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:

Reaction products of diphenylamine with nonene, branched

EC Number: [1]

CAS Number:

Index Number: Not listed in Annex VI of CLP

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[1] No EC or CAS number allocated. To be specified when allocated.

CONTENTS

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Unpublished Study Report (2020a) Reaction products of benzeneamine, N-phenyl with nonene (branched), Reproduction/Developmental Toxicity Screening Test in Wistar Rats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
- 100% (UVCB)
- 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
		F_1	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 [µkat/L] day 123	Mean	0.63 v	0.71	0.76 **	0.95 **
	S.d.	0.05	0.15	0.15	0.49
	N	10	10	10	10
	Median	0.60	0.66	0.70	0.75
	Deviation Vs Control [%]		13.42	21.25	51.60
AST [µkat/L] day 123	Mean	1.67 k	1.65	1.71	1.83
	S.d.	0.27	0.28	0.22	0.27
	N	10	10	10	10
	Median	1.65	1.58	1.67	1.81
	Deviation Vs Control [%]		-1.44	2.51	9.58
ALP [µkat/L] day 123	Mean	1.00 v	1.26 *	1.94 **	2.66 **
	S.d.	0.16	0.22	0.15	0.36
	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 [nkat/L] day 123	Mean	25 x+	25	25	25
	S.d.	0	0	0	1
	N	10	10	10	10
	Median	25	25	25	25
	Deviation Vs Control [%]		0	0	1
UREA [mmol/L] day 123	Mean	4.60 k	5.08	4.34	4.77
	S.d.	0.64	1.40	0.41	0.83
	N	10	10	10	10
	Median	4.66	4.80	4.30	4.43
	Deviation Vs Control [%]		10.43	-5.65	3.56
CREA [µmol/L] day 123	Mean	30.2 k	29.9	28.1	28.1
	S.d.	5.8	6.7	2.5	2.5
	N	10	10	10	10
	Median	30.6	28.8	28.0	27.9
	Deviation Vs Control [%]		-1.1	-7.1	-6.9
GLUC [mmol/L] day 123	Mean	6.42 k	6.48	6.64	6.51
	S.d.	0.63	0.83	0.68	0.47
	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 [µmol/L] day 123	Mean	1.60 v	1.36 *	1.36	1.15 **
	S.d.	0.23	0.34	0.27	0.25
	N	10	10	10	10
	Median	1.48	1.26	1.35	1.17
	Deviation Vs Control [%]		-14.97	-14.65	-27.85
TPROT [g/L] day 123	Mean	62.96 v	62.82	61.90	60.87 *
	S.d.	1.74	1.37	1.52	2.52
	N	10	10	10	10
	Median	63.40	62.56	61.58	60.86
	Deviation Vs Control [%]		-0.22	-1.68	-3.31
ALB [g/L] day 123	Mean	37.11 v	37.20	36.74	35.09 **
	S.d.	0.98	0.67	0.59	1.00
	N	10	10	10	10
	Median	37.08	37.22	36.58	35.14
	Deviation Vs Control [%]		0.24	-0.99	-5.44
GLOB [g/L] day 123	Mean	25.85 k	25.63	25.16	25.78
	S.d.	1.00	0.95	1.26	2.01
	N	10	10	10	10
	Median	26.18	25.40	24.93	25.30
	Deviation Vs Control [%]		-0.87	-2.67	-0.26
CHOL [mmol/L] day 123	Mean	1.62 k	1.56	1.44	1.52
	S.d.	0.34	0.24	0.28	0.29
	N	10	10	10	10
	Median	1.54	1.50	1.41	1.46
	Deviation Vs Control [%]		-3.71	-11.00	-6.00
TRIG [mmol/L] day 123	Mean	0.88 v	1.34 **	1.49 **	1.77 *
	S.d.	0.25	0.38	0.46	0.86
	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 [µkat/L] day 132	Mean	0.55 k	0.59	0.60	0.64
	S.d.	0.06	0.12	0.20	0.18
	N	10	10	10	10
	Median	0.55	0.55	0.56	0.62
	Deviation Vs Control [%]		7.60	9.26	15.97
AST [µkat/L] day 132	Mean	1.56 k	1.47	1.67	1.64
	S.d.	0.35	0.27	0.28	0.33
	N	10	10	10	10
	Median	1.47	1.50	1.68	1.64
	Deviation Vs Control [%]		-5.59	7.45	5.39
ALP [µkat/L] day 132	Mean	0.73 v	1.21 **	1.62 **	2.98 **
	S.d.	0.25	0.28	0.36	0.99
	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 [nkat/L] day 132	Mean	25 x+	25	25	40 **
	S.d.	0	0	0	21
	N	10	10	10	10
	Median	25	25	25	32
	Deviation Vs Control [%]		0	0	62
