RAWG

RAWG - Amphocarboxylates

EXPERT PANEL MEETING
SEPTEMBER 8-9, 2025

Cosmetic Ingredient Review

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Memorandum

To: Expert Panel for Cosmetic Ingredient Safety Read-Across Working-Group Members
From: Jinqiu Zhu, PhD, DABT, ERT, DCST, CIR Toxicologist
Priya Ferguson, M.S., Senior Scientific Analyst/Writer, CIR
Date: August 15, 2025
Subject: New Read-Across Source Material Submission for Fatty Amphocarboxylates

At the June 2024 meeting, the Panel issued an IDA on fatty amphocarboxylates, requesting the following data:

- Dermal absorption data
- Developmental and reproductive toxicity data on Disodium Cocoamphodiacetate
- Further information regarding the composition and impurities of these ingredients as used in cosmetics (particularly percentage of actives in ingredients, fatty acid compositions, and degrees of esterification (e.g., how much of Sodium Cocoamphoacetate has 0, 1, or 2 acetate substitutions)
- Sensitization data on Sodium Lauroamphoacetate and Disodium Lauroamphodiacetate at maximum use concentrations
- Any information (e.g., clarifications on compositions) to support the use of previously suggested read-across sources

Since the last review of these ingredients, additional documentation related to Fatty Amphocarboxylates was received (*data_FattyAmphocarboxylates_092025* in this pdf; please note that these data are also part of the Fatty Amphoacetate report pdf so that you have them readily available when reviewing the document named *data10_FattyAmphocarboxylates_092025* in that pdf). These materials include the following:

- EU Alkylamphoacetates Consortium. 2025. OECD 414 Prenatal Developmental Toxicity Study of Amphoacetates C8-C18 by Oral Gavage in Time-Mated New Zealand White Rabbits.
- Lavin Williams A, DeSesso JM, and Richmond E. 2024. Expert Opinion Regarding Impact of the Rabbit OECD 414 Study of Amphoacetates C8-C18 on Potential Need for Reproductive Toxicity Classification in Accordance with GHS and EU CLP.
- Lavin Williams A and DeSesso JM. 2024. Review of results from the OECD 414 Study in Rabbits to Assess Whether the Increase in Post-Implantation Loss at the Mid-Dose is Secondary to Maternal Toxicity.
- EU Alkylamphoacetates Consortium. 2025. OECD 443 Extended One Generation Reproductive Study (including Cohort 1) of Amphoacetates C8-C18 (diacetate form) by Oral Gavage in Rats.
- Charles River Laboratories. 2025. Analogue Approach for REACH Registration of Alkylamphoacetates version March 2025.
- Bigorra J, Amela C, Bonastre N, et al. 2000. Amphoteric Surfactants: Structure-Performance Correlation. Proceedings (Vol 2) of the 5th World Surfactants Conference.
- DeSesso JM and Lavin Williams A. 2023. Expert Review of Available Repeat-Dose and Developmental and Reproductive Toxicity (DART) Studies for Amphoacetates.

Two new DART studies were provided (on "amphoacetates C8-C18") in response to the Panel's IDA for developmental and reproductive toxicity data on Disodium Cocoamphodiacetate. These include a prenatal developmental toxicity study in rabbits (OECD TG 414) and an extended one-generation reproductive toxicity study in rats (OECD TG 443). The rabbit study identified maternal toxicity at 175 and 350 mg/kg/d, with increases in resorptions and post-implantation loss observed at 175 mg/kg/d. The NOAEL for both maternal and developmental toxicity was determined to be 75 mg/kg/d. Expert reviewers from Exponent concluded that the increased early resorptions were likely secondary to maternal toxicity and did not warrant self-classification for adverse effects on development in accordance with GHS and EU CLP. The rats study further supported the absence of reproductive toxicity: no adverse effects on reproductive performance, fertility, or developmental endpoints–including offspring

viability, growth, sexual maturation, organ weights, and histopathology–were observed at doses up to the highest level tested. An NOAEL of 1000 mg/kg/d was established for developmental and reproductive toxicity. The updated Charles River document (March 2025), titled *Analogue Approach for REACH Registration of Alkylamphoacetates* reflects these new findings. The RAWG should determine if inclusion of these 2 studies is appropriate based off of the test substance used (amphoacetates C8-C18). Experts from Exponent's broader review of available repeat-dose and DART studies for amphoacetates found no evidence of adverse effects on fertility, reproductive organs, or fetal development at non-maternally toxic dose of the four commercial amphoacetates surfactant products tested (Dehyton® DC (INCI name, Disodium Cocoamphodiacetate); Miranol Ultra C32 (INCI name, Sodium Cocoamphoacetate); PC-2020-926 (no INCI name); and Sodium Lauroamphoacetate), with exposures up to 1000 mg/kg bw/d.

Additionally, the submission included a technical paper titled *Amphoteric Surfactants: Structure–Performance Correlation*, which was provided in partial response to the IDA for further information on ingredient composition and impurities. This paper presents a structural-performance analysis of amphoacetate surfactants using NMR-based techniques, identifying monoacetate, diacetate, and quaternized species formed under varying synthetic and hydrolysis conditions. It indicates that the chemical structure and functional properties of these surfactants are influenced by multiple factors, including the type of amine (e.g., AEEA), the alkylating agent, and most critically, the source of fatty acid (e.g., coconut oil, hydrogenated coconut oil, lauric acid). These variables affect the distribution of molecular species and, consequently, alter key performance attributes such as surface activity and foaming. The paper also describes the formation of substituted imidazolines and their subsequent ring-opening reactions (amidation and carboxymethylation), which depend on reaction parameters like pH, time, and molar ratios.

The RAWG has reviewed data regarding read-across for this ingredient group at previous meetings. For the Panel's recollection, links to these meetings have been provided below:

- June 2023 Wave 2 (https://www.cir-safety.org/sites/default/files/w2_FA_1.pdf)
- June 2024 Wave 2 (https://www.cir-safety.org/sites/default/files/Data%20Supplement_Wave%202_062024.pdf), starting on p 4

The RAWG is requested to review the new submissions, and to reassess the appropriateness of the proposed read-across strategies in light of the additional source materials and external expert evaluations.

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: May 21, 2025

SUBJECT: Fatty Acid Amphocarboxylates

EU Alkylamphoacetates Consortium. 2025. OECD 414 Prenatal Developmental Toxicity Study of Amphoacetates C8-C18 by Oral Gavage in Time-Mated New Zealand White Rabbits.

Lavin Williams A. DeSesso JM and Richmond E. 2024. Expert Opinion Regarding Impact of the Rabbit OECD 414 Study of Amphoacetates C8-C18 on Potential Need for Reproductive Toxicity Classification in Accordance with GHS and EU CLP.

Lavin Williams A and DeSesso JM. 2024. Review of results from the OECD 414 Study in Rabbits to Assess Whether the Increase in Post-Implantation Loss at the Mid-Dose is Secondary to Maternal Toxicity.

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Bigorra J, Amela C, Bonastre N, et al. 2000. Amphoteric Surfactants: Structure-Performance Correlation. Proceedings (Vol 2) of the 5th World Surfactants Conference.

DeSesso JM and Lavin Williams A. 2023. Expert Review of Available Repeat-Dose and Developmental and Reproductive Toxicity (DART) Studies for Amphoacetates.

Name: OECD / Developmental toxicity / teratogenicity / Rabbit developmental toxicity / teratogenicity. CRL20346091 / Alkylamidoamine glycinate majority C12, 14 (amphoacetate)/ Amphoacetates C8-C18 / Reaction products o

Printing date: 2025-04-25T07:58:34.595Z

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ENDPOINT_STUDY_RECORD: Rabbit developmental toxicity / teratogenicity. CRL20346091

UUID: aebc00bd-494c-415e-9673-95aa9ebbb1e2
Dossier UUID:
Author: DLO
Date: 2025-03-10T14:34:59.368Z
Remarks:

Administrative data

Endpoint
developmental toxicity

Type of information
experimental study

Adequacy of study
key study

Robust study summary
true

Used for classification
true

Used for SDS
true

Study period: start date
2023-06-28

End date
2023-08-29

Reliability
1 (reliable without restriction)

Rationale for reliability incl. deficiencies
guideline study
Reliability 1

Justification for type of information
Testing was initiated as requested in ECHA decision ECHA decision TPE-D-2114539402-55-01/F. The total number of animals used in this study was considered to be the minimum required to properly characterize the effects of the test item. This study has been designed in such a way that it does not require an unnecessary number of animals to accomplish its objectives. At this time, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models which do not use live animals currently do not exist.

Data source

Reference

Prenatal Developmental Toxicity Study of Amphoacetates C8-C18 by Oral Gavage in Time-Mated New Zeala / Charles River Laboratories Den Bosch / study report

Data access
data submitter is data owner

Data protection claimed
yes, but willing to share

Materials and methods

Test guideline

Qualifier
according to guideline

Guideline
OECD Guideline 414 (Prenatal Developmental Toxicity Study)

Version / remarks
2018

Deviations
no

Qualifier
according to guideline

Guideline
EU Method B.31 (Prenatal Developmental Toxicity Study)

Version / remarks
2008

Deviations
no

Qualifier
according to guideline

Guideline
EPA OPPTS 870.3700 (Prenatal Developmental Toxicity Study)

Version / remarks
1998

Deviations
no

GLP compliance
yes (incl. QA statement)

Limit test
no

Test material

Test material information
Alkylamphoacetates C8-C18 (Diacetate form)

Specific details on test material used for the study
Purity/Composition correction factor: 2.1 (based on solid content);
The formulations prepared in water (Elix) (1-200 mg/mL; 4000 mL) were found to be stable when stored at room temperature (15 to 25°C) under normal laboratory light conditions for at least 24 hours, in a refrigerator (set to maintain 4°C) for at least 10 days and in a freezer (set to maintain -20°C) for at least 21 days (3 weeks). Test Facility Study No. 20346081.

Test animals

Species
rabbit

Strain
New Zealand White
rabbit

Details on test animals or test system and environmental conditions
TEST ANIMALS
- Age at study initiation: 17-21 weeks
- Weight at study initiation: 3094 – 4395 g
- Untreated females were mated at the Supplier and were at Day 1 or 2 of gestation on arrival at the Test Facility (Day 0 of gestation is the day of successful mating).
- Fasting period before study: No
- Housing: Individually in cages with perforated floors (Zoonlab, Germany, dimensions 67 x 62 x 55 cm) equipped with water bottles.
- Diet: Pelleted KLIBA NAFAG Rabbit Diet 3409 maintenance and breeding, from Granovit AG, Kaiseraugst, Switzerland). On arrival animals received approximately 25 grams pelleted diet and on subsequent days 170-190 grams were supplied.
- Water: Municipal tap water, ad libitum
- Enrichment: For psychological/environmental enrichment, animals were provided with shelters (Zoonlab, Germany, dimensions 40 x 32 x 23 cm) and wooden sticks (Swedish aspen wood, Bioservices, Schaijk, The Netherlands).
- Contaminants: Results of analysis for contaminants in food and enrichment materials were provided by the Suppliers, periodic analysis of the water is performed at the Test Facility. It is considered that there were no known contaminants in any of these that would interfere with the objectives of the study.
- Acclimation period: At least 6 days

ENVIRONMENTAL CONDITIONS
- Temperature (°C): 17-21 (target); 19-20 (actual)
- Humidity (%): 40-70 (target); 56-94 (actual)
- Air changes (per hr): at least 10
- Photoperiod (hrs dark / hrs light): 12/12

IN-LIFE DATES: From: 03 July 2023 To: 28 July 2023

Administration / exposure

Route of administration
oral: gavage

Vehicle
water Elix

Details on exposure
PREPARATION OF DOSING SOLUTIONS:

Trial preparations were performed to select the suitable vehicle and to establish a suitable form ulation procedure.
Test material dosing formulations were homogenized to visually acceptable levels at appropriate concentrations to meet dose level requirements. The dosing formulations were prepared at least weekly. Dose formulations were divided into aliquots where required to allow to be dispensed on each dosing occasion. When stored in the refrigerator after preparations, formulations were removed from the refrigerator and stirred at room temperature for at least 30 minutes before dosing. Formulations were dosed within 24 hours after removal from the refrigerator.
The dosing formulations were kept at room temperature until dosing. If practically possible, the dosing formulations were continuously stirred until and during dosing.

Analytical verification of doses or concentrations
yes

Details on analytical verification of doses or concentrations
Dose formulation samples were collected for analysis as indicated in table 1.
Analyses were performed using a validated analytical procedure (Test Facility Study No. 20346081). Concentration results were regarded to be acceptable if mean sample concentration results were within or equal to ± 10% for solutions and ± 15% suspensions of theoretical concentration. Homoge neity results were regarded to be acceptable if the relative standard deviation (RSD) of concentrations were ≤ 10% for each group.
Stability analyses performed previously in conjunction with the method development and validation study (Test Facility Study No. 20346081) demonstrated that the test material is stable in the vehicle when prepared and stored under the same conditions at concentrations bracketing those used in the present study.

RESULTS OF THE DOSE FORMULATION ANALYSES:
- Accuracy: The concentrations analyzed in the formulations of Groups 2, 3 and 4 were in agreement with target concentrations (i.e., mean sample concentration results were within or equal to 90-110% of target concentration). Mean recovery %: group 1: n.a.; group 2 96%; group 3 98%; group 4 99%.
A small response at the retention time of the test material was observed in the chromatograms of the Group 1 (control) formulation. Based on the magnitude of the response
(0.00011-0.00041 mg/mL) this was considered negligible and of no impact on the study results.
- Homogeneity: The formulations of Groups 2 and 4 were homogeneous (i.e., coefficient of variation ≤ 10%). Actual values for CoV: group 2: 0.82%; group 4: 1.1%.

Details on mating procedure
Not relevant, females were mated at the breeder.

Duration of treatment / exposure
22 days (Day 7 to Day 28 of gestation, inclusive)

Frequency of treatment
Once daily

Duration of test
Animals were sacrificed Day 29 of gestation

Doses / concentrations

Dose / conc.	
0	mg/kg bw/day (actual dose received)

Remarks
Group 1

Dose / conc.	
75	mg/kg bw/day (actual dose received)
Remarks	
Group 2	
Dose / conc.	
175	mg/kg bw/day (actual dose received)
Remarks	
Group 3	
Dose / conc.	
350	mg/kg bw/day (actual dose received)
Remarks	
Group 4	

No. of animals per sex per dose
22

Control animals
yes, concurrent vehicle

Details on study design
- Dose selection rationale:
The dose levels were selected based in consultation with the Sponsor and based on the results of the dose range finding study in pregnant rabbits with oral exposure of Amphoacetates C8-C18 (Test Facility Study No. 20346088) and in an attempt to produce graded responses to the test material. The high-dose level should produce some toxic effects, but not excessive lethality that would prevent meaningful evaluation. The mid-dose level is expected to produce minimal to moderate toxic effects. The low-dose level should produce no observable indications of toxicity.
In this dose range finding study, dose levels of 250, 350 and 450 mg a.i./kg bw/day were tested. At 450 mg a.i./kg bw/day, 2/6 females aborted their litter on Day 19 or 20 of gestation and were therefore euthanized. Besides that, one female of this dose group was euthanized for humane reasons as it did not consume feed for seven consecutive days. Therefore, this dose level exceeded the maximum tolerated dose level and was not suitable as high dose for the Main study. At 250 and 350 mg a.i./kg bw/day, one female each was euthanized for humane reasons on Day 19 or 21 of gestation as they did not consume feed for seven days. At 350 mg a.i./kg bw/day, slight body weight loss (up to -3.8 g) was noted over Days 7-9 and 18-21 of gestation and was considered within normal values during other intervals. Gravid uterus adjusted body weight change was considered within normal values. Food consumption was lower than control over Days 7-21 of gestation. Overall food consumption was above the control group (124 g vs. 116 g, respectively). No test material-related macroscopic abnormalities were noted.
Despite the euthanasia of 1/6 females at 350 mg a.i./kg bw/day, this dose level was selected as high dose level for the main study as other parameters did not show dose limiting effects. Dose levels were selected in consultation with the Sponsor as 0, 75, 175 and 350 mg a.i./kg bw/day.

High dose level used
no

Justification for deviation from the high dose level
Please, see "details on study design"

Examinations

Maternal examinations
MORTALITY: Yes
- Time schedule: At least twice daily beginning upon arrival through termination/release. Except on days of receipt and necropsy where frequency was at least once daily.
Animals were observed within their cage unless necessary for identification or confirmation of possible findings.

CLINICAL OBSERVATIONS: Yes
- Time schedule: At least once daily (0 to 1 hour post-dose) starting on Day 7 of gestation up to the day prior to necropsy. On the day of necropsy, an additional clinical observation was performed.
Animals were observed during the dosing procedure. Cage debris were examined to detect premature birth, if applicable.

BODY WEIGHT: Yes
- Time schedule for examinations: Animals were weighed individually On Days 3, 7, 9, 12, 15, 18, 21, 24, 27 and 29 of gestation.

FOOD CONSUMPTION: Yes
- Time schedule for examinations: Quantitatively measured daily from Day 3 of gestation onwards.

WATER CONSUMPTION: Yes
- Monitored by visual inspection of on a regular basis throughout the study.

POST-MORTEM EXAMINATIONS: Yes
- Scheduled euthanasia on gestation day 29 by intravenous injection of sodium pentobarbital. Unsch eduled Euthanasia were also carried out by intravenous injection of sodium pentobarbital
- All animals were subjected to an external, thoracic and abdominal examination, with special attent ion being paid to the reproductive organs.

Ovaries and uterine content
Each ovary and uterine horn of all animals were dissected and examined as quickly as possible to det ermine:
- The number of corpora lutea;
- The weight of the uterus (not for animals found dead, euthanized before planned necropsy or that st arted to deliver);
- The number of implantation sites;
- The number and distribution of live and dead fetuses;
- The number and distribution of early and late resorptions.
- Placental morphology.

Fetal examinations
External, visceral, and skeletal findings were recorded as developmental variations (alterations in anatomic structure that are considered to have no significant biological effect on animal health or body conformity and/or represent slight deviations from normal) or malformations (those structural a nomalies that alter general body conformity, disrupt or interfere with normal body function, or may be incompatible with life).
EXTERNAL EXAMINATIONS
Live fetuses (including those of females found dead or euthanized before planned necropsy) or pups from females that delivered early were euthanized by administration of sodium pentobarbital into the oral cavity using a small metal feeding tube.
Viable and non-viable fetuses of dams surviving until scheduled necropsy, late resorptions from dams euthanized from GD22 onwards, and fetuses of dams not surviving until Day 29 of gestation or dams that started to deliver before day 29; were subjected to external examinations. Body weights were recorded for viable and non-viable fetuses of dams surviving until scheduled necropsy.
Malformed late resorptions were collected and fixed in 10% buffered formalin. Late resorptions wi thout malformations were discarded.

FETAL VISCERAL AND SKELETAL EXAMINATIONS

All viable and non-viable fetus of dams surviving until scheduled necropsy on GD29 were subjected to:
- Internal Sex Determination: Yes, all per litter.
- Visceral Body Examination: Yes, all per litter. Fresh (non-fixed state) (Stuckhardt and Poppe, 1984; Woo and Hoar,1972), abnormalities were collected and fixed at the discretion of the Study Director. Fetuses were eviscerated, fixed in 96% aqueous ethanol, macerated in potassium hydroxide and sta ined (Alizarin Red S) (Dawson, 1926).
- Visceral Head Examination: Yes, all per litter. The heads of approximately 50% of fetuses were fix ed in Bouin's solution for soft-tissue examination (Wilson,1965), tissues without incidental finding s, variations or malformations were discarded. Tissues with incidental findings, variations or malfo rmations were stored in 10% buffered formalin. The heads of other 50% of fetuses were examined by mid-coronal slice.
- Skeletal Examination: Yes, all per litter. Specimens were archived in glycerin with bronopol as pres ervative.

Statistics
All statistical tests were conducted at the 5% significance level. All pairwise comparisons were con ducted using two sided tests and reported at the 1% and 5% levels, unless otherwise noted.
The pairwise comparisons of interest are listed below:
Group 2 vs. Group 1
Group 3 vs. Group 1
Group 4 vs. Group 1
Analyses were performed according to table 2, when possible, but excluded any group with less than 3 observations.
The overall incidence of number of fetuses and litters affected was calculated.

-Parametric/Non-Parametric
Levene's test was used to assess the homogeneity of group variances.
The groups were compared using an overall one-way ANOVA F-test if Levene's test was not significa nt or the Kruskal-Wallis test if it was significant. If the overall F-test or Kruskal-Wallis test was found to be significant, then pairwise comparisons were conducted using Dunnett's or Dunn's test, respective ly.

-Non-Parametric
The groups were compared using an overall Kruskal-Wallis test. If the overall Kruskal-Wallis test was found to be significant, then the above pairwise comparisons was conducted using Dunn's test.

-Incidence
A Fisher's exact test was used to conduct pairwise group comparisons of interest.

Indices
- Body Weight Gains: Calculated for the following intervals: Days 3 to 7, 7 to 9, 9 to 12, 12 to 15, 15 to 18, 18 to 21, 21 to 24, 24 to 27, 27 to 29, and 7 to 29 of gestation;
- Gravid Uterus Adjusted Body Weight Gain: Body weight on Day 29 of gestation - body weight on Day 7 of gestation - gravid uterus weight;
- Overall Food Consumption: Calculated between each scheduled interval (individual data only) and calculated over the complete Dosing Period. Summarization and statistical analysis intervals reflect the same intervals as the body weight gains;
- Pregnancy Rate (%): (No. of pregnant females / No. of mated females) x 100
- Pre-implantation loss (%): ((number of corpora lutea - number of implantations)/number of corpora lutea) x 100;
- Post-implantation loss (%): ((number of implantation sites - number of live fetuses)/number of imp lantation sites) x 100.
- Live Male Fetus/Litter (%): No. of live male fetuses x 100 / (No. of live male fetuses + number of live female fetuses)
- Live Female Fetus/Litter (%): No. of live female fetuses x 100 / (No. of live male fetuses + number of live female fetuses)
- Litter % of Fetuses with Abnormalities: No. of fetuses in litter with a given finding x 100 / No. of fetus es in litter examined

Historical control data
Uterine content Historical Control Data New Zealand White CRL Rabbit (Jan 2020-Jul 2023; n= 636 litters):
- Post-Implantation Loss (%) – mean: 5.83, mean±2SD: 0.77-10.89, min-max: 2.60-14.47
- Early Resorptions (%) – mean: 3.95, mean±2SD: 0.59-7.32, min-max: 1.72-9.35
- Late Resorptions (%) – mean: 1.63, mean±2SD: 0.00-4.22, min-max: 0.00-6.85

Additional relevant Historical Control Data New Zealand White CRL Rabbit (Jan 2020-Jul 2023) on external, visceral and skeletal malformations and variations are included in the report and attached in the summary.

Any other information on materials and methods incl. tables

Table 1. Dose Formulation Sample Collection Schedule

Sample Type	Group	Stratum	Sampling (into)	Number of Samples per Group			Sample Amount (mg)	Interval(s)
				Collected	Analyzed	Backup		
Concentration	Groups 1 and 3	Middle	From dosing container into clear glass container	2	2	-	500	Week 1 (range: 03-09 Jul 2023)
Concentration and Homogeneity Analyses[a]	Groups 2 and 4	Top		2	2	-	500	
		Middle		2	2	-	500	
		Bottom		2	2	-	500	

[a] The averaged result from homogeneity analysis will serve as concentration verification. - Sampling not applicable

Table 2. Statistical Matrix

Variables for Inferential Analysis[a]	Statistical Method		
	Parametric/ Non-Parametric	Non-Parametric	Incidence
Body Weight	X	-	-
Body Weight Gains	X	-	-
Food Consumption	X	-	-
Caesarean-Section Late Gestation[b]			

Parental Indices and Mortality	-	-	X
Gravid Uterine Weight and Gravid Uterus Adjusted Body Weights	X	-	-
Ovarian and Uterine Examinations	-	X	-
Litter Observations (Litter Means)[c]	X	-	-
Litter % of Fetuses with Gross/External/ Visceral/Skeletal Abnormalities[c]	-	X	-

[a] Excludes animals not pregnant from the gestation phase summarization and statistical analysis.

[b] Excludes animals euthanized pre-terminally from summarization and statistical analysis.

[c] Presented for sexes combined; live fetuses only.

Results and discussion

Results: maternal animals

General toxicity (maternal animals)

Clinical signs
effects observed, treatment-related

Description (incidence and severity)
No test material-related clinical signs were noted during the treatment period up to 175 mg/kg bw/day.
At 350 mg/kg bw/day, erected fur was noted in 8/12 females surviving until scheduled necropsy on multiple days from Day 19 of gestation onwards. Slightly softer feces were noted for 2/12 females on a single day. This change in fecal consistency was also observed in 4/10 premature deaths at 350 mg/kg bw/day. However, as this sign was only observed on a single day in a minority of the females with no relation to duration of treatment or day of death, this clinical sign was considered incidental and therefore unrelated to treatment with the test material.
At 175 mg/kg bw/day, slightly softer feces and erected fur were noted for 1/21 females on a single day. Based on the occurrence in a single female on a single day, these signs were considered incidental at this dose level and therefore unrelated to treatment with the test material. Furthermore, 4/21 females surviving until scheduled necropsy were noted with clinical signs considered related to the gavage procedure (i.e., labored breathing, liquid discharge from mouth and/or nostrils, sneezing, increased activity, salivation and/or excessive grooming), and were therefore considered unrelated to treatment with the test material.
Any other clinical signs noted during the treatment period occurred within the range of background findings to be expected for rabbits of this age and strain which are housed and treated under the conditions in this study, were noted in absence of a dose related response or were noted in the control g

roup only. At the incidence observed, these were considered to be unrelated to treatment with the test material.

Mortality
mortality observed, treatment-related

Description (incidence)
In total, 13 females (2/22 females, 1/22 female and 10/22 females at 75, 175 and 350 mg/kg bw/day, respectively) did not survive until scheduled necropsy.
At 175 and 350 mg/kg bw/day, all unscheduled euthanasia females were euthanized for humane rea sons based on clinical signs, body weight loss and/or prolonged (near) absent food consumption. Based on the results in the females surviving until scheduled necropsy at 175 mg/kg bw/day and the high incidence of early termination at 350 mg/kg bw/day, these preterm deaths were considered r elated to treatment with the test material. Details on these females are shown in table 3.
Female No. 44 (75 mg/kg bw/day) was euthanized for humane reasons on Day 20 of gestation based on absent food consumption for seven consecutive days. The animal also had a body weight loss (7%) over Days 9-20 of gestation and erected fur on Days 19 and 20 of gestation. At necropsy, the li ver was noted with a prominent lobular architecture. The female was pregnant and had 11 embryos in normal development. The euthanasia was considered isolated to this single female based on t he results of the females of this dose group surviving until scheduled necropsy. Therefore, it was c onsidered unlikely that this death was related to treatment with the test material.
-Euthanasia Not Related to Treatment with the Test Material
Female No. 23 (75 mg/kg bw/day) was euthanized for humane reasons on Day 21 of gestation as it presented with labored breathing and increased respiratory rate after the gavage procedure. Body weight and food consumption on the days prior to euthanasia were considered normal and no oth er clinical signs were noted. At necropsy, it was confirmed that this death was related to the gavage procedure and not to treatment with the test material as the thoracic cavity and trachea contained red or clear fluid and dark-red foci were noted for the lungs. The female was pregnant and had nine embryos in normal development and three early resorptions.
Note: All relevant findings for these preterminally euthanized animals are included in this mortality section and will not be included in the subsequent results sections.

Body weight and weight changes
effects observed, treatment-related

Description (incidence and severity)
Body weights, body weight gain and weight gain adjusted for gravid uterus were considered to be unaf fected at 75 mg/kg bw/day.
At 175 and 350 mg/kg bw/day, body weight loss or lower body weight gain were noted over Days 9-15 and Days 18-21 of gestation, resulting in a lower overall body weight gain (186 and 255 g, resp ectively, vs. 326 g in control; not statistically significant at 350 mg/kg bw/day) with no effect on body weight at end of treatment on Day 29 of gestation. The changes in body weight gain at 350 mg/kg bw/day were mainly due to body weight losses in prematurely euthanized females (not included in overall body weight gain). Gravid uterus adjusted body weight loss was also larger at 175 mg/kg bw/ day (-330 g vs. -162 g in control). At 350 mg/kg bw/day, gravid uterus adjusted body weight loss for fe males surviving until scheduled necropsy (n=12) was comparable to the control group.
Any other statistically significant changes in body weights and body weight gain were considered to be unrelated to treatment with the test material as no trend was apparent regarding dose and d uration of treatment or were only observed during a single interval with no overall effect.
See also tables 4 to 6.

Food consumption and compound intake (if feeding study)
effects observed, treatment-related

Description (incidence and severity)
No test material-related changes in food consumption were noted at 75 mg/kg bw/day.
At 175 and 350 mg/kg bw/day, food consumption was lower than control during the majority of the treatment period, resulting in a lower overall food consumption (15% and 10% lower than control, res pectively). The lower food consumption at 350 mg/kg bw/day was mainly due to minimal to absent food consumption in prematurely euthanized females (not included in overall food consumption).

Any other statistically significant changes in food consumption were considered to be unrelated to t reatment with the test material as no trend was apparent regarding dose and duration of treatment. See also table 7.

Gross pathological findings
no effects observed

Description (incidence and severity)
Macroscopic observations at necropsy did not reveal any alterations that were considered to be relat ed to treatment with the test material.
Findings that were noted among control and/or treated animals were considered to be unrelated to the test material, as they remained within the range of biological variation for rabbits of this age and strain.

Details on results
Data from females that were not pregnant were excluded from summary tables and statistical analysis, with the exception of macroscopic pathology. Data from animals that did not survive until scheduled necropsy were included in summary tables up to the point where they were removed from the study. Data on uterine and ovarian examinations and litter observations, as well as fetal abnormalities from females that did not survive until scheduled necropsy were not part of the stati stical analysis nor reported in summary tables.

Maternal developmental toxicity

Pre- and post-implantation loss
effects observed, treatment-related

Description (incidence and severity)
The number of corpora lutea and implantation sites, and pre-implantation loss in the control and test material-treated groups was considered unaffected by treatment with the test material up to 175 mg/ kg bw/day.
At 175 mg/kg bw/day, a higher post-implantation loss was noted (12% vs. 5% in controls, not statis tically significant), caused by a higher percentage of both early and late resorptions (8 and 4% vs. 3 and 2% in controls, respectively, not statistically significant). Mean values were above the historical control data range (mean±2SD), except for the percentage of late resorptions (please, see post-implantation loss and resorption Historical control data in the Materials and Methods section).

-TOP DOSE RESULTS - NOT USED TO DRAW CONCLUSIONS ON DEVELOPMENTAL TOXICITY: At 350 mg/kg bw/day, number of pregnant females, corpora lutea and implantation sites, and pre-implantati on loss were considered comparable to the control group. A higher post-implantation loss was noted (11% vs. 5% in control), which was due to a higher percentage of early resorptions (10% vs. 3% in con trol).

Please, see also table 9.

Total litter losses by resorption
no effects observed

Description (incidence and severity)
No total litter loss observed up to and including 175 mg/kg bw/day.
Please see also table 8.

-TOP DOSE RESULTS - NOT USED TO DRAW CONCLUSIONS ON DEVELOPMENTAL TOXICITY: One female at 350 mg/kg bw/day had total litter loss by resorption.

Early or late resorptions
effects observed, treatment-related

Description (incidence and severity)
Please see "Pre- and post-implantation loss - Description (incidence and severity)" and table 9.

Dead fetuses
effects observed, non-treatment-related

Description (incidence and severity)
There were no test material-related effects on litter size up to and including 175 mg/kg bw/day.
Any statistically significant changes in litter size were considered to be unrelated to treatment with the test material as no trend was apparent regarding dose level.

-TOP DOSE RESULTS - NOT USED TO DRAW CONCLUSIONS ON DEVELOPMENTAL TOXICITY: :
At 350 mg/kg bw/day, the litter size was comparable to the control group.

Please, see also table 9

Changes in number of pregnant
no effects observed

Description (incidence and severity)
The number of pregnant females in the control and test material-treated groups was considered unaffected by treatment with the test material up to 175 mg/kg bw/day.
All females, including those euthanized for humane reasons (see mortality results), were pregnant except for one female at 75 mg/kg bw/day, two females at 175 mg/kg bw/day and one female at 350 mg/kg bw/day.
As a result, there were 22, 19, 19 and 12 females with litters in the control, 75, 175 and 350 mg/kg bw/day groups, respectively, available for evaluation.

Please see also table 8.

Details on maternal toxic effects
Any statistically significant changes in maternal pregnancy data were considered to be unrelated to treatment with the test material as no trend was apparent regarding dose level.
According to the guidelines, a minimum of 16 females with implantations at termination is required in each group for an adequate evaluation. The 12 animals available at 350 mg/kg bw/day were considered not sufficient for a robust and valid evaluation of the developmental data. Therefore, the developmental data from the 350 mg/kg bw/day group was not used for any conclusions and is reported separately under the title "TOP DOSE RESULTS - NOT USED TO DRAW CONCLUSIONS ON DEVELOPMENTAL TOXICITY: " in the "Description (incidence and severity)" fields.

Effect levels (maternal animals)

Key result
true

Dose descriptor
NOAEL (Maternal)

Effect level

75	mg/kg bw/day (actual dose received)

Based on
test mat.

Basis for effect level
body weight and weight gain
food consumption and compound intake
mortality

Maternal abnormalities

Key result
true

Abnormalities
effects observed, treatment-related

Localisation
uterus

Description (incidence and severity)
Higher post-implantation loss caused by a higher number of early and late resorptions starting at 175 mg/kg bw/day. significant). Mean values were above the historical control data range (mean ±2SD), except for the percentage of late resorptions.

Results (fetuses)

Fetal body weight changes
no effects observed

Description (incidence and severity)
There were no test material-related effects on fetal body weights (both sexes) up to and including 175 mg/kg bw/day.

TOP DOSE RESULTS - NOT USED TO DRAW CONCLUSIONS ON DEVELOPMENTAL TOXICITY: At 350 mg/kg bw/day, fetal body weights were within normal ranges.

Changes in sex ratio
no effects observed

Description (incidence and severity)
The male:female ratio was unaffected by treatment with the test material up to and including175 mg/kg bw/day.

-TOP DOSE RESULTS - NOT USED TO DRAW CONCLUSIONS ON DEVELOPMENTAL TOXICITY: At 350 mg/kg bw/day, the male:female distribution was shifted in favor of female fetuses (61.4%).

Changes in litter size and weights
effects observed, non-treatment-related

Description (incidence and severity)
There were no test material-related effects on litter size up to and including 175 mg/kg bw/day. Any statistically significant changes in litter size were considered to be unrelated to treatment with the test material as no trend was apparent regarding dose level.

-TOP DOSE RESULTS - NOT USED TO DRAW CONCLUSIONS ON DEVELOPMENTAL TOXICITY: At 350 mg/kg bw/day, the litter size was comparable to the control group.

Please, see also table 9

External malformations
effects observed, non-treatment-related

Description (incidence and severity)
The numbers of fetuses (litters) submitted to external examination for groups 1, 2, 3 and 4 were 188 (22), 198 (19), 174 (19) and 97 (12) respectively.

No test material-related external malformations and variations were noted up to and including 175 mg/kg bw/day.

External malformations were noted in one fetus at 75 mg/kg bw/day (No. 33 L3) which was noted w ith omphalocele. Based on its single occurrence, this was considered not to be related to treatment with the test material.

TOP DOSE RESULTS - NOT USED TO DRAW CONCLUSIONS ON DEVELOPMENTAL TOXICITY:
At 350 mg/kg bw/day, no external malformations and variations were noted.

Please, see also tables 10, 11 under "Any other information on results incl. tables" and attached "HCD" and "Summary of Fetal Abnormalities by Finding" tables.

Skeletal malformations
effects observed, non-treatment-related

Description (incidence and severity)
The numbers of fetuses (litters) submitted to skeletal examination for groups 1, 2, 3 and 4 were 188 (22), 198 (19), 174 (19) and 97 (12) respectively.

No test material-related skeletal malformations or variations were noted up to 175 mg/kg bw/day.
Skeletal malformations were observed in one fetus in each of the control and the 175 mg/kg bw/day groups, and in two fetuses at 75 mg/kg bw/day.
At 75 mg/kg bw/day, one fetus with lumbar hemivertebra (Fetus 39-L3) and one fetus with sternos chisis (Fetus 33-L3; which also presented with omphalocele) were noted. Due to the single occurrence and group distribution, all these malformations were considered not test material-related.
In the control group, the fetus with multiple visceral abnormalities (No. 6-R9) also had two vertebral thoracic malformations.
All recorded skeletal variations occurred in a diverse array of skeletal structures, including fore- and hindlimbs, pelvic girdle, (supernumerary) rib and vertebra. These variations were either scored infrequently, comparable to the control group, in the absence of a dose relationship, in the control group only and/or within the range of available historical control data and therefore considered not related to treatment with the test material.

TOP DOSE RESULTS - NOT USED TO DRAW CONCLUSIONS ON DEVELOPMENTAL TOXICITY:
At 350 mg/kg bw/day, fused caudal vertebra was noted in a single fetus.

Please, see also tables 10, 11 under "Any other information on results incl. tables" and attached "HCD" and "Summary of Fetal Abnormalities by Finding" tables.

Visceral malformations
effects observed, non-treatment-related

Description (incidence and severity)
The numbers of fetuses (litters) submitted to visceral examination for groups 1, 2, 3 and 4 were 188 (22), 198 (19), 174 (19) and 97 (12) respectively.

No test material-related visceral malformations were noted up to and including 175 mg/kg bw/day.
Visceral malformations were observed in one fetus in each of the control, 75 and 175 mg/kg bw/day groups.
At 75 mg/kg bw/day, Fetus No. 38-R9 was noted with a diaphragmatic hernia and misshapen spleen. At 175 mg/kg bw/day, Fetus No. 45-R16 was noted with Tetralogy of Fallot. As these malformations occurred for single fetuses, they were considered chance findings.
Control Fetus No. 6-R9 was noted with malformations in various areas. These malformations included abdominal situs inversus, malpositioned adrenal gland and several cardiovascular abnormalities. As this concerned a control fetus this was considered spontaneous of origin.
Visceral variations were observed in a wide range of structures including adrenal gland, gallbladder, abdomen, gonad, liver, lung, spleen, stomach and (subclavian) artery. In all cases, these were either scored infrequently, comparable to the control group and/or within the range of available historical control data and therefore considered not related to treatment with the test material.
Incidental findings (not otherwise classified as malformation or variation) only involved a single case of discolored liver lobe in a control fetus.

TOP DOSE RESULTS - NOT USED TO DRAW CONCLUSIONS ON DEVELOPMENTAL TOXICITY:At 350 mg/kg bw/day, the mean litter incidence of fetuses with the variation retrocaval ureter was higher (not statistically significant).

Please, see also tables 10, 11 under "Any other information on results incl. tables" and attached "HCD" and "Summary of Fetal Abnormalities by Finding" tables.

Details on embryotoxic / teratogenic effects

According to the guidelines, a minimum of 16 females with implantations at termination is required in each group for an adequate evaluation. The 12 animals available at 350 mg/kg bw/day were considered not sufficient for a robust and valid evaluation of the developmental data. Therefore, the developmental data from the 350 mg/kg bw/day group was not used for any conclusions and is reported separately under the title "TOP DOSE RESULTS - NOT USED TO DRAW CONCLUSIONS ON DEVELOPMENTAL TOXICITY: " in the "Description (incidence and severity)" fields.
Historical Control Data regarding Fetal Pathology available as an attachment.

Effect levels (fetuses)

Key result
true

Dose descriptor
NOAEL (Developmental)

Effect level

75 mg/kg bw/day (actual dose received)

Based on
test mat.

Sex
male/female

Basis for effect level
other: Increased post-implantation loss caused by a higher number of early and late resorptions

Fetal abnormalities

Key result
true

Abnormalities
no effects observed

Description (incidence and severity)
There were no fetal abnormalities in this study

Overall developmental toxicity

Key result
true

Developmental effects observed
yes

Lowest effective dose / conc.

75 mg/kg bw/day (actual dose received)

Treatment related
yes

Relation to maternal toxicity
developmental effects as a secondary non-specific consequence of maternal toxicity effects

Dose response relationship
yes

Relevant for humans
not specified

Any other information on results incl. tables

Table 3. Test Material-Related Euthanasia

Dose Level	Female No.	Day of Euthanasia	Reason for Termination and Maternal Results
175 mg/kg bw/day	63	Day 20 of gestation	Absent food consumption for seven consecutive days. In addition, erected fur was noted on Day 20 of gestation and body weight loss (3%) was noted over Days 15-20 of gestation. No macroscopic abnormalities were noted at necropsy. This female was pregnant with nine embryos in normal development.
350 mg/kg bw/day	69	Day 18 of gestation	Body weight loss (12%) was noted over Days 12-18 of gestation. Moreover, absent food consumption was noted for five consecutive days and the animal was noted with erected fur and a thin appearance from Day 15 of gestation onwards. At necropsy, the thyroid gland was pale discolored and pale-

			red foci were noted on the liver. This female was not pregnant.
	84	Day 18 of gestation	Absent food consumption was noted for seven consecutive days and body weight loss (9%) was noted over Days 7-18 of gestation. Moreover, the animal had a pale skin, thin appearance and decreased activity on Day 18 of gestation, and slightly softer feces on Day 14 of gestation. At necropsy, pale discoloration of the lungs and liver and a prominent lobular architecture of the liver was noted. Not test material-related macroscopic observations included clear watery cysts on the oviducts and ectopic splenic tissues. This female was pregnant and had 11 embryos in normal development.
	70	Day 19 of gestation	Absent food consumption was noted for seven consecutive days. In addition, erected fur was noted on the day of necropsy and body weight gain was absent from the initiation of treatment on Day 7 of gestation onwards. At necropsy, pale discoloration of the lungs and liver were noted. Not test material-related macroscopic observations included clear watery cysts on the oviducts. This female was pregnant and had eight embryos in normal development and three late resorptions.

	75	Day 19 of gestation	Absent food consumption was noted for seven consecutive days. Erected fur was noted on Days 18 and 19 of gestation. Body weight loss (3%) was noted over Days 9-18 of gestation. No abnormalities were noted at necropsy. This female was pregnant and had nine embryos in normal development.

Absent food consumption is defined as less than 10% of the maximum allotted daily amount of feed (i.e., less than 19 g/day).

Table 4. Summary of Body Weights (g): Gestation

		Day(s) Relative to Mating								
		7	9	12	15	18	21	24	27	29
0 mg/kg bw/day Group 1	Mean	3508.7	3539.8	3580.0	3692.5	3674.0	3695.8	3732.8	3797.3	3835.1
	SD	298.8	296.3	291.4	299.5	279.3	294.2	285.9	288.0	304.4
	N	22	22	22	22	22	22	22	22	22
75 mg/kg bw/day Group 2	Mean	3623.9	3674.9	3715.4	3772.8	3772.6	3803.0	3852.6	3907.6	3982.3
	SD	293.2	291.3	284.9	303.5	300.9	309.6	314.2	313.6	315.2
	N	21	21	21	21	21	20	19	19	19
	%Diff	3.3	3.8	3.8	2.2	2.7	2.9	3.2	2.9	3.8
175 mg/kg bw/day Group 3	Mean	3766.9*	3798.2*	3790.3	3819.6	3833.4	3870.6	3914.6	3933.3	3986.3
	SD	360.2	357.0	345.6	337.2	360.4	319.0	315.3	322.6	351.2
	N	20	20	20	20	20	19	19	19	19
	%Diff	7.4	7.3	5.9	3.4	4.3	4.7	4.9	3.6	3.9
350 mg/kg bw/day Group 4	Mean	3531.8	3549.7	3528.1	3575.6	3561.4	3606.0	3687.3	3746.7	3780.8
	SD	298.9	291.7	316.7	303.0	300.1	330.4	369.2	367.8	375.9
	N	21	21	21	21	21	15	12	12	12

	%Diff	0.7	0.3	-1.4	-3.2	-3.1	-2.4	-1.2	-1.3	-1.4

* = p ≤ 0.05

Table 5. Summary of Body Weight Gains (g): Gestation

		Day(s) Relative to Mating								
		7 → 9	9 → 12	12 → 15	15 → 18	18 → 21	21 → 24	24 → 27	27 → 29	7 → 29
0 mg/kg bw/day Group 1	Mean	31.0	40.3	112.5	-18.6	21.9	37.0	64.5	37.9	326.4
	SD	61.0	64.5	50.6	59.7	37.7	47.5	47.9	49.2	139.6
	N	22	22	22	22	22	22	22	22	22
75 mg/kg bw/day Group 2	Mean	51.0	40.5	57.4*	-0.1	23.5	62.3	54.9	74.7	371.2
	SD	44.3	41.1	91.4	70.7	55.8	44.6	49.1	24.8	121.8
	N	21	21	21	21	20	19	19	19	19
175 mg/kg bw/day Group 3	Mean	31.4	-7.9*	29.3**	13.9	-1.3	43.9	18.7	52.9	186.0**
	SD	36.7	67.8	52.5	89.2	60.9	59.8	74.7	61.6	133.7
	N	20	20	20	20	19	19	19	19	19
350 mg/kg bw/day Group 4	Mean	17.9	-21.5**	47.5**	-14.2	-18.9	53.1	59.3	34.2	255.0
	SD	44.9	51.3	79.5	94.3	95.7	63.2	39.5	48.5	181.4
	N	21	21	21	21	15	12	12	12	12

* = p ≤ 0.05; ** = p ≤ 0.01

Table 6. Summary of Gravid Uterine Weights and Gravid Uterus Adjusted Body Weights: Gestation

Day(s) Relative to Mating		0 mg/kg bw/day Group 1	75 mg/kg bw/day Group 2	175 mg/kg bw/day Group 3	350 mg/kg bw/day Group 4
Bodyweight on Day 7 (g)	Mean	3508.7	3611.1	3800.3 *	3525.8

	SD	298.8	306	336.7	349.8
	N	22	19	19	12
	%Diff	-	2.9	8.3	0.5
Terminal Body Weight (g)	Mean	3835.1	3982.3	3986.3	3780.8
	SD	304.4	315.2	351.2	375.9
	N	22	19	19	12
	%Diff	-	3.8	3.9	-1.4
Gravid Uterus Weight (g)	Mean	488.82	558.48	515.65	449.42
	SD	103.23	69.79	108.42	100.41
	N	22	19	19	12
	%Diff	-	14.25	5.49	-8.06
Adjusted BWG (7-abw) (g)	Mean	-162.4	-187.3	-329.6 **	-194.4
	SD	150.2	104.1	144.1	135
	N	22	19	19	12
	%Diff	-	15.3	103	19.7

* = p ≤ 0.05; ** = p ≤ 0.01

Table 7. Summary of Food Consumption: Gestation

Food Mean Daily Consumption (g/animal/day)		Day(s) Relative to Mating								
		7 → 9	9 → 12	12 → 15	15 → 18	18 → 21	21 → 24	24 → 27	27 → 29	7 → 29
0 mg/kg bw/day Group 1	Mean	148.57	139.62	104.52	105.76	113.20	97.95	106.64	117.02	115.19
	SD	26.46	23.04	31.68	41.63	26.56	24.08	30.83	35.47	19.39
	N	22	22	22	22	22	22	22	22	22

75 mg/kg bw/day Group 2	Mean	159.60	135.59	77.10	103.98	115.58	121.98*	97.35	115.45	112.82
	SD	23.84	38.18	46.37	50.62	30.90	28.14	30.98	25.15	18.73
	N	21	21	21	21	20	19	19	19	19
	%Diff	7.42	-2.89	-26.24	-1.68	2.11	24.53	-8.71	-1.35	-2.06
175 mg/kg bw/day Group 3	Mean	157.50	122.88	65.65*	74.42	99.61	98.72	85.72	92.21	98.44*
	SD	21.72	44.55	43.63	41.70	41.91	29.83	39.24	44.67	19.17
	N	20	20	20	20	19	19	19	19	19
	%Diff	6.01	-11.99	-37.19	-29.63	-12.00	0.78	-19.62	-21.20	-14.54
350 mg/kg bw/day Group 4	Mean	127.52*	86.76**	53.68**	53.92**	91.64	122.97	109.03	102.71	103.16
	SD	35.26	41.23	49.82	59.16	57.32	40.34	38.45	42.94	26.77
	N	21	21	21	21	15	12	12	12	12
	%Diff	-14.16	-37.86	-48.64	-49.01	-19.04	25.54	2.24	-12.23	-10.45

* = p ≤ 0.05; ** = p ≤ 0.01

Table 8. Summary of Maternal Performance

		0 mg/kg bw/day Group 1	75 mg/kg bw/day Group 2	175 mg/kg bw/day Group 3	350 mg/kg bw/day Group 4
Group Size - Females		22	22	22	22
Number of Females Pregnant [f]	N+ve	22	21	20	21
	%	100	95.5	90.9	95.5
Female with Live Fetuses [f]	N+ve	22	21	20	20
	%	100	100	100	95.2
Total Resorptions [f]	N+ve	0	0	0	1
	%	0	0	0	4.8
Female with all Nonviable [f]	N+ve	0	0	0	1

	%	0	0	0	4.8

Table 9. Summary of Ovarian and Uterine Examinations and Litter Observations

		0 mg/kg bw/ day Group 1	75 mg/kg bw/ day Group 2	175 mg/kg bw/ day Group 3	350 mg/kg bw/ day Group 4
Female with Live Fetuses	N+ve	22	19	19	12
	%	100	100	100	100
Number of Corpora Lutea	Mean	10.9	11.4	11.3	10.4
	SD	2.4	1.7	2.1	1.4
	N	22	19	19	12
	%Diff	-	4.6	4.2	-4.1
Number of Implantations	Mean	9	10.8*	10.5	9.2
	SD	2.4	1.5	2.2	1.6
	N	22	19	19	12
	%Diff	-	19.9	15.8	1.3
Pre-implantation Loss (%)	Mean	15.07	4.27	7.22	10.63
	SD	19.15	6.29	10	18.11
	N	22	19	19	12
	%Diff	-	-71.64	-52.09	-29.46
Total Number of Fetuses	Mean	8.5	10.4*	9.2	8.2
	SD	2.3	1.5	1.8	2.4
	N	22	19	19	12
	%Diff	-	21.9	7.2	-4.4

Number of Live Fetuses	Mean	8.5	10.4*	9.2	8.1
	SD	2.3	1.5	1.8	2.4
	N	22	19	19	12
	%Diff	-	21.9	7.2	-5.4
Number of Dead Fetuses	Mean	0	0	0	0.1
	SD	0	0	0	0.3
	N	22	19	19	12
	%Diff	-	-	-	-
Number of Early Resorptions	Mean	0.3	0.3	0.9	1
	SD	0.6	0.7	1.4	2.1
	N	22	19	19	12
	%Diff	-	-0.8	181.2	214.3
Early Resorption/ Implants (%) -	Mean	3.41	2.6	8.1	10.45
	SD	6.01	6.22	12.42	20.93
	N	22	19	19	12
Number of Late Resorptions	Mean	0.2	0.1	0.4	0
	SD	0.4	0.5	0.7	0
	N	22	19	19	12
	%Diff	-	-42.1	131.6	-100
Late Resorption/ Implants (%) -	Mean	1.79	0.96	3.63	0
	SD	3.95	4.17	5.95	0
	N	22	19	19	12
Total Number of Resorptions	Mean	0.5	0.4	1.3	1
	SD	0.6	1	1.4	2.1

	N	22	19	19	12
	%Diff	-	-15.8	163.2	100
Post-implantation Loss (%)	Mean	5.2	3.56	11.74	11.21
	SD	6.24	8.32	12.18	20.68
	N	22	19	19	12
	%Diff	-	-31.48	125.82	115.65
Number of Live Male Fetuses	Mean	4.3	4.7	4.9	3.3
	SD	2	1.5	1.9	1.8
	N	22	19	19	12
	%Diff	-	9.7	14.6	-24.7
Number of Live Female Fetuses	Mean	4.2	5.7*	4.2	4.8
	SD	2	1.3	1.8	1.6
	N	22	19	19	12
	%Diff	-	34.5	-0.4	14.3
Live Male Fetus/Litter (%)	Mean	50.8	45.29	53.05	38.63
	SD	20.28	11.96	18.98	17
	N	22	19	19	12
	%Diff	-	-10.84	4.43	-23.97
Live Female Fetuses/Litter (%)	Mean	49.2	54.71	46.95	61.37
	SD	20.28	11.96	18.98	17
	N	22	19	19	12
	%Diff	-	11.2	-4.57	24.75
Mean Fetal Weight males (g)	Mean	41.55	39.2	39.85	38.29
	SD	5.61	4.48	3.4	4.63

	N	22	19	18	11
	%Diff	-	-5.67	-4.09	-7.86
Mean Fetal Weight females (g)	Mean	39.83	38.26	38.7	38.61
	SD	5.23	4.43	4.86	4.97
	N	22	19	19	12
	%Diff	-	-3.94	-2.82	-3.05
Mean Fetal Weight all (g)	Mean	40.7	38.63	39.49	38.97
	SD	5.05	3.93	3.69	5.1
	N	22	19	19	12
	%Diff	-	-5.09	-2.97	-4.25

* = $p \leq 0.05$

* = $p \leq 0.05$

Table 10. Summary of Malformations - Individual Descriptions

Dose Level (mg/kg bw/ day)	Female No.	Fetus No.	Malformation(s)#
0	6	R9	Adrenal gland, Malpositioned (V) Aorta, Overriding (V) Abdomen, Situs, inversus (V) Heart, Large (V) Ventricular septum, Absent (V) Rib, Fused (S) Thoracic arch, Fused (S) Thoracic centrum, Absent (S)

75	33	L3	Trunk, Omphalocele (E) Sternebra, Sternoschisis (S)
	38	R9	Diaphragm, Hernia (V) Spleen, Misshapen (V)
	39	L3	Lumbar vertebra, Hemivertebra (S)
175	45	R16	Great vessels, Tetralogy of Fallot (V)
350	*86*	*L6*	*Caudal vertebra, Fused (S)*

#: Including external (E), visceral (V) and skeletal (S) examinations.

Table 11. Summary of Fetal Abnormalities by Classification

Exam Type: External	Number of Fetuses Examined:	0 mg/kg bw/ day Group 1	75 mg/kg bw/ day Group 2	175 mg/kg bw/ day Group 3	350 mg/kg bw/ day Group 4
		188	198	174	97
	Number of Fetuses Evaluated:	192	200	182	98
	Number of Litters Examined:	22	19	19	12
	Number of Litters Evaluated:	22	19	19	12
• Malformation	Number of Fetuses	0	1	0	0
	Litter % of Fetuses [k]	0.00	0.53	0.00	0.00
	Number of Litters	0	1	0	0

• All classifications	Number of Fetuses	0	1	0	0
	Litter % of Fetuses [k]	0.00	0.53	0.00	0.00
	Number of Litters	0	1	0	0
Exam Type: Visceral Body	Number of Fetuses Examined:	0 mg/kg bw/ day Group 1	75 mg/kg bw/ day Group 2	175 mg/kg bw/ day Group 3	350 mg/kg bw/ day Group 4
		188	198	174	97
	Number of Fetuses Evaluated:	192	200	182	98
	Number of Litters Examined:	22	19	19	12
	Number of Litters Evaluated:	22	19	19	12
• Incidental	Number of Fetuses	1	0	0	0
	Litter % of Fetuses [k]	0.51	0.00	0.00	0.00
	Number of Litters	1	0	0	0
• Variation	Number of Fetuses	21	23	12	19
	Litter % of Fetuses [k]	10.47	11.83	7.07	24.42
	Number of Litters	11	12	8	8
• Malformation	Number of Fetuses	1	1	1	0
	Litter % of Fetuses [k]	0.51	0.66	0.40	0.00

	Number of Litters	1	1	1	0
• All classifications	Number of Fetuses	22	24	13	19
	Litter % of Fetuses [k]	10.98	12.49	7.48	24.42
	Number of Litters	11	12	9	8
Exam Type: Skeletal Head	Number of Fetuses Examined:	0 mg/kg bw/ day Group 1	75 mg/kg bw/ day Group 2	175 mg/kg bw/ day Group 3	350 mg/kg bw/ day Group 4
		94	99	85	48
	Number of Fetuses Evaluated:	192	200	182	98
	Number of Litters Examined:	22	19	19	12
	Number of Litters Evaluated:	22	19	19	12
• Variation	Number of Fetuses	4	1	2	1
	Litter % of Fetuses [k]	4.70	0.88	2.63	1.67
	Number of Litters	4	1	2	1
• All classifications	Number of Fetuses	4	1	2	1
	Litter % of Fetuses [k]	4.70	0.88	2.63	1.67
	Number of Litters	4	1	2	1
Exam Type: Skeletal Body	Number of Fetuses Examined:	0 mg/kg bw/ day	75 mg/kg bw/ day	175 mg/kg bw/ day	350 mg/kg bw/ day

		Group 1	Group 2	Group 3	Group 4
		188	198	174	97
	Number of Fetuses Evaluated:	192	200	182	98
	Number of Litters Examined:	22	19	19	12
	Number of Litters Evaluated:	22	19	19	12
• Variation	Number of Fetuses	152	152	143	75
	Litter % of Fetuses [k]	82.39	76.79	81.75	77.97
	Number of Litters	22	19	19	12
• Malformation	Number of Fetuses	1	2	0	1
	Litter % of Fetuses [k]	0.51	0.93	0.00	0.76
	Number of Litters	1	2	0	1
• All classifications	Number of Fetuses	152	153	143	75
	Litter % of Fetuses [k]	82.39	77.32	81.75	77.97
	Number of Litters	22	19	19	12

DISCUSSION ON THE RESULTS AS IN STUDY REPORT:

Time-mated female New Zealand White rabbits were treated with Amphoacetates C8-C18 from Day 7 to 29 of gestation, inclusive, by daily oral gavage at dose levels of 75, 175 and 350 mg/kg bw/day. The rabbits of the control group received the vehicle, Elix water, alone.

Maternal Results

One female at 175 mg/kg bw/day and ten females at 350 mg/kg bw/day were prematurely euthanized based on clinical signs, body weight loss and/or prolonged (near) absent food consumption.

The death of one female at 75 mg/kg bw/day was unlikely to be related to treatment with the test material based on the absence of any effects on maternal and developmental parameters in the remaining animals of this dose group.

In the surviving females at 175 and 350 mg/kg bw/day, body weight loss or lower body weight gain accompanied by lower food consumption were observed during the majority of the treatment period. At 350 mg/kg bw/day, this was mainly caused by changes in prematurely euthanized females. In females surviving until scheduled necropsy, a lower overall body weight gain and food consumption were noted, which was accompanied by the presence of erected fur on multiple days at 350 mg/kg bw/day only. Despite the limited effect size in surviving females, the effects noted at these dose levels were considered adverse as this led to the euthanasia of one and ten females at 175 and 350 mg/kg bw/day, respectively.

Developmental results

As in the high dose group (350 mg/kg bw/day) only 12 females with viable litters were available for evaluation, the available data were considered not sufficient for a robust and valid evaluation of the developmental data.

At 175 mg/kg bw/day, a higher post-implantation loss was noted, which was caused by a higher percentage of early and late resorptions. Mean values of post-implantation loss and early resorptions were above the historical control data range (mean±2SD), while the control group and values at 75 mg/kg bw/day were within normal ranges. In order to exclude for a potential effect of the litter size that might be affected by the number of implantations in general, the post-implantation loss was calculated as mean number as well as percentage per implantations per litter. With this correction for the litter size, the early resorptions noted at 175 mg/kg bw/day are still above the mean±2SD of the historical control data and therefore considered adverse.

Overall remarks, attachments

Overall remarks

A Review of the Results of this study as well as an Expert Opinion Regarding on the Potential Need for Reproductive Toxicity Classification, have been comissioned to Exponent, Inc. (see attachments from 2024 in section 13.2) to clarify the relevance of the increases in early resorptions and post-implantation loss. The conclusions of these evaluations are briefly summarized in the paragraph below:

The increases in early resorptions and post-implantation loss at 175 mg/kg/day were relatively minor compared to control values, not statistically significant, and although slightly outside of the laboratory's mean ± 2 SD historical control data (HCD) range, the values were within the laboratory's observed range of HCD based on reported minimum and maximum values. Obvious and substantial maternal toxicity was also present at the mid and high dose levels. While the available data are highly suggestive of a relationship between maternal systemic toxicity and increased post-implantation loss, these findings could not be clearly linked with maternal toxicity on an individual animal basis. However, the diets of the rabbits were supplemented with hay and vegetables and no records of this additional food consumption are available, which complicates making associations between developmental outcomes and maternal toxicity on an individual animal basis. Additionally, the existing and extensive DART database for amphoacetates does not show other evidence of increased resorptions (early or late) or increased post-implantation loss as a result of exposure to Amphoacetates C8-C18 or other structurally related amphoacetates. In summary, after careful and detailed review and evaluation, it is the opinion of Exponent scientists with significant expertise in DART that the minor increases in resorptions and post-implantation loss observed in the mid-dose group at 175 mg/kg/day in the rabbit OECD 414 study do not warrant self-classification for adverse effects on development in accordance with GHS and EU CLP.

Attachments

Type other: Summary of Fetal Abnormalities by Finding
Type other: Relevant Historical Control Data

Applicant's summary and conclusion

Conclusions

In conclusion, based on the results of this prenatal developmental toxicity study in time-mated fema le New Zealand White rabbits the following maternal and developmental No Observed Adverse Effect Levels (NOAELs) for Amphoacetates C8-C18 were established:

Maternal NOAEL: 75 mg/kg bw/day (based on mortality at 175 and 350 mg/kg bw/day and cor relating clinical signs and lower body weight gain and food consumption in females surviving until s cheduled necropsy).
Developmental NOAEL: 75 mg/kg bw/day (based on the higher post-implantation loss at 175 mg/ kg bw/day).

Executive summary

The objectives of this study were to determine the potential of Amphoacetates C8-C18 to induce developmental toxicity after maternal exposure during the critical period of organogenesis and to characterize maternal toxicity at the exposure levels tested when given orally by gavage to time-mated female New Zealand White rabbits from Days 7 to 28 of gestation, inclusive. In addition, the No Observed Adverse Effect Levels (NOAELs) for maternal toxicity and developmental toxicity were evaluated.

The dose levels in this study were selected to be 0, 75, 175, 350 mg/kg bw/day, based on the results of the Dose Range Finder study (Test Facility Study No. 20346088).

Chemical analyses of formulations were conducted once during the study and confirmed that formulations of test material in Elix water were prepared accurately and homogenously.

The following parameters and end points were evaluated in this study for the F_0-generation: mortality/ moribundity, clinical signs, body weights, food consumption, macroscopic examination, and uterine contents (including corpora lutea, implantation sites, pre- and postimplantation loss and number of live and dead fetuses).

In addition, the following parameters were determined for the F_1-generation: fetal body weights, sex ratio, external, visceral and skeletal malformations and developmental variations.

In total, 13 females did not survive until scheduled necropsy. One female at 175 mg/kg bw/day and ten females at 350 mg/kg bw/day were prematurely euthanized based on clinical signs, body weight loss and/or prolonged (near) absent food consumption. The euthanasia of one female at 75 mg/kg bw/day considered to be an incidental finding and was unlikely to be related to treatment with the test material based on the absence of any maternal and developmental parameters in the remaining animals of the dose group. Furthermore, the euthanasia of the second female at 75 mg/kg bw/day was considered related to be related to the gavage procedure.

As in the high dose group (350 mg/kg bw/day) only 12 females with viable litters were available for evaluation, the available data was considered not sufficient for a robust and valid evaluation of the developmental data.

At 175 mg/kg bw/day, an adverse higher post-implantation loss, caused by higher percentages of early and late resorptions, was noted.

No test material-related changes were noted in any of the remaining maternal parameters investigated in this study (i.e., macroscopic evaluation, corpora lutea, uterine contents including implantation sites and pre-implantation loss).

No test material-related changes were noted in any of the remaining developmental parameters investigated up to 175 mg/kg bw/day (i.e., litter size, sex ratio, fetal body weights, external, visceral and skeletal malformations and developmental variations).

In conclusion, based on the results in this prenatal developmental toxicity study the following

maternal and developmental No Observed Adverse Effect Levels (NOAELs) for Amphoacetates C8-C18 were established:

Maternal NOAEL:	75 mg/kg bw/day (based on mortality observed at 175 and 350 mg/kg bw/day, and the effects noted on body weight and food consumption of females surviving until scheduled necropsy).
Developmental NOAEL:	75 mg/kg bw/day (based on the higher post-implantation loss noted at 175 mg/kg bw/day).

References

TEST_MATERIAL_INFORMATION: Alkylamphoacetates C8-C18 (Diacetate form)

UUID: 30fe3d1d-0b19-4c73-a833-6fc5dc023606
Dossier UUID:
Author: DLO
Date: 2025-02-21T10:58:58.508Z
Remarks:

Name
Alkylamphoacetates C8-C18 (Diacetate form)

Composition

Composition

Type
Constituent

Reference substance
Alkylamidoamine glycinate majority C12, 14 (amphoacetate) / Reaction products of 1H-Imidazole-1-ethanol, 4,5-dihydro-, 2-(C7-C17 odd-numbered, C17-unsatd. alkyl / 931-291-0

EC number	EC name
931-291-0	EC Inventory

CAS number	CAS name

IUPAC name

Reaction products of 1H-Imidazole-1-ethanol, 4,5-dihydro-, 2-(C7-C17 odd-numbered, C17-unsatd. alkyl) derivs. and sodium hydroxide and chloroacetic acid

Remarks
Aqueous solution

Other characteristics

Test material form
liquid: viscous

Details on test material
- Physical appearance: clear yellow liquid
- Storage conditions: At room temperature

LITERATURE: Prenatal Developmental Toxicity Study of Amphoacetates C8-C18 by Oral Gavage in Time-Mated New Zealand White Rabbits.

UUID: 9720788e-4a3b-42d2-8abc-a8c4d1308aae
Dossier UUID:
Author: DLO
Date: 2024-09-02T12:49:39.720Z
Remarks:

General information

Reference Type
study report

Title
Prenatal Developmental Toxicity Study of Amphoacetates C8-C18 by Oral Gavage in Time-Mated New Zealand White Rabbits.

