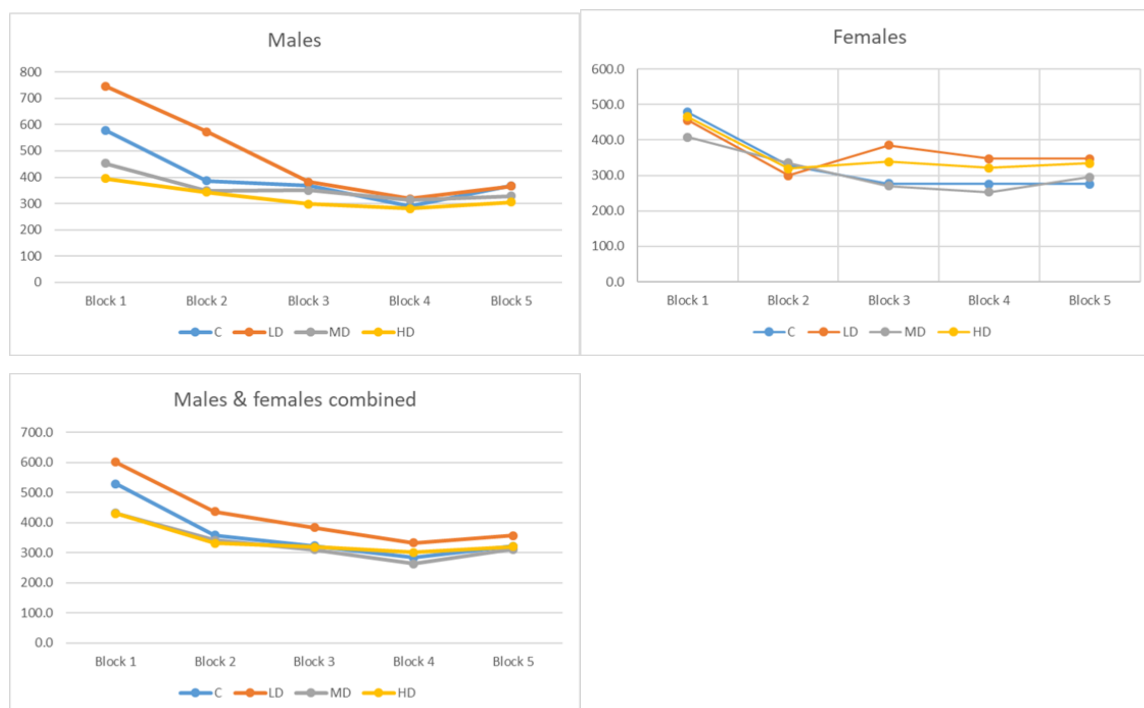


Figure 1: Mean maximal amplitude over times showing decreased habituation (flat curves) in males and in males and females combined from the mid-dose.

Functional observational battery (FOB) and motor activity in cohort F1C2A animals on PND69

No test substance-related or spontaneous findings were observed in male and female animals of all test groups during the home cage observation.

No statistically significant changes on motor activity data (summation of all intervals) was observed in the male and female animals of all dose groups in comparison to the concurrent control group.

The DS notes that the motor activity was measured in the dark, as numbers of horizontal and vertical movements during 12 intervals, each lasting 5 minutes. There is no explanation on the fact that the investigation was performed in the dark during their normal light-phase of their circadian cycle. Statistical analysis was not performed according to the NAFTA guidance and no HCD or positive control are available. However, the CV seems appropriate and the assessment of the endpoints indicates no effect.

Neuropathology

- Brain weight

The mean absolute and relative brain weight did not show significant differences when compared to the control group in F1C2A animals.

In F1C2B (terminated at PND22), absolute brain weight was unaffected and the increased relative brain weights from the mid dose level in males and at the high dose level in females groups were assessed as a secondary effect to the body weight decrease.

- Brain length and width

In F1C2A animals, the length of the brain of high dose males was significantly decreased (-3.2%). Width measurements were without any findings.

In F1C2B animals, al length and width measurements were without any findings.

- Morphometry F1C2A animals (not performed in F1C2B animals)

According to the study author, there were no statistically significant changes.

However, the corpus callosum width was non-significantly increased in the high dose group by 17 and 16%, in males and females, respectively (low- and mid-dose levels not analysed). From the DS view, this is a rather large and biologically significant change in the size of a brain region. Furthermore, a two-way anova (sex, treatment) statistical analysis of the data performed by the DS showed that the effect is actually statistically significant. It is noteworthy that the corpus callosum is the principal inter-hemispheric myelinated tract (white matter) and histopathological findings linked to myelin degeneration in the cord white matter were observed in C2A animals.

Table 48: Brain morphometrics

Male:

Group		Measurements									
		1	2	3	4	5	6	7	8	9	10
10	Measurement (mm)	1.81	1.82	3.95	3.90	1.85	1.99	0.59	1.60	1.52	0.82
	Percent deviation (Control set to 100%)	100	100	100	100	100	100	100	100	100	100
13	Measurement (mm)	1.75	1.78	4.00	3.79	1.78	1.85	0.69	1.54	1.56	0.88
	Percent deviation (Control set to 100%)	97	98	101	97	96	93	117	96	103	107
	Statistical Significance										

Female:

Group		Measurements									
		1	2	3	4	5	6	7	8	9	10
10	Measurement (mm)	1.71	1.74	3.87	3.74	1.81	1.83	0.56	1.58	1.58	0.90
	Percent deviation (Control set to 100%)	100	100	100	100	100	100	100	100	100	100
13	Measurement (mm)	1.77	1.77	4.05	3.90	1.77	1.86	0.65	1.66	1.65	0.88
	Percent deviation (Control set to 100%)	104	102	105	104	98	102	116	105	104	98
	Statistical Significance										

1 Frontal cortex left
2 Frontal cortex right
3 Nucleus caudatus width left
4 Nucleus caudatus width right
5 Parietal cortex left
6 Parietal cortex right
7 Corpus callosum width
8 Hippocampus left
9 Hippocampus right
10 Base of lobus vermis cerebelli No 8
N/A No measurement possible (tear in section)

- Histopathological findings

<u>F1C2A animals</u>

In the thoracic cord of high dose males, there was an increased incidence of focal to multifocal axonal degeneration in the white matter. This was characterized by digestion chambers with occasional pyknotic nuclei and presence of gitter cells (macrophages with foamy cytoplasm interpreted as ingested myelin debris).

According to the study author, the pathogenesis of this finding might be linked to thyroid and suggested that axonal degeneration observed in this study might be an exacerbation of the spontaneous finding as the repair mechanism controlled by thyroid hormones might be impaired.

While not discussed in the study report and not graded, axonal degenerations were also slightly increased in other areas (2 vs 0 tibial nerve degeneration in high-dose males; lumbar cord axonal degeneration and sciatic nerve degeneration in 2 high-dose females vs 0 in controls).

Table 42: Neurohistopathological findings in F1C2A animals

		Males				**Females**			
	Dose level (ppm)	**0**	**200**	**600**	**1800**	**0**	**200**	**600**	**1800**
	No. of animals	10	10	10	10	10	10	10	10
Thoracic cord	Degeneration, axonal	2	5	4	**9**	4	3	1	4
	• Grade 1	2	5	4	**8**	4	3	1	4
	• Grade 2	1	0	0	1	0	0	0	0
	Gitter cells	1	2	1	**5**	2	1	0	0
	• Present	1	2	1	**5**	2	1	0	0
Lumbar cord	Degeneration, axonal	1	–	–	0	0	–	–	**2**
Prox. sciatic nerve	Degeneration, axonal	1	–	–	1	0	–	–	**2**
Prox. tibial nerve	Degeneration, axonal	**0**	**–**	**–**	**2**	**1**	**–**	**–**	**1**

A follow-up analysis of thoracic spinal cord of F1C2B males (Amendment 2022) has been performed and no incidence of axonal degeneration was found. Other areas in F1C2B males were not examined and F1C2B females were not investigated.

In the absence of effects in F1C2B males, the study author interpreted the axonal degeneration in thoracic cord of F1C2A males to be a chronic toxic effect rather than a developmental effect.

However, the DS considers that exposure during the developmental period could have contributed to the delayed effects observed in F1C2A animals on PND77 even if not observed at an earlier time point (PND22). According to RAC note (RAC/62/2022/05) addressing developmental neurotoxicity and neurotoxicity under the current CLP hazard classes, adverse effects on the nervous system investigated or detected at any point in the life span of the organism exposed during the developmental period, covering both prenatal and postnatal development until sexual maturation (determined by preputial separation and vaginal opening), should be addressed under developmental toxicity (DNT), even if the exposure had also continued after sexual maturation. Furthermore, in the single available study (i.e. OECD TG 422) where the spinal cord and the sciatic nerve of adults (not exposed during developmental phases) were processed for histopathological investigation, no axonal degeneration was observed in males or females which further supports the involvement of developmental exposure in the occurrence of this lesion.

Conclusion

The study author proposed the following NOAELs.

The NOAEL for general, systemic toxicity is below 200 ppm (about 18 mg/kg bw/d) in the F0 parental rat, based on evidence for liver toxicity and corresponding thyroid histopathology and thyroid hormone changes in all test groups. The NOAEL in the F1 adult rats is 200 ppm. At 1800 ppm (about 167 mg/kg bw/d) distinct toxicity such as decreased body weight/body weight gain, anemia as well as liver and thyroid toxicity was noted in the F0 parental animals as well as adolescent and adult F1 offspring, including F1B parental rats
The NOAEL for fertility and reproductive performance for the F0 and F1 parental rats is 600 ppm (about 54 mg/kg bw/d), based on lower numbers of implants and subsequently smaller litters at the LOAEL (Lowest Observed Adverse Effect Level) of 1800 ppm (about 167 mg/kg bw/d).
The NOAEL for developmental toxicity in the F1 and F2 progeny is 200 ppm (about 18 mg/kg bw/d), based on reduced preweaning body weight gain, which was observed at the LOAEL (Lowest Observed Adverse Effect Level) of 600 ppm (about 54 mg/kg bw/d).
The NOAEL for developmental neurotoxicity for the F1 progeny is 600 ppm (about 54 mg/kg bw/d), based on an increased incidence of focal to multifocal axonal degeneration in the white matter of thoracic cord, which was observed at the LOAEL (Lowest Observed Adverse Effect Level) of 1800 ppm (about 167 mg/kg
When compared to control animals no findings were noted in the thoracic spinal cord of male weanling rats (Cohort 2B) after perfusion fixation. The increased incidence of axonal degeneration in the thoracic cord of male animals of the Cohort 2A (day 77) is therefore interpreted to be a chronic toxic effect rather than a developmental effect (Amendment, 20222).

- ➢ The DS considers that effects relevant for classification for fertility are observed (decreased number of implantation and consequent decreased litter size) from the mid dose level.
- ➢ The DS considers the neurohistopathological findings, morphometric changes and the effects on ASR as relevavnt for developmental classification.

3.10.1.4 Study 4

Unpublished Study Report (2014). Combined 28-day repeated dose toxicity study with the reproduction/developmental toxicity screening test of Benzenamine, N-phenyl-reaction products with 2,4,4- timethylpentene in rats by oral gavage.

Test type

- GLP-study
- OECD TG 422 (1996)
- Deviations

Compared to current OECD TG 422 (2016): Pups terminated before PND 13, AGD and nipple retention not investigated. Estrous cycle not monitored.

Test substance

- Test material used in the study is equivalent **to Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene** identified in the CLH dossier

- Name of test substance: Benzenamine, N-phenyl-, reaction products with 2,4,4- trimethylpentene
- Batch No.: 40401913D
- Purity: 100 % (UVCB)

Test animals

- Rat: Crl:WI(Han) (outbred, SPF-Quality). Male/Female
- 10 per sex per dose
- Age and weight at the study initiation: 10-12 weeks

Administration/exposure and description of test design

- Oral gavage, corn oil 5 mL/kg body weight
- Males were exposed for 28 days, i.e. 2 weeks prior to mating, during mating, and up to the day prior to scheduled necropsy. Females were exposed for 39-45 days, i.e. during 2 weeks prior to mating, during mating, during post-coitum, and during at least 4 days of lactation (up to the day prior to scheduled necropsy).
- Actual doses (mg/kg bw/day):
  Test group 1: 0
  Test group 2: 25 mg/kg bw/d
  Test group 3: 75 mg/kg bw/d
  Test group 4: 225 mg/kg bw/d
- Historical control data if available

Description of test design:

The following observations and examinations were evaluated: mortality / viability, clinical signs (daily), functional observations and locomotor activity (end of treatment), body weight and food consumption (at least at weekly intervals), clinical pathology (end of treatment), macroscopy at termination, organ weights and histopathology on a selection of tissues, and reproduction/developmental parameters, consisting of mating, fertility and conception indices, precoital time, number of corpora lutea and implantation sites, gestation index and duration, parturition, maternal care, sex ratio and early postnatal pup development (mortality, clinical signs, body weights and macroscopy). Formulations were analyzed to assess accuracy, homogeneity and stability**.**

Results

- No treatment-related death was observed. One control female (no. 50) was euthanized in extremis after a prolonged parturition.
- Salivation was seen after dosing for animals at 75 and 225 mg/kg bw/day after dosing. No other clinical signs of toxicity were noted during the observation period including during the functional observations battery.
- Food consumption, body weights and body weight gain of treated animals remained in the same range as controls over the treatment period.