UREA [mmol/L] day 132	Mean	6.32 k	6.00	6.77	6.43
	S.d.	0.60	0.82	1.03	0.90
	N	10	10	10	10
	Median	6.23	5.75	7.08	6.70
	Deviation Vs Control [%]		-5.08	7.18	1.72
CREA [µmol/L] day 132	Mean	30.9 v	34.9 **	35.0 **	31.6
	S.d.	2.1	5.3	3.8	3.8
	N	10	10	10	10
	Median	30.5	34.7	35.4	31.2
	Deviation Vs Control [%]		13.1	13.3	2.3
GLUC [mmol/L] day 132	Mean	5.28 k	5.25	5.38	5.59
	S.d.	0.43	0.54	0.55	0.43
	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 [µmol/L] day 132	Mean	1.93 k	1.62	1.74	1.73
	S.d.	0.53	0.38	0.26	1.07
	N	10	10	10	10
	Median	1.86	1.53	1.72	1.49
	Deviation Vs Control [%]		-16.19	-9.94	-10.34
TPROT [g/L] day 132	Mean	63.84 k	61.89	60.46	63.22
	S.d.	3.59	1.68	1.88	2.89
	N	10	10	10	10
	Median	62.82	62.20	60.71	63.17
	Deviation Vs Control [%]		-3.06	-5.29	-0.98
ALB [g/L] day 132	Mean	39.04 v	36.44 **	35.62 **	35.06 **
	S.d.	1.85	1.19	1.07	1.16
	N	10	10	10	10
	Median	38.84	36.46	35.47	34.67
	Deviation Vs Control [%]		-6.66	-8.77	-10.19
GLOB [g/L] day 132	Mean	24.80 v	25.45	24.85	28.16 **
	S.d.	2.03	1.04	1.22	2.05
	N	10	10	10	10
	Median	24.14	24.88	24.63	27.84
	Deviation Vs Control [%]		2.60	0.18	13.51
CHOL [mmol/L] day 132	Mean	1.40 v	1.28	1.62	2.66 **
	S.d.	0.36	0.32	0.22	0.63
	N	10	10	10	10
	Median	1.40	1.14	1.67	2.48
	Deviation Vs Control [%]		-8.29	15.73	90.49
TRIG [mmol/L] day 132	Mean	0.77 v	1.04	1.24 *	3.48 **
	S.d.	0.26	0.57	0.45	1.56
	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 [µkat/L] day 123	Mean	0.63 v	0.71	0.76 **	0.95 **
	S.d.	0.05	0.15	0.15	0.49
	N	10	10	10	10
	Median	0.60	0.66	0.70	0.75
	Deviation Vs Control [%]		13.42	21.25	51.60
AST [µkat/L] day 123	Mean	1.67 k	1.65	1.71	1.83
	S.d.	0.27	0.28	0.22	0.27
	N	10	10	10	10
	Median	1.65	1.58	1.67	1.81
	Deviation Vs Control [%]		-1.44	2.51	9.58
ALP [µkat/L] day 123	Mean	1.00 v	1.26 *	1.94 **	2.66 **
	S.d.	0.16	0.22	0.15	0.36
	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 [nkat/L] day 123	Mean	25 x+	25	25	25
	S.d.	0	0	0	1
	N	10	10	10	10
	Median	25	25	25	25
	Deviation Vs Control [%]		0	0	1
UREA [mmol/L] day 123	Mean	4.60 k	5.08	4.34	4.77
	S.d.	0.64	1.40	0.41	0.83
	N	10	10	10	10
	Median	4.66	4.80	4.30	4.43
	Deviation Vs Control [%]		10.43	-5.65	3.56
CREA [µmol/L] day 123	Mean	30.2 k	29.9	28.1	28.1
	S.d.	5.8	6.7	2.5	2.5
	N	10	10	10	10
	Median	30.6	28.8	28.0	27.9
	Deviation Vs Control [%]		-1.1	-7.1	-6.9
GLUC [mmol/L] day 123	Mean	6.42 k	6.48	6.64	6.51
	S.d.	0.63	0.83	0.68	0.47
	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 [µmol/L] day 123	Mean	1.60 v	1.36 *	1.36	1.15 **
	S.d.	0.23	0.34	0.27	0.25
	N	10	10	10	10
	Median	1.48	1.26	1.35	1.17
	Deviation Vs Control [%]		-14.97	-14.65	-27.85
TPROT [g/L] day 123	Mean	62.96 v	62.82	61.90	60.87 *
	S.d.	1.74	1.37	1.52	2.52
	N	10	10	10	10
	Median	63.40	62.56	61.58	60.86
	Deviation Vs Control [%]		-0.22	-1.68	-3.31
ALB [g/L] day 123	Mean	37.11 v	37.20	36.74	35.09 **
	S.d.	0.98	0.67	0.59	1.00
	N	10	10	10	10
	Median	37.08	37.22	36.58	35.14
	Deviation Vs Control [%]		0.24	-0.99	-5.44
GLOB [g/L] day 123	Mean	25.85 k	25.63	25.16	25.78
	S.d.	1.00	0.95	1.26	2.01
	N	10	10	10	10
	Median	26.18	25.40	24.93	25.30
	Deviation Vs Control [%]		-0.87	-2.67	-0.26
CHOL [mmol/L] day 123	Mean	1.62 k	1.56	1.44	1.52
	S.d.	0.34	0.24	0.28	0.29
	N	10	10	10	10
	Median	1.54	1.50	1.41	1.46
	Deviation Vs Control [%]		-3.71	-11.00	-6.00