Author
Charles River Laboratories Den Bosch

Year
2024

Testing facility
Charles River Laboratories Den Bosch BV, Hambakenwetering 7, 5231 DD ‘s-Hertogenbosch, The Netherlands

Report date
2024-06-04

Report number
20346091

Study sponsor
Verdant Specialty Solutions (on behalf of the Amphoacetates consortium). 811 Main Street, 18th Floor Houston, TX 77002. United States of America

Exponent®

Expert Opinion Regarding Impact of the Rabbit OECD 414 Study of Amphoacetates C8-C18 on Potential Need for Reproductive Toxicity Classification in Accordance with GHS and EU CLP

Exponent®

Expert Opinion Regarding Impact of the Rabbit OECD 414 Study of Amphoacetates C8-C18 on Potential Need for Reproductive Toxicity Classification in Accordance with GHS and EU CLP

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25 September 2024

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Executive Summary

An OECD 414 prenatal developmental toxicity study of Amphoacetates C8-C18 (EC 931-291-0) was recently conducted in the New Zealand White (NZW) rabbit via oral gavage at doses of 75, 175, and 350 mg/kg/day (CRL, 2024). Multiple females, particularly in the high dose group, did not survive until scheduled necropsy; this finding precludes use of data from the high dose group for making regulatory decisions regarding developmental toxicity. Increased resorptions and post-implantation loss were observed in the mid-dose group at 175 mg/kg/day. Exponent scientists were requested to assess whether the results of this study warrant classification and labelling of the tested substance in accordance with GHS and EU CLP Regulation (EC) No 1272/2008 for adverse effects on development. Exponent was also requested to determine whether any mechanistic studies may be recommended to further investigate the reported findings at 175 mg/kg/day.

Based on our review, Exponent scientists are of the opinion that the results from the rabbit OECD 414 study of Amphoacetates C8-C18 do not warrant self- classification and labelling in accordance with GHS and EU CLP for adverse effects on development. The increases in early resorptions and post-implantation loss at 175 mg/kg/day were relatively minor compared to control values, not statistically significant, and although slightly outside of the laboratory's mean ± 2 SD historical control data (HCD) range, the values were within the laboratory's observed range of HCD based on reported minimum and maximum values. Obvious and substantial maternal toxicity was also present at the mid-dose and may account for the increased early resorptions and post-implantation loss, although these findings could not be clearly linked with maternal toxicity on an individual animal basis. Moreover, the existing and extensive DART database for amphoacetates does not show other evidence of increased resorptions (early or late) or increased post-implantation loss as a result of exposure to Amphoacetates C8-C18 or other structurally related amphoacetates.

The client should be aware that, although Exponent is of the opinion that these findings do not warrant classification, in the rather conservative environment currently prevalent in Europe, it is possible that regulatory bodies may interpret the findings as being borderline Reproductive Toxicity Cat 2 (H361d). Should such a determination be made, conducting mechanistic studies to

further investigate this issue may not be helpful because the effect signal is so weak as to possibly not be related to treatment, and there is no information available from the rabbit OECD 414 studies of Amphoacetates C8-C18 or from the scientific literature to suggest a specific mechanism for these findings. Further, it is unlikely that running an additional rabbit OECD 414 study with Amphoacetates C8-C18 (or a new rabbit OECD 414 study of Amphoacetates C12 or C12-14) would negate the findings that have been already shown in rabbits. Thus, even if no adverse effect is shown in a new study, it is unlikely that this would change any interpretation of existing data for Amphoacetates C8-C18.

Purpose

An OECD 414 prenatal developmental toxicity study of Amphoacetates C8-C18 (EC 931-291-0) was recently conducted in the New Zealand White (NZW) rabbit via oral gavage at doses of 75, 175, and 350 mg/kg/day (CRL, 2024). Multiple females, particularly in the high dose group, did not survive until scheduled necropsy; this finding precludes use of data from the high dose group for making regulatory decisions regarding developmental toxicity (Annex I: 3.7.2.4.4 of the 2024 update to the CLP guidance; ECHA, 2024). Increased resorptions and post-implantation loss were observed in the mid dose group at 175 mg/kg/day.

Exponent scientists were previously requested to review the results of this study to assess whether the effects on pregnancy at the mid-dose group were likely secondary to maternal toxicity. The results of that evaluation have been reported in a technical report issued on 06 March 2024 (DeSesso and Lavin Williams, 2024). In that analysis, it was concluded that while the available data are highly suggestive of a relationship between maternal systemic toxicity and increased post-implantation loss at the mid-dose, an obvious relationship between the indicators of maternal systemic toxicity (i.e., clinical signs, reduced food consumption, and reduced body weight gains) and post-implantation loss could not be clearly shown on an individual animal basis. Exponent scientists have been subsequently requested to assess whether the results of this study warrant self-classification and labelling of the tested substance in accordance with GHS and EU CLP Regulation (EC) No 1272/2008 for adverse effects on development. If a Reproductive Toxicity Cat 2 (H361d) classification was determined to be appropriate, Exponent was also requested to address whether any mechanistic studies could be recommended to confirm whether a Reproductive Toxicity Cat 1B (H360d) classification is required.

For this assessment, Exponent relied upon the following study reports, which were provided by the client for review:

- Charles River Laboratories (CRL). 2024. Prenatal Developmental Toxicity Study of Amphoacetates C8-C18 by Oral Gavage in Time-Mated New Zealand White Rabbits. Charles River Laboratories Den Bosch BV. Study No. 20346091. Final report, amended.

- Charles River Laboratories (CRL). 2023a. Dose Range Finding Study of Amphoacetates C8-C18 by Oral Gavage in Pregnant New Zealand White Rabbits. Charles River Laboratories Den Bosch BV. Study No. 20346088. Final report.
- Charles River Laboratories (CRL). 2023b. Tolerability Study with Amphoacetates C8-C18 by Oral Gavage in Non-Pregnant New Zealand White Rabbits. Charles River Laboratories Den Bosch BV. Study No. 20346091. Final report.

Exponent scientists also relied on additional information requested from the laboratory regarding mating and dietary supplementation on the study; preliminary data available from an on-going OECD 443 EOGRT toxicity study; study reports previously provided to Exponent for the purpose of other analyses; information available in the published literature; and our expertise in developmental and reproductive toxicology (DART).

Rabbit OECD 414 study – Further evaluation for maternal toxicity

The rabbit OECD 414 study of Amphoacetates C8-C18 (CRL, 2024) was summarized in our previous technical report (DeSesso and Lavin Williams, 2024). Briefly, pregnant NZW rabbits (N=22/grp) were dosed by oral gavage with 0, 75, 175, or 350 mg/kg/day of Amphoacetates C8-C18 in water (dosing volume: 5 mL/kg)[1] on gestation days (GDs) 7 to 28; these doses were selected based on results of a dose range-finding (DRF) study (CRL, 2023a), which is discussed in a subsequent section of this report.

Mortality. In the definitive study, 10 rabbits at 350 mg/kg/day did not survive to the scheduled necropsy (Table 1); another 1 and 2 rabbits died in the mid- and low dose groups, respectively. Two of the deaths (one at the low dose and one at the high dose) were considered likely related to gavage error. Based on body weight losses and negligible food consumption during the 5 to 9 days preceding death, the remaining deaths were thought to be due to treatment.

As shown in Table 1, among the decedents, rabbits that lost the most body weight prior to death (shown in bold italics) also had the greatest numbers of resorptions, suggesting a relation to maternal toxicity.

[1] Although a dosing volume of 5 mL/kg was not well-tolerated in the first rat study of Amphoacetates C8-C18 (Pels Rijcken, 2018), resulting in the dosing volume being reduced to <2 mL/kg for subsequent studies in the rat, 5 mL/kg was found to be tolerated in rabbits in both the tolerability study (CRL, 2023b) as well as the DRF study (CRL, 2023a) conducted prior to the definitive rabbit OECD 414 study.

Table 1. Mortality and resorptions in the rabbit OECD 414 study of Amphoacetates C8-C18 (CRL, 2024).

Dose (mg/kg/day)	Animal #	Associated findings	# Resorptions
0	---	---	---
75	44	No food consumption for 7 days preceding; body weight loss (7%)	0
	23	Normal food consumption and body weight; gavage error	3
175	63	No food consumption for 7 days preceding; body weight loss (3%)	0
350	69	No food consumption for 5 days preceding; body weight loss (12%); not pregnant	---
	84	No food consumption for 7 days preceding; body weight loss (9%);	0
	70	No food consumption for 7 days preceding	3
	75	No food consumption for 7 days preceding; body weight loss (3%)	0
	80	No food consumption for 7 days preceding; no body weight gain	0
	83	No food consumption for 7 days preceding; body weight loss (3%)	2
	85	No food consumption for 7 days preceding; no body weight gain	1
	71	***No food consumption for various days preceding; body weight loss (12%)***	***11***
	81	No food consumption for 7 days preceding; normal body weight gain (4%); possible gavage error	1
	67	***No food consumption for 9 days preceding; body weight loss (12%)***	***9***

Body weight gain. Body weight gain (BWG) data from the rabbit OECD 414 study are shown in Table 2. BWG over the dosing period (GD 7-29) is calculated based on the body weight measured on GD 29 minus the body weight measured on GD 7. We have also presented these data as a percentage of the body weight of the animals on GD 7, the day dosing began. The adjusted BWG is the BWG over the dosing period (GD 7-29) minus the weight of the gravid uterus (which accounts for the weight of the litter).

Table 2. Body weight gain from the rabbit OECD 414 study of Amphoacetates C8-C18 (CRL, 2024).

Dose (mg/kg/day):	0	75	175	350
BWG – GD 7-29 (g)	326.4 22[c]	371.2 14%[b] 19[c]	186.0** -43%[b] 19[c]	255.0 -22%[b] 12[c]
GD 7-29 as % of GD 7 BW	9.3 22[c]	10.3 11%[b] 19[c]	4.9 -47%[b] 19[c]	7.2 -23%[b] 12[c]
Adjusted BWG (g)	-162.4 22[c]	-187.3 -15%[b] 19[c]	-329.6** -103%[b] 19[c]	-194.4 -20%[b] 12[c]
BWG – GD 7-9 (g)	31.0 ± 61.0[a] 22[c]	51.0 ± 44.3[a] 165%[b] 21[c]	31.4 ± 36.7[a] 101%[b] 20[c]	17.9 ± 44.9[a] 58%[b] 21[c]
BWG – GD 9-12 (g)	40.3 ± 64.5 22[c]	40.5 ± 41.1 100%[b] 21[c]	-7.9* ± 67.8 -120%[b] 20[c]	-21.5** ± 51.3 -153%[b] 21[c]
BWG – GD 12-15 (g)	112.5 ± 50.6 22[c]	57.4* ± 91.4 51%[b] 21[c]	29.3** ± 52.5 26%[b] 20[c]	47.5** ± 79.5 42%[b] 21[c]

* p≤0.05; ** p≤0.01.
[a] Mean ± SD.
[b] Percent of control.
[c] Number of rabbits.
BWG = body weight gain; GD = gestation day.

From the data in Table 2, it can be seen that rabbits in the mid-dose group at 175 mg/kg/day gained significantly less weight over the dosing period than did controls (43% less). When these data are presented as a percentage of the doe's body weight on GD 7 (initiation of dosing), this effect is slightly more pronounced. Moreover, when the weight of the resulting litters is considered in the adjusted BWGs, rabbits in the mid-dose group at 175 mg/kg/day showed a significant and substantially greater mean body weight loss compared to controls (103% difference from control). These data thus demonstrate substantial maternal toxicity at the mid-dose.

Reductions in body weight gains at or shortly after the initiation of dosing can be particularly informative regarding maternal toxicity. From the data in Table 2, it can be seen that, at 175 mg/kg/day, body weight gains in the GD 9-21 and GD 12-15 intervals were 120% less and 84% less than control, respectively.

In the above analysis, the effect on body weight gains at the high dose over the full dosing interval (GD 7-29) was not as pronounced as that of the mid-dose group. However, it is noted that the above data were calculated based only on those animals that survived to necropsy on GD 29. The most severely affected animals at 350 mg/kg/day died before term and as shown in Table 1, exhibited substantial body weight losses prior to death. Thus, the data in Table 2 should not be interpreted as showing the absence of a dose-response relationship.

Food consumption. Select food consumption data for the study are shown in Table 3.

Table 3. Mean food consumption (g/animal/day) in the rabbit OECD 414 study of Amphoacetates C8-C18 (CRL, 2024).

Dose (mg/kg/day):	0	75	175	350
GD 7-9	148.57 ± 26.46[a] 22[c]	159.60 ± 23.84 107%[b] 21[c]	157.50 ± 21.72 106%[b] 20[c]	127.52* ± 35.26 86%[b] 21[c]
GD 9-12	139.62 ± 23.04 22[c]	135.59 ± 38.18 97% [b] 21[c]	122.88 ± 44.55 88%[b] 20[c]	86.76** ± 41.23 62%[b] 21[c]
GD 12-15	104.52 ± 31.68 22[c]	77.10 ± 46.37 74%[b] 21[c]	65.65* ± 43.63 63%[b] 20[c]	53.68** ± 49.82 51%[b] 21[c]
GD 15-18	105.76 ± 41.63 22[c]	103.98 ± 50.62 98%[b] 21[c]	74.42 ± 41.70 70%[b] 20[c]	53.92** ± 59.16 51%[b] 21[c]
GD 18-21	113.20 ± 26.56 22[c]	115.58 ± 30.90 98%[b] 20[c]	99.61 ± 41.91 88%[b] 19[c]	91.64 ± 57.32 81%[b] 15[c]
GD 7-29	115.19± 19.39 22[c]	112.82 ± 18.73 98%[b] 19[c]	98.44* ± 19.17 85%[b] 19[c]	103.16 ± 26.77 90%[b] 12[c]

* p≤0.05; ** p≤0.01.
[a] Mean ± SD.
[b] Percent of control.
[c] Number of rabbits.
GD = gestational day; SD = standard deviation.

The data presented in Table 3 above, show that Amphoacetates C8-C18 caused a treatment-related reduction in mean food consumption. In the high dose group at 350 mg/kg/day, this effect was seen immediately upon the initiation of treatment on GD 7; mean food consumption was decreased relative to control through GD 21, by which time most of the premature deaths had occurred and, thus, data for the sickest rabbits were no longer included in the food consumption

calculations. Consequently, high-dose data in Table 3 should not be interpreted as showing the absence of a dose-response relationship. In the mid dose group at 175 mg/kg/day, decreased mean food consumption began slightly later (GD 9), but also continued through GD 21. Thus, significant reductions in food consumption are apparent at the mid-dose that coincide with the reduced body weight gains observed for this treatment group.

Resorptions and post-implantation loss. Mean resorptions and post-implantation loss data from the rabbit OECD 414 study of Amphoacetates C8-C18 are shown in Table 4. Note that, because of the substantial maternal mortality present at 350 mg/kg/day, which exceeded 10%, this dose should not be used for making regulatory decisions regarding developmental toxicity in accordance with GHS and EU CLP guidance for classification and labelling in Europe (ECHA, 2024). These data are nonetheless presented in Table 4 for the purposes of drawing conclusions regarding maternal toxicity.

Table 4. Mean resorptions and post-implantation loss data from the rabbit OECD 414 study of Amphoacetates C8-C18 (CRL, 2024).

Dose (mg/kg/day):	0	75	175	350 (survivors only)	350 (decedents only)	HCD[b]
# Females with live litters	22	19	19	12	9	
Percent early resorptions	3.41 ± 6.01[a]	2.60 ± 6.22[a]	8.10 ± 12.42[a]	10.45 ± 20.93[a]	15.47	3.95[c] ±2SD (0.59-7.32) Range: 1.72-9.35
Percent late resorptions	1.79 ± 3.95[a]	0.96 ± 4.17[a]	3.63 ± 5.95[a]	0.00 ± 0.00[a]	12.47	1.63[c] ±2SD (0.00-4.22) Range: 0.00-6.85
Percent post-implantation loss	5.20 ± 6.24[a]	3.56 ± 8.32[a]	11.74 ± 12.18[a]	11.21 ± 20.68[a]	27.93	5.83[c] ±2SD (0.77-10.89) Range: 2.60-14.47
Live litter size	8.5 ± 2.3[a]	10.4* ± 1.5[a]	9.2 ± 1.8[a]	8.1 ± 2.4[a]	---	9.35[c] ±2SD (8.1-10.59) Range: 8.38-10.67

[a] Mean ± SD.
[b] Historical control data reported based on 636 litters from NZW rabbit studies conducted Jan 2020 through Jul 2023.
[c] Historical control data reported as mean data (mean ± 2 SD) and the range of all reported values (minimum to maximum).
HCD = historical control data; SD = standard deviation.

None of the resorption or post-implantation values calculated based on surviving does (presented in the non-shaded cells) was found to be statistically significantly different from control. The lack of statistical significance appears to be related to the high variation in the data (as demonstrated

by the large standard deviation values), which makes interpretation rather challenging. The percentages of early resorptions and post implantation loss at the mid-dose were slightly outside of the ±2 standard deviations (SD) historical control data (HCD) range provided by the laboratory. However, the mid-dose values were within the laboratory's observed range of minimum and maximum control values (1.72-9.35 for early resorptions; 2.60-14.47 for post-implantation loss). Percentages of early and late resorptions and post implantation loss in the mid-dose group at 175 mg/kg/day were also within minimum/maximum HCD ranges for rabbits at other Charles River sites.[2] It is also noteworthy that there was no adverse effect of treatment on litter size and all reported values were within the laboratory's HCD range.

For the purposes of comparison, Exponent scientists calculated the percentages of early/late resorptions and post-implantation loss for the animals that died in the high dose group at 350 mg/kg/day (shown in the shaded cells in Table 4). These data demonstrate that early resorptions and post-implantation loss increased in the mid- and high dose groups in a dose-related manner. Additionally, the percentages of early resorptions and post-implantation loss were greater in the decedents in the high dose group at 350 mg/kg/day compared to the survivors. Moreover, in the high dose group at 350 mg/kg/day, late resorptions were observed only in those animals that died on study.

Conclusion. Overall, Exponent's independent expert review of the above summary data indicates that, in the definitive rabbit OECD 414 study of Amphoacetates C8-C18, substantial maternal toxicity was present both at the high dose of 350 mg/kg/day and at the mid-dose of 175 mg/kg/day. Maternal toxicity in the mid-dose group at 175 mg/kg/day was demonstrated primarily by the substantially reduced maternal body weight gain and adjusted maternal body weight loss, which coincided with reduced food intake. Maternal toxicity at 350 mg/kg/day was evidenced by high mortality preceded by absent food consumption and body weight loss. Despite these signs of obvious maternal toxicity in the mid- and high dose groups at ≥175 mg/kg/day, the percentages of early resorptions and post-implantation loss were minimally affected, not statistically significantly different from control, and within the bounds of the historical control range observed

[2] https://www.criver.com/products-services/safety-assessment/toxicology-services/developmental-and-reproductive-toxicology-dart/historical-control-data?region=3696

at the laboratory. Moreover, live litter size was unaffected by treatment. It should be additionally noted that no effect of treatment on fetal weights or the incidences of fetal external, visceral and skeletal anomalies was reported up to 175 mg/kg/day (*i.e.*, there is no indication that increases in early resorptions and post-implantation loss were the result of teratogenic effects). Thus, other than the minimal increases in (mainly early) resorptions and post-implantation loss in the mid-dose group at 175 mg/kg/day, no other indications of developmental toxicity were observed in this study.

In our previous report (DeSesso and Lavin Williams, 2024), the rabbit OECD 414 data for Amphoacetates C8-C18 were evaluated to assess whether a relationship between maternal systemic toxicity and post-implantation loss could be demonstrated on an individual animal basis. However, no clear associations could be shown between increased early (or late) resorptions and clinical signs of toxicity, maternal food consumption in early organogenesis, or body weight gains (either for the entire dosing period or for specific dosing intervals). For the purposes of this report, additional analyses were undertaken. Again, no clear relationships could be found on an individual animal basis between increased post-implantation loss (mostly, early resorptions) and indications of maternal systemic toxicity or other data, including adjusted body weight gain, body weight gain as a percentage of the animal's body weight at the initiation of dosing (GD 7), food efficiency, macroscopic findings, parentage, or mating partners (data not shown). Due to issues with reduced food intake, the rabbit diets were supplemented with hay and vegetables; however, individual animal records of how much was provided/eaten were not available, which confounds interpretation of these data.

Even if the available individual animal data cannot be used to conclude definitively that the observed increases in early resorptions and post-implantation loss at 175 mg/kg/day were secondary to maternal toxicity, a relationship with maternal toxicity cannot be discarded given the clear systemic toxicity seen at ≥175 mg/kg/day and manifested as mortality at 350 mg/kg/day. This conclusion is further supported by the fact that the percentages of early resorptions and post-implantation loss were substantially larger in the decedents in the high dose group at 350 mg/kg/day compared to other treatment groups and compared to high dose group survivors.

Other data for context

Rabbit dose range-finding (DRF) study

In the dose range-finding (DRF) study (CRL 2023a), Amphoacetates C8-C18 doses of 0, 250, 350, and 450 mg/kg/day were administered via oral gavage on GD 7-28 to groups of pregnant NZW rabbits (N=6/grp). Two females in the high dose group at 450 mg/kg/day aborted; one of these animals showed substantial body weight loss and negligible food consumption GD 7-18. Another rabbit in the high dose group at 450 mg/kg/day also exhibited minimal to no food consumption and substantial body weight loss over GD 7-20. These three animals were euthanized for humane reasons. Because the high dose group at 450 mg/kg/day was determined to be too high, the other three rabbits in this group were euthanized on GD 23. In the mid-dose group at 350 mg/kg/day, a single female was euthanized on GD 19 following 7 days of negligible food consumption and body weight loss of 5%; additionally, clinical signs of toxicity were seen in this dose group along with reduced food consumption and minimal body weight losses (2-4 g) on GD 7-9 and GD 18-21. In the low dose group at 250 mg/kg/day, a single female was euthanized on GD 21 after 7 days of negligible food consumption and body weight loss (6%); additionally, a mean body weight loss of 30 g was noted for the dose group on GD 15-18.

It is noteworthy that only 1 of 6 animals died in the mid-dose group at 350 mg/kg/day in the DRF study while 10 of 22 animals died in the definitive rabbit OECD 414 study at this same dose. The reason for the discrepancy across the two studies is not fully obvious as the two studies were conducted in the same laboratory using rabbits from the same breeder, test material from the same lot, the same vehicle, at the same volume, and for the same period of gestation. Therefore, in the absence of any other obvious reason, it is assumed that the difference in mortality across the two studies likely reflects the variation in individual animal sensitivities, which is more apparent in the definitive rabbit study with a larger group size. Nonetheless, all animals in the mid-dose group at 350 mg/kg/day in the DRF study showed clinical signs or died in the course of this study. Additionally, the mean percent of post-implantation loss was increased at this dose compared to control. However, this was in a study with a low number of animals per group, which impacts the

mean percentages; thus, the DRF data should not be considered directly equivalent to that reported in the definitive study.

In the low-dose group at 250 mg/kg/day, the percent post-implantation loss was not substantially different from control (7.22 ± 11.0 vs. 6.00 ± 13.42 in the control group). Further, the percent post-implantation loss at 250 mg/kg/day was within the laboratory's HCD range (mean ± 2SD = 0.77-10.89). Thus, the data available from the DRF study do not support an effect of Amphoacetates C8-C18 treatment on the percent resorptions or percent post-implantation loss at a dose of 175 mg/kg/day (*i.e.,* the mid-dose) in the definitive rabbit OECD 414 study.

Rat OECD 414 study

A rat OECD 414 study of Amphoacetates C8-C18 was conducted in Wistar rats (Bressers, 2019). This study was previously reviewed in detail in the Exponent technical review of the developmental and reproductive toxicity (DART) data available for amphoacetates (DeSesso and Lavin Williams, 2023). In this study, Amphoacetates C8-C18 doses of 0, 100, 300, and 1000 mg/kg/day (the limit dose) were administered via oral gavage on GD 6-20 to groups of Wistar Han rats (N=22/grp). No maternal toxicity was observed at all dose levels. Rates of resorptions (early and late), post-implantation loss, and live litter size were unaffected by treatment (Table 5). Additionally, no effects were observed on fetal body weights or the rates of fetal external, visceral, and skeletal anomalies.

Table 5. Mean resorptions and post-implantation loss data from the rat OECD 414 study of Amphoacetates C8-C18 (Bressers, 2019).

Dose (mg/kg/day):	0	100	300	1000	HCD[b]
# Females with live litters	22	22	21	22	
Percent early resorptions	2.7 ± 5.41[a]	3.2 ± 6.29[a]	4.1 ± 6.52[a]	4.5 ± 8.27[a]	5.0[c] 95% CI (1.9-9.9) Range: 1.5-11.6
Percent late resorptions	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.1[c] 95% CI (0.0-0.4) Range: 0.0-1.0
Percent post-implantation loss	2.7 ± 5.41[a]	3.2 ± 6.29[a]	4.1 ± 6.52[a]	4.5 ± 8.27[a]	5.1[c] ±2SD (1.9-10.1) Range: 1.5-11.6
Live litter size	10.4 ± 2.91[a]	10.4 ± 2.63[a]	10.7 ± 1.75[a]	10.3 ± 2.12[a]	10.7[c] 95% CI (9.6-11.7) Range: 9.1-11.7

[a] Mean ± SD.
[b] Historical control data reported based on 49 studies conducted 2014-2018.
[c] Historical control data reported as mean data (mean ± 2 SD) and the range of all reported values (minimum to maximum).
HCD = historical control data; SD = standard deviation.

OECD 422 study

The Exponent technical review of available amphoacetate DART data (DeSesso and Lavin Williams, 2023) also discussed a combined repeat dose/DART screening (OECD 422) study of Amphoacetates C8-C18 in rats (Pels Rijcken, 2018). In this study, Amphoacetates C8-C18 doses of 0, 100, 300, and 1000 mg/kg/day (the limit dose) were administered via oral gavage to Wistar Han rats (N=10/sex per grp). Dosing began 14 days prior to mating and continued through 4 weeks of exposure (males) or through gestation and an additional 13 days of lactation (females). In this study, one male and four females in the high-dose group at 1000 mg/kg/day died or were sacrificed *in extremis* with respiratory or other clinical signs noted prior to death. These deaths were considered due to regurgitation of the test material after gavage dosing. As a result, the volume of test material dosed in later rat studies was reduced, and other such deaths were not observed in subsequent studies. However, because of the large number of premature deaths at the high dose, the remaining females in the high-dose group at 1000 mg/kg/day were sacrificed on GD 14. Thus, no littering data are available for the high-dose group. At the remaining doses of 0, 100 and 300 mg/kg/day, the mean numbers of live pups at the first litter check on postnatal day

(PND) 1 were 12.1 ± 2.3, 10.9 ± 4.1, and 11.6 ± 2.4, respectively. Thus, because there is no significant decrease in mean live litter size, these data do not infer an increase in post-implantation loss in the rat as a result of treatment at doses of ≤300 mg/kg/day.

OECD 443 study

An extended one-generation reproductive toxicity (EOGRT; OECD 443) study of Amphoacetates C8-C18 is currently in progress (at the time of writing). Preliminary data from this study were shared with Exponent. Amphoacetates C8-C18 doses of 0, 100, 300, and 1000 mg/kg/day (the limit dose) were administered via oral gavage to groups of rats (Wistar Han; N=25/sex per grp). Dosing began 10 weeks premating, and for females, continued through gestation and lactation. Although salivation was noted for 16 males and 9 females in the high dose group, no other clinical signs were noted, and no treatment-related mortality was observed in the premating or gestation periods. For females, no toxicologically relevant changes in body weights/gains or food consumption were observed.

At littering, no effect of treatment was observed on the live litter size (Table 6). Although post-implantation loss/litter was higher in the high-dose group at 1000 mg/kg/day, this finding was considered to be primarily due to a single dam that had an exceptionally high post-implantation loss (13 implantation sites but only 5 pups). The rates of post-implantation loss for the other dams in the high-dose group at 1000 mg/kg/day were within normal limits. Pup weights at birth were unaffected by treatment and mating of the second generation was not triggered. Thus, these data do not support there being an effect of Amphoacetates C8-C18 treatment on post-implantation loss in the rat.

Table 6. Preliminary mean resorptions and post-implantation loss data from the rat OECD 443 study of Amphoacetates C8-C18.

Dose (mg/kg/day):	0	100	300	1000
# Females with live litters	24	24	22	20
Percent post-implantation loss	8.65 ± 9.80[a]	4.93 ± 8.24[a]	7.16 ± 8.45[a]	12.76 ± 16.45[a]
Live litter size	10.2 ± 3.4[a]	10.8 ± 3.4[a]	10.0 ± 3.5[a]	10.6 ± 2.8[a]
[a] Mean ± SD. SD = standard deviation.				

DART studies of other amphoacetates

As discussed in the previous Exponent technical review (DeSesso and Lavin Williams, 2023), DART data are available for other compositionally and structurally related amphoacetates. These compounds, which represent either mono- or diacetate forms of amphoacetate, predominantly differ from each other in terms of their carbon (C) chain lengths (Table 7).

Table 7. Amphoacetates for which DART data are available.

	C8-C18	C12-C14	C12
Monoacetates:	OECD 422		OECD 414 (Rat)
Diacetates:	OECD 422 OECD 414 (Rat) OECD 414 (Rabbit) OECD 443	OECD 414 (Rat)	

The existing DART studies for the other amphoacetates include a combined repeat dose/DART screening (OECD 422) study of Amphoacetates C8-C18 Monoacetate form (contains appr. 95% monoacetates and 5% diacetates) (De Raat-Beekhuijzen, 2018); and a DRF and definitive rat OECD 414 study of Amphoacetates C12-C14 Diacetate form (contains appr. 40% to 45% monoacetate and 55% to 60% diacetates) (Vriends, 2022a, 2022b); and DRF and definitive rat OECD 414 studies of Amphoacetates C12 Monoacetate form (contains appr. 75% to 100% monoacetate and 0% to 25% diacetates) (Langedijk, 2022; van Otterdijk, 2022). The data available from these studies related to resorptions and post-implantation loss are shown in Table 8. No other rabbit DART data are available for amphoacetates.

From the data in Table 8, it can be seen that, in the rat 414 studies (both DRF and definitive) at doses up to the limit dose of 1000 mg/kg/day, treatment with amphoacetates had no effect on the rates of resorptions (early and late) and percentages of post-implantation loss. These data are consistent with those of the rat 414 study of Amphoacetates C8-C18. Thus, the DART data available for the other amphoacetates do not indicate a class effect of amphoacetates on resorptions or post-implantation loss.

Table 8. Select data from the DART studies of other amphoacetates.

Amphoacetates C8-C18 Monoacetate form (contains appr. 95% monoacetates and 5% diacetates)					
OECD 422	**Doses (mg/kg/day) (N)**	**0 (10)**	**100 (10)**	**300 (10)**	**1000 (10)**
	Mean # implantations	13.6 ± 1.8[a]	13.5 ± 2.1[a]	11.1 ± 3.9[a]	13.1 ± 2.9[a]
	Mean # live pups at 1st litter check	13.3± 2.0[a]	11.4 ± 1.6[a]	11.5 ± 1.1[a]	11.0 ± 2.6[a]
Amphoacetates C12-C14 Diacetate form (contains appr. 40% to 45% monoacetates and 55% to 60% diacetates)					
DRF OECD 414 (rat)	**Doses (mg/kg/day) (N)**	**0 (6)**	**100 (5)**	**300 (4)**	**1000 (6)**
	# early resorptions per litter	0.5 ± 0.5[a]	1.2 ± 2.2[a]	0.3 ± 0.5[a]	0.2 ± 0.4[a]
	# late resorptions per litter	0.0 ± 0.00[a]	0.0 ± 0.00[a]	0.0 ± 0.00[a]	0.0 ± 0.00[a]
	Percent post-implantation loss	4.23 ± 4.71[a]	8.81 ± 15.47[a]	1.92 ± 3.85[a]	1.28 ± 3.14[a]
OECD 414 (rat)	**Doses (mg/kg/day) (N)**	**0 (21)**	**100 (22)**	**300 (20)**	**1000 (22)**
	# early resorptions per litter	0.6 ± 0.9[a]	0.7 ± 0.9[a]	0.8 ± 1.0[a]	0.5 ± 0.7[a]
	# late resorptions per litter	0.0 ± 0.00[a]	0.0 ± 0.00[a]	0.0 ± 0.00[a]	0.0 ± 0.00[a]
	Percent post-implantation loss	4.64 ± 7.23[a]	6.03 ± 7.80[a]	6.12 ± 7.96[a]	3.66 ± 5.83[a]
Amphoacetates C12 Monoacetate form (contains appr. 75% to 100% monoacetate and 0% to 25% diacetates)					
DRF OECD 414 (rat)	**Doses (mg/kg/day) (N)**	**0 (5)**	**300 (6)**	**600 (5)**	**1000 (6)**
	# early resorptions per litter	0.7 ± 0.8[a]	1.3 ± 1.4[a]	0.4 ± 0.5[a]	0.3 ± 0.5[a]
	# late resorptions per litter	0.0 ± 0.00[a]	0.0 ± 0.00[a]	0.0 ± 0.00[a]	0.2 ± 0.4[a]
	Percent post-implantation loss	20.40 ± 39.40[a]	10.96 ± 11.61[a]	3.33 ± 4.56[a]	3.51 ± 3.88[a]
OECD 414 (rat)	**Doses (mg/kg/day) (N)**	**0 (21)**	**100 (21)**	**300 (20)**	**1000 (22)**
	# early resorptions per litter	0.3 ± 0.7[a]	0.4 ± 0.8[a]	0.4 ± 0.5[a]	0.4 ± 1.1[a]
	# late resorptions per litter	0.0 ± 0.00[a]	0.0 ± 0.00[a]	0.0 ± 0.00[a]	0.0 ± 0.00[a]
	Percent post-implantation loss	2.46 ± 4.75[a]	3.73 ± 8.24[a]	3.29 ± 4.15[a]	3.39 ± 9.85[a]

[a] Mean ± SD.
DRF = dose range-finding

Data analysis and conclusions

After thorough review of the available DART data regarding resorptions and post-implantation loss, Exponent scientists are of the opinion that the results from the rabbit OECD 414 study of Amphoacetates C8-C18 do not warrant self-classification for adverse effects on development in accordance with GHS and EU CLP Regulation (EC) No 1272/2008 . The percentages of early resorptions and post-implantation loss in the mid-dose group at 175 mg/kg/day were not statistically significantly different from control and were within the laboratory's observed range of HCD based on reported minimum and maximum values. Moreover, substantial maternal toxicity was present at the mid-dose of 175 mg/kg/day. This was particularly evident based on mean body weight gains observed in the GD 9-12 and GD 12-15 intervals, which at the mid-dose were 120% less and 84% less than control, respectively. Additionally, mean adjusted body weight loss over the entire dosing period (GD 7-29) was 103% more than that of the control group (losses of 162.4 grams in the control group and 329.6 grams in the mid-dose group). Working groups on maternal toxicity and the use of rabbits in DART studies have recommended that doses causing marked toxicity leading to mortality and/or decreased body weight gains of >20% for prolonged periods be avoided in prenatal developmental toxicity studies (Moxon et al., 2023; Beyer et al., 2011). Certainly, in the rabbit OECD 414 study of Amphoacetates C8-C18, the body weight losses observed at the mid-dose substantially exceeded this proposed limit.

After multiple evaluations of the evidence from the rabbit OECD 414 study of Amphoacetates C8-C18, a direct correlation between the post-implantation loss data with maternal toxicity indicators could not be shown on an individual animal basis. However, rabbits are known to exhibit substantial inter-animal variability, which confounds data interpretation (Moxon et al., 2023). For example, within a single study, rabbit body weights can vary by as much as 1000-1500 grams. Rabbits tend to gain little weight (or often lose weight) during gestation; they also frequently reduce their food intake just prior to giving birth. This phenomenon was evidenced in the rabbit OECD 414 study of Amphoacetates C8-C18 by the negative adjusted body weight gains at termination in all dose groups, including control. Rabbits additionally exhibit highly variable food intake from one day to the next. To reduce this variability, restricting the amount of food available on a daily basis has been suggested (Moxon et al., 2023). To address the problem

of reduced feed intake in the rabbit OCED 414 study of Amphoacetates C8-C18, the diets were supplemented with hay and vegetables; however, individual animal records of the amounts of these materials provided/consumed were not available, which further confounds interpretation of the data from this study. Overall, the preceding issues complicate making associations between developmental outcomes and maternal toxicity on an individual animal basis.

The existing DART database for amphoacetates is relatively rich, as there are multiple DART studies available for Amphoacetates C8-C18 and compositionally and structurally related amphoacetates. No consistent findings of increased resorptions and post-implantation loss were observed across these studies. In the DRF rabbit OECD 414 study of Amphoacetates C8-C18, increased early resorptions and post-implantation loss were not observed at 250 mg/kg/day – a dose which is notably higher than the mid-dose of 175 mg/kg/day used in the definitive study. While increases were observed at 350 mg/kg/day, this is the same dose at which severe maternal toxicity occurred in the definitive study that precluded the use of data from that group for making regulatory decisions. Other developmental toxicity data in rabbits are not available in the current database. Increased resorptions and post-implantation loss were not seen in any of the rat OECD 414 studies (DRF or definitive) of Amphoacetates C8-C18, Amphoacetates C12-14, and Amphoacetates C12, despite the fact that all of these studies were conducted up to the limit dose of 1000 mg/kg/day. While a slightly elevated post-implantation loss was reported at 1000 mg/kg/day in the OCED 443 study of Amphoacetates C8-C18, this was due primarily to a single dam with an exceptionally high post-implantation loss (8/13 implantation sites) and this single instance cannot be considered related to treatment. In the OECD 422 study of Amphoacetates C8-C18 Monoacetate form, mean litter sizes in the treated groups were all near the HCD mean, while the control group litter size was considered high. Finally, in the OECD 422 study of Amphoacetates C8-C18, increases in post-implantation loss were not seen at doses of ≤1000 mg/kg/day. Thus, the available DART data for amphoacetates does not provide additional evidence for classification in accordance with GHS and EU CLP.

In summary, after careful and detailed review and evaluation, it is the opinion of Exponent scientists with significant expertise in DART that the minor increases in resorptions and post-implantation loss observed in the mid-dose group at 175 mg/kg/day in the rabbit OECD 414 study

do not warrant self-classification for adverse effects on development in accordance with GHS and EU CLP. Conducting mechanistic studies to further investigate the potential cause of these findings are unlikely to be informative because the increases in early resorptions and post-implantation loss are slight and possibly not related to treatment. Moreover, there is no information available from the rabbit OECD 414 studies of Amphoacetates C8-C18 or from the scientific literature that points to a specific mechanism for further investigation. Repeating the rabbit OECD 414 study (or conducting a rabbit OECD 414 study on Amphoacetates C12 or C12-14) is also not recommended. Even if a new study were completely definitive of no developmental effects, it would not erase the findings that have been already reported. Thus, unless further information comes to light to suggest a potential mechanism of action, we advise against any mechanistic studies.

Limitations

The purpose of this analysis is limited to a review of the results from the OECD 414 rabbit study of Amphoacetates C8-C18. This assessment is based on review of the study data, and the authors' combined expertise in developmental and reproductive toxicology (DART). The opinions presented herein are made to a reasonable degree of scientific certainty. Exponent reserves the right to supplement this report and to expand or modify the conclusions and findings based on the review of additional materials as they become available through additional work, or through the review of additional work performed by others. The scope of services performed during this investigation may not adequately address the needs of other users of this report, and any re-use of this report or its findings, conclusions or recommendations as presented herein are at the sole risk of the user.

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Review of Results from the OECD 414 Study in Rabbits to Assess Whether the Increase in Post-Implantation Loss at the Mid-Dose is Secondary to Maternal Toxicity

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Review of Results from the OECD 414 Study in Rabbits to Assess Whether the Increase in Post-Implantation Loss at the Mid-Dose is Secondary to Maternal Toxicity

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Executive Summary

An OECD 414 prenatal developmental toxicity study of Dehyton DC (C8-18 amphoacetates) was recently conducted in the New Zealand White (NZW) rabbit via oral gavage at doses of 75, 175 and 375 mg/kg/day. Multiple females, particularly in the high dose group, did not survive until scheduled necropsy. One female at 75 mg/kg/day, one female at 175 mg/kg/day, and ten females at 350 mg/kg bw/day were prematurely euthanized based on clinical signs, body weight loss, and/or prolonged (near) absent food consumption; another death at 75 mg/kg/day was due to gavage error only. Increased resorptions and post-implantation loss were observed at 175 mg/kg/day. Exponent scientists were requested to review the results of this study to assess whether the effects on pregnancy in the mid-dose group occurred secondary to maternal systemic toxicity.

Based on evaluation of mean food consumption and body weight gains data, maternal toxicity appeared to be evident at the mid-dose. However, further analysis shows that, while the available data are highly suggestive of a relationship between maternal systemic toxicity and increased post-implantation loss at the mid-dose, the data fail to demonstrate an obvious relationship between indicators of maternal systemic toxicity (i.e., clinical signs, reduced food consumption, and reduced body weight gains) and post-implantation loss on an individual animal basis. Thus, the available data cannot be used to conclude definitively that the observed increases in early resorptions and post-implantation loss at 175 mg/kg/day are secondary to maternal toxicity.

Purpose

An OECD 414 prenatal developmental toxicity study of Dehyton DC (C8-18 amphoacetates) was recently conducted in the New Zealand White (NZW) rabbit via oral gavage at doses of 75, 175 and 375 mg/kg/day. Multiple females, particularly in the high dose group, did not survive until scheduled necropsy. One female at 75 mg/kg/day, one female at 175 mg/kg/day, and ten females at 350 mg/kg bw/day were prematurely euthanized based on clinical signs, body weight loss, and/or prolonged (near) absent food consumption; another death at 75 mg/kg/day was due to gavage error only. Increased resorptions and post-implantation loss were observed at 175 mg/kg/day. Exponent scientists were requested to review the results of the study to assess whether the effects on pregnancy at the mid-dose group occurred secondary to maternal toxicity.

For this assessment, Exponent relied upon the draft study report provided for review as follows:

- Charles River Laboratories. 2023. Prenatal Developmental Toxicity Study of Amphoacetates C8-C18 by Oral Gavage in Time-Mated New Zealand White Rabbits. Charles River Laboratories Den Bosch BV. Study No. 20346091. Draft report.

Exponent scientists also relied on additional information as available in the published literature and their own expertise in developmental and reproductive toxicology.

Below, the results of the OECD 414 rabbit study of Dehyton DC are briefly summarized, after which our analysis of these data are described in detail.

Study summary

Dehyton DC was administered to NZW rabbits (n=22/grp) in water (dosing volume of 5 mL/kg) via oral gavage on gestation days (GDs) 7 to 28. The doses of 0, 75, 175, and 350 mg/kg/day were selected based on results of a dose range-finding (DRF) study in which Dehyton DC doses of 0, 250, 350, and 450 mg/kg/day were administered via oral gavage on GD 7-28 to groups of NZW rabbits (n=6/grp). In the DRF study, no females survived to study termination at 450 mg/kg/day. At 350 mg/kg/day, a single female was euthanized on GD 19 following 7 days of negligible food consumption and body weight loss of 5%; additionally, clinical signs of toxicity were seen in this dose group along with reduced food consumption (14%) and minimal body weight loss (2-4 g) on GD 7-9 and GD 18-21. At 250 mg/kg/day, a single female was euthanized on GD 21 after 7 days of negligible food consumption and body weight loss (6%); additionally, body weight loss of 30 g was noted for the dose group on GD 15-18.

In the main study, a total of 13 rabbits did not survive until the scheduled necropsy, as shown in Table 1 below. Two deaths were considered likely related to gavage error. Based on negligible food consumption and body weight gains preceding euthanization, the other deaths appear to be due to treatment-related toxicity.

Table 1. Mortality on the OECD 414 rabbit study of Dehyton DC.

Dose (mg/kg/day)	GD	Animal #	Associated findings	# Fetuses present	# Resorptions present
0	---	---	---	---	---
75	20	#44	No food consumption for 7 days preceding; body weight loss (7%)	11	0
	21	#23	Normal food consumption and body weight; gavage error	9	3
175	20	#63	No food consumption for 7 days preceding; body weight loss (3%)	9	0
350	18	#69	No food consumption for 5 days preceding; body weight loss (12%); not pregnant	---	---
		#84	No food consumption for 7 days preceding; body weight loss (9%);	11	0
	19	#70	No food consumption for 7 days preceding	8	3 (L)
		#75	No food consumption for 7 days preceding; body weight loss (3%)	9	0

Dose (mg/kg/day)	GD	Animal #	Associated findings	# Fetuses present	# Resorptions present
	20	#80	No food consumption for 7 days preceding; no body weight gain	10	0
		#83	No food consumption for 7 days preceding; body weight loss (3%)	9	2 (E)
		#85	No food consumption for 7 days preceding; no body weight gain	13	1 (L)
	21	#71	No food consumption for various days preceding; body weight loss (12%)	1	11 (E)
		#81	No food consumption for 7 days preceding; normal body weight gain (4%); possible gavage error	13	1 (E)
	23	#67	No food consumption for 9 days preceding; body weight loss (12%)	0	2 (E) 7 (L)
E = early resorption; GD = gestational day; L = late resorption					

The laboratory concluded that, among the animals that survived until study termination, clinical signs observed at doses of ≤175 mg/kg/day were not due to treatment. At 350 mg/kg/day, erect fur, which was noted in 8 of 12 rabbits on multiple days, was considered treatment related.

Food consumption data for this study are shown in Table 2. From these data, the following can be concluded:

- Treatment with Dehyton DC caused a treatment-related reduction in mean food consumption.
- Effects were seen immediately in the high dose group with the initiation of treatment on GD 7 and continued through GD 21, by which time most of the rabbits that died in this treatment group were no longer included in the food consumption calculations.
- The mid-dose group showed effects on mean food consumption beginning GD 12-15; these effects also continued through GD 21.
- The low dose group showed a transient (albeit not statistically significant) 26% reduction in mean food consumption GD 12-15.
- In general, food consumption was much more variable in the treated groups compared to control; it is likely that this variability is related to differing sensitivities of the rabbits to the effects of Dehyton DC.

Table 2. Mean food consumption (g/animal/day) in the OECD 414 rabbit study of Dehyton DC.

Dose (mg/kg/day):	0	75	175	350
GD 7-9	147.57 ± 26.46[a] 22[c]	159.60 ± 23.84 107%[b] 21	157.50 ± 21.72 106% 20	127.52* ± 35.26 86% 21
GD 9-12	139.62 ± 23.04 22	135.59 ± 38.18 97% 21	122.88 ± 44.55 88% 20	86.76** ± 41.23 62% 21
GD 12-15	104.52 ± 31.68 22	77.10 ± 46.37 74% 21	65.65* ± 43.63 63% 20	53.68** ± 49.82 51% 21
GD 15-18	105.76 ± 41.63 22	103.98 ± 50.62 98% 21	74.42 ± 41.70 70% 20	53.92** ± 59.16 51% 21
GD 18-21	113.20 ± 26.56 22	115.58 ± 30.90 98% 20	99.61 ± 41.91 88% 19	91.64 ± 57.32 81% 15
GD 21-24	97.95 ± 24.08 22	121.98* ± 28.14 125% 19	98.72 ± 29.83 99% 19	122.97 ± 40.34 126% 12
GD 24-27	106.64 ± 30.83 22	97.35 ± 30.98 91% 19	85.72 ± 39.24 80% 19	109.03 ± 38.45 98% 12
GD 27-29	117.02 ± 35.47 22	115.45 ± 25.15 99% 19	92.21 ± 44.67 79% 19	102.71 ± 42.94 88% 12

* p≤0.05; ** p≤0.01.
[a] Mean ± SD.
[b] Percent of control.
[c] Number of rabbits.
GD = gestational day; SD = standard deviation.

Among those rabbits that survived to the end of gestation, body weights were not significantly different on GD 29 (Table 3). However, body weight gains were significantly affected in all dose groups towards the beginning of gestation (Table 4). This response is most likely secondary to the reduction in food consumption that was observed with treatment. As the study progressed, body weight gains became more similar across all dose groups, including control (data not shown); it is not clear, however, whether this is because the rabbits on study adapted to treatment and/or if it is because the female rabbits that did not eat had been euthanized or died on study and, thus, no longer included in the calculations for body weight gains.

Table 3. Mean body weight (g) at the end of gestation in the OECD 414 rabbit study of Dehyton DC.

Dose (mg/kg/day):	0	75	175	350
GD 29	3835.1 ± 304.4[a] 22[c]	3982.3 ± 315.2 104%[b] 19	3986.3 ± 351.2 104% 19	3780.8 ± 375.9 99% 12

No statistical differences from control.
[a] Mean ± SD.
[b] Percent of control.
[c] Number of rabbits.
GD = gestational day; SD = standard deviation.

Table 4. Body weight gains (g) from GD 7 to GD 21 in the OECD 414 rabbit study of Dehyton DC.

Dose (mg/kg/day):	0	75	175	350
GD 7-9	31.0 ± 61.0[a] 22[c]	51.0 ± 44.3 165%[b] 21	31.4 ± 36.7 101% 20	17.9 ± 44.9 58% 21
GD 9-12	40.3 ± 64.5 22	40.5 ± 41.1 100% 21	-7.9* ± 67.8 -120% 20	-21.5** ± 51.3 -153% 21
GD 12-15	112.5 ± 50.6 22	57.4* ± 91.4 51% 21	29.3** ± 52.5 26% 20	47.5** ± 79.5 42% 21
GD 15-18	-18.6 ± 59.7 22	-0.1 ± 70.7 [d] 21	13.9 ± 89.2 20	-14.2 ± 94.3 21
GD 18-21	21.9 ± 37.7 22	23.5 ± 55.8 107% 20	-1.3 ± 60.9 -106% 19	-18.9 ± 95.7 -186% 15

* p≤0.05; ** p≤0.01.
[a] Mean ± SD.
[b] Percent of control.
[c] Number of rabbits.
[d] Percent of control values not calculated based on negative body weight gain in control group.
GD = gestational day; SD = standard deviation.

Pregnancy and litter data are shown in Table 5. The number of pregnant females that survived to study termination in the high dose group (n=12) was below the minimum required according to the OECD No. 414 study test guideline in order to provide a robust statistical evaluation (n=16). Nevertheless, for the purposes of this evaluation, the data from the high dose group are also considered.

The mean numbers of implantations and live/total fetuses per litter were significantly increased at the low dose compared to control. However, these differences were not considered adverse, nor treatment related. Although not statistically different from control, the percentages of post-implantation loss and early resorptions in the mid- and high dose groups were above the laboratory's historical control data (HCD) range. The percentage of late resorptions was greater than that in the control group but still within the laboratory's historical control range. Nonetheless, the mean number of fetuses per litter at these doses were similar to control. No effect of treatment was observed on the fetal sex ratio or mean fetal weights.

Table 5. Mean pregnancy and litter data from the OECD 414 rabbit study of Dehyton DC.

Dose (mg/kg/day):	0	75	175	350	HCD[b]
# Females on study	22	22	22	22	
# Pregnant females	22	21	20	21	
# Females with live fetuses	22	21	20	20	
# Females with live fetuses on GD 29	22	19	19	12	
Number corpora lutea	10.9 ± 2.4[a]	11.4 ± 1.7	11.3 ± 2.1	10.4 ± 1.4	
Number implantations	9.0 ± 2.4	10.8* ± 1.5	10.5 ± 2.2	9.2 ± 1.6	
Percent preimplantation loss	15.07 ± 19.15	4.27 ± 6.29	7.22 ± 10.00	10.63 ± 18.11	
Number of live fetuses per litter	8.5 ± 2.3	10.4* ± 1.5	9.2 ± 1.8	8.1 ± 2.4	
Percent early resorptions	3.41 ± 6.01	2.60 ± 6.22	8.10 ± 12.42	10.45 ± 20.93	3.95[c] (0.59-7.32)
Percent late resorptions	1.79 ± 3.95	0.96 ± 4.17	3.63 ± 5.95	0.00 ± 0.00	1.63 (0.00-4.22)
Percent post-implantation loss	5.20 ± 6.24	3.56 ± 8.32	11.74 ± 12.18	11.21 ± 20.68	5.83 (0.77-10.89)
Percent male fetuses per litter	50.80 ± 20.28	45.29 ± 11.96	53.05 ± 18.98	38.63 ± 17.00	
Mean male fetal weight (g)	41.55 ± 5.61	39.20 ± 4.48	39.85 ± 3.40	38.29 ± 4.63	
Mean female fetal weight (g)	39.83 ± 5.23	38.26 ± 4.43	38.70 ± 4.86	38.61 ± 4.97	

* p≤0.05.
[a] Mean ± SD.
[b] Historical control data reported based on 636 litters from NZW rabbit studies conducted Jan 2020 through Jul 2023.
[c] Historical control data reported as mean data (mean ± 2 SD).
GD = gestational day; HCD = historical control data; NZW = New Zealand White; SD = standard deviation.

Malformation data from the fetal examinations are shown in Table 6. As can be seen from these data, there were no treatment-related increases in fetal malformations. Similarly, no treatment-related increases in fetal variations were observed (data not shown).

Table 6. Malformations observed in the OECD 414 rabbit study of Dehyton DC.

Dose (mg/kg/day):		0	75	175	350
# Fetuses (litters) examined:		188 (22)	198 (19)	174 (19)	97 (12)
External examination					
Trunk, omphalocele	**N**	---	1 (1)[a]	---	---
	%	---	0.53 (5.3)	---	---
TOTAL FETUSES WITH EXTERNAL MALFORMATIONS	**N**	---	1 (1)	---	---
	%	---	0.53	---	---
Visceral examination					
Adrenal gland, malpositioned	**N**	1 (1)	---	---	---
	%	0.51 (4.5)	---	---	---
Aorta, overriding aorta	**N**	1 (1)	---	---	---
	%	0.51 (4.5)	---	---	---
Diaphragm, hernia	**N**	---	1 (1)	---	---
	%	---	0.66 (5.3)	---	---
Abdomen, situs inversus	**N**	1 (1)	---	---	---
	%	0.51 (4.5)	---	---	---
Great vessels, Tetralogy of Fallot	**N**	---	---	1 (1)	---
	%	---	---	0.40 (5.3)	---
Ventricular septum, absent	**N**	1 (1)	---	---	---
	%	0.51 (4.5)	---	---	---
Spleen, misshapen	**N**	---	1 (1)	---	---
	%	---	0.66 (5.3)	---	---
TOTAL FETUSES WITH VISCERAL MALFORMATIONS	**N**	1 (1)	1 (1)	1 (1)	---
	%	0.51	0.66	0.40	---
Skeletal examination					
Rib, fused	**N**	1 (1)	---	---	---
	%	0.51 (4.5)	---	---	---
Sternebra, sternoschisis	**N**	---	1 (1)	---	---
	%	---	0.53 (5.3)	---	---
Caudal vertebra, fused	N	---	---	---	1 (1)
	%	---	---	---	0.76 (8.3)
Lumbar vertebra, hemivertebra	**N**	---	1 (1)	---	---
	%	---	0.40 (5.3)	---	---
Thoracic arch, fused	**N**	1 (1)	---	---	---
	%	0.51 (4.5)	---	---	---
Thoracic centrum, absent	**N**	1 (1)	---	---	---
	%	0.51 (4.5)	---	---	---
TOTAL FETUSES WITH SKELETAL MALFORMATIONS	**N**	1 (1)	2 (2)	---	1 (1)
	%	0.51	0.93	---	0.76

* $p \leq 0.05$; ** $p \leq 0.01$.
[a] # affected fetuses (# affected litters).
[b] Litter percent affected fetuses (% affected litters).

Data analysis

The question to be addressed is whether the effects on pregnancy observed at the mid-dose (i.e., increased early resorptions and increased post-implantation loss) occurred secondary to maternal toxicity.

At the mid-dose, the single pregnant female that died had 9 fetuses and no resorptions (0.0%). However, among the pregnant does that died at 350 mg/kg/day, the average live litter size was 8.22 and the average percentage of resorptions was 27.9%. When broken down according to early versus late resorptions, at 350 mg/kg/day, the percentage of early resorptions was 15.5% and the percentage of late resorptions was 12.5%. These rates of resorption are above those of observed in the animals that survived at the high dose (Table 7). Thus, those animals at the high dose that died as a result of treatment also showed greater rates of resorption than those animals that survived treatment. These data suggest that the increased resorptions observed in the study could be related to maternal toxicity.

Table 7. Mean early and late resorptions, including data from decedents.

Dose (mg/kg/day):	0	75	175 (survivors only)	350 (survivors only)	350 (decedents only)
Number of fetuses per litter	8.5	10.4	9.2	8.2	11.2
Number of live fetuses per litter	8.5	10.4	9.2	8.1	8.2
Percent early resorptions	3.41	2.60	8.10	10.45	15.47
Percent late resorptions	1.79	0.96	3.63	0.00	12.47
Percent post-implantation loss	5.20	3.56	11.74	11.21	27.93

Instances of resorptions in the control group, when they occurred, typically involved single fetuses per litter. Ten control females showed resorptions; with one exception (a female that had two resorptions), these all involved single fetuses that were resorbed (data not shown). Further, seven of the resorptions were early; four were late.

The data that contributed to post-implantation loss at the mid-dose are shown in the Table 8. In this case, 13 pregnant females experienced resorptions. These included 9 with early resorptions and 6 with late resorptions (2 pregnant females had both early and late resorptions). Further, as highlighted in yellow, the pregnant females that had more than two total resorptions at the mid-dose were #45, #46, #62, and #65.

Table 8. Resorptions and post-implantation loss at 175 mg/kg/day.

Female	Total # fetuses	# early resorptions	% early resorptions	# late resorptions	% late resorptions	Total resorptions	% post-impl loss
45	13	3	18.8	0	0.0	3	18.8
46	10	1	7.7	2	15.4	3	23.1
47	8	0	0.0	0	0.0	0	0.0
48	9	1	10.0	0	0.0	1	10.0
49	8	0	0.0	1	11.1	1	11.1
50	7	0	0.0	0	0.0	0	0.0
51	10	0	0.0	0	0.0	0	0.0
52	8	0	0.0	0	0.0	0	0.0
53	10	0	0.0	0	0.0	0	0.0
54	10	1	8.3	1	8.3	2	16.7
55	11	0	0.0	1	8.3	1	8.3
56	9	0	0.0	2	18.2	2	18.2
57[a]	---	---	---	---	---	---	---
58	9	1	10.0	0	0.0	1	10.0
59[a]	---	---	---	---	---	---	---
60	12	0	0.0	1	7.7	1	7.7
61	10	0	0.0	0	0.0	0	0.0
62	5	3	37.5	0	0.0	3	37.5
63[b]	9	0	0.0	0	0.0	0	0.0
64	9	1	10.0	0	0.0	1	10.0
65	7	5	41.7	0	0.0	5	41.7
66	9	1	10.0	0	0.0	1	10.0

[a]This female was not pregnant.
[b]This female died on study.
Yellow highlighting indicates females that had >2 resorptions.

To assess whether the increased early resorption rate and post-implantation loss observed at the mid-dose were secondary to maternal toxicity, the available data are evaluated below in relation to the clinical signs, food consumption, and body weight gains observed at the mid-dose. Particular attention is given to the four females at the mid-dose that experienced >2 resorptions as well as the female that died at this dose.

Clinical signs

Limited clinical signs were observed at 175 mg/kg/day. The clinical signs observed in those animals at the mid-dose that exhibited more than two resorptions are shown in Table 9. It should be noted that two other females at the mid-dose were reported with clinical signs: #60 (labored breathing; feces abnormal; sneezing; increased activity) and #63, which died on study (fur erect). While 3 of the 4 females that experienced >2 resorptions at 175 mg/kg/day showed clinical signs, the clinical signs were typically limited to only 1-2 days during gestation. Further, the signs that were observed in these animals generally were not consistent with those associated with toxicity at the high dose (i.e., erect fur). Additionally, the clinical signs were not consistent with those typically associated with reduced food consumption in rabbits: reduced and/or abnormal feces, depression, lethargy, dehydration, teeth grinding, hunched posture, and reduced activity (Moxon et al., 2023). Thus, it cannot be concluded based on review of the clinical signs data that the increased early resorptions and post-implantation loss observed at 175 mg/kg/day were secondary to maternal systemic toxicity.

Table 9. Clinical signs observed in females with >2 resorptions at 175 mg/kg/day.

Female	Clinical signs
45	---
46	Labored breathing (2)[a]; mouth discharge (2); nostril discharge (1)
62	Excessive grooming (1)
65	Salivation (3); mouth discharge (1)
[a]Number of days on which the clinical sign was observed.	

Food consumption

Rabbits are highly sensitive for gastrointestinal (GI) disturbance, which can reduce motility of the GI tract and cause rabbits to stop eating (Moxon et al., 2023). As discussed in the study summary above, negligible food consumption was observed in decedents of the OECD 414 rabbit study of Dehyton DC. Further, the period of greatest sensitivity for treatment-related effects on food consumption was GD 12-15.

Table 10 shows the measured food consumption for this study interval as well as for the two preceding study intervals (beginning with the initiation of treatment) and the study interval that follows GD 12-15. The females that experienced >2 resorptions or that died on study are highlighted in yellow. As can be seen from these data, two females (#45 and #46) experienced very low total food consumption in the GD 12-15 study interval, as did the female that died on study (#63). Female #45 also consumed little food in the preceding study interval (GD 9-12). However, food consumption was unaffected for the other two females that experienced >2 resorptions (#62 and #65). Moreover, there are other females in the mid-dose group that also consumed very little food in the GD 12-15 study interval, including females #51, #60 and #61. Female #60 had a single late resorption, while the other two females did not have any post-implantation loss. Thus, limited food consumption during the GD 12-15 study interval is not predictive of post-implantation loss in the OECD 414 rabbit study of Dehyton DC.

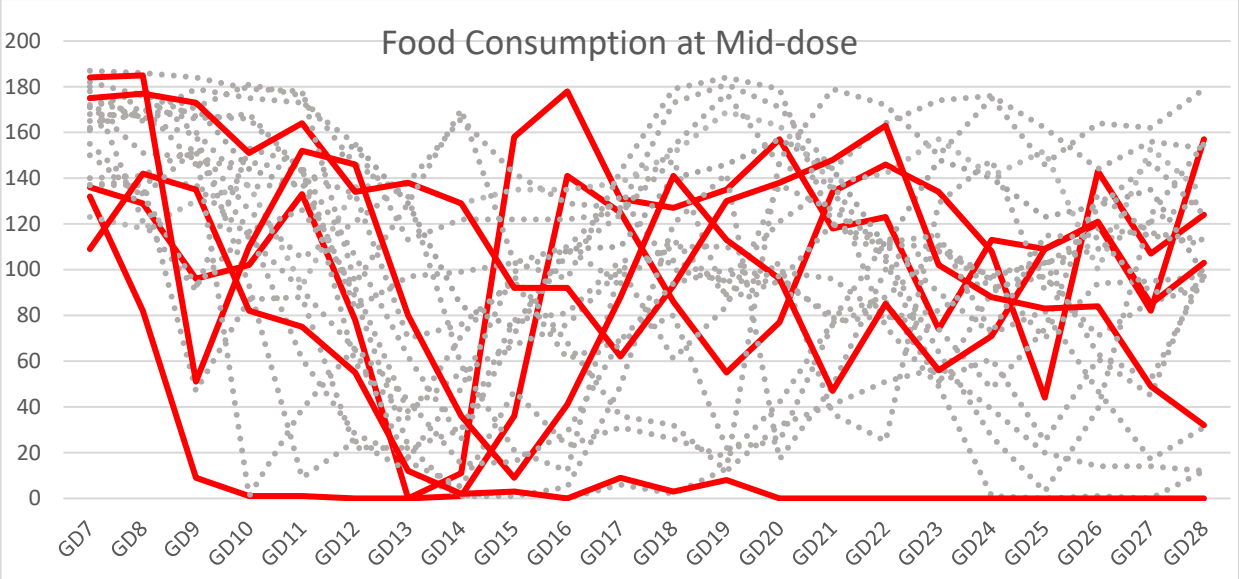
Table 10. Total food consumption (g) for different study intervals for pregnant females[a] at 175 mg/kg/day.

Female	GD 7-9	GD 9-12	GD 12-15	GD 15-18
45	214	11	11	467
46	265	331	79	302
47	349	451	368	294
48	335	527	390	369
49	339	475	279	225
50	331	477	279	345
51	274	222	74	263
52	288	336	185	243
53	301	390	165	201
54	341	528	336	134
55	358	434	112	9
56	323	327	94	302
58	336	436	194	303
60	343	261	38	55
61	276	342	86	101
62	352	488	401	246
63[b]	251	292	69	12
64	373	541	393	342
65	369	313	262	138
66	282	193	124	114

[a]Females #57 and # 59 were not pregnant and, thus, are not included in the table.
[b]This female died on study.
GD = gestational day.
Yellow highlighting indicates females that had >2 resorptions or that died on study.

The figure below depicts food consumption in grams/day for the mid-dose animals over the course of the study beginning on GD 7 when dosing was initiated. The four females that experienced >2 resorptions and the female that died on study are shown in red; the other females in the mid-dose group are shown in grey. While most (but not all) of the females that died or had >2 resorptions at 175 mg/kg/day experienced intervals of reduced daily food consumption over the course of the study, many other females in the mid-dose group also experienced reductions in daily food consumption during the study. Thus, it cannot be concluded based on review of the daily food consumption data that increased early resorptions and post-implantation loss observed at 175 mg/kg/day were secondary to maternal systemic toxicity.

Figure 1. Daily food consumption (g/day) at 175 mg/kg/day. Solid red line represents females that died on study or experienced >2 resorptions; dotted grey lines represent other females in the mid-dose group (GD = gestational day).



Body weight gains

Body weight gains are often linked to food consumption such that reduction in food intake may result in reduced body weight gains. In the OECD 414 rabbit study of Dehyton DC, most of the decedents in the high dose group exhibited negligible body weight gains or body weight losses prior to death. Further, as detailed in the study summary above, the study intervals in which the most substantial effect on body weight gains were observed in the mid-dose group were GD 9-12 and GD 12-15.

Table 11 shows the body weight gains for the mid-dose animals for these two study intervals as well as the adjusted body weight gain over the course of dose administration (i.e., body weight gain GD 7-29 minus gravid uterine weight). Females that experienced >2 resorptions or that died on study are highlighted in yellow. As can be seen from these data, there is no relationship between amount of body weight gained or lost in a single study interval or over the course of the study and increased resorptions and post-implantation loss. Thus, it cannot be concluded based on review of the body weight gains data that the increased early resorptions and post-implantation loss observed at 175 mg/kg/day were secondary to maternal systemic toxicity.

Table 11. Body weight gains (g) for different study intervals for pregnant females[a] at 175 mg/kg/day.

Female	Body weight gain (g)		Adjusted BWG GD 7-29
	GD 9-12	GD 12-15	
45	-177	0	-322
46	42	-43	-453
47	57	69	-66
48	79	111	-155
49	13	96	-342
50	11	126	-196
51	-65	17	-278
52	-7	-20	-310
53	44	25	-630
54	11	-35	-396
55	-68	-17	-369
56	19	43	-302
58	46	-7	-399
60	-123	-38	-564
61	40	-14	-331
62	16	23	-194
63[b]	-17	39	---[c]
64	48	109	-139
65	-16	46	-329
66	-111	55	-489

[a]Females #57 and # 59 were not pregnant and, thus, are not included in the table.
[b]This female died on study.
[c]As this female died early, terminal body weight gains and adjusted body weight gain could not be calculated.
BWG = body weight gain; GD = gestational day.
Yellow highlighting indicates females that had >2 resorptions or that died on study.