- *Males reproductive data*

No toxicologically relevant effects on reproductive parameters were noted with treatment up to 225 mg/kg bw/day. Furthermore, spermatogenic staging profiles were normal for all males examined.

- *Female reproduction and delivery data*

The mating, fertility and conception indices, precoital time, and number of corpora lutea and implantation sites were unaffected by treatment.

There were 10, 10, 10 and 9 pregnant females in the control, 25, 75 and 225 mg/kg bw/day groups, respectively.

No toxicologically relevant effects on the gestation index and duration, parturition, maternal care or on most aspects of early postnatal pup development (clinical signs, body weight and macroscopy) were observed up to 225 mg/kg bw/day.

The gestation index and duration of gestation were similar between all groups.

While not statistically significant, the mean number of implantation sites was decreased (-16%) in the high-dose group (9.2 vs 10.9 in controls).

Table 43: Litter report

		GROUP 1 CONTROL	GROUP 2 25 MG/KG	GROUP 3 75 MG/KG	GROUP 4 225 MG/KG
NECROPSY					
Corpora Lutea	MEAN	13.2	13.7	12.1	12.2
	ST.DEV	2.3	3.7	2.0	2.4
	N	10	10	10	9
Implantations	MEAN	10.9	11.5	10.5	9.2
	ST.DEV	2.4	2.2	1.7	2.3
	N	10	10	10	9

LITTERS				
TOTAL	9	10	10	9
DURATION OF GESTATION				
MEAN (+)	21.3	21.2	21.1	21.1
ST.DEV.	0.5	0.6	0.6	0.8
N	9	10	10	9
DEAD PUPS AT FIRST LITTER CHECK				
LITTERS AFFECTED (#)	0	1	0	0
TOTAL	0	1	0	0
MEAN (+)	0.0	0.1	0.0	0.0
ST.DEV.	0.0	0.3	0.0	0.0
N	9	10	10	9
LIVING PUPS AT FIRST LITTER CHECK				
% OF MALES / FEMALES (#)	57 / 43	55 / 45	51 / 49	45 / 55
TOTAL	98	105	94	71
MEAN (+)	10.9	10.5	9.4	7.9 +
ST.DEV.	2.6	2.5	2.0	1.8
N	9	10	10	9
POSTNATAL LOSS				
% OF LIVING PUPS	0.0	1.0	0.0	11.3
LITTERS AFFECTED (#)	0	1	0	3
TOTAL (#)	0	1	0	8 ##
MEAN (+)	0.0	0.1	0.0	0.9
ST.DEV.	0.0	0.3	0.0	1.7
N	9	10	10	9
VIABILITY INDEX (#)	100.0	99.0	100.0	88.7 ##

Viability index = (Number of alive pups before planned necropsy / Number of pups born alive) *100
+/++ Steel-test significant at 5% (+) or 1% (++) level
/ ## Fisher's Exact test significant at 5% (#) or 1% (##) level

- *F1 generation litter/pups*

The number of dead pups at first litter check and sex ratio were unaffected by treatment, and clinical signs, body weight and external macroscopy did not reveal treatment-related findings.

At 225 mg/kg bw/day the mean number of living pups at first litter check (7.9) was significantly lower than for controls (10.9). Female no. 80 had only 4 pups, which contributed to this slightly low mean.

Discounting her data, there were a mean of 8.4 pups/litter, which was also lower than controls and an effect of treatment could not be excluded.

The postnatal loss was also significantly higher for animals at 225 mg/kg bw/day with 11.3% of living pups lost (8 pups over 3 litters) than controls (no pups lost). High dose animals also had a correspondingly low viability index of 88.7%. The majority of the pups (5) came from a single litter and there were no signs of ill health in other pups from this dose level.

However, when taken together with the lower mean number of pups at first litter check, an effect of treatment could not be excluded.

Body weights of pups were unaffected by treatment up to 225 mg/kg bw/day.

- *Clinical pathology*

Haemathology: No treatment-related changes among hematological parameters were observed.

Clinical chemistry

Compared to controls, animals at 225 mg/kg bw/day had relevant increases in alkaline phosphatase (ALP, both sexes), bilirubin (both sexes), glucose (males), cholesterol (females) and decreased albumin (both sexes), total protein (females) inorganic phosphate (both sexes) and total thyroxine (T4, both sexes).

At 75 mg/kg bw/day, animals had increased ALP (both sexes, not significantly different for males), glucose (males, not statistically significant), total bilirubin (both sexes) and decreased albumin (both sexes) and total protein (females).

Calcium was lower for all treated animals of both sexes with no clear dose-response relationship.

The statistically significant decrease in cholesterol seen for males at 75 mg/kg bw/day was not considered toxicologically relevant as it occurred in the absence of a dose-dependent distribution.

Thyroid hormones

T4 measurements were significantly lower for females of Groups 2 and 3 as well, with no clear dose-response relationship.

Thyroid stimulating hormone (TSH) was much higher than controls for all groups (both sexes, not always statistically significant). These data showed high variability with one or more individuals in each treated group showing extremely high values (Group 2, male no. 11, Group 3 male nos. 22, 25, and female no. 62 and Group 4 male no. 33). When recalculated excluding the outliers, group means remained higher than controls (males Group 2: 0.518, Group 3: 0.548, Group 4: 0.475; females Group 3: 0.386); though no dose-dependent distribution was apparent. In the absence of a clear relationship with total T3 or T4, and in the absence of adverse findings seen in the thyroids during the microscopic examination, no toxicological relevance was attributed to higher TSH and T4 values by the study author. The DS considers that a treatment related cannot be excluded.

Table 44: Thyroid hormones in males

		GROUP 1 CONTROL	GROUP 2 25 MG/KG	GROUP 3 75 MG/KG	GROUP 4 225 MG/KG
END OF TREATMENT					
Inorg.Phos	MEAN	2.08	1.88	1.90	1.79 *
mmol/L	ST.DEV	0.25	0.05	0.14	0.07
	N	5	5	5	5
TSH	MEAN	0.143	1.116	0.706	0.794
uIU/mL	ST.DEV	0.070	1.347	0.253	0.740
	N	5	5	5	5
Total T3	MEAN	85.1	74.3	77.9	73.6
ng/dL	ST.DEV	9.8	4.4	5.9	12.8
	N	5	5	5	5
Total T4	MEAN	4.28	3.97	3.79	3.04 *
ug/dL	ST.DEV	1.10	0.74	0.27	0.24
	N	5	5	5	5

Table 45: Thyroid hormones in females

		GROUP 1 CONTROL	GROUP 2 25 MG/KG	GROUP 3 75 MG/KG	GROUP 4 225 MG/KG
END OF TREATMENT					
Inorg.Phos	MEAN	2.54	2.21	2.16	1.86 **
mmol/L	ST.DEV	0.29	0.16	0.29	0.16
	N	5	5	5	5
TSH	MEAN	0.146	0.490 *	0.488 *	0.224
uIU/mL	ST.DEV	0.037	0.233	0.282	0.075
	N	5	5	5	5
Total T3	MEAN	71.1	62.2	72.6	73.3
ng/dL	ST.DEV	8.0	6.0	8.0	9.0
	N	5	5	5	5
Total T4	MEAN	3.15	1.96 *	2.05 *	2.02 *
ug/dL	ST.DEV	0.44	0.49	0.58	0.71
	N	5	5	5	5

- *Pathology*

Organ weights

Absolute and relative liver weights were significantly higher for males of all treatment groups (approximately 20-54% increase in relative weight for males). Liver weights were also higher for females at 75 and 225 mg/kg bw/day, but the difference from controls was only significantly different for females at 75 mg/kg bw/day.

Relative kidney weights were significantly higher for males at 225 mg/kg bw/day only.

Other organ weights and organ to body weight ratios among the dose groups were similar to control levels.

Histopathology

Test item-related microscopic findings were noted in the liver of both sexes starting at 75 mg/kg bw/day, the thyroid gland of the males in all treated groups, and in the kidney of females at 225 mg/kg bw/day.

Hepatocellular hypertrophy of the liver was noted in 6/6 males (6 minimal) and 1/5 females (1 minimal) treated at 75 mg/kg bw/day and in 8/8 males (2 minimal, 6 slight) and 6/6 females (2 minimal, 4 slight) at 225 mg/kg bw/day.

Hepatocellular vacuolation of zone 1 (periportal) and/or zone 2 (midzonal) of the liver was noted in 2/5 females (2 minimal) treated at 75 mg/kg bw/day and in 8/8 males (3 minimal, 5 slight) and 6/6 females (3 minimal, 1 slight, 2 moderate) at 225 mg/kg bw/day.

This finding consisted of microvesicular vacuolation of the midzonal hepatocytes (zone 2) in the males, whereas in the females the vacuolation ranged from microvesicular vacuolation of the periportal hepatocytes (zone 1) to combined micro-and macrovesicular vacuolation of periportal and midzonal hepatocytes (recorded as slight and moderate degrees) in the 225 mg/kg bw/day group.

Follicular cell hypertrophy of the thyroid gland was noted at an increased incidence in 4/5 males (2 minimal, 2 slight) treated at 25 mg/kg bw/day, in 5/5 males (2 minimal, 3 slight) at 75 mg/kg bw/day and in 5/5 males (minimal) at 225 mg/kg bw/day compared to 1/5 males (minimal) of the control group. The study author considered the increased incidence of hypertrophy of the follicular epithelium was considered to be non-adverse based on the absence of a clear dose response and/or relation in severity and being within background levels noted in male rats of this strain and age. The DS considered that taking into account hormonal changes, the histopathological findings observed in thyroid glands are considered treatment-related.

Accumulation of brown pigment in the cortical tubular epithelium of the kidneys of females was noted in 3/5 rats (minimal) treated at 225 mg/kg bw/day. At the low severity and in absence of other kidney pathology (like inflammation, degeneration or necrosis) was regarded to be non-adverse in nature.

No effect was observed at histopathological examination of the spinal cord (cervical, thoracic and lumbar) and the sciatic nerve.

Table 46: Summary of histopathological findings

	Males				Females			
Dose level:	**0**	**25**	**75**	**225**	**0**	**25**	**75**	**225**
Liver [a]	**5**	**5**	**6**	**8**	**5**	**5**	**5**	**6**
Hepatocellular hypertrophy								
Minimal	-	-	6	2	-	-	1	2
Slight	-	-	-	6	-	-	-	4
Vacuolation zone 1/2								
Minimal	-	-	-	*3*	-	-	*2*	*3*
Slight	-	-	-	*5*	-	-	-	*1*
Moderate	-	-	-	-	-	-	-	*2*
Thyroid gland [a]	**5**	**5**	**5**	**5**	**5**	**0**	**0**	**5**
Hypertrophy follicular cell								
Minimal	1	2	2	5	2	-	-	1
Slight	-	2	3	-	-	-	-	-
Kidney [a]	**5**	**1**	**0**	**5**	**5**	**5**	**5**	**5**
Accumulation brown pigment								
Minimal	-	-	-	-	-	-	-	3

Conclusion

The study author proposed the following NOAELs

Parental NOAEL: 25 mg/kg bw/day, based on hepatic toxicity.

Reproduction NOAEL: 225 mg/kg bw/day in the absence of reproductive toxicity

Developmental NOAEL: 75 mg/kg bw/day based on lower mean number of pups at the first litter check compared to controls (7.9 versus 10.9) and an increase in postnatal loss and a correspondingly lower viability index.

- The DS considers that it cannot be excluded that the decreased mean number of implantation sites in high dose females.