TRIG [mmol/L] day 123	Mean	0.88 v	1.34 **	1.49 **	1.77 *
	S.d.	0.25	0.38	0.46	0.86
	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 [µkat/L] day 132	Mean	0.55 k	0.59	0.60	0.64
	S.d.	0.06	0.12	0.20	0.18
	N	10	10	10	10
	Median	0.55	0.55	0.56	0.62
	Deviation Vs Control [%]		7.80	9.26	15.97
AST [µkat/L] day 132	Mean	1.56 k	1.47	1.67	1.64
	S.d.	0.35	0.27	0.28	0.33
	N	10	10	10	10
	Median	1.47	1.50	1.68	1.64
	Deviation Vs Control [%]		-5.59	7.45	5.39
ALP [µkat/L] day 132	Mean	0.73 v	1.21 **	1.62 **	2.98 **
	S.d.	0.25	0.28	0.36	0.99
	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 [nkat/L] day 132	Mean	25 x+	25	25	40 **
	S.d.	0	0	0	21
	N	10	10	10	10
	Median	25	25	25	32
	Deviation Vs Control [%]		0	0	62
UREA [mmol/L] day 132	Mean	6.32 k	6.00	6.77	6.43
	S.d.	0.60	0.82	1.03	0.90
	N	10	10	10	10
	Median	6.23	5.75	7.08	6.70
	Deviation Vs Control [%]		-5.08	7.18	1.72
CREA [µmol/L] day 132	Mean	30.9 v	34.9 **	35.0 **	31.6
	S.d.	2.1	5.3	3.8	3.8
	N	10	10	10	10
	Median	30.5	34.7	35.4	31.2
	Deviation Vs Control [%]		13.1	13.3	2.3
GLUC [mmol/L] day 132	Mean	5.28 k	5.25	5.38	5.59
	S.d.	0.43	0.54	0.55	0.43
	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 [µmol/L] day 132	Mean	1.93 k	1.62	1.74	1.73
	S.d.	0.53	0.38	0.26	1.07
	N	10	10	10	10
	Median	1.86	1.53	1.72	1.49
	Deviation Vs Control [%]		-16.19	-9.94	-10.34
TPROT [g/L] day 132	Mean	63.84 k	61.89	60.46	63.22
	S.d.	3.59	1.68	1.88	2.89
	N	10	10	10	10
	Median	62.82	62.20	60.71	63.17
	Deviation Vs Control [%]		-3.06	-5.29	-0.98
ALB [g/L] day 132	Mean	39.04 v	36.44 **	35.62 **	35.06 **
	S.d.	1.85	1.19	1.07	1.16
	N	10	10	10	10
	Median	38.84	36.46	35.47	34.67
	Deviation Vs Control [%]		-6.66	-8.77	-10.19
GLOB [g/L] day 132	Mean	24.80 v	25.45	24.85	28.16 **
	S.d.	2.03	1.04	1.22	2.05
	N	10	10	10	10
	Median	24.14	24.88	24.63	27.84
	Deviation Vs Control [%]		2.60	0.18	13.51
CHOL [mmol/L] day 132	Mean	1.40 v	1.28	1.62	2.66 **
	S.d.	0.36	0.32	0.22	0.63
	N	10	10	10	10
	Median	1.40	1.14	1.67	2.48
	Deviation Vs Control [%]		-8.29	15.73	90.49
TRIG [mmol/L] day 132	Mean	0.77 v	1.04	1.24 *	3.48 **
	S.d.	0.26	0.57	0.45	1.56
	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	Mean	24.28 k	23.28	23.85	27.68
[nmol/L]	S.d.	3.86	4.10	5.81	10.22
day 132	N	10	10	10	10
	Median	24.40	24.06	22.66	25.75
	Deviation Vs Control [%]		-4.12	-1.77	13.98
TSH	Mean	5.19 v	8.22 **	6.55 *	9.87 **
[µg/L]	S.d.	1.14	3.08	1.41	3.94
day 132	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	Mean	66.23 v	59.94	50.88 **	48.22 **
[nmol/L]	S.d.	7.15	9.66	7.57	7.42
day 90	N	10	10	10	10
	Median	63.95	61.01	50.48	50.22
	Deviation Vs Control [%]		-9.50	-23.18	-27.19
TSH	Mean	6.96 v	6.90	7.57	9.59 *
[µg/L]	S.d.	1.81	1.98	3.38	2.20
day 90	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	Mean	41.52 v	38.97	32.08 *	44.71
[nmol/L]	S.d.	6.15	5.44	9.43	5.82
day 90	N	10	10	10	10
	Median	41.56	40.34	31.26	43.44
	Deviation Vs Control [%]		-6.13	-22.73	7.70
TSH	Mean	4.55 v	4.86	6.16	7.24 **
[µg/L]	S.d.	0.70	0.99	1.98	2.67
day 90	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	Mean	16.02 k	15.76	14.12	10.14 X
[nmol/L]	S.d.	5.36	2.78	2.30	2.50
day 4	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	Mean	4.43 k	4.50	4.47	3.98 X
[µg/L]	S.d.	0.33	0.44	0.42	0.32
day 4	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