Conclusions

In the OCED 414 rabbit study of Dehyton DC, substantial maternal toxicity was observed at the high dose of 350 mg/kg/day, which resulted in mortality of 10 females in this dose group and an insufficient number of survivors (n=12) at this dose for a robust statistical analysis. At the mid-dose of 175 mg/kg/day, increased early resorptions and post-implantation loss were observed; although these endpoints were not significantly different from control, they were above the laboratory's HCD range. Based on evaluation of mean food consumption and body weight gains data, maternal toxicity occurred at the mid-dose. While the available data are suggestive of a relationship between maternal systemic toxicity and increased post-implantation loss, examination of individual animal data for the mid-dose group fails to demonstrate an obvious relationship between the indicators of maternal systemic toxicity (i.e., clinical signs, reduced food consumption, and reduced body weight gains) and post-implantation loss. Thus, based on the available data, it cannot be concluded that the observed increases in early resorptions and post-implantation loss at the mid-dose are secondary to maternal system toxicity.

Limitations

The purpose of this analysis is limited to a review of the results from the OECD 414 rabbit study of Dehyton DC. This assessment is based on review of the study data, and the authors' combined expertise in developmental and reproductive toxicology. The opinions presented herein are made to a reasonable degree of scientific certainty. Exponent reserves the right to supplement this report and to expand or modify the conclusions and findings based on the review of additional materials as they become available through additional work, or through the review of additional work performed by others. The scope of services performed during this investigation may not adequately address the needs of other users of this report, and any re-use of this report or its findings, conclusions or recommendations as presented herein are at the sole risk of the user.

References

Charles River Laboratories. 2023. Prenatal Developmental Toxicity Study of Amphoacetates C8-C18 by Oral Gavage in Time-Mated New Zealand White Rabbits. Charles River Laboratories Den Bosch BV. Study No. 20346091. Draft report.

Moxon M, M Beekhuijzen, B Hannas, J Manton, J French, L Malley. 2023. An overview of the current challenges when using rabbits for prenatal developmental toxicity studies with consideration of the impact on data interpretation. Reproductive Toxicology 118: 108386. doi: 10.1016/j.reprotox.2023.108386.

Name: OECD / Toxicity to reproduction / Toxicity to reproduction. EOGRTS_OECD 443_CRL_20346108 / Alkylamidoamine glycinate majority C12, 14 (amphoacetate)/ Amphoacetates C8-C18 / Reaction products o

Printing date: 2025-04-25T07:58:22.182Z

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ENDPOINT_STUDY_RECORD: Toxicity to reproduction. EOGRTS_OECD 443_CRL_20346108

UUID: 5c2e86ea-c7c9-45af-bfa8-3349b079a82a
Dossier UUID:
Author: DLO
Date: 2025-03-18T11:28:02.680Z
Remarks:

Administrative data

Endpoint
extended one-generation reproductive toxicity - basic test design (Cohorts 1A, and 1B without extension)

Type of information
experimental study

Adequacy of study
key study

Robust study summary
true

Used for classification
true

Used for SDS
true

Study period: start date
2023-09-27

End date
2025-02-15

Reliability
1 (reliable without restriction)

Rationale for reliability incl. deficiencies
guideline study
Reliability 1

Data source

Reference

Extended One Generation Reproductive Toxicity Study (including Cohort 1) of Amphoacetates C8-C18 by / Charles River Laboratories Den Bosch BV / study report

Data access
data submitter is data owner

Data protection claimed
yes, but willing to share

Materials and methods

Test guideline

Qualifier
according to guideline

Guideline
OECD Guideline 443 (Extended One-Generation Reproductive Toxicity Study)

Version / remarks
June 2018

Qualifier
according to guideline

Guideline
EU Method B.56 (Extended One-Generation Reproductive Toxicity Study)

Version / remarks
15 July 2014

Principles of method if other than guideline
OECD guidance document supporting OECD test guideline 443 on the extended onegeneration re productive toxicity test, No. 151, July 2013

GLP compliance
yes (incl. QA statement)

Limit test
no

Justification for study design
The design of this study was based on the final decision on a compliance check of the test ite by EC HA (Decision No. TPE-D-2114539402-55-01/F, date 27-Jan-2021)

Test material

Test material information
Alkylamphoacetates C8-C18 (Diacetate form)

Test animals

Species
rat

Strain
Wistar Han Crl: WI(Han).
rat

Details on species / strain selection
The Wistar Han rat was chosen as the animal model for this study as it is an accepted rodent species for reproduction and developmental toxicity testing by regulatory agencies.

Sex
male/female

Details on test animals or test system and environmental conditions
TEST ANIMALS
- Age at study initiation: (F0) Males/Females: 6.2 weeks old
- Weight at study initiation: (F0) Males: 105 – 187 g; Females: 94 – 134 g
- Fasting period before study: No
- Housing: Prior to mating and during the post-weaning period, animals were group housed (up to 5 animals of the same sex and same dosing group and cohort together) in polycarbonate cages (Makrolon type IV; height 18 cm or type 2000P; 61x43.5x21.5 cm depending on body weight). During the mating phase, males and females were cohabitated on a 1:1 basis in Makrolon plastic cages (type III; height 18 cm). During the post-mating phase, males were housed in Makrolon type IV or type 2000P cages with a maximum of 5 males/cage. Females were individually housed in Makrolon plastic cages (type III, height 18 cm).
During the lactation phase, females were housed in Makrolon plastic cages (type III, height 18 cm). Pups were housed with the dam until termination or until weaning on PND 21. Cages contained sterilized wooden fibers as bedding material (Safe S 8-15, JRS - J.Rettenmaier & Söhne GmbH + CO. KG, Rosenberg, Germany) and were equipped with water bottles.
Animals were socially housed for psychological/environmental enrichment and provided with items such as devices for hiding in, paper and/or objects for chewing, except when interrupted by study procedures/activities.
- Diet: SM R/M-Z from SSNIFF, pelleted maintenance diet ad libitum (SSNIFF Spezialdiäten GmbH, Soest, Germany).
- Water: tap water ad libitum, except during designated procedures.
- Acclimation period: 12 days
Analysis confirmed that there were no known contaminants in the water, diet or enrichment materials that could interfere with the outcome of the study.

ENVIRONMENTAL CONDITIONS SET TO MANTAIN (F0 and F1)
- Temperature (°C): 20 - 24 (actual 21 - 22)
- Humidity (%): 40 - 70 (actual 44 - 70)
- Air changes (per hr): at least 10
- Photoperiod (hrs dark / hrs light): 12h / 12h

IN-LIFE DATES: From: 09 October 2023 To: 01 May 2024

Administration / exposure

Route of administration
oral: gavage

Vehicle
water Elix

Details on exposure
PREPARATION OF DOSING SOLUTIONS: Dosing solutions were prepared at least twice weekly by mixing the test item and the vehicle for groups 2-3. For Group 4, the test material was administered as received.
For Groups 2-3, test material l dosing formulations (w/w) were homogenized to visually acceptable levels at appropriate concentrations to meet dose level requirements. Dose formulations were divided into aliquots where required to allow to be dispensed on each dosing occasion. Dosing solutions and test material were stored at 4°C. The dosing formulations were removed from the refrigerator and stirred at room temperature for at least 30 minutes before dosing and dosed within 24 hours after removal from the refrigerator. The dosing formulations were kept at room temperature until dosing. The dosing formulations were swirled shortly before use for dosing. Adjustment were made for specific gravity of the test material. A correction was made for the purity/composition of the test material. A factor of 2.1 was used to correct for the purity/composition of the test material.

VEHICLE
- Justification for use and choice of vehicle: Trial preparations were performed to select the suitable vehicle and to establish a suitable formulation procedure. Stability analyses performed previously in conjunction with the method validation study (Test Facility Study No. 20346081) demonstrated that the test material is stable in the vehicle when prepared and stored under the same conditions at co ncentrations bracketing those used in the present study.
- Adjusted (with correction factor: 2.1) concentration in vehicle: 0, 111.5, 334.6 and 115.2 mg/mL
- Amount of vehicle: 1.883 mL/kg bw

Details on mating procedure
- M/F ratio per cage: 1:1
- Length of cohabitation: after a minimum of 10 weeks of treatment and until mating occurred, or 14 days had elapsed.
- Proof of pregnancy: presence of an intravaginal copulatory plug or sperm in a vaginal lavage. The day of confirmed mating was designated as post-coitum (p.c) day 0.
- Once mating has occurred, the males and females were separated. After successful mating each pregnant female was caged individually.
- When mating had not occurred after 14 days, the animals were separated without further opportunity for mating.
- After successful mating each pregnant female was caged individually.

Analytical verification of doses or concentrations
yes

Details on analytical verification of doses or concentrations
Analyses were performed using a validated analytical procedure (Test Facility Study No. 20346081).

Dose formulation samples were collected for analysis on weeks 1, 8, 16 and 24 of treatment.
Acceptance criteria: For concentration, mean sample concentration results within or equal to ± 10% for solutions or ±15% for suspensions of theoretical concentration are considered accepta ble.For homogeneity, a relative standard deviation (RSD) of concentrations of ≤ 10% for each group is considered acceptable.

Accuracy results:
The concentrations analyzed in the formulations of Groups 2 and 3 were in agreement with target concentrations (i.e. mean sample concentration results were within or equal to 90-110% of target concentration). Group 4 was used as received and therefore not analyzed. Small responses at the retention time of the test material were observed in all chromatograms of the Group 1 formulation prepared for use in Weeks 1, 8, 16 and 24. These responses were determined to have no impact on the outcome of the study since the maximum contribution to the Group 2 samples for all Group 1 formulations was ≤ 0.01%.

Homogeneity:
The formulations of Group 2 and Group 3 were homogeneous (i.e. coefficient of variation ≤ 10%).

Duration of treatment / exposure
F0-males: 7 days a week for a minimum of 11 weeks, including 10 weeks prior to mating (with the objective of covering at least one spermatogenic cycle) and during the mating period, up to and including the day before scheduled necropsy.

F0-females: 7 days a week for a minimum of 16 weeks, including 10 weeks prior to mating, the variable time to conception, the duration of pregnancy and at least 21 days after delivery, up to and including the day before scheduled necropsy. Females were not be dosed during littering.

Pups: Prior to weaning, pups were not treated directly but could potentially be exposed to the test material in utero, via maternal milk, or from exposure to maternal urine/ feces.

From weaning onwards (PND 21), F1-animals of Cohorts 1A, 1B and 1C were dosed up to and including the day before scheduled necropsy. The F1-animals of Cohort Surplus and Spares (not assigned to one of the cohorts) were not dosed.

Frequency of treatment
Once daily

Doses / concentrations

Dose / conc.	
100	mg/kg bw/day (actual dose received)
Remarks	
Group 2	
Dose / conc.	
300	mg/kg bw/day (actual dose received)
Remarks	
Group 3	
Dose / conc.	
1000	mg/kg bw/day (actual dose received)
Remarks	
Group 4	

No. of animals per sex per dose
F0: 25 Males + 25 Females
F1: F1: 20 males + 20 females were assigned to each group and Cohort for cohorts 1A, 1B and 1C. 10 males + 10 females per group were assigned to Cohort surplus (cohort surplus animals were not directly dosed).

Please refer also to table 1.

Control animals
yes
Group 1

Details on study design
- Dose selection rationale:
The oral route of administration was selected because this was specified in the final decision by ECHA.
The dose levels were selected based on the results of a preliminary reproductive toxicity study (reproduction/developmental toxicity screening test) with oral exposure of Amphoacetates C8-C18 in rats, Test Facility Study No. 518366 and a 90-day repeated dose toxicity study with oral exposure of Amphoacetates C8-C18 in rats, Test Facility Study No. 20164357, and in an attempt to produce graded responses to the test material. In the reproductive toxicity study, three females had to be prematurely euthanized and one female was found dead at 1000 mg/kg bw/day. The deaths/morbidity of these animals were likely caused by regurgitation and were triggered by the physical/chemical properties of the test material dose formulation in combination with the oral gavage administration. Based on the early termination of these females, the study group (1000 mg/kg bw/day, the remaining 6 out of 10 females) was terminated on Day 14 post-coitum. These females were all pregnant with a normal number of foetuses and did not show any direct test material-related morphological changes. No developmental toxicity was noted up to 300 mg/kg bw/day.

In the 90-day repeated dose toxicity study, findings noted were a lower body weight compared with control from the second week of treatment onwards, with corresponding lower food consumption at 100, 300 and 1000 mg/kg bw/day. No mortality occurred.
As no mortality occurred in the 90-day repeated dose toxicity study and no other signs of toxicity were noted in this study and in the reproduction/developmental toxicity screening test, a dose of 1000 mg/kg bw/day was considered a suitably high dose. To prevent the mortality that occurred in the reproduction/developmental toxicity screening test, the dose formulations were prepared and treated in a comparable manner as for the 90-day repeated dose toxicity test (i.e. with the lowest dose volu me as possible, administration of undiluted test item at the high dose and no stirring but swirling of formulations prior to use for dosing to prevent foaming).
The high-dose level should produce some toxic effects, but not death nor obvious suffering. The mi d-dose level is expected to produce minimal to moderate toxic effects. The low-dose level should pro duce no observable indications of toxicity.

High dose level used
yes

Justification for deviation from the high dose level
n.a

Examinations

Parental animals: Observations and examinations
MORTALITY: Yes
- Time schedule: At least twice daily beginning upon arrival through termination/release. Except on days of receipt and necropsy where frequency was at least once daily.

CAGE SIDE OBSERVATIONS: Yes
- Time schedule: At least once daily, up to the day prior to necropsy. These clinical observations were at least conducted prior to dosing and after dosing.
Animals were observed for specific clinical signs within their cage unless necessary for identification or confirmation of possible findings.

ARENA OBSERVATIONS: Yes
- Time schedule: Once before the first administration of the test material and at weekly intervals during the treatment period.
Animals were observed outside the home cage in a standard arena pre-dose.

DETAILED CLINICAL OBSERVATIONS: Yes
- Time schedule: On the day of scheduled necropsy. Animals were removed from the cage.

BODY WEIGHT: Yes
- Time schedule for examinations: On the first day of treatment (prior to dosing), and weekly thereafter. Mated females were weighed on Days 0, 4, 7, 11, 14, 17, and 20 post-coitum and during la ctation on PND 1, 4, 7, 14 and 21.

FOOD CONSUMPTION: Yes, food consumption was quantitatively measured per cage.
- Time schedule for examinations: Weekly, except for males and females which are housed together for mating and for females without evidence of mating. Food consumption of mated females was measured on Days 0, 4, 7, 11, 14, 17, and 20 of gestation and during lactation on PND 1, 4, 7, 14 and 21.

WATER CONSUMPTION: Yes
- Water consumption was monitored on regular basis throughout the study by visual inspection of the water bottles. If inter group differences were noted, consumption may be assessed by weight.

REPRODUCTIVE DATA: Yes
- Time schedule for examinations: Daily from the mating period onwards.

Male paired with, mating date, confirmation of pregnancy, and delivery day were recorded. Palpation a nd/or body weight measurement may be used to aid in confirmation of pregnancy.
The females were allowed to litter normally. Postnatal day (PND) 1 was defined as the day when a litt er was found completed (i.e. membranes and placentas cleaned up, nest built and/or feeding of pups started). The day prior to PND 1 was considered to be the day when the female started to deliver and was defined as Lactation Day (LD) 0 for the dam and PND 0 for the offspring, and used for recording of delivery. Females that were littering were left undisturbed. Cage debris of pregnant females wer e examined for evidence of premature delivery. Signs of difficult or prolonged parturition were reco rded, if applicable. Deficiencies in maternal care, such as inadequate construction or cleaning of t he nest, pups left scattered and cold, physical abuse of pups or apparently inadequate lactation or feeding, were recorded, if applicable.

HAEMATOLOGY: Yes
- Time schedule for collection of blood: On the day of scheduled necropsy (between 07:30 and 10:30 a.m.).
- Anesthetic used for blood collection: Yes (isoflurane)
- Animals fasted: The selected animals were fasted overnight with a maximum of 24 hours before blo od sampling.
- How many animals: 10 males + 10 females per group
- Parameters: White Blood Cell Count (WBC), Neutrophils (absolute), Lymphocytes (absolute), Mo nocytes (absolute), Eosinophils (absolute), Basophils (absolute),Large unstained cells (LUC), (absol ute) Red Blood Cell Count (RBC), Reticulocytes (absolute), Red Blood Cell Distribution Width (RDW), Hemoglobin, Hematocrit, Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Platelets
COAGULATION: yes
- Time schedule for collection of blood: On the day of scheduled necropsy (between 07:30 and 10:30 a.m.).
- Anesthetic used for blood collection: Yes (isoflurane)
- Animals fasted: The selected animals were fasted overnight with a maximum of 24 hours before blo od sampling.
- How many animals: 10 males + 10 females per group
- Parameters: Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT)

CLINICAL CHEMISTRY: Yes
- Time schedule for collection of blood: On the day of scheduled necropsy (between 07:30 and 10:30 a.m.).
- Anaesthetic used for blood collection: Yes (isoflurane)
- Animals fasted: The selected animals were fasted overnight with a maximum of 24 hours before blood sampling.
- How many animals: 10 males + 10 females per group
- Parameters checked : Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkalin e Phosphatase (ALP), Total protein, Albumin, Bile Acids, Total Bilirubin, Urea, Creatinine, Glucose, Cholesterol, Sodium, Potassium, Chloride, Calcium, Inorganic Phosphate (Inorg. Phos).

THYROID HORMONES: Yes
- Time schedule for collection of blood: On the day of scheduled necropsy (between 07:30 and 10:30 a.m.).
- Anaesthetic used for blood collection: Yes (isoflurane)
- Animals fasted: The selected animals were fasted overnight with a maximum of 24 hours before blood sampling.
- How many animals: serum of 10 males + 10 females per group
- Parameters checked : Thyroxine (T4), Thyroid-Stimulating Hormone (TSH)

URINALYSIS: Yes
- Time schedule for collection of urine: on day of necropsy
- Metabolism cages used for collection of urine: Yes
- Animals fasted: Urine was collected overnight (approx. 15-20 hours) with absence of food but with water available.

- How many animals: 10 males + 10 females per group
- Parameters: Volume, Specific gravity, Clarity, Colour, pH, Blood, Leukocyte esterase, Bilirubin, Protein, Ketones, Glucose, Sediment (White blood cells (WBC-sed.), Red blood cells (RBC-sed.), Casts, Epithelial cells, Crystals, Bacteria, Other)

Oestrous cyclicity (parental animals)
Daily vaginal lavage was performed beginning 14 days prior to mating and during mating until evidence of copulation is observed. Vaginal lavage continued for those females with no evidence of copulation until termination of the mating period. On the day of scheduled necropsy, a vaginal lavage was also taken to determine the stage of estrous. This was done for all females, except for females that had to be euthanized in extremis, die spontaneously or with total litter loss.
Estrous stages were determined by examining the cytology of vaginal lavage samples.

Sperm parameters (parental animals)
Sperm samples were taken from the proximal part of the vas deferens (right) at necropsy. Sperm motility and progressive motility were assessed from all samples. Sperm smears for morphological evaluation were fixed from all samples and stained with hematoxylin and eosin. Abnormal forms of sperm from a differential count of at least 200 spermatozoa (if possible) per animal were recorded. Evaluation was performed for all samples.
One epididymis (right) was removed, placed in labeled bags, and kept in the freezer set to maintain -20°C. After thawing, the right epididymis was weighed, homogenized and evaluated for sperm numbers. Evaluation was performed for all samples.
In the case of any abnormalities in the right epididymis, the right side organ(s) was fixed in modified Davidson's solution, and the left side organ was used for evaluation of sperm numbers. If abnormalities are found in both epididymes, both these organs were fixed in modified Davidson's solution and no evaluation of sperm numbers was performed.

Litter observations
STANDARDISATION OF LITTERS
- Performed on day 4 postpartum: yes
- The size of each litter was adjusted by randomly culling extra pups to obtain as nearly as possible 4 males and 4 females per litter.

PARAMETERS EXAMINED IN F1 OFFSPRING:
- Mortality: For F1 animals before weaning, the number of live and dead pups was determined on PND 1 and daily thereafter. After weaning all animals assigned to cohort animals were observed for general health/mortality and moribundity twice a day throughout the study.
- Clinical observations: On all pups, at least once daily, including the day of necropsy. These clinical observations were at least be conducted prior to dosing and after dosing.
- Detailed Clinical Observations: F1 animals were observed weekly for specific clinical signs in a standard arena. Once the first animals have reached PND 21 and thereafter at weekly intervals during the treatment period. On the day of scheduled necropsy, animals were also removed from the cage to perform detailed clinical observations.
- Body weight: For F1 before weaning on PND 1, 4, 7, 13 and 21. Weekly from weaning onwards. This started on a specific date on which all pups were at least at PND 21. If necessary for scheduling purposes, weekly body weights may have been collected at a slightly different interval in the week prior to or of scheduled necropsies. In addition, the body weight was recorded of each female on the day of acquisition of vaginal patency and of each male on the day of acquisition of balano-preputial separation.
- Food consumption: At weekly intervals from weaning onwards. Quantitatively measured per cage.
- Water consumption: On a regular basis throughout the study by visual inspection of the water bottles. If inter group differences were noted, consumption may be assessed by weight.
- Sex determination: Sex was externally determined on PND 1, 4 and 13.
- Anogenital distance (AGD) was measured for all live pups on PND1 and normalized to the cube root of body weight.
- Areola/Nipple retention was examined on all male pups in each litter on PND13.
- Vaginal Patency was monitored daily for all females in Cohorts 1A, 1B and 1C from PND25 onwards. Vaginal patency (vaginal opening) was monitored by visual inspection of the vaginal area. Body weight was recorded on the day of acquisition of vaginal patency.

- Balano-preputial Separation was monitored daily for all males in Cohorts 1A, 1B and 1C from PND 35 onwards. Balano-preputial separation (prepuce opening) was monitored by visual inspection of the genital area. Body weight was recorded on the day of acquisition of balano-preputial separation.
- Stage of Estrous Determination was carried out though vaginal lavage on the day of scheduled necropsy for all females in Cohort 1A. Daily vaginal lavage was performed starting on the first da y after onset of vaginal patency and continued until the first estrus was determined, in order to de termine the time interval between these two events. After that daily vaginal lavage was performed fr om PND 75 to 88. Finally, on the day of scheduled necropsy, a vaginal lavage was taken from all surv iving females.

HAEMATOLOGY IN COHORT 1A: Yes
- Time schedule for collection of blood: On the day of scheduled necropsy (between 07:30 and 10:30 a.m.).
- Anesthetic used for blood collection: Yes (isoflurane).
- Animals fasted: Yes, overnight with a maximum of 24 hours before blood sampling.
- How many animals: 10 males + 10 females per group.
- Parameters: White Blood Cell Count (WBC), Neutrophils (absolute), Lymphocytes (absolute), M onocytes (absolute), Eosinophils (absolute), Basophils (absolute),Large unstained cells (LUC), (abso lute) Red Blood Cell Count (RBC), Reticulocytes (absolute), Red Blood Cell Distribution Width (RDW), Hemoglobin, Hematocrit, Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Platelets.

COAGULATION IN COHORT 1A: yes
- Time schedule for collection of blood: On the day of scheduled necropsy (between 07:30 and 10:30 a.m.).
- Anesthetic used for blood collection: Yes (isoflurane)
- Animals fasted: Yes, overnight with a maximum of 24 hours before blood sampling.
- How many animals: 10 males + 10 females per group
- Parameters: Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT)

CLINICAL CHEMISTRY IN COHORT 1A: Yes
- Time schedule for collection of blood: at termination
- Anaesthetic used for blood collection: Yes (isoflurane)
- Animals fasted: Yes, overnight with a maximum of 24 hours before blood sampling.
- How many animals: 10 males + 10 females per group.
- Parameters checked: Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alka line Phosphatase (ALP), Total protein, Albumin, Bile Acids, Total Bilirubin, Urea, Creatinine, Glucose, Cholesterol, Sodium, Potassium, Chloride, Calcium, Inorganic Phosphate (Inorg. Phos).

THYROID HORMONES: Yes
- Time schedule for collection of blood: For Cohort 1A animals on the day of scheduled necropsy (between 07:30 and 10:30 a.m.), for F1 pups culled on PND4 (from 7:00 to 10:30 a.m., samples were pooled per litter.), for F1 animals of Cohort Surplus on PND 22-24 (between 08:00 and 11:30 a.m.).
- Anesthetic used for blood collection: Yes (isoflurane) for Cohort 1A and Cohort Surplus animals. In pups culled on PND 4, blood was collected by decapitation.
- Animals fasted: Cohort 1A animals were fasted overnight with a maximum of 24 hours before blood sampling.
- How many animals: 10 males + 10 females per group from Cohort 1A and 10 males + 10 females per group F1 animals of Cohort Surplus. Additionally, 2 culled pups per litter (1 female and one male if possible).
- Parameters checked: TSH, T4

URINALYSIS IN COHORT 1A: Yes
- Time schedule for collection of urine: on day of necropsy
- Metabolism cages used for collection of urine: Yes
- Animals fasted: Urine was collected overnight (approx. 15-20 hours) with absence of food.
- How many animals: 10 males + 10 females of Cohort 1A

- Parameters: Volume, Specific gravity, Clarity, Colour, pH, Blood, Leukocyte esterase, Bilirubin, Protein, Ketones, Glucose, Sediment (White blood cells (WBC-sed.), Red blood cells (RBC-sed.), Casts, Epithelial cells, Crystals, Bacteria, Other).

SPERM PARAMETERS IN COHORT 1A: Yes

- Sperm samples were taken from the proximal part of the vas deferens (right) at necropsy. Sperm motility and progressive motility were assessed from all samples. Sperm smears for morphological evaluation were fixed from all samples and stained with hematoxylin and eosin. Abnormal forms of sperm from a differential count of at least 200 spermatozoa (if possible) per animal were recorded. One epididymis (right) was removed, placed in labeled bags, and kept in the freezer set to maintain -20°C. After thawing, the right epididymis was weighed, homogenized and evaluated for sperm n umbers. Evaluation was performed for all samples. In the case of any abnormalities in the right epid idymis, the right side organ(s) was fixed in modified Davidson's, and the right side organ was used for evaluation of sperm numbers. If abnormalities were found in both epididymes, both these organs w ere fixed in modified Davidson's solution and no evaluation of sperm numbers was performed.

LYMPHOCYTE SUBTYPING IN COHORT 1A: Yes

- From 10 selected animals/sex/group of Cohort 1A, splenic lymphocyte subpopulation analysis was performed at termination. One half of the spleen was kept on ice until splenic lymphocytes were isola ted using 70 µm cell strainers. The other half of the spleen was preserved for histopathological eva luation. Splenocytes were counted with with a Countess™ 3 automated cell counter. Subpopulations of T-cells, T-helper cells, T-cytotoxic cells, B-cells and NK-cells were determined in isolated splenic lymphocytes using the BD FACSCanto™ I and/or BD FACSLyric™ flow cytometer system on the day of necropsy. The % lymphoid cells of splenocytes was determined using the Forward Scatter and Side Scatter.

Postmortem examinations (parental animals)

SACRIFICE:

Animals surviving until scheduled euthanasia or euthanized for humane reasons were deeply anesthetized using isoflurane and subsequently exsanguinated. Animals surviving until scheduled eut hanasia were fasted overnight with a maximum of 24 hours before necropsy and with water available.

- Male animals surviving until scheduled euthanasia: After successful mating and a minimum of 10 weeks of treatment. For males which fail to sire, at the end of the mating period and after a minimum of 10 weeks of treatment.
- Female animals surviving until scheduled euthanasia: on LD 23-25 for females which deliver, on post-coitum days 25-27 for females with evidence of mating, or approximately 24-26 days after the last day of the mating period for females without evidence of mating.
- Dams with total litter loss were euthanized within 24 hours after the last pup is found dead or missing. In this case, females were not fasted before necropsy.

GROSS NECROPSY

- All animals were subjected to a full post-mortem examination, with special attention being paid to the reproductive organs. The numbers of former implantation sites were recorded for all paired females.

ORGAN WEIGHTS / HISTOPATHOLOGY

-The organs identified for weighing in Table 2 (under ''Any other information on materials and me thods incl. tables'') were weighed at necropsy for all scheduled euthanasia animals and for females with total litter loss. Terminal body weights were also recorded for all females with total litter loss and for all animals surviving until scheduled euthanasia.

Organ weights were not recorded for animals found dead or euthanized in poor condition or in extre mis.

-A microscopic examination was performed on all tissues listed in Table 2 (under ''Any other i nformation on materials and methods incl. tables'') for all control and group 4 animals surviving until scheduled necropsy and for all unscheduled deaths. Microscopic examination of gross lesions, target tissues and reproductive tissues was performed for all group 2 and groups 3 animals surviving until scheduled necropsy. Microscopic examination of reproductive tissues was performed for females wi th total litter loss and males that failed to sire.

Postmortem examinations (offspring)
SACRIFICE
-F1 pups sacrificed before weaning:
• Pups sacrificed in extremis younger than 7 days and live fetuses of females that die spontaneously (or were euthanized in extremis), were euthanized by decapitation. Pups sacrificed on or after PND 7 were euthanized by an intraperitoneal injection of sodium pentobarbital.
• On PND 4, the pups scheduled for culling (> 8 pups per litter) were euthanized by decapitation.
-F1 generation animals sacrificed after weaning:
• Cohort 1A animals were sacrificed on PND 89-95 by isoflurane anesthesia followed by exsang uination (only animals from this cohort were fasted before sacrifice).
• Cohort 1B animals were sacrificed on or after PND ≥ 97 by isoflurane anesthesia followed by exsan guination (not fasted before sacrifice).
• Cohort 1C animals were sacrificed after positive determination of vaginal patency or balano-prepu tial separation by carbon dioxide inhalation (gradual fill procedure).
• Cohort surplus animals and spare F1 animals were sacrificed on PND 22-24 by intraperitoneal injec tion of sodium pentobarbital.

GROSS NECROPSY
-F1 pups sacrificed before weaning:
• Stillborn pups and pups found dead between birth and PND 13 were sexed (both externally and i nternally, if possible) and externally examined with emphasis on developmental morphology. For pups found dead or sacrificed in extremis from PND 14 onwards a limited necropsy was performed including sex determination (both externally and internally, if possible).
• For pups culled on PND4 sex was be determined both externally and internally (if possible). Pups were externally examined, with particular attention to the external reproductive genitals to examine signs of altered development.
-F1 generation animals sacrificed after weaning:
• Spare F1-animals which are not assigned to one of the Cohorts were subjected to a limited exa mination, with special attention being paid to the reproductive organs. Descriptions of all macrosco pic abnormalities were recorded.
• Cohort 1A animals were subjected to a full post-mortem examination, with special attention being paid to the reproductive organs.
• Cohort 1B animals were subjected to a limited examination, with special attention being paid to the r eproductive organs.
• Cohort 1C and cohort surplus animals were subjected to a limited examination, with special attenti on being paid to the reproductive organs.

HISTOPATHOLOGY / ORGAN WEIGHTS
-The organs identified for weighing in Tables 3 to 5 (under "Any other information on materials and methods incl. tables") were weighed at necropsy for all animals in cohorts 1A, 1B and Surplus and fe males with total litter loss. Organ weights were not recorded for animals found dead or euthanized in poor condition or in extremis
-A microscopic examination was performed on all tissues listed in Tables 3 to 5 (under "Any other information on materials and methods incl. tables") for animals in cohorts 1A and 1B.
- In addition to the procedures described above, for Cohort 1A animals of Groups 1 and 4, HE stained s tep sections of both ovaries and corpora lutea were be prepared. One of the ovaries was quantitatively evaluated for follicles (primordial and small growing follicles counted together), as well as corpora lutea initially.

Statistics
General variables:
- Body Weight Gains: Males and F1-animals: Calculated between each scheduled interval as well as overall premating period and overall dosing period. Females: Premating period: Calculated between each scheduled interval as well as overall premating period until initiation of cohabitation. Gestation and Lactation: Calculated between each scheduled interval as well as GD 0-20 and LD 1-21.
- Food Consumption: Calculated between each scheduled interval.
- Mean Overall Food Consumption: For males: Calculated over the complete Treatment Period.

For females: Calculated overall food consumption was also calculated over the premating period, GD 0-20 and LD 1-21.
- Organ Weight Relative to Body Weight: Calculated against the terminal body weight.

All statistical tests were conducted at the 5% significance level. All pairwise comparisons were conducted using two sided tests and were reported at the 1% and 5% levels, unless otherwise noted.
The pairwise comparisons of interest are listed below:
Group 2 vs. Group 1
Group 3 vs. Group 1
Group 4 vs. Group 1

Analyses were performed according to the matrix in table 6, but excluded any group with less than 3 observations.
• Parametric / Non-Parametric: Levene's test was used to assess the homogeneity of group variances. The groups were compared using an overall one-way ANOVA F-test if Levene's test was not significant or the Kruskal-Wallis test if it was significant. If the overall F-test or Kruskal-Wallis test was found to be significant, then pairwise comparisons were conducted using Dunnett's or Dunn's test, respectively.
• Incidence: A Fisher's exact test was used to conduct pairwise group comparisons of interest.

Reproductive indices
• Pre-coital interval = Number of days between initiation of cohabitation and confirmation of mating
• Female Mating Index (%) = (Number of females with evidence of mating [or no confirmed mating date and pregnant]/ Number of females paired) x 100
• Female Fertility Index (%) = (Number of pregnant females/ Number of females with evidence of mating [or no confirmed mating date and pregnant]) x 100
• Female Pregnancy Index (%) = (Number of pregnant females/ Number of females paired) x 100
• Male Mating Index (%) = (Number of males with evidence of mating [or female partner confirmed pregnant])/ Number of males paired) x 100
• Male Fertility Index (%) = (Number of males impregnating a female/ Number of males with evidence of mating [or female partner confirmed pregnant]) x 100
• Male Pregnancy Index (%) = (Number of males impregnating a female/ Number of males paired) x 100
• Gestation Length: The gestation length is calculated from GD 0 to the day the first pup is observed
• Gestation index (%) = (Number of females with live offspring/ Number of pregnant females) x 100

Offspring viability indices
• Live birth index (%) = (Number of live newborn pups. / Number of newborn pups) x 100
• Sex Ratio (% Males) = (Number of live male pups / Total number of live pups) x 100
• Viability index (%; Day 4 after littering)) = (Number of live pups on Day 4 (before culling) /Number of live newborn pups) x 100
• Lactation index (%) = (Number of live pups on Day 21 after littering / Number live pups on Day 4 [after culling]) x 100
• Post-implantation loss/litter = ([number of implantation sites – total newborn pups]/ Number of implantation sites) x 100

Any other information on materials and methods incl. tables

Table 1. Experimental design

Generation	Group No.	Dose Level (mg/kg bw/day)	*Dose Volume (mL/kg bw)*[a]	Adjusted Dose Conc. (mg/mL)[b]	Cohort	Number of F_0-Animals		Animal Numbers	
						Males	Females	Males	Females

F0	1	0 (Vehicle)	1.883	0	-	25	25	*01-25*	*101-125*
	2	100	1.883	111.5	-	25	25	*26-43, 45-50, 3001*	*126-150*
	3	300	1.883	334.6	-	25	25	*51-75*	*151-175*
	4	1000	1.883	1115.2	-	25	25	*76-100*	*176-200*
F1	1	*0 (Vehicle)*	1.883	*0*	*1A*	20	20	*201-220*	*481-500*
					1B	20	20	*221-240*	*501-520*
					1C	20	20	*241-260*	*521-540*
					Surplus[d]	10	10	*261-270*	*541-550*
	2	*100*	1.883	111.5	*1A*	20	20	*271-290*	*551-570*
					1B	20	20	*291-310*	*571-590*
					1C	20	20	*311-330*	*591-610*
					Surplus[d]	10	10	*331-340*	*611-620*
	3	*300*	1.883	334.6	*1A*	20	20	*341-360*	*621-640*
					1B	20	20	*361-380*	*641-660*
					1C	20	20	*381-400*	*661-680*
					Surplus[d]	10	10	*401-410*	*681-690*
	4	*1000*	1.883	1115.2	*1A*	20	20	*411-430*	*691-710*
					1B	20	21	*431-450*	*711-730*
									761[d]
					1C	20	21	*451-470*	*731-750*
									762[e]
					Surplus[d]	10	10	*471-480*	*751-760*

[a] The dose level were corrected for purity with a factor 2.1.
[b] Based on the most recent body weight measurement and calculated as ((dose level (g/kg)/specific gravity)*(100/purity of test material (%))
[c] Dose concentration adjusted with correction factor: 2.1 (100/purity of test material (%)).

[d] The F1-animals of Cohort Surplus were not dosed.

[e] Female No. 716 was found dead shortly after dosing on 07 Feb 2024. A spare female from the same dose group was used to complement Cohort 1B andwas therefore re-allocated on 12 Feb 2024 as Female. No. 761.
f Female No. 742 was sacrificed for humane reasons on 08 Feb 2024. A spare female from the same dose group was used to complement Cohort 1C and was therefore re-allocated on 12 Feb 2024 as Female. No. 762.

Table 2. Tissue Collection and Preservation for the F0-Generation

Tissue	***Weigh*** [a]	***Macroscopic Evaluation and Collection***	***Histology Processing***	***Microscopic Evaluation*** [b, c]
Animal identification	-	*X*	-	-
Body cavity, nasal	**-**	***X***	**-**	**-**

Bone marrow, sternum	-	*X*	*X*	*X*
Bone, sternum	-	*X*	*X*	*X*
Brain	*X*	*X*	*X* d	*X* d
Epididymis	*X* (2)	*X* (2)	*X* (1)	*X (1) e,* f
Eye	-	*X* (2)	*X* (2)	*X* (2)
Gland, adrenal	*X* (2)	*X* (2)	*X* (2)	*X* (2)
Gland, harderian	-	*X (2)* h	-	-
Gland, mammary	-	*X*	*X*	*X* g
Gland, parathyroid	*X (2)* i	*X (2)* i	*X* (2)	*X (2)* i
Gland, pituitary	*X*	*X*	*X*	*X*
Gland, prostate	*X* j	*X* j	*X*	*X* f
Gland, seminal vesicle including coagulating gland and fluid	*X* (2)	*X* (2)	*X* (2)	*X (2)* f
Gland, thyroid	*X* (2)	*X* (2)	*X* (2)	*X* (2)
Gross lesions/ masses	-	*X*	*X*	*X*
Heart	*X*	*X*	*X*	*X*
Kidney	*X* (2)	*X* (2)	*X* (2)	*X* (2)
Large intestine, caecum	-	*X*	*X*	*X*
Large intestine, colon	-	*X*	*X*	*X*
Large intestine, rectum	-	*X*	*X*	*X*
Liver	*X*	*X*	*X*	*X*
Lung	-	*X* k	*X*	*X*
Muscle, skeletal	-	*X*	*X*	*X*

Nerve, optic	-	*X* l	*X*	*X* l
Nerve, sciatic	-	*X* (2)	*X* (1)	*X* (1)
Ovaries including oviducts	*X* (2)	*X* (2)	*X* (2)	*X (2)* f
Skin	-	*X*	-	-
Small intestine, duodenum	-	*X*	*X*	*X*
Small intestine, ileum	-	*X*	*X*	*X*
Small intestine, jejunum	-	*X*	*X*	*X*
Spinal cord	-	*X*	*X* m	*X* m
Spleen	*X*	*X*	*X*	*X*
Stomach	-	*X*	*X*	*X*
Testes	*X* (2)	*X* (2)	*X* (2)	*X (2) f,* n
Thymus	*X*	*X*	*X*	*X*
Trachea	-	*X*	*X*	*X*
Urinary bladder	-	*X*	*X*	*X*
Uterus/Cervix	*X*	*X*	*X*	*X* f
Vagina	-	*X*	*X*	*X* f
Vas deferens	-	*X (1)* o	*X (1)* o	*X (1)* o

X = Procedure to be conducted; - = Not applicable. (1) = one side. (2) = both sides.

[a] Organ weights were not determined for animals which die spontaneously or are sacrificed in extremis.

[b] In first instance, histopathological examination was performed on tissues of animals in the control and high dose group (Groups 1 and 4). In case a treatment-related effect is suspected, histopathological examination was extended to animals in the intermediate dose groups (Groups 2 and 3).

[c] Efforts were made to evaluate all protocol-required tissues microscopically; however, it is not always feasible for every protocol-required tissue to be present on every slide. Protocol-required tissues that are not examined were documented in the histopathology data and the impact of these missing tissues on the study was documented in the pathology report.

[d] Eight brain levels were examined including cerebellum, midbrain and cortex.

[e] As default, the right epididymidis was used for evaluation of sperm parameters and the left side was collected for histopathological evaluation. If there were gross findings in the right epididymidis, the abnormal side was fixed and the left epididymidis was used for evaluation of sperm parameters. If

abnormalities are found in both epididymes, both sides were fixed. For males found dead or sacrificed in extremis, both sides were fixed.

[f] Procedure conducted for all animals of Groups 1 and 4, all males that fail to sire, females that fail to deliver pups and females with total litter loss.

[g] Procedure conducted for all animals of Groups 1 and 4, and all females with total litter loss. Collect inguinal region with skin.

[h] Examined only if present in the routine section of the eye.

[i] Collected and weighed with gland, thyroid. Examined only if present in the routine section of thyroid.

[j] Collected and weighed dorsolateral and ventral parts combined. In the event of a treatment-related effect on total prostate weight, dissect (after fixation) and weigh separately.

[k] Infused with formalin.

[l] Examined only if present in the routine section of the eye. Part of the optic nerve remained attached to the eye and fixed in Modified Davidsons's fixative. The remaining part of the optic nerve was placed in formalin.

[m] Examined one transverse and one longitudinal section from each of the following areas: cervical, mid-thoracic, lumbar.

[n] For the testes a detailed qualitative examination was made taking into account the tubular stages of the spermatogenic cycle. The examination was conducted in order to identify treatment related effects such as missing germ cell layers or types, retained spermatids, multinucleate or apoptotic germ cells and sloughing of spermatogenic cells into the lumen. Any cell- or stage-specificity of testicular findings was noted.

[o] Left side only.

Table 3. Tissue Collection and Preservation for the F1-Generation – Cohort 1A and all F1-animals which die spontaneously or are sacrificed in extremis from weaning onwards.

Tissue	**Weigh [a]**	**Macroscopic Evaluation and Collection**	**Histology Processing**	**Microscopic Evaluation [b, c]**
Animal identification	-	X	-	-
Body cavity, nasal	-	X	-	-
Bone marrow, sternum	-	X	X d	X d
Bone, sternum	-	X	X	X
Brain	X	X	X e	X e
Epididymis	X (2)	X (2)	X (1)	X (1) f
Eye	-	X (2)	X (2)	X (2)

Gland, adrenal	X (2)	X (2)	X (2)	X (2)
Gland, harderian	-	X (2) g	-	-
Gland, mammary	-	X	X	X h
Gland, parathyroid	X (2) i	X (2) i	X (2)	X (2) i
Gland, pituitary	X	X	X	X
Gland, prostate	X j	X j	X	X
Gland, seminal vesicle including coagulation gland and fluid	X (2)	X (2)	X (2)	X (2)
Gland, thyroid	X (2)	X (2)	X (2)	X (2)
Gross lesions/ masses	-	X	X	X
Heart	X	X	X	X
Kidney	X (2)	X (2)	X (2)	X (2)
Large intestine, caecum	-	X	X	X
Large intestine, colon	-	X	X	X
Large intestine, rectum	-	X	X	X
Liver	X	X	X	X
Lung	-	X k	X	X
Lymph node	X l	X l	X l	X l
Muscle, skeletal	-	X	X	X
Nerve, optic	-	X m	X	X m
Nerve, sciatic	-	X (2)	X (1)	X (1)
Ovaries including oviducts	X (2)	X (2)	X (2) n	X (2) n

Skin	-	X	-	-
Small intestine, duodenum	-	X	X	X
Small intestine, ileum	-	X	X	X
Small intestine, jejunum	-	X	X	X
Spinal cord	-	X	X o	X o
Spleen	X	X	X	X p
Stomach	-	X	X	X
Testes	X (2)	X (2)	X (2)	X (2) q
Thymus	X	X	X	X
Trachea	-	X	X	X
Urinary bladder	-	X	X	X
Uterus/Cervix	X	X	X	X
Vagina	-	X	X	X
Vas deferens	-	X (1) r	X (1) r	X (1) r

X = Procedure conducted; - = Not applicable. (1) = one side. (2) = both sides.

[a] Organ weights were not determined for animals which die spontaneously or are sacrificed in extremis.

[b] In first instance, histopathological examination was performed on tissues of animals in the control and high dose groups (Groups 1 and 4), unless otherwise indicated (i.e. bone marrow, lymph nodes, spleen; see specific footnotes below). In case a treatment-related effect is suspected, histopathological examination was extended to animals in the intermediate dose groups (Groups 2 and 3).

[c] Efforts were made to evaluate all protocol-required tissues microscopically; however, it was not always feasible for every protocol-required tissue to be present on every slide. Protocol-required tissues that are not examined were documented in the histopathology data and the impact of these missing tissues on the study was documented in the pathology report.

[d] From all animals of Groups 1 and 4, and from 10 selected animals/sex of Groups 2 and 3, bone marrow were evaluated histopathologically.

[e] Eight brain levels were examined including cerebellum, midbrain and cortex.

[f] As default, the right epididymis were used for evaluation of sperm parameters and the left side were collected for histopathology. If there were gross findings in the right epididymis, the abnormal side was fixed and the left epididymidis was used for evaluation of sperm parameters. If abnormalities were

found in both epididymes, both sides were fixed. For males found dead or sacrificed in extremis, both sides were fixed.

[g] Examined only if present in the routine section of the eye.

[h] Collected inguinal region with skin. Examined for both males and females.

[i] Collected and weighed with gland, thyroid. Examined only if present in the routine section of thyroid.

[j] Collected and weighed dorsolateral and ventral parts combined. In the event of a treatment-related effect on total prostate weighted, dissected (after fixation) and weighed separately.

[k] Infused with formalin.

[l] From 10 selected animals/sex of all groups, the lymph nodes were collected (axillary and mesenteric site), weighed (axillary lymph nodes were weighed paired) and evaluated histopathologically.

[m] Examined only if present in the routine section of the eye. Part of the optic nerve remained attached to the eye and fixed in Modified Davidsons's fixative. The remaining part of the optic nerve was placed in formalin.

[n] From all females of Groups 1 and 4, one ovary was examined for ovarian follicle counts and a quantitative evaluation of primordial and small growing follicles, as well as corpora lutea.

[o] Examined one transverse and one longitudinal section from each of the following areas: cervical, mid-thoracic, lumbar.

[p] From 10 selected animals/sex of all groups, after determination of spleen weight, half of the spleen was kept on ice until splenic lymphocyte subpopulation analysis and the other half of the spleen was used for histopathology evaluation. From the remaining 10 animals/sex/group, the total spleen was used for histopathology.

[q] For the testes a detailed qualitative examination was made taking into account the tubular stages of the spermatogenic cycle. The examination was conducted in order to identify treatment related effects such as missing germ cell layers or types, retained spermatids, multinucleate or apoptotic germ cells and sloughing of spermatogenic cells into the lumen. Any cell- or stage-specificity of testicular findings was noted.

[r] Left side only.

Table 4. Tissue Collection and Preservation F1-Generation – Cohort 1B

Tissue	Weigh a	Collect	Histology e	Microscopic Evaluation
Animal identification	-	X	-	-
Epididymes	X (2)	X (2)	X (1) e	-
Gland, adrenal	X	X	X e	-
Gland, mammary	-	X b	X e	-
Gland, parathyroid	X (2) c	X (2) c	X (2) e	-
Gland, pituitary	X	X	X e	-

Gland, prostate	X d	X d	X e	-
Gland, seminal vesicle including coagulation gland and fluid	X (2)	X (2)	X (2) e	-
Gland, thyroid	X (2)	X (2)	X (2) e	-
Gross lesions/ masses	-	X	X e	-
Kidney	X (2)	X (2)	X (2) e	-
Liver	X	X	X e	-
Ovaries including oviducts	X (2)	X (2)	X (2) e	-
Testes	X (2)	X (2)	X (2) e	-
Uterus/Cervix	X	X	X e	-
Vagina	-	X	X e	-

X = Procedure conducted; - = Not applicable. (1) = one side. (2) = both sides.

[a] Organ weights were not determined for animals which die spontaneously or are sacrificed for humane reasons

[b] Collected inguinal region with skin. Examined for females with total litter loss only.

[c] Collected and weigh with gland, thyroid. Examined only if present in the routine section of thyroid.

[d] Collected and weighed dorsolateral and ventral parts combined. In the event of a treatment-related effect on total prostate weighted, dissected (after fixation) and weighed separately.

[e] The tissues of all Cohort 1B animals were processed to block stage.

Table 5. Tissue Collection and Preservation for the F1-Generation – Cohort 1C

Tissue	Weigh	Collect	Histology	Microscopic Evaluation
Animal identification	-	X [a]	-	-
Gross lesions/ masses	-	X	-	-

X = Procedure conducted; - = Not applicable.

[a] Only in case of gross lesions. Cohort 1C: Chip. Positive controls: location ear and tail.

Table 6. Tissue Collection and Preservation F1-Generation – Cohort Surplus

Tissue	Weigh a	Collect	Histology	Microscopic Evaluation
Animal identification	-	X	-	-
Brain [b]	X	X	-	-
Gland, mammary (both sexes) [c]	-	X	-	-
Gross lesions/ masses	-	X	-	-
Spleen	X	X	-	-
Thymus	X	X	-	-

X = Procedure conducted; - = Not applicable.

[a] Organ weights were not determined for animals which die spontaneously or are sacrificed in extremis.

[b] Seven brain levels to be examined including cerebellum, midbrain and cortex.

[c] Collect inguinal region with skin. Examine for both males and females.

Results and discussion

Results: P0 (first parental generation)

General toxicity (P0)

Clinical signs
effects observed, treatment-related

Description (incidence and severity)
No test material-related clinical signs were noted during the study period up to 300 mg/kg bw/day.
Salivation (up to moderate degree) was seen occasionally after dosing during Days 9-62 and Days 9-51 of premating in males and females, respectively, of the 1000 mg/kg bw/day dose group.
One female at 1000 mg/kg bw/day was noted with abnormal breathing sounds on multiple days (pre - and post-dose) throughout the treatment period. At the single incidence, this was considered unrel ated to treatment with the test material.
Any other clinical signs noted during the treatment period occurred in control animals or were within the range of background findings to be expected for rats of this age and strain which are housed and treated under the conditions in this study and did not show any apparent dose-related trend. At the incidence observed, these were considered not test material-related.
Please refer to tables 7a, 7b, 7c, 7d.

Mortality
mortality observed, non-treatment-related

Description (incidence)
No test material-related mortality occurred during the study period.
In total, one control female, one male at 100 mg/kg bw/day, one female at 300 mg/kg bw/day and one female at 1000 mg/kg bw/day were euthanized for humane reasons or found dead.
Female No. 116 (control) was euthanized for humane reasons on Day 24 of gestation due to diffi culties at delivery. This female was in progress of delivery during parturition check. It was noted that it had a dark purple membrane in the vulva that appeared to block the exit and the vulva area was severely swollen. Additionally, this female presented with erected fur and hunched posture. At ne cropsy, black mucoid content and two live fetuses were noted in the uterus. Main microscopic finding s included mild erosion/ulceration of the uterus, marked extramedullary hematopoiesis of the spleen and mild decreased lymphoid cellularity in the thymus. Delivery difficulties were regarded as the ca use of moribundity of this control female. As this was a control animal, its death was unrelated to treatment with the test material.
Female No. 161 (300 mg/kg bw/day) was found dead on Day 24 of gestation. No clinical signs were noted for this female, and body weight (gain) was normal up to Day 20 of gestation. At nec ropsy, autolysis, dark red foci on the thymus and 10 dead fetuses in the uterus were noted. Main mic roscopic findings included moderate extramedullary hematopoiesis in the spleen and mild multifocal hepatocellular necrosis in the liver. The necrosis in the liver might be related to the presence of dead material (fetuses) in the uterus and the combination of these findings was regarded the cause of death for this female. The hepatocellular necrosis and dead fetuses were regarded as an incidental finding and therefore, unrelated to the treatment with the test material.
Female No. 198 (1000 mg/kg bw/day) was found dead in the morning on Day 13 of treatment. At ne cropsy, a perforation of the esophagus was noted. Therefore, this mortality was considered procedure -related.
Male No. 44 (100 mg/kg bw/day) was euthanized for humane reasons on Day 2 of treatment due to elongated upper incisors. This finding, and therefore the death of this animal, was considered not related to treatment with the test material. The animal was replaced with spare Male No. 3001.
Finally, total litter loss was noted at 100 mg/kg bw/day (Female No. 142 on PND 4), 300 mg/kg bw/ day (Female Nos. 152 on PND 1 and 157 on PND 3) and 1000 mg/kg bw/day (Female No. 176 on PND 1).

Body weight and weight changes
no effects observed

Description (incidence and severity)
Body weights and body weight gain of test material-treated animals remained in the same range as controls over the treatment period.
Any variations in body weights and body weight gain were considered not to be related to treatment with the test material since no trend was apparent regarding dose and duration of treatment.
Please refer to tables 8a, 8b, 8c, 8d, 9a, 9b, 9c and 9d.

Food consumption and compound intake (if feeding study)
no effects observed

Description (incidence and severity)
Food consumption of test material-treated animals remained in the same range as controls over the tr eatment period.
Any changes in food consumption were considered not to be related to treatment with the test m aterial since no trend was apparent regarding dose and duration of treatment.
Refer to table 10a, 10b, 10c, 10d.

Haematological findings
no effects observed

Description (incidence and severity)
Hematological parameters were considered unaffected by treatment with the test material.

Any differences in hematology parameters, regardless of statistical significance, were considered not test material-related, based on the absence of a dose response and/or general overlap of individual values with the range of control values.
Please refer to table 11a, 11b.
Coagulation parameters were considered unaffected by treatment with the test material.
Please refer to table 12a, 12b.

Clinical biochemistry findings
effects observed, treatment-related

Description (incidence and severity)
Clinical chemistry parameters were considered unaffected by treatment with the test material in males up to 300 mg/kg bw/day and in females up to the highest dose level tested (1000 mg/kg bw/day).
In males, statistically significantly decreased urea concentrations were noted at 1000 mg/kg bw/day (0.86x of control).
Remaining differences in clinical chemistry parameters, regardless of statistical significance, were considered not test material-related, based on the absence of a dose response, general overlap of individual values with the range of control values, and/or were of a magnitude of change commonly observed in rats under similar study conditions.
Please refer to tables 13a, 13b.

Endocrine findings
effects observed, treatment-related

Description (incidence and severity)
Serum levels of T4 and TSH were considered unaffected by treatment with the test material in females at 100 and 1000 mg/kg bw/day and in males up to the highest dose level tested (1000 mg/kg bw/day).
In females at 300 mg/kg bw/day, T4 levels were increased (1.22x of control, not statistically significant). Mean value remained within the historical control range (table 13c) and thus, this slight increase is considered to be not related to the test material. TSH levels were unaffected.
Please refer to tables 13a, 13b.

Urinalysis findings
no effects observed

Description (incidence and severity)
Urinalysis parameters were considered unaffected by treatment with the test material.
The statistically significantly increased specific gravity in females at 1000 mg/kg bw/day was considered not test material related based on a general overlap of individual values with the range of control values.
Please refer to tables 14a and 14b.

Organ weight findings including organ / body weight ratios
effects observed, treatment-related

Description (incidence and severity)
There were no test material-related changes in organ weights up to 300 mg/kg bw/day.
There were statistically significantly increased relative kidney and liver weights at 1000 mg/kg bw/day which were regarded test material-related.

- Kidney: Statistically significantly increased kidney weights (relative to body weight) were noted in males and females at 1000 mg/kg bw/day.
- Liver: Statistically significantly increased liver weights (relative to body weight) were noted in males and females at 1000 mg/kg bw/day.

There were no other test material-related organ weight changes.

Please refer to tables 15a and 15b.

Gross pathological findings
effects observed, non-treatment-related

Description (incidence and severity)
There were no test material-related macroscopic findings up to 1000 mg/kg bw/day.
Watery fluid in the uterus, observed in one, four, five and seven females of the control, 100, 300 and 1000 mg/kg bw/day groups, respectively, is related to a stage in the estrous cycle and is not a sign of toxicity.
All of the remaining recorded macroscopic findings were within the range of background gross observations encountered in rats of this age and strain.
Please refer to table 16.

Histopathological findings: non-neoplastic
no effects observed

Description (incidence and severity)
There were no test material-related microscopic findings in the F0-animals up to 1000 mg/kg bw/day.
All the recorded microscopic findings were within the range of background pathology encountered in rats of this age and strain. There was no test material related alteration in the prevalence, severity, or histologic character of those incidental tissue alterations.
Please refer to table 17.

Reproductive function / performance (P0)

Reproductive function: oestrous cycle
effects observed, non-treatment-related

Description (incidence and severity)
Length and regularity of the estrous cycle were considered unaffected by treatment with the test mat erial.
Most females had regular cycles of 4 to 5 days. A regular cycle followed by an extended di-estrus was noted in Female No. 106 (control) and an irregular cycle was noted in Female No. 116 (control). Both females were pregnant. As these findings occurred in control females only, they were not test materi al-related.
Note: Estrous cycle data is available from the start of the mating period until mating of the animals was confirmed by evidence of sperm in the vaginal lavage or by the appearance of an intravaginal copulatory plug. This data was only collected for confirmation of mating and was not used for evalu ation of the estrous cycle regularity.

Reproductive function: sperm measures
effects observed, non-treatment-related

Description (incidence and severity)
Sperm motility, concentration and morphology parameters were considered unaffected by treatment with the test material.
For two males at 1000 mg/kg bw/day (Male Nos. 86 and 90), very low sperm motility (1%) and no progressive sperm were noted. These findings are occasionally noted in rats under similar study conditions and at the incidence noted, were considered not test material-related. It should be noted that both males were able to sire and produced normal offspring.
The statistically significantly increased sperm density noted at 1000 mg/kg bw/day was considered not test material-related based on a general overlap of individual values with the range of control values and as it was at a magnitude of change commonly observed in rats under similar conditions.
Any other differences noted in sperm parameters, regardless of statistical significance, were con sidered not test material-related based on the absence of a dose response
Please refer to tables 18 and 19.

Reproductive performance
effects observed, non-treatment-related

Description (incidence and severity)
REPRODUCTIVE DATA:
-Mating index for males and females was unaffected by treatment with the test material.

-Precoital time was considered not to be affected by treatment with the test material. Most females showed evidence of mating within 5 days, except for one control female (No. 119), two females at 100 mg/kg bw/day (Nos. 145 and 150) and one female at 1000 mg/kg bw/day (No. 178), which were confirmed mated after 13 or 14 days. The incidence of animals with a longer precoital interval was considered not related to treatment with the test material as this is commonly observed in rats under similar study conditions and was noted in absence of a dose response relationship.
-Number of implantation sites was considered unaffected by treatment with the test material. One control female (No. 116) with an incomplete delivery and one female at 100 mg/kg bw/day (No. 142) with a total litter loss on Day 4 of lactation (for details, see Section 7.4.8) had two implantation sites only. As this was noted in absence of a dose response relationship, this was considered unrelated to treatment with the test material. Note: Due to the morphology of the uterus, the complete uterus could not be checked for implantation sites. Only the implantation sites of the fetuses could be counted for Female No. 116.
-Fertility index was considered unaffected by treatment with the test material.
One female at 100 and 300 mg/kg bw/day (Nos. 148 and 156, respectively) and three females at 1000 mg/kg bw/day (Nos. 187, 188 and 189) were not pregnant. Incidental histopathological findings were present in the reproductive organs of Male No. 48 (which mated with Female No. 148) and of Female No. 189 (see Section 7.3.6). As these cases of non-pregnancy remained within the normal range and in absence of test material related histopathological findings, they were considered unrelated to treatment with the test material.
- Pregnancy index for males and females was considered unaffected by treatment with the test material.
- Microscopic Evaluation of Reproductive Performance: Couples that did not succeed in producing healthy offspring are summarized in table 20. There were 1/25 couples at 100 mg/kg bw/day (Male No. 48/Female No. 148), 1/25 couples at 300 mg/kg bw/day (Male No. 56/Female No. 156) and 3/24 couples at 1000 mg/kg bw/day (Male Nos. 87, 88, 89/Female Nos. 187, 188, 189) that failed to deliver pups. The findings in the reproductive organs of Male No. 48 (moderate degeneration of the seminiferous tubules in the testis and marked decreased sperm in the epididymis) and of Female No. 189 (membrane between vagina/cervix) were regarded the cause of infertility of these respective couples. These incidental findings were regarded unrelated to the treatment with the test material. No abnormalities were seen in the reproductive organs of the remaining couples which could account for the lack of offspring. There were no findings suggestive of infertility in the reproductive organs of Male No. 98 which did not mate because the female was found dead before mating. Female No. 142 at 100 mg/kg bw/day, Female Nos. 152 and 157 at 300 mg/kg bw/day and Female No. 176 at 1000 mg/kg bw/day had a total litter loss. There were no findings in the reproductive tract or mammary gland of these females that could explain the early death of their offspring. Stage dependent qualitative evaluation of spermatogenesis was performed in the testis of males of Groups 1 and 4 and males that did not produce offspring. The testis revealed normal progression of the spermatogenic cycle and the expected cell associations and proportions in the various stages of spermatogenesis were present.
Please, see also tables 20 and 21a and 21b for a summary of reproductive data.

DEVELOPMENTAL DATA:
-Gestation index (females with living pups on Day 1 compared to the number of pregnant females) and duration of gestation were considered unaffected by treatment with the test material. One control female (No. 116) had only two live fetuses in the right uterine horn and one female at 300 mg/kg bw/day (No. 161) had only five dead fetuses in both the right and left uterine horn, but no live offspring. In addition, all 13 pups of one female at 300 mg/kg bw/day (No. 152) were stillborn and all pups of one female at 1000 mg/kg bw/day (No. 176) were presumed to be cannibalized before PND 1. In the absence of a dose response relationship, these cases without live offspring were considered unrelated to treatment with the test material.
-Parturition/Maternal Care: No signs of difficult or prolonged parturition were noted among the pregnant females, except for control Female No. 116, who was euthanized for humane reasons due to labor difficulties. Examination of cage debris of pregnant females revealed no signs of abortion or premature birth. No deficiencies in maternal care were observed.
-Post-Implantation Loss: The total number of offspring born compared to the total number of uterine implantation sites (post-implantation loss) was considered unaffected by treatment with the test material.The higher post-implantation loss at 1000 mg/kg bw/day was mainly due to one female

(No. 196) with 13 implantations and 5 newborn pups only. Individual values of other females were generally within the range of control values and therefore this change was considered unrelated to treatment with the test material.
-Litter Size F0-Generation: Litter size was considered unaffected by treatment with the test material. One control female (No. 120), one female at 100 mg/kg bw/day (No. 142), two females at 300 mg/kg bw/day (Nos. 152 and 157) and one female at 1000 mg/kg bw/day (No. 196) had none or up to three live newborn pups only. As this was noted in the absence of a dose response relationship, this was considered unrelated to treatment with the test material.
- Sex ratio was considered unaffected by treatment with the test material. Note: The sex of 19 pups at 300 mg/kg bw/day (Pup Nos. 1-13 from Litter No. 152 and Pup Nos. 2 7 from Litter No. 157) and two pups at 1000 mg/kg bw/day (Pup No. 9 from Litter No. 181 and Pup No. 14 from Litter No. 184) that were found dead at first litter check could not be determined due to cannibalism or autolysis.
-Live Birth Index: The number of live offspring on Day 1 after littering compared with the total number of offspring born (live birth index) was considered unaffected by treatment with the test material. One control pup, one pup at 100 mg/kg bw/day, 20 pups (including all 13 pups from Litter No. 152, see below for details of dam, and six pups from Litter No. 157) at 300 mg/kg bw/day and three pups at 1000 mg/kg bw/day were found dead at first litter check. Autolysis or cannibalism precluded determination of the presence of milk in the stomach at necropsy for all pups. As such, it could not be determined whether the pups were stillborn or died after birth. These dead pups were considered unrelated to treatment with the test material, since the mortality incidence did not show a dose related trend and remained within the range considered normal for pups of this age. Female No. 152 (300 mg/kg bw/day) had a total litter loss on Day 1 of lactation. No clinical signs were noted for this female and at necropsy an enlarged spleen and gelatinous consistency of the thymus were noted. For Female No. 176 (1000 mg/kg bw/day) a total litter loss was presumed on Day 1 of lactation as the start of the delivery was noted, but no pups were found during the pup observations. This female had 13 implantation sites. Pups were presumably cannibalized before the pup observations were performed. Erected fur and hunched posture were noted for this female on the last days of gestation and pale discoloration of the adrenal glands and liver, an enlarged spleen and a small thymus were noted at necropsy. These single cases of (suspected) total litter loss were considered incidental findings in absence of histopathological findings and unrelated to treatment with the test material. Please see also table 22 and 23 for a summary of developmental data.

Please, see F1 mortality results for viability and weaning indexes.

Effect levels (P0)

Key result
true

Dose descriptor
NOAEL Reproductive toxicity

Effect level

>=	1000	mg/kg bw/day (nominal)

Based on
test mat.

Sex
male/female

Basis for effect level
other: No effects seen up to the highest dose level tested (1000 mg/kg bw/day)

Remarks on result
not determinable due to absence of adverse toxic effects

Key result
true

Dose descriptor
NOAEL General toxicity

Effect level

>=	1000	mg/kg bw/day (nominal)

Based on
test mat.

Sex
male/female

Basis for effect level
other: No effects seen up to the highest dose level tested (1000 mg/kg bw/day)

Remarks on result
not determinable due to absence of adverse toxic effects

Target system / organ toxicity (P0)

Key result
true

Critical effects observed
no

Results: F1 generation

General toxicity (F1)

Clinical signs
effects observed, treatment-related

Description (incidence and severity)
Until Weaning:
No clinical signs occurred among pups that were considered related to treatment with the test material. The nature and incidence of clinical signs remained within the range considered normal for pups of this age, and were therefore considered unrelated to treatment with the test material.