3.10.1.5 Study 5

Study reference

Unpublished study report, 2014c. Reaction products of benzeneamine, N-phenyl with nonene (branched) **ORAL PRENATAL DEVELOPMENTAL TOXICITY STUDY IN RATS.**

Test type

- GLP-study
- OECD TG 414 (2001)
- Deviations None

Test substance

- Test material used in the study is equivalent to **Reaction products of diphenylamine with nonene, branched** identified in the CLH dossier
- Name of test substance: Reaction products of benzeneamine, N-phenyl with nonene (branched)
- Batch identification.: 240312/k7
- Purity: 100 % (UVCB)

Test animals

- Wistar IGS Crl: WI (Han)
- 24 mated females per sex per dose
- Virgin female rats 10 weeks old and with a weight range of 177-196 g The male rats to be used were from the same supplier and were at least 11 weeks old (at least 298 g).

Administration/exposure

- All animals were administered during the gestation period, starting from Day 6 through Day 19 post coitum at the dosing volume of 4 mL/kg.
- Vehicle: corn oil. The formulations were prepared daily (concentrations of 12.5, 37.5 and 125 mg/mL) and the concentrations were calculated and expressed in terms of test item as supplied
- Actual doses (mg/kg bw/day):

Test group 1 (control, vehicle alone): 0

Test group 2: 50 mg/kg bw/d

Test group 3: 150 mg/kg bw/d

Test group 4: 500 mg/kg bw/d

- HCD from 6 studies between 2004 and 2014. All studies (867 fetuses out of 138 litters) used Wistar Hannover virgin female rats of at least 9 weeks.

Description of test design:

- Females were paired one to one in the home cage of the male and left overnight. Vaginal smears were taken daily in the morning from the day after pairing until a positive identification of mating was made. The day of mating, as judged by the presence of sperm in the vaginal smear or by the presence of a copulation plug, was considered as Day 0 of gestation (or Day 0 post coitum).
- All animals were administered during the gestation period, starting from Day 6 through Day 19 post coitum at the dosing volume of 4 mL/kg. Body weight, daily clinical signs and food consumption were recorded during the in vivo phase. All females were caesarean-sectioned on Day 20 post coitum and subjected to post mortem examination. The number of corpora lutea, implantations, early and late intrauterine deaths, live and dead fetuses, uterus weight, fetal weight and fetal sex were recorded. All fetuses were examined for external abnormalities. Approximately one half of the fetuses in each litter was examined for fixed-visceral and skeletal abnormalities

Results and discussion

- No animal died during the study. 8 females were found not pregnant at necropsy: 1 each in the control and mid-dose groups, 4 in the low dose group and 2 in the high dose group.
  The number of females with live fetuses on gestation Day 20 was 23 in each of the control and mid-dose groups, 20 in the low dose group and 22 in the high dose group.
- No signs of toxicological significance were noted during the study and no signs of reactions to treatment were observed during the dosing period.
- Statistically significant decrease in body weight (up to 7%) and body weight gain was observed in treated females receiving 500 mg/kg bw/day, starting from Day 9 post coitum. Statistically significant decrease in corrected body weight and corrected body weight gain was noted in treated females receiving 150 and 500 mg/kg bw/day. However, the difference of corrected body weight at 150 mg/kg bw/day versus control was minor (258 g versus 270 g) and therefore not considered adverse.
- Statistically significant decrease in food consumption (up to 22%) was observed in treated females receiving 500 mg/kg bw/day, starting from Day 9 post coitum.

Litter data

Litter data, mean fetal weight and sex ratio were unaffected by treatment.

Table 47: Litter data

Group(s)		Corpora Lutea	Implan-tations	Uterine Deaths Early	Uterine Deaths Late	Uterine Deaths Total	Viable young Total	Viable young M	Viable young F	% Males	Implantation loss (%) Pre	Implantation loss (%) Post	Implantation loss (%) Total	Litter Weight (g)	Mean Foetal Weight (g)
1	Mean	12.87	12.26	0.52	0.04	0.57	11.70	6.00	5.70	50.96	4.68	4.15	8.52	43.10	3.69
	SD	1.63	1.63	1.47	0.21	1.50	1.77	2.24	2.05	17.27	5.13	10.27	11.78	6.71	0.34
	(n)	23	23	23	23	23	23	23	23	23	23	23	23	23	23
2	Mean	12.26	11.47	0.21	0.05	0.26	11.21	5.67	5.84	49.82	6.56	4.12	10.05	39.88	3.63
	SD	2.58	2.44	0.54	0.23	0.56	2.84	2.22	2.29	14.18	6.13	11.67	13.68	9.32	0.40
	(n)	19	19	19	19	19	19	18	19	18	19	19	19	19	19
3	Mean	13.09	12.22	0.52	0.00	0.52	11.70	5.78	5.91	49.19	6.28	4.73	10.86	42.41	3.65
	SD	2.70	2.35	0.73	0.00	0.73	2.55	1.98	1.98	12.79	6.68	6.74	7.39	8.85	0.28
	(n)	23	23	23	23	23	23	23	23	23	23	23	23	23	23
4	Mean	12.27	11.73	0.14	0.00	0.14	11.59	5.36	6.23	46.95	6.11	1.18	7.23	40.11	3.51
	SD	2.66	3.06	0.35	0.00	0.35	3.08	2.06	2.25	12.97	10.60	3.07	10.96	9.86	0.39
	(n)	22	22	22	22	22	22	22	22	22	22	22	22	22	22

* = Statistically significantly different from control group value at p< 0.05

External examination of fetuses

15 small fetuses (fetal weight < 2.7 g) were detected: 2 out of 269 in the control group, 2 out of 214 in the low dose group, 1 out of 269 in the mid-dose group and 10 out of 254 in the high dose group. This higher number of small fetuses was due to one litter (7 fetuses from female no. 147). Systemic toxicity was particularly marked in this female (corrected terminal BW -21% as compared to controls and negative corrected BW gain, hunched posture and piloerection at GD20).

One fetus in the high dose group showed malrotation of the hindlimb, considered incidental.

Table 48: External examination of fetuses

Group	Organ	Cat	Observation(s)	No. Foetuses Observed	No. Foetuses Affected	%	No. Litters Observed	No. Litters Affected	%
1	Whole foetus		No abnormalities detected	269	267	99.26	23	23	
	Whole foetus	AN	Small	269	2	0.74	23	2	8.70
2	Whole foetus		No abnormalities detected	214	212	99.08	20	20	
	Whole foetus	AN	Small	214	2	0.93	20	1	5.00
3	Whole foetus		No abnormalities detected	269	268	99.63	23	23	
	Whole foetus	AN	Small	269	1	0.37	23	1	4.35
4	Whole foetus		No abnormalities detected	254#	243	95.67	22	22	
	Hindlimb	MA	Malrotated, left	254#	1	0.39	22	1	4.55
	Whole foetus	AN	Small	254#	10	3.92	22	3	13.64

One foetus was erroneously not examined (see section 4.8)

Visceral examination of fetuses

No relevant findings that could be considered treatment-related were observed at visceral examination of fetuses in the treated groups, compared to controls.

Table 49: Visceral examination of fetuses

Group	Organ	Cat	Observation(s)	No. Foetuses Obs	No. Foetuses Aff	No. Foetuses %	No. Dams Obs	No. Dams Aff	No. Dams %
1	Abdomen	VA	Haemorrhagic	129	3	2.33	23	2	8.70
	Kidneys	AN	Ectopic	129	1	0.78	23	1	4.35
	Testis	AN	Displaced	129	2	1.55	23	1	4.35
	Ureter	VA	Enlarged slight	129	1	0.78	23	1	4.35
	Whole foetus	-	No abnormalities detected	129	122	94.57	-	-	-
2	Kidneys	AN	Ectopic	100	3	3.00	19	2	10.53
	Ureter	VA	Enlarged slight	100	1	1.00	19	1	5.26
	Whole foetus	-	No abnormalities detected	100	96	96.00	-	-	-
3	Heart	AN	Haemorrhagic	128	1	0.78	23	1	4.35
	Kidneys	AN	Ectopic	128	1	0.78	23	1	4.35
	Testis	AN	Displaced	128	3	2.34	23	3	13.04
	Whole foetus	-	No abnormalities detected	128	122	95.31	-	-	-
4	Abdomen	VA	Haemorrhagic	122	2	1.64	22	2	9.09
	Kidneys	AN	Ectopic	122	6	4.92	22	3	13.64
	Kidneys	VA	Pelvic dilatation slight	122	1	0.82	22	1	4.55
	Testis	AN	Displaced	122	2	1.64	22	2	9.09
	Ureter	AN	Enlarged moderate	122	1	0.82	22	2	9.09
	Ureter	VA	Enlarged slight	122	3	2.46	22	2	9.09
	Whole foetus	-	No abnormalities detected	122	108	88.52	-	-	-

Skeletal examination of fetuses

The alterations recorded at skeletal examinations of fetuses were noted in both control and treated groups with a similar incidence.

Table 50: Skeletal examination of fetuses

Group	Organ	Cat	Observation(s)	No. Foetuses Obs	No. Foetuses Aff	No. Foetuses %	No. Dams Obs	No. Dams Aff	No. Dams %
1	Forepaw(s)	AN	Metacarpal(s) no ossification 4th	140	25	17.86	23	12	52.17
	Forepaw(s)	AN	Abnormal shape	140	2	1.43	23	2	8.70
	Lumbar vertebrae	AN	Arch(es) incomplete ossification	140	1	0.71	23	1	4.35
	Ribs	AN	Wavy	140	7	5.00	23	3	13.04
	Ribs	VA	Rudimentary 14th	140	72	51.43	23	20	86.96
	Ribs	VA	14 ribs	140	7	5.00	23	4	17.39
	Ribs	VA	Short 14th	140	6	4.29	23	2	8.70
	Skull	AN	Temporal incomplete ossification	140	17	12.14	23	7	30.43
	Skull	AN	Hyoid no ossification	140	1	0.71	23	1	4.35
	Skull	AN	Zygomatic incomplete ossification	140	5	3.57	23	2	8.70
	Skull	VA	Parietal incomplete ossification	140	15	10.71	23	6	26.09
	Skull	VA	Supraoccipital incomplete ossification	140	9	6.43	23	5	21.74
	Skull	VA	Interparietal incomplete ossification	140	16	11.43	23	6	26.09
	Sternebrae	AN	Asymmetrical ossification 5th	140	3	2.14	23	3	13.04
	Sternebrae	AN	Asymmetrical ossification	140	6	4.29	23	6	26.09
	Sternebrae	VA	No ossification 5th	140	5	3.57	23	5	21.74
	Sternebrae	VA	Incomplete ossification	140	4	2.86	23	4	17.39
	Sternebrae	VA	Incomplete ossification 5th	140	34	24.29	23	15	65.22
	Sternebrae	VA	Incomplete ossification 6th	140	38	27.14	23	16	69.57
	Thoracic vertebrae	VA	Centrum incomplete ossification	140	6	4.29	23	6	26.09
	Thoracic vertebrae	VA	Centrum dumb-bell shaped	140	2	1.43	23	2	8.70
	Whole foetus	-	No abnormalities detected	140	21	15.00	-	-	-

Group	Organ	Cat	Observation(s)	No. Foetuses Obs	No. Foetuses Aff	No. Foetuses %	No. Dams Obs	No. Dams Aff	No. Dams %
2	Forepaw(s)	AN	Metacarpal(s) no ossification 4th	114	28	24.56	20	10	50.00
	Lumbar vertebrae	AN	Centrum dumb-bell shaped	114	1	0.88	20	1	5.00
	Lumbar vertebrae	VA	Centrum incomplete ossification	114	1	0.88	20	1	5.00
	Ribs	AN	Thickened	114	1	0.88	20	1	5.00
	Ribs	AN	Wavy	114	8	7.02	20	5	25.00
	Ribs	VA	14 ribs	114	12	10.53	20	5	25.00
	Ribs	VA	Rudimentary 14th	114	77	67.54	20	19	95.00
	Ribs	VA	Short 14th	114	9	7.89	20	7	35.00
	Skull	AN	Zygomatic incomplete ossification	114	3	2.63	20	3	15.00
	Skull	AN	Frontal incomplete ossification	114	1	0.88	20	1	5.00
	Skull	AN	Temporal incomplete ossification	114	8	7.02	20	7	35.00
	Skull	AN	Hyoid no ossification	114	6	5.26	20	5	25.00
	Skull	VA	Parietal incomplete ossification	114	8	7.02	20	6	30.00
	Skull	VA	Supraoccipital incomplete ossification	114	4	3.51	20	3	15.00
	Skull	VA	Interparietal incomplete ossification	114	12	10.53	20	8	40.00
	Sternebrae	AN	Asymmetrical ossification	114	6	5.26	20	4	20.00
	Sternebrae	AN	No ossification 6th	114	5	4.39	20	3	15.00
	Sternebrae	AN	Asymmetrical ossification 5th	114	2	1.75	20	2	10.00
	Sternebrae	VA	Incomplete ossification 6th	114	33	28.95	20	13	65.00
	Sternebrae	VA	No ossification 5th	114	9	7.89	20	6	30.00
	Sternebrae	VA	Incomplete ossification 5th	114	35	30.70	20	13	65.00
	Sternebrae	VA	Incomplete ossification	114	3	2.63	20	3	15.00
	Thoracic vertebrae	AN	Centrum bipartite	114	3	2.63	20	3	15.00
	Thoracic vertebrae	VA	Centrum incomplete ossification	114	5	4.39	20	3	15.00
	Thoracic vertebrae	VA	Centrum dumb-bell shaped	114	1	0.88	20	1	5.00
	Whole foetus	-	No abnormalities detected	114	4	3.51	-	-	-