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

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

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	Mean	18.74 k	17.95	14.89	15.71 X
[nmol/L]	S.d.	4.62	5.00	2.66	5.49
day 4	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	Mean	4.46 k	4.72	4.74	3.83 X
[µg/L]	S.d.	0.41	0.81	0.60	0.81
day 4	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

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

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

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

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

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

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

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

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

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			

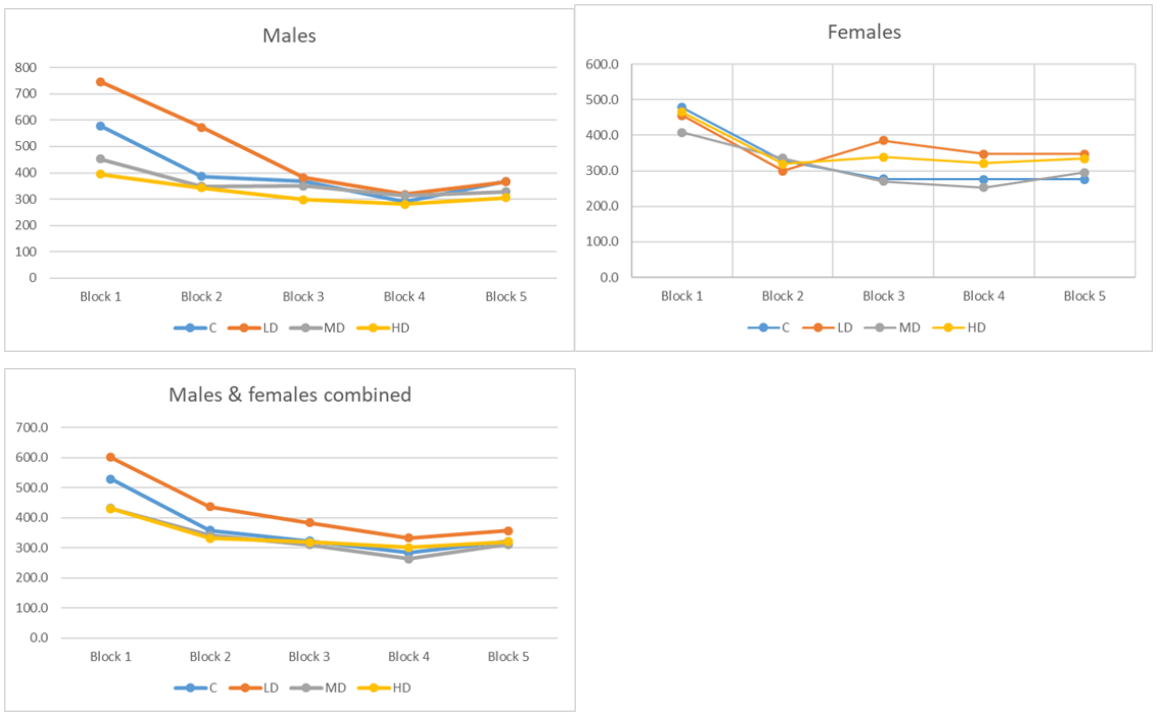

Figure 1: Mean maximal amplitude over times showing decreased habituation (flat curves) in males and in males and females combined from the mid-dose.

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*

Haematology: 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	Late	Total	Viable young Total	M	F	% Males	Implantation loss (%) Pre	Post	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	Affected	%	No. Litters Observed	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	Aff	%	No. Dams Obs	Aff	%
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	Aff	%	No. Dams Obs	Aff	%
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	Aff	%	No. Dams Obs	Aff	%
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≤ 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 99-01 F	multiple external malformations 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
* = $p \leq 0.05$ (Wilcoxon-test [one-sided]) ** = $p \leq 0.01$ (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

	significantly decreased metabolites
	not significantly but ≤20% decreased
	significantly increased metabolites
	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)

4.1.2 BOD_5/COD

No data available.

4.1.3 Aquatic simulation tests

- **Study 1**

Study reference:

Unpublished study report, 2020

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 **UVCB substance** (EC 701-385-4).

Test type:

OECD Guideline 309 (Aerobic Mineralisation in Surface Water - Simulation Biodegradation Test), GLP compliant. The study report menitonned the following deviation: FOCUS and CAKE software are validated by the manufactures of these tools and no further validation according to GLP was conducted at the test facility. The biochemical oxygen demand in surface water was measured at the test facility according to ISO 17025 but without a GLP status. Inorganic nitrogen forms, total phosphorus and orthophosphate in sediments were measured at the test facility without a GLP status.