From Weaning onwards:
No test material-related clinical signs were noted during clinical observations at 100 mg/kg bw/day.
Salivation (up to severe degree) was seen occasionally after dosing and incidentally also before do sing in males and females at 1000 mg/kg bw/day. Salivation was also noted in a few animals at 300 mg/kg bw/day on one or two days. Based on the low incidence at this dose level, this was considered unrelated to treatment with the test material.
Abnormal breathing sounds (pre- and post-dose) were transiently (up to 6 days) observed in males and females at 300 and 1000 mg/kg bw/day with a dose-related increase in the number of affected animals and with more affected males than females. Abnormal breathing sounds were also noted once in one female at 100 mg/kg bw/day. At this single incidence, this was considered unrelated to treatment with the test material.
Any other clinical signs noted during the treatment period occurred in control animals or were within the range of background findings to be expected for rats of this age and strain which are housed and

treated under the conditions in this study, did not show any apparent dose-related trend and/or occurred in a single animal only. At the incidence observed, these were considered not test material-related. Please refer to table 26.

Mortality / viability
mortality observed, non-treatment-related

Description (incidence and severity)
Until Weaning:
- Viability index: The number of live pups on Day 4 before culling compared to the number of pups on Day 1 (viability index) was considered unaffected by treatment with the test material. Five, one and three pups at 100, 300 and 1000 mg/kg bw/day, respectively, were found dead or missing on PND 1, 3 or 4. No abnormalities or autolysis were noted at necropsy for the dead pups. Pups missing were most likely cannibalized. On the days before missing, the pup at 300 mg/kg bw/day was noted with moderate up to severe limited usage of the paws, thin appearance, slight suspected dehydration and no milk band present. Additionally, one pup at 100 mg/kg bw/day was euthanized for humane reasons on PND 1 as it had a severe skin lesion, located dorsal cervical. These dead, missing or euthanized pups were considered unrelated to treatment with the test material, since the mortality incidence did not show a dose-related trend and remained within the range considered normal for pups of this age. Female No. 157 (300 mg/kg bw/day) had a total litter loss on Day 3 of lactation. At necropsy an enlarged spleen and small thymus were noted. Based on the single incidence in the mid dose group only and in absence of histopathological (and other relevant) findings, this total litter loss was considered unrelated to treatment with the test material.
- Lactation index: The number of live offspring at weaning (PND 21) compared to the number of live offspring on Day 4 (after culling) was considered unaffected by treatment with the test material.
At 100 mg/kg bw/day, the last remaining pup of Litter No. 142 was euthanized for humane reasons as it presented with a thin appearance, no milk band present, cold to touch and decreased activity on PND 4, resulting in a total litter loss on Day 4 of lactation for Female No. 142. No abnormalities were noted for both the pup and dam at necropsy.
The death of this pup and resulting total litter loss were considered unrelated to treatment with the test material, since the mortality incidence did not show a dose-related trend and remained within the range considered normal for pups of this age.

Please refer to table 23.

From weaning onwards:
No test material-related mortality occurred during the study period.
In total, one male at 100 mg/kg bw/day, two females at 300 mg/kg bw/day and one male and three females at 1000 mg/kg bw/day were euthanized for humane reasons or found dead.
Male No. 324 (Cohort 1C, 100 mg/kg bw/day) was found dead shortly after dosing on Day 21 relative to start of the F1-generation. No clinical signs were noted on the days before its death and body weights and body weight gain were considered normal up to Day 15. Relevant findings at necropsy consisted of lung findings (failed to collapse, dark red foci and pale discoloration), without microscopic correlates. There were no microscopic findings of note, and the cause of death was undetermined.
Female No. 635 (Cohort 1A, 300 mg/kg bw/day) was found dead prior to dosing on Day 22 relative to start of the F1-generation. Abnormal breathing sounds and hunched posture were noted from Day 18 onwards. Low body weight gain between Days 8-15 resulting in a low body weight in Day 15 were also noted for this female. At necropsy, failure to collapse of the lungs were noted. At microscopic examination the lungs showed post-mortem changes and no cause of death could be determined.
Male No. 414 (Cohort 1A, 1000 mg/kg bw/day) was found dead shortly after dosing on Day 19 relative to start of the F1-generation. No clinical signs were noted on the days before its death and body weights and body weight gain were considered normal up to Day 15. At necropsy, failure to collapse the lungs were noted. There was no microscopic correlate for the lung finding or other microscopic findings of note and the cause of death was undetermined.
Female No. 716 (Cohort 1B, 1000 mg/kg bw/day) was found dead shortly after dosing on Day 9 relative to start of the F1-generation. No clinical signs were noted on the days before its death and body weights and body weight gain were considered normal up to Day 8. At necropsy, failure to co

llapse the lungs and clear red fluid in the thoracic cavity were noted. There were no microscopic co rrelates for these findings and no microscopic findings that could explain the death of this animal.
Female No. 718 (Cohort 1B, 1000 mg/kg bw/day) was found dead shortly after dosing on Day 17 relative to start of the F1-generation. No clinical signs were noted on the days before its death and body weights and body weight gain were considered normal up to Day 15. At necropsy, dark red fo ci on the lungs and pale discoloration of the lungs and lacrimal gland were noted. There was no micr oscopic correlate for the lung findings or other microscopic findings of note and the cause of death was undetermined.
The few deaths with undetermined cause of death occurred in young animals of different dose groups without a clear dose-related trend were considered unrelated to treatment with the test material.
Female No. 643 (Cohort 1B, 300 mg/kg bw/day) was euthanized for humane reasons on Day 70 relat ive to start of the F1-generation based on severe body weight loss (20%) between Days 64-70. Clinica l signs noted for this female were hunched posture (from Day 66 onwards) and abnormal breathing sounds (Day 70). Findings at necropsy consisted of pale discolored lungs that failed to collapse and decreased adipose tissue. Microscopic findings included hypertrophy/hyperplasia of the epithelium and presence of foreign material in the trachea and bronchus, moderate mononuclear inflammation and mild erosions in the lung, all suggestive of a gavage procedure-related cause of death. This death was therefore considered unrelated to treatment with the test material.
Female No. 742 (Cohort 1C, 1000 mg/kg bw/day) was euthanized for humane reasons on Day 10 relative to start of the F1-generation. Clinical signs noted on the day of death consisted of abnormal breathing sounds and hunched posture and severe body weight loss (21%) was also observed be tween Days 8-10. At necropsy, dark red foci on the glandular stomach were noted. Main microscopic findings included an erosion/ulcer (minimal) and mild diffuse hyperplasia of the epithelium of the non -glandular stomach. These stomach lesions are likely gavage procedure-related and contributed to the poor condition of this animal. This death was therefore considered unrelated to treatment with the test material.

Note: For the pup(s) found dead, missing or that were preterminally euthanized after day 1, all relevant findings mentioned under F1 mortality results will not be included under F1 clinical signs, body we ights, macroscopic findings, etc., unless specified otherwise. Likewise, for pups found dead on day 1 all relevant findings are mentioned under Live Birth Index (in F0 reproductive performance results).

Body weight and weight changes
effects observed, treatment-related

Description (incidence and severity)
Until Weaning:
Body weights of pups were considered unaffected by treatment with the test material.

From weaning onwards:
Body weights and body weight gain were considered unaffected by treatment with the test material in males up to 300 mg/kg bw/day and in females up to the highest dose level tested (1000 mg/kg bw/ day).
In males at 1000 mg/kg bw/day, a statistically significantly decreased body weight gain was noted during multiple weeks throughout the treatment period resulting in a decreased overall body weight gain (Days 1-70) and a 4% body weight decrease compared to control from Day 64 of treatment.
Any other variations in body weights and body weight gain were considered unrelated to treatment with the test material since no trend was apparent regarding dose and/or duration of treatment.
Please refer to tables 27a, 27b and 28.

Food consumption and compound intake (if feeding study)
no effects observed

Description (incidence and severity)
Food consumption of test material-treated animals remained in the same range as controls over the tr eatment period.

Haematological findings
no effects observed

Description (incidence and severity)
Haematology of Cohort 1A:
Hematological parameters were considered unaffected by treatment with the test material. Any differences in hematology parameters, regardless of statistical significance, were considered not test material-related, based on the absence of a dose response, general overlap of individual values with the range of control values, and/or were of a magnitude of change commonly observed in rats under similar study conditions.

Coagulation parameters of Cohort 1A:
Coagulation parameters were considered unaffected by treatment with the test material. Any di fferences in coagulation parameters were considered unrelated to treatment with the test material in absence of a dose-related trend.

Clinical biochemistry findings
effects observed, treatment-related

Description (incidence and severity)
Clinical biochemistry:
Clinical chemistry parameters were considered unaffected by treatment with the test material. Any differences in hematology parameters, regardless of statistical significance, were considered not test material-related, based on the absence of a dose response, general overlap of individual values with the range of control values, a relative low control value and/or were of a magnitude of change commonly observed in rats under similar study conditions.
Please refer to tables 30a and 30b.

Thyroid hormone analyses:
- PND 4- F1 Pups:
Serum T4 levels in male and female pups, culled at PND 4, were considered unaffected by treatment with the test material. Please refer to table 25.
- Cohort 1A (PND 89-95)
Serum levels of T4 were considered unaffected by treatment with the test material in females at 100 and 1000 mg/kg bw/day and in males up to the highest dose level tested (1000 mg/kg bw/day) and TSH levels were unaffected in males and females up to the highest dose level tested (1000 mg/kg bw/day).
In females at 300 mg/kg bw/day, T4 level was increased when compared with controls (1.20x of control, not statistically significant). Mean level remained within the historical control range (table 30c).
- Cohort Surplus (PND 22-24)
Serum levels of T4 and TSH were considered unaffected by treatment with the test material.
Please refer to tables 30a, 30b and 31.

Urinalysis findings
effects observed, non-treatment-related

Description (incidence and severity)
Urinalysis parameters were considered unaffected by treatment with the test material. One male and one female at 1000 mg/kg bw/day had a very high urine volume (35 and 30 mL, respectively). At the single incidence noted for each sex, this finding was considered not test material-related.

Sexual maturation
effects observed, non-treatment-related

Description (incidence and severity)
Sexual maturation was considered unaffected by treatment with the test material.

In males at 300 mg/kg bw/day, balanopreputial separation was delayed (42.0 vs 40.6 days for the control) but this was considered to be unrelated to treatment with the test material since no dose-related trend was noted and values remained within normal range [BPS Historical control data Wistar Han (Year 2018-September 2022): Mean PND=41.4; P5-P95 = 39.00-45.00 (n=1477)].
A longer time from vaginal opening to first estrus was noted in females at 100, 300 and 1000 mg/kg bw/day (3.8, 3.7 and 3.9 vs 2.8 for the control at 100, 300 and 1000 mg/kg bw/day, respectively), although this increase in time to first estrus was not statistically significant. This was considered to be the result of a relatively low control value compared with historical control data (table 29b) and to be unrelated to treatment with the test material.
Please refer to table 29a.

Anogenital distance (AGD)
effects observed, non-treatment-related

Description (incidence and severity)
Anogenital distance (absolute and normalized for body weight) in male and female pups was considered unaffected by treatment with the test material.
For Litter No. 173 (1000 mg/kg bw/day), remarkable large anogenital distances (absolute and normalized for body weight) in female pups were noted. As this was noted for one litter only, this was considered an incidental finding and unrelated to treatment with the test material.
Please refer to table 24.

Nipple retention in male pups
no effects observed

Description (incidence and severity)
Treatment with the test material had no effect on areola/nipple retention. No male pups were observed on PND 13 to have retained nipples.

Organ weight findings including organ / body weight ratios
effects observed, treatment-related

Description (incidence and severity)
There were no test material-related changes in organ weights up to 300 mg/kg bw/day in the F1 animals of Cohort 1A and 1B.
There were increased kidney and liver weights in the F1-animals at 1000 mg/kg bw/day which were regarded test material-related.

- Cohort 1A (PND 89-95)
  - Kidney: Statistically significantly increased kidney weights were noted at 1000 mg/kg bw/day in males (relative to body weight) and females (absolute) of Cohort 1A.
  - Liver: Statistically significantly increased liver weights were noted at 1000 mg/kg bw/day in males (relative to body weight) and females (absolute) of Cohort 1A.

The statistically significantly decreased thyroid weights (absolute and relative to body weight) in females at 300 mg/kg bw/day and the lower axillary lymph node weights in females of all treatment groups (statistically significant as absolute value at 100 and 1000 mg/kg bw/day) of Cohort 1A were considered unrelated to treatment with the test material, in absence of a dose-related pattern.
- Cohort 1B (≥ PND 90)
  - Kidney: Statistically significantly increased kidney weights (absolute and relative to body weight) were noted in females at 1000 mg/kg bw/day of Cohort 1B.
  - Liver: Statistically significantly increased liver weights were noted in males (relative to body weight) and in females (absolute and relative to body weight) at 1000 mg/kg bw/day of Cohort 1B.

The statistically significantly decreased thyroid gland and liver weights in males of Cohort 1B at 100 mg/kg bw/day were considered unrelated to treatment with the test material in absence of a dose-related pattern.

Cohort Surplus (PND 22-24)
There were no test material-related organ weight changes in males and females up to 1000 mg/kg bw/day.The statistically significantly increased spleen weights (relative to body weight) in males at 300

mg/kg bw/day were considered unrelated to treatment with the test material in absence of a dose-related pattern.

Please refer to tables 35a and 35b.

Gross pathological findings
effects observed, non-treatment-related

Description (incidence and severity)
Until Weaning:
No macroscopic findings were noted among pups sacrificed at culling (PND 4) or at the end of the lactation period that were considered to be related to treatment with the test material. The nature and incidence of macroscopic findings remained within the range considered normal for pups of this age, and were therefore considered unrelated to treatment with the test material.

From Weaning onwards:
- Cohort 1A (PND 89-95)
There were no test material-related macroscopic findings in males and females up to 1000 mg/kg bw/day. Watery fluid in the uterus, observed in three, five, six and two females for the control, 100, 300 and 1000 mg/kg bw/day groups, respectively, is related to a stage in the estrous cycle and is not a sign of toxicity. All macroscopic findings were within the range of background gross observations encountered in rats of this age and strain.

- Cohort 1B (≥ PND 90)
There were no test material-related macroscopic findings in males and females up to 1000 mg/kg bw/day. Watery fluid in the uterus, observed in five, two, two and four females for the control, 100, 300 and 1000 mg/kg bw/day groups, respectively, is related to a stage in the estrous cycle and is not a sign of toxicity.All remaining macroscopic findings were within the range of background gross observations encountered in rats of this age and strain.

- Cohort 1C (males: ≥ PND 35; females: ≥ PND 25)
There were no test material-related macroscopic findings in males and females up to 1000 mg/kg bw/day. All macroscopic findings were within the range of background gross observations encountered in rats of this age and strain.

- Cohort Surplus (PND 22-24)
There were no test material-related macroscopic findings in males and females up to 1000 mg/kg bw/day. All macroscopic findings were within the range of background gross observations encountered in rats of this age and strain.

Histopathological findings
effects observed, non-treatment-related

Description (incidence and severity)
There were no test material-related microscopic findings in the F1-animals of Cohort 1A up to 1000 mg/kg bw/day. All recorded microscopic findings were regarded within the range of background pathology encountered in rats of this age and strain. There was no test material related alteration in the prevalence, severity, or histologic character of those incidental tissue alterations.

- Spermatogenesis
Stage dependent qualitative evaluation of spermatogenesis was performed in the testis of males of the control group and the males treated at 1000 mg/kg bw/day. The testis revealed normal progression of the spermatogenic cycle and the expected cell associations and proportions in the various stages of spermatogenesis were present.

- Ovarian Follicle and Corpora Lutea Counts (cohort 1A)

There were no test material-related effects on the ovarian follicle counts and the corpora lutea counts in females of Cohort 1A in the 1000 mg/kg bw/day group when compared to control Group. Any variation between group mean counts represented biological variability and were not statistically si gnificant.

Other effects
effects observed, non-treatment-related

Description (incidence and severity)
- Estrous Cycle in Cohort 1A animals:
Length and regularity of the estrous cycle were considered unaffected by treatment with the test mate rial. Most females had regular cycles of 4 to 5 days. An irregular cycle was noted in Female Nos. 621 and 633 (300 mg/kg bw/day) and 706 (1000 mg/kg bw/day). As these findings occurred in absence of a dose-related trend, they were considered not test material-related.

- Sperm Analysis in Cohort 1A animals:
Sperm motility, concentration and morphology parameters were considered unaffected by treatment with the test material. For Male Nos. 202, 218 (control), 286, 287 (100 mg/kg bw/day) and 430 (1000 mg/kg bw/day), very low (1%) or no motile and progressive sperm were noted. These findings are occasionally noted in rats under similar study conditions and at the incidence noted, and in absence of a dose response, were considered not test material-related.
The statistically significantly increased sperm density noted at 100 mg/kg bw/day was considered not test material-related in absence of a dose-related trend.
An increased number of sperm with coiled tail noted at 1000 mg/kg bw/day (not statistically s ignificant) was mainly due to one male with a very high number. Based on the single incidence, this was considered unrelated to treatment with the test material.
Note: For three, three, two and three males of the control, 100, 300 and 1000 mg/kg bw/day groups, respectively, no sperm morphology data was available as less than 200 cells could be counted. This is occasionally noted in rats under similar study conditions. As males were evenly distributed over the groups and there were sufficient number of animals still available, this was without an effect on the interpretation of the study results.
Please refer to tables 32 and 33.

Developmental immunotoxicity (F1)

Developmental immunotoxicity
effects observed, non-treatment-related

Description (incidence and severity)
There were no test material-related effects on splenic lymphocyte subpopulations observed.
A slightly decreased T-cytotoxic cell splenic subpopulation was noted for females at 1000 mg/kg bw/day (not statistically significant), resulting in an increased T-helper cells/ T-cytotoxic cells ratio. This was mainly caused by two females (Nos. 699 and 709). As these shifts were noted in two fem ales only and occurred in the absence of corroborative findings in spleen, this was considered to re present biological variability and was regarded unrelated to treatment with the test material.

Please refer to table 34.

Details on results (F1)

For Tables and Appendices presented as “Day(s): - relative to start date”, the start date, or “Day 1” is the first PND 21 (i.e. 30 Jan 2024) for the entire F1-generation. For Tables and Appendices presented as “Day(s): - relative to birth date”, Day 0 is the respective birth date (PND 0) of each individual an imal.

Effect levels (F1)

Key result
true

Dose descriptor
NOAEL Developmental toxicity

Generation
F1

Effect level

>=	1000	mg/kg bw/day (nominal)

Based on
test mat.

Sex
male/female

Basis for effect level
other: No effects seen (before or after weaning) up to the highest dose level tested (1000 mg/kg bw/day)

Remarks on result
not determinable due to absence of adverse toxic effects

Key result
true

Dose descriptor
NOAEL General toxicity

Generation
F1

Effect level

>=	1000	mg/kg bw/day (nominal)

Based on
test mat.

Sex
male/female

Basis for effect level
other:
No effects seen up to the highest dose level tested (1000 mg/kg bw/day)

Remarks on result
not determinable due to absence of adverse toxic effects

Target system / organ toxicity (F1)

Key result
true

Critical effects observed
no

Overall reproductive toxicity

Key result
true

Reproductive effects observed
no

Any other information on results incl. tables

Table 7a. Summary of Clinical Observations: F0 Generation - Males

Observation Type: All Types	*Male*			
From Day 1 (Start Date) to 89 (Start Date)	*0 mg/kg bw/day Group 1*	*100 mg/kg bw/day Group 2*	*300 mg/kg bw/day Group 3*	*1000 mg/kg bw/day Group 4*
Salivation				
Number of Animals Affected	*0*	*0*	*1*	*16*
Number of Times Recorded	*0*	*0*	*2*	*49*
First to Last seen	*-*	*-*	*21 - 42*	*9-62*
Breathing, Abnormal Sounds				
Number of Animals Affected	*0*	*1*	*3*	*2*
Number of Times Recorded	*0*	*3*	*10*	*2*
First to Last seen	*-*	*62 - 63*	*6-56*	*23 - 55*
Skin, Lesion, Dorsal Cervical				
Number of Animals Affected	*0*	*0*	*0*	*1*
Number of Times Recorded	*0*	*0*	*0*	*3*
First to Last seen	*-*	*-*	*-*	*55 - 56*
Skin, Lesion, Scapular, Left				
Number of Animals Affected	*1*	*0*	*0*	*0*
Number of Times Recorded	*6*	*0*	*0*	*0*
First to Last seen	*37 - 40*	*-*	*-*	*-*
Skin, Scab, Base of Tail				

Number of Animals Affected	*0*	*1*	*0*	*0*
Number of Times Recorded	*0*	*3*	*0*	*0*
First to Last seen	*-*	*87 - 88*	*-*	*-*
Skin, Scab, Dorsal Cervical				
Number of Animals Affected	*0*	*1*	*0*	*1*
Number of Times Recorded	*0*	*41*	*0*	*22*
First to Last seen	*-*	*50 - 71*	*-*	*57 - 67*
Skin, Scab, Scapular, Left				
Number of Animals Affected	*2*	*1*	*0*	*0*
Number of Times Recorded	*168*	*45*	*0*	*0*
First to Last seen	17 - 87	23 - 45	-	-
Skin, Scab, Scapular, Right				
Number of Animals Affected	*0*	*0*	*1*	*0*
Number of Times Recorded	*0*	*0*	*61*	*0*

7b. Summary of Clinical Observations: F0 Generation - Premating

Observation Type: All Types	*Female*			
From Day 1 (Start Date (A)) to -1 (Mating)	*0 mg/kg bw/day Group 1*	*100 mg/kg bw/day Group 2*	*300 mg/kg bw/day Group 3*	*1000 mg/kg bw/day Group 4*
Salivation				
Number of Animals Affected	*0*	*0*	*1*	*9*
Number of Times Recorded	*0*	*0*	*1*	*29*
First to Last seen	*-*	*-*	*43 - 43*	*9-51*
Breathing, Abnormal Sounds				
Number of Animals Affected	*0*	*0*	*1*	*1*
Number of Times Recorded	*0*	*0*	*1*	*34*
First to Last seen	*-*	*-*	*9-9*	*27 - 74*

Hunched Posture				
Number of Animals Affected	*0*	*0*	*0*	*1*
Number of Times Recorded	*0*	*0*	*0*	*5*
First to Last seen	*-*	*-*	*-*	*93 - 95*
Fur, Erected				
Number of Animals Affected	*0*	*0*	*0*	*1*
Number of Times Recorded	*0*	*0*	*0*	*3*
First to Last seen	*-*	*-*	*-*	*93 - 94*
Skin, Scab, Dorsal Cervical				
Number of Animals Affected	*0*	*0*	*1*	*0*
Number of Times Recorded	*0*	*0*	*52*	*0*
First to Last seen	*-*	*-*	*38 - 64*	*-*
Skin, Scab, Scapular, Right				
Number of Animals Affected	*0*	*0*	*1*	*0*
Number of Times Recorded	*0*	*0*	*4*	*0*
First to Last seen	*-*	*-*	*36 - 57*	*-*
Swollen, Ventral Cervical				
Number of Animals Affected	*0*	*0*	*1*	*0*
Number of Times Recorded	*0*	*0*	*1*	*0*
First to Last seen	*-*	*-*	*72-72*	*-*

7c. Summary of Clinical Observations: F0 Generation - Gestation

Observation Type: All Types Sex: Female From Day 0 (Mating (A)) to 0 (Littering)	*0 mg/kg bw/day Group 1*	*100 mg/kg bw/day Group 2*	*300 mg/kg bw/day Group 3*	*1000 mg/kg bw/ day Group 4*
Breathing, Abnormal Sounds				
Number of Animals Affected	*0*	*0*	*1*	*1*
Number of Times Recorded	*0*	*0*	*1*	*11*
First to Last seen	*-*	*-*	*20 - 20*	*0 - 11*

Hunched Posture				
Number of Animals Affected	*1*	*0*	*0*	*0*
Number of Times Recorded	*1*	*0*	*0*	*0*
First to Last seen	*24 - 24*	*-*	*-*	*-*
Fur, Erected				
Number of Animals Affected	*1*	*0*	*0*	*0*
Number of Times Recorded	*1*	*0*	*0*	*0*
First to Last seen	*24 - 24*	*-*	*-*	*-*
Fur, Loss				
Number of Animals Affected	*1*	*1*	*1*	*0*
Number of Times Recorded	*10*	*23*	*12*	*0*
First to Last seen	*17 - 21*	*10-22*	*16 - 21*	*-*
Skin, Flaking, Lumbar				
Number of Animals Affected	*0*	*1*	*0*	*0*
Number of Times Recorded	*0*	*3*	*0*	*0*
First to Last seen	*-*	*16 - 17*	*-*	*-*
Swollen, Ventral Cervical				
Number of Animals Affected	*0*	*0*	*1*	*0*
Number of Times Recorded	*0*	*0*	*30*	*0*
First to Last seen	*-*	*-*	*0 - 13*	*-*
Swollen, Vulva				
Number of Animals Affected	*1*	*0*	*0*	*0*
Number of Times Recorded	*1*	*0*	*0*	*0*
First to Last seen	*24 - 24*	*-*	*-*	*-*
Other (see comment), Vulva				
Number of Animals Affected	*1*	*0*	*0*	*0*
Number of Times Recorded	*1*	*0*	*0*	*0*

7d. Summary of Clinical Observations: F0 Generation - Lactation

Observation Type: All Types Sex: Female From Day 1 (Littering (A)) to 25 (Littering)	*0 mg/kg bw/day Group 1*	*100 mg/kg bw/day Group 2*	*300 mg/kg bw/day Group 3*	*1000 mg/kg bw/day Group 4*
Breathing, Abnormal Sounds				
Number of Animals Affected	*0*	*0*	*1*	*1*
Number of Times Recorded	*0*	*0*	*1*	*23*
First to Last seen	*-*	*-*	*10-10*	*9-21*
Fur, Erected				
Number of Animals Affected	*0*	*0*	*0*	*1*
Number of Times Recorded	*0*	*0*	*0*	*1*
First to Last seen	*-*	*-*	*-*	*23 - 23*
Fur, Loss				
Number of Animals Affected	*1*	*1*	*1*	*0*
Number of Times Recorded	*52*	*53*	*53*	*0*
First to Last seen	*1-25*	*1-25*	*1-25*	*-*
Skin, Scab, Dorsal Cervical				
Number of Animals Affected	*0*	*1*	*0*	*0*
Number of Times Recorded	*0*	*24*	*0*	*0*
First to Last seen	*-*	*6-24*	*-*	*-*

Table 8a. Summary of Body Weights: F0 Generation - Males

Sex: Male		*Day(s) Relative to Start Date*												
		1	*8*	*15*	*22*	*29*	*36*	*43*	*50*	*57*	*64*	*71*	*78*	*85*
0 mg/kg bw/day Group 1	*Mean*	*157.6*	*199.4*	*239.4*	*266.6*	*293.4*	*309.6*	*326.8*	*339.7*	*353.2*	*362*	*368.5*	*373.5*	*382.2*
	SD	*12*	*13.7*	*15.2*	*17.8*	*19.7*	*22.4*	*24.5*	*24.6*	*25.8*	*27.5*	*27.7*	*26.8*	*26.5*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
100 mg/kg bw/day Group 2	*Mean*	*152.8*	*195.2*	*236.1*	*264*	*292.6*	*308.8*	*326.3*	*338.6*	*350.8*	*361.1*	*368.5*	*373.8*	*383.3*
	SD	*15.5*	*20.1*	*23.8*	*25.6*	*29.5*	*31.5*	*32.9*	*33.8*	*35.6*	*37*	*37.5*	*37.8*	*37.5*
	N	*24*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
	%Diff	*-3.1*	*-2.1*	*-1.4*	*-1*	*-0.3*	*-0.3*	*-0.2*	*-0.3*	*-0.7*	*-0.2*	*0*	*0.1*	*0.3*

300 mg/kg bw/day Group 3	*Mean*	*143.8**	*195.2*	*235.8*	*264.9*	*292.7*	*310.7*	*327.9*	*341.9*	*353*	*363.4*	*372.1*	*379.5*	*389.4*
	SD	*19.6*	*13.7*	*15.5*	*16*	*17.6*	*20.7*	*22.4*	*24.1*	*25.2*	*25.8*	*26.4*	*27.7*	*28.2*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
	%Diff	*-8.8*	*-2.1*	*-1.5*	*-0.6*	*-0.2*	*0.4*	*0.3*	*0.6*	*-0.1*	*0.4*	*1*	*1.6*	*1.9*
1000 mg/kg bw/day Group 4	*Mean*	*150.6*	*196.6*	*234.4*	*262*	*286.6*	*301.7*	*319.9*	*330.6*	*341*	*349.4*	*355.4*	*360.9*	*365.6*
	SD	*14.5*	*15.5*	*18*	*20.2*	*22.4*	*25*	*26.7*	*27.5*	*26.6*	*26.6*	*27.6*	*28.5*	*28.1*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
	%Diff	*-4.4*	*-1.4*	*-2.1*	*-1.7*	*-2.3*	*-2.6*	*-2.1*	*-2.7*	*-3.4*	*-3.5*	*-3.6*	*-3.4*	*-4.4*

* = p ≤ 0.05

Table 8b. Summary of Body Weights: F0 Generation - Premating

Sex: Female		*Day(s) Relative to Start Date*										
		1	*8*	*15*	*22*	*29*	*36*	*43*	*50*	*57*	*64*	*71*
0 mg/kg bw/day Group 1	*Mean*	*114.6*	*132.3*	*150*	*163.8*	*179*	*187*	*194.8*	*200.2*	*207.7*	*212.9*	*214.2*
	SD	*6.1*	*7.1*	*9.1*	*9.1*	*8.9*	*11.7*	*11.9*	*11.1*	*10.9*	*13.2*	*13.5*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
100 mg/kg bw/day Group 2	*Mean*	*113*	*129.4*	*148.1*	*161.5*	*176.4*	*186.8*	*192.4*	*198.7*	*203.3*	*209.3*	*209.9*
	SD	*7.5*	*10.1*	*11.4*	*11.7*	*12.5*	*13.4*	*14.7*	*14.2*	*15.2*	*15.2*	*15.8*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
	%Diff	*-1.4*	*-2.2*	*-1.3*	*-1.4*	*-1.4*	*-0.1*	*-1.3*	*-0.7*	*-2.1*	*-1.7*	*-2*
300 mg/kg bw/day Group 3	*Mean*	*115.6*	*134.4*	*153.2*	*167.1*	*182.6*	*194.2*	*199.9*	*206.8*	*211.7*	*218.2*	*218.8*
	SD	*7.6*	*8.4*	*10.5*	*12*	*13*	*14.2*	*14.1*	*14.8*	*14.2*	*14.8*	*15.4*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
	%Diff	*0.9*	*1.6*	*2.1*	*2*	*2.1*	*3.8*	*2.6*	*3.3*	*1.9*	*2.5*	*2.1*
1000 mg/kg bw/day Group 4	*Mean*	*115.9*	*135.4*	*154.5*	*169.4*	*183.8*	*192.3*	*201*	*207.3*	*213.8*	*217.1*	*219.3*
	SD	*6.7*	*6.8*	*6.5*	*8.2*	*8.4*	*8.4*	*7.5*	*8.7*	*9.2*	*9.3*	*9.9*
	N	*25*	*25*	*24*	*24*	*24*	*24*	*24*	*24*	*24*	*24*	*24*
	%Diff	*1.2*	*2.4*	*3*	*3.4*	*2.7*	*2.8*	*3.1*	*3.5*	*2.9*	*2*	*2.4*

Table 8c. Summary of Body Weights: F0 Generation - Gestation

Sex: Female		*Day(s) Relative to Mating (Litter: A)*						
		0	*4*	*7*	*11*	*14*	*17*	*20*
0 mg/kg bw/day Group 1	*Mean*	*215.9*	*228.8*	*234.5*	*247.3*	*257.5*	*278.3*	*308.8*
	SD	*14.7*	*13.8*	*14.1*	*14.9*	*15.9*	*21*	*27*

	N	24	24	24	24	24	24	24
100 mg/kg bw/day Group 2	Mean	213.5	225.5	231.5	246.6	255.7	281.1	315.3
	SD	16.6	16.5	16.4	17.7	17	18	22.5
	N	21	21	21	21	21	23	20
	%Diff	-1.1	-1.5	-1.3	-0.3	-0.7	1	2.1
300 mg/kg bw/day Group 3	Mean	220.9	233.2	239.6	253.4	262.9	286.5	324
	SD	16.9	16.2	17.6	18.8	20.1	21.4	25.3
	N	24	24	24	24	24	24	24
	%Diff	2.3	1.9	2.2	2.4	2.1	3	4.9
1000 mg/kg bw/day Group 4	Mean	219.5	232.2	236.8	252.5	263	287.6	325.3
	SD	13.9	14.3	13.1	13.8	15.1	17	19.2
	N	20	20	20	20	20	20	20
	%Diff	1.6	1.4	1	2.1	2.1	3.4	5.3

Table 8d. Summary of Body Weights: F0 Generation - Lactation

Sex: Female		Day(s) Relative to Littering (Litter: A)				
		1	4	7	14	21
0 mg/kg bw/day Group 1	Mean	244.3	257.2	265.3	281.3	272.3
	SD	14	14.6	14.8	19	17.4
	N	24	24	24	24	24
100 mg/kg bw/day Group 2	Mean	241	251.2	260.7	275.7	273.9
	SD	18.5	15.5	14.9	16.5	16.9
	N	24	24	23	23	23
	%Diff	-1.4	-2.3	-1.7	-2	0.6
300 mg/kg bw/day Group 3	Mean	247	257.4	265.6	283.1	275.2
	SD	18.1	19.5	19	20.2	20.1
	N	23	21	21	21	21
	%Diff	1.1	0.1	0.1	0.7	1.1
1000 mg/kg bw/day Group 4	Mean	248.9	260.3	267.5	286.1	279.2
	SD	16.4	14.1	18.3	16.2	13.8
	N	20	20	20	20	20
	%Diff	1.9	1.2	0.8	1.7	2.5

Table 9a. Summary of Body Weight Gains (g): F0 Generation - Males

Sex: Male		Day(s) Relative to Start Date													
		1 → 8	8 → 15	15 → 22	22 → 29	29 → 36	36 → 43	43 → 50	50 → 57	57 → 64	64 → 71	1 → 71	71 → 78	78 → 85	1 → 85
0 mg/	Mean	41.7	40.1	27.2	26.8	16.2	17.2	12.9	13.5	8.8	6.6	210.9	5	8.7	224.6

kg bw/ day Group 1	*SD*	*3.9*	*4.3*	*5.9*	*5.5*	*4.7*	*3.9*	*3.5*	*3.1*	*3.2*	*2.9*	*23.7*	*4.4*	*4.5*	*22.7*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
100 mg/ kg bw/ day Group 2	*Mean*	*42.4*	*41*	*27.9*	*28.6*	*16.2*	*17.5*	*12.4*	*12.2*	*10.2*	*7.4*	*216.6*	*5.3*	*9.5*	*231.5*
	SD	*7.5*	*4.6*	*5.1*	*5.2*	*5.3*	*3.3*	*3.4*	*3.3*	*3.7*	*3.3*	*27.6*	*4.7*	*4.7*	*28.4*
	N	*24*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*24*	*25*	*25*	*24*
300 mg/ kg bw/ day Group 3	*Mean*	*51.4***	*40.6*	*29.1*	*27.8*	*18*	*17.2*	*14*	*11.1*	*10.5*	*8.6*	*228.3**	*7.4*	*9.9*	*245.7**
	SD	*12.1*	*3.9*	*3.9*	*4.3*	*5*	*4.8*	*3.2*	*4.7*	*3.4*	*3.3*	*28.7*	*4.4*	*3.5*	*29.4*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
1000 mg/ kg bw/ day Group 4	*Mean*	*46*	*37.8*	*27.6*	*24.6*	*15.1*	*18.2*	*10.7*	*10.4**	*8.4*	*6*	*204.8*	*5.5*	*4.6***	*214.9*
	SD	*9.7*	*3.9*	*5.4*	*5*	*4.6*	*3.9*	*3.8*	*3.3*	*3.4*	*3*	*22.2*	*3.1*	*4.8*	*22.5*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*

* = p ≤ 0.05; ** = p ≤ *0.01*

Table 9b. Summary of Body Weight Gains (g): F0 Generation - Premating

Sex: Female		*Day(s) Relative to Start Date*										
		1 → 8	*8 → 15*	*15 → 22*	*22 → 29*	*29 → 36*	*36 → 43*	*43 → 50*	*50 → 57*	*57 → 64*	*64 → 71*	*1 → 71*
0 mg/ kg bw/ day Group 1	*Mean*	*17.7*	*17.7*	*13.8*	*15.2*	*8.1*	*7.8*	*5.4*	*7.5*	*5.2*	*1.3*	*99.7*
	SD	*3.8*	*4.6*	*5.2*	*4.5*	*4.6*	*4.8*	*4.6*	*4.4*	*3.8*	*3.3*	*10.9*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
100 mg/kg bw/ day Group 2	*Mean*	*16.4*	*18.7*	*13.4*	*15*	*10.4*	*5.5*	*6.4*	*4.6*	*6*	*0.6*	*96.9*
	SD	*5.2*	*6.6*	*3.4*	*4*	*3.1*	*3.7*	*4.7*	*4.9*	*4.4*	*3.2*	*10.7*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
300 mg/kg bw/ day Group 3	*Mean*	*18.8*	*18.8*	*13.9*	*15.6*	*11.6**	*5.7*	*6.9*	*4.9*	*6.6*	*0.5*	*103.2*
	SD	*3.5*	*3.5*	*4.5*	*6.1*	*4.2*	*3.2*	*4.5*	*5.5*	*4*	*3.2*	*11.2*
	N	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*25*
1000 mg/kg	*Mean*	*19.5*	*18.7*	*14.9*	*14.3*	*8.5*	*8.7*	*6.3*	*6.5*	*3.3*	*2.2*	*103*

bw/ day Group 4	*SD*	*2.6*	*3.2*	*4.3*	*5.4*	*4.5*	*4.1*	*4.3*	*3.6*	*3.1*	*3.1*	*8.3*
	N	*25*	*24*	*24*	*24*	*24*	*24*	*24*	*24*	*24*	*24*	*24*

* = p ≤ *0.05*

Table 9c. Summary of Body Weight Gains (g): F0 Generation - Gestation

Sex: Female		**Day(s) Relative to Start Date**						
		0 → 4	4 → 7	7 → 11	11 → 14	14 → 17	17 → 20	0 → 20
0 mg/ kg bw/day Group 1	Mean	12.9	5.7	12.8	10.2	20.8	34.6	95.6
	SD	4.9	2.6	4.3	3	6.9	8.1	16.8
	N	24	24	24	24	24	23	23
100 mg/ kg bw/day Group 2	Mean	12	6	15	9.1	25	36	101.3
	SD	3.6	2.4	3	4.3	6	8.3	13.7
	N	21	21	21	21	21	20	20
300 mg/ kg bw/day Group 3	Mean	12.3	6.4	13.8	9.5	23.6	37.5	103
	SD	2.2	3.5	3.3	3.2	4.6	5.4	12.4
	N	24	24	24	24	24	24	24
1000 mg/ kg bw/day Group 4	Mean	12.7	4.7	15.7*	10.5	24.6	37.7	105.9
	SD	4.2	3.5	2.9	2.9	4.2	5.4	9.7
	N	20	20	20	20	20	20	20

***** = p ≤ *0.05***

Table 9d. Summary of Body Weight Gains (g): F0 Generation - Lactation

Sex: Female		*Day(s) Relative to Littering (Litter: A)*				
		1 → 4	*4 → 7*	*7 → 14*	*14 → 21*	*1 → 21*
0 mg/kg bw/ day Group 1	*Mean*	*12.9*	*8.1*	*16*	*-9*	*28*
	SD	*7*	*6.4*	*7.1*	*9.8*	*8.5*
	N	*24*	*24*	*24*	*24*	*24*

100 mg/kg bw/day Group 2	Mean	10.2	9.3	14.9	-1.7*	32.8
	SD	6.4	5.7	7.2	9.2	9.2
	N	24	23	23	23	23
300 mg/kg bw/day Group 3	Mean	10.4	8.1	17.6	-7.9	28.2
	SD	8	8	10.3	9.6	12.7
	N	21	21	21	21	21
1000 mg/kg bw/day Group 4	Mean	11.4	7.2	18.6	-6.9	30.3
	SD	7.8	10.9	12.2	8.1	12.2
	N	20	20	20	20	20

* = p ≤ 0.05

Table 10a. Summary of Food Consumption: F0 Generation - Males

Sex: Male		Day(s) Relative to Animal Start Date												
		1 → 8	8 → 15	15 → 22	22 → 29	29 → 36	36 → 43	43 → 50	50 → 57	57 → 64	64 → 71	71 → 78	78 → 85	1 → 85
0 mg/kg bw/day Group 1	Mean	20.6	22.1	23.1	23.1	22.9	22.2	22.3	22	22.1	21.9	21.7	21.5	22.1
	SD	1	0.6	0.9	1.3	1.1	0.9	0.8	0.6	0.6	0.7	0.7	0.7	0.7
	N	5	5	5	5	5	5	5	5	5	5	5	5	5
100 mg/kg bw/day Group 2	Mean	20.1	22	23	23.4	23.3	22.6	22.6	22	22.2	22	22.4	22.4	22.3
	SD	1.3	1.3	1.1	1.5	1.3	1.1	1.2	1.1	1.1	1.2	1.1	1.5	1.2
	N	5	5	5	5	5	5	5	5	5	5	5	5	5
	%Diff	-2.4	-0.1	-0.3	1	1.8	1.5	1.4	0.4	0.6	0.4	3	4.3	0.9
300 mg/kg bw/day Group 3	Mean	20.3	22.4	23.2	23.8	23.7	23.1	23.3	22.6	22.7	23	23.3	22.8	22.8
	SD	1.1	0.8	0.8	0.7	0.8	0.6	0.5	0.5	0.4	0.4	1.2	0.4	0.5
	N	5	5	5	5	5	5	5	5	5	5	5	5	5
	%Diff	-1.4	1.7	0.5	2.7	3.6	3.9	4.7	2.8	2.6	4.7	7.5	5.9	3.1
1000 mg/kg bw/day Group 4	Mean	20.4	22.5	23.3	23.7	23.7	22.9	23.3	22.5	22.2	21.9	22.6	22.1	22.6
	SD	0.9	1	1.1	1.3	1.4	1.4	1.1	1.1	1.1	0.8	2.5	1.6	1.1
	N	5	5	5	5	5	5	5	5	5	5	5	5	5
	%Diff	-0.9	2.1	0.7	2.4	3.4	3.2	4.7	2.3	0.4	-0.2	4.3	2.7	2

* = p ≤ 0.05

***Table* 10b. Summary of Food Consumption: F0 Generation - Premating**

Sex: Female	Day(s) Relative to Animal Start Date

		1 → 8	8 → 15	15 → 22	22 → 29	29 → 36	36 → 43	43 → 50	50 → 57	57 → 64	64 → 71	1 → 71
0 mg/kg bw/day Group 1	Mean	13.5	13.9	14.8	15.4	15.9	15.2	15.6	15.5	15.2	14.9	15
	SD	0.7	0.6	0.7	0.6	0.8	0.7	0.5	0.5	0.8	0.8	0.6
	N	5	5	5	5	5	5	5	4	5	5	5
100 mg/kg bw/day Group 2	Mean	13.1	13.8	14.8	15.2	15.8	14.9	15.5	15.1	14.6	14.4	14.7
	SD	0.5	0.4	0.5	0.3	0.6	0.7	0.7	0.6	0.7	0.9	0.5
	N	5	5	5	5	5	5	5	4	5	5	5
	%Diff	-2.8	-1.1	0.1	-0.8	-0.5	-2.2	-0.4	-3.1	-3.7	-3.4	-1.6
300 mg/kg bw/day Group 3	Mean	13.3	14.3	15.8*	16	16.8	15.6	16.6*	16.1	15.8	15.4	15.6
	SD	0.2	0.3	0.7	0.6	0.7	0.7	0.7	0.5	0.6	0.4	0.4
	N	5	5	5	5	5	5	5	5	5	5	5
	%Diff	-0.8	2.9	6.6	4	5.5	2.6	6.5	3.7	4.1	2.9	4
1000 mg/kg bw/day Group 4	Mean	13.9	14.4	15.6	15.6	16.4	15.5	16.2	15.7	15.4	14.9	15.3
	SD	0.4	0.4	0.3	0.4	0.4	0.4	0.3	0.4	0.5	0.5	0.3
	N	5	5	5	5	5	5	5	5	5	5	5
	%Diff	3.4	3.2	5	1.7	2.9	2.2	4	0.7	1.9	-0.1	2.5

Table 10c. Summary of Food Consumption: F0 Generation - Gestation

Sex: Female		Day(s) Relative to Mating (Litter: A)						
		0 → 4	4 → 7	7 → 11	11 → 14	14 → 17	17 → 20	0 → 20
0 mg/kg bw/day Group 1	Mean	17.6	19.21	19.38	20.39	21.14	22.91	19.82
	SD	1.77	1.58	1.72	1.69	1.89	2.23	1.42
	N	24	24	24	24	24	23	23
100 mg/kg bw/day Group 2	Mean	17.54	19.16	19.35	20.19	21.48	23.58	19.98
	SD	1.48	1.29	1.51	1.51	1.69	1.81	1.2
	N	21	21	21	21	21	20	20
	%Diff	-0.39	-0.26	-0.15	-0.97	1.6	2.93	0.81
300 mg/kg bw/day Group 3	Mean	17.96	19.76	19.92	20.74	21.92	24.69	20.64
	SD	1.93	2.26	2.33	2.17	2.11	2.53	2
	N	24	24	24	24	24	24	24
	%Diff	2.01	2.89	2.8	1.7	3.68	7.77	4.16
1000 mg/kg bw/day Group 4	Mean	16.91	18.87	19.4	20.52	21.92	23.43	19.97
	SD	3.06	2.11	2.09	1.77	1.94	2.13	1.84
	N	20	20	20	20	20	20	20
	%Diff	-3.93	-1.78	0.13	0.63	3.68	2.27	0.78

***Table 10*d. Summary of Food Consumption: F0 Generation - Lactation**

Sex: Female		Day(s) Relative to Littering (Litter: A)				
		1 → 4	4 → 7	7 → 14	14 → 21	1 → 21
0 mg/kg bw/day Group 1	Mean	30.15	40.89	53.27	63.6	51.56
	SD	5.76	5.75	8.55	10.67	8.16
	N	24	24	24	24	24
100 mg/kg bw/day Group 2	Mean	28.63	41.23	54.89	67.77	53.5
	SD	5.1	3.78	3.71	4.59	3.1
	N	24	23	23	23	23
	%Diff	-5.07	0.84	3.04	6.55	3.75
300 mg/kg bw/day Group 3	Mean	29.24	41.02	54.99	66.4	53.02
	SD	4.57	3.95	4.59	7.17	4.9
	N	21	21	21	21	21
	%Diff	-3.03	0.31	3.21	4.4	2.83
1000 mg/kg bw/day Group 4	Mean	29.77	40.48	53.05	64.58	51.71
	SD	6.03	6.34	7.55	8.65	7.2
	N	20	20	20	20	20
	%Diff	-1.28	-0.99	-0.42	1.54	0.28

***Table* 11a. Summary of Hematology Values: F0 Generation- Males**

Sex: Male		Reporting Hematology															
		WBC (10^9/L)	NEUT (10^9/L)	LYMPH (10^9/L)	MONO (10^9/L)	EOS (10^9/L)	BASO (10^9/L)	LUC (10^9/L)	RBC (10^12/L)	RETIC (10^9/L)	RDW (%)	HGB (g/L)	HCT (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	PLT (10^9/L)
0 mg/kg bw/day Group 1	Mean	4.415	0.867	3.34	0.09	0.082	0.005	0.032	8.48	210.74	11.97	153.5	0.4421	52.16	18.12	347.5	717.5
	SD	1.368	0.263	1.081	0.036	0.038	0.007	0.026	0.433	44.68	0.57	6	0.018	0.67	0.62	10	89.1
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
100 mg/kg bw/day Group 2	Mean	5.601	1.289	4.072	0.115	0.077	0.007	0.038	8.975	223.15	12.35	155.5	0.4545	50.68**	17.34*	341.9	689.8
	SD	0.848	0.384	0.974	0.034	0.026	0.008	0.015	0.449	26.55	0.52	6.5	0.0212	0.79	0.58	9.2	101
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	tCtrl	1.27	1.49	1.22	1.28	0.94	1.4	1.19	1.06	1.06	1.03	1.01	1.03	0.97	0.96	0.98	0.96
300 mg/kg	Mean	5.694*	1.109	4.329	0.112	0.089	0.006	0.052	8.846	207.52	12.07	156	0.4536	51.26	17.65	344.3	649.2
	SD	1.172	0.403	0.828	0.04	0.039	0.005	0.038	0.54	36.14	0.31	8.6	0.0292	0.77	0.33	8.5	73.7

bw/ day Group 3	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	tCtrl	1.29	1.28	1.3	1.24	1.09	1.2	1.63	1.04	0.98	1.01	1.02	1.03	0.98	0.97	0.99	0.9
1000 mg/ kg bw/ day Group 4	Mean	4.653	1.092	3.355	0.106	0.062	0.007	0.034	8.257	187.11	1.69	150.9	0.4348	52.69	18.31	347.5	691.8
	SD	0.865	0.295	0.68	0.025	0.015	0.005	0.024	0.502	25	0.47	9.9	0.0279	1.86	1	9.4	92.8
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	tCtrl	1.05	1.26	1	1.18	0.76	1.4	1.06	0.97	0.89	0.98	0.98	0.98	1.01	1.01	1	0.96

** = p ≤ 0.05; ** = p ≤* 0.01

Table 11b. Summary of Hematology Values: F0 Generation - Females

Sex: Female		Reporting Hematology															
		WBC	NEUT	LYMPH	MONO	EOS	BASO	LUC	RBC	RETIC	RDWC	HGB	HCT	MCV	MCH	MCHC	PLT
		(10^9/ L)	(10^9/ L)	(10^9/ L)	(10^9/ L)	(10^9/ L)	(10^9/ L)	(10^9/ L)	(10^12/ L)	(10^9/ L)	(%)	(g/ L)	(L/ L)	(fL)	(pg)	(g/ L)	(10^9/ L)
0 mg/ kg bw/ day Group 1	Mean	4.486	1.204	3.034	0.133	0.078	0.007	0.03	8.792	188.82	1.39	167.4	0.4895	55.72	19.08	342.3	861.1
	SD	1.502	0.683	0.772	0.074	0.045	0.007	0.02	0.618	57.11	0.74	6.6	0.0254	4.73	0.92	9.3	121.6
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
100 mg/ kg bw/ day Group 2	Mean	3.677	1.009	2.43	0.122	0.084	0.002	0.026	8.375	174.46	1.55	159.8	0.4635	55.29	19.08	345.3	818.3
	SD	0.558	0.28	0.415	0.033	0.028	0.004	0.018	0.535	33.7	0.83	9.1	0.0329	1.52	0.7	9.5	74.7
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	tCtrl	0.82	0.84	0.8	0.92	1.08	0.29	0.87	0.95	0.92	1.01	0.95	0.95	0.99	1	1.01	0.95
300 mg/ kg bw/ day Group 3	Mean	3.987	0.893	2.859	0.096	0.11	0.004	0.025	8.261	170.39	1.25	157.1	0.4568	55.3	19.05	344.7	850.3
	SD	1.531	0.446	1.149	0.044	0.185	0.005	0.014	0.809	35.26	0.71	14.3	0.0473	3.65	0.65	7.5	115.2
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	tCtrl	0.89	0.74	0.94	0.72	1.41	0.57	0.83	0.94	0.9	0.99	0.94	0.93	0.99	1	1.01	0.99
1000 mg/ kg bw/ day Group 4	Mean	4.522	1.204	2.997	0.15	0.139	0.003	0.029	8.609	188.82	1.64	164.5	0.4858	56.45	19.13	338.7	857
	SD	1.257	0.474	0.806	0.055	0.146	0.005	0.017	0.397	44.11	0.6	7.1	0.0212	2.29	0.91	7.8	140.5
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	tCtrl	1.01	1	0.99	1.13	1.78	0.43	0.97	0.98	1	1.02	0.98	0.99	1.01	1	0.99	1

Table 12a. Summary of Coagulation Values: F0 Generation - Males

Sex: Male	Reporting Coagulation

		PT	APTT
		(sec)	(sec)
0 mg/kg bw/day Group 1	Mean	20.2	12.83
	SD	1.43	2.31
	N	9	9
100 mg/kg bw/day Group 2	Mean	21	13.8
	SD	0.81	1.56
	N	9	9
	tCtrl	1.04	1.08
300 mg/kg bw/day Group 3	Mean	20.52	13.81
	SD	0.99	1.96
	N	10	10
	tCtrl	1.02	1.08
1000 mg/kg bw/day Group 4	Mean	19.58	11.91
	SD	1.05	1.47
	N	8	8
	tCtrl	0.97	0.93

Table 12b. Summary of Coagulation Values: F0 Generation- Females

Sex: Female		Reporting Coagulation	
		PT	APTT
		(sec)	(sec)
0 mg/kg bw/day Group 1	Mean	21.52	13.25
	SD	1.07	1.91
	N	10	10
100 mg/kg bw/day Group 2	Mean	21.44	13.15
	SD	2.06	1.81
	N	10	10
	tCtrl	1	0.99
300 mg/kg bw/day Group 3	Mean	20.97	12.44
	SD	2.02	0.91
	N	10	10
	tCtrl	0.97	0.94
1000 mg/kg bw/day Group 4	Mean	21.58	13.78
	SD	1.35	2.23
	N	10	10
	tCtrl	1	1.04

***Table 1*3a. Summary of Clinical Chemistry Values: F0 Generation- Males.**

Sex: Male		Reporting Biochemistry																Hormone Analysis	
Day: 89 Relative to Start Date		ALT (U/L)	AST (U/L)	ALP (U/L)	TPROT (g/L)	ALB (g/L)	BILE (umol/L)	TBIL (umol/L)	UREA (mmol/L)	CREAT (umol/L)	GLUC (mmol/L)	CHOL (mmol/L)	NA (mmol/L)	K (mmol/L)	CL (mmol/L)	CA (mmol/L)	PHOS (mmol/L)	T4 (ng/mL)	TSH (mU/L)
0 mg/kg bw/day Group 1	Mean	37.5	77.3	73.7	63.85	38.75	10.26	0.66	4.26	22.3	8.085	1.616	146.4	4.1	109.8	2.596	1.905	53.13	0.15
	SD	2.8	6.4	15.2	2.71	1.1	2.16	0.26	0.55	2.2	0.926	0.289	1	0.18	1.1	0.046	0.152	4.97	0.0988
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
100 mg/kg bw/day Group 2	Mean	41.7	80.5	69	63.66	38.35	8.02	0.63	4.2	21.2	8.7	1.653	144.4 **	4.12	107.8 **	2.687	1.99	55.41	0.2676
	SD	5.3	12.6	10.2	3.39	1.8	2.79	0.28	0.48	2.8	1.249	0.36	0.5	0.21	1	0.112	0.235	8.87	0.3101
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	9	10	10	10
	hCtrl	1.11	1.04	0.94	1	0.99	0.78	0.95	0.99	0.95	1.08	1.02	0.99	1	0.98	1.03	1.04	1.04	1.72
300 mg/kg bw/day Group 3	Mean	39.2	75.8	69.5	61.99	38.09	11.29	0.5	4.04	20.5	8.616	1.65	144.4 **	4.08	108.4 **	2.765 **	1.969	58.12	0.1463
	SD	4.9	10.8	9.7	1.27	1.31	4.4	0	0.51	1.8	1.408	0.265	0.8	0.16	0.7	0.118	0.213	10.43	0.0813
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	hCtrl	1.05	0.98	0.94	0.97	0.98	1.1	0.76	0.95	0.92	1.07	1.02	0.99	1	0.99	1.07	1.03	1.09	0.94
1000 mg/kg bw/day Group 4	Mean	45.1 **	82.6	78.9	60.97	37.65	15.9	0.67	3.66 *	20.4	7.731	1.673	145.5	4.08	108.8	2.697	1.922	52.53	0.1121
	SD	6.3	10.2	13.4	1.83	0.78	10.85	0.22	0.48	2.5	1.008	0.232	1.3	0.15	1	0.093	0.21	6.57	0.0553
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	hCtrl	1.2	1.07	1.07	0.95	0.97	1.55	1.02	0.86	0.91	0.96	1.04	0.99	1	0.99	1.04	1.01	0.99	0.72

p ≤ 0.05; ** = p ≤ 0.01

Table 13b. Summary of Clinical Chemistry Values: F0 Generation - Female

Sex: Female		Reporting Biochemistry																Hormone Analysis	
Day: 23 Relative to Littering (Litter: A)		ALT (U/L)	AST (U/L)	ALP (U/L)	TPROT (g/L)	ALB (g/L)	BILE (umol/L)	TBIL (umol/L)	UREA (mmol/L)	CREAT (umol/L)	GLUC (mmol/L)	CHOL (mmol/L)	NA (mmol/L)	K (mmol/L)	CL (mmol/L)	CA (mmol/L)	PHOS (mmol/L)	T4 (ng/mL)	TSH (mU/L)
0 mg/kg bw/day	Mean	40.1	83.2	55.2	61.84	38.81	10.49	0.64	6.22	22.3	7.225	1.556	143.2	3.62	105.2	2.504	2	38.18	0.2657
	SD	5.3	13.7	13.2	2.86	1.75	8.41	0.31	0.94	3.2	0.727	0.211	1.6	0.23	3.2	0.145	0.38	9.75	0.2322
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Group 1																			
100 mg/kg bw/day Group 2	*Mean*	*47*	*90.5*	*55.4*	*61.34*	*38.2*	*13*	*0.92*	*6.85*	*21*	*7.427*	*1.637*	*142.2*	*3.77*	*105.6*	*2.462*	*1.993*	*45.31*	*0.14999*
	SD	*7.5*	*14.2*	*11.6*	*2.13*	*1.2*	*4.68*	*0.36*	*1.15*	*4.8*	*0.846*	*0.356*	*1.6*	*0.32*	*3.6*	*0.124*	*0.476*	*7.44*	*0.11146*
	N	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
	tCtrl	*1.17*	*1.09*	*1*	*0.99*	*0.98*	*1.24*	*1.44*	*1.1*	*0.94*	*1.03*	*1.05*	*0.99*	*1.04*	*1*	*0.98*	*1*	*1.19*	*0.56*
300 mg/kg bw/day Group 3	*Mean*	*42.3*	*89.7*	*56.5*	*61.59*	*39.26*	*25.9*	*0.89*	*6.56*	*19.8*	*7.827*	*1.711*	*141.4**	*3.88*	*104.4*	*2.59*	*2.07*	*46.41*	*0.17994*
	SD	*4.4*	*13.5*	*14.4*	*2.63*	*1.89*	*25.53*	*0.51*	*0.96*	*2.9*	*1.431*	*0.371*	*1*	*0.39*	*2.7*	*0.079*	*0.351*	*12.11*	*0.08037*
	N	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
	tCtrl	*1.05*	*1.08*	*1.02*	*1*	*1.01*	*2.47*	*1.39*	*1.05*	*0.89*	*1.08*	*1.1*	*0.99*	*1.07*	*0.99*	*1.03*	*1.04*	*1.22*	*0.68*
1000 mg/kg bw/day Group 4	*Mean*	*45.7*	*86.8*	*54*	*61.42*	*38.36*	*22.63*	*0.62*	*6.88*	*20.2*	*8.071*	*1.743*	*141.6**	*3.61*	*104.1*	*2.643**	*2.039*	*41.15*	*0.18077*
	SD	*6.2*	*11.6*	*12.4*	*2.28*	*1.74*	*16.49*	*0.27*	*1.02*	*2.3*	*1.231*	*0.469*	*1.3*	*0.32*	*2.1*	*0.115*	*0.322*	*12.98*	*0.19*
	N	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
	tCtrl	*1.14*	*1.04*	*0.98*	*0.99*	*0.99*	*2.16*	*0.97*	*1.11*	*0.91*	*1.12*	*1.12*	*0.99*	*1*	*0.99*	*1.06*	*1.02*	*1.08*	*0.68*

*= p ≤ *0.05*

Table 13c. Historical Data F0 Generation- Hormone values - Females

FEMALES						
2.1 CLINICAL BIOCHEMISTRY						
MEASUREMENT	***UNITS***	***MEAN***	***STD.DEV.***	***N***	***P5***	***P95***
END OF TREATMENT						
TSH	*uIU/mL*	*0.202*	*0.1764*	*126*	*0.0330*	*0.6260*
Total T4	*ug/dL*	*4.64*	*1.331*	*128*	*2.680*	*7.350*
PND 4 END OF IN-LIFE *Total T4*	*ug/dL*	*1.45*	*0.421*	*228*	*0.500*	*2.100*

Table 14a. Summary of Urinalysis Values: F0 Generation - Males

Sex: Male Day: 89 Relative to Start *Date*	*Reporting Urinalysis*		
	VOLUME	*SPECIFIC*	*URINE*
	(mL)	*GRAVITY*	*pH*

0 mg/kg bw/day Group 1	*Mean*	*8.7*	*1.0287*	*6.95*
	SD	*6.15*	*0.0126*	*0.69*
	N	*10*	*10*	*10*
100 mg/kg bw/day Group 2	*Mean*	*9.2*	*1.0278*	*6.95*
	SD	*3.77*	*0.0125*	*0.28*
	N	*10*	*10*	*10*
	tCtrl	*1.06*	*1*	*1*
300 mg/kg bw/day Group 3	*Mean*	*6.9*	*1.0332*	*7*
	SD	*3.31*	*0.0083*	*0.53*
	N	*10*	*10*	*10*
	tCtrl	*0.79*	*1*	*1.01*
1000 mg/kg bw/day Group 4	*Mean*	*8.4*	*1.0319*	*7.15*
	SD	*5.93*	*0.0141*	*0.67*
	N	*10*	*10*	*10*
	tCtrl	*0.97*	*1*	*1.03*

Table 14b. Summary of Urinalysis Values: F0 Generation - females

Sex: Female *Day: 23 Relative to Littering (Litter: A)*		*Reporting Urinalysis*		
		VOLUME (mL)	*SPECIFIC GRAVITY*	*URINE pH*
0 mg/kg bw/day Group 1	*Mean*	*10*	*1.0243*	*6.55*
	SD	*3.53*	*0.0067*	*0.37*
	N	*10*	*10*	*10*
100 mg/kg bw/day Group 2	*Mean*	*8.2*	*1.0331*	*6.4*
	SD	*4.83*	*0.0092*	*0.39*
	N	*10*	*10*	*10*
	tCtrl	*0.82*	*1.01*	*0.98*
300 mg/kg bw/day Group 3	*Mean*	*11.1*	*1.0284*	*6.65*
	SD	*4.98*	*0.0057*	*0.47*
	N	*10*	*10*	*10*
	tCtrl	*1.11*	*1*	*1.02*
1000 mg/kg bw/day Group 4	*Mean*	*8.6*	*1.0373 **	*6.6*
	SD	*4.12*	*0.0115*	*0.21*
	N	*10*	*10*	*10*
	tCtrl	*0.86*	*1.01*	*1.01*

** = $p \leq 0.05$*

Table 15a. Summary of Organ Weights: F0 Generation - Males

Sex: Male		0 mg/kg bw/day Group 1	100 mg/kg bw/day Group 2	300 mg/kg bw/day Group 3	1000 mg/kg bw/day Group 4
Day(s) Relative to Start Date					
Terminal Body Weight (g)	Mean	365.4	364.9	369.8	350.5
	SD	25.2	35.1	27.9	27.5
	N	25	25	25	25
	%Diff	-	-0.1	1.2	-4.1
Brain (g)	Mean	2.0469	2.0438	2.0378	2.0051
	SD	0.0552	0.0831	0.0841	0.0792
	N	25	25	25	25
	%Diff	-	-0.1524	-0.4456	-2.0421
Brain (%bw)	Mean	0.56228	0.56421	0.55322	0.57451
	SD	0.03424	0.04911	0.03618	0.03849
	N	25	25	25	25
	%Diff	-	0.34309	-1.61066	2.17581
Epididymis Weight (g)	Mean	1.1369	1.1296	1.127	1.1296
	SD	0.0759	0.1101	0.1369	0.091
	N	25	25	25	25
	%Diff	-	-0.6438	-0.869	-0.6474
Epididymis (%bw)	Mean	0.31257	0.31137	0.30537	0.32393
	SD	0.02969	0.0342	0.03784	0.03426
	N	25	25	25	25
	%Diff	-	-0.38394	-2.30354	3.63538
Gland, Adrenal Weight (g)	Mean	0.05257	0.05219	0.0504	0.05496
	SD	0.00825	0.00796	0.00764	0.00583
	N	25	25	25	25
	%Diff	-	-0.71526	-4.13179	4.5503
Gland, Adrenal (%bw)	Mean	0.01444	0.01439	0.01366	0.01573
	SD	0.00243	0.00236	0.0021	0.00169
	N	25	25	25	25
	%Diff	-	-0.38381	-5.39105	8.9099
Gland, Pituitary Weight (g)	Mean	0.00903	0.00881	0.00862	0.00856
	SD	0.00082	0.00114	0.00144	0.00094
	N	25	25	25	25
	%Diff	-	-2.48007	-4.56156	-5.27015
Gland, Pituitary (%bw)	Mean	0.00248	0.00242	0.00234	0.00244

	SD	*0.00024*	*0.00027*	*0.00039*	*0.00022*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*-2.40358*	*-5.66822*	*-1.39435*
Gland, Prostate Weight (g)	*Mean*	*0.9275*	*0.9001*	*0.8876*	*0.8383*
	SD	*0.1984*	*0.1627*	*0.1023*	*0.12*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*-2.9542*	*-4.2955*	*-9.6131*
Gland, Prostate (%bw)	*Mean*	*0.25469*	*0.24852*	*0.24102*	*0.24009*
	SD	*0.05654*	*0.04814*	*0.03034*	*0.03509*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*-2.42182*	*-5.36707*	*-5.73079*
Seminal Vesicle Weight (g)	*Mean*	*1.3473*	*1.3233*	*1.328*	*1.3786*
	SD	*0.3771*	*0.2028*	*0.2262*	*0.2848*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*-1.7814*	*-1.4281*	*2.3217*
Seminal Vesicle (%bw)	*Mean*	*0.3706*	*0.36477*	*0.36146*	*0.3954*
	SD	*0.10466*	*0.05874*	*0.06789*	*0.08928*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*-1.5731*	*-2.46566*	*6.69427*
Thyroid/ Parathyroid (g)	*Mean*	*0.01616*	*0.01668*	*0.01812*	*0.01597*
	SD	*0.00276*	*0.00325*	*0.00352*	*0.00382*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*3.21862*	*12.15647*	*-1.1389*
Thyroid/ Parathyroid (%bw)	*Mean*	*0.00442*	*0.00459*	*0.00492*	*0.00455*
	SD	*0.00071*	*0.00093*	*0.00096*	*0.00098*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*3.86454*	*11.16008*	*2.80188*
Heart Weight (g)	*Mean*	*0.9676*	*0.9803*	*0.9806*	*0.9434*
	SD	*0.0601*	*0.0862*	*0.0828*	*0.0902*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*1.3104*	*1.3352*	*-2.5092*
Heart (%bw)	*Mean*	*0.26513*	*0.26938*	*0.26568*	*0.26928*
	SD	*0.01058*	*0.01716*	*0.01957*	*0.01589*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*1.60077*	*0.20585*	*1.56323*

Kidney Weight (g)	*Mean*	*2.2423*	*2.3324*	*2.3373*	*2.324*
	SD	*0.216*	*0.2671*	*0.254*	*0.165*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*4.0155*	*4.2367*	*3.6444*
Kidney (%bw)	*Mean*	*0.61442*	*0.63929*	*0.63269*	*0.66480 **
	SD	*0.05043*	*0.04792*	*0.0575*	*0.04453*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*4.04758*	*2.97335*	*8.19922*
Liver Weight (g)	*Mean*	*8.5536*	*8.7339*	*9.1074*	*8.7793*
	SD	*0.777*	*1.0929*	*0.829*	*0.6332*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*2.1072*	*6.474*	*2.6379*
Liver (%bw)	*Mean*	*2.34244*	*2.39294*	*2.46913*	*2.50779 **
	SD	*0.16355*	*0.17453*	*0.22968*	*0.10399*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*2.15571*	*5.40851*	*7.0587*
Spleen (g)	*Mean*	*0.5263*	*0.5681*	*0.5805*	*0.5386*
	SD	*0.0526*	*0.0858*	*0.145*	*0.0619*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*7.9501*	*10.2987*	*2.3486*
Spleen (%bw)	*Mean*	*0.14443*	*0.15596*	*0.15764*	*0.15401*
	SD	*0.01526*	*0.01958*	*0.04305*	*0.01661*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*7.98586*	*9.14502*	*6.63253*
Testis Weight (g)	*Mean*	*3.5287*	*3.4294*	*3.4925*	*3.4976*
	SD	*0.2433*	*0.3564*	*0.4981*	*0.2993*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*-2.8147*	*-1.0247*	*-0.8819*
Testis (%bw)	*Mean*	*0.97078*	*0.94514*	*0.94591*	*1.00388*
	SD	*0.10058*	*0.1071*	*0.14078*	*0.12028*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*-2.64111*	*-2.56204*	*3.40899*
Thymus Weight (g)	*Mean*	*0.2594*	*0.2665*	*0.2837*	*0.2319*
	SD	*0.0469*	*0.0474*	*0.0541*	*0.0434*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*2.729*	*9.3586*	*-10.6229*
Thymus (%bw)	*Mean*	*0.07104*	*0.0732*	*0.07668*	*0.06619*
	SD	*0.01225*	*0.01205*	*0.01344*	*0.01159*
	N	*25*	*25*	*25*	*25*
	%Diff	*-*	*3.04993*	*7.93652*	*-6.82895*

** = p ≤ *0.01*

Table 15b. Summary of Organ Weights: F0 Generation - Females

Sex: Female		*0 mg/kg bw/day Group 1*	*100 mg/kg bw/day Group 2*	*300 mg/kg bw/day Group 3*	*1000 mg/kg bw/day Group 4*
Day(s) Relative to Littering (Litter: A)					
Terminal Body Weight (g)	*Mean*	*226.3*	*226.9*	*225.9*	*228.5*
	SD	*14.1*	*16.2*	*17.4*	*11.9*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*0.3*	*-0.2*	*1*
Brain (g)	*Mean*	*1.905*	*1.8749*	*1.9054*	*1.8799*
	SD	*0.0761*	*0.0675*	*0.0871*	*0.0549*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*-1.5817*	*0.02*	*-1.3176*
Brain (%bw)	*Mean*	*0.84442*	*0.82901*	*0.84694*	*0.82465*
	SD	*0.05392*	*0.0466*	*0.05947*	*0.04275*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*-1.82438*	*0.2986*	*-2.34074*
Gland, Adrenal Weight (g)	*Mean*	*0.06684*	*0.06886*	*0.06611*	*0.07097*
	SD	*0.01318*	*0.00746*	*0.00978*	*0.0124*
	N	*23*	*23*	*21*	*20*
	%Diff	*-*	*3.02478*	*-1.09159*	*6.18032*
Gland, Adrenal (%bw)	*Mean*	*0.02945*	*0.03043*	*0.02928*	*0.03108*
	SD	*0.00526*	*0.00341*	*0.00401*	*0.00533*
	N	*23*	*23*	*21*	*20*
	%Diff	*-*	*3.33945*	*-0.56213*	*5.5523*
Gland, Pituitary Weight (g)	*Mean*	*0.01122*	*0.01229*	*0.0111*	*0.01109*
	SD	*0.00133*	*0.00437*	*0.00147*	*0.00162*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*9.50128*	*-1.03443*	*-1.16599*
Gland, Pituitary (%bw)	*Mean*	*0.00496*	*0.00545*	*0.00492*	*0.00486*
	SD	*0.00055*	*0.0021*	*0.00054*	*0.00071*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*9.89586*	*-0.93201*	*-2.02352*
Thyroid/ Parathyroid (g)	*Mean*	*0.01463*	*0.01463*	*0.01592*	*0.01504*
	SD	*0.00289*	*0.00295*	*0.00316*	*0.00301*

	N	*24*	*23*	*21*	*19*
	%Diff	*-*	*0.00743*	*8.88075*	*2.852*
Thyroid/ Parathyroid (%bw)	*Mean*	*0.00646*	*0.00648*	*0.00707*	*0.00662*
	SD	*0.00121*	*0.00141*	*0.00143*	*0.00137*
	N	*24*	*23*	*21*	*19*
	%Diff	*-*	*0.23562*	*9.47339*	*2.47721*
Heart Weight (g)	*Mean*	*0.814*	*0.8238*	*0.8513*	*0.8281*
	SD	*0.0499*	*0.0632*	*0.0615*	*0.0509*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*1.2018*	*4.5864*	*1.7322*
Heart (%bw)	*Mean*	*0.36024*	*0.36355*	*0.37783*	*0.36297*
	SD	*0.01992*	*0.02175*	*0.02656*	*0.02249*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*0.91807*	*4.88242*	*0.75692*
Kidney Weight (g)	*Mean*	*1.841*	*1.8673*	*1.8969*	*1.9572*
	SD	*0.158*	*0.1889*	*0.1716*	*0.1382*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*1.4335*	*3.0364*	*6.3142*
Kidney (%bw)	*Mean*	*0.81399*	*0.82224*	*0.84*	*0.85786 **
	SD	*0.05569*	*0.04717*	*0.04824*	*0.06053*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*1.01345*	*3.19553*	*5.39026*
Liver Weight (g)	*Mean*	*8.1707*	*8.2214*	*8.2765*	*8.838*
	SD	*0.8439*	*0.8221*	*0.9261*	*0.9565*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*0.6213*	*1.2956*	*8.1668*
Liver (%bw)	*Mean*	*3.60706*	*3.62253*	*3.65988*	*3.86751 ***
	SD	*0.25012*	*0.23879*	*0.22641*	*0.3492*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*0.42881*	*1.46423*	*7.22069*
Ovary / Oviduct (g)	*Mean*	*0.1361*	*0.1416*	*0.1377*	*0.1365*
	SD	*0.0222*	*0.0193*	*0.0188*	*0.0199*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*4.0284*	*1.1325*	*0.2755*
Ovary/Oviduct (%bw)	*Mean*	*0.0602*	*0.06279*	*0.06128*	*0.05972*
	SD	*0.00936*	*0.01*	*0.00956*	*0.00795*
	N	*24*	*23*	*21*	*20*

	%Diff	*-*	*4.31673*	*1.79928*	*-0.78402*
Spleen (g)	*Mean*	*0.5034*	*0.5089*	*0.4946*	*0.4957*
	SD	*0.0723*	*0.0612*	*0.0818*	*0.0692*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*1.1002*	*-1.7489*	*-1.5247*
Spleen (%bw)	*Mean*	*0.2219*	*0.22409*	*0.21836*	*0.21698*
	SD	*0.02429*	*0.01971*	*0.02708*	*0.02762*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*0.98736*	*-1.59707*	*-2.21836*
Thymus Weight (g)	*Mean*	*0.2026*	*0.2057*	*0.1963*	*0.1951*
	SD	*0.039*	*0.0469*	*0.04*	*0.0408*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*1.5369*	*-3.1051*	*-3.7138*
Thymus (%bw)	*Mean*	*0.0896*	*0.09086*	*0.08718*	*0.08555*
	SD	*0.01625*	*0.01999*	*0.01837*	*0.01788*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*1.40464*	*-2.70693*	*-4.52304*
Uterus/Cervix (g)	*Mean*	*0.5882*	*0.6564*	*0.6662*	*0.7057*
	SD	*0.1386*	*0.2121*	*0.2055*	*0.2183*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*11.5995*	*13.2737*	*19.9745*
Uterus/Cervix (%bw)	*Mean*	*0.2598*	*0.2882*	*0.29744*	*0.31159*
	SD	*0.05775*	*0.08602*	*0.09951*	*0.10386*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*10.93253*	*14.49085*	*19.9357*

p ≤ 0.05; ** = p ≤ *0.01*

Table 16. Summary of Macroscopic Pathology: F0 Generation - Scheduled Euthanasia animals.