Group	Organ	Cat	Observation(s)	No. Foetuses Obs	Aff	%	No. Dams Obs	Aff	%
3	Cervical vertebrae	AN	Arches incomplete ossification	141	1	0.71	23	1	4.35
	Cervical vertebrae	AN	Cervical rib(s)	141	1	0.71	23	1	4.35
	Forepaw(s)	AN	Metacarpal(s) no ossification 4th	141	17	12.06	23	10	43.48
	Forepaw(s)	AN	Abnormal shape	141	1	0.71	23	1	4.35
	Forepaw(s)	AN	All metacarpal incomplete ossification	141	1	0.71	23	1	4.35
	Hindpaw(s)	AN	Metatarsal(s) no ossification 4th	141	1	0.71	23	1	4.35
	Hindpaw(s)	AN	Metatarsal(S) incomplete ossification	141	1	0.71	23	1	4.35
	Lumbar vertebrae	AN	Arch(es) incomplete ossification	141	1	0.71	23	1	4.35
	Pelvic girdle	AN	Pubis incomplete ossification	141	1	0.71	23	1	4.35
	Pelvic girdle	AN	Ischium incomplete ossification	141	1	0.71	23	1	4.35
	Ribs	AN	Wavy	141	3	2.13	23	3	13.04
	Ribs	VA	Rudimentary 14th	141	103	73.05	23	23	100.00
	Ribs	VA	Short 14th	141	11	7.80	23	7	30.43
	Ribs	VA	14 ribs	141	3	2.13	23	3	13.04
	Sacral vertebrae	AN	Arch(es) incomplete ossification	141	1	0.71	23	1	4.35
	Skull	AN	Zygomatic incomplete ossification	141	2	1.42	23	2	8.70
	Skull	AN	Palatine incomplete ossification	141	1	0.71	23	1	4.35
	Skull	AN	Hyoid no ossification	141	5	3.55	23	4	17.39
	Skull	AN	Temporal incomplete ossification	141	7	4.96	23	4	17.39
	Skull	AN	General incomplete ossification	141	1	0.71	23	1	4.35
	Skull	VA	Supraoccipital incomplete ossification	141	3	2.13	23	3	13.04
	Skull	VA	Parietal incomplete ossification	141	5	3.55	23	5	21.74
	Skull	VA	Interparietal incomplete ossification	141	6	4.26	23	6	26.09
	Sternebrae	AN	Asymmetrical ossification	141	3	2.13	23	2	8.70
	Sternebrae	AN	Asymmetrical ossification 5th	141	3	2.13	23	3	13.04
	Sternebrae	VA	Incomplete ossification	141	2	1.42	23	2	8.70
	Sternebrae	VA	Incomplete ossification 5th	141	29	20.57	23	15	65.22
	Sternebrae	VA	No ossification 5th	141	1	0.71	23	1	4.35
	Sternebrae	VA	Incomplete ossification 6th	141	46	32.62	23	18	78.26
	Thoracic vertebrae	AN	Centrum asymmetrical ossification	141	1	0.71	23	1	4.35
	Thoracic vertebrae	AN	Centrum bipartite	141	2	1.42	23	2	8.70
	Thoracic vertebrae	VA	Centrum incomplete ossification	141	4	2.84	23	4	17.39
	Thoracic vertebrae	VA	Centrum dumb-bell shaped	141	1	0.71	23	1	4.35
	Whole foetus	-	No abnormalities detected	141	10	7.09	-	-	-

Group	Organ	Cat	Observation(s)	No. Foetuses Obs	Aff	%	No. Dams Obs	Aff	%
4	Forepaw(s)	AN	Abnormal shape	133	1	0.75	22	1	4.55
	Forepaw(s)	AN	Metacarpal(s) no ossification 4th	133	14	10.53	22	8	36.36
	Ribs	AN	Wavy	133	1	0.75	22	1	4.55
	Ribs	VA	Short 14th	133	7	5.26	22	5	22.73
	Ribs	VA	14 ribs	133	12	9.02	22	6	27.27
	Ribs	VA	Rudimentary 14th	133	95	71.43	22	22	100.00
	Skull	AN	Temporal incomplete ossification	133	7	5.26	22	6	27.27
	Skull	AN	Frontal incomplete ossification	133	1	0.75	22	1	4.55
	Skull	AN	Hyoid no ossification	133	6	4.51	22	4	18.18
	Skull	VA	Interparietal incomplete ossification	133	7	5.26	22	4	18.18
	Skull	VA	Parietal incomplete ossification	133	3	2.26	22	3	13.64
	Sternebrae	AN	Asymmetrical ossification 5th	133	5	3.76	22	4	18.18
	Sternebrae	AN	Additional ossification site	133	1	0.75	22	1	4.55
	Sternebrae	AN	Asymmetrical ossification	133	10	7.52	22	5	22.73
	Sternebrae	VA	Incomplete ossification 5th	133	37	27.82	22	15	68.18
	Sternebrae	VA	No ossification 5th	133	3	2.26	22	2	9.09
	Sternebrae	VA	Incomplete ossification 6th	133	51	38.35	22	17	77.27
	Sternebrae	VA	Incomplete ossification	133	5	3.76	22	3	13.64
	Thoracic vertebrae	AN	Centrum bipartite	133	3	2.26	22	3	13.64
	Thoracic vertebrae	VA	Centrum incomplete ossification	133	3	2.26	22	3	13.64
	Whole foetus	-	No abnormalities detected	133	13	9.77	-	-	-

Conclusion

Based on the results obtained in this study, the mid-dose level (150 mg/kg bw/day) was considered the NOAEL for maternal toxicity and the high dose level (500 mg/kg bw/day) was considered the NOAEL for developmental toxicity by the study author.

- The DS considers that the increased number of small fetuses in the high-dose group is rather considered as secondary to the poor condition of female no.147 as a specific landmark of developmental toxicity.

3.10.1.6 Study 6

Study reference:

Unpublished study report, 2019a. Reaction products of benzeneamine, N-phenyl with nonene (branched) Prenatal Developmental Toxicity Study in New Zealand White Rabbits Oral Administration (Gavage).

Test type

- GLP-study
- OECD TG 414 (2018)
- Deviations: None

Test substance

- Test material used in the study is equivalent to **Reaction products of diphenylamine with nonene, branched** identified in the CLH dossier
- Name of test substance: Reaction products of benzeneamine, N-phenyl with nonene (branched)
- Batch identification.: 0016046440

Test animals

- New Zealand White rabbits (Crl:KBL(NZW))
- 25 inseminated females per sex per dose
- 15-16 weeks old at the study initiation.
- Body weight on GD 0 varied between 3397 – 4185 g

Administration/exposure

- The test substance was administered as an aqueous suspension to groups of 25 inseminated female New Zealand White rabbits orally by gavage on gestation days (GD) 6 through 28.
- The vehicle control group, consisting of 25 females, was dosed with the vehicle (0.5% Sodium carboxymethyl cellulose [CMC] suspension in deionized water (with 10 mg/100 mL Cremophor EL) in parallel.
- Actual doses (mg/kg bw/day):

Test group 0 (control, vehicle alone): 0

Test group 1: 10 mg/kg bw/d

Test group 2: 30 mg/kg bw/d

Test group 3: 100 mg/kg bw/d

- A standard dose volume of 10 mL/body weight was used for each test group.
- HCD report based on 14 studies (2014-2017), 13 by gavage, one by diet.

Description of test design:

- Food consumption and body weight of the animals were recorded regularly throughout the study period. The state of health of the animals was checked each day.
- On GD 29, all females were sacrificed and assessed by gross pathology (including weight determinations of the unopened uterus and placentas). For each doe, corpora lutea were counted and number and distribution of implantation sites (differentiated between resorptions, live and dead fetuses) were determined.
- At terminal sacrifice on GD 29, 20-24 females per group had implantation sites.
- The fetuses were removed from the uterus, sexed, weighed and further investigated for any external, soft tissue and skeletal (inclusive cartilage) findings.

Results and discussion

- One female of the control group (No. 21) died after gavage error.
- Two control (Nos. 7 [GD 20] and 11 [GD 28]) and four high-dose females (Nos. 78 [GD 24], 79 [GD 26], 84 [GD 28] and 94 [GD 27] - 100 mg/kg bw/d) were sacrificed after abortion. In the HCD from 14 studies (2014-2017), only 4 abortions out 350 dams (1, 1%) were reported (the incidence by study was not reported). The high-dose cases may already represent exaggerated maternal toxicity, given also the distinct drop in food consumption and body weight gain as well as an exceptionally high number of does showing reduced defecation.
- In total, reduced defecation was observed in four control, two low-dose, eleven mid-dose and twenty high-dose females (0, 10, 30 and 100 mg/kg bw/d). The high incidence of reduced defecation in the high dose group, along with reduced food consumption indicates a treatment-related effect.
- In high dose does (100 mg/kg bw/d), food consumption was distinctly and statistically significantly reduced from GD 7-23 (up to -59% in comparison to the control). Overall, the high-dose does consumed 31% less food than the concurrent control does during the treatment period (GD 6-28).
- At this dose level there was a reduced mean body weights (BW), reduced average body weight gain (BWC), weight loss during the treatment period (GD 6-28, -24.0 g vs. +104.4 g in control) and lower net weight gain (-421.0g) in comparison to the concurrent control (-322.2 g).
- At the lower dose levels, no test substance-related adverse effects on food consumption and body weight were noted.

Table 51: Effects in does

Dose mg/kg bw/d	**0**	**10**	**30**	**100**
Mean food consumption/d GD6-28 (g)	118.2	123	114.1	81.6 (-31%)
Mean BW GD6 (g)	3932	3914	3908	3910
Mean BW GD14 (g)	4036	4022	4020	3888*
Mean BW GD28 (g)	4030	4071	4018	3864
Mean corrected BW GD28 (g)	3610.3	3663.4	3591.9	3473.3 (-4%)
Mean BW change GD6-28 (g)	104.4	156.9	110.4	-24
Mean corrected BW change GD6-28 (g)	-322.2	-250.2	-316	-421
Pregnant females N	23	24	24	24
Abortion	2	0	0	**4**

* : $p \leq 0.05$

Litter data

- The conception rate was 92% in the control group (0 mg/kg bw/d) and 96% in the low-, mid- and high-dose groups (10, 30 and 100 mg/kg bw/d).
- There were no test substance-related and/or biologically relevant differences between the different test groups in conception rate, in the mean numbers of corpora lutea and implantation sites or in the

values calculated for the pre- and the post-implantation losses, the numbers of resorptions and viable fetuses.

External examination of fetuses

- The sex distribution of the fetuses in test groups 1-3 (10, 30 and 100 mg/kg bw/d) was comparable to the control fetuses. Any observable differences were without biological relevance.
- The mean fetal weight of test group 3 (100 mg/kg bw/d) was statistically significantly lower than control in male fetuses and when both sexes were combined (-12% in comparison to the concurrent control). The mean weight of the female high-dose fetuses was also slightly lower but without attaining statistical significance.
- Four fetuses in one single litter (high-dose doe No. 76) had multiple external malformations, i.e. domed head, cleft palate and small tongue, partly associated with visceral or skeletal malformations. One of these four fetuses had also a hydrocephaly (visceral malformation) and another one malformed skull bone (skeletal malformation). All of them had paw hyperflexion (variation) and empty stomach (unclassified soft tissue observation).
- No statistically significant differences of overall incidences were noted between the groups.