Test substance:

The test material is indicated as radiolabelled 14C-Reaction products of benzeneamine, N-phenyl with nonene (branched) (EC 701-385-4, CAS 36878-20-3[2]) which contains 2 major isomers groups: monosubstituted C9DPA and disubstituted C9C9DPA.

Materials and methods:

Surface water and sediments were collected from the same site at the river arm "Ranschgraben" west of Schifferstadt (21 November 2018). A 5 mm layer of sediment from the top surface was collected. Aerobic conditions were maintained during the transport of water and sediment samples to the laboratory.

A stock solution with 0.7266 g test item was prepared in 20 mL ethanol and the application of the test substance to the test flasks was made by aliquots of stock solution. Two different concentrations of test substance differing by a factor of 5 were used (2 and 10 µg/L). The volume of the test solution corresponded to 333 mL + ca. 0.764 mL suspended solids suspension (equivalent to ca. 15 mg dw/L). The test flasks were incubated at 12 ±1 °C under dark conditions for 60 days. pH (8.2-9.4) and oxygen content (9.2-10.7 mg/L) were measured in all test assays on each scheduled sampling day (0, 7, 14, 27, 49, 55 and 60).

One test flask with only the test system (inoculum blank) was set up and was used for physico-chemical on every sampling point and at the end of exposure, also for microbiological analysis. Duplicate test vessels containing sterile test system and high-test concentration, 10 µg/L were set up as sterile controls. The test water was autoclaved three times 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 possible abiotic degradation or other non-biological removal of the test substance. The reference substance aniline at a concentration 10µg/L was added to the test system for the reference control assay. Finally, the solvent control consist of duplicate flasks containing the reference substance at a final nominal concentration of 10 µg/L treated with the same amount of solvent (10.8 µL ethanol) that was present in the highest test substance concentration used in this test (10 µg/L test substance). After setting up the test assays, a suitable amount of 1 M NaOH solution was placed in the internal container as the absorbing solution in each flask. The flasks were closed with polypropylene matching screw cap with pouring ring impermeable to air and CO_2 and were placed on magnetic stirrers and gently and continuously stirred (ca. 100 rpm agitation).

The rate of degradation and DT50 and DT90 calculations of the test item in the water phase was calculated under consideration of recommendations of the FOCUS kinetics workgroup with the measured LSC (Liquid Scintillation Counter) values for radioactivity in comparison to %TAR (total applied radioactivity) using software tool CAKE. The fit of observations was performed by non-linear regression using the software tool CAKE.

Results:

The calculated DT50 (50% disappearance time) values were 0.3 d for C9DPA and 4.2 d for C9C9DPA at 12°C for the highest concentration of 10 µg/L. Kinetic evaluations in low test concentration (2µg/L) did not

[2] Those identifiers are mentionned in the publically disseminated lead Registration Dossier but are incorrect as they are corresponding to a substance without branched alkyl chain.

yield a good visual fit in any of the models for isomer group 2 and due to the faster disappearance of isomer group 1, a half-life determination was not possible. Based on CO_2 evolution, at a concentration of 10µg/L, 0.4% biodegradation was reached after 60 days (0.6% for 2 µg/L after 60 days). Mean total recovery was 89% for the 2µg/L concentration and 75% for the 10 µg/L concentration. Metabolites could be detected but were pooled in one group by calculating the sum of peak areas. Identification of these metabolites was not possible due to the low overall radioactivity. The results of the radio-HPLC measurements of the lower test concentration show appearance of the metabolites in the dichloromethane extracts from day 0 up to day 55 in the low dose and up to day 60 in the high dose. The LOD was fixed at approximately 1% of the applied total radioactivity. A lower LOD could not be established because the sensitivity of the radio-HPLC system as well as the preconcentration factor were at their maximum.

A significant decline of isomer group 1 and 2 in the parent substance was found. Disappearance of both isomer groups from the test system indicates physical or chemical processes as primary degradation processes. During the experimental exposure, a significant portion of the applied activity was removed from the wall of the test vessels after different extraction procedures. No significant mineralization (<1%) was measured within the tested duration of 60 days. The activity of the microbial biomass at the beginning and at the end of exposure as well as pH and oxygen measured during the exposure indicated a relatively stable system with enough oxygen for the microbes to sustain. Biodegradation of 14C-aniline as control substance in control assays (25%) as well as solvent control assays (37%) at the end of the study confirmed the biological activity in the test system and the degradation of aniline in solvent control assay indicated that no toxicity to the microorganism because of the presence of ethanol in the test substance assays.

4.1.4 Other degradability studies

- **Study 2**

Study reference:

Unpublished study report, 2003

Detailed study summary and results:

One inherent biodegradability – Concawe test (according to the OECD TG 302D Draft) is performed on **one constituent of the UVCB substance** (EC 701-385-4[3]). The study is GLP compliant and no deviations are mentioned in the study report. This study was realized with the test material "Reaction products of Benzenamine, N-phenyl, and Nonene, branched" (EC 253-249-4; CAS 36878-20-3[4]), followed by purification (distillation) with a 96% C9DPA content (EC 248-295-7; CAS 27177-41-9[5]).