Removal Reason(s): TERMINAL EUTHANASIA		*Male*				*Female*		
Summary: Incidence	*0 mg/ kg bw/day Group 1*	*100 mg/ kg bw/day Group 2*	*300 mg/ kg bw/day Group 3*	*1000 mg/ kg bw/day Group 4*	*0 mg/ kg bw/day Group 1*	*100 mg/ kg bw/day Group 2*	*300 mg/ kg bw/day Group 3*	*1000 mg/ kg bw/day Group 4*
Number of Animals:	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
ADIPOSE TISSUE, ABDOMINAL								
Submitted	*0*	*0*	*0*	*1*	*.*	*.*	*.*	*.*

Nodule; firm, yellow	.	.	.	*1*	.	.	.	.
BONE MARROW, STERNUM								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
BONE, STERNUM								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
BRAIN								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
CERVIX								
Submitted	.	.	.	.	*24*	*24*	*22*	*23*
No Visible Lesions	.	.	.	.	*24*	*24*	*22*	*21*
Abnormal appearance	.	.	.	.	*0*	*0*	*0*	*1*
Nodule; tan	.	.	.	.	*0*	*0*	*0*	*1*
EPIDIDYMIS								
Submitted	*25*	*25*	*25*	*25*	.	.	.	.
No Visible Lesions	*25*	*25*	*24*	*25*	.	.	.	.
Small	*0*	*0*	*1*	*0*	.	.	.	.
EYE								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*24*	*25*	*24*	*24*	*22*	*23*
Protrusion	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*
GLAND, ADRENAL								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*23*	*24*	*22*	*21*
Discoloration, pale	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
Enlargement	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*1*
Focus, dark; black	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*

GLAND, CLITORAL								
Submitted	*.*	*.*	*.*	*.*	*1*	*0*	*1*	*1*
Focus, dark; brown	*.*	*.*	*.*	*.*	*1*	*.*	*0*	*0*
Focus, dark; tan	*.*	*.*	*.*	*.*	*0*	*.*	*1*	*1*
GLAND, COAGULATING								
Submitted	*25*	*25*	*25*	*25*	*.*	*.*	*.*	*.*
No Visible Lesions	*25*	*25*	*25*	*25*	*.*	*.*	*.*	*.*
GLAND, HARDERIAN								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
GLAND, MAMMARY								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
GLAND, PARATHYROID								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
GLAND, PITUITARY								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
GLAND, PROSTATE								
Submitted	*25*	*25*	*25*	*25*	*.*	*.*	*.*	*.*
No Visible Lesions	*24*	*25*	*25*	*24*	*.*	*.*	*.*	*.*
Small Enlargement	*0*	*0*	*0*	*1*	*.*	*.*	*.*	*.*
	1	*0*	*0*	*0*	*.*	*.*	*.*	*.*
GLAND, SEMINAL VESICLE								
Submitted	*25*	*25*	*25*	*25*	*.*	*.*	*.*	*.*
No Visible Lesions	*23*	*25*	*25*	*25*	*.*	*.*	*.*	*.*
Small	*2*	*0*	*0*	*0*	*.*	*.*	*.*	*.*

GLAND, THYROID								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*24*	*24*	*25*	*24*	*24*	*22*	*21*
Enlargement	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*
Small	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*
Abnormal appearance	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
HEART								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
KIDNEY								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*23*	*24*	*25*	*25*	*24*	*24*	*22*	*22*
Dilatation; pelvis	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Small	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*
Discoloration, dark; brown	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
LARGE INTESTINE, CECUM								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
LARGE INTESTINE, COLON								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
LARGE INTESTINE, RECTUM								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
LIVER								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*24*	*24*	*23*	*24*	*23*	*22*	*23*
Small; lateral lobe	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*

Small; papillary process	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*
Discoloration, dark; red, medial lobe	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*
Discoloration, dark; brown, papillary process	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*
Abnormal appearance; medial lobe	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*
Hernia; medial lobe	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*
Focus, raised; yellow, medial lobe	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*
LUNG								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
LYMPH NODE, ILIAC								
Submitted	*.*	*.*	*.*	*.*	*0*	*0*	*0*	*1*
No Visible Lesions	*.*	*.*	*.*	*.*	*.*	*.*	*.*	*1*
LYMPH NODE, MEDIASTINAL								
Submitted	*0*	*1*	*0*	*0*	*.*	*.*	*.*	*.*
Discoloration, dark; red		*1*	*.*	*.*	*.*	*.*	*.*	*.*
LYMPH NODE, MESENTERIC								
Submitted	*0*	*1*	*0*	*0*	*.*	*.*	*.*	*.*
No Visible Lesions	*.*	*1*	*.*	*.*	*.*	*.*	*.*	*.*
LYMPH NODE, PANCREATIC								
Submitted	*0*	*0*	*1*	*0*	*.*	*.*	*.*	*.*
Discoloration, dark; red		*.*	*1*	*.*	*.*	*.*	*.*	*.*

LYMPH NODE, RENAL								
Submitted	*0*	*0*	*0*	*1*	*0*	*1*	*0*	*0*
Discoloration, dark; red		*.*	*.*	*1*	*.*	*1*	*.*	*.*
MUSCLE, SKELETAL								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
NERVE, OPTIC								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
NERVE, SCIATIC								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*21*	*23*
Abnormal appearance	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*
OVARY								
Submitted	*.*	*.*	*.*	*.*	*24*	*24*	*22*	*23*
No Visible Lesions	*.*	*.*	*.*	*.*	*24*	*24*	*22*	*23*
OVIDUCT								
Submitted	*.*	*.*	*.*	*.*	*24*	*24*	*22*	*23*
No Visible Lesions	*.*	*.*	*.*	*.*	*24*	*24*	*22*	*23*
SKIN								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*23*	*22*	*23*
Thin hair coat; abdominal	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*
Thin hair coat; forelimb, left	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*
Thin hair coat; forelimb, right	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*
Thin hair coat; lumbar	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*

SMALL INTESTINE, DUODENUM								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
SMALL INTESTINE, ILEUM								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
SMALL INTESTINE, JEJUNUM								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
SPINAL CORD								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
SPLEEN								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*23*	*24*	*25*	*23*	*24*	*22*	*23*
Abnormal appearance	*0*	*2*	*0*	*0*	*1*	*0*	*0*	*0*
Enlargement	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*
STOMACH								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*23*	*25*	*24*	*23*	*24*	*22*	*21*
Focus, dark; black, glandular	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*
Focus, dark; red, glandular	*0*	*2*	*0*	*0*	*0*	*0*	*0*	*2*
Abnormal appearance; gelatinous, glandular	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*
TESTIS								
Submitted	*25*	*25*	*25*	*25*	.	.	.	.
No Visible Lesions	*25*	*24*	*24*	*25*	.	.	.	.

Small	*0*	*0*	*1*	*0*	*.*	*.*	*.*	*.*
Abnormal appearance	*0*	*1*	*0*	*0*	*.*	*.*	*.*	*.*
THYMUS								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*24*	*24*	*25*	*24*	*23*	*24*	*22*	*23*
Focus, dark; red	*1*	*1*	*0*	*1*	*1*	*0*	*0*	*0*
TRACHEA								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
URINARY BLADDER								
Submitted	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
No Visible Lesions	*25*	*25*	*24*	*25*	*24*	*24*	*22*	*23*
Abnormal content; mucoid, yellow	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*
UTERUS								
Submitted	*.*	*.*	*.*	*.*	*24*	*24*	*22*	*23*
No Visible Lesions	*.*	*.*	*.*	*.*	*23*	*20*	*17*	*16*
Fluid accumulation, pale	*.*	*.*	*.*	*.*	*1*	*4*	*5*	*7*
VAGINA								
Submitted	*.*	*.*	*.*	*.*	*24*	*24*	*22*	*23*
No Visible Lesions	*.*	*.*	*.*	*.*	*24*	*24*	*22*	*23*
VAS DEFERENS								
Submitted	*25*	*25*	*25*	*25*	*.*	*.*	*.*	*.*
No Visible Lesions	*25*	*25*	*25*	*25*	*.*	*.*	*.*	*.*

Table 17. Summary of Microscopic Pathology: F0 Generation - Scheduled Euthanasia animals.

Removal Reason(s): TERMINAL EUTHANASIA		*Male*				*Female*		
Summary: Incidence	*0 mg/ kg bw/day Group 1*	*100 mg/ kg bw/day Group 2*	*300 mg/ kg bw/day Group 3*	*1000 mg/ kg bw/day Group 4*	*0 mg/ kg bw/day Group 1*	*100 mg/ kg bw/day Group 2*	*300 mg/ kg bw/day Group 3*	*1000 mg/ kg bw/day Group 4*

Number of Animals:	*25*	*25*	*25*	*25*	*24*	*24*	*22*	*23*
ADIPOSE TISSUE,								
ABDOMINAL								
Examined	*0*	*0*	*0*	*1*	*.*	*.*	*.*	*.*
Necrosis; adipose tissue	*.*	*.*	*.*	*1*	*.*	*.*	*.*	*.*
.... mild	*.*	*.*	*.*	*1*	*.*	*.*	*.*	*.*
BONE MARROW, STERNUM								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*23*	*.*	*.*	*23*	*21*	*.*	*.*	*21*
Hematopoietic cells, decreased	*2*	*.*	*.*	*1*	*0*	*.*	*.*	*0*
.... minimal	*2*	*.*	*.*	*1*	*0*	*.*	*.*	*0*
Cellularity, increased; adipocytes	*0*	*.*	*.*	*1*	*3*	*.*	*.*	*2*
.... minimal	*0*	*.*	*.*	*1*	*3*	*.*	*.*	*2*
BONE, STERNUM								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*25*	*.*	*.*	*25*	*24*	*.*	*.*	*23*
BRAIN								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*25*	*.*	*.*	*25*	*24*	*.*	*.*	*22*
Gliosis	*0*	*.*	*.*	*0*	*0*	*.*	*.*	*1*
.... mild	*0*	*.*	*.*	*0*	*0*	*.*	*.*	*1*
CERVIX								
Examined	*.*	*.*	*.*	*.*	*24*	*1*	*1*	*23*
No Visible Lesions	*.*	*.*	*.*	*.*	*24*	*1*	*1*	*22*
Cyst; squamous	*.*	*.*	*.*	*.*	*0*	*0*	*0*	*1*
.... mild	*.*	*.*	*.*	*.*	*0*	*0*	*0*	*1*
EPIDIDYMIS, LEFT								
Examined	*25*	*1*	*2*	*25*	*.*	*.*	*.*	*.*

No Visible Lesions	*23*	*0*	*1*	*23*	.	.	.	.
Infiltration, mononuclear cell; interstitial	*1*	*0*	*0*	*1*	.	.	.	.
.... minimal	*1*	*0*	*0*	*1*	.	.	.	.
Vacuolation, epithelial	*1*	*0*	*0*	*1*	.	.	.	.
.... minimal	*1*	*0*	*0*	*1*	.	.	.	.
Cellularity, decreased; sperm	*0*	*1*	*1*	*0*	.	.	.	.
.... mild	*0*	*0*	*1*	*0*	.	.	.	.
.... marked	*0*	*1*	*0*	*0*	.	.	.	.
Cellular debris	*0*	*1*	*0*	*0*	.	.	.	.
.... moderate	*0*	*1*	*0*	*0*	.	.	.	.
EYE								
Examined	*25*	*0*	*1*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*23*	.	*0*	*23*	*23*	.	.	*21*
Hemorrhage, retro-orbital	*2*	.	*1*	*2*	*1*	.	.	*2*
.... minimal	*2*	.	*0*	*1*	*1*	.	.	*2*
.... mild	*0*	.	*1*	*1*	*0*	.	.	*0*
GLAND, ADRENAL								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*24*	.	.	*23*	*23*	.	.	*22*
Vacuolation, medullary	*0*	.	.	*1*	*0*	.	.	*0*
.... minimal	*0*	.	.	*1*	*0*	.	.	*0*
Vacuolation, cortical, zonafasciculata	*1*	.	.	*0*	*0*	.	.	*0*
.... minimal	*1*	.	.	*0*	*0*	.	.	*0*
Hemorrhage, acute, cortical	*0*	.	.	*0*	*0*	.	.	*1*

.... moderate	0	.	.	0	0	.	.	1
Hypertrophy, cortical, zona fasciculata	0	.	.	1	1	.	.	0
.... minimal	0	.	.	1	1	.	.	0
GLAND, CLITORAL								
Examined	.	.	.	.	1	0	1	1
Dilatation; ductular	.	.	.	.	1	.	1	1
.... mild	.	.	.	.	1	.	0	1
.... moderate	.	.	.	.	0	.	1	0
GLAND, COAGULATING								
Examined	24	1	1	25	.	.	.	.
No Visible Lesions	24	1	1	25	.	.	.	.
Not Examined: Not Present In Section.	1	0	0	0	.	.	.	.
GLAND, MAMMARY								
Examined	25	0	0	25	24	0	0	23
No Visible Lesions	25	.	.	25	0	.	.	3
Infiltration, mononuclear cell	0	.	.	0	0	.	.	1
.... minimal	0	.	.	0	0	.	.	1
Development; lobuloalveolar	0	.	.	0	24	.	.	20
GLAND, PARATHYROID								
Examined	25	0	0	25	23	0	0	22
No Visible Lesions	25	.	.	25	23	.	.	22
Not Examined: Not Present In Section.	.	.	.	.	1	0	0	1

GLAND, PITUITARY								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*16*	*.*	*.*	*19*	*18*	*.*	*.*	*22*
Cyst; pars distalis	*2*	*.*	*.*	*2*	*2*	*.*	*.*	*0*
Cyst; pars intermedia	*7*	*.*	*.*	*4*	*4*	*.*	*.*	*1*
GLAND, PROSTATE								
Examined	*25*	*1*	*1*	*25*	*.*	*.*	*.*	*.*
No Visible Lesions	*21*	*0*	*0*	*25*	*.*	*.*	*.*	*.*
Infiltration, mononuclear cell; interstitial	*4*	*1*	*1*	*0*	*.*	*.*	*.*	*.*
.... minimal	*4*	*1*	*1*	*0*	*.*	*.*	*.*	*.*
Infiltration, neutrophilic; lumen	*1*	*0*	*0*	*0*	*.*	*.*	*.*	*.*
.... minimal	*1*	*0*	*0*	*0*	*.*	*.*	*.*	*.*
GLAND, SEMINAL VESICLE								
Examined	*25*	*1*	*1*	*25*	*.*	*.*	*.*	*.*
No Visible Lesions	*25*	*1*	*1*	*25*	*.*	*.*	*.*	*.*
GLAND, THYROID								
Examined	*25*	*1*	*1*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*18*	*1*	*1*	*15*	*20*	*.*	*.*	*21*
Cyst; ultimobranchial	*2*	*0*	*0*	*5*	*2*	*.*	*.*	*1*
Hypertrophy, follicular cell	*4*	*0*	*0*	*4*	*2*	*.*	*.*	*0*
.... minimal	*4*	*0*	*0*	*4*	*2*	*.*	*.*	*0*
Ectopia; thymic	*1*	*0*	*0*	*1*	*0*	*.*	*.*	*1*
Colloid alteration	*1*	*0*	*0*	*1*	*0*	*.*	*.*	*0*
.... minimal	*1*	*0*	*0*	*1*	*0*	*.*	*.*	*0*

HEART								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*25*	*.*	*.*	*23*	*24*	*.*	*.*	*23*
Infiltration, mononuclear cell; ventricular	*0*	*.*	*.*	*2*	*0*	*.*	*.*	*0*
.... minimal	*0*	*.*	*.*	*2*	*0*	*.*	*.*	*0*
KIDNEY								
Examined	*25*	*1*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*13*	*0*	*.*	*15*	*22*	*.*	*.*	*18*
Cast; hyaline	*0*	*0*	*.*	*0*	*0*	*.*	*.*	*1*
.... minimal	*0*	*0*	*.*	*0*	*0*	*.*	*.*	*1*
Cast; hyaline, medullary	*1*	*0*	*.*	*1*	*1*	*.*	*.*	*0*
.... minimal	*1*	*0*	*.*	*1*	*1*	*.*	*.*	*0*
Cast; hyaline, papillary	*0*	*0*	*.*	*1*	*0*	*.*	*.*	*1*
.... minimal	*0*	*0*	*.*	*1*	*0*	*.*	*.*	*1*
Infiltration, mononuclear cell; interstitial	*6*	*1*	*.*	*5*	*1*	*.*	*.*	*2*
.... minimal	*6*	*1*	*.*	*5*	*1*	*.*	*.*	*2*
Basophilia; cortical, tubular	*5*	*1*	*.*	*7*	*0*	*.*	*.*	*2*
.... minimal	*5*	*1*	*.*	*7*	*0*	*.*	*.*	*2*
Accumulation; cortical, hyaline droplets, tubular	*5*	*0*	*.*	*1*	*0*	*.*	*.*	*0*
.... minimal	*5*	*0*	*.*	*1*	*0*	*.*	*.*	*0*
Dilatation; pelvis	*2*	*0*	*.*	*0*	*0*	*.*	*.*	*0*

.... minimal	1	0	.	0	0	.	.	0
.... mild	1	0	.	0	0	.	.	0
Cyst; medullary	0	0	.	0	0	.	.	1
.... mild	0	0	.	0	0	.	.	1
LARGE INTESTINE, CECUM								
Examined	25	0	0	25	24	0	0	23
No Visible Lesions	25	.	.	25	24	.	.	23
LARGE INTESTINE, COLON								
Examined	25	0	0	25	24	0	0	23
No Visible Lesions	25	.	.	25	23	.	.	23
Erosion	0	.	.	0	1	.	.	0
.... minimal	0	.	.	0	1	.	.	0
LARGE INTESTINE, RECTUM								
Examined	25	0	0	25	24	0	0	23
No Visible Lesions	25	.	.	25	24	.	.	23
LIVER								
Examined	25	0	1	25	24	1	0	23
No Visible Lesions	13	.	1	15	21	0	.	18
Necrosis; hepatocellular	0	.	0	0	1	1	.	0
.... minimal	0	.	0	0	1	0	.	0
.... mild	0	.	0	0	0	1	.	0
Necrosis; lobar	0	.	0	1	0	0	.	0
.... moderate	0	.	0	1	0	0	.	0
Infiltration, mononuclear cell	12	.	0	9	3	0	.	5
.... minimal	12	.	0	9	3	0	.	5
LUNG								
Examined	25	0	0	25	24	0	0	23
No Visible Lesions	18	.	.	18	16	.	.	16

Infiltration, mixed cell; alveolar	*0*	*.*	*.*	*2*	*0*	*.*	*.*	*0*
.... minimal	*0*	*.*	*.*	*2*	*0*	*.*	*.*	*0*
Infiltration, mixed cell; peribronchial, perivascular	*7*	*.*	*.*	*4*	*3*	*.*	*.*	*7*
.... minimal	*7*	*.*	*.*	*4*	*3*	*.*	*.*	*7*
Accumulation; alveolar, macrophage	*1*	*.*	*.*	*1*	*6*	*.*	*.*	*0*
.... minimal	*1*	*.*	*.*	*1*	*6*	*.*	*.*	*0*
LYMPH NODE, MEDIASTINAL								
Examined	*0*	*1*	*0*	*0*	*.*	*.*	*.*	*.*
Erythrophagocytosis	*1*	*.*	*.*	*.*	*.*	*.*	*.*	*.*
.... minimal	*.*	*1*	*.*	*.*	*.*	*.*	*.*	*.*
LYMPH NODE, PANCREATIC								
Examined	*0*	*0*	*1*	*0*	*.*	*.*	*.*	*.*
Normal morphology	*.*	*.*	*1*	*.*	*.*	*.*	*.*	*.*
LYMPH NODE, RENAL								
Examined	*0*	*0*	*0*	*1*	*0*	*1*	*0*	*0*
Erythrophagocytosis	*.*	*.*	*.*	*1*	*.*	*1*	*.*	*.*
.... mild	*.*	*.*	*.*	*1*	*.*	*1*	*.*	*.*
MUSCLE, SKELETAL								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*18*	*.*	*.*	*20*	*24*	*.*	*.*	*23*
Infiltration, mononuclear cell	*6*	*.*	*.*	*4*	*0*	*.*	*.*	*0*
.... minimal	*6*	*.*	*.*	*4*	*0*	*.*	*.*	*0*
Degeneration/ necrosis; myofiber	*1*	*.*	*.*	*1*	*0*	*.*	*.*	*0*
.... minimal	*1*	*.*	*.*	*1*	*0*	*.*	*.*	*0*

NERVE, OPTIC								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*24*	*.*	*.*	*23*	*23*	*.*	*.*	*23*
Hemorrhage	*1*	*.*	*.*	*2*	*1*	*.*	*.*	*0*
.... minimal	*1*	*.*	*.*	*2*	*1*	*.*	*.*	*0*
NERVE, SCIATIC								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*1*	*23*
No Visible Lesions	*25*	*.*	*.*	*25*	*24*	*.*	*1*	*23*
OVARY								
Examined	*.*	*.*	*.*	*.*	*24*	*1*	*1*	*23*
No Visible Lesions	*.*	*.*	*.*	*.*	*24*	*1*	*1*	*23*
OVIDUCT								
Examined	*.*	*.*	*.*	*.*	*24*	*1*	*1*	*23*
No Visible Lesions	*.*	*.*	*.*	*.*	*24*	*1*	*1*	*23*
SKIN								
Examined	*.*	*.*	*.*	*.*	*0*	*1*	*0*	*0*
No Visible Lesions	*.*	*.*	*.*	*.*	*.*	*1*	*.*	*.*
SMALL INTESTINE, DUODENUM								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*25*	*.*	*.*	*25*	*24*	*.*	*.*	*23*
SMALL INTESTINE, ILEUM								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*25*	*.*	*.*	*25*	*24*	*.*	*.*	*23*
SMALL INTESTINE, JEJUNUM								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*25*	*.*	*.*	*25*	*24*	*.*	*.*	*23*
SPINAL CORD								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*

No Visible Lesions	*25*	*.*	*.*	*25*	*24*	*.*	*.*	*23*
SPLEEN								
Examined	*25*	*0*	*1*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*2*	*.*	*0*	*1*	*0*	*.*	*.*	*0*
Pigment	*23*	*.*	*0*	*24*	*24*	*.*	*.*	*23*
.... minimal	*21*	*.*	*0*	*24*	*13*	*.*	*.*	*18*
.... mild	*2*	*.*	*0*	*0*	*11*	*.*	*.*	*5*
Extramedullary hematopoiesis	*11*	*.*	*1*	*9*	*2*	*.*	*.*	*1*
.... minimal	*8*	*.*	*0*	*8*	*2*	*.*	*.*	*1*
.... mild	*3*	*.*	*0*	*1*	*0*	*.*	*.*	*0*
.... moderate	*0*	*.*	*1*	*0*	*0*	*.*	*.*	*0*
STOMACH								
Examined	*25*	*2*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*19*	*1*	*.*	*17*	*17*	*.*	*.*	*16*
Hemorrhage; mucosal, glandular	*0*	*1*	*.*	*0*	*0*	*.*	*.*	*1*
.... minimal	*0*	*1*	*.*	*0*	*0*	*.*	*.*	*1*
Ulceration; non-glandular	*0*	*0*	*.*	*2*	*0*	*.*	*.*	*1*
.... minimal	*0*	*0*	*.*	*1*	*0*	*.*	*.*	*1*
.... mild	*0*	*0*	*.*	*1*	*0*	*.*	*.*	*0*
Vacuolation; epithelial, limiting ridge	*1*	*0*	*.*	*1*	*0*	*.*	*.*	*1*
.... minimal	*1*	*0*	*.*	*1*	*0*	*.*	*.*	*1*
Erosion; glandular	*2*	*0*	*.*	*0*	*1*	*.*	*.*	*0*
.... minimal	*2*	*0*	*.*	*0*	*0*	*.*	*.*	*0*
.... mild	*0*	*0*	*.*	*0*	*1*	*.*	*.*	*0*
Infiltration, mononuclear cell; glandular	*4*	*0*	*.*	*5*	*2*	*.*	*.*	*2*

.... minimal	4	0	.	5	2	.	.	2
Infiltration, mixed cell; glandular	1	0	.	0	1	.	.	2
.... minimal	1	0	.	0	1	.	.	2
Dilatation; glandular; mucosa	0	0	.	0	1	.	.	0
.... minimal	0	0	.	0	1	.	.	0
Edema; submucosal, glandular	1	0	.	1	1	.	.	2
.... minimal	0	0	.	1	1	.	.	2
.... mild	1	0	.	0	0	.	.	0
Edema; submucosal, non-glandular	0	0	.	2	2	.	.	0
.... minimal	0	0	.	0	2	.	.	0
.... mild	0	0	.	2	0	.	.	0
TESTIS								
Examined	25	1	2	25	.	.	.	.
No Visible Lesions	22	0	1	21	.	.	.	.
Degeneration; seminiferous tubule	1	1	1	3	.	.	.	.
.... minimal	1	0	0	3	.	.	.	.
.... mild	0	0	1	0	.	.	.	.
.... moderate	0	1	0	0	.	.	.	.
Atrophy; seminiferous tubule	2	0	0	1	.	.	.	.
.... minimal	2	0	0	0	.	.	.	.
.... mild	0	0	0	1	.	.	.	.
THYMUS								
Examined	25	1	0	25	24	0	0	23
No Visible Lesions	23	0	.	22	18	.	.	17

Cellularity, increased; epithelial, tubules and cords	*2*	*0*	*.*	*2*	*5*	*.*	*.*	*5*
.... minimal	*2*	*0*	*.*	*2*	*4*	*.*	*.*	*5*
.... mild	*0*	*0*	*.*	*0*	*1*	*.*	*.*	*0*
Congestion; hemorrhage	*0*	*1*	*.*	*1*	*1*	*.*	*.*	*1*
.... minimal	*0*	*1*	*.*	*1*	*1*	*.*	*.*	*1*
TRACHEA								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*21*	*.*	*.*	*18*	*19*	*.*	*.*	*19*
Dilatation; submucosal gland	*4*	*.*	*.*	*7*	*5*	*.*	*.*	*4*
.... minimal	*3*	*.*	*.*	*4*	*4*	*.*	*.*	*3*
.... mild	*1*	*.*	*.*	*3*	*1*	*.*	*.*	*1*
URINARY BLADDER								
Examined	*25*	*0*	*0*	*25*	*24*	*0*	*0*	*23*
No Visible Lesions	*25*	*.*	*.*	*24*	*24*	*.*	*.*	*23*
Infiltration, mononuclear cell; submucosal	*0*	*.*	*.*	*1*	*0*	*.*	*.*	*0*
.... minimal	*0*	*.*	*.*	*1*	*0*	*.*	*.*	*0*
UTERUS								
Examined	*.*	*.*	*.*	*.*	*24*	*1*	*1*	*23*
No Visible Lesions	*.*	*.*	*.*	*.*	*6*	*1*	*1*	*4*
Dilatation; lumen	*.*	*.*	*.*	*.*	*0*	*0*	*0*	*1*
.... severe	*.*	*.*	*.*	*.*	*0*	*0*	*0*	*1*
Implantation site(s)		*.*	*.*	*.*	*18*	*0*	*0*	*18*
VAGINA								
Examined	*.*	*.*	*.*	*.*	*24*	*1*	*1*	*23*
No Visible Lesions	*.*	*.*	*.*	*.*	*24*	*1*	*1*	*22*

Infiltration, mixed cell; lumen	.	.	.	.	*0*	*0*	*0*	*1*
.... minimal	.	.	.	.	*0*	*0*	*0*	*1*
VAS DEFERENS								
Examined	*25*	*1*	*1*	*25*	.	.	.	.
No Visible Lesions	*24*	*1*	*1*	*25*	.	.	.	.
Vacuolation epithelial	*1*	*0*	*0*	*0*	.	.	.	.
.... minimal	*1*	*0*	*0*	*0*	.	.	.	.

Table 18. Summary of Sperm Morphology Evaluation: F0 Generation

Sex: Male			*0 mg/kg bw/day Group 1*	*100 mg/kg bw/day Group 2*	*300 mg/kg bw/day Group 3*	*1000 mg/kg bw/day Group 4*
Day(s) Relative to Start Date						
>=200 Sperm	*86*	*N-ve*	*0*	*0*	*0*	*0*
Evaluated		*N+ve*	*25*	*25*	*25*	*24*
Normal Sperm/Total Sperm (%)	*86*	*Mean*	*92.06*	*85.24 **	*91.7*	*93.21*
		SD	*8.43*	*20.11*	*5.78*	*4.03*
		N	*25*	*25*	*25*	*24*
		%Diff	*-*	*-7.41*	*-0.39*	*1.25*
Normal Sperm	*86*	*Mean*	*184.1*	*170.5*	*183.4*	*186.4*
		SD	*16.9*	*40.2*	*11.6*	*8.1*
		N	*25*	*25*	*25*	*24*
		%Diff	*-*	*-7.4*	*-0.4*	*1.2*
Total Sperm Abnormal	*86*	*Mean*	*15.9*	*29.5*	*16.6*	*13.6*
		SD	*16.9*	*40.2*	*11.6*	*8.1*
		N	*25*	*25*	*25*	*24*
		%Diff	*-*	*85.9*	*4.5*	*-14.5*
Abnormal Head(s)	*86*	*Mean*	*0.8*	*1.4*	*1.3*	*1.3*
		SD	*1.2*	*2*	*2*	*1.4*
		N	*25*	*25*	*25*	*24*
		%Diff	*-*	*71.4*	*57.1*	*48.8*
Detached Head	*86*	*Mean*	*2.9*	*9.6*	*3.4*	*3.9*
		SD	*2.7*	*29.7*	*4.2*	*3.7*
		N	*25*	*25*	*25*	*24*
		%Diff	*-*	*231.9*	*16.7*	*36*
Abnormal Midpiece	*86*	*Mean*	*1*	*2.3*	*1.6*	*1.1*
		SD	*1.4*	*3.9*	*2.2*	*1.1*

		N	*25*	*25*	*25*	*24*
		%Diff	*-*	*119.2*	*57.7*	*8.2*
Coiled Tail	*86*	*Mean*	*10.6*	*12.5*	*8.4*	*6.4*
		SD	*15.8*	*14.3*	*8.9*	*6.2*
		N	*25*	*25*	*25*	*24*
		%Diff	*-*	*17.7*	*-20.8*	*-39.5*
Other Tail	*86*	*Mean*	*0.5*	*2.9 **	*1.6 **	*0.8*
		SD	*0.7*	*5.8*	*1.7*	*1.2*
		N	*25*	*25*	*25*	*24*
		%Diff	*-*	*500*	*241.7*	*73.6*
Abnormal Sperm Combo	*86*	*Mean*	*0*	*0.9*	*0.2*	*0*
		SD	*0.2*	*3.3*	*0.5*	*0.2*
		N	*25*	*25*	*25*	*24*
		%Diff	*-*	*2100*	*500*	*4.2*

** = p ≤* 0.05

Table 19. Summary of Sperm Motility and Density: F0 Generation

Sex: Male		*0 mg/kg bw/day Group 1*	*100 mg/kg bw/ day Group 2*	*300 mg/kg bw/ day Group 3*	*1000 mg/kg bw/ day Group 4*
Motile Sperm/ Total (%)	*Mean*	*73.1*	*66.5*	*68.3*	*66.2*
	SD	*20.7*	*25*	*20.5*	*23.3*
	N	*25*	*25*	*25*	*25*
Progressive Sperm/Total (%)	*Mean*	*35*	*35.5*	*31.5*	*29.6*
	SD	*16.1*	*17.4*	*18.7*	*15.9*
	N	*25*	*25*	*25*	*25*
Sperm Density (10^6/g)	*Mean*	*553.76*	*597.84*	*592.65*	*654.84 ***
	SD	*72.79*	*155.44*	*105.8*	*87.53*
	N	*24*	*25*	*25*	*25*
	%Diff	*-*	*7.96*	*7.02*	*18.25*

No Significance at p ≤ 0.05
**** = p ≤ 0.01

Table 20. *Summary Table F0-Generation– Males that Failed to Sire, Females that Failed to Deliver Healthy* Pups.

Group Number	*Dose Level (mg/kg bw/day)*	*Male/Female Nos.*	*In-Life Reason*	*Histopathology Related to Reproductive Failure*
1	*0*	*-/-*	*-*	*-*
2	*100*	*-/142*	*Total litter loss*	*No histopathological correlate*

		48/148	*Not pregnant*	*Male: moderate degeneration seminiferous tubule, marked decreased sperm epididymis*
3	*300*	*-/152*	*Total litter loss*	*No histopathological correlate*
		56/156	*Not pregnant*	*No histopathological correlate*
		-/157	*Total litter loss*	*No histopathological correlate*
4	*1000*	*-/176*	*Total litter loss*	*No histopathological correlate*
		87/187	*Not pregnant*	*No histopathological correlate*
		88/188	*Not pregnant*	*No histopathological correlate*
		89/189	*Not pregnant*	*Membrane between vagina and cervix*
		98/-	*Female found dead prior to mating; male was not paired*	*No indication of infertility*

Table 21a. Summary of Reproductive Performance: F0 Generation - Males

Sex: Male		*0*	*100*	*300*	*1000*
Day(s) Relative to Pairing (Litter: A)		*mg/kg bw/day Group 1*	*mg/kg bw/day Group 2*	*mg/kg bw/day Group 3*	*mg/kg bw/day Group 4*
Group Size - Males		*25*	*25*	*25*	*25*
Paired - Males	*N+ve*	*25*	*25*	*25*	*24*
Mated Males	*N+ve*	*25*	*25*	*25*	*24*
Male Impregnated a Female	*N+ve*	*25*	*24*	*24*	*21*
Male Mating Index	*%*	*100*	*100*	*100*	*100*
	ProA	*25/25*	*25/25*	*25/25*	*24/24*
Male Fertility Index	*%*	*100*	*96*	*96*	*87.5*
	ProA	*25/25*	*24/25*	*24/25*	*21/24*

Male Pregnancy Index	*%*	*100*	*96*	*96*	*87.5*
	ProA	*25/25*	*24/25*	*24/25*	*21/24*

Table 21b. Summary of Reproductive Performance: F0 Generation - Females

Sex: Female		*0*	*100*	*300*	*1000*
Day(s) Relative to Pairing (Litter: A)		*mg/kg bw/day Group 1*	*mg/kg bw/day Group 2*	*mg/kg bw/day Group 3*	*mg/kg bw/day Group 4*
Group Size - Females		*25*	*25*	*25*	*25*
Paired - Females	*N+ve*	*25*	*25*	*25*	*24*
Mated Females	*N+ve*	*25*	*25*	*25*	*23*
Pregnant	*N+ve*	*25*	*24*	*24*	*21*
Pre-coital Interval (Days)	*Mean*	*3.2*	*3.8*	*2.7*	*3*
	SD	*2.3*	*3.1*	*0.9*	*2.6*
	N	*25*	*25*	*25*	*23*
	%Diff	*-*	*20.3*	*-15.2*	*-3.7*
Confirmed Mating Days 1-7	*N+ve*	*24*	*23*	*25*	*22*
	%	*96*	*92*	*100*	*95.7*
Confirmed Mating Days 8-14	*N+ve*	*1*	*2*	*0*	*1*
	%	*4*	*8*	*0*	*4.3*
Female Mating Index	*%*	*100*	*100*	*100*	*100*
	ProA	*25/25*	*25/25*	*25/25*	*24/24*
Female Fertility Index	*%*	*100*	*96*	*96*	*87.5*
	ProA	*25/25*	*24/25*	*24/25*	*21/24*
Female Pregnancy Index	*%*	*100*	*96*	*96*	*87.5*
	ProA	*25/25*	*24/25*	*24/25*	*21/24*

Table 22. Natural Delivery Observations: F0 Generation

Sex: Female		*0*	*100*	*300*	*1000*
Day(s) Relative to Littering (Litter: A)		*mg/kg bw/day Group 1*	*mg/kg bw/day Group 2*	*mg/kg bw/day Group 3*	*mg/kg bw/day Group 4*
Group Size - Females		*25*	*25*	*25*	*25*
Number of Females Pregnant	*N+ve*	*25*	*24*	*24*	*21*

	%	100	96	96	87.5
Gestation Index	%	96	100	91.7	95.2
	ProA	24/25	24/24	22/24	20/21
Females Completing Delivery	N+ve	24	24	23	20
Female with Liveborn	N+ve	24	24	22	20
Female with no Liveborn	N+ve	0	0	1	0
Fem w/ Stillborn Pups	N+ve	1	1	3	2
Stillborn Pups/ Litter (%)	Mean	0.38	0.35	8.44	2.36
	SD	1.86	1.7	26.78	9
	N	24	24	23	20
	%Diff	-	-8.33	2127.33	522.29
Number Pups Stillborn	Mean	0	0	0.9	0.2
	SD	0.2	0.2	2.9	0.5
	N	24	24	23	20
	%Diff	.	0	1987	260
Live Newborn Pups	Mean	10.2	11.4	10	10.6
	SD	3.4	2.5	3.5	2.8
	N	24	24	23	20
	%Diff	-	11.4	-1.6	3.3
Live Birth Index (%)	Mean	99.62	99.65	91.56	97.64
	SD	1.86	1.7	26.78	9
	N	24	24	23	20
	%Diff	-	0.03	-8.09	-1.99
Post-implant Loss/Litter (%)	Mean	8.29	4.9	7.16	12.48
	SD	9.74	7.98	8.45	16.06
	N	24	24	23	20
	%Diff	-	-40.93	-13.62	50.5
Implantation Sites - Total	Mean	10.7	12	11.7	12.2
	SD	3.8	2.5	1.7	1.5
	N	25	24	24	21
	%Diff	-	12.3	8.8	13.7
Gestation Length (Days)	Mean	21.4	21.4	21.6	21.8
	SD	0.7	0.8	0.7	0.5

	N	*24*	*24*	*23*	*20*
	%Diff	*-*	*0.2*	*0.9*	*2*

Table 23. Litter Observations: F0 Generation

Sex: Female		*0*	*100*	*300*	*1000*
Day(s) Relative to Littering (Litter: A)		*mg/kg bw/day Group 1*	*mg/kg bw/day Group 2*	*mg/kg bw/day Group 3*	*mg/kg bw/day Group 4*
Group Size - Females		*25*	*25*	*25*	*25*
Female with Livebon	*N+ve*	*24*	*24*	*22*	*20*
Viability Index (Birth-4) (%)	*Mean*	*100*	*96.16*	*95.45*	*98.5*
	SD	*0*	*11.42*	*21.32*	*5.17*
	N	*24*	*24*	*22*	*20*
	%Diff	*-*	*-3.84*	*-4.55*	*-1.5*
Lactation Index (%) 4Postcull-21	*Mean*	*100*	*95.83*	*100*	*100*
	SD	*0*	*20.41*	*0*	*0*
	N	*24*	*24*	*21*	*20*
	%Diff	*-*	*-4.17*	*0*	*0*
Live Male Pups/ Litter (%) 1	*Mean*	*49.26*	*50.68*	*52.87*	*52.69*
	SD	*16.39*	*15.48*	*15.72*	*9.08*
	N	*24*	*24*	*22*	*20*
	%Diff	*-*	*2.87*	*7.32*	*6.96*
Live Male Pups/ Litter (%) 21	*Mean*	*48.78*	*52.17*	*51.19*	*51.67*
	SD	*12.88*	*6.14*	*6.74*	*7.21*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*6.95*	*4.93*	*5.92*

Table 24. Summary of Litter Mean Pup Physical Development: F1 Generation - until weaning.

Sex: Female *Day(s) Relative to Littering (Litter:* A)		*0* *mg/kg bw/day Group 1*	*100* *mg/kg bw/day Group 2*	*300* *mg/kg bw/day Group 3*	*1000* *mg/kg bw/day Group 4*
Mean Pup AGD male (mm) d1	*Mean*	*2.882*	*2.767*	*2.782*	*2.815*
	SD	*0.297*	*0.301*	*0.213*	*0.423*

	N	*24*	*23*	*22*	*20*
	%Diff	*-*	*-3.999*	*-3.462*	*-2.314*
Mean Pup AGD females	*Mean*	*1.081*	*1.038*	*1.131*	*1.128*
	SD	*0.189*	*0.150*	*0.374*	*0.299*
	N	*24*	*24*	*21*	*20*
	%Diff	*-*	*-4.018*	*4.621*	*4.301*
Mean Normalized Pup AGD m d1	*Mean*	*1.523*	*1.487*	*1.485*	*1.486*
	SD	*0.148*	*0.168*	*0.117*	*0.200*
	N	*24*	*23*	*22*	*20*
	%Diff	*-*	*-2.363*	*-2.505*	*-2.385*
Mean Normalized Pup AGD f d1	*Mean*	*0.584*	*0.567*	*0.615*	*0.611*
	SD	*0.099*	*0.082*	*0.214*	*0.159*
	N	*24*	*24*	*21*	*20*
	%Diff	*-*	*-2.926*	*5.218*	*4.528*
Mean Pup A/N Count males d13	*Mean*	*0.0*	*0.0*	*0.0*	*0.0*
	SD	*0.0*	*0.0*	*0.0*	*0.0*
	N	*24*	*23*	*21*	*20*
	%Diff	*-*	*-*	*-*	*-*

Table 25. Summary of Pup Thyroid Hormone Values: F1 Generation - PND4

Sex: Both	*Thyroid* Hormones
	T4

		(ng/mL)
0 *mg/kg bw/day Group 1*	*Mean* *SD* *N*	*13.80* *2.20* *17*
100 *mg/kg bw/day* *Group 2*	*Mean* *SD* *N*	*13.09* *2.03* *22*
	tCtrl	*0.95*
300 *mg/kg bw/day* *Group 3*	*Mean* *SD* *N*	*12.65* *1.72* *18*
	tCtrl	*0.92*
1000 *mg/kg bw/day* *Group 4*	*Mean* *SD* *N*	*14.13* *1.46* *16*
	tCtrl	*1.02*

Table 26. Summary of Clinical Observations: F1 Generation - from weaning onwards - Cohort 1A-1B-1C

Observation Type: All Types	*Male*				*Female*			
From Day 1 (Start Date) to 93 (Start Date)	*0*	*100*	*300*	*1000*	*0*	*100*	*300*	*1000*
	mg/kg bw/day Group 1	*mg/kg bw/day Group 2*	*mg/kg bw/day Group 3*	*mg/kg bw/day Group 4*	*mg/kg bw/day Group 1*	*mg/kg bw/day Group 2*	*mg/kg bw/day Group 3*	*mg/kg bw/day Group 4*
Salivation								
Number of Animals Affected	*0*	*0*	*2*	*42*	*0*	*0*	*2*	*39*

Number of Times Recorded	*0*	*0*	*2*	*76*	*0*	*0*	*4*	*80*
First to Last seen	*-*	*-*	*36 - 36*	*05-71*	*-*	*-*	*36 - 83*	*07-89*
Breathing, Labored								
Number of Animals Affected	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
Number of Times Recorded	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
First to Last seen	*-*	*-*	*-*	*-*	*-*	*-*	*-*	*21 - 21*
Breathing, Abnormal Sounds								
Number of Animals Affected	*0*	*0*	*7*	*9*	*0*	*1*	*3*	*3*
Number of Times Recorded	*0*	*0*	*33*	*47*	*0*	*1*	*23*	*11*
First to Last seen	*-*	*-*	*20 - 49*	*04-30*	*-*	*37 - 37*	*13 - 70*	*21 - 89*
Tail, Bent (PT)								
Number of Animals Affected	*0*	*1*	*0*	*1*	*0*	*0*	*1*	*0*
Number of Times Recorded	*0*	*44*	*0*	*62*	*0*	*0*	*108*	*0*
First to Last seen	*-*	*52 - 74*	*-*	*50 - 79*	*-*	*-*	*22 - 73*	*-*
Hunched Posture								
Number of Animals Affected	*0*	*0*	*1*	*0*	*0*	*0*	*2*	*2*
Number of Times Recorded	*0*	*0*	*1*	*0*	*0*	*0*	*18*	*3*
First to Last seen	*-*	*-*	*22 - 22*	*-*	*-*	*-*	*18 - 70*	*20 - 41*
Tail, Missing (PT)								
Number of Animals Affected	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*

Number of Times Recorded	*0*	*0*	*0*	*0*	*0*	*0*	*39*	*0*
First to Last seen	*-*	*-*	*-*	*-*	*-*	*-*	*3-21*	*-*
Fur, Erected								
Number of Animals Affected	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*
Number of Times Recorded	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*3*
First to Last seen	*-*	*-*	*-*	*-*	*-*	*-*	*-*	*20 - 41*
Fur, Loss								
Number of Animals Affected	*1*	*2*	*0*	*0*	*0*	*0*	*0*	*0*
Number of Times Recorded	*7*	*23*	*0*	*0*	*0*	*0*	*0*	*0*
First to Last seen	*5-8*	*05-69*	*-*	*-*	*-*	*-*	*-*	*-*
Skin, Lesion, Dorsal Cervical								
Number of Animals Affected	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*
Number of Times Recorded	*0*	*48*	*0*	*0*	*0*	*0*	*0*	*0*
First to Last seen	*-*	*42 - 69*	*-*	*-*	*-*	*-*	*-*	*-*
Skin, Scab, Cranium								
Number of Animals Affected	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*
Number of Times Recorded	*0*	*0*	*26*	*0*	*0*	*0*	*0*	*0*
First to Last seen	*-*	*-*	*15 - 46*	*-*	*-*	*-*	*-*	*-*
Skin, Scab, Dorsal Cervical								
Number of Animals Affected	*2*	*3*	*0*	*0*	*0*	*1*	*0*	*0*

Number of Times Recorded	*61*	*185*	*0*	*0*	*0*	*44*	*0*	*0*
First to Last seen	*12-60*	*35 - 77*	*-*	*-*	*-*	*47 - 69*	*-*	*-*
Skin, Scab, Ventral Cervical								
Number of Animals Affected	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*
Number of Times Recorded	*0*	*0*	*15*	*0*	*0*	*0*	*0*	*0*
First to Last seen	*-*	*-*	*17 - 24*	*-*	*-*	*-*	*-*	*-*
Eye Closed, Site Not Recorded								
Number of Animals Affected	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
Number of Times Recorded	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
First to Last seen	*-*	*-*	*-*	*-*	*-*	*-*	*-*	*21 - 21*
Eyeball, Abnormal Size, Right								
Number of Animals Affected	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*
Number of Times Recorded	*0*	*15*	*0*	*0*	*0*	*0*	*0*	*0*
First to Last seen	*-*	*22 - 29*	*-*	*-*	*-*	*-*	*-*	*-*
Heart Rate Abnormal								
Number of Animals Affected	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
Number of Times Recorded	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
First to Last seen	*-*	*-*	*-*	*-*	*-*	*-*	*-*	*21 - 21*
Discharge, Color, Eye, Left								
Number of Animals Affected	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*

Number of Times Recorded	*0*	*0*	*0*	*0*	*0*	*0*	*140*	*0*
First to Last seen	*-*	*-*	*-*	*-*	*-*	*-*	*5-71*	*-*
Teeth, Missing								
Number of Animals Affected	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
Number of Times Recorded	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*13*
First to Last seen	*-*	*-*	*-*	*-*	*-*	*-*	*-*	*41 - 47*
Other (see comment)								
Number of Animals Affected	*0*	*0*	*0*	*1*	*1*	*0*	*1*	*1*
Number of Times Recorded	*0*	*0*	*0*	*53*	*173*	*0*	*63*	*1*
First to Last seen	*-*	*-*	*-*	*9-42*	*9-93*	*-*	*42 - 71*	*43 - 43*
Activity Decreased								
Number of Animals Affected	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*1*
Number of Times Recorded	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*2*
First to Last seen	*-*	*-*	*-*	*35 - 35*	*-*	*-*	*-*	*20 - 21*
Twitches (Generalized)								
Number of Animals Affected	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
Number of Times Recorded	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
First to Last seen	*-*	*-*	*-*	*-*	*-*	*-*	*-*	*21 - 21*

Table 27a. Summary of Body Weights: F1 Generation - from weaning onwards - Cohort 1A-1B-1C - Male

Sex: Male	*Day(s) Relative to Start Date*

		1	8	15	22	29	36	43	50	57	64	70
0 mg/kg bw/day Group 1	Mean	53.7	74	119.5	168.1	210.6	251.4	285.9	309.4	330.5	348.2	361.1
	SD	2.9	5.8	7.7	9.7	12.6	15.7	16.1	17.3	18.5	20.4	21.9
	N	60	60	60	60	60	40	40	40	40	40	40
100 mg/kg bw/day Group 2	Mean	52.8	71.7	115.7	163.5	**203.9****	247.7	278.6	303.5	321.8	338.5	350.8
	SD	4.2	5.1	7.1	9.2	10.7	13.1	15.8	18.1	21.6	24.1	24.7
	N	60	60	60	59	59	40	40	40	40	40	40
	%Diff	-1.6	-3.1	-3.2	-2.7	-3.2	-1.5	-2.6	-1.9	-2.6	-2.8	-2.9
300 mg/kg bw/day Group 3	Mean	53.1	73.2	117.6	165.2	206.7	251.1	283	308.5	329.2	348.3	361.7
	SD	4.8	6	8.5	11	14	13.3	16.2	21.4	25.5	28.8	30.8
	N	60	60	60	60	60	40	40	40	40	40	40
	%Diff	-1.2	-1.1	-1.6	-1.7	-1.9	-0.1	-1	-0.3	-0.4	0	0.2
1000 mg/kg bw/day Group 4	Mean	54.9	74.1	117.7	166.1	207.4	246.8	277.3	301.1	319.4	**334.2***	**345.4***
	SD	5.1	7.8	9.7	10.4	12	14.4	15.6	18.2	20.9	22	23.4
	N	60	60	60	59	59	39	39	39	39	39	39
	%Diff	2.1	0.2	-1.5	-1.2	-1.5	-1.8	-3	-2.7	-3.4	-4	-4.4

* = p ≤ 0.05; ** = p ≤ 0.01

Table 27b. Summary of Body Weights: F1 Generation - from weaning onwards -Cohort 1A-1B-1C - Female

Sex: Female		Day(s) Relative to Start Date											
		1	8	14	15	22	29	36	43	50	57	64	70
0 mg/kg bw/day Group 1	Mean	51.7	69.1	-	104.3	135.4	155.1	174.3	186.9	197.6	208.1	216.1	222.5
	SD	2.8	6.1	-	8.1	9.2	9.9	10.9	11	11.9	12.8	12.9	13.4
	N	60	60	-	60	45	45	40	40	40	40	40	40
100 mg/kg bw/day Group 2	Mean	51	68.6	-	104.9	135.8	156.2	174.8	187.7	195.7	209	217.4	222.9
	SD	3.5	5	-	6.7	7.4	8.8	9.9	11	11.2	12.1	12.8	12.4
	N	60	60	-	60	40	40	40	40	40	40	40	40
	%Diff	-1.4	-0.8	-	0.6	0.3	0.7	0.3	0.5	-0.9	0.4	0.6	0.2
300 mg/kg	Mean	51.4	69.8	-	106	137.2	156.8	178	189.2	196.7	209.1	218.4	221.8
	SD	4.4	6.2	-	8	8.4	9.5	10.9	12.5	12.4	13.6	15	17.1

bw/ day Group 3	N	60	60	-	60	39	39	39	39	39	39	39	39
	%Diff	-0.7	0.9	-	1.7	1.3	1.1	2.1	1.2	-0.4	0.5	1.1	-0.3
1000 mg/ kg bw/ day Group 4	Mean	53	70.1	46.0n	103.4	134	155.6	177.6	190.1	200.3	209.4	220.2	224.6
	SD	4.7	6.9	0	13.9	15.2	13.1	12	13.1	14	13	13.6	14.5
	N	58	58	2	60	44	44	39	39	39	39	39	39
	%Diff	2.6	1.5	-	-0.8	-1.1	0.3	1.9	1.7	1.4	0.6	1.9	0.9

Table 28. Summary of Body Weight Gains (g): F1 Generation - from weaning onwards - Cohort 1A-1B-1C

Sex: Male		Day(s) Relative to Start Date										
		1 → 8	8 → 15	15 → 22	22 → 29	29 → 36	36 → 43	43 → 50	50 → 57	57 → 64	64 → 70	1 → 70
0 mg/ kg bw/ day Group 1	Mean	20.3	45.5	48.6	42.5	41.2	34.5	23.5	21.1	17.7	12.9	307.5
	SD	5.7	3.2	3.9	5	4.9	4.7	4.7	5.4	4.7	4.5	21.2
	N	60	60	60	60	40	40	40	40	40	40	40
100 mg/ kg bw/ day Group 2	Mean	18.9	44	47.8	40.4	43.4	30.9**	25	18.3	16.7	12.3	297.8
	SD	3.9	3.3	4.2	5.2	4.7	5.5	5.9	5.7	4.6	4	23.2
	N	60	60	59	59	40	40	40	40	40	40	40
300 mg/ kg bw/ day Group 3	Mean	20.1	44.4	47.6	41.5	42.1	31.9	25.5	20.7	19.1	13.4	308.4
	SD	4.5	3.8	4.5	4.6	4.3	6.4	7.1	5.9	5.1	3.6	29.5
	N	60	60	60	60	40	40	40	40	40	40	40
1000 mg/ kg bw/ day Group 4	Mean	19.3	43.5**	48.4	41.3	40	30.5**	23.8	18.3	14.8*	11.1	290.3**
	SD	5.7	3.8	4.6	3.5	4.2	4.6	5.5	5.1	4.4	3.9	21.2
	N	60	60	59	59	39	39	39	39	39	39	39
Sex: Female												
0 mg/ kg bw/ day Group 1	Mean	17.4	35.2	31.3	19.7	18.4	12.6	10.7	10.6	8	6.5	170.6
	SD	5.6	3.7	4.3	3.9	3.5	4.4	4.1	3.8	3.9	3.7	12.3
	N	60	60	45	45	40	40	40	40	40	40	40
100	Mean	17.6	36.4	31.1	20.4	18.6	13	8.0**	13.3*	8.4	5.5	172
	SD	4.1	3.4	3.3	4.6	2.8	3.9	4	4.1	3.8	4.4	12.2

mg/kg bw/day Group 2	*N*	*60*	*60*	*40*	*40*	*40*	*40*	*40*	*40*	*40*	*40*	*40*
300 mg/kg bw/day Group 3	*Mean*	*18.4*	*36.3*	*31.2*	*19.7*	*21.2***	*11.2*	*7.5***	*12.5*	*9.3*	*3.4*	*170.5*
	SD	*4.9*	*3.5*	*3.9*	*4.4*	*3.7*	*4.2*	*4.3*	*5.4*	*3.6*	*9*	*15.6*
	N	*60*	*60*	*39*	*39*	*39*	*39*	*39*	*39*	*39*	*39*	*39*
1000 mg/kg bw/day Group 4	*Mean*	*17.1*	*35.3*	*32.1*	*21.6*	*21.3***	*12.5*	*10.2*	*9.1*	*10.7***	*4.4*	*172.2*
	SD	*5.4*	*3.5*	*3.5*	*4.4*	*4.3*	*4.3*	*3.8*	*4.5*	*3.7*	*4*	*11.6*
	N	*58*	*58*	*44*	*44*	*39*	*39*	*39*	*39*	*39*	*39*	*38*

* = p ≤ 0.05; ** = p ≤ 0.01

Table 29a. Summary of Sexual Maturation and First Estrus: F1 Generation - from weaning onwards Cohort 1A-1B-1C

Sex: Male		*0*	*100*	*300*	*1000*
		mg/kg bw/day Group 1	*mg/kg bw/day Group 2*	*mg/kg bw/day Group 3*	*mg/kg bw/day Group 4*
Preputial Separation (Days)	*Mean*	*40.6*	*41.2*	*42.0***	*41.2*
	SD	*1.4*	*2*	*2.5*	*2*
	N	*60*	*59*	*60*	*59*
	%Diff	*-*	*1.4*	*3.5*	*1.5*
Bodyweight at Maturity (g)	*Mean*	*173.4*	*171.6*	*178.1*	*174.4*
	SD	*12.4*	*12.9*	*16.1*	*12.8*
	N	*60*	*59*	*60*	*59*
	%Diff	*-*	*-1*	*2.7*	*0.6*
Sex: Female		*0*	*100*	*300*	*1000*
		mg/kg bw/day Group 1	*mg/kg bw/day Group 2*	*mg/kg bw/day Group 3*	*mg/kg bw/day Group 4*
Vaginal Opening (Days)	*Mean*	*30.3*	*30*	*30.8*	*30.8*
	SD	*1.7*	*1.7*	*1.7*	*1.7*
	N	*60*	*60*	*60*	*60*
	%Diff	*-*	*-0.9*	*1.8*	*1.7*
Bodyweight at Maturity (g)	*Mean*	*92.6*	*91.1*	*95.2*	*94.9*
	SD	*9.4*	*10*	*9.6*	*10.2*
	N	*60*	*60*	*60*	*60*
	%Diff	*-*	*-1.7*	*2.7*	*2.4*
Age at First Estrus (Days)	*Mean*	*32.7*	*33.8*	*34.8*	*34.7*
	SD	*2.7*	*3.1*	*3.2*	*2.7*
	N	*20*	*20*	*20*	*20*

	%Diff	*-*	*3.2*	*6.4*	*6*
Vag. Open to 1st Estrus (Days)	*Mean*	*2.8*	*3.8*	*3.7*	*3.9*
	SD	*1.8*	*2.5*	*2.5*	*2*
	N	*20*	*20*	*20*	*20*
	%Diff	*-*	*36.4*	*34.5*	*41.8*

*** = p ≤* 0.01

Table 29b. Historical Data Sexual Maturation and First Estrus - Females

VAGINAL PATENCY AND FIRST ESTRUS						
	UNITS	***MEAN***	***STD.DEV.***	***N***	***P5***	***P95***
First estrus	*PND*	*35.1*	*3.20*	*459*	*30.00*	*40.00*
Time to first E	*DAYS*	*3.5*	*1.64*	*20*	*1.00*	*5.50*
Time to first E	*Days*	*3.8*	*2.13*	*439*	*1.00*	*7.00*
BW at *VO*	*gram*	*95*	*12.6*	*1356*	*73.0*	*115.0*
VO	*PND*	*31.4*	*2.43*	*1359*	*27.00*	*35.00*

Table 30a. Summary of Selected Clinical Chemistry Values: F1 Generation - from weaning onwards - Cohort 1A - Males

Sex: Male Day: 71 Relative to Start Date		Reporting Biochemistry	Hormone Analysis	
		CL	T4	TSH
		(mmol/L)	(ng/mL)	(mU/L)
0 mg/kg bw/day Group 1	Mean	106.8	61.82	0.1383
	SD	1.3	8.49	0.0706
	N	10	10	10
100 mg/kg bw/day Group 2	Mean	106.1	54.64	0.1524
	SD	0.9	13.68	0.0722
	N	10	10	10
	tCtrl	0.99	0.88	1.1
300 mg/kg bw/day Group 3	Mean	105.7	57.39	0.1396
	SD	1.2	13.18	0.0956
	N	10	10	10
	tCtrl	0.99	0.93	1.01
1000 mg/kg bw/day Group 4	Mean	105.5 *	54.45	0.1575
	SD	0.7	11.52	0.1176

	N	10	10	10
	tCtrl	0.99	0.88	1.14

* = p ≤ 0.05

Table 30b. Summary of selected Clinical Chemistry Values: F1 Generation - from weaning onwards - Cohort 1A - Females

Sex: Female Day: 71 Relative to Start Date		Reporting Biochemistry	Hormone Analysis	
		BILEAC	T4	TSH
		(umol/L)	(ng/mL)	(mU/L)
0 mg/kg bw/day Group 1	Mean	9.27	30.12	0.1814
	SD	2.71	10.68	0.2238
	N	10	10	10
100 mg/kg bw/day Group 2	Mean	11.57	32.25	0.1002
	SD	4.02	9.59	0.0534
	N	10	10	10
	tCtrl	1.25	1.07	0.55
300 mg/kg bw/day Group 3	Mean	23.42	36.06	0.1263
	SD	18.25	11.92	0.0892
	N	10	10	10
	tCtrl	2.53	1.2	0.7
1000 mg/kg bw/day Group 4	Mean	30.20 **	30.44	0.0934
	SD	28.87	6.13	0.0723
	N	10	10	10
	tCtrl	3.26	1.01	0.51

** = p ≤ 0.01

Table 30c. Historical Data - Hormone analysis- Females - Cohort 1A.