Table 52: Individual fetal external malformations

Test group	Doe No.-Fetus No., Sex	Finding
0 (0 mg/kg bw/d)	25-10 F a)	domed head
1 (10 mg/kg bw/d)	38-05 D	open eye
2 (30 mg/kg bw/d)	none	
3 (100 mg/kg bw/d)	76-04 M a), 76-06 F, 76-11 F b), 76-12 M	multiple external malformations
	99-01 F	umbilical hernia

Table 53: Malformations in the four polymarformed feteses from doe No.76

3 (100 mg/kg bw/d)	76-04 M	multiple external malformations (domed head, cleft palate, small tongue), hydrocephaly
	76-06 F	multiple external malformations (domed head, cleft palate, small tongue)
	76-11 F	multiple external malformations (domed head, cleft palate, small tongue), severely malformed skull bones
	76-12 M	multiple external malformations (domed head, cleft palate, small tongue)

No. = number; M = male; F = female; D = dead
a) fetus with additional soft tissue malformations, b) fetus with additional skeletal malformations

Skeletal examination of fetuses

- Regarding skeletal malformations, no significant differences between the groups were note. The overall incidences were well within the historical control range of the test facility.

Table 54: Individual fetal skeletal malformations

Test group	Doe No.-Fetus No., Sex	Finding
0 (0 mg/kg bw/d)	9-03 M	thoracic hemivertebra
1 (10 mg/kg bw/d)	26-10 M 46-04 M	thoracic hemivertebra, misshapen thoracic vertebra thoracic hemivertebra
2 (30 mg/kg bw/d)	68-07 F 75-06 F	exoccipital fused with 1st cervical arch, cervical hemivertebra thoracic hemivertebra, branched rib
3 (100 mg/kg bw/d)	76-11 F [a)]	severely malformed skull bones

- The observed skeletal variations were related to several parts of fetal skeletons and appeared in the majority of cases without a relation to dosing.

  However, irregular ossification of interparietal was increased and outside the historical control range in the mid- and high-dose groups and 'unossified talus (with present cartilage)' was statistically significantly increased and outside the historical control range in test group 3 (100 mg/kg bw/d). This finding may represent slight delays of ossification, which did not affect morphology, as the underlying cartilage model was intact in all these cases.

Table 55: Occurrence of statistically significantly increased fetal skeletal variations (expressed as mean percentage of affected fetuses/litter)

Finding	Test group 0 0 mg/kg bw/d	Test group 1 10 mg/kg bw/d	Test group 2 30 mg/kg bw/d	Test group 3 100 mg/kg bw/d	HCD Mean % (range)
Irregular ossification of interparietal	0.6	1.6	***2.9****	***2.6****	0.8 (0.0 - 1.7)
Misshapen sacral vertebra	2.4	3.8	***8.7*****	5.9	4.4 (1.9 - 8.6)
Unossified sternebra; unchanged cartilage	9.3	10.3	***24.5****	11.7	13.5 (7.7 - 23.5)
Unilateral ossification of sternebra; unchanged cartilage	0.7	1.6	3.2*	0.9	2.7 (0.5 - 5.4)
Unossified talus; cartilage present	0.0	0.8	0.0	***4.4*****	1.0 (0.0 - 2.6)

mg/kg bw/d = milligram per kilogram body weight per day; HCD = Historical control data; % = per cent
* = p ≤ 0.05 (Wilcoxon-test [one-sided]) ** = p ≤ 0.01 (Wilcoxon-test [one-sided])

Visceral examination of fetuses

- The distribution of the visceral mafformations about the test groups does not indicate an association to the treatment and no statistically significant differences between the groups were noted.

Table 56: Individual fetal visceral malformations

Test group	Doe No.-Fetus No., Sex	Finding
0 (0 mg/kg bw/d)	25-06 F 25-10 F [a)]	persistent truncus arteriosus hydrocephaly
1 (10 mg/kg bw/d)	29-02 F 37-02 M 44-01 F	persistent truncus arteriosus absent subclavian malpositioned kidney, short ureter
2 (30 mg/kg bw/d)	69-03 M 75-02 F 75-08 M	multiple malformations of the great vessels aortic arch atresia, malpositioned kidney malpositioned kidney
3 (100 mg/kg bw/d)	76-04 M [a)]	hydrocephaly

- Absent lung lobe (Lobus inferior medialis) was observed in all test groups including the control. (0, 10, 30 and 100 mg/kg bw/d). For the affected fetuses/litter incidence in the high-dose group (100 mg/kg bw/d) the difference to the concurrent control was not statistically significant, however, it was outside the historical control range (HCD: mean% 0.9, range 0.0 - 2.0). On the other hand the findings were clustered in only 3 litters resulting in a litter incidence of 15% which is inside the historical control range (HCD: mean% 7.0, range 0.0 – 17.4).
- Other variations, such as dilated cerebral ventricle, malpositioned carotid branches narrowed pulmonary trunk, dilated aorta and dilated renal pelvis occurred in individual fetuses of test groups 0, 1 and/or 3 and not considered related to treatment.

Conclusion

The study author proposed the following NOAELs.

NOAEL for maternal toxicity of 30 mg/kg bw/d based on evidence of systemic maternal toxicity at the high-dose level of 100 mg/kg bw/d, such as a slightly higher incidence of abortions and reduced defecation in almost all females of this group, along with a distinct decrease of food consumption as well as body weight/body weight gain.

NOAEL for prenatal developmental toxicity of 30 mg/kg bw/d based on slightly reduced fetal weights and evidence for a delay in ossification (the respective findings are not considered as independent, toxicologically relevant adverse effects of the test substance on embryofetal development).

- The DS considers that the increased number of abortions (4 vs. 2 in control), the decreased fetal weight associated to delays of ossification at the high dose levelmay be at least partly secondary to drop in food consumption observed at this dose level as supported by studies on caloric restriction during pregnancy in rabbit.

3.10.2 Other data

3.10.2.1 Study 7

Study reference

Unpublished Study Report (2013) Reaction products of benzeneamine, N-phenyl with nonene (branched) - Repeated-dose 90-Day Toxicity Study in Wistar Rats Administration by Gavage (study report).

Test type

- GLP-study
- OECD TG 408 (1998)
- Deviations: broadly compliant to the current version of OECD 408 (2018) except that serum total T4, T3 and TSH not measured.

Test substance

- Test material used in the study is equivalent to **Reaction products of diphenylamine with nonene, branched** identified in the CLH dossier
- Name of test substance: Reaction products of benzeneamine, N-phenyl with nonene (branched)
- Batch identification: 240312/K7

Test animals

- Crl:WI(Han) rats
- 10 animals/sex/dose
- 42 ± 1 day(s) old at the study initiation.

Administration/exposure

- Reaction products of benzeneamine, N-phenyl with nonene (branched) was administered orally (by gavage) to groups of 10 male and 10 female Wistar rats at concentration levels of 100, 300 and 1000 mg/kg bw/d over a period of at least 3 months. Control animals received respective vehicle only.
- The vehicle: corn oil. Dose volume: 4 ml/kg body weight
- Actual doses (mg/kg bw/day):

Test group 0 (control, vehicle alone): 0

Test group 1: 100 mg/kg bw/d

Test group 2: 300 mg/kg bw/d

Test group 3: 1000 mg/kg bw/d

- HCD report based on 34 studies (2004-2010), by gavage, diet, drinking water or inhalation

Description of test design

- Body weight and food consumption were determined once weekly. All animals were checked for any signs of toxicity, moribund state or mortality daily prior application and within 2 and 5 hours post application.
- Detailed clinical examinations (DCO) in an open field, were conducted prior the start of the administration period and subsequently once weekly (in the morning) thereafter.

- Functional observational battery (FOB) and measurement of Motor activity (MA) were carried out separately by gender, in all animals and randomized sequence towards the end of administration period.
- Ophthalmological examinations were performed before the beginning and at the end of the administration period.
- Clinico-chemical and haematological examination, urinalysis, as well as, sperm assessment were performed towards the end of administration period.
- After the administration period, all surviving rats were sacrificed and assessed by gross pathology, followed by histopathological examinations.

Results and discussion

- There was no mortality. Salivation after dosing was observed in all treated groups.
- There was no effect on food consumption.
- Body weight (-14.6% on day 91) and body weight gain (-24.2% on day 91) were affected in high dose males.
- There was no treatment related changes on FOB or motor activity.
- Haematology: the mean haemoglobin in rats of test group 3 (1000 mg/kg bw/d) compared to controls was slightly decreased (males 3 %; females 7 %).
- Liver effects

A change in biochemical parameters began at 100 mg/kg bw/d (increase of alkaline phosphatase (ALP) levels in females (+ 68%) ; and albumin in females (-10%)) and became more pronounced at higher doses (with, decreased total bile acid in both sexes, prolonged prothrombin time in males increased γ-glutamyltransferase (GGT) activities, glucose and triglyceride levels in females.

Histopathological findings in liver were also observed from 100 mg/kg/d (increased liver weight, centrilobular hypertrophy (minimal to slight in males and females, single cell necrosis and midzonal fatty change in males).

- Thyroid effects

There was an increased relative weight of thyroid gland in males and hypertrophy/hyperplasia of thyroid follicular cells (minimal to slight) and altered colloid (minimal) which affected an increasing number of animals with increasing doses in both sexes.

- **There were no treatment-related changes regarding the weight or histopathological examination of the reproductive organs**.

The study author concluded that under the conditions of this study, the NOAEL (no observed adverse effect level) could not be determined for the orally (by gavage) administered test substance and was therefore
< 100 mg/kg bw/d in male and female animals.

- ➢ The DS concurs with the study author that no NOAEL can be set considering liver and thyroid changes observed from the low dose level.

3.10.2.2 Study 8

Unpublished Study Report (2014a) Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene Metabolome analysis conducted for a screening study in Wistar rats Administration by gavage for 29 days.

Test type

- Non-GLP
- The metabolome investigations as described in section 2.1.2 were carried out in accordance with the Standard Operating Procedures

Test substances

- Test material used in the study is equivalent to the substances identified in the CLH dossier

Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene

- Name of test substance: Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene
- CAS No.: 68411-46-1
- Batch identification: 13/0227-1

Reaction products of diphenylamine with nonene, branched

- Name of test substance: Benzeneamine, N-phenyl with nonene (branched)
- CAS No.: *6878-20-3 (old CAS)*
- Batch identification: 240312/K7

Test animals

- Wistar rats (Crl:Wi(Han))
- 5 males/dose
- 6 weeks old at the study initiation.

Administration/exposure

- Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene (Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene) was administered daily for 29 days to 5 male Wistar rats per dose group via gavage at dose levels of **125 and 300 mg/kg body weight/day.** A group of 5 untreated males served as control.
- Results were compared to the metabolome profile from a 90-day study with reaction products of benzeneamine, N-phenyl with nonene (branched) Benzeneamine, N-phenyl with nonene (branched) at dose level **1000 and 300 mg/kg body weight/day (from study 7)**

Description of test design

- After the dosing period, blood samples for metabolome analysis were taken retroorbitally from overnight fasted animals under isoflurane anaesthesia and the obtained EDTA-plasma was covered with nitrogen and frozen at -80°C.
- The plasma metabolome was examined by metanomics GmbH following proprietary sample work up using GC/MS and LC/MS-MS techniques.

- The sex- and day-stratified heteroscedastic t-test ("Welch test") was applied to compare metabolite levels of dose groups with respective controls. A significance of $p < 0.05$ was applied.
  On the basis of 297 analytes, 15 significant metabolite changes can be expected on a significance level of 0.05 (“false positive” rate). Therefore, up to 15 significantly changed metabolites, the metabolome is considered as not affected by the test compound.
- Test substance related changes in the metabolome were analyzed as follows:

1) Analysis of specific metabolic changes for each dose group
2) Using an established algorithm, the similarity of the test compound metabolic profile with the predefined patterns in MetaMap®Tox (> 120 patterns currently covering 42 modes of action) was determined and evaluated by an expert panel.

The outcome of this assessment is one of four defined categories: The metabolite changes match a certain mode of action (“match”), the metabolic change is weakly associated with a mode of action (“weak match”), no conclusion is possible (“equivocal”) or the metabolic change does not match with a mode of action (“mismatch”).

3) Comparison with the entire metabolome of reference compounds, called “profile comparison” using Spearman and Pearson correlations. Based on the reference database, a threshold value of 0.50 for male animals and 0.60 for female animals displays approximately the 95th percentile of all correlation coefficients obtained by the profile comparison. Correlation coefficients above these values are considered as indicating a clear match between two treatments.

Results and discussion

With substance Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene, no effects on body weight and food consumption were observed.

- CHANGED METABOLITES - ASSESSMENT OF KEY CHANGES

At 300 mg/kg, Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene showed strong changes in the metabolite profile compared to the control for male animals (59 metabolite changes relative to the control group, 24 increased and 35 decreased).

Testosterone and androstenedione were significantly increased. This increase was considered incidental, as no matches with patterns corresponding to the adrenals aromatase inhibitors or compounds sharing adrenal effects by comparing against other compounds in the database were found.