[3] This is a list number

[4] These identifiers have been deleted

[5] Those identifiers are mentionned in the publically disseminated lead Registration Dossier but are incorrect as they are corresponding to a substance without branched alkyl chain.

The test material, at a nominal concentration of 23.4 mg/L, was exposed to a mixed population of soil microorganisms and activated sewage sludge (predominantly domestic sewage) with culture medium. The aerobic soil microorganisms were obtained from Allestree Park, Derby, Derbyshire, UK. The soil surface was cleared of leaf litter and a sample of soil was collected from a depth of approximately 10 cm. The mixed population of activated sewage sludge micro-organisms was obtained from the aeration stage of the Severn Trent Water Plc sewage treatment plant at Loughborough, Leicestershire, UK, which treats predominantly domestic sewage. The sealed culture vessels were placed in the dark at 21°C for 56 days. The inoculum used in the biodegradation test was pre-exposed to the test material in order to enhance the biodegradative potential of the inoculum for 14 days prior to the start of the biodegradation test. The test material is poorly soluble (water solubility=0.0113 mg/L), therefore the test material was dissolved in diethylether and an aliquot of the solvent stock solution was applied to a glass fiber filter paper. After evaporation of the solvent, the filter paper containing the test material was added to the test medium.

The degradation of the test material was assessed by the determination of carbon dioxide produced on days 0, 7, 14, 21, 28, 35, 42, 49 and 56, and by compound specific analyses (HPLC) on days 0, 28 and 56. Control solutions with inoculum and the standard material, n-Hexadecane, together with a toxicity control were used for validation purposes. N-Hexadecane attained 105% degradation after 56 days thereby confirming the suitability of the inoculum and test conditions. The toxicity control attained 44% degradation after 56 days thereby confirming that the test material was not toxic to the sewage treatment micro-organisms used in the study. The mean amount of inorganic carbon produced in the control vessels at the end of the test is 1.7 % (< 15%) of the organic carbon added initially as test material to the test vessels thereby confirming the validity of the biodegradation test. Compound-specific analyses conducted on day 0, 28 and 56 indicated that the test material attained 3% degradation after 56 days. No degradation of the test material based on carbon dioxide production was observed after 56 days.

4.2 Bioaccumulation

4.2.1 Bioaccumulation test on fish

- **Study 3**

Study reference:

Unpublished study report, 2000

Detailed study summary and results:

The bioaccumulation potential in aquatic species of **one constituent (C9DPA) of the UVCB substance** was experimentally measured.

Test type:

The study follows the guideline of the test methods designated for New Chemical Substances (1974, amended 1998) under Chemical Substances Control Law of Japan (MITI). Deviations are not mentioned in the study report although some elements deviated from the standard OECD TG 305 (use of surfactant and dissolvent, measurements were made for a group of 2 fish instead of individually). The test is GLP compliant.

Test substance:

The test material is 4-nonyl-N-phenylaniline (EC 248-295-7, CAS 27177-41-9[6]). The test material is indicated in the registration dossier as one constituent of the registred substance.

Materials and methods:

The study was realised on *Cyprinus carpio* in continuous flow-through system for 42 days of exposure followed by additional 42 days of depuration duration. Mean lipid content of the fish at the beginning of the study was 4.8% and was 4.9% at the end of the study. Individual fish weight at the beginning of the experiment was not available in the study report and start on day 7 for 2 specimens at each concentrations tested. The pH range observed for the media during the study was 7.1 – 7.6, O_2 levels were 6.7 – 8.3 mg/L, and temperature was 23.6 – 24.9°C.

The C9DPA constituent was prepared by addition of test substance to HCO-30 surfactant which was then dissolved in 2-methoxyethanol and fish were exposed at two nominal concentrations: a high exposure level of 100 µg/L (HCO-30, 4 mg/L, 2-methoxyethanol, < 25 ppm [v/v]) and a low exposure level of 10 µg/L (HCO-30, 0.4 mg/L, 2-methoxyethanol, < 25 ppm [v/v]). Thus, the higher concentration was well above the water solubility of the test item (11.3 µg/L).

Sampling for test organisms were on days 7, 14, 21, 28, and 35 of the exposure period. Six fish were taken from each exposure group and four of them were analyzed two by two. The BCF exceeded a prescribed limit of 1000, and an elimination test was conducted using the remaining fish. On days 4, 10, 20, and 42 of elimination, four fish were taken from each exposure levels and analyzed two by two. In addition, the remainder of the fish samples taken on day 42 of exposure (two fish each from high and low exposure levels) were dissected to fish tissue and analyzed by HPLC. The final analytical solutions of the fish exposed for 42 days was directly analyzedy HPLC to determine the concentrations of the compounds corresponding to the peaks that appear at retention times of 2.3 minutes and about 29 minutes in the chromatogram of a 100-mg/L standard solution. The 29-minute peak, which was probably due to dialkylphenylamine, was not found in the chromatograms of the exposed fish. The 2.3-minute peak, which was probably due to diphenylamine, appeared in the chromatograms of the exposed fish, but the BCF of the compound was estimated at less than about one-fifth the BCF of nonyldiphenylamine for high exposure level and less than about one tenth of that for low exposure level.