MEASUREMENT	UNITS	MEAN	STD.DEV.	N	P5	P95
COHORT 1A; END OF IN-LIFE						
TSH	uIU/mL	0.072	0.0598	130	0.011	0.195
Total T4	ug/dL	3.4	1.113	130	1.81	5.92

Table 31. Summary Clinical Chemistry Values: F1 Generation - from weaning onwards - Cohort Surplus

Sex: Male *Day: 22 Relative to Birth Date*	*Hormone Analysis*	
	T4	*TSH*
	(ng/mL)	*(mU/L)*

0 mg/kg bw/day Group 1	*Mean*	*39.42*	*0.0601*
	SD	*5.84*	*0.0375*
	N	*10*	*10*
100 mg/kg bw/day Group 2	*Mean*	*39.11*	*0.0698*
	SD	*5.25*	*0.0436*
	N	*10*	*10*
	tCtrl	*0.99*	*1.16*
300 mg/kg bw/day Group 3	*Mean*	*36.96*	*0.0737*
	SD	*6.33*	*0.0456*
	N	*10*	*10*
	tCtrl	*0.94*	*1.23*
1000 mg/kg bw/day Group 4	*Mean*	*38.51*	*0.0569*
	SD	*8.92*	*0.0333*
	N	*10*	*10*
	tCtrl	*0.98*	*0.95*
Sex: Female Day: 22 Relative to Birth Date		*T4 (ng/ml)*	*TSH (mU/L)*
0 mg/kg bw/day Group 1	*Mean*	*36.22*	*0.0607*
	SD	*6.63*	*0.0351*
	N	*10*	*10*
100 mg/kg bw/day Group 2	*Mean*	*36.27*	*0.0397*
	SD	*5.21*	*0.0178*
	N	*10*	*10*
	tCtrl	*1*	*0.65*
300 mg/kg bw/day Group 3	*Mean*	*35.07*	*0.051*
	SD	*6.63*	*0.0213*
	N	*10*	*10*
	tCtrl	*0.97*	*0.84*
1000 mg/kg bw/day Group 4	*Mean*	*41.42*	*0.0483*
	SD	*7.45*	*0.0164*
	N	*10*	*10*
	tCtrl	*1.14*	*0.8*

Table 32. Summary of Sperm Motility and Density: F1 Generation - from weaning onwards - Cohort 1A

Sex: Male		*0 mg/kg bw/day Group 1*	*100 mg/kg bw/day Group 2*	*300 mg/kg bw/day Group 3*	*1000 mg/kg bw/day Group 4*
Motile	*Mean*	*48.8*	*46.1*	*54.3*	*53.8*
Sperm/Total	*SD*	*23.5*	*24.6*	*22.2*	*23.9*

(%)	*N*	*20*	*20*	*20*	*19*
Progressive	*Mean*	*25.6*	*19.6*	*24.2*	*28.0*
Sperm/Total	*SD*	*15.5*	*13.0*	*11.4*	*13.7*
(%)	*N*	*20*	*20*	*20*	*19*
Sperm [	*Mean*	*633.09*	*800.35 **	*692.36*	*623.57*
Density	*SD*	*138.19*	*250.93*	*108.39*	*104.32*
(10^6/g)	*N* *%Diff*	*20* *-*	*20* *26.42*	*20* *9.36*	*19* *-1.50*

*= p ≤ 0.05

Table 33. Summary of Sperm Morphology Evaluation: F1 Generation - from weaning onwards - Cohort 1A

Sex: Male			*0*	*100*	*300*	*1000*
Day(s) Relative to Start Date			*mg/kg bw/ day Group 1*	*mg/kg bw/ day Group 2*	*mg/kg bw/ day Group 3*	*mg/kg bw/ day Group 4*
>=200 Sperm	*71*	*N-ve*	*0*	*0*	*0*	*0*
Evaluated		*N+ve*	*17*	*17*	*18*	*16*
Normal Sperm	*71*	*Mean*	*92.68*	*92.85*	*93*	*92.44*
/Total Sperm		*SD*	*4.12*	*3.92*	*4.86*	*5.45*
(%)		*N*	*17*	*17*	*18*	*16*
		%Diff	*-*	*0.19*	*0.35*	*-0.26*
Normal	*71*	*Mean*	*185.4*	*185.7*	*186*	*184.9*
Sperm		*SD*	*8.2*	*7.8*	*9.7*	*10.9*
		N	*17*	*17*	*18*	*16*
		%Diff	*-*	*0.2*	*0.3*	*-0.3*
Total Sperm	*71*	*Mean*	*14.6*	*14.3*	*14*	*15.1*
Abnormal		*SD*	*8.2*	*7.8*	*9.7*	*10.9*
		N	*17*	*17*	*18*	*16*
		%Diff	*-*	*-2.4*	*-4.4*	*3.3*
Abnormal	*71*	*Mean*	*0.9*	*0.8*	*0.6*	*0.7*
Head(s)		*SD*	*1.1*	*1.3*	*0.7*	*1*
		N	*17*	*17*	*18*	*16*
		%Diff	*-*	*-12.5*	*-41*	*-27*
Detached	*71*	*Mean*	*5.6*	*3.5*	*4.7*	*3.1*
Head		*SD*	*4.7*	*2.4*	*4*	*2.4*

		N	*17*	*17*	*18*	*16*
		%Diff	*-*	*-37.5*	*-16.4*	*-44.7*
Abnormal	*71*	*Mean*	*1.3*	*1.5*	*1.5*	*1.2*
Midpiece		*SD*	*1.8*	*2.2*	*2.1*	*1.6*
		N	*17*	*17*	*18*	*16*
		%Diff	*-*	*13.6*	*15.9*	*-8.2*
Coiled	*71*	*Mean*	*5.6*	*7.1*	*6.7*	*9.8*
Tail		*SD*	*5.2*	*4.6*	*4.7*	*9.2*
		N	*17*	*17*	*19*	*16*
		%Diff	*-*	*27.4*	*20.6*	*75.6*
Other	*71*	*Mean*	*1.1*	*1.2*	*0.2*	*0.2*
Tail		*SD*	*1.6*	*1.9*	*0.4*	*0.4*
		N	*17*	*17*	*18*	*16*
		%Diff	*-*	*16.7*	*-84.3*	*-82.3*
Abnormal	*71*	*Mean*	*0.1*	*0.1*	*0.1*	*0.1*
Sperm Combo		*SD*	*0.3*	*0.3*	*0.2*	*0.3*
		N	*17*	*17*	*18*	*16*
		%Diff	*-*	*0*	*-52.8*	*6.2*

Table 34. Summary of Immunophenotyping Values: F1 Generation - from weaning onwards

Sex: Male Day: 71 Relative to Start Date		*DB Flow Cytometry Rat Spleen*					
		%CD3+CD45RA-	*%CD3-CD45RA+*	*%CD3-CD161a+*	*%CD3+CD4+*	*%CD3+CD4-*	*%CD4:%CD8*
		Spleen (%)	*Spleen (%)*	*Spleen (%)*	CD8-Spleen (%)	*CD8+ Spleen (%)*	*Ratio Spleen*
0 mg/kg bw/day Group 1	*Mean*	*43.51*	*38.82*	*5.78*	*28.59*	*15.19*	*2.04*
	SD	*7.45*	*6.19*	*1.55*	*3.5*	*5.14*	*0.587*
	N	*10*	*10*	*10*	*10*	*10*	*10*
100 mg/kg bw/day Group 2	*Mean*	*42.62*	*38.16*	*6.55*	*28.29*	*14.18*	*2.178*
	SD	*7.73*	*7.01*	*1.36*	*5.59*	*4.47*	*0.836*
	N	*10*	*10*	*10*	*10*	*10*	*10*
	tCtrl	*0.98*	*0.98*	*1.13*	*0.99*	*0.93*	*1.07*
300 mg/kg bw/day Group 3	*Mean*	*45.02*	*38.02*	*4.86*	*30.4*	*14.47*	*2.157*
	SD	*7.07*	*6.33*	*1.28*	*4.77*	*3.16*	*0.425*
	N	*10*	*10*	*10*	*10*	*10*	*10*
	tCtrl	*1.03*	*0.98*	*0.84*	*1.06*	*0.95*	*1.06*
1000 mg/kg bw/day Group 4	*Mean*	*43.09*	*38.65*	*5.61*	*29.17*	*14.05*	*2.168*
	SD	*7.4*	*7.07*	*1.79*	*4.75*	*3.9*	*0.503*
	N	*10*	*10*	*10*	*10*	*10*	*10*
	tCtrl	*0.99*	*1*	*0.97*	*1.02*	*0.92*	*1.06*
Sex: Female		*DB Flow Cytometry Rat Spleen*					

Day: 71 Relative to Start Date		%CD3+CD45RA- Spleen (%)	%CD3-CD45RA+ Spleen (%)	%CD3-CD161a+ Spleen (%)	%CD3+CD4+CD8- Spleen (%)	%CD3+CD4-CD8+ Spleen (%)	%CD4:%CD8 Ratio Spleen
0 mg/kg bw/day Group 1	Mean	42.51	39.23	6.11	28.29	14	2.058
	SD	7.17	6.05	1.08	4.68	2.6	0.365
	N	10	10	10	10	10	10
100 mg/kg bw/day Group 2	Mean	42.69	39	6.08	27.26	14.92	1.964
	SD	7.52	5.53	1.5	4.5	4.2	0.633
	N	10	10	10	10	10	10
	tCtrl	1	0.99	1	0.96	1.07	0.95
300 mg/kg bw/day Group 3	Mean	40.81	40.12	6.22	26.64	14.49	1.904
	SD	4.93	6.44	1.96	2.77	3.21	0.393
	N	10	10	10	10	10	10
	tCtrl	0.96	1.02	1.02	0.94	1.04	0.93
1000 mg/kg bw/day Group 4	Mean	37.94	40.03	6.55	27.37	11.15	2.628
	SD	5	5.2	2.1	2.83	2.79	0.827
	N	12	12	12	10	10	10
	tCtrl	0.89	1.02	1.07	0.97	0.8	1.28

Table 35a. F1-Generation Cohort 1A: Mean Percent Organ Weight Differences from Control Groups – Males and Females

	Males			Females		
Dose Level (mg/kg bw/day):	100	300	1000	100	300	1000
KIDNEY						
Absolute (%)	0	4	2	1	5	8**
Relative to body weight (%)	1	5	8**	2	4	7
LIVER						
Absolute (%)	0	2	0	-3	0	7*
Relative to body weight (%)	1	3	6*	-2	-1	6

*: P≤0.05, **: P≤0.01

Table 35b. F1-Generation Cohort 1B: Mean Percent Organ Weight Differences from Control Groups – Males and Females

	Males			Females		
Dose Level (mg/kg bw/day):	100	300	1000	100	300	1000

KIDNEY						
Absolute (%)	*-2*	*5*	*4*	*1*	*4*	*9***
Relative to body weight (%)	*3*	*4*	*8*	*-1*	*3*	*6**
LIVER						
Absolute (%)	*-11***	*3*	*3*	*1*	*3*	*15***
Relative to body weight (%)	*-7*	*2*	*7***	*-1*	*1*	*11***

*: P≤0.05, **: P≤0.01

Overall remarks, attachments

Overall remarks

Discussion on results

This Extended One Generation Reproductive Toxicity Study included a 10-week premating period and Cohort 1. Wistar Han rats were treated with Amphoacetates C8-C18 by daily oral gavage at dose levels of 100, 300 and 1000 mg/kg bw/day. The animals of the control group received the vehicle, Water (Elix), alone.

General toxicity F0-Generation
Salivation was noted in males and females at 1000 mg/kg bw/day. Taking into account the nature and its time of occurrence (i.e., after dosing), this sign was considered to be a physiological response rather than a sign of systemic toxicity.
Statistically significantly decreased urea concentrations were noted in males at
1000 mg/kg bw/day. In the absence of a microscopic correlate, the lower urea concentrations were considered non-adverse.
Statistically significantly increased kidney and liver weights (relative to body weight) were noted in males and females at 1000 mg/kg bw/day. As these were of low magnitude and in the absence of a microscopic correlate, these changes were considered non-adverse.
T4 levels were increased in females at 300 mg/kg bw/day (not statistically significant). As the mean value remained within the normal range of biological variation, this was considered non-adverse.

Reproductive toxicity F0-Generation and Developmental toxicity F0/1-Generation – until weaning and post-weaning
There were no test material-related effects on any of the reproductive and developmental parameters (until weaning and post-weaning) investigated in this study up to the highest dose tested (1000 mg/kg bw/day).

General toxicity F1-Generation – post-weaning
Salivation was noted in males and females at 1000 mg/kg bw/day. Taking into account the nature and its time of occurrence (i.e., mostly after dosing), this sign was considered to be a physiological response rather than a sign of systemic toxicity.
Abnormal breathing sounds were noted in males and females at 300 and 1000 mg/kg bw/day. As the effect was noted incidental and transient, this was considered non-adverse.
Statistically significantly decreased body weight gain and body weight was noted in males at 1000 mg/kg bw/day. At the minor magnitude of the effect, this was considered non-adverse.
T4 level was increased in females at 300 mg/kg bw/day (not statistically significant). As the mean value remained within the normal range of biological variation, this was considered non-adverse.

Statistically significantly increased kidney and liver weights (absolute and/or relative to body weight) were noted in males and females of Cohort 1A and B at 1000 mg/kg bw/day. As these were of low magnitude and in the absence of a microscopic correlate, these changes were considered non-adverse.

Applicant's summary and conclusion

Conclusions

In conclusion, based on the results of this Extended One Generation Reproductive Toxicity Study (inc luding Cohort 1), the following No Observed Adverse Effect Levels (NOAELs) of Amphoacetates C8-C18 were established:

- General Toxicity (F0 and F1): at least 1000 mg/kg bw/day
- Reproductive Toxicity (F0): at least 1000 mg/kg bw/day
- Developmental Toxicity (F0/1) – until weaning: at least 1000 mg/kg bw/day
- Developmental – post-weaning: at least 1000 mg/kg bw/day

Executive summary

An Extended One Generation Reproductive Toxicity Study which included a 10-week premating period in F0-animals and investigation of F1-animals for at least 10 weeks after weaning (Cohort 1) was performed according to OECD/EC guidelines and GLP principles. Wistar Han rats were treated with the test item by daily oral gavage at dose levels of 100, 300 and 1000 mg/kg bw/day. The rats of the control group received the vehicle, water, alone. F0 Males were treated for 10 weeks prior to mating, during mating, and up to termination. F0 Females were trated 10 weeks prior to mating, the variable time to conception, the duration of pregnancy and at least 21 days after delivery, up to and including the day before scheduled necropsy. Chemical analyses of formulations were conducted at four occasions during the study and confirmed accuracy and homogeneity. No test material-related findings were noted at 100 mg/kg bw/day. For F0 Generation, at 300 mg/kg bw/day, non-adverse, statistically not significantly increased T4 levels in females were noted. At 1000 mg/kg bw/day, non-adverse salivation and statistically significantly increased kidney and liver weights in males and females were noted. Additionally in males, a non-adverse, statistically significantly decreased urea concentration was noted. No test material-related changes were noted in any of the remaining parameters investigated in this study (i.e., body weight, food consumption, hematology, coagulation, urinalysis, sperm analysis, estrous cycle and macroscopic and microscopic examination). The NOAEL for General Toxicity (F0) has been stablised at least 1000 mg/kg bw/day. No test material-related changes were noted in any of the reproductive parameters investigated in this study for F0 generation (i.e., mating, fertility and pregnancy indices, precoital interval, number of implantation sites, and microscopic examination of reproductive organs). The NOAEL for Reproductive toxicity F0-Generation has been stablised at least 1000 mg/kg bw/day. No test material-related changes were noted in any of the developmental parameters investigated in this study for F0/1 generation until weaning (i.e., gestation index and length, parturition, maternal care, post-implantation loss, litter size, sex ratio, live birth, viability and lactation indices, and early postnatal pup development consisting of mortality, clinical signs, body weight, anogenital distance, areola/nipple retention, T4 thyroid hormone levels and macroscopic examination). The NOAEL for Developmental Toxicity (F0/1) – until weaning has been stablised at least 1000 mg/kg bw/day. No test material related changes were noted in any of the post weaning developmental parameters investigated in this study (i.e., vaginal patency, balanopreputial separation and day of first estrus). The NOAEL for Developmental Toxicity - post weaning has been stablised at least 1000 mg/kg bw/day. For F1 generation, No test material-related findings were noted at 100 mg/kg bw/day. At 300 mg/kg bw/day, non-adverse abnormal breathing sounds in males and females and statistically significantly increased T4 level in females were noted. At 1000 mg/kg bw/day, non-adverse salivation, abnormal breathing sounds and statistically significantly increased liver and kidney weights were noted in males and females. A non-adverse, statistically significantly decreased body weight gain and body weights were additionally noted in males. No test material-related changes were noted in any of the remaining parameters investigated in this study (i.e., food consumption, estrous cycle, sperm analysis, hematology, coagulation, clinical chemistry, urinalysis, splenic lymphocyte subpopulation and macroscopic and microscopic examination). The NOAEL for General Toxicity (F1) has been stablised at least 1000 mg/kg bw/day.

References

TEST_MATERIAL_INFORMATION: Alkylamphoacetates C8-C18 (Diacetate form)

UUID: 30fe3d1d-0b19-4c73-a833-6fc5dc023606
Dossier UUID:
Author: DLO
Date: 2025-02-21T10:58:58.508Z
Remarks:

Name
Alkylamphoacetates C8-C18 (Diacetate form)

Composition

Composition

Type
Constituent

Reference substance
Alkylamidoamine glycinate majority C12, 14 (amphoacetate) / Reaction products of 1H-Imidazole-1-ethanol, 4,5-dihydro-, 2-(C7-C17 odd-numbered, C17-unsatd. alkyl / 931-291-0

EC number	EC name
931-291-0	EC Inventory

CAS number	CAS name

IUPAC name

Reaction products of 1H-Imidazole-1-ethanol, 4,5-dihydro-, 2-(C7-C17 odd-numbered, C17-unsatd. alkyl) derivs. and sodium hydroxide and chloroacetic acid

Remarks
Aqueous solution

Other characteristics

Test material form
liquid: viscous

Details on test material
- Physical appearance: clear yellow liquid
- Storage conditions: At room temperature

LITERATURE: Extended One Generation Reproductive Toxicity Study (including Cohort 1) of Amphoacetates C8-C18 by Oral Gavage in Rats

UUID: 45146d18-bd59-45ae-89e0-468d23bfe62f
Dossier UUID:
Author: DLO
Date: 2025-03-14T09:46:55.505Z
Remarks:

General information

Reference Type
study report

Title
Extended One Generation Reproductive Toxicity Study (including Cohort 1) of Amphoacetates C8-C18 by Oral Gavage in Rats

Author
Charles River Laboratories Den Bosch BV

Year
2025

Testing facility
Charles River Laboratories Den Bosch BV, Hambakenwetering 7, 5231 DD ‘s-Hertogenbosch
The Netherlands.
Charles River Laboratories Den Bosch BV, Nistelrooise Baan 3, 5374 RE Schaijk, The Netherlands

Report date
2025-02-21

Report number
20346108

Study sponsor
Verdant Specialty Solutions For account of the Members of the Amphoacetates C8-C18 Consortium, Oil Sites Road, Ellesmere Port, Cheshire CH65 4EY, United Kingdom

charles river

Test Facility Study No. 20342906

Analogue Approach for REACH Registration of ALKYLAMPHOACETATES- version March 2025

SPONSOR
(on behalf of the alkylamphoacetates consortium):
Innospec Performance Chemicals Europe Limited
Oil Sited Road, Ellesmere Port
Cheshire, CH65 4EY
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TEST FACILITY:
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The Netherlands

28 March 2025
(revised version of Charles River report finalized on April 29, 2019)

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1. RESPONSIBLE PERSONNEL

1.1. Test Facility

Test Facility	Charles River Laboratories Den Bosch BV Hambakenwetering 7 5231 DD ‘s-Hertogenbosch The Netherlands

1.2. Sponsor

Sponsor	(on behalf of the Amphoacetates consortium): Innospec Performance Chemicals Europe Limited Oil Sited Road, Ellesmere Port Cheshire, CH65 4EY United Kingdom

2. SUMMARY

This report describes the results of an *analogue approach* applied for the REACH registration of alkylamphoacetates with varying alkyl chain lengths covering volume bands of 10-100, 100-1000 or >1000 tonnes/year (Annex VII, VIII, IX and Annex X data requirements), as summarized below:

Substance name	EC no.	Highest tonnage band in the SIEF
Amphoacetates C8-C18	931-291-0	>1000
Amphoacetates C12-C14	938-645-3	10-100
Amphoacetates C12	271-794-6	100-1000

The analogues were identified based on comparable manufacturing processes, structural similarity (shared core structure, main components C12 and/or C14 linear alkyl chain derivatives) and resulting similar physico-chemical and (eco)toxicological properties.

The current update was initiated following ECHA decisions on testing proposals on the three analogues. The testing outline followed was chosen to strengthen the Read-Across hypothesis, by substantiating the data-set with new test data and by confirming the scientific validity of the historic data-set. The strategy followed was outlined in the document "Update on category approach and testing strategy for REACH registration of ALKYLAMPHOACETATES" (06 October 2016).

Alkylamphoacetates can be divided into two forms: the mono-acetate form in which mainly the mono-acetate molecules are present (>80%); and the di-acetate form, in which both mono-acetates and di-acetates are present at approximately 50% (see figure 1). Attempts were made to isolate the mono- and diacetate forms of a relatively narrow C-chain distribution amphoacetate by aid of preparative chromatography, and to use the isolated forms as standard in further HPLC-tests to gain insights a.o. on elution order and (UV) response factors. These attempts have been more successful than they were in the past and more in-depth investigations may be considered in future test programmes.

The additional data include physico-chemical parameters (vapour pressure and CMC), ecotoxicological tests (algae tests, acute *Daphnia* and fish tests, a *Daphnia* reproduction test and a fish early life stage test). The conclusions of the new studies, which were done including analytical verification of the test concentrations, were comparable to the conclusions of the old studies (effect concentrations based on nominal test concentrations). The test work was planned in a step-wise approach: chronic test work was performed with the analogue that showed the highest toxicity in the acute tests. A step-wise approach was also followed for human toxicity testing. Twenty-eight day repeated dose studies were performed with the mono- and the diacetate forms of alkylamphoacetates C8-C18, the analogue in which all alkyl lengths covering the spectrum/chemical space of the category are present. These studies (in which no adverse effects were seen apart from a secondary effect (regurgitation)) were followed up by a 90-day repeated dose study and a prenatal developmental study on the C8-C18 alkylampho(di)actetates. No adverse effects were observed after sub-chronic exposure up to and including the highest dose tested, resulting in a NOAEL of 1000 mg/kg bw/day. In the prenatal developmental toxicity study, low incidence of adverse effects were observed (cardiovascular/abdomen malformations in all dose groups and abnormal lung lobation in low and mid dose), however an in-depth analysis of the available developmental and reproductive data (DeSesso & Williams, 2023) did not support that C8-C18 alkylampho(di)actetates (nor any of the other analogues) caused cardiovascular/abdomen malformations. Based on their

overall assessment and opinion that the fetal findings are not treatment-related, and in absence of maternal adverse effects, the maternal and developmental NOAELs were both established as being at least 1000 mg/kg bw/day. To get a better understanding of the effect of alkyl chain length (distribution) and mono- and diacetate forms on toxicity, a 90-day repeated dose toxicity study and a prenatal developmental toxicity study with C12-14 alkylampho(di)acetates were also conducted. In the 90-day study no adverse test-item related effects were seen at any dose level, the NOAEL for sub-chronic exposure was found to be 1000 mg/kg bw/day. Similarly, no maternal or developmental toxicity were observed up to the highest dose level tested in the prenatal developmental toxicity study with C12-14 alkylampho(di)acetate. The maternal and developmental NOAELs were both established as being at least 1000 mg/kg bw/day. An additional prenatal developmental toxicity study has been conducted with C12 alkylampho(mono)acetates, in this study maternal and developmental toxicity were not observed up to the highest dose level tested, and the maternal and developmental NOAELs were all established as being at least 1000 mg/kg bw/day. In a prenatal developmental toxicity study conducted in New Zealand White rabbits with alkylampho(di)acetates, a maternal NOAEL of 75 mg/kg bw/day (based on mortality and lower body weights and food consumption) and a developmental NOAEL of 75 mg/kg bw/day (based on higher post-implantation loss) were established. A review of these results conducted by DART experts (DeSesso & Williams, 2024a and 2024b) concluded that the available data is highly suggestive of a relationship between maternal systemic toxicity and the developmental effects seen in rabbits and that these results do not warrant self-classification for adverse effects on development in accordance with EU CLP. Finally, in an Extended One Generation Reproductive Toxicity Study (EOGRTS) conducted also with alkylampho(di)acetates, the maternal, reproductive and developmental NOAELs were all set at 1000 mg/kg bw/day (highest dose tested) based on the absence of adverse and treatment related effects at all dose levels.

3. INTRODUCTION

The aim of this document is to provide the scientific basis and rationale for a read-across analogue approach used for the REACH registration of three alkylamphoacetate surfactant substances. The rationale was created based on the ECHA Guidance for the implementation of REACH, Guidance on information requirements and chemical safety assessment, Chapter R.6 (reporting format for a chemical category) and the Read-Across Assessment Framework (RAAF).[1,2,3] A substance-based structural and compositional analogue approach for read-across was followed, meaning that results are obtained with the source substance as such, and the result of the tests are used to predict the properties for the target substance(s).

The following substances are currently considered as analogues:

Table 1: Alkylamphoacetate analogues

Substance name	EC no.	Highest tonnage band in the SIEF

[1] Guidance on information requirements and chemical safety assessment, Chapter R.6, May 2008; http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_r6_en.pdf?vers=20_08_08
[2] Read-Across Assessment Framework (RAAF), ECHA-17-R-01-EN, March 2017
[3] Read-Across Assessment Framework (RAAF)_Considerations on multi-constituent substances and UVCBs, ECHA-17-R-04-EN, March 2017

Amphoacetates C8-C18	931-291-0	>1000
Amphoacetates C12-C14	938-645-3	10-100
Amphoacetates C12	271-794-6	100-1000

4. ANALOGUE DEFINITION

4.1. Definition of analogues

4.1.1. Read-across hypothesis

The read-across hypothesis is that the organism is not exposed to common compounds (metabolites/degradation products) but rather, as a result of structural similarity, that different compounds have similar (eco)toxicological and fate properties (i.e. RAAF Scenario 2). The properties investigated in a study conducted with one source substance are used to predict properties that would be observed in a study with the target substance if it were to be conducted. Qualitatively similar properties or absence of effect are predicted. The predicted property may be similar or based on a worst-case approach.

The analogues are alkylamphoacetates, which are amphoteric surfactants. The analogues are manufactured, marketed and used in aqueous solutions. The solid(s) content in a manufactured commercial product corresponds to the substance to be registered in accordance with REACH Article 3(1), in case water can be removed from the products without affecting or impacting upon the stability of the substance and/or its composition. For some alkylamphoacetate analogues the water cannot be removed without changing their chemical identity and/or composition and is thus part of the registered substance. The solid concentration in the commercial products generally ranges from 30 to 95%.

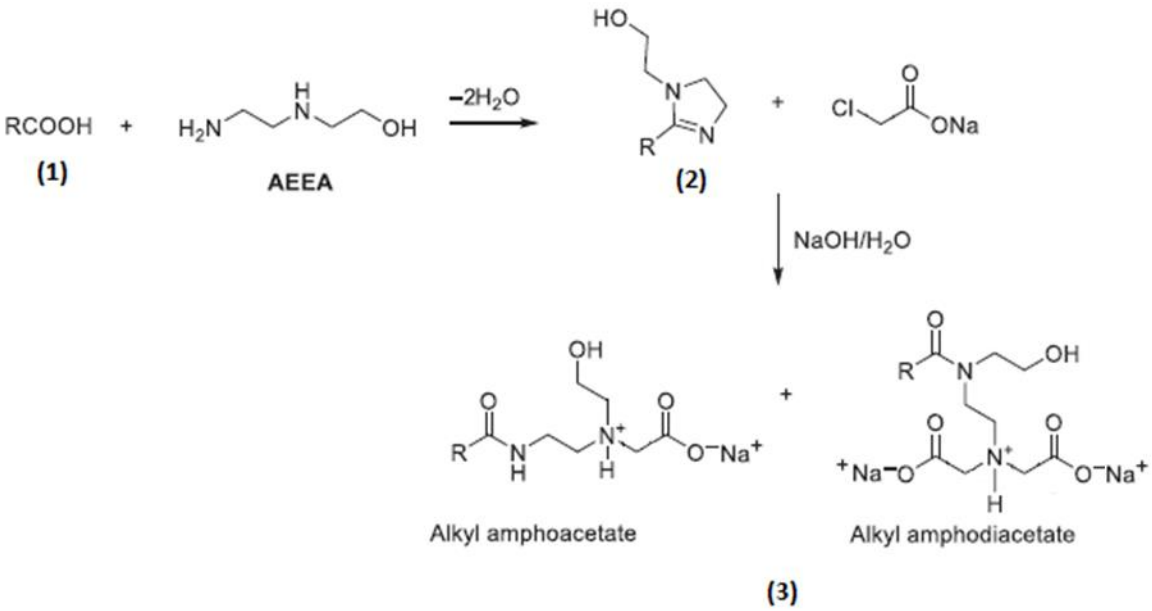
The analogues in this read-across justification report are identified and grouped based on the following characteristics: similarities in the general manufacturing process (including identical and/or comparable starting materials), functional groups, and general composition. The main variable resides in the alkyl chain distribution present in the raw starting materials.

1) Chemistry

Synthesis

The general chemistry of the manufacture of alkylamphoacetates is depicted in Figure 1 below.

Figure 1: Chemistry of the manufacture of alkylamphoacetates



Whereby:
AEEA is Aminoethylethanolamine (2-(2-aminoethylamino)ethanol; CAS: 111-41-1) and
R is the alkyl chain distribution derived from fatty acids or oils (see Table 2 and Appendix I)

The substances are manufactured in a batch-wise process, under similar reaction conditions. The imidazoline intermediate (2) (1H-Imidazole-1-ethanol, 4,5-dihydro-, 2-(Cx-y odd-numbered, alkyl) derivatives) is synthesised from the raw material fatty alkyl carboxylic acids (1) with aminoethylethanolamine and is often isolated.

The alkylampho(di)acetates (3) are subsequently synthesised in water, at ambient pressure and typically at a temperature of 80°C (cooled). The imidazoline intermediate (2) is reacted with chloroacetic acid in the presence of sodium hydroxide (alternatively, sodium chloroacetate can be used) and water. The amount of sodium hydroxide is as much as needed to have the pH well above 7, to start the exothermic reaction between the anion of chloroacetic acid that is formed in the water and the intermediate (2). The molar ratio between the intermediate and chloroacetic acid ranges from 1:1 to 1:2. The 1:1 molar ratio results in a monoacetate, while an excess of sodium chloroacetate/chloroacetic acid in the 1:2 molar ratio favours the formation of the diacetate. As a by-product, hydrochloric acid is formed during the reaction, that is neutralized via the addition of sodium hydroxide (pH is monitored and remains above 7 to keep the reaction going). The reaction terminates with complete consumption of chloroacetic acid resp. its anion. The resulting reaction mixture is neutralized to a pH of 9 or lower, by adding any acid (e.g. hydrochloric acid). The by-product sodium chloride is formed from the reaction of sodium hydroxide with hydrochloric acid.

Functional groups

Figure 2 presents the structural information of the alkylampho(di)acetates. The common structural features present in the surfactant are an amide bond and a hydroxyl group both originating from the reaction of the carboxylic acids and the AEEA and the presence of aminoglycinate function(s) (or “acetate”) originating from the reaction of the imidazole intermediate with the chloroacetic acid.

The upper two structures of Figure 2 are representative for the alkylampho(mono)acetates and the lower two structures the alkylampho(di)acetates. These are theoretical structures based on the knowledge of the chemistry (Uphues, 1998; Behler et al., 2001). The NMR spectra show peaks that are characteristics of these structures but the difficulty in identifying the precise structures present has been discussed in the document “interpretation of NMR spectra” attached to the Amphoacetates C8-18 and Amphoacetates C12 submissions (section 1.4). Their precise structure (i.e. positioning of the acetate and hydroxyl groups) and respective percentages are variable and cannot be analytically determined due to the lack of a suitable analytical method for these UVCB substances.

Attempts were undertaken to isolate the mono- and diacetate forms of a relatively narrow C-chain distribution amphoacetate by aid of preparative chromatography, and to use the isolated forms as standard in further HPLC-tests to gain insights amongst others on elution order and (UV) response factors. Although the separation and isolation appeared to be reasonably successful based on chromatograms, further attempts to crystallize and identify the collected fractions were less successful. Evaporation of preparative chromatography solvents from the collected fractions caused amongst others foaming and never yielded dry residues, which possibly could have been re-crystallized to yield purer acetate forms. In a best case, an

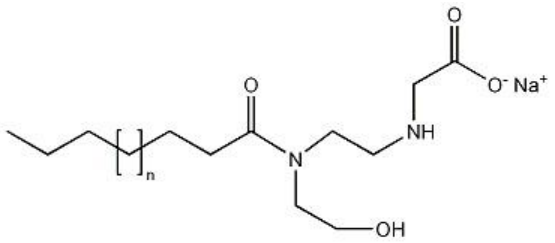
amorphous monoacetate solid could be isolated (confirmed by 1H-NMR analysis), but it proved impossible to isolate a diacetate solid.

Composition

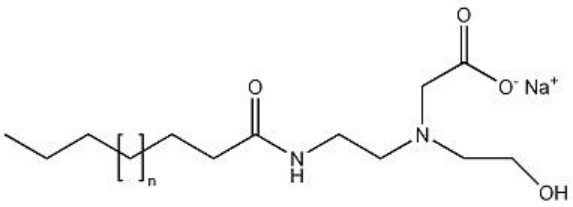
The alkylamphoacetates are UVCB substances and contain multiple constituents. The substances contain an alkylamphoacetate fraction, which consists of a group of various alkyl derived constituents bearing aminoglycinate functional group(s) (the "active surfactant fraction") and the by-product sodium chloride. The substances also contain some other constituents, such as residual water and the by-product sodium glycolate ($C_2H_4O_3Na$). The typical compositions of the analogues are reported in Table 2 (section 4.2).

Figure 2: General structures of the main constituents of the alkylamphoacetates fraction of the substances

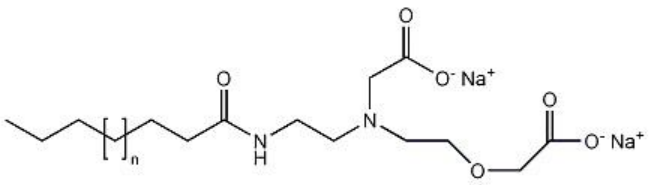
Alkyl-monoacetate 1



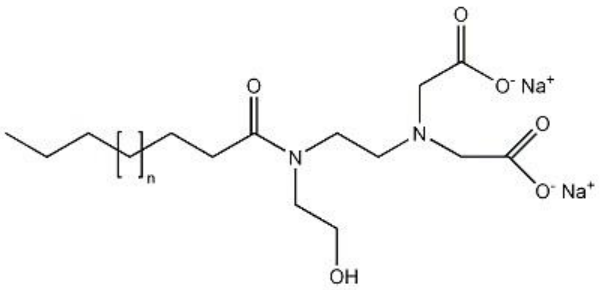
Alkyl-monoacetate 2



Alkyl-diacetate 1



Alkyl-diacetate 2



Variability/differences

An important difference is the use of various types of raw materials, differing mainly in the C-Chain length of the linear alkyl carboxylic acid starting material. UVCB-type substances derived from oleochemicals consist as mixtures of various alkyl-chain lengths at varying

concentrations (OECD 193). The amount of each chain length depends on the source of fatty acids, which usually originates from natural fats and oils (containing for example the alkyl chain length range from C8 to C18), but can also be from synthetic origin. As it is in general derived from a natural origin, the C8-18 alkyl distribution is variable, and can only be given as a range of chain lengths with the main constituents being C12 and C14. Fractionation can increase the concentration of a specific C-chain length cut (for example, to > 90% C12-alkyl for Alkylamphoacetates C12).

All analogue substances contain mono- and diacetate structures and are mainly comprised of the C12 and C14 forms. The ratio of mono- and diacetate constituents differ as a consequence of the relative amount of chloroacetic acid used in the manufacturing process as described above. The more chloroacetic acid is used, the more diacetate constituents will be present in the resulting product.

Besides differences in the number of incorporated carboxymethyl-groups (mono- and diacetates) and in their alkyl constituents, differences in the position of the acetate and hydroxyl groups may give rise to substructures 1 and 2 (see figure 2). While mono- and diacetates with substructure 2 predominate, mono- and diacetates with substructure 1, cannot be ruled out.

The ratio of the (potential) structures contained in the surfactant part of the substance have been found to influence the (eco)toxicological properties of the substances. For certain endpoints this influence is addressed, and follow-up testing (where applicable) is carried out, using the worst-case approach (i.e. the analogue with the least favourable properties will be tested and/or considered the source). Furthermore, all analogous structures have the same functional groups, i.e. one or two aminoglycinate (-NH-CH_2-COONa) functions (i.e. terminal acetate) and hydroxyl, linked to a fatty chain by an amide bond. The structural and compositional similarity is expected to result in similar behaviour of the analogues upon exposure to eco-system/environment and exposure to and uptake in the human body.

All analogue substances contain a main alkylamphoacetate ‘active surfactant’ fraction, as well as sodium chloride, sodium glycolate and residual water as impurities/by-products, all in comparable amounts (see Table 2). Because of the decreasing proportion of other alkyl chains, the Amphoacetates C12-C14 and Amphoacetates C12 have an increasing content in the C12 alkyl structures compared to the Amphoacetates C8-18.

2) Physicochemical properties and distribution

The alkylamphoacetate analogues are designed to be surface active, exhibit low vapour pressure (due to their relatively high molecular weight and presence of polar groups) and a high-water solubility (amphiphilic and polar groups). As the alkylamphoacetates are mainly present as sodium carboxylates at environmental pH (pKa of carboxylic acids is approx. 4 – 5), these constituents are expected to partition predominantly to the aquatic compartment and minimally adhere to organic matter. As the vapour pressure is expected to be low, the substances do not volatilize. Based on their amphiphilic structure together with a high-water solubility and moderate lipophilic character, the amphoacetates are expected to be systemically absorbed to some extent by the oral or dermal route (REACH guidance R7c, 2017). However, the presence of charged functional groups in substances has been shown to reduce dramatically the passage across the skin (Schaefer et al., 1996). As produced or under the use conditions the surfactant part of the substances will be either as a sodium salt, or as an amphoteric (zwitterionic) form with positive and negatively charged functional groups present.

It is concluded that based on the compositional and structural similarity of the components present and their respective water solubility, partition coefficient, vapour pressure and surface activity, the alkylamphoacetate analogues will be distributed similarly upon exposure to environment and in the human body and are expected to exhibit similar (eco)toxicological properties.

4.1.2. Applicability domain (AD) of the analogues

The alkylamphoacetates are defined as amphoteric surfactants. The proportion of alkyl chain lengths which comprise the substances can vary between C8 and C18. The alkylamphoacetate analogues all share the same key functional groups, i.e. one or two aminoglycinate (-NH-CH_2-COONa) functionalities (i.e. terminal acetate) and a single N-hydroxyethyl, linked to a fatty acid chain by an amide bond. In one of the diacetate structure forms, the hydroxyethyl group is converted to an ether bond after reaction with a second chloroacetate molecule. In view of their potential chemical reactivity these structural features are considered to define the toxicological profile to a higher extent than the alkyl chain length and/ or presence of mono- or diacetate forms.

It is obvious that a more detailed determination and discrimination of the compositional profile of the individual substances which comprise the alkylamphoacetate analogues would strengthen the read across hypothesis. To this end, a programme to generate new analytical data fulfilling the requirements of ECHA's Advice on using read-across for UVCB substances[4] is being developed and discussed by the alkylamphoacetate consortium at the moment.

4.1.3. List of endpoints covered

A RAAF Scenario 2 analogue approach (read-across) was applied to the following endpoints (nb: read across can differ per endpoint for each analogue):

- self-ignition temperature;
- biodegradability;
- algae toxicity;
- acute toxicity to *Daphnia* and/ or fish;
- activated sludge respiration inhibition;
- *Daphnia* reproduction toxicity testing;
- fish chronic testing;
- acute dermal toxicity;
- skin and eye irritation;
- skin sensitization;
- *in vitro* gene mutation in mammalian cells;
- sub-chronic repeated dose toxicity;
- toxicokinetic assessment.

4.2. Analogues

Substance identifiers for all alkylamphoacetate analogues are presented in Table 2. It should be noted that no molecular weight range can be accurately defined for these complex UVCB

[4] ECHA Advice on using read-across for UVCB substances, May 2022; https://www.echa.europa.eu/documents/10162/11395738/advice_uvcb_read-across_en.pdf

substances containing multiple constituents. The molecular weight mentioned is the molecular weight used for the chemical safety assessment (CSA). In case of Amphoacetates C12-C14 and Amphoacetates C12, a possible C12 monoacetate constituent and in case of Amphoacetates C8-C18, a possible C12 diacetate constituent (representing a worst-case approach for the CSA for human health) were considered for calculation of molecular weights.

The solid(s) content corresponds to the substance to be registered in accordance with REACH Article 3(1), for some alkylamphoacetate analogues the water cannot be removed and is thus part of the registered substance .

The NaCl content was determined by the determination of chloride by titration with silver nitrate. Based on these results, the alkylamphoacetate derivatives (active surfactant) fraction and the solid content are determined (see also previous section). The percentual composition of the alkylamphoacetate derivatives fraction (or alkyl chain distribution) presented in table 2 is based on the known C-Chain distribution of the fatty acid starting material(s).

Table 2 Substance identifiers for all analogues

Identification: Amphoacetates C8-C18		
	Type of substance:	UVCB Monoacetate form (contains appr. 95% monoacetates and 5% diacetates) and diacetate form (contains appr. 40% monoacetates and 60% diacetates)
	IUPAC name:	Reaction products of 1H-Imidazole-1-ethanol, 4,5-dihydro-, 2-(C7-C17 odd-numbered, C17-unsatd. alkyl) derivs. and sodium hydroxide and chloroacetic acid
	CAS Number:	-
	Alternative CAS numbers[5]	68650-39-5; 68334-21-4; 68390-66-9; 61791-32-0; 90387-76-1; 68608-65-1
	EC/List Number:	931-291-0
	Molecular Weight (for the CSA):	446 g/mol
	Compositional information (as manufactured, w/w)	
	Water	47-64%
	Total solids:	36-53%
	Total alkylamphoacetate derivatives	27-43%
	NaCl	0-15%
	Sodium glycolate	0-6%

[5] See Annex I for the SIEF merging justification document (as submitted with the registration of this substance)

<table>
<tr><th colspan="3">Identification: Amphoacetates C8-C18</th></tr>
<tr><td></td><td>Amido hydroxyethyl ethylenediamines</td><td>0-3%</td></tr>
<tr><td></td><td>Sodium chloroacetate</td><td>0-600 ppm</td></tr>
<tr><td></td><td>2-(2-aminoethylamino)ethanol</td><td>0-6 ppm</td></tr>
<tr><td></td><td>Compositional information (solvent free condition, w/w)</td><td></td></tr>
<tr><td></td><td>Total alkylamphoacetate derivatives</td><td>65-86%[6]</td></tr>
<tr><td></td><td>Alkyl chain distribution, Cn</td><td>
<table>
<tr><th>Cn</th><th>Mono#</th><th>Di#</th><th>Total</th></tr>
<tr><td>C8</td><td>0-11%</td><td>0-2%</td><td>0-11%</td></tr>
<tr><td>C10</td><td>0.1-10%</td><td>0-2%</td><td>0-11%</td></tr>
<tr><td>C12</td><td>16-56%</td><td>0-36%</td><td>42-64%</td></tr>
<tr><td>C14</td><td>5-20%</td><td>0-15%</td><td>6-26%</td></tr>
<tr><td>C16</td><td>1-22%</td><td>0-8%</td><td>4-22%</td></tr>
<tr><td>C18</td><td>0.1-16%</td><td>0-7%</td><td>0.1-18%</td></tr>
<tr><td>C18:1 and/or C18:2 [7]</td><td>0-9%</td><td>0-12%</td><td>0-20%</td></tr>
</table>
</td></tr>
<tr><td></td><td>NaCl</td><td>0-26%</td></tr>
<tr><td></td><td>Sodium glycolate</td><td>0-12%[8]</td></tr>
<tr><td></td><td>Amido hydroxyethyl ethylenediamines</td><td>0-6%</td></tr>
<tr><td></td><td>Sodium chloroacetate</td><td>0-1500ppm</td></tr>
<tr><td></td><td>2-(2-aminoethylamino)ethanol</td><td>0-14ppm</td></tr>
</table>

Identification: Amphoacetates C12-C14	
Type of substance:	UVCB Diacetate form only (contains appr. 40 to 45% monoacetate and 55 to 60 % diacetates)
IUPAC name:	Reaction products of 1H-Imidazole-1-ethanol, 4,5-dihydro-, 2-(C11-C13 odd-numbered alkyl) derivs. and sodium hydroxide and chloroacetic acid
CAS Number:	1689515-39-6

[6] The lower range figure for the surfactant fraction is due to the greater difficulty in drying the C8-18 substance and residual water

[7] Number of unsaturations per C18 alkyl chain: 0.001 - 0.15

[8] Analysed as glycolic acid and converted to sodium glycolate as this is the form more likely present in the UVCB substance. Compositional information in registration dossiers may be given as glycolic acid (due to the analytical method) and/or can be also converted to the sodium salt.

<table>
<tr><th colspan="3">Identification: Amphoacetates C12-C14</th></tr>
<tr><td></td><td>Alternative CAS numbers[9]</td><td>66161-62-4; 68608-66-2</td></tr>
<tr><td></td><td>EC/List Number:</td><td>938-645-3</td></tr>
<tr><td></td><td>Molecular Weight (for the CSA):</td><td>367 g/mol</td></tr>
<tr><td></td><td>Compositional information (as manufactured, w/w)</td><td></td></tr>
<tr><td></td><td>Water</td><td>50-51%</td></tr>
<tr><td></td><td>Total solids:</td><td>49-50%</td></tr>
<tr><td></td><td>Total alkylamphoacetate derivatives</td><td>≥39%</td></tr>
<tr><td></td><td>NaCl</td><td>0-10%</td></tr>
<tr><td></td><td>Sodium glycolate</td><td>2-4%</td></tr>
<tr><td></td><td>Amido hydroxyethyl ethylenediamines</td><td>0-2%</td></tr>
<tr><td></td><td>Sodium chloroacetate</td><td>0-65 ppm</td></tr>
<tr><td></td><td>2-(2-aminoethylamino)ethanol</td><td>0-5 ppm</td></tr>
<tr><td></td><td>Compositional information (solvent free condition, w/w)</td><td></td></tr>
<tr><td></td><td>Total alkylamphoacetate derivatives</td><td>≥78%</td></tr>
<tr><td></td><td>Alkyl chain distribution, Cn</td><td>
<table>
<tr><th>Cn</th><th>mono</th><th>di</th><th>total</th></tr>
<tr><td>C8</td><td>n.d.[10]</td><td>n.d.</td><td>n.d.</td></tr>
<tr><td>C10</td><td>≤2%</td><td>≤2%</td><td>≤4%</td></tr>
<tr><td>C12</td><td>26-37%</td><td>36-49%</td><td>67-80%</td></tr>
<tr><td>C14</td><td>7-16%</td><td>10-20%</td><td>20-32%</td></tr>
<tr><td>C16</td><td>≤2%</td><td>≤2%</td><td>≤4%</td></tr>
<tr><td>C18</td><td>n.d.</td><td>n.d.</td><td>n.d.</td></tr>
<tr><td>C18:1 and/or C18:2</td><td>n.d.</td><td>n.d.</td><td>n.d.</td></tr>
</table>
</td></tr>
<tr><td></td><td>NaCl</td><td>0-20%</td></tr>
<tr><td></td><td>Sodium glycolate[11]</td><td>6 -11%</td></tr>
<tr><td></td><td>Amido hydroxyethyl ethylenediamines</td><td>0-6%</td></tr>
<tr><td></td><td>Sodium chloroacetate</td><td>0-130ppm</td></tr>
<tr><td></td><td>2-(2-aminoethylamino)ethanol</td><td>0-14ppm</td></tr>
</table>

[9] The SIEF merging justification document is submitted with the registration of this substance
[10] n.d – Not determined.
[11] Analysed as glycolic acid and converted to sodium glycolate as this is the form more likely present in the UVCB substance. Compositional information in registration dossiers may be given as glycolic acid (due to the analytical method) and/or can be also converted to the sodium salt.

<table>
<tr><th colspan="3">Identification: Amphoacetates C12</th></tr>
<tr><td></td><td>Type of substance:</td><td>UVCB
Monoacetate form only (contains appr. 75 to 100% monoacetate and 0 to 25% diacetates)</td></tr>
<tr><td></td><td>IUPAC name:</td><td>Reaction products of 1H-Imidazole-1-ethanol, 4,5-dihydro-, 2-(C11 alkyl) derivs. and sodium hydroxide and chloroacetic acid</td></tr>
<tr><td></td><td>CAS Number:</td><td>68608-66-2</td></tr>
<tr><td></td><td>EC Number:</td><td>271-794-6</td></tr>
<tr><td></td><td>Molecular Weight (for the CSA):</td><td>367 g/mol</td></tr>
<tr><td></td><td>Compositional information (as manufactured, w/w)</td><td></td></tr>
<tr><td></td><td>Water</td><td>60-70%</td></tr>
<tr><td></td><td>Total solids:</td><td>30-40%</td></tr>
<tr><td></td><td>Total alkylamphoacetate derivatives</td><td>23-31%</td></tr>
<tr><td></td><td>NaCl</td><td>5-8%</td></tr>
<tr><td></td><td>Sodium glycolate</td><td>0.5-4%</td></tr>
<tr><td></td><td>Amido hydroxyethyl ethylenediamines</td><td>0-0.3%</td></tr>
<tr><td></td><td>Sodium chloroacetate</td><td>0-5000 ppm</td></tr>
<tr><td></td><td>2-(2-aminoethylamino)ethanol</td><td>0-4 ppm</td></tr>
<tr><td></td><td>Compositional information (solvent free condition, w/w)</td><td></td></tr>
<tr><td></td><td>Total alkylamphoacetate derivatives</td><td>76-80%</td></tr>
<tr><td></td><td>Alkyl chain distribution, Cn</td><td><table><tr><th>Cn</th><th>mono</th><th>di</th><th>total</th></tr><tr><td>C12</td><td>61-93%</td><td>0.1-21%</td><td>80-99.9%</td></tr><tr><td>Unknown</td><td>-</td><td>-</td><td>0.1-20%</td></tr></table></td></tr>
<tr><td></td><td>NaCl</td><td>16-20%</td></tr>
<tr><td></td><td>Sodium glycolate</td><td>4-8%</td></tr>
<tr><td></td><td>Amido hydroxyethyl ethylenediamines</td><td>0-0.5%</td></tr>
<tr><td></td><td>Sodium chloroacetate</td><td>0-9000ppm</td></tr>
<tr><td></td><td>2-(2-aminoethylamino)ethanol</td><td>0-10ppm</td></tr>
</table>

4.3. Purity/Impurities

The water content of the registered substances is determined by Karl-Fischer titration after the drying procedure.

The NaCl content was determined by the determination of chloride by titration with silver nitrate.

5. READ-ACROSS JUSTIFICATION

5.1. Physico-chemical properties

The assumption/hypothesis that the properties of the analogues are similar was in the first instance verified with respect to the physico-chemical parameters (

Table 4).

The substances are structurally very similar and are designed to exhibit surface-active properties. The surface tension was measured for all analogues and found to be in the same range (29.1 – 35.4 mN). Since this property influences how the water solubility is interpreted, the Critical Micelle Concentration (CMC) was determined in GLP-compliant studies performed in accordance with OECD Guideline No. 115 for all of the analogues, by measuring the surface tension of test item solutions at different test item concentrations. The Critical Micelle Concentration (CMC) of the mono- and the diacetate forms were found to be in a similar range: 160/150 and 239/262 mg solids/L for the monoacetate/diacetate form of Amphoacetates C8-C18 and Amphoacetates C12-C14, respectively. The CMC of Amphoacetates C12 (mono-acetate) was also determined and found to be higher, at 718 mg solids/L. The bulk water solubility of the different Amphoacetates was determined in GLP-compliant studies performed in accordance with EC A.8 method and OECD Guideline No. 105, by means of visual observations; the water solubility of all analogues was high (> 1000g solids/L).

For all analogues, it was considered justified to study the n-octanol/water partition coefficient (log Kow) by the estimation method, as all other methods were assessed to be not adequate due to their surface-active nature. However, based on the estimation method it was concluded that it is technically not possible to determine a reliable estimate of the log Kow for these complex and variable substances. Nevertheless, the water solubility was measured to be more than 1,000,000 mg solids/L and the solubility in octanol was found to be less than 82 mg/L. For these analogues, based on the complex and incompletely defined composition which contain variable alkyl chain lengths and a high concentration of NaCl and taking into account the tensio-active properties of the surfactant fraction and the fact that classical empirical methods cannot be used, it was deemed reasonable to assume a log Kow value of -1. This log Kow of -1 is justified on the basis of the experimentally determined solubility data in water and in octanol solvents, and on the basis of the calculated log Kow values of –0.64 to -4.19 for the main part of the surfactant fraction obtained with QSAR (US EPA Episuite KOWWIN v1.68).

All analogues were found to exhibit low vapour pressure. Measured vapour pressure values determined at 20 ºC for Amphoacetates C8-C18 was $1.4*10^{-7}$ Pa (monoacetate form) and < $8.4*10^{-7}$ Pa (diacetate form). For Amphoacetates C12-C14 the vapour pressure was $1.8*10^{-8}$ - $1.3*10^{-6}$ Pa (monoacetate form) and $2.6*10^{-6}$ Pa (diacetate form). For Amphoacetates C12 the vapour pressure was also very low with $1.5*10^{-7}$ Pa.

The density of the analogues in aqueous solutions is very similar and none of the analogues are highly flammable or exhibit pyrophoric or explosive properties.

All analogues decompose before reaching their boiling temperature. The differences observed in their melting point and decomposition temperatures can be explained by the difference in the extent of a more or less heterogeneous series of molecules present. The higher the content of molecules with a similar alkyl chain length (e.g. C12 alkyl), the easier the molecules can organize themselves to crystallize and melting at a specific temperature can be observed.

In view of the physico-chemical properties discussed above and the compositional and structural similarity of the components present in the analogues, it can be concluded that read-across between alkylamphoacetate analogues is justified and that they will be distributed similarly upon exposure to environment and in the human body and thus are expected to exhibit similar (eco)toxicological properties.

5.2. Environmental fate and eco-toxicological properties

The environmental properties of the analogues are presented in

Table 5. To allow comparison between the analogues, all concentrations mentioned in this chapter are expressed based on solids content (i.e. the pure active surfactant test item and salt, corrected for water content).

Aquatic toxicity: acute and chronic toxicity to invertebrates

Several older studies were available on the acute toxicity of alkylamphoacetates to the freshwater invertebrate *Daphnia magna* (see Appendix III). In short, the data indicated that the acute toxicity of Ampho(mono)acetates C12 to *Daphnia* (48h-EC_{50}'s: 89 – >100 mg/L) was lower than the toxicity of Amphoacetates C8-C18 (surrogate with unknown mono- to diacetate ratio) towards *Daphnia* (48h-EC_{50}'s: 2.5 – 18.5 mg/L). No data were available to address differences in toxicity profile between mono- and diacetate form of the analogues.

The historical tests were run without analytical verification of exposure concentrations, therefore ECHA concluded that the results were not adequate to fulfil the endpoint information requirement(s).

In order to provide more robust and high-quality data to cover this endpoint, in 2017 four acute *Daphnia magna* toxicity studies were performed in parallel, which included analytical verification of the test concentrations. The substances tested were Alkylamphoacetates C8-C18 (monoacetate and the diacetate form) and Amphoacetates C12-C14 (monoacetate and the diacetate forms). Analytical monitoring data collected during the test period showed that all substances were stable in aqueous solution (>80% of nominal concentrations at test end in all test solutions relevant for calculation of effect concentrations). Therefore, nominal concentrations were used to express the effect parameters in the final tests.

The results of the acute *Daphnia* tests performed in 2017 are summarized in Table 3 below.

Table 3 Acute *Daphnia* toxicity of Alkylamphoacetates C8-C18 and Amphoacetates C12-C14 tested in 2017

Test item	48h-EC_{50} (*Daphnia*; concentration based on solid fraction)
Alkylamphoacetates C8-C18	
Monoacetate form	25.4 mg/L
Diacetate form	56.6 mg/L
Alkylamphoacetates C12-C14	
Monoacetate form	67.3 mg/L
Diacetate form	> 100 mg/L

The alkyl chain distribution of the alkylamphoacetate analogues can be found in Appendix I. Comparison of the EC_{50} values with the alkylchain distribution reveals that the acute toxicity to daphnids is slightly higher in presence of alkylderivatives with a greater proportion of the longer carbon chains. Furthermore, the daphnids appear to be slightly more sensitive to the monoacetate form in comparison to the diacetate form. By adopting a conservative approach, the lowest 48h-EC_{50} values are considered worst-case to fill this endpoint. Therefore, the results

of the monoacetate forms were used as the key study values to cover this endpoint/information requirement for both C8-C18 and C12-C14 analogues, respectively.

The results of the acute *Daphnia* studies performed in 2017 yielded slightly higher EC_{50} values (i.e. lower toxicity) for all test substances compared to the old data (all data are given in Appendix III). The results from the new acute daphnid toxicity studies with alkylamphoacetates are considered more relevant, accurate and reliable than the historical data. However the old data covers a relatively large range (2.5-18.5 mg/L) of which one data point falls nearly within the new data range.

As analytical verification of exposure concentrations in the more recent studies demonstrate the C12-C14 and C8-C18 alkylamphoacetate test substance concentrations were within 20% of nominal and stable throughout the exposure period, this new data validates the reliability of the available historical acute daphnia toxicity study result with Amphoacetates C12. The more recent data for the C8-C18 and C12-C14 analogues combined with historical data on C12, allow, in a weight-of-evidence approach, to conclude on this endpoint for the C12 analogue: 48h-EC_{50} = 89 mg/L.

Long-term toxicity to aquatic invertebrates was determined for the alkylamphoacetate analogue that caused the highest toxicity in the acute tests(Alkylampho(mono)acetates C8-C18). The C8-C18 alkylampho(mono) acetate substance was subject to *Daphnia magna* reproduction study in accordance with OECD 211 under GLP conditions.

Ten neonates (<24 h old) were individually exposed in a semi-static system to nominal test concentrations of 0.46, 1.0, 2.2, 4.6 and 10 mg/L for 21 days with test solutions renewed every 48 hours. Additionally, a blank control was included with 20 neonates. Parental mortality, number of living offspring, immobile young and appearance of unhatched (aborted) eggs were recorded and the lengths of the surviving parental daphnids were measured at the end of the test.

Samples taken at the beginning and the end of three 48-hour renewal intervals were analysed. The concentrations measured in the freshly prepared solutions ranged between 47 - 133% of nominal, with the majority of results being within 87 -120% of nominal. Since the concentrations appeared to be unstable during the refreshment periods, Time Weighted Average concentrations were calculated to be 0.075, 0.15, 0.45, 1.6 and 3.7 mg/L.

Mortality in the controls did not exceed 20%, mortality in the test item groups ranged from 10 to 40% but was not statistically different from the control treatment. An increase of reproduction, rather than a reduction was observed in all concentrations tested. The onset of reproduction was not delayed in any of the test concentrations when compared to the controls (onset at day 7), except at the highest treatment group (at day 8). The change in mean body length of parent daphnids ranged between -1.0 and 1.8% in the four lowest concentrations and was not dose-related. At the highest concentration, a statistically significant reduction in mean body length of 4.6% was observed.

The 21-d NOEC for reproduction of *Daphnia magna* exposed to Amphoacetates C8-C18 (monoacetate form) was set at 3.7 mg/L (TWA concentration). The 21-d NOEC for growth reduction was set at 1.6 mg/L.

This result is read across to the other analogues, as Amphoacetates C8-C18 (monoacetate form) was concluded to represent the worst-case for the analogues based on the acute toxicity studies, but similar toxicity was expected.

Aquatic toxicity: algae

Similar to acute toxicity testing with the freshwater invertebrate *Daphnia magna*, toxicity of various alkylamphoacetates to aquatic plants was previously tested in historical studies (see Appendix III). These studies revealed toxicity in the same concentration range for mono- and diacetate forms (72h ErC50 = 28.5 mg/L for C8-C18 alkylampho(mono)acetates and 72h ErC50 = 30 mg/L for C8-C18 alkylampho(di)acetates). Furthermore, the algal toxicity studies indicated that Amphoacetates C8-C18 (monoacetate form) caused the highest toxicity compared to the other analogues (72h ErC50 = 10 mg/l for C8-18 alkylampho(mono)acetates and 72h ErC50 = 14.8 mg/L for C12 alkylampho(mono)acetate).

New algal toxicity testing was initiated in 2018 to verify these data. Alkylampho(mono)acetates C8-C18 and alkylampho(mono)acetates C12 (monoacetate form) were subject to 72h algae toxicity studies conducted in accordance with OECD guideline 201 under GLP conditions.

For Alkylampho(mono)acetates C8-C18 (monoacetate form), freshwater algae (*Pseudokirchneriella subcapitata*) were exposed to individually prepared Water Accommodated Fractions (WAF) of the test item prepared at nominal loading rates of 1.0, 3.2, 10, 32, and 100 mg/L (3 replicates per concentration) and an untreated control (6 replicates). Measured concentrations were not stable throughout the exposure period, therefore Time Weighted Average concentrations were calculated to be 0.23, 0.87, 2.6, 22 and 81 mg/L in WAFs prepared at loading rates of 1.0, 3.2, 10, 32 and 100 mg/L. A concentration-related increase of growth rate inhibition was observed at all test concentrations. Statistically significant growth rate inhibition was observed at the four highest WAFs tested, but a biologically relevant growth rate inhibition was observed only in the three highest WAFs tested. The 72h ErC_{50} and ErC_{10} for growth rate inhibition were 13 and 1.7 mg/L, respectively. The 72h-NOEC was 0.23 mg/L based on statistical significance and 0.87 mg/L based on biological relevance.

For Alkylampho(mono)acetates C12, freshwater algae (*Pseudokirchneriella subcapitata*) were exposed to individually prepared Water Accommodated Fractions of the test item prepared at nominal loading rates of 1.0, 3.2, 10, 32, and 100 mg/L (3 replicates per concentration) and an untreated control (6 replicates). Measured concentrations were not stable throughout the exposure period, therefore Time Weighted Average concentrations were calculated to be 0.015, 0.0025, 0.27, 210 and 21000 µg/L in WAFs prepared at loading rates of 1.0, 3.2, 10, 32 and 100 mg/L. A concentration-related increase of growth rate inhibition was observed with increasing test concentration, resulting in 100% inhibition of growth rate at the highest test concentration. The analytical results indicated a rapid decrease (to undetectable levels) of test concentrations within the first 24 hours of the test. As this decrease was seen independent of the presence of algae, and also observed in pre-coated test vessels, this indicates that the absence of the substance in the test solutions was not caused by sorption to the test vessels. At this point, it appears to be the case that the algae medium is not compatible with the test item and that the measured concentrations are not a reliable base to determine the NOEC value. Instead, the effect parameters for growth rate inhibition based on nominal loading rate were considered to be more relevant. This is also considered justified based on the OECD Guidance document on aquatic toxicity testing of difficult substances and mixtures number 23 (2019), which states that effect parameters can be calculated from the loading rates of the entire UVCB/multi-constituent substances when WAFs are tested. The NOELR for growth rate was determined to be 10 mg/L (nominal), the ErL_{10} 7.3 mg/L (nominal) and the ErL_{50} 44 mg/L (nominal).