Creatine, creatinine, phosphocreatine and urea were significantly decreased whereas ornithine and phosphate (inorganic and from organic phosphates) were increased but not significantly.

Many complex lipids, fatty acids and related (as nervonic acid, ceramides, lysophosphatidylcholines and phosphatidylcholines) were significantly increased; whereas many amino acids and homovanillic acid were downregulated.

At a dose level of 125 mg/kg, N-phenyl-, reaction products with 2,4,4- trimethylpentene showed also clear changes in the metabolite profile compared to the control for male animals (43 metabolite changes relative to the control group, 25 increased and 18 decreased).

Citrulline, uric acid, ornithine and phosphate (inorganic and from organic phosphates) were significantly increased

Many amino acids as well as complex lipids, fatty acids and related were significantly increased.

- DETECTION OF TOXICOLOGICAL MODES OF ACTION

The comparison of the metabolite changes induced by Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene treatment ag**ainst the established specific metabolite patterns** present in MetaMap® Tox **did not yield in any matches**, however some indications were apparent for liver and kidney toxicity.

- PROFILE COMPARISON WITH REFERENCE COMPOUNDS

Using total profile comparison, the metabolite profile of 300 mg/kg and 125 mg/kg Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene in male animals **did not show matches** (with those of compounds present in MetaMap®Tox) which would give **a clear indication for a certain toxicological mode of action.**

The best match in the database after comparison against more than 700 substances was observed for the metabolome profile from a 90-day study with reaction products of **benzeneamine, N-phenyl with nonene (branched) (Reaction products of diphenylamine with nonene, branched) (r = 0.489)).** It was found that a majority of metabolome changes were similar for both compounds in terms of significance and direction of the regulation (either increased or decreased).

Conclusion

Taken together, the metabolome analysis and evaluation for Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene in plasma after administration of 300 and 125 mg/kg bw/day of the test substance via gavage for 29 days, gives evidence for effects on liver function as well as slight effects on kidney function or slightly altered urea cycle. The Pearson-based correlation analysis showed no matches, which would give a clear indication for a certain toxicological mode of action. The best match was observed for Reaction products of benzeneamine, N-phenyl with nonene.

Table 57: Metabolite changes relative to controls after treatment with Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene or Reaction products of benzeneamine, N-phenyl with nonene

	Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene (300 mg/kg bw)	Reaction products of benzeneamine, N-phenyl with nonene (branched) (1000 mg/kg bw)	Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene (125 mg/kg bw)	Reaction products of benzeneamine, N-phenyl with nonene (branched) (300 mg/kg bw)
Compound	**m29**	**m92**	**m29**	**m92**
alpha-Tocopherol	0,67	0,61	0,90	0,89
Pantothenic acid	0,38	0,61	0,55	0,65
Fructose-6-phosphate	0,93	0,69	0,87	0,97
Salicylic acid	1,26	1,10	1,16	1,05
Coenzyme Q9	0,82	0,71	1,05	0,91
Cholic acid	1,03	NA	0,96	NA
Creatine	0,82	0,25	0,95	0,45
Taurocholic acid	0,05	0,16	0,16	0,33
Glycochenodeoxycholic acid	0,58	1,09	0,90	1,03
Glycerol, lipid fraction	0,41	0,88	0,37	1,21
Linoleic acid (C18:cis[9,12]2)	0,17	0,13	0,24	0,30
Stearic acid (C18:0)	2,69	1,32	1,46	1,96
Arachidonic acid (C20:cis[5,8,11,14]4)	2,24	1,65	1,26	2,07
Docosahexaenoic acid (C22:cis[4,7,10,13,16,19]6)	1,19	1,14	1,19	1,33
Campesterol	0,86	0,67	1,07	0,96
Heneicosanoic acid (C21:0)	0,40	0,17	0,57	0,43
Heptadecanoic acid (C17:0)	0,49	0,48	0,67	0,75
Eicosanoic acid (C20:0)	1,11	1,15	1,11	1,01
Tricosanoic acid (C23:0)	1,01	0,77	0,88	0,94
Nervonic acid (C24:cis[15]1)	1,36	0,97	1,64	1,19
Urea	0,65	0,86	0,67	1,23
Unknown lipid (28000473)	2,11	2,60	2,58	2,29
14-Methylhexadecanoic acid	0,88	1,10	0,92	1,08
gamma-Linolenic acid (C18:cis[6,9,12]3)	0,34	0,52	0,61	0,78
16-Methylheptadecanoic acid	1,31	1,15	0,98	1,14
17-Methyloctadecanoic acid	1,44	0,44	0,86	0,96
dihomo-gamma-Linolenic acid (C20:cis[8,11,14]3)	0,50	0,76	0,56	0,87
Elaidic acid (C18:trans[9]1)	3,03	1,06	1,82	1,23
myo-Inositol, lipid fraction	1,46	0,61	1,18	0,75
Lysine	1,11	1,07	1,12	1,13
Sucrose	0,81	0,73	0,98	0,82
Mannose	1,69	1,23	0,89	1,31
Proline	1,14	0,95	1,10	0,97
Threonic acid	1,03	0,89	1,07	0,94
Unknown polar (38000433)	1,96	1,79	1,58	1,77
Glucuronic acid	0,86	1,12	1,11	0,88
2-Hydroxybutyrate	1,27	1,56	1,26	1,50
Indole-3-lactic acid	0,62	0,47	0,77	0,52
Unknown polar (58000010)	0,79	0,82	0,88	0,98
Hippuric acid	0,96	2,31	1,56	1,47
Unknown polar (58000144)	1,33	0,44	1,05	0,68
Unknown polar (58000157)	0,94	1,29	0,92	1,12
Unknown polar (58000158)	0,86	0,83	0,94	0,79
Lysophosphatidylcholine (C18:2)	2,11	NA	1,34	NA
Lysophosphatidylcholine (C18:1)	1,11	1,36	1,02	1,33
Sphingomyelin (d18:2,C18:0)	1,00	0,92	0,99	0,95
Phosphatidylcholine (C16:0,C22:6)	1,12	1,77	1,17	1,37
Phosphatidylcholine No 02	0,89	0,83	0,95	0,93
Phosphatidylcholine (C16:0,C18:2)	0,89	0,65	0,97	0,69
Choline plasmalogen No 02 (putative)	0,92	0,88	0,95	0,91
Phosphatidylcholine (C16:0,C16:0)	1,07	0,82	1,03	0,97
Choline plasmalogen (C36:2) (putative)	0,80	0,87	0,83	0,99
Choline plasmalogen (C18,C20:4)	1,02	1,47	0,97	1,47
PC No 04 (putative)	0,85	0,77	0,82	0,87
TAG (C16:0,C18:2)	0,60	0,67	0,80	0,85
Unknown lipid (68000033)	0,97	0,99	0,97	1,04
Unknown lipid (68000034)	0,61	0,71	0,71	0,73
Lysophosphatidylcholine (C17:0)	0,50	NA	0,56	NA
Lyso PE (C22:0) (putative)	0,62	0,69	0,78	0,80
Lysophosphatidylcholine (C20:4)	0,45	0,52	0,62	0,77
TAG (C18:2,C18:3)	1,08	0,92	1,05	0,96
TAG (C42:9) (DAG-Fragment) (putative)	1,84	1,70	1,32	1,83
Choline plasmalogen No 03 (putative)	1,31	0,89	1,07	1,05
Phosphatidylcholine (C18:0,C18:2)	0,94	0,95	0,95	0,97
Phosphatidylcholine (C18:0,C18:1)	1,06	1,15	1,03	1,14
Phosphatidylcholine (C18:0,C20:3)	1,07	1,01	1,07	1,01
Sphingomyelin (d18:1,C24:0)	0,94	0,99	0,98	1,05
Phosphatidylcholine (C18:0,C22:6)	0,49	0,53	0,73	0,68
TAG (C52:5 (H) or C50:2 (Na)) (putative)	1,69	1,64	1,47	1,47
TAG (C16:0,C18:1,C18:3)	1,01	1,18	1,03	1,14
TAG (C18:1,C18:2,C18:3)	1,03	1,26	1,06	1,33
TAG (putative)	0,98	0,78	1,03	1,00
Unknown lipid (68000060)	0,92	0,86	0,97	0,89
Homovanillic acid (HVA)	0,70	0,89	0,90	0,98
Androstenedione	3,06	2,07	1,21	2,23
Testosterone	4,47	1,55	1,02	1,99

Legend (colour)	
yellow	significantly decreased metabolites
light yellow	not significantly but ≤20% decreased
red	significantly increased metabolites
light red	not significantly but ≥20% increased

3.11 Specific target organ toxicity – single exposure

Evaluation not performed for these substances.

3.12 Specific target organ toxicity – repeated exposure

Evaluation not performed for these substances.

3.13 Aspiration hazard

Evaluation not performed for these substances.

4 ENVIRONMENTAL HAZARDS

4.1 Degradation

4.1.1 Ready biodegradability (screening studies)

- **Study 1**

Study reference:

Unpublished study report, 1988

Detailed study summary and results:

The ready biodegradation test (OECD TG 301B) was performed using the **UVCB substance** (EC 270-128-1).

Test type:

OECD Guideline 301 B, Ready Biodegradability: CO_2 Evolution Test, modified Sturm test, GLP compliant. Derivations mentioned in the study report included: The volume of the test solution was reduced from 3.0 L to 1.5 L. The CO_2 formed by biodegradation was absorbed with NaOH and determined on a carbon analyzer. Due to the poor solubility of the test substance in water, an emulsifier was used to achieve a better distribution in the medium. The test substance was added to the medium, homogenized with Nonylphenol 10E05P0.

Test substance:

Test substance is indicated in the registration dossier as Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene (EC 270-128-1, CAS 68411-46-1).

Materials and methods:

In this study, the determination of the biodegradability was made by measurement of the carbon dioxide formation (% of $ThCO_2$ (Theoretical Carbon dioxide)) calculated from the ThOC (Theoretical organic carbon) or TOC (Total organic carbon) for 28 days. The bacteria were collected from a activated sludge of a sewage treatment plant in Reinach (CH) on 18/08/1988. The test medium was prepared following the guideline and the 2L flasks were maintained at 22 ± 2°C with addition of 25 ml/min air free of carbon dioxide. 20 mg/L of reference substance aniline and 2 concentrations of the substance (10.6 mg/L and 20.1 mg/L) were tested. One blank containing water and 0.5 mL of the Nonylphenol solution, and a second blank

containing only water were used. Measurments were maded to determine the initial CO_2 of the 0.05 N sodium hydroxide and the CO_2 absorbed in the absorbers filled with 200 ml 0.05 N sodium hydroxide on days 5, 11, 14, 18, 21, 24, 27 and 28. The biodegradation was calculated on the basis of the theoretical carbon content of the test substance and the cumulative quantities of carbon dioxide determined on the days of measurements. For the calculation the formula given in the guideline was used. For the blank values, blank 1 (with the emulgator) were used.

Results:

Based on theoretical carbon dioxide formation the biodegradation was calculated as 0% in 28 days for the tested concentrations 10.6 mg/L and 1% in 28 days for the tested concentration 20.1 mg/L. Negative degradation values were obtained for several sample days although they are within the variation of the method. The biodegradation was calculated as 80.8% after 14 days and 94.4% in 28 days for the reference substance aniline.

4.1.2 BOD_5/COD

Not assessed in this dossier

4.1.3 Aquatic simulation tests

- **Study 2**

Study reference:

Unpublished study report, 2023

Detailed study summary and results:

A simulation testing on ultimate degradation in surface water according to OECD TG 309 (GLP compliant) was performed for the for the constituent 4-tert-butyl-N-(4-tert-butylphenyl)aniline OR C4C4DPA (CAS 4627-22-9)

Test type:

OECD Guideline 309 (Aerobic Mineralisation in Surface Water - Simulation Biodegradation Test), GLP compliant.

Test substance:

The test material is indicated as 14C-Bis(4-tert-butylphenyl)amine, Batch No: 1254-1101 Chemical purity: 97.3 %

Materials and methods:

Surface water from river arm “Ranschgraben’’ west of Schifferstadt was collected on 13th April 2021. After reaching the laboratory, the coarse particles were removed from the water with a nylon sieve (100 µm) prior

to use in the test. Subsamples from the collected water were taken for determination of physico-chemical and biological parameters. Afterwards, fresh surface water sample was stored under dark conditions for one day at a temperature of ~ 5.8 °C – 6.9 °C before preparation of test assays.The test was performed in 1 L GL45 cylindrical spinner flasks with 2 angled sidearms (dimension (H x B): 23 cm x 10 cm). In total, 82 test vessels were set up for this study. To each of these 1 L test vessels, about 500 mL surface water was added and also a Teflon coated magnet bar. Separate closed tests vessels were set up for planned sampling point. Through the sampling port inlet, air was supplied after acidification process to remove the dissolved CO_2.