Results:

[6] Those identifiers are mentionned in the publically disseminated lead Registration Dossier but are incorrect as they are corresponding to a substance without branched alkyl chain.

It could not be concluded from the measurements that the bioconcentration had reached a steady state by day 35. It was stated in the study that, for this reason, an additional sampling and analysis measurement was conducted on day 42.

The bioconcentration factors at steady state (BCFSS) were used to evaluate the potential of bioaccumulation. At a concentration of 10 µg/L, a BCF for the whole body of 1730 L/kg w.w. was calculated by the authors (BCF=411 L/kg w.w. for 100 µg/L). Mean measured concentrations in water were constant throughout the exposure period (101.0 µg/L high exposure group and 9.46 µg/L low exposure group after 42 days). Analysis of the fish tissues indicated that, at a concentration of 10 µg/L, viscera is the tissue with the highest BCF value (4760) followed by skin with scale, head, and muscle and bone (BCF value of 2490, 2280 and 1520 repectively).

There was no dead fish or abnormal condition throughout the exposure period of 42 days and the elimination period of 42 days. There was no abnormality in shape of the body or in swimming and feeding behavior.

4.2.2 Bioaccumulation test with other organisms

No data available

4.3 Acute toxicity

4.3.1 Short-term toxicity to fish

- **Study 4**

Study reference:

Unpublished study report, 2000

Detailed study summary and results:

The bioaccumulation potential in aquatic species of **one constituent (C9DPA) of the UVCB substance** was experimentally measured. This test was composed of two parts and the first part consisted in an acute toxicity testing using fish.

Test type:

The study follows the guideline of the acute toxicity testing (according to the Japanese Industrial Standard (J I S) Method "K0102-1993, Industrial Waste Water Testing Method, 71, Acute toxicity study using fish). No deviation was mentioned and the study followed the GLP principles. This study is considered reliable with restrictions: the study report mentioned the use of a solvent and a dispersant, and 10% mortality was observed in the control group at the end of the 96 hours.

Test substance:

The test material is 4-nonyl-N-phenylaniline (EC 248-295-7, CAS 27177-41-9[7]). The test material is indicated in the registration dossier as one constituent of the registred substance.

Materials and methods:

An acute toxicity test was conducted for 96 hours using Japanese Medaka (Oryzias latipes). .The fish were acclimatated for 2 weeks at 24±2°C. The length of fish was around 3 cm and the weight around 0.2g. Five different concentrations (0 (control), 10, 20, 40 and 80 mg/L) and 10 fish/groups were tested. Water temperature during the test was 23-24°C and renewal happened every 48h. Aeration was continuous and the concentration of dissolved oxygen measured value was 6.0-8.3 mg/L. No feeding occurs during this 96h test period. The test substance, 800 mg, was dissolved i n 40 mL of 40 w/v% HCO-30 in 2-methoxyethanol, and diluted to 50 mL with 2-methoxyethanol. Each of 1.25, 2.5, 5, and 10 mL from the solution was added dropwise to dilution water with stirring to disperse the test substance, and diluted to 2 liters . (Concentration of the test substance: 10, 20, 40, and 80 mg/L and concentration of HCO-30: 200, 400, 800, and 1600 mg/L). For control, eight milliliters of 40 w/v% HCO-30 in 2-methoxyethanol was diluted with 2-methoxyethanolto 10 mL, which was then diluted to 2L with dilution water. (Concentration of HCO-30: 1600 mg/L)

Results:

No analytical measurements were reported and the LC50 (96 h) was calculated to be 52 mg/L (nominal). 10 % mortality was observed in the control and at 10 mg/L, 0% at 20 mg/L, 20% at 40 mg/L and 100%at 80 mg/L. After 24 and 48 hours no mortality was observed except for the 80 mg/L goup were mortality reached 100%.

4.3.2 Short-term toxicity to aquatic invertebrates

4.3.3 Algal growth inhibition tests

- **Study 5**

Study reference:

Unpublished study report, 1997

Detailed study summary and results:

The toxicity to aquatic algae study was performed with **the UVCB substance** (EC 701-385-4).

Test type:

The study follows the OECD Guideline 201 (Alga, Growth Inhibition Test) is GLP compliant without analytical monitoring**.**

Test substance:

[7] Those identifiers are mentionned in the publically disseminated lead Registration Dossier but are incorrect as they are corresponding to a substance without branched alkyl chain.

The test substance is mentioned in the registration dossier as Reaction products of Benzeneamine, N-phenyl- with nonene (branched) [1] (EC 701-385-4). No detail was given regarding the identity of the test substance in the study report.