The 72h-ErC_{50} for Alkylampho(mono)acetates C8-C18 of 13 mg/L based on the TWA concentrations corresponds to a nominal loading rate 72h ErL50 value of 19.3 mg/L. This is in

the same order of magnitude as the 72h-ErL_{50} value of 44 mg/L (nominal) for Amphoacetates C12. The fact that the 72h ErL50 value for Alkylampho(mono)acetates C8-C18 is somewhat lower (i.e. slightly more toxic) than the result for Alkylampho(mono)acetates C12 is in line with the pattern of results from aquatic toxicity studies with other trophic level species. Therefore, it is reasonable to use the algal toxicity data for the C8-C18 Alkylampho(mono)acetate as read across to Alkylamphoacetates C12-C14. The 72h-ErC_{50}, 72h-ErC_{10} and 72h-NOErC values for the C8-C18 alkylampho(mono)acetate were determined to be 13, 1.7 and 0.87 mg/L, respectively. Since the NOErC is lower than the ErC_{10}, this value is used as a worst-case key read-across value for chemical safety assessment.

Aquatic toxicity: acute and chronic toxicity to fish

Several historical studies with Alkylampho(mono and di)acetates C8-C18 available covering acute toxicity to fish, and one historical, unreliable, study with Alkylampho(mono)acetates C12. An overview of these older studies can be found in Appendix III.

The acute toxicity of Alkylampho(mono)acetates C12 and Alkylampho(mono)acetates C8-C18 to the freshwater fish *Cyprinus carpio* were investigated in more recent studies in 2018. Both studies included analytical verification of test item concentrations and were performed in accordance with OECD 203 under GLP conditions.

In a semi-static acute toxicity test with Alkylampho(mono)acetates C12, carp were exposed for 96 hours to an untreated control and nominal test item concentrations of 5.0, 10, 20, 40 and 80 mg/L. Measured concentrations at the start and end of the first and last renewal periods were at 116 -154% of the nominal test concentrations. Based on these results, the effect parameters were expressed based on nominal exposure concentrations. No mortality or other effects were observed in the control and at the three lowest concentrations tested during the exposure period. All fish exposed to the highest concentration were found dead after the first 24 hours of exposure. All fish exposed to 40 mg/L were found dead after 48 hours of exposure. The 96h-LC_{50} for Alkylampho(mono)acetates C12 was determined to be 28 mg/L based on analytically confirmed nominal exposure concentrations.

In a semi-static test with Alkylampho(mono)acetates C8-C18 , carp were exposed for 96 hours to an untreated control and nominal test concentrations of 0.46, 1.0, 2.2, 4.6 and 10 mg/L. Measured test item concentrations were at the level of nominal concentrations (99 -115%) in freshly prepared test medium and at 95 - 142% of nominal in spent solutions. Because of the unknown reason for the observed increase in measured concentrations, the average nominal exposure concentrations were calculated to be 0.54, 1.1, 2.5, 5.1 and 11 mg/L. No mortality or other effects were observed in the control and at the three lowest concentrations tested during the exposure period. All fish exposed to the highest concentration were found dead after the first 24 hours of exposure. Six of the 7 fish exposed to 5.1 mg/L were found dead after 96 hours of exposure. The 96h-LC_{50} was determined to be 4.0 mg/L based on analytically confirmed average nominal exposure concentrations.

In line with the pattern observed with the acute toxicity testing on *Daphnia* and algae tests, Alkylampho(mono)acetates C8-C18 exhibited higher toxicity to fish than the C12 Alkylampho(mono)acetate analogue. Therefore, it is reasonable to use the acute fish toxicity data for C8-C18 Alkylampho(mono)acetate as read across to Amphoacetates C12-C14.

Following the conduct of a new suite of acute toxicity studies on fish, it was deemed appropriate to consider a flow-through fish early-life stage (ELS) toxicity test in order to assess possible

lethal and sub-lethal effects of alkylamphoacetate substances through exposure during embryonic and early larval development of the fathead minnow (*Pimephales promelas*). As Alkylampho(mono)acetates C8-C18 had been found to exhibit the highest acute toxicity to fish of all the alkylamphoacetate analogues, this substance was selected as a conservative worst-case representative substance from the category.

The chronic fish study was conducted in accordance with OECD 210 and in compliance with GLP. Fertilized eggs (80 eggs per group, divided into four replicates) were exposed to an untreated control and the test item at mean measured concentrations of 0.035, 0.090, 0.22, 0.61 and 1.6 mg/L. Nominal concentrations were 0.05, 0.13, 0.31, 0.78 and 2.0 mg/L and were selected based on the results of a range-finding test (with target concentrations of 0.050, 0.50 and 5.0 mg solids/L) in which no mortality of the newly hatched larvae was observed in the control and 0.050 mg /L group, while mortality of 5.3% and 75% was observed at concentrations of 0.50 and 5.0 mg/L, respectively. Embryonic and larval survival was not affected at concentrations up to and including 1.6 mg/L; EC_{10} values were >1.6 mg/L (measured). It should be noted that consistent malformations of the caudal fin were observed in the highest test concentration of 1.6 mg/L (measured), and thus the NOEC was considered to be 0.61 mg/L (measured) based on malformation effects. EC_{10} values for growth reduction based on weight and length were 0.80 and 0.79 mg/L (measured), respectively. The NOEC for growth reduction based on weight and length was 0.61 mg/L (measured).

Since Alkylampho(mono)acetates C8-C18 exhibited higher toxicity than the other alkylamphoacetate analogues in all aquatic toxicity tests, it is considered both reasonable and scientifically justified to use these results as worst-case read across values to cover the chronic fish toxicity endpoint/information requirements for Amphoacetates C12-C14 and Amphoacetates C12.

Aquatic toxicity: microorganisms

With Amphoacetates C12 (monoacetate form) an activated sludge respiration inhibition study (OECD 209) has been performed. A NOEC of 560 mg/L was determined and this value is used as read across to the other C12-C14 and C8-C18 Alkylamphoacetate analogues.

Stability & biotic degradation

Amphoacetates C8-C18 and Amphoacetates C12 were tested for biodegradation (OECD 301 (mono- and diacetate form), OECD 302 (monoacetate form) and OECD 311 (mono- and diacetate form) for Amphoacetates C8-C18) (OECD 301 for Amphoacetates C12) and were found to be readily biodegradable. As Amphoacetates C12-C14 also contains mainly C12 and C14 mono- and diacetate constituents, similar to the tested substances, Amphoacetates C12-C14 are also considered to be ready biodegradable.

No hydrolysis data are available for any of the alkylamphoacetates. In accordance with column 2 of REACH Annex VIII, Hydrolysis as a function of pH does not have to be addressed in case the substances are readily biodegradable (study scientifically not necessary).

Bioaccumulation

As the analogues of the alkylamphoacetate consortium do not have a log Kow ≥4, the substances are considered to have a low potential for bioaccumulation. In accordance with column 2 of REACH Annex IX, the study bioaccumulation in aquatic species does not need to be conducted for any of the analogues.

Transport and distribution

As the screening study for adsorption and desorption behavior (OECD 121) is technically not feasible (due to the surface active nature of the alkylamphoacetates) and the adsorption/desorption using batch-equilibrium method (OECD 106) study has not been performed (due to the UVCB nature of alkylamphoacetates), the alternative option is to calculate the organic carbon-normalized sorption coefficient for soil and sediment (Koc) using an *in silico* (QSAR) approach. As the surfactant part of the analogues is present as sodium carboxylates at environmental pH, the substance is expected to partition predominantly in the aquatic compartment and to minimally adhere to organic matter. For the environmental CSA, the Koc of the substances has been calculated based on log Kow. The Koc of the substance has been calculated with EUSES version 2.1, based on log Kow, by using the in EUSES default QSAR for the chemical class non-hydrophobics:

$Koc = (10.47 \times Kow^{0.52})/1000$ (with Koc in m^3/kg)

For Amphoacetates C8, the Koc is thus 236.4 L/kg (based on a Kow of 401.2). The calculated Koc value for the other analogues is 3.16 L/kg (based on a Kow of 0.1).

Adsorption is thus considered to be negligible for the analogues of the alkylamphoacetate consortium. Modelling the distribution is not possible for this specific UVCB substance, but due to its extreme high water solubility, low vapour pressure and low LogKow, it can be concluded that the substance will predominantly distribute to the freshwater compartment. Distribution to other environmental compartments is considered to be negligible.

5.3. Toxicological properties

With regards to mammalian toxicological endpoints, the hypothesis/assumption that the biological effect properties of the Alkylamphoacetate analogues are similar has been verified (

Table 6 and Appendix V).

Acute tox (oral, dermal and/or inhalation)

For all alkylamphoacetate analogues the acute oral toxicity was tested in accordance with OECD test guideline 401 for the mono- and diacetate, and the LD50 was found to be at least 5000 mg/kg bw. For the monoacetate of the C8-C18 alkylamphoacetate also an LD50 >5000 mg/kg bw was determined. Based on the data for the mono- and diacetate of C12-C14 alkylamphoacetate and the monoacetate of C8-C18 alkylamphoacetate the LD50 of the diacetate of C8-C18 is also expected to be >5000 mg/kg bw. For the monoacetate of C12 alkylamphoacetate an LD50 of 3422 mg/kg bw was found.

For the analogue alkylampho(mono)acetates C8-C18, the acute dermal toxicity (OECD 402) was determined to be above 2612 mg/kg in a limit test. As alkylamphoacetates C12-C14 and alkylamphoacetates C12 have also mainly C12 and C14 mono- and diacetates similar to the tested substance, it is reasonable and scientifically justified to read-across the data to these substances, resulting in an acute dermal LD50 of >2612 mg/kg for all members of the alkylamphoacetates category.

No acute inhalation toxicity studies are available for any of the analogues. Testing for acute inhalation toxicity is not considered necessary as the exposure of humans via inhalation is not likely due to low vapour pressure of the alkylamphoacetate substances.

Corrosion/irritation (skin, eye)

Alkylamphoacetates C8-C18 (monoacetate and surrogate with unknown mono- to diacetate ratio)and alkylampho(mono)acetates C12 were tested for skin irritation/corrosion (OECD 404). Based on the results of the available studies, these 2 substances do not need to be classified as irritating to skin. As alkylamphoacetates C12-C14 has also mainly C12 and C14 mono- and diacetates similar to the tested substances, it is considered that alkylamphoacetates C12-C14 does not need to be classified as irritating to skin either.

With regard to eye irritation, all substances have been tested (according to or equivalent to OECD 405), in various concentrations in water. Aqueous solutions of the substance alkylamphoacetates C8-C18 (mono- and diacetate and surrogate with unknown ratio) were shown to be irritating to eyes (reversible effects in 3 studies; at a concentration of ≥ 38.9%), or corrosive to eyes (in 2 studies, irreversible similar grading of effects in 1 animal out of 3 in one study conducted with a diacetate form, or 1 animal out of 4 tested in a study conducted with a monoacetate form; at a concentration of ≥ 31%). Alkylampho(mono)acetates C8-C18 has also been studied as a 50% aqueous solution and in this study the observed effects do not warrant classification. Based on a worst-case approach, the substance Amphoacetates C8-18 is classified as causing irreversible effects on the eye (Category 1; H318).

In one study (OECD 405), a solution of 50% alkylampho(mono)acetates C12 showed slight irritation to the eyes, below classification criteria. In another study (OECD 405), an aqueous solution of 50% alkylampho(mono)acetates C12 was shown to be irritating to eyes. Based on a worst-case approach, the substance Alkylamphoacetates C12 is classified as irritating to eyes (Category 2; H319). In two studies at concentrations of ≤15% alkylampho(mono)acetates C12, ocular effects were below threshold criteria to warrant classification in accordance with EU CLP.

Alkylampho(mono)acetates C12-C14 has been tested for eye irritation at a concentration of approximately 16% in water, in this study observed effects do not warrant classification. The composition of this substance is more closely related to the composition of the substance alkylampho(mono)acetates C12; as it contains 69-78% of the C12 alkyl derivatives and lacks the shorter and longer alkyl chain derivatives and unsaturated C18 alkyl chain derivatives. For these reasons, it is therefore considered scientifically justified and appropriate that the classification of irritating to eyes (Category 2; H319) is read across to alkylampho(mono)acetates C12-C14.

Based on the data, >16% might be considered as a specific concentration limit for EU CLP classification of alkylamphoacetates C12 and alkylamphoacetates C12-C14 as irritating to eyes. When the eye irritation studies in rabbits in which the substances are tested at similar concentration (~50%) are compared, the average intensity of the ocular lesions are considered relatively similar. Only in 2 of the studies performed with alkylamphoacetates C8-C18 (mono- and diacetate form), the effects were irreversible which cannot be explained by differences in pH of the used solutions and also not by differences in surfactant, impurity and/or sodium chloride contents.

Skin sensitization

All analogues are surfactants, therefore results of a local lymph node assay performed with an analogue should be considered with care as it can be expected to result in a false positive outcome (OECD 336, Annex VI, Roberts et al., 2016, Ball et al., 2011). Indeed, in a LLNA performed with alkylampho(mono)acetates C12 and according to OECD guideline 429, the test item was found to induce proliferation of lymph node lymphocytes at the concentration of 50% (v/v) with an SI of >3. However, since the substance is a surfactant and showed clear irritating effects (described in scientific literature as confounding factors for false positives (OECD 336, Annex VI, Roberts et al., 2016, Ball et al., 2011), the result is considered inconclusive.

A guinea pig maximization test (GPMT), which is the preferred test for surfactants, was performed according to OECD guideline 406 with an alkylampho(mono)acetate C8-C18 (100% mono amphoacetate form). The lowest irritating concentration was chosen at the induction phase and the maximal non-irritating concentration was used at challenge. After epidermal induction performed on test day 8, slight to well-defined erythematous reactions were observed in all test animals treated with the test article at 75 % in bi-distilled water. After challenge, none of the animals of the test group were observed with positive skin reactions after treatment with the maximum non-irritant concentration of the test article of 1 % in water. Based on these results, alkylampho(mono)acetates C8-C18 was considered to be not sensitising.

Alkylampho(mono)acetate C12 (≥ 95% monoacetate form), was also tested in a GPMT in accordance with a test method equivalent to the OECD 406 guideline. Since 5 of the 20 animals (25%) were observed to have a positive response during challenge, the substance is not classified as a skin sensitiser in accordance with the CLP Regulation. It is of note that the choice for the intradermal induction exposure was based on the minimal irritating exposure in the range finding test, while it should have been the highest exposure to cause mild-to-moderate skin irritation. Therefore, it cannot be excluded that the outcome may be an underestimation of the actual skin sensitisation potential of the substance.

In order to substantiate the findings of the GPMT with alkylampho(mono)acetate C12, *in silico* (QSAR) predictions on the skin sensitizing potential for 4 representative C12-alkyl derivatives (mono-amphoacetate 1 and 2, and di-amphoacetate 1 and 2) and 3 potential minor constituents

(by-products) present in alkylamphoacetate C12 were performed with the DEREK NEXUS program (v 5.0.2). DEREK NEXUS is a knowledge-based system that contains more than 80 alerts specific to skin sensitization and it is recommended for KE 1 predictions in OECD TG 497. The rules are based on the presence of specific sub-structures, or chemical classes related to potential mechanisms for skin sensitization. Eight representative mono- and diacetate structures with the outer end alkyl chains, i.e. 4 representative C8-alkyl derivatives and 4 representative C18-alkyl derivatives, were investigated with DEREK NEXUS (see Appendix III: Chemical structures evaluated by DEREK NEXUS (version 5.0.2) for structures investigated). For all of these structures DEREK NEXUS did not find any sub-structures in its database that triggered an alert for sensitisation potential.

In the conclusions of a safety assessment of Cocoamphoacetates published in the Journal of the American College of Toxicology in 1990), cocoampho(mono)acetate and cocoamphodiacetate were reported to be, at concentrations of 10% and 5% respectively, neither a skin irritant nor skin sensitizer based on a human repeated insult patch test (HRIPT) with 141 subjects. Although these results were published in a peer-reviewed journal, which can be regarded to be scientific expert judgement, the data are not found reliable due to the fact that no information was given on the exact study outline, identity and purity of the test substance and that no results were included in the report. Cocoamphoacetates were later re-assessed by the cosmetic ingredient review (CIR) expert panel in in 2005/2006. The Panel reviewed newly available studies and confirmed the safety of Cocoamphoacetate and Cocoamphodiacetate at concentrations as high as 18 and 12% respectively.

In order to conclude on the skin sensitising potential of the Alkylamphoacetates, the following aspects are considered to be crucial:

- For two analogues (alkylampho(mono)acetates C8-18 and alkylampho(mono)acetates C12), it has been shown that a GPMT study results in a negative outcome.
- DEREK NEXUS did not find any substructures in its database that triggered an alert for skin sensitisation potential for the chemical structures present in the amphoacetates, indicating both mono- and diacetates do not have functional groups in them that are known to induce skin sensitization.
- In spite of wide spread use of the alkylamphoacetates, no reports on cases of skin sensitization in the public domain or in company-owned data can be found.
- All major constituents of alkylamphoacetates C8-C18, alkylamphoacetates C12-14 and alkylamphoacetates C12 are ionized at all realistic pH levels; based on this dermal absorption is expected to be negligible or very limited (WHO, 2006) and thus sensitization events are unlikely to occur (Basketter, 2008).

Based on this information, it is considered scientifically valid to read across the data on skin sensitization potential to all other alkylamphoacetate analogues within the category.

Genotoxicity

The substances alkylampho(di)acetates C8-C18, alkylampho(di)acetates C12-C14 and alkylampho(mono)acetates C12 tested negative (with and without metabolic activation) in the *Salmonella typhimurium* reverse mutation assay conducted in accordance with OECD 471. Alkylampho(di)acetates C12-14 and alkylampho(mono)acetates C12 tested also negative (with and without metabolic activation) in the *Escherichia coli* reverse mutation assay (OECD 471).

Only alkylamphoacetates C8-C18 (monoacetate) was tested in an *in vitro* mouse lymphoma assay (OECD 476) and was shown to be negative (with and without metabolic activation). Alkylamphoacetates C12-C14 and alkylamphoacetates C12 are mainly comprised of C12 and C14 mono- and diacetates similar to the alkylamphoacetate C8-C18 tested substance. However, amphoacetates C8-C18 cover a wider distribution of chain lengths than C12-C14 or C12. Fatty alkyl chains are not electrophilic functional groups and do not exert any potential for DNA- or protein-binding; but their length may impact molecular weight and log Kow, which in turn may affect passive permeation through lipid bilayers and into mammalian cell nuclei. While cell membrane permeability is a direct function of log Kow and an indirect function of molecular size/weight, a far higher dependence on the octanol:water partition coefficient than on the latter is usually observed (Rowland, 2011). All analogues contain C12 and/or C14 alkyl chains. As C8-18 has C8 and C10 as only minor constituents, while C16 and C18 are major constituents next to C12 and C14, C8-18 can be considered a worst case for access to a cell. Conversely, diacetate forms are both bigger and more hydrophilic molecules than their monoacetate counterparts, thus they can be assumed to have lower capacity to reach the nuclei of mammalian cells than smaller and more lipophilic monoacetates. The source substance contains mainly monoacetate structures and in similar or higher ratios than the target substances. It is thus concluded that the data on substances with longer alkyl chains and a higher or similar monoacetate to diacetate ratio [i.e. alkylampho(mono)acetates C8-C18] can be used to predict the effects of alkylamphoacetates with shorter chain lengths and lower or similar monoacetate to diacetate ratios [i.e. alkylampho(di)acetates C8-C18, alkylamphoacetates C12-C14 (mono- and diacetate forms) and alkylamphoacetates C12 (mono- and diacetate forms)]. In addition, for each of the four possible C12 alkyl amphoacetate constituents (mono- or diacetate forms 1 and 2), OECD QSAR Toolbox v4.3 predicts a negative outcome of the *in vitro* mutation study in mammalian cells (Kavanagh, 2021; www.oecd.org/ chemicalsafety/oecd-qsar-toolbox.htm). I*n silico* predictions using DEREK Nexus version 5.0.2 and VEGA QSAR have also been carried out. A DEREK report (Barentsen, 2017a, Barentsen, 2017b) concluded that the representative constituents, mono- and diacetate structures with the outer end alkyl chains, i.e. C8 and C18 and the minor constituent of C8-18 amphoacetate with oleic acid the monoacetate (see Appendix II for structures investigated) , do not show a potential for either mutagenicity or carcinogenicity. The VEGA QSAR software was run with two representatives of C12 Amphoacetates (C12 Alkyl amphoacetate Form 1 Monoacetate and C12 Alkyl amphoacetate Form 2 Diacetate), the VEGA models confirm absence of genotoxic activity for all tested constituents (Kavanagh, 2021; vegahub.eu; Benfenati, 2013).

Moreover, retrospective comparisons have shown a comparable (Kirkland, 2005; Matthews, 2006) if not better (Zeiger, 1998) performance of the Ames test in comparison to *in vitro* mutagenicity assays in mammalian cells for the prediction of rodent carcinogens. The added value of the *in vitro* mammalian gene mutation assay in the absence of positive findings with an Ames test and an *in vitro* assay in mammalian cells to identify numerical and/or structural chromosomal aberrations has been questioned "*because the bacterial gene mutation test detects all relevant modes of action specifically leading to gene mutations. Moreover, most of the substances positive in mammalian gene mutation tests also induce clastogenic effects*" (Pfuhler, 2005). The combination of the Ames test with an *in vitro* chromosomal aberration assay or the *in vitro* micronucleus test are sufficiently sensitive to predict *in vivo* genotoxins, with the *in vitro* mammalian gene mutation assay only increasing the sensitivity of the test battery from 78 % to 79 % (Kirkland, 2011). In Chapter R7.a (Endpoint-specific guidance) (ECHA, 2017b), it is acknowledged that other regulatory frameworks do not require *in vitro* mammalian gene mutation assays to confirm the absence of mutagenic potential. A reason why this approach has not been adopted for EU REACH is not provided in this guidance document.

The applicant acknowledges the information requirement for *in vitro* mammalian gene mutation for regulatory purposes, but believes that the negative outcomes in the two *in vitro* genotoxicity assays confirm that read-across from the source substance Amphoacetate C8-C18 to the target substances Amphoacetate C12 and C12-C14, together with the supporting information from several QSARs on genotoxic potential, fulfils the information requirements for *in vitro* mammalian gene mutation is sufficiently justified.

The substances alkylamphoacetates C8-C18 (monoacetate form), alkylamphoacetates C12-C14 (diacetate form) and alkylamphoacetates C12 (monoacetate form) exhibited no clastogenic effects (with and without metabolic activation) when tested in the *in vitro* chromosome aberration assay (OECD 473). In the studies with alkylamphoacetates C8-C18 and C12-C14, a dose-dependent increase in the number of polyploid cells was noted with and without the use of a metabolic activation system.

In order to determine if the positive responses seen *in vitro* for Amphoacetates C8-C18 and C12-14 were indicative for *in vivo* genotoxicity, a mouse bone marrow cytogenetic assay (OECD 475) was performed with C8-18 alkylampho(mono)acetates. Male mice (5/group) were exposed orally (gavage) to 500, 1000 or 2000 mg/kg bw/day and bone marrow was sampled 12-18 (all doses, vehicle control group and positive control group (treated with cyclophosphamide) or 36-44 (highest dose only) hours after dosing. No mortality occurred, no clinical signs were noted in any of the mice. The number of cells with chromosome aberrations found in the vehicle control animals was within the laboratory historical control data range. The positive control animals treated with cyclophosphamide induced a statistically significant increase in the number of cells with chromosome aberrations, indicating that the test conditions were adequate. C8-18 Alkylampho(mono)acetates did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations, at both sampling times. Based on these results it is concluded that C8-18 alkylampho(mono)acetates does not disturb mitotic processes and cell cycle progression and does not induce numerical chromosome aberrations *in vivo*.

Since C8-C18 contains longer alkyl chains and a higher monoacetate to diacetate ratio than ampho(di)acetates C12-C14 (this argument was explained more profoundly on the previous page for gene mutation test in mammalian cells), it is considered appropriate to read-across the available genotoxicity data to cover this substance and conclude that Ampho(di)acetates C12-C14 is negative for disturbing mitotic processes and cell cycle progression and inducing numerical chromosome aberrations in vivo.

Toxicokinetics

An assessment of the toxicokinetic behaviour of alkylamphoacetates C8-C18, alkylamphoacetates C12-14 and alkylamphoacetates C12 to the extent that can be derived from the relevant available information has been performed in accordance with ECHA Guidance on information requirements and chemical safety assessment, Chapter R.7c (May 2008). Oral, dermal and inhalation absorption rates of 100%, 10% and 100% were estimated for each of the routes, respectively. Slight variations observed in the liver weights and/or clinical chemistry in the 28-day repeated dose toxicity study (OECD 407) with alkylampho(mono)acetates C8-C18 and the 90-day repeated dose (OECD 408) studies with alkylampho(di)acetates C12-C14 and alkylampho(di)acetates C8-C18, provided evidence of absorption by the oral route. The dermal absorption rate of 10% is supported by experimental data on a structurally related amphoteric surfactant, dodecylamidopropylbetaine (CAS# 4292-10-8) showing a dermal absorption of less than 3.5% in Wistar rats (HERA 2005). All major constituents of alkylamphoacetates C8-C18, alkylamphoacetates C12-14 and alkylamphoacetates C12 are ionized at all physiological pH levels due to their amphoteric nature, which influences the ability to cross hydrophobic

membrane barriers such as skin (WHO, 2006); based on this, 10 % dermal absorption can be considered a highly conservative assumption.

Alkyl amphoacetates consist of hydrophilic constituents, with predicted Log Kow ranges of -3.58 to +1.33 (monoacetates), and -6.15 to -0.75 (diacetates), respectively (EPIsuite Kowwin v1.67). The molecular weight range of monoacetates is 310-394 g/mol, while for diacetates it is 390-474 g/mol. As discussed in the genotoxicity section, the log Kow increases with the molecular weight. It can be safely assumed that amphoacetates with a higher alkyl chain length have a similar or higher bioavailability than that of the shorter alkyl chain lengths, thus alkyl amphoacetates C8-C18 can be considered a worst-case. The values also indicate that diacetates, which are both bigger and more hydrophilic molecules, can be assumed to have lower bioavailability compared to the smaller and more lipophilic monoacetates.

The alkylamphoacetates may be distributed throughout the body based on the relatively low molecular weight. It may be expected that the amphoacetates undergo metabolic transformation and/or conjugation prior to elimination, although no empirical data is available to substantiate this.

Repeated dose toxicity

Two sub-acute repeated dose toxicity studies combined with screening for reproduction and developmental effects were performed according to OECD 422 with two representative alkylamphoacetates C8-C18. One study was conducted with C8-C18 alkylampho(mono)acetates whilst the second study was conducted with C8-18 alkylampho(di)acetates. The rationale to perform the test with both forms was to investigate whether the structural and compositional difference in chemistry exhibited an impact on systemic and reproductive/developmental effects. Treatment (oral, gavage) of test animals with C8-18 alkylampho(mono)acetates was associated with a few minor non-adverse changes at the highest dose group i.e. slight salivation in both sexes, lower food consumption in females in the last week of gestation and during lactation, and lower activated partial thromboplastin time in males. Serum levels of T4 in males were not affected by treatment (not measured in females or pups), and no changes in thyroid weight or histopathology were observed. No treatment-related or toxicologically relevant changes were noted in the other parameters investigated in this study. Based on the absence of adverse effects up to 1000 mg/kg bw/day, a parental No Observed Adverse Effect Level (NOAEL) C8-18 alkylampho(mono)acetates of 1000 mg/kg bw/day was established. For C8-18 alkylampho(di)acetates, there was a high mortality in the females (4/10) and one premature death in the males at 1000 mg/kg bw/day. These deaths were concluded to be related to gavage errors (test item administration-related regurgitation) and thus secondary to the test item (possibly triggered by physical/chemical properties of the test-item solution in combination with the route of administration). Serum levels of T4 in males were not affected by treatment (not measured in females or pups). No changes in thyroid weight were observed, follicular cell hypertrophy of the thyroid gland was found in males at the 1000 mg/kg bw/day dose group. Similar findings were observed at the 300 mg/kg bw/day dose group but at a slightly lower severity. These findings were considered to be non-adverse based on its low severity (up to mild) and absence of any additional degenerative, inflammatory or proliferative findings and changes in T4 hormone levels. Due to the high mortality caused by the gavage-related incidents secondary to test item administration, the high dose group (1000

mg/kg bw/day) could not be assessed and the parental No Observed Adverse Effect Level (NOAEL) of 300 mg/kg bw/day was established for C8-18 alkylampho(di)acetates[12].

To follow-up the results seen with C8-18 alkylampho(di)acetates, a sub-chronic 90-day toxicity study (oral, gavage) was performed according to OECD guideline 408. In the sub-chronic study, the dosing volume (1.895 ml/kg bw/day) was decreased compared to the 28-day sub-acute study (5 ml/kg bw/day) in order to minimize risk of regurgitation. In this study no mortality was seen at all doses (100, 300 and 1000 mg/kg bw/d), which suggests the mortalities which occurred in the high dose group in the 28-day sub-acute study were indeed likely secondary to the test item. Lower TSH values in all test item-treated male groups and lower T4 values in high-dose males were observed, achieving a level of statistical significance when compared to controls. The values remained however within the Historical Control Data Range. In the absence of a dose response relationship, effects on thyroid weight or macroscopic/microscopic correlates; these values were considered to be of no toxicological significance. No toxicologically significant changes were noted in any of the parameters investigated in this study. No adverse effects were seen on reproduction parameters (estrous cycle length, spermatogenesis, weight, appearance and histopathology of reproduction organs). Based on these results, the no observed adverse effect level (NOAEL) for sub-chronic exposure was found to be 1000 mg/kg bw/day.

Furthermore, a historical 28-day study is available for alkylampho(mono)acetates C8-C18. Following repeated oral (gavage) administration to rats for 28 days, the NOAEL was found to be 92.5 mg/kg bw/day (active substance basis equivalent to 250 mg/kg bw/d test item), based on a dose-dependent effect on liver weight (without histopathological changes). At 92.5, 185 and 370 mg/kg bw/day, increase in absolute and relative liver weights was noted (+10, +21 and +21% (absolute), +4, +9 and +12% (relative to body weight) compared to controls, respectively). These effects were not seen in males and there were no other toxicologically relevant findings in both sexes up to and including 370 mg/kg bw/day (active substance basis equivalent to 1000 mg/kg bw/d active substance basis). As the parameters included in this study are limited and doses were not analytically confirmed, this study is used as supporting evidence only.

To get a better understanding of the effect of alkyl chain length (distribution) on toxicity, a sub-chronic 90-day toxicity study (oral, gavage) has been performed according to OECD guideline 408 with C12-C14 alkylampho(di)acetates. The clinical signs seen were non-adverse and comparable with those found in the 90-day study on amphoacetates C8-18 (diacetate form). Non-adverse squamous cell hyperplasia in the stomach (with hyperkeratosis) was observed in females at 300 and 1000 mg/kg bw/day, while non-adverse goblet cell hyperplasia (without cellular atypia , inflammatory or degenerative changes) of the rectum was observed in a few high-dose males, these tissues fully recovered and were consistent with a local reaction to irritation (presumptively by the test material), these effects were not seen in the 90-day study on ampho(di)acetates C8-18 (although in this study histopathological examination of stomach and rectum was only conducted in the control an high dose groups) but similar effects were seen in dams in a OECD 414 study with alkylampho(mono)acetates C12. Haematological findings comprised of decreased red blood cell count and red blood cell distribution width and increased mean corpuscular volume and mean corpuscular haemoglobin in males at 1000

[12] Please note that there is a discrepancy in the derivation of this systemic NOAEL between the study report and the Expert DART Review (DeSesso & Williams 2023). While the Expert DART Review considers that deaths at 1000 mg/kg bw/day were likely result of the gavage procedure, that they should not be considered as the basis for maternal/systemic and that the NOAEL should thus be set at the highest dose; the conclusions of the study report (i.e. considering these deaths as secondary to the test item and thus test item-related), are preferred for this study as the represent a more conservative approach.

mg/kg bw/day, based on the absence of a histopathological correlation and/or full recovery, these changes were considered to be non-adverse; the 90-day study on amphoacetates C8-18 (diacetate form) also reports haematological changes in males (increases in platelet numbers and prothrombin time) however in this study they were considered non-test item related. Non-adverse (reversible) clinical chemistry findings (increase in triglyceride concentration) were observed in males treated with 1000 mg/kg bw/day of C12-C14 alkylampho(di)acetates. Conversely in the 90-day study on amphoacetates C8-18 (diacetate form), and non-adverse increases were observed in alkaline phosphatase cholesterol (HDL and LDL). Hormone analysis showed no effect of C12-C14 alkylampho(di)acetates on T3 and TSH levels in males and females, and no effect on T4 levels in males. Increased T4 levels were seen in females at 100 and 1000 mg/kg bw/day, but these changes remained within historical control range, were reversible and not accompanied by changes in thyroid weight or histopathology; changes in T4 levels were thus considered non-adverse. Finally higher kidney and liver weight was noted in females treated with 1000 mg/kg bw/day of C12-C14 alkylampho(di)acetate (increases in liver weight were also observed in the 28-day study that is available for alkylampho(mono)acetates C8-C18). No test material-related changes were noted in any of the remaining parameters investigated in this study (i.e., mortality, body weight, food consumption, ophthalmoscopy, coagulation and macroscopic pathology). Based on these results, the no observed adverse effect level (NOAEL) for sub-chronic exposure was found to be 1000 mg/kg bw/day[13].

Additionally, in the OECD 443 study conducted with alkylampho(di)acetates the dose levels were 0, 100, 300, and 1000 mg/kg bw/day. No treatment-related adverse effects on any of the parameters related to systemic toxicity studied (i.e mortality/moribundity, clinical signs, body weight, food consumption, clinical pathology including measurement of thyroid hormones and urinalysis, sperm analysis, estrous cycle, macroscopic findings, organ weights and microscopic examinations in F0 and F1 and splenic lymphocyte subpopulation analysis in F1) were seen up to (and including) the highest dose tested of 1000 mg/kg bw/day. In F0 animals at 1000 mg/kg bw/day, salivation, higher kidney and liver weights (in males and females), and lower urea concentrations (in males) were observed. Salivation was considered to be a physiological response rather than a sign of systemic toxicity, while higher kidney and liver weights and lower urea concentrations were considered non-adverse based on the effect size and the absence of a microscopic correlate. Additionally, T4 levels were increased in F0 females at 300 mg/kg bw/day (not statistically significant). As the mean value remained within historical control data range and there is not dose-response relationship, this was considered non-adverse. F0 TSH levels were unaffected. In F1 animals, non-adverse abnormal breathing sounds (in males and females) and higher T4 level (in females) were noted at 300 mg/kg bw/day; while salivation, abnormal breathing sounds, higher liver and kidney weights (in males and females) and lower body weight gain and body weights (in males) were noted at 1000 mg/kg bw/day. Salivation was again not considered a sign of systemic toxicity; abnormal breathing sounds were incidental and transient and thus considered not-adverse; lower body weight gain and body weight were also considered not-

[13]Please note that there is a discrepancy in the derivation of the systemic NOAEL between the study report and the Expert DART Review (DeSesso & Williams 2023). In the Expert DART Review, squamous cell hyperplasia in females and goblet cell hyperplasia in males were considered adverse effects, leading to the establishment of lower systemic NOAELs for males and females. However, in this case the conclusions of the study report (i.e. systemic NOAEL of 1000 mg/kg bw/day for males and females) are preferred and have been maintained, given that: the irritation effects in the non-glandular forestomach are not relevant for humans (due to their location), were reversible, and are due to irritating nature of the test material rather than systemic toxicity; and given that the goblet cell hyperplasia of the rectum in males was fully reversible by the end of the recovery period, was seen without cellular atypia, and thus considered adaptive rather than adverse.

adverse based on the effect size. Higher T4 levels in F1 animals were not statistically significantly different from controls, remained within historical control data range, did not follow a dose-response curve and thus were considered non-adverse. F1 TSH levels were unaffected. Higher kidney and liver weights were also considered non-adverse based on the effect size and the absence of a microscopic correlate. A NOAEL of 1000 mg/kg bw/day was established for systemic/general toxicity.

As amphoacetates C12 is mainly comprised of C12 mono- ,and the diacetate of the alkylamphoacetates C8-C18 was less or just as toxic as the monoacetate of the alkylamphoacetates C8-C18, the alkylamphomonoacetate C12 can be considered similar to the tested C12-C14 alkylampho(di)acetate substance, and it is considered appropriate and justified to read-across the 90-day oral NOAEL of 1000 mg/kg bw/day to this substance. In order to substantiate the read across hypothesis for the alkylamphoacetates, predictive toxicity assessments of C8-monoacetates and diacetates and of C18-monoacetates and diacetates were performed using the *in silico* model DEREK NEXUS. In this assessment version 5.0.2 of DEREK NEXUS was used. The exact chemical structures assessed are included in Appendix III: Chemical structures evaluated by DEREK NEXUS (version 5.0.2). DEREK analysis predicts no toxicity for any endpoint present in the DEREK database that is relevant for humans for "C8- monoacetate1", "C8-diacetate1" and "C8-diacetate2", or for "C18-monoacetate1", "C18-diacetate1" and "C18-diacetate2". Also, for the minor constituent of alkylamphoacetates C8-C18 with oleic acid, "C18unsat monoacetate2" no toxicity was predicted. Since the structures assessed have the same functional groups present in all analogues, it can be concluded that none of the analogues exhibits any structural alerts for adverse toxic effects. This *in silico* (QSAR) assessment is in line with the general outcome of repeated dose toxicity and reproductive/developmental screening on C8-C18 alkylamphoacetates and serves as important supporting information to justify the category and read-across approach employed.

Reproductive/developmental toxicity

As discussed above, two screening level studies for reproduction and developmental effects were performed in accordance with OECD 422 using two representative forms of alkylamphoacetates C8-C18 (high diacetate form and predominantly monoacetate form). In the study with alkylampho(mono)acetates C8-C18, no reproductive toxicity effects were observed up to the highest dose level tested (1000 mg/kg bw/day). No treatment-related changes were noted in the reproductive parameters examined (i.e. mating and fertility indices, precoital time, number of implantation sites, oestrous cycle, spermatogenic profiling, and histopathological examination of reproductive organs). However, in the study with alkylampho(di)acetates C8-C18, reproductive and developmental parameters could not be completely assessed for all groups [14], due to excessive mortalities in the dams caused by secondary effects via regurgitation.

In neither of the OECD 422 studies was any adverse developmental toxicity effect observed up to the highest dose level at which developmental parameters were assessed (1000 mg/kg bw/day or 300 mg/kg bw/day). No treatment-related changes were noted in the developmental parameters investigated in the studies (i.e. gestation, viability and lactation indices, duration of gestation, parturition, sex ratio, maternal care and early postnatal pup development consisting

[14] It must be noted that this is in disagreement with the study report conclusions that no reproduction toxicity was observed up to the highest dose level tested (1000 mg/kg). It is however in agreement with the more conservative interpretation of the data made in the Expert DART Review (DeSesso & Williams 2023), where it is considered that reproductive parameters could not be assessed at 1000 mg/kg bw/day due to absence of complete reproductive data from maternal animals that reached full term in that treatment group.

of mortality, clinical signs, body weight, anogenital distance (PND 1), areola/nipple retention (PND 13 males), and macroscopy).

Furthermore, no effects on body weight, macroscopy or histopathology were seen in the sub-chronic 90-day study on male and female reproductive organs performed with alkylampho(di)acetates C8-C18. Stage dependent qualitative evaluation of spermatogenesis in the testes was performed. The testes revealed normal progression of the spermatogenic cycle and the expected cell associations and proportions in the various stages of spermatogenesis were present.

Finally, in the OECD 443 study conducted with Amphoacetate C8-C18 (diacetate form), no treatment related effects were observed on any of the reproductive parameters studied (*i.e* mating, fertility and pregnancy indices, precoital interval, number of implantation sites, gestation index and length, parturition, maternal care) up to (and including) the highest dose tested of 1000 mg/kg bw/day. T4 levels were increased in F0 and F1 females at 300 mg/kg bw/day (not statistically significant), as the mean value remained within historical control data range and in absence of a clear dose-response relationship, this was considered non-adverse. Based on the absence of effects no extension of cohort 1B to include an F2-generation was considered necessary. A NOAEL of 1000 mg/kg bw/day was established for general, reproductive and developmental toxicity.

A summary of thyroid-related effects is given in Appendix IV. Given the fact that all mean hormone levels measured in exposed animals were within Historical Control Data Range (HCDR) and the large natural variability in thyroid hormone/TSH measurements (Li *et al.* 2019; Beekhuijzen *et al.* 2019), and effects were contradictory for T4 in different studies, these differences are likely caused by chance, rather than the result of a toxicological effect of the test item. Thyroid weight and histopathology should be evaluated in conjunction with changes in serum thyroid hormones (both T3 and T4) and TSH to allow correct interpretation of changes (Li *et al.* 2019), especially since the thyroid gland of rodents is much more sensitive than that of humans to loss of colloid and induction of hypertrophy and hyperplasia from a TSH increase. Following this argumentation, the fact that no effects on thyroid weight or histopathology in combination with an effect in hormone levels were observed in any of the OECD414 or OECD408 studies indicates that there were no adverse effects on the thyroid system.

Taken together, there are no indications that alkylamphoacetates C8-C18 have an adverse effect on reproduction.

Developmental toxicity was tested for all alkylamphoacetate analogues in accordance with OECD test guideline 414.

A prenatal developmental toxicity study was performed with alkylampho(di)acetate C8-C18 in the rat. No treatment-related mortality occurred during the study period and no test item-related changes were noted in clinical appearance, body weight, food consumption, macroscopic examination, thyroid hormone levels, thyroid weights, microscopic appearance of the thyroids, and fertility parameters (pregnancy rate, numbers of corpora lutea and implantation sites, and pre-implantation loss). Visceral examination of foetuses revealed severe cardiovascular malformations at 100, 300 and 1000 mg/kg bw/day, in 4 (4) foetuses (litters) in total. At 1000 mg/kg bw/day, one foetus had a right-sided aortic arch, ventricular septum defect and no eyes. At 300 mg/kg bw/day, one foetus had a ventricular septum defect, absence of the ductus arteriosus, situs inversus and abnormal lung lobation. At 100 mg/kg bw/day, two foetuses were viscerally malformed: one foetus had abnormal lung lobation and transposition of the great vessels, and the other foetus presented itself with situs inversus, abnormal lung lobation, interrupted aortic arch (between right subclavian and right carotid), retro-oesophageal ductus

arteriosus and a ventricular septum defect. These cardiovascular malformations were considered to be unrelated to treatment with the test item, as described in more detail at the end of this section. Mean litter incidences of a 7th cervical ossification site were 1.5%, 5.2%, 4.6% and 11.3% per litter in the control, 100, 300 and 1000 mg/kg bw/day groups, respectively. The increase at the high dose was not statistically significant but was outside the historical control data range. Therefore, a test item-related effect cannot be excluded for this type of skeletal variation. Slightly lower serum levels of thyroid stimulating hormone (TSH) were noted at 300 and 1000 mg/kg bw/day, however without reaching statistical significance and within the historical control range. This slight difference in TSH levels was therefore regarded to be unrelated to the test-item. Serum levels of total T3 and T4 were regarded to be unaffected by treatment up to 1000 mg/kg bw/day. Additionally, no test item related effects on thyroid weight or histopathology were noted. No test item-related changes were noted in litter size, post-implantation loss, sex ratio, foetal body weights, foetal ano-genital distance, external and skeletal malformations and variations. In conclusion, the maternal and developmental No Observed Adverse Effect Levels (NOAELs) for alkylampho(di)acetates C8-C18 were determined to be at least 1000 mg/kg bw/day[15].

To better understand the effect of alkyl chain length (distribution) and mono- and diacetate forms on prenatal developmental toxicity in the rat, additional studies according to OECD TG 414 and in accordance with GLP were also conducted with alkyl ampho(di)acetate C12-14 and with alkyl ampho(mono)acetate C12.

In the first study, time-mated female Wistar Han rats were administered alkylampho(di)acetate C12-14 (100, 300, 1000 mg/kg bw/day). Test item related clinical signs (abnormal breathing sounds) were recorded in all treatment groups but were considered non-adverse. T4 and TSH serum levels were considered to be unaffected by treatment with the test material, decreased serum levels of T3 were noted at 1000 mg/kg bw/day, but remained within the historical control data range, additionally no test item related changes in thyroid weight or pathology were noted. Cardiovascular malformations were observed in 2 foetuses from two different dams at 100 mg/kg (1 fetus with interrupted aortic arch, ventricular septal defect and absent tracheal cartilage rings (next to omphalocele); 1 fetus with transposition of the great vessels and a ventricular septal defect), due to the exclusive occurrence of these heart malformations in the low-dose group, they were considered not to be related to treatment with the test material. These heart defects resemble those found in the OECD 414 study with alkylampho(di)acetates C8-C18 and will be discussed together further below. A fetus from the high-dose group (1000 mg/kg bw/day) had sternoschisis and a control fetus missed half of a lumbar centrum. The singular occurrence of these malformations and/or occurrence in a control fetus did not indicate a relationship with the test material and as such they were ruled as chance findings. No test item-related changes were observed in maternal parameters: mortality, body weight, food consumption, gross pathology or histopathology. No test item-related changes were noted in litter size, post-implantation loss, sex ratio, foetal body weights, foetal ano-genital distance, or in external and skeletal malformations and variations. In conclusion, maternal and developmental toxicity were not observed up to the highest dose level tested. The maternal and developmental No Observed Adverse Effect Levels (NOAELs) were all established as being at least 1000 mg/kg bw/day.

[15] Please note that in the study report no developmental NOAEL for alkylampho(di)acetates C8-C18 was determined, as the cardiovascular malformations observed at all dose levels were considered as the basis for LOAEL setting. However, based on DeSesso & Williams 2023, these malformations have been considered unrelated to treatment administration.

In a Prenatal Developmental Toxicity Study according to OECD TG 414, time-mated female Wistar Han rats were administered alkylampho(mono)acetates C12 (100, 300, 1000 mg/kg bw/day). Test item related clinical signs (abnormal breathing sounds) were recorded at 100 and 1000 mg/kg bw/day but were considered non-adverse. Slight weight loss and lower food consumption were recorded at 300 and 1000 mg/kg bw/day for some animals over the first three days of the administration period which recovered as treatment proceeded, so it was considered non-adverse. Most females at 1000 mg/kg bw/day showed an irregular surface of the non-glandular stomach, on one occasion accompanied by dark red foci on the glandular stomach, these findings were considered to represent irritating properties of the test material following gavage administration and were considered non-adverse. Similar effects were seen in the OECD 408 study with C12-C14 alkylampho(di)acetates. At 1000 mg/kg bw/day, a lower mean Total T3 concentration was recorded, mean T3 levels remained however within the historical control data range and were considered non adverse. Serum levels of T4 and TSH were considered not to be affected by treatment with the test material up to the highest dose tested. There were no test material-related alterations in thyroid gland weights, macroscopic thyroid gland lesions (including the small size of the left thyroid gland for two females at 1000 mg/kg bw/day) occurred without microscopic correlates, and all macroscopic findings remained within the range of background gross observations encountered in rats of this age and strain. No test item-related changes were observed in maternal mortality or histopathology. No test item-related changes were noted in litter size, post-implantation loss, sex ratio, foetal body weights, foetal ano-genital distance, or in external, skeletal and visceral malformations and variations. In conclusion, maternal and developmental toxicity were not observed up to and including the highest dose level tested. The maternal and developmental No Observed Adverse Effect Levels (NOAELs) were all established as being at least 1000 mg/kg bw/day.

Lastly, a prenatal developmental toxicity study according to OECD 414 and GLP principles was conducted in New Zealand White rabbits. In this study animals were treated with alkylampho(di)acetate C8-C18 (the dose levels were selected to be 0, 75, 175, 350 mg/kg bw/day). One female at 175 mg/kg bw/day and ten females at 350 mg/kg bw/day were prematurely euthanized based on clinical signs, body weight loss and/or prolonged (near) absent food consumption. Two females were euthanized at 75 mg/kg bw/day although these deaths were unrelated to treatment with the test material or related to the gavage procedure itself and were considered toxicologically irrelevant. Due to the high number of deaths at 350 mg/kg bw/day, data from this group was not taken into account when deriving conclusions on developmental toxicity. At 175 mg/kg bw/day, an adverse higher post-implantation loss, caused by higher percentages of early and late resorptions, was noted. No other test material-related changes were noted in any of the remaining maternal and developmental parameters investigated in this study (*i.e.* macroscopic evaluation, corpora lutea, uterine contents including implantation sites and pre-implantation loss, litter size, sex ratio, fetal body weights, external, visceral and skeletal malformations and developmental variations). Based on this a maternal NOAEL of 75 mg/kg bw/day (based on mortality observed at 175 and 350 mg/kg bw/day, and the effects noted on body weight and food consumption of females surviving until scheduled necropsy) and a developmental NOAEL of 75 mg/kg bw/day (based on the higher post-implantation loss noted at 175 mg/kg bw/day) were established.

Because a low incidence of cardiac / great vessel malformations was observed in two out of the four developmental toxicity studies conducted and an increased incidence of post-implantation loss was observed in the rabbit developmental study, DART Reviews by globally recognised DART experts (DeSesso & Williams 2023, 2024a and 2024b) were commissioned to specifically clarify the importance of these findings. The authors of these reviews concluded that:

- None of the cardiac / great vessel malformations was significantly increased and, within each study, the greatest number of malformations occurred in the low dose group. Increased maternal toxicity and/or resorptions/post-implantation loss did not occur at higher doses; thus, there is no evidence to support this being a low-dose effect. In order to discern if there might be a trend for production of cardiovascular malformations, the data for all three definitive prenatal developmental toxicity studies were combined. Whether the combined data were assessed based on the incidences of malformations, number of malformed fetuses, or underlying perturbed morphogenetic processes, there was neither statistical significance nor a dose responsive increase.
- In-depth analysis of the cardiac and great vessel systems of fetuses exposed to alkylampho(di)acetates C8-C18, alkylampho(di)acetate C12-14 and alkylampho(mono)acetates C12 at doses as high as the limit dose does not support that these substances cause malformations of the target area. This conclusion is also supported by the absence of any treatment-related cardiac abnormalities in both the FELS toxicity test of alkylampho(mono)acetates C8-C18 and the dose range-finding studies for alkylampho(di)acetate C12-14 and alkylampho(mono)acetates C12 (which included visceral examinations of fetal hearts).The increases in early resorptions and post-implantation loss seen in rabbits at 175 mg/kg bw/day were relatively minor compared to control values, not statistically significant, and within the laboratory's observed range of HCD based on reported minimum and maximum values. Obvious and substantial maternal toxicity was also present at the mid and high dose levels. While the available data are highly suggestive of a relationship between maternal systemic toxicity and increased post-implantation loss, these findings could not be clearly linked with maternal toxicity on an individual animal basis. However, no records for diet supplementation with hay and vegetables are available, which complicates making associations between developmental outcomes and maternal toxicity on an individual animal basis.
- The existing and extensive DART database for amphoacetates does not show other evidence of increased resorptions (early or late) or increased post-implantation loss as a result of exposure to Amphoacetates C8-C18 or other structurally related amphoacetates.
- Taken together, in-depth analyses of the available developmental and reproductive data for alkylamphoacetates C8-C18 (mono- and diacetate forms), alkylampho(di)acetate C12-14 and alkylampho(mono)acetates C12 do not support the classification of these substances for reproductive or developmental hazards,

Finally, in the OECD 443 study conducted with alkylampho(di)acetate C8-C18 (the dose levels were 0, 100, 300, and 1000 mg/kg bw/day), no treatment related effects on any of the developmental parameters studied (*i.e* post-implantation loss, litter size, sex ratio, live birth, viability and lactation indices, early postnatal pup development [mortality, clinical signs, body weights, anogenital distance, areola/nipple retention, macroscopic findings, measurement of thyroid hormones, and organ weights], vaginal patency, balanopreputial separation and day of first estrus) were seen up to (and including) the highest dose tested of 1000 mg/kg bw/day. Based on the absence of effects no extension of cohort 1B to include an F2-generation was considered necessary. A NOAEL of 1000 mg/kg bw/day was established for developmental and reproductive toxicity

In conclusion, no effects on reproduction were observed for any of the substances tested, there are also no indications that the registered substance has a direct adverse effect on development,

as any effects seen are either not treatment-related (*i.e.* cardiac malformations in the rat) or considered secondary to maternal toxicity (*i.e.* increased post-implantation loss in rabbits).

6. DATA MATRIX

The key data for the analogues are presented in Table 4-6. All available studies, both key and supporting, are included in the tables in Appendix III, IV and V. The use of read across to meet a particular endpoint requirement is indicated in the tables by 'RA'. QSAR calculated values for physico-chemical data are presented for some endpoints next to the measured values to indicate that the difference between measured and calculated is in the same range of the variation between substances.

If for a particular endpoint no reliable data were available, this is indicated by not determined "n.d." in the tables. If filling of an endpoint is not relevant for one of the analogues related to its tonnage band, this is indicated with not applicable ("N.A.").

Endpoints with a waiving statement not relevant for read-across, such as flash point, stability in organic solvents, dissociation constant and viscosity, have not been included.

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Table 4 Data Matrix, Physico-chemical Properties for the Alkylamphoacetates

REACH	Endpoint	Amphoacetates C8-C18	Amphoacetates C12-C14	Amphoacetates C12
7.1	State of the substance at ambient conditions	Pasty orange solid (containing approximately 6% residual water)	Light yellow crystals (containing approximately 2% residual water)	Light yellow powder with lumps (containing approximately 4% residual water)
7.2	Melting/freezing point [ºC]	The substance has no melting temperature, substance decomposes starting at 160°C	The substance has no melting temperature, substance decomposes starting at 150°C	40 (see comment in text)
7.3	Boiling point [ºC]	The substance has no boiling temperature, decomposition starts at 160 °C	The substance has no boiling temperature, decomposition starts at 150 °C	The substance has no boiling temperature, decomposition starts at 75 °C
7.4	Relative density at 20 ºC	**RA from C12-C14:** 1.33	1.33	**RA from C12-C14:** 1.33
7.5	Vapour pressure at 20 ºC [Pa]	$1.4*10^{-7}$ (monoacetate); $< 8.4*10^{-7}$ (diacetate)	$1.8*10^{-8}$ - $1.3*10^{-6}$ (monoacetate); $2.6*10^{-6}$ (diacetate)	$1.5*10^{-7}$
7.6	Surface tension [mN/m]	34 (concentration: 0.5 g/L) Surface active	35.4 (concentration: 1 g/L) Surface active	31.9 (concentration: 1 g/L) Surface active
7.7	Water solubility at 20 ºC	> 1000 g/L (bulk)	Between 206.4 g/L and 1032 g/L (bulk)	> 1000 g/L (bulk)
	Critical Micelle Concentration [mg solids/L]	160 (monoacetate); 158 (diacetate)	239 (monoacetate); 262 (di-acetate)	718
7.8	Partition coefficient n-octanol/water [log Pow]	-1	-1	-1
	n-octanol solubility (visually determined) [g/L]	< 0.082	< 1.1	< 1.08
7.9	Flash point [ºC]	Aqueous solutions have no flashpoint A 50% aqueous solution has a boiling temperature of approximately 105 ºC	Aqueous solutions have no flashpoint	Aqueous solutions have no flashpoint. A 39% aqueous solution has a boiling temperature of approximately 94 ºC

REACH	Endpoint	Amphoacetates C8-C18	Amphoacetates C12-C14	Amphoacetates C12
7.10	Flammability (in contact with water and pyrophoric properties)	The substance does not ignite spontaneously in contact with water and has no pyrophoric properties (based on the molecular structure of the constituents of the substance)	The substance does not ignite spontaneously in contact with water and has no pyrophoric properties (based on the molecular structure of the constituents of the substance)	The substance does not ignite spontaneously in contact with water and has no pyrophoric properties (based on the molecular structure of the constituents of the substance)
7.11	Explosive properties (based on the molecular structure of the constituents of the substance)	Negative	Negative	Negative
7.12	Self-ignition temperature	**RA from C12-C14:** not self-ignitable	Not self-ignitable	**RA from C12-C14:** not self-ignitable
7.13	Oxidising properties (based on the molecular structure of the constituents of the substance)	Negative	Negative	Negative
7.14	Granulometry	Waived: The substance is marketed and used in aqueous solutions (non-granular form)	Waived: The substance is marketed and used in aqueous solutions (non-granular form)	Waived: The substance is marketed and used in aqueous solutions (non-granular form)
7.15	Stability in organic solvents	Waived: Not a critical property	Waived: Not a critical property	Waived: Not a critical property
7.16	Dissociation constant	Waived: Test technically not feasible	Waived: Test technically not feasible	Waived: Test technically not feasible
7.17	Viscosity	Waived: Substance is a solid	Waived: Substance is a solid	Waived: Substance is a solid

Table 5 Data Matrix, Environmental Fate/Toxicity for the Alkylamphoacetates

REACH	Endpoint	Amphoacetates C8-C18	Amphoacetates C12-C14	Amphoacetates C12
9.2.1.1	Ready biodegradability[16]	Readily biodegradable under both aerobic (OECD 301 A, D, E and F) and anaerobic conditions (OECD 311)	**RA:** Readily biodegradable under aerobic conditions	Readily biodegradable under aerobic conditions (OECD 301B)
9.2.2.1	Hydrolysis as function of pH	Waived: the substance is readily biodegradable (study is scientifically not necessary)	Waived: the substance is readily biodegradable (study is scientifically not necessary)	Waived: the substance is readily biodegradable (study is scientifically not necessary)
9.3.1	Adsorption/desorption [log Koc]	The screening study is technically not feasible due to the surface active nature of the substance (OECD 121) 3.16 L/kg, calculated using the QSAR: $Koc = \frac{10.47 \cdot Kow^{0.52}}{1000}$ (with Koc in m^3/kg)	The screening study is technically not feasible due to the surface active nature of the substance (OECD 121) 3.16 L/kg, calculated using the QSAR: $Koc = \frac{10.47 \cdot Kow^{0.52}}{1000}$ (with Koc in m^3/kg)	The screening study is technically not feasible due to the surface active nature of the substance (OECD 121) 3.16 L/kg, calculated using the QSAR: $Koc = \frac{10.47 \cdot Kow^{0.52}}{1000}$ (with Koc in m^3/kg)
9.3.2	Bioaccumulation in aquatic species	Waived: low potential for bioaccumulation based on log Pow ≤ 3	Waived: low potential for bioaccumulation based on log Pow ≤ 3	Waived: low potential for bioaccumulation based on log Pow ≤ 3
9.1.1	Acute toxicity to *Daphnia*, 48h-EC50 [mg/L, based on solid content]	EC50 = 25.4; NOEC = 9.4 (monoacetate) (OECD 202)	EC50 = 67.3; NOEC = 45.5 (monoacetate) (OECD 202)	EC50 = 89 (monoacetate) (OECD 202)
9.1.2	Growth inhibition algae, 72h-ErC50, NOErC [mg/L, based on solid content]	72h-ErC50 = 13 72h-NOErC = 0.87 72h-ErC10 = 1.7 (monoacetate) (OECD 201)	**RA from C8-C18:** 72h-ErC50 = 13 72h-NOErC = 0.87 72h-ErC10 = 1.7 (OECD 201)	72h-ErL50 = 44 (monoacetate) (OECD 201)

[16] In accordance with the REACH Guidance and the OECD Guidelines, the 10-day window should not be applied to interpret the results of the tests. The substances consist of homologues of surfactants composed of alkyl chains of varying length. It is anticipated that a sequential biodegradation of the individual structures takes place.

REACH	Endpoint	Amphoacetates C8-C18	Amphoacetates C12-C14	Amphoacetates C12
9.1.3	Acute toxicity to fish, LC50 [mg/L, based on solid content]	96h-LC50: 4.0 (monoacetate) (OECD 203)	**RA from C8-C18:** 96h-LC50: 4.0 (OECD 203)	96h-LC50: 28 (monoacetate) (OECD 203)
9.1.4	Activated sludge respiration inhibition, EC50 [mg/L, based on solid content]	**RA from C12:** NOEC: 560 (OECD 209)	**RA from C12:** NOEC: 560 (OECD 209)	NOEC: 560 (monoacetate) (OECD 209)
9.1.5	Long-term toxicity to *Daphnia*, NOEC [mg/L, based on solid content]	$NOEC_{repro}$ = 3.7 $EC10_{repro}$ = >3.7 $NOEC_{growth\ reduction}$ = 1.6 $EC10_{growth\ reduction}$ = >3.7 (monoacetate) (OECD 211)	**RA from C8-C18:** $NOEC_{repro}$ = 3.7 $EC10_{repro}$ = >3.7 $NOEC_{growth\ reduction}$ = 1.6 $EC10_{growth\ reduction}$ = >3.7 (OECD 211)	**RA from C8-C18:** $NOEC_{repro}$ = 3.7 $EC10_{repro}$ = >3.7 $NOEC_{growth\ reduction}$ = 1.6 $EC10_{growth\ reduction}$ = >3.7 (monoacetate) (OECD 211)
9.1.6	Long-term toxicity to fish, NOEC [mg/L, based on solid content]	$NOEC_{growth}$ = 0.61 $EC10_{length}$ = 0.79 $EC10_{weight}$ = 0.80 (monoacetate) (OECD 210)	**RA from C8-C18:** $NOEC_{growth}$ = 0.61 $EC10_{length}$ = 0.79 $EC10_{weight}$ = 0.80 (OECD 210)	**RA from C8-C18:** $NOEC_{growth}$ = 0.61 $EC10_{length}$ = 0.79 $EC10_{weight}$ = 0.80 (monoacetate) (OECD 210)

Table 6 Data Matrix, Toxicological endpoints for the Alkylamphoacteates

REACH	Endpoint	Amphoacetates C8-C18	Amphoacetates C12-C14	Amphoacetates C12
8.5.1	Acute oral, LD50 [mg/kg bw]	>5000 (equivalent or similar to OECD 401; monoacetate)Diacetate: no data	>5939 (equivalent or similar to OECD 401; surrogate monoacetate) 7935 (equivalent or similar to OECD 401; diacetate)	3422 (equivalent or similar to OECD 401; monoacetate)
8.5.2	Acute inhalation, LC50 [mg/L]	waived	waived	waived
8.5.3	Acute dermal, LD50 [mg/kg bw]	> 2612 (OECD 402/EU Method B.3; monoacetate)	**RA from C8-C18:** > 2612	**RA from C8-C18:** > 2612
8.1	Skin irritation/corrosion	Not classified as irritating to skin (similar or according to OECD 404/EU Method B.4; monoacetate and surrogate of unknown mono-/diacetate ratio) Diacetate: no data	**RA:** not classified as irritating to skin	Not classified as irritating to skin (OECD 404/EU Method B.4; monoacetate)
8.2	Eye irritation	Classified as causing irreversible effects on the eye (Category 1), based on worst-case results (variable results were observed) (OECD 405/EU Method B.5; mono and diacetate form)	**RA from C12:** Classified as irritating to eyes (Category 2) [Conc. >16%]	Classified as irritating to eyes (Category 2) [Conc. >16%] (OECD 405/EU Method B.5; monoacetate)
8.3	Skin sensitisation	Not classified (OECD 406/EU Method B.6; monoacetate)	**RA from C8-C18 and/or C12:** not classified	Not classified (OECD 406; monoacetate)
8.4.1	In vitro gene mutation in bacteria (*Salmonella typhimurium* reverse mutation assay)	Not mutagenic with/without S9 (similar to OECD 471; diacetate)	Not mutagenic with/without S9 (OECD 471; diacetate)	Not mutagenic with/without S9 (OECD 471; monoacetate)
	In vitro gene mutation in bacteria (*Escherichia coli* reverse mutation assay)	**RA from C12-C14 and/or C12:** not mutagenic with/without S9	Not mutagenic with/without S9 (OECD 471; diacetate)	Not mutagenic with/without S9 (OECD 471; monoacetate)

REACH	Endpoint	Amphoacetates C8-C18	Amphoacetates C12-C14	Amphoacetates C12
8.4.2	*In vitro* cytogenicity in mammalian cells	Not clastogenic with/without S9 (OECD 473/EU Method B.10; monoacetate)	Not clastogenic with/without S9 (OECD 473; diacetate)	Not clastogenic with/without S9 (OECD 473/EU Method B.10; monoacetate)
8.4.3.	*In vitro* gene mutation in mammalian cells	Not mutagenic with/without S9 (OECD 476/EU Method B.17; monoacetate)	**RA from C8-C18:** not mutagenic with/without S9	**RA from C8-C18:** not mutagenic with/without S9
	In vivo mammalian bone marrow chromosome aberration test	Negative (OECD 475; monoacetate)	**RA from C8-C18:** Negative	N.A.
8.6.1	28-day repeated dose toxicity	NOAEL = 1000 mg/kg bw/day (OECD 422; monoacetate); NOAEL ≥ 300 mg/kg bw/day[17] (OECD 422; diacetate);	No data	No data
8.6.2	90-day repeated dose toxicity	NOAEL = 1000 mg/kg bw/day (OECD 408; diacetate)	NOAEL = 1000 mg/kg bw/day (OECD 408; diacetate)	**RA from C12-C14:** 1000 mg/kg bw/day (OECD 408)
8.7.1	Screening reproductive/developmental toxicity	$NOAEL_{repro/dev}$ = 1000 mg/kg bw/day (OECD 422, monoacetate); $NOAEL_{repro/development}$ ≥ 300 mg/kg bw/day[17] (OECD 422, diacetate)	Waived: the study does not need to be conducted because a pre-natal developmental toxicity study is available	Waived: the study does not need to be conducted because a pre-natal developmental toxicity study is available
8.7.2	Pre-natal developmental toxicity, one species	$NOAEL_{parental}$ = 1000 mg/kg bw/day; $NOAEL_{development}$ = 1000 mg/kg bw/day (OECD 414, rat, diacetate)	$NOAEL_{parental}$ = 1000 mg/kg bw/day; $NOAEL_{development}$ = 1000 mg/kg bw/day (OECD 414, rat, diacetate)	$NOAEL_{parental}$ = 1000 mg/kg bw/day; $NOAEL_{development}$ = 1000 mg/kg bw/day (OECD 414, rat, monoacetate)

[17] Nb: High dose group excluded from study due to mortality related to regurgitation (secondary effect)

REACH	Endpoint	Amphoacetates C8-C18	Amphoacetates C12-C14	Amphoacetates C12
	Pre-natal developmental toxicity, second species	$NOAEL_{parental}$ = 75 mg/kg bw/day; $NOAEL_{development}$ = 75 mg/kg bw/day (OECD 414, rabbit, diacetate)	**RA from C8-C18:** $NOAEL_{parental}$ = 75 mg/kg bw/day; $NOAEL_{development}$ = 75 mg/kg bw/day (OECD 414, rabbit, diacetate)	**RA from C8-C18:** $NOAEL_{parental}$ = 75 mg/kg bw/day; $NOAEL_{development}$ = 75 mg/kg bw/day (OECD 414, rabbit, diacetate)
8.7.3	Extended One Generation Toxicity Study	$NOAEL_{parental}$ = 1000 mg/kg bw/day; $NOAEL_{reproductive}$ = 1000 mg/kg bw/day $NOAEL_{development}$ = 1000 mg/kg bw/day (OECD 443, rat, diacetate)	N.A.	**RA:** $NOAEL_{parental}$ = 1000 mg/kg bw/day; $NOAEL_{reproductive}$ = 1000 mg/kg bw/day $NOAEL_{development}$ = 1000 mg/kg bw/day (OECD 443, rat, diacetate)
8.8.1	Toxicokinetic assessment	For risk assessment purposes: oral absorption 100%, inhalation absorption 100% and dermal absorption 10% (expert statement)	**RA:** For risk assessment purposes: oral absorption 100%, inhalation absorption 100% and dermal absorption 10%	**RA:** For risk assessment purposes: oral absorption 100%, inhalation absorption 100% and dermal absorption 10%

7. RETENTION OF RECORDS

The final report generated by Charles River from this study will be transferred to a Charles River archive no later than the date of final report issue.