Test assays with test substance: Two different concentrations of test substance differed by a factor of 10 were used. Low concentration of test substance designated by acronym 'L' is intended for measuring the degradation kinetics and high concentration designated by 'H' for identification and quantification of major transformation products.

Low concentration: 4 µg test substance per liter
High concentration. 40 µg test substance per liter

Test assays with test substance for biodegradation kinetics and metabolite identification (FT): These test assays were set up in duplicates at two different test concentrations, namely low concentration FT-L assays and high concentration FT-H assays.
Test assays with test substance for mass balance calculation (FM): Additionally, test flasks with test substance were set up for mass balance calculation at the end of exposure in duplicate flasks for each test concentration (low concentration FM-L, high concentration FM-H)).
The test substance is added from the stock solution, into the test substance assays (FT and FM). The organic solvent will not exceed 1% by volume in the water phase. Low concentration flasks (FT-L & FM-L): 45.8 µL from corresponding test substance stock solution per 500 mL test water. High concentration flasks (FT-H & FM-H): 45.8 µL from corresponding test substance stock solution per 500 mL test water.
Sterile control assay (FS) with test substance for abiotic transformation: Duplicate test vessels containing sterile test system and high-test concentration test substance (40 µg/L) were set up as sterile controls. For this, surface water was autoclaved at 121°C for 20 minutes prior to the addition of test substance. These test assays were sampled only at the end of exposure to examine the possible abiotic degradation or other nonbiological removal of the test substance. No water exchange was conducted on day 59 as these are sterile samples.
Sterile control flasks (FS): 45.8 µL from corresponding test substance stock solution per 500 mL test water.
Reference control assay (FC): 14C reference substance was added from the stock solution to the test system to confirm a minimum of microbial activity. This assay was set up in duplicates for each sampling day.
Reference assay flasks (FC): 550 µL of the reference substance stock solution.

Solvent control assay (FOC): Duplicate flasks containing test system containing reference substance at a inal nominal concentration of 10 µg/L treated with the approximate same amount of solvent (50 µL acetonitrile) that was present in the highest test substance concentration used in this test (40 µg/L test substance). This was to examine possible adverse effects of the solvent by determining the degradation of the reference substance.

Solvent control (FSC) : Test flasks containing test system and used solvent but without test substance. The highest solvent volume used for adding the test substance to the test assays (50 µL) was tested in these assays.

Blank control assay (FB): Test flask with only test system was set up and was used only for the physico-chemical analysis on the samplings days as well as for the microbiological analysis at the end of exposure.

Parameter control assay with test substance (FP): These test assays were dosed with high concentration test substance (40 µg/L) and test system.

High-12C (FTMET-H): These additional test assays were prepared with 49.8 µL of non labelled test substance at high concentration of 40 µg/L and test system.

Solvent control (FSC-MET): Additional set of solvent control test flasks containing test system and solvent (50 µL) but without test substance for 12C experiment. The highest solvent volume used for adding the test substance to the test assays was dosed to these assays.

After setting up the test assays, about 5 mL of 2 M NaOH solution was placed in the internal container as the absorbing solution in each test flask. The flasks were closed with polypropylene matching screw cap with pouring ring impermeable to air and CO2 and were placed on magnetic stirrers and gently and continuously stirred while still maintaining a homogeneous suspension (approx. 100 rpm agitation) until the end of exposure. No contact between test solution and lid of the test flask is made during the preparation of test assays and also during incubation. The test vessels were connected with two CO2 traps filled with 2 M NaOH of 100 mL and a trap filled with ethylene glycol of 50 mL to capture organic volatiles.

Test vessels were filled with 500 mL surface water on 14th April 2021 and incubated 12 ± 2°C in the dark for 5 days and was aerated by a gentle orbital shaking of the test vessel on magnetic stirrer under constant air flow. This equilibration procedure was done due to very low temperature in the test system (about 7.2 °C) at the time of collection. All these test flasks were incubated at 12 ±1 °C from day 0 of exposure in a thermostat enabled water bath under dark conditions for 91 days. The test flasks were placed on magnetic stirrers and gently and continuously stirred while still maintaining a homogeneous suspension (approx. 100 rpm agitation) until the end of exposure. Temperature was checked and documented from the water bath on sampling days.

Samplings were conducted from the corresponding test assays by harvesting the whole flasks at the start of exposure (Day 0, after complete homogenization), Day 1, Day 7, Day 14, Day 21, Day 28 and on Day 49. Because of very low $^{14}CO_2$ evolution detected within the first 49 days, it was decided to extend the batch test from 60 to 91 days as a semicontinuous procedure. Reserve bottles (FTLA and FTHA, FML and FMH, FMET-H and FSC-MET) were used for sampling in the remaining 31 days. On day 59 before surface water

removal and water replenishment, direct LSC, evolved $^{14}CO_2$ from NaOH traps and organic volatiles measurements were conducted from the reserve flasks. After these measurements, water (1/3, about 165 mL) was removed from the test flasks and the residual test substance (theoretic) concentration lost in this 1/3rd water will be replenished for the initial amounts of test substance by adding adequate aliquots (approx.15.3 µL of 14C labelled test substance stock solution and approx. 16.6 µL unlabeled test substance stock solution) from corresponding stock solution with freshly collected water. Every two weeks, three additional samplings after Day 59 were conducted such as on Day 71, Day 80 and on Day 91. Since the test substance is degrading slower without significant CO2 production, long interval between sampling days is chosen after water change. No water change was needed after day 59 as the metabolite formation is detected (preliminary info from NOACK institute) and the pH and oxygen parameters were stable. Analytical samples from these additional sampling points were also sent to NOACK for substance specific analytics.
A general scheme of sampling is provided in table 3a and 3b. Sampling was conducted as described in the following steps after harvesting the whole flasks at each sampling time from the corresponding test assays and the samples are represented in mass instead of volume unless otherwise stated.

Direct LSC measurements in the test substance assays (FTL, FTLA, FTH, FTHA, FS, FSA, FML, FMH) and reference assays (FC, FCA, FOC): For direct LSC measurements, duplicate samples (about 5 mL) were taken (except on day 7 in FCA where only 2.73 g was taken) through the inlet and after combining with 15 mL of Ultima Gold scintillation cocktail, these samples were assayed by LSC.

Sampling of $_{14}CO^2$ for quantification of mineralization in the test substance assays (FTL, FTLA, FTH, FTHA, FS, FSA, FML, FMH) and reference assays (FC, FCA, FOC):
The mineralization of the test substance was determined by "direct $^{14}CO_2$ measurement" from duplicate test assays on each sampling day. The $^{14}CO_2$ generated from mineralization of test substance during experimental exposure is trapped into the absorption liquid (2 M NaOH solution) placed in the internal container attached in the test vessel.
Dissolved $^{14}CO_2$
In addition to this, there can be CO_2 dissolved in the test solution. In order to remove this dissolved CO_2 from the surface water, about 50 mL of the water phase in the test flasks was removed through the sampling port constructed of Teflon tubing that extended to the bottom of all test vessels connected to a stopcock. Prior to the removal of samples, the stopcock was opened, and the test mixture was repeatedly pulled up and pushed back into the flask using a syringe to clear the line of the test flask. The samples were added to another flask containing a compartment filled with 5 mL of 2 M NaOH and were acidified by adding about 1.5 mL of 6N HCl to lower the pH of the samples to ~ 1 without opening the vessels to the atmosphere. After acidification, the test vessel was connected to the air supply and is lightly aerated for 24 hours to allow CO_2 to diffuse from solution into the headspace. The dissolved CO_2 was captured in the absorption liquid (2 M NaOH solution) placed in the internal container in the test vessel.

After 24 hrs., a suitable aliquot from this NaOH solution (~ 1 mL) was mixed with 15 mL LSC scintillation cocktail and was measured by LSC as double determination for radioactivity. Remaining radioactivity in the acidified sample was measured in duplicates without any further treatment.

After closing the valve in the gas transferring tubes, two NaOH traps with 100 mL of 2 M solution were removed and quickly capped. Duplicate samples from the sodium hydroxide traps were transferred to scintillation vials and combined with a suitable scintillation cocktail and analyzed by LSC.
Confirmation of the analyte were originally planned to be performed in representative samples through precipitation with barium chloride if [14C]-CO2 occurred in amounts higher than 5% of applied radioactivity. For this, NaOH sample (40 mL) on each day of sampling were retained in freezer. These NaOH samples were however, not sent for substance specific analytics since the activity was very low.

Organic volatiles in the test substance assays (FTL, FTLA, FTH, FTHA, FS, FSA, FML,FMH) and reference assays (FC, FCA, FOC):
Organic volatiles that might have been formed are absorbed into the 100 mL ethylene glycol absorption flask connected to the test flasks. The aeration was stopped, and a suitable volume from this was taken after opening the valve. The samples were mixed with LSC scintillation cocktail and was measured by LSC as double determination for radioactivity. Retain sample (40 mL) on each day of sampling were stored in the freezer. These samples were discarded without further analysis, since the activity was very low.

Chemical analyses for the measurement of parent and degradation products (test substance assays only ((FTL, FTLA, FTH, FTHA, FS, FSA):
After sampling for direct LSC measurement and dissolved $^{14}CO_2$ sample from each of the test substance assays FTL, FTH, FS, the rest test water was transferred to 500 mL polypropylene flasks. After keeping in the freezer (about -18°C) until shipment, the processed samples were sent in dry ice or under cooling conditions to the test site Noack Laboratorien GmbH Käthe-Paulus-Str. 1, 31157 Sarstedt, Germany for substance specific analysis .

Chemical analyses for the measurement of parent and degradation products (test substance assays on Day 91, FM (FML & FMH):
After direct LSC analysis, following additional extraction steps were performed mass balance test assays on Day 91, FM (FML & FMH). Due to the affinity of the test item to the test vessel, on day 60 about 8 g of C18 adsorbent powder (Agilent C18 End capped)) was added as adsorber to the water phase and shaken vigorously for five minutes. The mixture of water, sediment and C18 powder were separated from the water phase by filtration through a funnel attached with fluted filter to separate the C18 material from the water phase. The funnel attached with fluted filter containing the C18 adsorbent was placed on a centrifuge vessel. The test vessel was first flushed with 100 mL of dichloromethane (DCM) and later this liquid was transferred

to the centrifuge vessel through the filter containing the C18 adsorbent. The filter containing the C18 powder was extracted additionally by adding it to the centrifuge vessel and by shaking the mixture for five minutes. This vessel was centrifuged for 4 minutes at 4000 rpm to separate the extract from filter and C18 adsorbent. The above extraction step was repeated two more times (3 extraction steps in total) with 100 mL dichloromethane in each step. An aliquot of each extraction step was analyzed by LSC and after pooling all the three extracts, the pooled extract was measured by LSC.
An aliquot was withdrawn from the water phase after filtration and analyzed by LSC. Both the combined dichloromethane extracts and water phase after filtration were sent at room temperature to the test site Noack Laboratorien GmbH Käthe-Paulus-Str. 1, 31157 Sarstedt, Germany for substance specific analysis as precautionary samples, if the analytics of the FTH do not work out due to technical issues. The extraction phases were not analyzed due to the poor mass balance recovery after this extraction procedure which is comparable to the recovery derived from direct analysis of water phase.

Residual activity in the test flasks on Day 91, FM (FML & FMH after dichloromethane extraction)
Since the mass balance recovery after summing up the contents in the test solution with the contents in the absorption bottles does not reach the required areas of activity (90%- 100%), it was assumed that the test substance and possibly formed metabolites adhered to the walls of the test vessels. Therefore, these additional following extractions were conducted intending to close the mass balance. The test vessels of the test assays FML1, FML2, FMH1 and FMH2 were extracted with various solvents to remove the attached test substance and possible metabolites from the walls of the test vessels. For this purpose, 50 mL of the corresponding solvent was heated to about 50 °C and given to the appropriate test vessel. Then the test vessels were shaken for about 30 minutes at 125 rpm on a horizontal lab shaker. This procedure was done 3 times per solvent. The individual extracts were measured in the LSC. After sampling for LSC measurement, the extracts for each solvent were combined separately. The combined extracts were also measured in the LSC. These extracts were stored at <-18°C. The weights of the amounts of solvent added and those of the extracts were documented.The extractions are carried out with the following solvents in the order indicated. 1.Acetone, 2.Acetonitrile 3.Ethanol 4.Chloroform 5.Toluene 6.Heptane. Since the recovery was poor (1%) after each solvent step except with acetone where up to 5%TAR was recovered in both concentration groups, no additional analyses were conducted on these extracts.