Materials and methods:

Five WAFs were individual prepared. The test material and dilution water were mixed and stirred for approx. 20 hours. After settling for 4 hours the water phase containing the WAF was siphoned. This study was performed with Pseudokirchneriella subcapitata at 24°C under static conditions. pH at test initiation was between 7.6 - 8.1 and 8.3 - 9.8 a the end of the test. Initial cells density was approximatively 10,000 cells/ml. The study does not mention the use of a dissolvent or solubiliser. The test was performed for 96 hours under static condition with a control (0 mg/L) and five nominal test concentrations (WAF): 0.3, 3.3, 33, 330, 3300 mg/L. Two replicates of each concentration and control used in the test.

Results:

No measured concentration of the test substance was available, thus the results are based on nominal concentrations. A 72 hour EC50 corresponding to 200 mg/L was calculated using the number of cells/mL (96 hour EC50=220 mg/L). A 72 hour EC50 corresponding to 600 mg/L was calculated using the average specific growth rate (96 hour EC50=870 mg/L). Finally, a NOEC(96h) of 33 mg/L defined as the highest concentration of test substance that allowed at least 90% of control growth was obtained both for the number of cell/mL and the specific growth rate. The study report indicated that, at the conclusion of the test, a sample of test media was used to determine wheter toxic effects were algicidal or algistatic. After 168h of incubation, it was reported that the effect of the test material was algistatic rather than algicidal No insoluble material was noted during the test that might explain physical effects.

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, 2020b

Detailed study summary and results:

The long-term toxicity to aquatic invertebrates study was performed with **the UVCB substance** (EC 701-385-4).

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 substance is mentioned in the registration dossier as Reaction products of Benzeneamine, N-phenyl- with nonene (branched) [1] (EC 701-385-4[8]).

Materials and methods:

In this study on *Daphnia magna STRAUS*, the test was conducted as a semi-static limit test at nominal loading rates (1.98 - 2.96 - 4.45 - 6.67 - 10.0 mg/L) for 21 days. The test item was mixed with water for a prolonged period sufficient to ensure equilibration between the test item and the water phase. At the completion of mixing and following a settlement period, the WAF was separated by siphon. This procedure was followed for each renewal of the test solutions. 10 daphnids and 10 replicates were used for all WAFs and the control. 1 daphnid was held individually per replicate. The temperature, pH and dissolved oxygen remain within the acceptable limits during the experiment. The chemical specific analysis showed that only one of the two main constituents (the C9DPA) could be determined. The measured concentrations of C9DPA in the fresh media, at the beginning of the exposure-renewal interval, were in the range of < LOQ (1 µg test item/L) to 4.65 µg/L. At the end of an exposure-renewal interval (24 hours), most of the measured concentrations were < LOQ.

Results:

The WAFs were checked for any undissolved or emulsified material by Tyndall effect, which was negative. Significant differences of reproduction were determined in comparison to the control using statistical standard procedures as normality test (Shapiro-Wilk's test), variance homogeneity test (Levene's test), Step-down Jonckheere-Terpstra test procedure for reproduction. The Fisher's exact binomial test with Bonferroni correction was used for adult mortality. A significant reduction in the reproduction per female parent animal inserted at the start of the exposure was observed at the nominal loading rates of 6.67 and 10.0 mg/L (NOELR of 4.45 mg/L). A significant trend in mortality was observed reaching 50% mortality at the highest

[8] This is a list number

loading rate tested (NOELR adult mortality: 6.67 mg/L). The calculated ELR10 (21d) for the Substance based on the nominal loading rate was 4.12 mg/L

Effects on Reproduction for all Introduced Parents

(based on the nominal loadings of the water accommodated fractions of the test item[mg/L])

Nominal loading rate of the test item [mg/L]	Mean number of offspring perintroducedparental daphnid		
	Mean	SD	CV
10.0	76.1	34.6	45.4
6.67	74.5	26.5	35.5
4.45	84.7	22.7	26.8
2.96	100.4	9.05	9.0
1.98	92.4	6.02	6.5
Control	96.9	12.3	12.7

Mortality [%] of the Adult Daphnids after 7, 14 and 21 Days of Exposure (n = 10)

Nominal loading rate of the test item [mg/L]	Adult Mortality [%]		
	7 days	14 days	21 days
10.0	0	20	50
6.67	0	10	10
4.45	0	10	10
2.96	0	0	10
1.98	0	0	0
Control	0	0	0

First Appearance of Living Juveniles in the Individual Groups

Nominal loading rate of the test item [mg/L]	Day of first appearance of living juveniles at the introduced parental daphnids in replicate no.										First appearance (mean day)
	1	2	3	4	5	6	7	8	9	10	
10.0	7	7	7	7	7	7	7	7	7	7	7.0
6.67	7	7	7	7	7	8	8	7	7	7	7.2
4.45	7	7	7	8	7	7	8	8	7	7	7.3
2.96	8	7	7	7	8	7	8	9	7	9	7.7
1.98	7	8	7	9	7	9	8	7	8	7	7.7
Control	7	7	7	8	7	8	8	7	7	7	7.3

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

No data available