Chemical analyses for the measurement of Parent and degradation products (FMETH and FSC-MET)
These samples are from additional test assays with unlabeled test material (FMET-H) and its control (FSC-MET). On each sampling day (including day 0), about 5 mL sample from the duplicate test vessels of FMET-H and FSC-MET were taken and were transferred to a polypropylene vial. After keeping in the freezer (about -18°C) until shipment, the processed samples were sent in dry ice or under cooling.

Results:

Mean recoveries of total applied radioactivity (TAR) in the water phase of the tests with the low (4.0 µg/L) and high (40 µg/L) test concentrations were within the range of 44% - 94% TAR and 40 - 90% TAR in LSC measurements, respectively. On Day 0 and on day 28, a mass balance recovery in the range of 90 - 100% TAR was achieved in low test concentrations and in high concentration, on day 0, day 28 as well as on day 71. Loss of radioactivity by adsorption to the test vessels in the range of 3 - 26% in FTL and 1 - 29% in FTH test assays were observed during the exposure period. In the sterile control FS, 36% activity was found on test flask surface and 45% in water and 0.2% $^{14}CO_2$ and thus an 80% total recovery was measured on day 59. About 55% TAR in total from test water and $^{14}CO_2$ were measured in FS on day 91. Since no test vessel extraction was taken place for the FS test assays on day 91, no exact mass balance can be given. In mass balance test assays with low, FML and high concentration, FMH respectively, an overall recovery of 93% and 94% TAR was achieved on day 91 after rigorous extraction procedure using different solvents.

The test substance14C-Bis(4-tert-butylphenyl)amine was removed from the surfacewater after application by degradation and abiotic elimination processes such as adsorption to the test vessel surface. During storage a significant amount of the test substance was absorbed to the storage vessel.

Substance specific analysis of the sterile control FS test assays indicated that about 66% of the total applied radioactivity was retrieved on day 59. Test substance of about 34% as well as about 3% M1, 27% M2 and 4% M3 were determined from this assay.

Radiolabeled benzoic acid, sodium salt was used as the reference substance in biological control assay for determining the presence of biological activity in the test water system used for this study. Mineralization of about 69% after 14 days in the reference substance assays indicated that the surface water test system was biologically active and therefore, the test is valid. Reference assay with solvent indicated a cumulative total mineralization of 67% of the total applied reference item after 14 days in presence of acetonitrile as solvent. This result indicated that the solvent did not pose a toxicity to the microorganisms present in surface water test system and the test system was biologically active during exposure. Kinetic analysis was not performed in this study due to the poor mass recovery after substance specific analytics and insufficient data for metabolites and test substance in each sampling points due to the loss of activity through adsorption and possibly other abiotic transformation.

Metabolite Identification and Transformation Pathway

The analysis of FTH and FS indicated the formation of metabolites and in total, three metabolites (M1, M2 and M3) were detected. The test item concentration declined from 58% to 1% and the concentration of M1 and M2 increased during the exposure. A maximum of 13% metabolite M1, 36% metabolite M2 and 12% metabolite M3 was observed (single values) during the exposure. In the low concentration test assay, metabolite determination was analytically not possible.Metabolites could be detected in the samples of the test group FT H and FS. 4-tert-Butylaniline (CAS-No. 769-92-6) and 4-tertButylcatechol (CAS-No. 98-29-3) were hypothesized as possible metabolites. These metabolites were also postulated by the software

CATALOGIC 301C (v.11.15, LMC Oasis). Samples from the extracts of the storage vessels for the water phase of the groups FTH and FS were selected for the metabolite analysis.

4-tert-Butylaniline could be detected as metabolite in the storage vessel extract of the water samples from day 59. This is in accordance with the radiometric analysis which showed an increase in % of M2 between day 49 and day 59.

Maximum mineralization ($^{14}CO_2$) found in the low concentration test assay was 5% TAR whereas in high concentration was only 1% TAR.

No organic volatiles were detected in high concentration test assays but up to 0.5% organic volatiles were observed in low concentration test assays.

4.1.4 Other degradability studies

4.2 Bioaccumulation

4.2.1 Bioaccumulation test on fish

4.2.2 Bioaccumulation test with other organisms

Not assessed in this dossier

4.3 Acute toxicity

4.3.1 Short-term toxicity to fish

4.3.2 Short-term toxicity to aquatic invertebrates

- **Study 3**

Study reference:

Unpublished study report, 2004

Detailed study summary and results:

The short-term toxicity to aquatic invertebrates study was performed with **the UVCB substance** (EC 270-128-1).

Test type:

The study follows the OECD Guideline 202 (*Daphnia sp*. Acute Immobilisation Test). No deviation was mentioned and the study is GLP compliant.

Test substance:

The test material is Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene (EC 270-128-1, CAS 68411-46-1). The purity was not indicated.

Materials and methods:

The study was conducted as a 48-hour static test on *Daphnia magna* aged 6-24h at study initiation. Test temperature was 19-20°C, pH 7.8-7.9 and dissolved oxygen 8.8-9.3 mg/L during the study. WAFs with loading rates of the test item at 4.6, 10, 22, 46 and 100 mg/L, and a control were tested. At each test concentration and for the control, 20 daphnids were used and divided into four replicates of five daphnids each. No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the entire test duration. Analytical monitoring showed that the mean measured test concentrations (0h and 48h) were 0.32 mg/L (loading rate 10 mg/L), 0.30 mg/L (loading rate 22 mg/L), 0.63 mg/L (loading rate 46 mg/L), and 0.36 mg/L (loading rate 100 mg/L).

Results:

Toxic effects were observed in the study and were increasing with increasing nominal concentrations. The measured concentrations of the test item were approximately in the same range at all loading rates and a dose-response curve for EC50 calculation based on measured concentrations could not be determined. Therefore the results were reported with the nominal loading rates of the substance. The EL50 (24h) for immobility was >100 mg/L based on nominal loading rate (WAF) and the EL50 (48h) was 51 mg/L (nominal loading rate WAF). No mortality was observed in the control group. All the daphnids were immobilised after 48h at 100 mg/L.

- **Study 4**

Study reference:

Unpublished study report, 1998

Detailed study summary and results:

The short-term toxicity to aquatic invertebrates study was performed with **the UVCB substance** (EC 270-128-1).

Test type:

The study follows the OECD Guideline 202 (*Daphnia sp*. Acute Immobilisation Test). No deviation was mentioned and the study is GLP compliant.

Test substance:

The test material is Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene (EC 270-128-1, CAS 68411-46-1). The test material is indicated as TK 12340.

Materials and methods:

The study was conducted as a 24-hour static test on *Daphnia magna* aged 6-24h at study initiation. A water accommodated fraction with a single loading rate of 100 mg/L was performed. A supersaturated stock suspension was prepared by dispersing 40 mg of the test item into 400 ml test water by ultrasonic treatment for 15 min. The stock suspension was stirred at room temperature in the dark over 96 h. The supersaturated stock suspension was then filtered. The first 50 ml of the filtrate were discarded. Test temperature was 20-21°C, pH 8.1-8.2 and dissolved oxygen 8.2-8.4 mg/L during the study. In the control group and the test group, 20 daphnids were tested and divided into two groups of ten animals. No auxiliary solvent or emulsifier was used.

Results:

The test was stopped after 24h, since 100% mortality was observed. No immobilisation was noted in the control group. No remarkable observations were made concerning the appearance of test medium and the control. No analytical measurement was performed and the EC50 could not be determined.

4.3.3 Algal growth inhibition tests

- **Study 5**

Study reference:

Unpublished study report, 2006

Detailed study summary and results:

The toxicity to aquatic algae study was performed with **the UVCB substance** (EC 270-128-1).

Test type:

The study follows the OECD Guideline 201 (Alga, Growth Inhibition Test) is not GLP compliant and was conducted without analytical monitoring.

Test substance:

The test material is Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene (EC 270-128-1, CAS 68411-46-1).

Materials and methods:

The study was conducted on *Desmodesmus subspicatus* at nominal concentrations (based on water accommodated fractions) of 1, 10 and 100 mg/L for 72h in static condition. The respective concentrations were moderately stirred in algal medium for 24 h at room temperature. After this incubation time, the undissolved materials were removed by sterile filtration (0.45 µm) to obtain sterile test substance solutions for the algal growth test. The test temperature was 22±0.5°C and the initial cell density was 10,000-20,000 cells/ml. Three test vessels (4 for the controls) were used for each concentrations of the tested substance. No analysis of the test concentrations was conducted.

Results:

The EC50(72h) value was >100 mg/L (nominal) based on both growth rate and biomass. The determined NOEC is 10 mg/L (nominal) as a significant growth rate inhibition of 14% was observed at 100 mg/L.

4.3.4 *Lemna* sp. growth inhibition test

4.4 Chronic toxicity

4.4.1 Fish early-life stage (FELS) toxicity test

4.4.2 Fish short-term toxicity test on embryo and sac-fry stages

4.4.3 Aquatic Toxicity – Fish, juvenile growth test

4.4.4 Chronic toxicity to aquatic invertebrates

- **Study 6**

Study reference:

Unpublished study report, 2016

Detailed study summary and results:

The long-term toxicity to aquatic invertebrates study was performed with **the UVCB substance** (EC 270-128-1).

Test type:

The study follows the OECD Guideline 211 (*Daphnia magna* Reproduction Test). The study is GLP compliant and no deviations were made from the guideline.

Test substance

The test material is Benzenamine, N-phenyl-, reaction products with 2,4,4-trimethylpentene (EC 270-128-1, CAS 68411-46-1).

Materials and methods:

This study was conducted with *Daphnia magna* STRAUS in semi-static condition (daily renewal of the test solution) for 21 days at 0 (control), 0.625, 1.25, 2.5, 5 and 10 mg/L nominal concentrations. Test solutions were prepared separately for each test loading rate as a water accommodated fraction (WAF) using a liquid-liquid saturator technique. For each test solution, a glass aspirator bottle (2-L) with a bottom side-outlet attached to a stopcock was filled with 2-L test media. Prior to use the test substance was homogenized by

shaking the container. Then the test substance was pipetted carefully on the water surface according to the loading rate. The bottle was closed and the solution was stirred on a magnetic plate for about 3 days. The stirring was slow (approx. 100 rpm) so that no vortex forms. Stirring was stopped for 1-hour and any undissolved material was allowed to separate. Undissolved test substance was only observed as an oily drop on the water surface. Then the required volume of the WAF (approx. 1.2-L per test solution) was removed from the bottom port. The water pH, temperature and dissolved oxygen were within acceptable guideline specifications. Each test group consisted of 10 replicates containing one juvenile *Daphnia* (<24h old) each. Measured concentration were not performed in this study.

Results:

Reduced reproduction was observed in all treatment groups and followed a concentration response pattern. No mortality was observed among parent animals over the 21d exposure period. The reported ELR10 of 1.68 mg/L (95% CI: 0.767-3.68) and NOELR of <0.625 mg/L is therefore based on the most sensitive endpoint (reproduction). No analytical monitoring was conducted. The solutions were visually inspected for the presence of any undissolved test substance. All test solutions were colorless clear and no undissolved test substance was visible.

Mortality and Reproduction after 21 days

Test Groups	Test Groups Nominal (mg/L)	Reproduction mean living young per surviving adult	Reproduction % effect b	Mortality Parent animals	Mortality% effect b
0	0 (control)	120.3 (9.5% a)	-	0	-
1	0.625	115.5*	3.99 *	0	-
2	1.25	110.4*	8.23 *	0	-
3	2.5	103.6**	13.9 **	0	-
4	5	100.6**	16.4 **	0	-
5	10	83.2**	30.8**	0	-

*: p<=0.05; **: p<=0.01

a: coefficient of variation for control fecundity based on surviving parent

b: Effect relative to control. Only calculated for statistically significant effects

Other biological observations among surviiving parent animals in each concentration afetr 21 days exposure:

Test Groups	Test Groups Nominal (mg/L)	Mean Growth (length, mm)	% ImmobileYoung	Mean Days to First Brood	% Aborted eggs
0	0 (control)	4.22	0.0	9.9	0.3
1	0.625	4.21	0.3	9.8	0.7
2	1.25	4.17	0.4	10.0	1.0
3	2.5	4.09	1.1	10.2	0.6
4	5	4.05	0.9	10.1	0.9
5	10	3.90*	4.8*	10.9	1.6

*p<=0.01

4.4.5 Chronic toxicity to algae or aquatic plants

[See short-term toxicity]

4.5 Acute and/or chronic toxicity to other aquatic organisms

Not assessed in this dossier