8. CLASSIFICATION AND LABELLING (C&L) AND PBT/PMT PROPERTIES

Classification and labelling

Based on the outcome of the available studies the analogues have been assessed to require the following classification and labelling according to Regulation (EC) No 1272/2008 including related amendments (e.g., the Commission Regulations (EU) No 286/2011, No 618/2012 and No 487/2013):

Table 7 Classification and labelling for the Alkylamphoacetates

Substance	Classification	Labelling
Amphoacetates C8-C18	Classified as causing irreversible effects on the eye (Category 1), based on worst-case results (variable results were observed) Aquatic Chronic 3	Pictogram: GHS05 Signal word: Danger Hazard statement: H318, H412 Precautionary Statements: P280; P305+P351+P338; P310
Amphoacetates C12-C14	Read Across from Amphoacetates C12: Classified as irritating to eyes (Category 2) Read Across from Amphoacetates C8-C18: Aquatic Chronic 3	Pictogram: GHS07 Signal word: Warning Hazard statement: H319, H412 Precautionary Statements: P280; P305+P351+P338; P337+P313 Specific concentration limits: Eye Irrit. 2: C >16%
Amphoacetates C12	Classified as irritating to eyes (Category 2) Read Across from Amphoacetates C8-C18: Aquatic Chronic 3	Pictogram: GHS07 Signal word: Warning Hazard statement: H319, H412 Precautionary Statements: P280; P305+P351+P338; P337+P313 Specific concentration limits: Eye Irrit. 2: C >16%

PBT/vPvB properties

As the available data does not allow a definitive conclusion on the PBT or vPvB properties of alkylamphoacetates, the screening criteria as mentioned in the ECHA Guidance on information requirements and chemical safety assessment Chapter R.11: PBT Assessment (Table R. 11-1) are used to decide whether the substances potentially fulfil the PBT or vPvB criteria.

As all alkylamphoacetates are concluded to be readily biodegradable and have a log Kow lower than 4.5, the substances do not fulfil the screening criteria for classification as P, vP, B or vB.

The alkylamphoacetate substances have been concluded to exhibit acute L(E)C50 values >0.1 mg/L to aquatic organisms. Therefore, the available data demonstrate that the substances do not fulfil criteria for classification as T related to eco-toxicity endpoints. Furthermore, the substances are not classified as carcinogenic, mutagenic, toxic for reproduction (CMR) or STOT-RE. The substances therefore do not fulfil the screening criteria for T related to human toxicity endpoints either.

PMT/vPvM properties

In accordance with EU CLP, a substance shall be considered a PMT substance when it fulfils the persistence, mobility **and** toxicity criteria. All alkylamphoacetate analogues are concluded to be readily biodegradable and have a log Kow lower than 4.5, the substances do not fulfil the screening criteria for classification as P, vP. As the calculated logKoc values for the substances is 0.5, they fulfil the criteria for M or vM. Since criteria for “persist”, “mobile”, “toxic”, “very persistent” and “very mobile” can be applied directly, there is no need to conduct a WoE analysis. The substance does not fulfils persistence and toxicity criteria, as such cannot be considered PMT or vP/vM.

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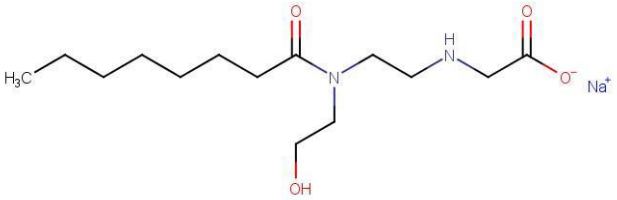
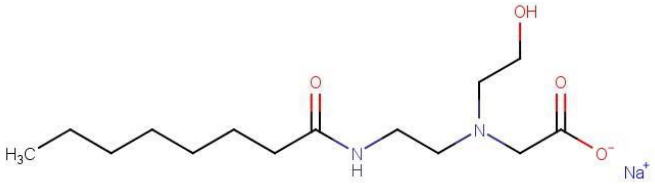
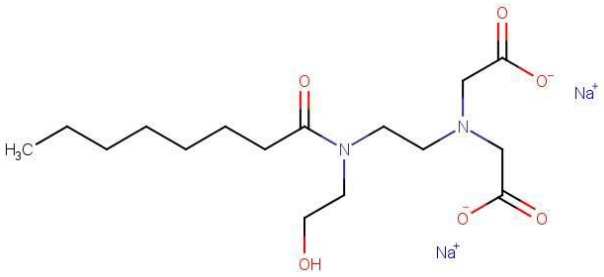
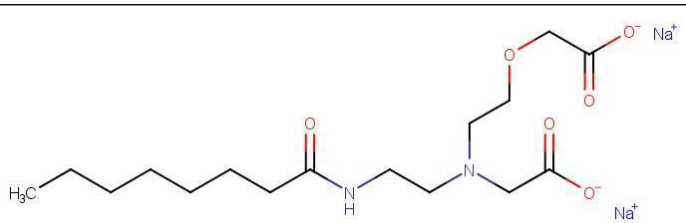
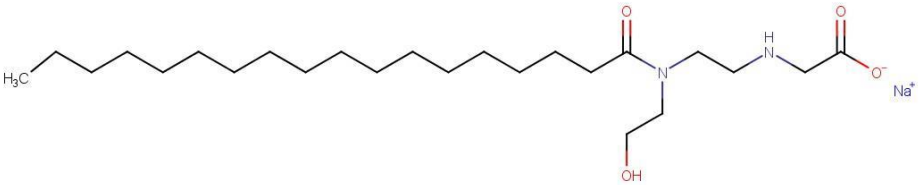
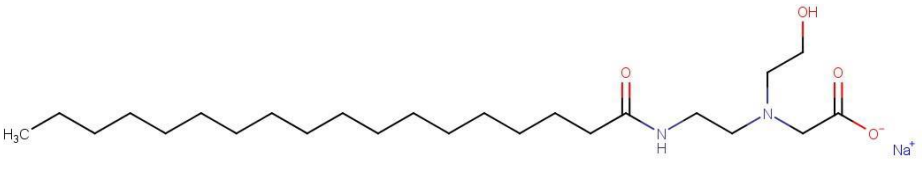
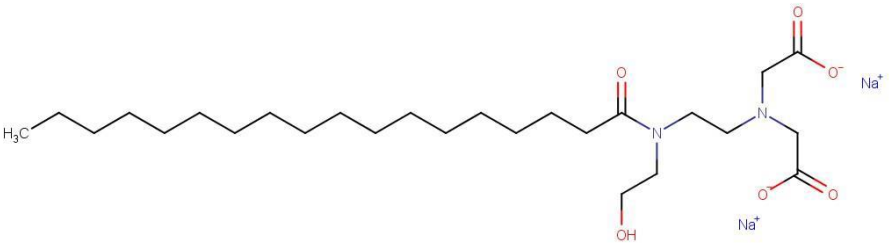
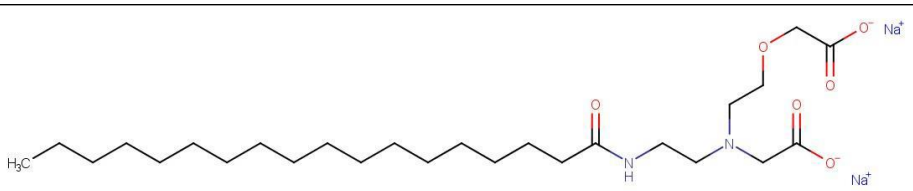
APPENDICES

Appendix I: Overview alkyl chain length distribution of the Alkylamphoacetate analogues

Alkyl chain length	Amphoacetates C8-C18	Amphoacetates C12	Amphoacetates C12-C14
C8	0-11%		
C10	0-11%		≤4%
C12	42-64%	80-99.9%	67–80%
C14	6-26%		20 –32%
C16	4-22%		≤ 4%
C18	0.1-18%		

Appendix III: Chemical structures evaluated by DEREK NEXUS (version 5.0.2)

C8-monoacetate1	
C8-monoacetate2	
C8-diacetate1	
C8-diacetate2	
C18-monoacetate1	
C18-monoacetate2	
C18-diacetate1	

C18-diacetate2	

charles river

Appendix IIIII: Overview of available ecotoxicity studies

Amphoacetates C8-18	Acute toxicity to fish	Chronic toxicity to fish	Acute toxicity to aquatic invertebrates	Chronic toxicity to aquatic invertebrates	Toxicity to aquatic plants	Activated Sludge Respiration inhibition
Mono- : 95-100% Di- : 0-5%	**96h-LC50 = 4.0 mg/L (CRL 2018)** --- 96h-LC50 = 4.2 mg/L (Bazin 1995) --- 96h-LC50 = 5.5 mg/L (Wetton 1996b) --- 96h-LC50 = 8.24 mg/L (Wehrhahn 2002)	**NOEC = 0.61 mg/L (CRL 2019)**	**48h-EC50 = 25.4 mg/L (ibacon 2017a)** --- 48h-EC50 = 8.2 mg/L (Vandendaele 2010a) --- 48h-EC50 = 6 mg/L (Vandendaele 2010b) --- 48h-EC50 = 18.5 mg/L (Wetton 1996) --- 48h-EC50 >100 mg/L (Bazin 1995)	**NOEC = 1.6 mg/L (CRL 2018)**	**72h-ErC50 = 13 mg/L (CRL 2018)** --- 72h-ErC50 = 10 mg/L (Cerbelaud 1995)	*No data*
Mono- : 80-85% Di- : 15-20%	96h-LC50 = 6.4 mg/L (Wetton 1996a) --- 96h-LC50 = 8.5 mg/L (Rudolf 2001) --- 96h-LC50 = 10 mg/L (Berger and Guhl 1998)	*No data*	48h-EC50 = 12.6 mg/L (Bazin 1994) --- 48h-EC50 = 17.9 mg/L (Wierich 2001) --- 48h-EC50 = 17.9 mg/L (Guhl 1993)	*No data*	72h-ErC50 = 28.5 mg/L (Pandard 2001) --- 72h-ErC50 = 3.7 mg/L (Safepharm 1996)	*No data*
Mono- : 50% Di- : 50%	96h-LC50 = 13.9 mg/L (Kamp 1996)	*No data*	48h-EC50 = 56.7 mg/L (ibacon 2017b)	*No data*	72h-ErC50 = 30 mg/L (Lebertz 1998)	*No data*
Undefined ratio	96h-LC50 = 23 mg/L (Burger 2002)	*No data*	48h-EC50 = 2.5 mg/L (Wetton 1992)	*No data*	*No data*	NOEC = 12.7 g/L (Weyandt 1991)

Note: bold indicate the studies used as Key studies in the IUCLID dossiers.

Amphoacetates C12	**Acute toxicity to fish**	**Chronic toxicity to fish**	**Acute toxicity to aquatic invertebrates**	**Chronic toxicity to aquatic invertebrates**	**Toxicity to aquatic plants**	**Activated Sludge Respiration inhibition**
Mono- : 95-100% Di- : 0-5%	**96h-LC50 = 28 mg/L (CRL 2018)** ---------- 96h-LC50 = 1.6 mg/L (Guhl 1993b)	*No data*	**48h-EC50 = 89 mg/L (Guhl 1993a)** --------- 48h-EC50 > 100 mg/L (Pandard 2001)	*No data*	**72h-ErL50 = 44 mg/L (CRL 2018)** ---------- 72h-ErC50 = 14.8 mg/L (Bätscher 2008)	**NOEC = 560 mg/L (Notox 2012)**
Mono- : 80-85% Di- : 15-20%	*No data*		*No data*	*No data*	*No data*	*No data*
Mono- : 50% Di- : 50%	*No data*		*No data*	*No data*	*No data*	*No data*

Note: bold indicate the studies used as Key studies in the IUCLID dossiers.

Amphoacetates C12-14	**Acute toxicity to fish**	**Chronic toxicity to fish**	**Acute toxicity to aquatic invertebrates**	**Chronic toxicity to aquatic invertebrates**	**Toxicity to aquatic plants**	**Respiration inhibition**
Mono- : 95-100% Di- : 0-5%	*No data*	*No data*	*No data*	*No data*	*No data*	*No data*
Mono- : 80-85% Di- : 15-20%	*No data*		**EC50 = 67.3 mg/L NOEC 45.5 mg/L (ibacon 2017)**	*No data*	*No data*	*No data*
Mono- : 50% Di- : 50%	*No data*		EC50 > 100 mg/L NOEC = 20.7 mg/L (ibacon, 2017)	*No data*	*No data*	*No data*

Note: bold indicate the studies used as Key studies in the IUCLID dossiers.

Appendix IVV: Overview of available thyroid-related data

	T3	T4	TSH	Thyroid weight/pathology	Notes
OECD 422 on C8-18 alkylampho(mono)acetates	not measured	unaffected	not measured	unaffected	
OECD 422 on C8-18 alkylampho(di)acetates	not measured	unaffected	not measured	**follicular cell hypertrophy of the thyroid gland was found in males**	follicular cell hypertrophy considered to be non-adverse based on low severity (up to mild) and absence of correlates
OECD 408 on C8-18 alkylampho(di)acetates	unaffected	**Lower plasma levels in males**	**Lower plasma levels in males**	unaffected	No dose response relationship for TSH effects. TSH and T4 within HCR
OECD 408 on C12-C14 alkylampho(di)acetates	unaffected	**Higher plasma levels in males and females**	unaffected	unaffected	Within historical control range
OECD 414 on alkylampho(di)acetates C12-14	**Decreased plasma levels in dams**	unaffected	unaffected	unaffected	within historical control range
OECD 414 on alkylampho(mono)acetates C12	**Decreased plasma levels in dams**	unaffected	unaffected	unaffected	within historical control range
OECD 414 on alkylampho(di)acetates C8-18 (rat)	unaffected	unaffected	**Lower plasma levels in dams**	unaffected	within historical control range, mainly caused by 4 animals in the control group, for which TSH serum levels were above the historical control range.
OECD 443 on alkylampho(di)acetates C8-18	not measured	**Higher plasma levels in in F0 and F1 females**	unaffected	unaffected	within historical control range and with no clear dose-response.

Appendix V: Overview of available toxicity studies

Amphoacetates C8-18	**Acute Toxicity - Oral**	**Acute Toxicity - Dermal**	**Skin Irritation**	**Eye Irritation /Corrosion**	**Skin Sens.**	**Repeat-dose Toxicity**	**Genetic Toxicity**	**Developmental toxicity**	**Toxicity to reproduction**
Mono- : 95-100% Di- : 0-5%	LD50 = 7956-9828 mg/kg bw (Gill 1977c) --- LD50 > 15 ml aqueous solution /kg bw (Middleton 1977)	*No data*	Non irritating (Harper , 1995b, 1995c and 1995d; **Haynes 1995**; Morris 1996)	*No data*	Non sensitizing (**Arcelin 1998**, Liebert 1990)	**NOAEL = 1000 mg/kg bw/day (OECD 422) (CRL, 2018**)	*No data*	*No data*	**NOAEL=1000 mg/kg bw/day (both parental & reproduction) (OECD 422) (CRL, 2018)**
Mono- : 80-85% Di- : 15-20%	LD50 = 10413 mg/kg (Gill 1977b) --- LD50 > 15 ml aqueous solution/kg bw (Middleton 1977) --- LD50 > 15 ml/kg bw (Levenstein 1977) LD50 > 500mg/kg bw (Gloxhuber 1977) --- LD50 = 10413 mg/kg bw (Gil 1977c) --- LD50 > 5 ml/kg bw (Levenstein 1975) --- LD50 = 28 ml aqueous solution/kg bw (Shapiro 1982) --- LD50 : < 15000 mg aqueous solution/kg bw (Shapiro 1987)	**LD50 > 2612 mg/kg (Notox 2010)**	Non irritating (**Dufour 1977a**)	Not-classified (Shapiro, 1990b) --- Irritating (Shapiro 1990a) --- Eye damage(Kastner, 1987) --- Irritating; (Dufour, 1997a)	*No data*	NOAEL = 92.5 mg/kg bw/day (Potokar 1990)	Non clastogenic (OECD 473) (Notox 2010) --- Non mutagenic (OECD 476) (Notox, 2010) --- negative (OECD 475) (CRL.2018)	*No data*	*No data*
Mono- : 50% Di- : 50%	*No data*	*No data*	*No data*	Serious eye damage (**Bien 1995)**	*No data*	**NOAEL = 1000 mg/kg bw/day (OECD 408) (CRL, 2019**) --- **NOAEL = 300 mg/kg bw/day (OECD 422) (CRL, 2018**) --- **NOAEL = 1000 mg/kg bw/day ; Parental NOAEL = 1000**	non mutagenic (OECD 471) (Grotsch 1994; **Hillmann 1991**)	**maternal NOAEL = 1000 mg/kg bw/day ; developmental NOAEL = 1000 mg/kg bw/day (OECD 414; rat) (CRL, 2019)** --- **maternal NOAEL = 75 mg/kg bw/day ; developmental NOAEL = 75 mg/kg bw/day (OECD 414; rabbit) (CRL, 2024)** --- **developmental NOAEL = 1000 mg/kg bw/day (OECD 443) (CRL, 2025)**	**reproductive NOAEL ≥ 300**[17] **mg/kg bw/day ; Parental NOAEL ≥ 300**[17] **mg/kg bw/day (OECD 422) (CRL, 2018)** --- **reproductive NOAEL = 1000 mg/kg bw/day ; Parental NOAEL = 1000 mg/kg bw/day (OECD 443) (CRL, 2025)**

						mg/kg bw/day (OECD 443) (CRL, 2025)			
Undefined ratio			Non Irritating (Harper 1995a; **Dufour 1977b**)	Irritating (**Dufour 1997b)**					

Note: bold indicate the studies used as Key studies in the IUCLID dossiers.

Amphoacetates C12-14	**Acute Toxicity - Oral**	**Acute Toxicity - Dermal**	**Skin Irritation**	**Eye Irritation /Corrosion**	**Skin Sens.**	**Repeat-dose Toxicity**	**Genetic Toxicity**	**Developmental toxicity**	**Toxicity to reproduction**
Mono- : 95-100% Di- : 0-5%	//	*No data*	*No data*	*No data*	*No data*	*No data*	*No data*	*No data*	*No data*
Mono- : 80-85% Di- : 15-20%	**LD50 > 5939 mg/kg (Levenstein 1980)**	*No data*	*No data*	*No data*	*No data*	*No data*	*No data*	*No data*	*No data*
Mono- : 50% Di- : 50%	LD50 = 7935 mg/kg bw (Sandhowe-Grote)	*No data*	*No data*	*No data*	*No data*	**NOAEL = 1000 mg/kg bw/day (OECD 408) (CRL, 2022**)	non clastogenic (OECD 473)\ (CRL,2021) --------- Non mutagenic (OECD 471) (CRL,2021)	**maternal NOAEL ≥ 1000 mg/kg bw/day ; developmental NOAEL ≥ 1000 mg/kg bw/day (OECD 414) (CRL, 2022)**	*No data*

Note: bold indicate the studies used as Key studies in the IUCLID dossiers.

Amphoacetates C12	**Acute Toxicity - Oral**	**Acute Toxicity - Dermal**	**Skin Irritation**	**Eye Irritation/ Corrosion**	**Skin Sens.**	**Repeat-dose Toxicity**	**Genetic Toxicity**	**Developmental toxicity**	**Toxicity to reproduction**
Mono- : 95-100% Di- : 0-5%	LD50>2000 mg/kg bw(aqueous solution with a solid content of approximately 35%.) (Potokar 1990)	*No data*	non-iritating; (Steiling 1990a, Krächter 1992)	Non-irritating (solid content approximately 12.6%) (Esdaile 1999a and 1999b)	Non sensitizing (Steiling 1990b)	*No data*	Non mutagenic (OECD 471) (Cinelli 2000, Banduhn 1990) ---------- Non clastogenic (OECD 473) (Roy, 2012)	**maternal NOAEL ≥ 1000 mg/kg bw/day ; developmental NOAEL ≥ 1000 mg/kg bw/day (OECD 414) (CRL, 2022)**	*No data*
Mono- : 80-85% Di- : 15-20%	**LD50 of 3422 mg/kg bw (Levenstein 1976)** **----------** LD50 = 6116 mg/kg bw (Levenstein 1978) ---------- LD50>2000 mg/kg bw(aqueous solution with a solid content of approximately	*No data*	Miranol H2M Conc; Non-irritating (**Longobardi 2001**)	Irritating (**Longobardi 2001b,** Levenstein 1976a and 1976b)	*No data*	*No data*	*No data*	*No data*	*No data*

	35%.) (Longobardi 2001)								
Mono- : 50% Di- : 50%	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*
Undefined ratio	LD50 >7500 mg/kg (Middleton 1977)			Irritant (Haynes 1985)					

Note: bold indicate the studies used as Key studies in the IUCLID dossiers.

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AMPHOTERIC SURFACTANTS: STRUCTURE-PERFORMANCE CORRELATION

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COGNIS[1], Universitat Autònoma de Barcelona[2]

Abstract
Amphoteric surfactants belong to a very mild surfactant class. They are regularly used in cosmetic and detergent formulations as co-surfactants. Raw materials for the manufacture of amphoterics are usual in the synthesis of surfactants, but the chemistry involved is complex.
Column chromatography along with ^{1}H-NMR and ^{13}C-NMR analysis allow us to separate, identify and characterize those compounds found in different synthetic pathways.
However, those products prepared from different fatty acid chains found within natural oils and fats, as well as the use of different manufacturing procedures, determine different physico-chemical and performance properties.
This paper highlights new aspects within the field of amphoterics and permits us to correlate structure with product functionality.

Résumé
Les tensioactifs amphotères appartiennent à une classe de tensioactifs très doux.
Ils sont utilisés en tant que co-tensioctifs dans la formulation de produits cosmétiques et détergents .
Leurs matières premières sont celles couramment utilisées dans la synthèse des tensioactifs, mais la chimie concernée n'est pas aisée.
La chromatographie sur colonne et la spectroscopie RMN (^{1}H et ^{13}C) permettent la séparation et l'identification des produits issus des différents modes de synthèse.
Selon la longueur de la chaîne des acides gras utilisés et le procédé de synthèse, les produits obtenus présentent des différences vis à vis des caractéristiques physico-chimiques et des performances applicatives.
Cette relation structure-propriétés apporte de nouvelles informations dans le domaine des amphotères.

Zusammenfassung
Amphotere Tenside stellen sehr milde oberflaechenaktive Verbindungen dar. Sie kommen hauptsaechlich als Co-Tenside in Kosmetik- und Waschmittelrezepturen zum Einsatz. Obwohl bei der Synthese dieser Stoffgruppe durchweg bekannte Tensidrohstoffe verwendet werden, ist die zugrundeliegende Chemie doch recht komplex.
Einsatz der Saeulenchromatographie sowie ^{1}H-NMR- und ^{13}C-NMR-Technik ermoeglichen die Abtrennung, Identifizierung und Charakterisierung der durch unterschiedliche Synthesewege erhaltenen Produktgemische, die sich sowohl in ihren physikalisch-chemischen Eigenschaften als auch in ihren Anwendungsprofilen unterscheiden.
Dieser Beitrag erlaeutert im besonderen den Zusammenhang zwischen Struktur und Anwendung von Amphoteren.

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1. INTRODUCTION

Amphoterics are surfactants widely used as co-surfactants in dishwashing products, shampoos, shower and bath formulations. Amongst the most interesting properties of these amphoterics are: mildness to eyes and skin, excellent performance concerning foaming power even in hard water, wetting power, good detergency, compatibility with other surfactants, biodegradability. In terms of amphoterics improving the dermatological properties in combinations with anionics (1), it is noteworthy that this combination accounts for 73% of 'care' formulations (2).

First proposed structures for amphoterics were cyclic alkylated imidazolines (3). Since a great deal of the analysis of amphoterics some years ago (4) was based on the different nitrogen atoms present in the molecule being correlated to product function, there has been considerable attention given to the mode of synthesis under differing conditions (5). Nowadays it is well accepted that amphoterics are products derived from the ring opening of the imidazoline (6). However, in recent publications (7) and catalogues (8) different structures are still assigned for mono- and diacetates.

In this contribution, we present results derived from chromatographic separations and NMR studies which bring new information about the structure and reaction mechanisms of the amphoterics and their performance properties specifically related to those molecular species which are present in commercial products as well as to the fatty acid chain distribution.

2. STRUCTURE ELUCIDATION

Synthetic pathways are well described in a recent publication (9). Raw materials for the first step of the synthesis of amphoterics are fatty acids and aminoethylethanolamine that generate the imidazoline ring using the right process conditions as well as the right ratios of reactants in order to avoid undesirable by-products (10). The second step is the alkylation of the imidazoline that can take place using different conditions in order to optimise the desired composition of the final product. It is relevant in this step to take into account that the imidazoline ring opens in aqueous solutions (11) and that the hydrolysis rate is different depending on the pH value (12). Thus, in order to obtain products with 'high' monocarboxylic content, the intermediate imidazoline has to be hydrolyzed before carrying out the reaction with sodium monochloroacetate, while the direct reaction of imidazoline and the chloroacetate increases considerably the amount of dicarboxylic products (9).

A pure imidazoline was hydrolyzed at different temperatures both in water and in aqueous sodium hydroxide. The reaction was followed up by using ^{1}H-NMR analysis. The species present in the different stages of the reaction are shown in Figure 1.

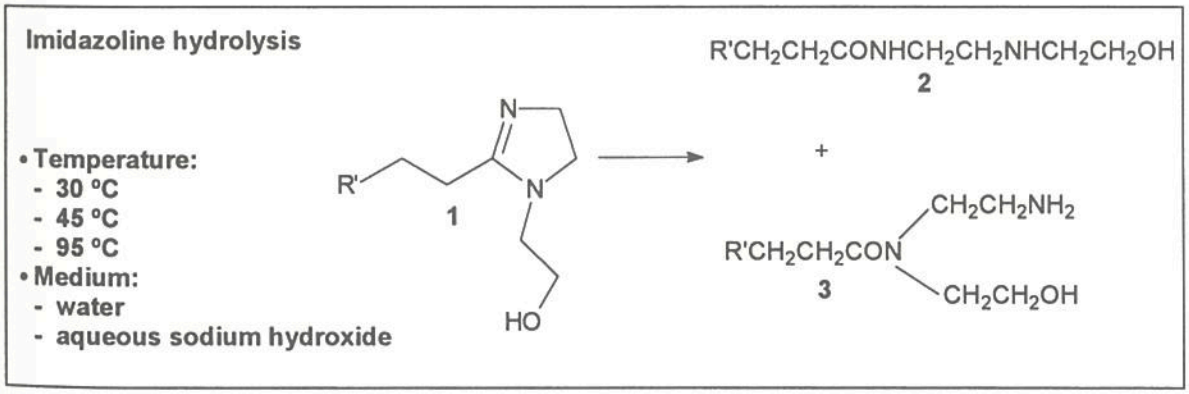

FIGURE 1. Hydrolysis reaction of imidazoline **1**: reaction conditions and formed species

Imidazoline **1** gives two products: the secondary amidoamine **2** and the tertiary amidoamine **3**. The assignment of the signals in the NMR spectrum was carried out using the data from the pure imidazoline and the pure secondary amidoamine registered previously. Figure 2 shows the spectra of the pure imidazoline (a), the reaction mixture after 45 minutes of hydrolysis at 45 ºC (b), and the crude material after 20 hours of reaction (c), which consists of almost pure amidoamine **2**.

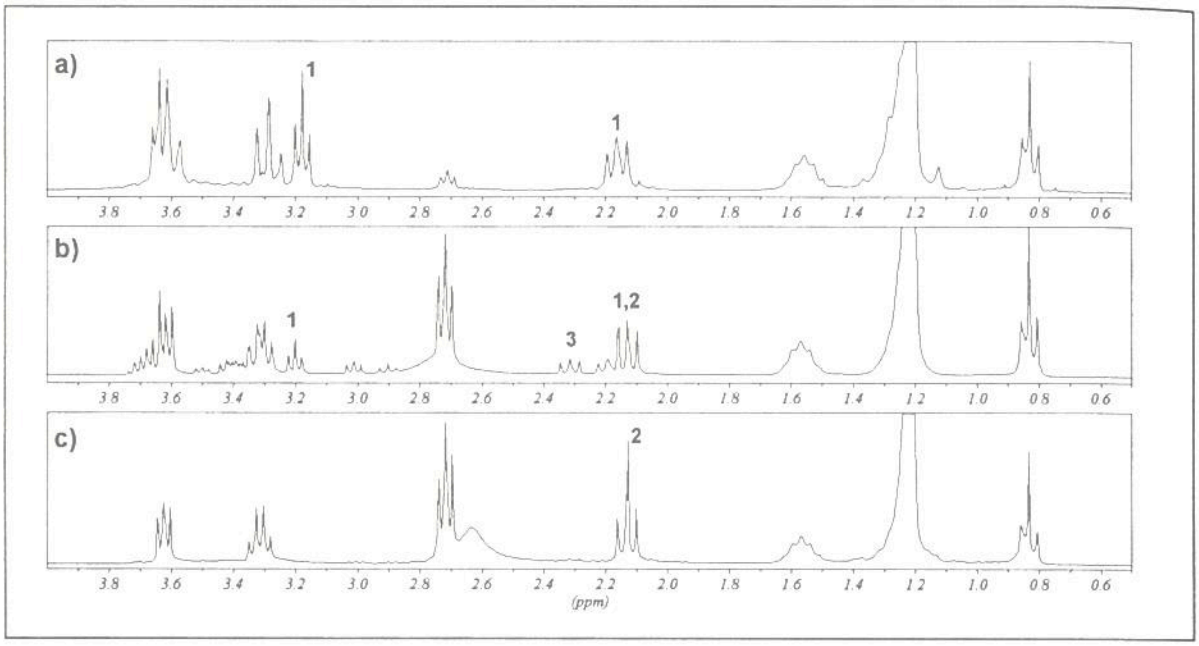

FIGURE 2. Spectra of (a) the imidazoline **1**, (b) reaction mixture after 45 min of hydrolysis at 45 ºC and (c) after 20 h

Table 1 shows molar percentages of the species present at different times during the hydrolysis at 45 ºC. A very similar result is obtained when the reaction is performed at 95 ºC. However, when the reaction was conducted at 30 ºC the final mixture composition was: 85% of the secondary amidoamine **2** and 15% of the tertiary amidoamine **3**.

TABLE 1
Imidazoline hydrolysis at 45ºC

Time	Imidazoline **1**	Secondary amidoamine **2**	Tertiary amidoamine **3**
5 minutes	52	39	9
15 minutes	45	44	11
30 minutes	31	55	14
45 minutes	23	60	17
1 hour	17	64	19
2 hours	7	69	24
3 hours	3	73	24
5 hours	0	78	22
7 hours	0	77	23
20 hours	0	>99	<1

Under alkaline conditions using sodium hydroxide at 95 ºC for 3 hours, the reaction crude contained >99% secondary amidoamine **2** and <1% of the tertiary amidoamine **3**.

According to these results, both secondary and tertiary amidoamine are formed during the hydrolysis process of the imidazoline. The fact that at the very beginning of the hydrolysis both products are already present in the medium and the secondary amidoamine is the major product, indicates that the two possible hydrolyses of the imidazoline take place simultaneously (9). The content of the tertiary product increases at the beginning, but then decreases forming the secondary amidoamine.

From these results, and depending on the hydrolysis conditions, the alkylation of the resulting reaction mixture will give different ratios of mono and diacetate derivatives.

The identification of the different components in amphodiacetates was studied in order to clarify their composition. Chromatographic separation (12) of the alkylated products derived from the reaction of sodium monochloroacetate with lauric imidazoline without previous hydrolysis leads to the identification of the products described in Figure 3.

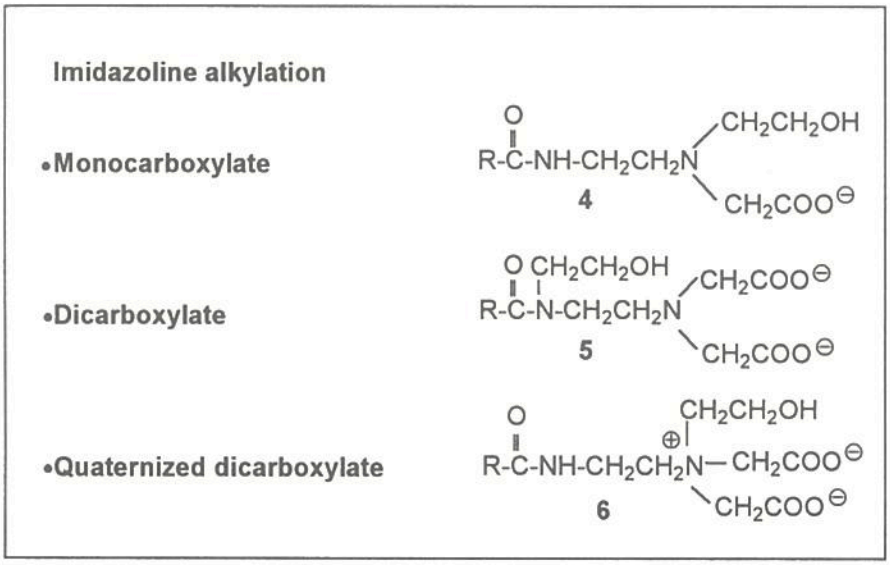

FIGURE 3. Products formed in the alkylation of a non hydrolized imidazoline

Identification of the monocarboxylic compound **4** has been carried out by means of ^{1}H-NMR, ^{13}C-NMR, COSY experiments, $^{1}H/^{13}C$ correlation and HMBC (Hetero Multiple Bond Correlation). Figure 4 shows some incompatible structures.

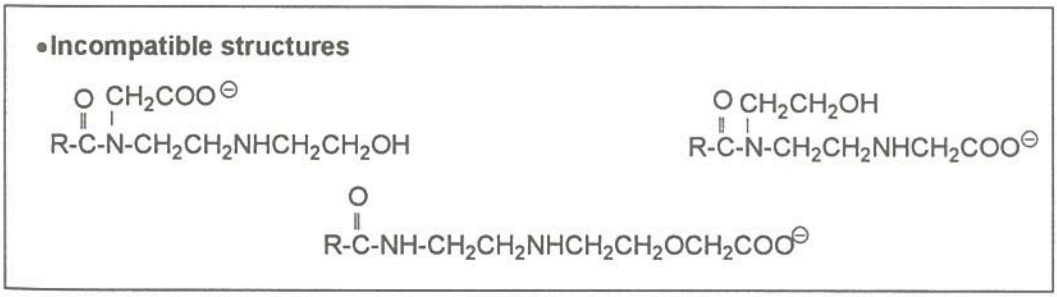

FIGURE 4. Monocarboxylic incompatible structures

Identification of the amphodicarboxylate **5** has been carried out by means of ^{1}H-NMR, ^{13}C-NMR, COSY experiments, NOESY-2D experiments and $^{1}H/^{13}C$ correlation. Figure 5 shows some incompatible structures.

•**Incompatible structures**

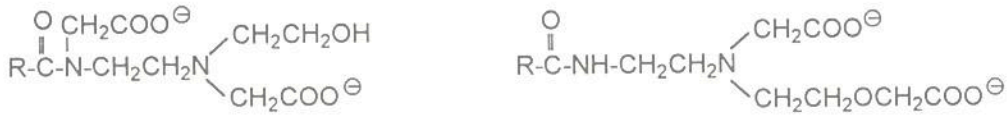

FIGURE 5. Dicarboxylic incompatible structures

The presence of a tertiary amide in compound **5** induces *cis/trans* isomerism and some protons are split in two absorptions. By heating at 330 K, some multiplets disappear becoming broad bands and some of them collapse into well defined signals. Figure 6 presents this behaviour.

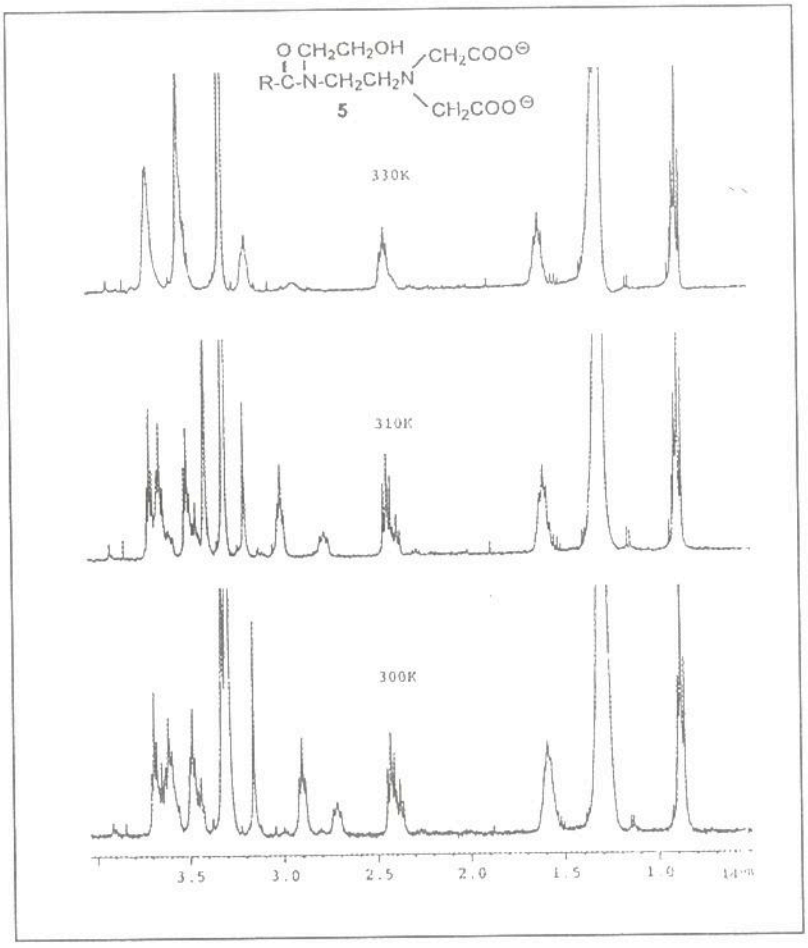

FIGURE 6. *cis/trans* isomerism of **5**

There is also a dialkylated minor product that is a quaternized ammonium salt, **6**. The chemical shifts in the ^{1}H-NMR spectrum are located to lower fields compared with the amphodiacetate **5** because of the positive charge present in the molecule. The addition of sodium hydroxide does not modify the ^{1}H-NMR spectrum thus confirming in this way the presence of a quaternized nitrogen atom. There is no *cis/trans* isomerism confirming the absence of a tertiary amide.

3. PERFORMANCE

It is possible to find in the market several products having different compositions depending on the manufacturing procedure. In this study, using experimental design methodology, we have prepared different products according to a full factorial design (13), and the factors being: product type and fatty chain. The levels of the product type are: pure monoacetate, monoacetate with a low amount of diacetate and the so-called diacetate (9).
The levels of the fatty chain are: coconut (C8-C18, C18'), hydrogenated coconut (C8-C18), cut coconut (C12-C18, C18') and lauric acid. In this way we got 12 different amphoterics to be tested in formulations. Figure 7 summarizes the design.

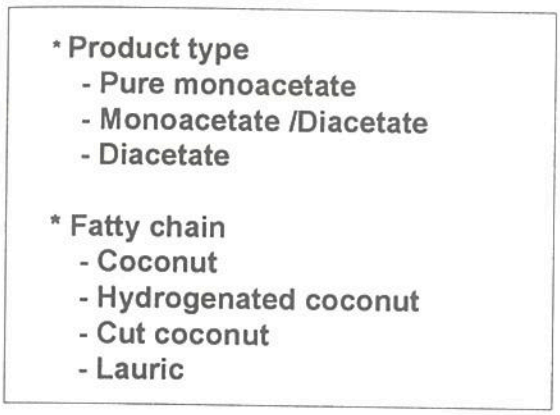

FIGURE 7. Full factorial design

The so-called diacetate contains both monoacetate and diacetate. In contrast to the monoacetates that exhibit high viscosity at 50% solids and which remain stable during storage, diacetates are low viscous liquids at the same concentration, but show increasing viscosity during storage. This is due to the presence of the tertiary amide group having an alcohol group that may undergo an intramolecular reaction (14). Figure 8 shows the differences between both product types concerning the manufacturing process and the appearance.

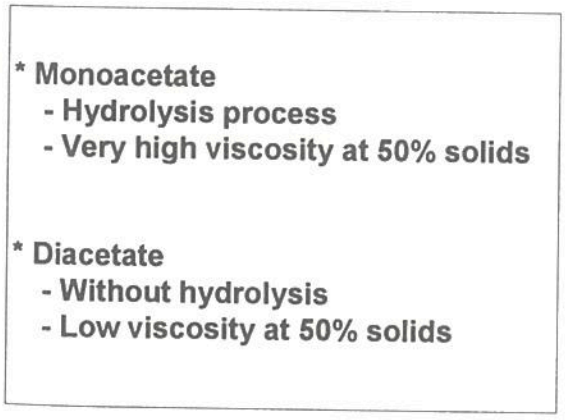

FIGURE 8. Physical properties

Amphoterics have been formulated in two concentrated dishwashing formulations. Table 2 shows both formulations:

TABLE 2
Dishwashing formulations

Component	Formulation A (%)	Formulation B (%)
LES	16	14
FAS	9	8
Amphoteric	7	7
APG	-	3

Both dishwashing formulations (A and B) were clear liquids. The same amount of ethanol was added to each formulation and the viscosity range was between 400 and 800 cps. Foaming power and cleansing performance were evaluated for all the products. Foaming power was evaluated using DIN 53902 and the cleansing performance using a standard soil. Table 3 summarizes products and results.

TABLE 3
Results of the experimental design

Product	Fatty chain	Viscosity A	Viscosity B	Foam A	Foam B	Cleansing A	Cleansing B
Mono	Coconut	700	650	230	240	33	33
Mono	Hydrog.Coconut	800	650	240	205	34.5	35
Mono	Cut coconut	750	600	240	225	34.5	33.5
Mono	Lauric	500	500	245	250	35	34
Mono/Di	Coconut	500	550	250	230	34	34.5
Mono/Di	Hydrog.Coconut	675	550	235	220	34	32.5
Mono/Di	Cut coconut	450	450	245	260	35	35
Mono/Di	Lauric	400	500	240	235	35	33
Di	Coconut	800	800	240	240	33	34
Di	Hydrog.Coconut	750	625	250	235	34.5	36
Di	Cut coconut	750	700	250	235	36	33
Di	Lauric	675	650	230	230	36	34

Viscosity is measured in mPa.s. There is a trend in the influence of fatty chain on viscosity: hydrogenated coconut > coconut > cut coconut > lauric. In the same way there is a trend in the influence of product type on viscosity: di>mono>mono/di.
Concerning foaming power and cleansing performance there are no significant influences coming from different fatty acid chains or different product type.

Amphoterics were formulated in two personal care formulations. Table 4 shows both formulations.

TABLE 4
Personal care formulations

Component	Formulation C (%)	Formulation D (%)
LES	7.5	8.1
Amphoteric	2.5	1.8
APG	-	2

All formulations were adjusted to pH 6.0 - 6.5 and the following parameters were checked: viscosity against sodium chloride concentration and foaming power (DIN 53902).

Table 5 summarizes the results and shows the highest viscosity reached by addition of sodium chloride.

TABLE 5
Results of test on cosmetic formulations

Product	Fatty chain	Viscosity C	NaCl %	Viscosity D	NaCl %	Foaming C	Foaming D
Mono	Coconut	21000	4.5	18700	4.5	200	220
Mono	Hydrog.Coconut	21000	4.5	15600	5.0	235	235
Mono	Cut coconut	25000	4.0	20200	4.0	190	230
Mono	Lauric	23000	4.0	17900	4.0	240	255
Mono/Di	Coconut	21000	4.0	17300	4.0	210	240
Mono/Di	Hydrog.Coconut	18000	4.0	16400	4.5	230	235
Mono/Di	Cut coconut	20000	3.5	18200	4.0	215	245
Mono/Di	Lauric	19500	3.0	16600	4.0	230	235
Di	Coconut	15500	5.5	12800	5.5	215	245
Di	Hydrog.Coconut	12500	4.5	17000	4.5	225	220
Di	Cut coconut	18000	4.5	17500	5.0	195	235
Di	Lauric	16000	6.0	11700	5.0	260	240

Monoacetates have similar viscosity to mono/di and these are higher than diacetates independent of the fatty chain. However to get a viscosity between 4000 and 5000 mPa.s, which is the usual viscosity range in these kinds of products both pure monoacetate and mono/di are suitable, both requiring the same amount of sodium chloride. The cut coconut fatty chain gives the higher viscosity in almost each amphoteric. Figure 9 shows viscosity plots for amphoterics (Formulation C). Foaming behaviour is very similar for all formulations and differences are not significant.

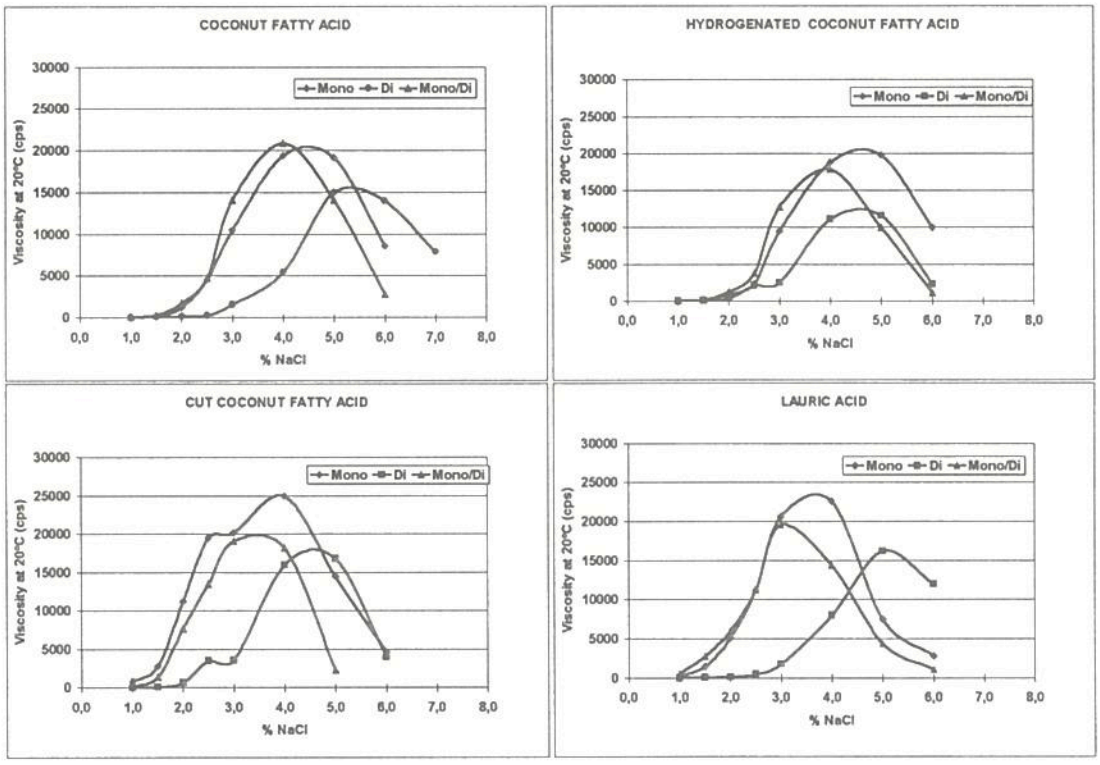

FIGURE 9. Viscosity plots

In respect of 'skin irritation', both monoacetates and diacetates exhibit a low score in the Het-Cam Test. A shampoo formulation has been prepared according to a 2^2 factorial design having the following factors and levels shown in Table 6:

TABLE 6.
2^2 factorial design

Factor	Level -	Level +
Amphotericc surfactant	Monoacetate	Diacetate
APG	No	Yes

Shampoo contained 9% a.s. LES and 3% a.s. co-surfactant. Testing was carried out using a 10% a.s.dilution of the shampoo. Table 7 shows the irritation score.

TABLE 7
Irritation potential

Formulation	Amphoteric	APG	Irritation
1	Monoacetate	No	0.90
2	Diacetate	No	0.79
3	Monoacetate	Yes	0.70
4	Diacetate	Yes	0.61

Low irritating anionic surfactant tested at 5% a.s. has a score of 1.0. APG improves the score of amphoacetates as shown in Table 7.

4. CONCLUSIONS

Monoacetate and diacetate are fully characterized using spectroscopic analysis. The hydrolysis products of the imidazoline are completely characterized by means of NMR spectroscopy. Different products are available to get the right performance properties both in diswashing and personal care formulations. Within the context of amphoterics, the use of these naturally-based fatty acids permits a flexibility in production which can be taylored to manufacture products with specific or optimised properties.

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Exponent®

Expert Review of Available Repeat-Dose and Developmental and Reproductive Toxicity (DART) Studies for Amphoacetates

Exponent®

Expert Review of Available Repeat-Dose and Developmental and Reproductive Toxicity (DART) Studies for Amphoacetates

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Executive Summary

Available repeat-dose and developmental and reproductive toxicity (DART) studies of the monoacetate and diacetate forms of C8-C18 amphoacetate, C12-C14 diamphoacetate, and C12 monoamphoacetate were assessed, paying particular attention to the incidence and dose-response of developmental cardiovascular findings. Any consistencies in findings across the body of studies and the potential influence of maternal toxicity were also investigated.

A total of four amphoacetates (Dehyton® DC, Miranol Ultra C32, PC-2020-926, and sodium lauroamphoacetate; refer to appendix for detailed information on substance identification) were evaluated at doses of 0 (control), 100, 300 or 1000 mg/kg/day. The parental and developmental no observed adverse effect levels (NOAELs) in the three definitive prenatal developmental toxicity studies (for Dehyton® DC, PC-2020-926, and sodium lauroamphoacetate) were the highest dose tested (1000 mg/kg/day). The maternal NOAELs for the prenatal developmental toxicity studies of Dehyton® DC and PC-2020-926 are generally supported by results from their respective 90-day repeat-dose studies. It is noted, however, that due to perceived maternally toxic effects at the high dose in the combined 28-day repeat-dose and reproduction/developmental toxicity screening test (OECD 422) of Dehyton® DC, the high dose dams were euthanized at GD 14, which precluded examination of fetuses at term and the call of NOAELs at the next lower dose (300 mg/kg/day). The developmental NOAEL for the fourth amphoacetate (Miranol Ultra C32) was also determined to be 1000 mg/kg/day, but that assessment was based on an OECD 422, which does not include visceral examination.

There was a low incidence of cardiac and great vessel malformations in two of the three definitive prenatal developmental toxicity studies. None of the malformations was significantly increased and, within each study, the greatest number of malformations occurred in the low dose group. In order to discern if there might be a trend for cardiovascular malformations in animals exposed to the test items, the data from all three studies were combined. Whether the combined data were assessed based on the incidences of malformations, number of malformed fetuses, or underlying perturbed morphogenetic processes, there was neither statistical significance nor a dose-responsive increase.

Aminoethylethanolamine (AEEA) is a starting material in the synthesis of amphoacetates, and small amounts of residual (non-reacted) AEEA can remain in the finished products. The potential for gestational exposure to AEEA to cause congenital cardiac defects was evaluated. High doses of AEEA caused aneurysms of the aorta and alterations in the pattern of great vessels but no defects of the heart. These defects were not observed in the prenatal developmental toxicity studies for the subject amphoacetates. Additionally, the NOAEL for AEEA developmental toxicity is two orders of magnitude above the highest potential AEEA exposure that might have occurred due to amphoacetate exposure in the studies reviewed herein. Thus, AEEA is unlikely to underlie the cardiac defects observed in the prenatal development studies of the subject amphoacetates.

Taken together, in-depth analyses of the available developmental and reproductive toxicity data for the four subject amphoacetates do not support the classification of these substance for reproductive or developmental hazard. Likewise, in-depth analysis of the cardiac and great vessel systems of fetuses exposed to Dehyton® DC, PC-2020-926, and sodium lauroamphoacetate at doses as high as the limit dose does not support that these substances cause malformation of the target area. This conclusion is also supported by the absence of any treatment-related cardiac abnormalities in both the fish early-life stage toxicity test of Miranol Ultra C32 and the dose range-finding studies for PC-2020-265, and sodium lauroamphoacetate (which included visceral examinations of fetal hearts).

1. Purpose

Amphoacetates comprise amphoteric surfactants that are mild detergents. They are formed by a two-step process in which fatty acids of various chain lengths are reacted with AEEA followed by reaction with chloroacetate (Farn, 2006). The resulting products are manifold and depend on the chain lengths of the starting products. An extensive table in the Appendix presents the names, synonyms, identification numbers and physical characteristics of amphoacetates with various chain lengths discussed within this review.

Exponent scientists were requested by the REACH amphoacetates consortium member companies to review the results from the available repeat-dose and developmental and reproductive toxicity (DART) studies of the C8-C18 amphoacetates Miranol Ultra C32 (90:10 monoacetate/diacetate ratio) and Dehyton® DC (50:50 monoacetate/diacetate ratio);a C12-C14 amphoacetates PC-2020-926 (60:40 monoacetate/diacetate ratio); and a C12 monoacetate sodium lauroamphoacetate. This review focuses specifically on developmental cardiovascular findings and assesses the incidence and dose-response of these findings within and across the studies. The potential influence of any maternal effects on the reported outcomes and any consistency in findings that exist across the body of studies are also addressed.

For this assessment, Exponent relied upon the specific studies provided for review by the client's representative at Charles River Laboratories in Den Bosch in the Netherlands. These studies are as follows:

- Bressers, S. 2019. Prenatal Developmental Toxicity Study of Dehyton® DC by Oral Gavage in Rats. Charles River Laboratories Den Bosch BV. Study No. 20164358. 19 July 2019.
- De Raat-Beekhuijzen, MEW. 2018. Combined 28-Day Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test of Miranol Ultra C32 by Oral Gavage in Rats. Charles River Laboratories Den Bosch BV. Study No. 518373. 09 July 2018.

- Gerding, L. 2022. A 90-Day Study of PC-2020-926 by Oral Gavage in Wistar Han Rats with a 28-Day Recovery Period. Charles River Laboratories Den Bosch BV. Study No. 20297957. 27 October 2022.
- Langedijk J. 2022. Dose Range Finding Study of Acetic acid, chloro-, sodium salt, reaction products with 4,5-dihydro-2-undecyl-1H-imidazole-1-ethanol and sodium hydroxide by Oral Gavage in Pregnant Wistar-Han Rats. Charles River Laboratories Den Bosch BV. Study No. 20293263. 16 February 2022.
- Pels Rijcken, WR. 2018. Combined 28-Day Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test of Dehyton® DC by Oral Gavage in Rats. Charles River Laboratories Den Bosch BV. Study No. 518366. 23 November 2018.
- Tobor-Kaplon, MA. 2019. Fish Early-Life Stage Toxicity Test with Miranol Ultra C32 (Flow-Through). Charles River Laboratories Den Bosch BV. Study No. 20177673. 19 August 2019.
- van Otterdijk, F. 2022. Prenatal Developmental Toxicity Study of Acetic acid, chloro-, sodium salt, reaction products with 4,5-dihydro-2-undecyl-1H-imidazole-1- ethanol and sodium hydroxide by Oral Gavage in Time-Mated Wistar Han Rats. Charles River Laboratories Den Bosch BV. Study No. 20293266. 29 August 2022.
- Vriends, A. 2022a. Dose Range Finding Study of PC-2020-926 by Oral Gavage in Pregnant Wistar-Han Rats. Charles River Laboratories Den Bosch BV. Study No. 20297966. 06 May 2022.
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- Wagenaar, L. 2019. A 90-Day Study of Dehyton® DC by Oral Gavage in Wistar Rats. Charles River Laboratories Den Bosch BV. Study No. 20164357. 23 July 2019.

Exponent scientists also relied on additional information as available in the published literature, the Charles River historical control database (Charles River, 2023), and their own expertise in developmental and reproductive toxicology.

Below, the results of the individual studies are first summarized according to test article and the chronological order in which the reports were issued. Next, a comprehensive evaluation of the combined results of these studies is provided that takes into consideration embryological development processes.

2. Study summaries

Dehyton® DC

Combined 28-Day Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (Pels Rijcken, 2018)

This study was conducted in compliance with good laboratory practices (GLP) and according to the Organization for Economic Cooperation and Development (OECD) test guideline (TG) No. 422 (2016). Male and female Wistar Han rats (10/sex per group) were dosed by oral gavage with 0, 100, 300 or 1000 mg/kg/day of Dehyton® DC in water at a dosing volume of 5 mL/kg. These formulations were adjusted to account for purity of the test article (47.6%) and the top dose administered (1000 mg/kg/day) is the limit dose for this test. Males were dosed for a minimum of 28 days beginning 14 days prior to mating. Females were dosed beginning 14 days prior to mating, through mating, gestation, and 13 days of the lactation period.

Methods in Brief. Parental animals were checked twice daily for mortality/morbidity, once daily for clinical signs, and once weekly for arena observations. Females were screened for estrous cyclicity during the first 14 days prior to mating, after which mating was conducted on a 1:1 basis until mating was confirmed (designated gestational day [GD] 0). Females were allowed to litter normally. Body weights and food consumption were measured weekly in males and in females prior to mating; after mating, female body weights and food consumption were measured on GDs 0, 4, 7, 11, 14, 17, and 20 and during lactation on postnatal days (PNDs) 1, 4, 7, and 13. Functional assessments were conducted on 5 rats/sex per group in their respective last weeks of treatment. At sacrifice, blood was collected from parental animals for assessment of hematology, clinical chemistry and thyroid hormone (thyroxine; T4). Pups were checked daily for mortality/morbidity and clinical signs, and weighed on PND 1, 4, 7, and 13. The numbers of live and dead pups were recorded on PND 1, sex was assessed on PNDs 1 and 4, anogenital distance (AGD; normalized to the cube root of body weight) was measured on PND 1, and nipple retention was evaluated in males on PND 13. On PND 4, litters were culled to 8 pups each (4 of each sex), when possible. Thyroid hormone (T4) was assessed in 2 pups/litter per group from culled animals on PND 4 and in remaining pups at the end of study. At necropsy, select organs from parental

animals were weighed in all dose groups, and tissues were evaluated for histopathology in the control and high dose groups.

Results. A single female at 300 mg/kg/day died as a result of blood sampling just prior to necropsy; this death was not considered treatment related. One male and four females at 1000 mg/kg/day died or were sacrificed *in extremis* during the course of the study. Respiratory and other clinical signs were noted in these animals prior to death/sacrifice. Macroscopic findings in the lungs were also observed in these animals; additionally, in one of the females, foreign material was noted in the tracheal lumen. The laboratory concluded that these deaths were likely due to regurgitation of the test article; however, in our opinion, it cannot be excluded that these deaths may have been related to gavage error. Nonetheless, it is noted that in the later studies described below, the dosing volume for Dehyton® DC in water was reduced from 5 mL/kg to 1.796-1.895mL/kg and no other such deaths were observed in the subsequent studies. It should also be noted that, because 4 of the 10 females at 1000 mg/kg/day died or were euthanized prematurely, the remaining 6 females in this dose group were sacrificed early on GD 14. Therefore, data for the high dose females should be interpreted with some caution as animals in the control and other treatment groups were sacrificed during the postnatal lactation period rather than in gestation.

Various clinical signs, including salivation, labored breathing rales, and piloerection were noted in most animals treated at 1000 mg/kg/day and occasionally in some animals treated at 300 mg/kg/day. Significantly reduced mean body weight gain was observed in males at 1000 mg/kg/day, resulting in a 6% decreased body weight at the end of treatment compared to controls. Female body weights were unaffected by treatment and no clearly treatment-related effect on food consumption was noted for either males or females. Functional observational parameters were not affected by treatment. Significant hematologic changes were noted for both males and females at 1000 mg/kg/day compared to controls. Although a number of clinical chemistry parameters in females of the 1000 mg/kg/day dose group were significantly different from control, the changes were minimal and interpreted by the laboratory as being likely a result of the difference in physiological status (pregnancy versus lactation) rather than an effect of treatment. In females at 300 mg/kg/day, total protein and albumin were significantly lower than

control, but at the lower limit of the normal range. No toxicologically relevant clinical chemistry changes were noted for males.

Serum T4 concentrations were unaffected by treatment in males. However, an increase in the incidence of minimal to slight thyroid follicular cell hypertrophy was noted at 300 and 1000 mg/kg/day. Serum T4 data were not available for parental females; however, no increased thyroid histopathology was observed in these animals at ≤1000 mg/kg/day.

Brain and kidney weights relative to body weight were significantly increased in high dose males compared to controls. In high dose females, numerous organ weights were significantly different from control (both absolute and relative to body weight); however, because these animals were sacrificed at a different stage of physiological development, no conclusions can be drawn regarding a relation to treatment. No significant organ weight differences from control were found for females in the low and mid-dose treatment groups.

Reproductive and litter parameters are presented in Table 1. Estrous cyclicity was unaffected by treatment. No significant effect of treatment was observed on mating, fertility or gestation indices, precoital time or the mean number of implantations per female. Because females at 1000 mg/kg/day were sacrificed prior to delivery, littering and pup developmental are only available for animals in the control, low and mid-dose groups. No effects of treatment were observed on gestation duration and no indications of prolonged parturition were noted. Litter size, live birth index, and viability and lactation indices were not significantly affected by treatment at ≤300 mg/kg/day. While the number of dead pups noted at the first litter check were 0, 0 and 9 in the control, low and mid-dose groups respectively, 7 of the 9 dead pups were in a single litter. The associated dam was the only one in the dose group to have lost weight during the postnatal period.

Table 1. Reproductive and litter parameters from the combined repeat-dose/DART screening test of Dehyton® DC (Pels Rijcken, 2018)

Dose (mg/kg/day):	0	100	300	1000
Mating index (%)	100	100	100	100
Fertility index (%)	90	80	100	100
Gestation index (%)	100	100	90	
Mean precoital time (days)	2.1	2.1	2.6	2.0
Mean # implantations (± SD)	12.9 ± 1.9	12.9 ± 3.0	12.5 ± 4.2	11.3 ± 4.4
Gestation duration (days ± SD)	21.2 ± 0.4	21.3 ± 0.5	21.2 ± 0.7	
# dead pups at 1st litter check (# affected litters)	0 (0)	0 (0)	9 (3)	
Mean # live pups at 1st litter check (± SD)	12.1 ± 2.3	10.9 ± 4.1	11.6 ± 2.4	
Mean postnatal loss (± SD)	0.3 ± 0.7	0.0 ± 0.0	0.1 ± 0.3	
Post-implantation survival index (%)	94	84	90	
Live birth index(%)	100	100	92	
Viability index (%)	97	100	99	
Lactation index (%)	100	100	99	

No effects of treatment were observed on pup clinical signs, body weights, sex ratio, anogenital distance, nipple retention, or serum T4 levels in male or female pups and no macroscopic findings related to treatment were observed at necropsy.

Based on these data, the laboratory concluded that the parental no observed adverse effect level (NOAEL) was 300 mg/kg/day based on mortality and regurgitation of the formulations. While the NOAEL for parental toxicity could have been called lower due to the increased incidence of thyroid hypertrophy in males at 300 mg/kg/day, the data presented in the 90-day study (see below) indicate that the thyroid finding was spurious. The laboratory indicated the reproductive NOAEL as ≥1000 mg/kg/day based on no effects observed; however, in the absence of complete reproductive data at 1000 mg/kg/day from maternal animals that reached full term, we consider the reproductive NOAEL in this study to be ≥300 mg/kg/day. The laboratory correctly indicated the developmental NOAEL as ≥300 mg/kg/day based on no effects observed.

Prenatal Developmental Toxicity Study (Bressers, 2019)

This study was conducted in compliance with GLP and according to OECD TG No. 414 (2018). Time-mated female Wistar Han rats (22/group) were dosed by oral gavage with 0, 100, 300 or 1000 mg/kg/day of Dehyton® DC in water at a dosing volume of 1.796 mL/kg. These formulations were adjusted to account for purity of the test article (48%) and the top dose administered (1000 mg/kg/day) is the limit dose for this test. Dosing was from GD 6 though GD 20.

Methods in Brief. Dams were checked twice daily for mortality/morbidity and at least once daily for clinical signs. Body weights and food consumption were measured on GDs 2, 6, 9, 12, 15, 18, and 21. At sacrifice on GD 21, blood was collected from the dams for assessment of thyroid hormones (triiodothyronine [T3], T4, and TSH). Dams were subjected to examination of the thoracic and abdominal cavities. Uteri and thyroid glands were weighed, and the thyroid prepared for histopathologic examination. Data regarding litter indices were collected. Uteri of apparently non-pregnant rats were stained for identification of potential implantation sites. All live fetuses were sexed, weighed and examined for external anomalies; AGD (normalized to the cube root of fetal body weight) was also measured. One half of the fetuses in each litter was examined by fresh dissection for visceral anomalies; this examination included the heart and major blood vessels. The heads were examined by Wilson sectioning. The other half of the fetuses in each litter was subjected to skeletal examination using Alizarin Red S staining. Fetal data were appropriately assessed based on litter means.

Results. No treatment-related mortality or clinical signs of toxicity were observed. At 300 mg/kg/day, a single dam was euthanized *in extremis* on GD 16 due to a non-treatment related spinal injury. Also, at ≥300 mg/kg/day, rats exhibited increased salivation after dosing; this finding was considered by the laboratory to be a physiological response to the test article rather than a clinical sign of toxicity. No treatment-related effects on body weight or food consumption were observed. Slightly lower serum TSH levels were seen at ≥300 mg/kg/day. However, the differences from control were not statistically significant and individual values were within the historical control data (HCD) range; therefore, the differences were not considered to be related

to treatment. No effects on T3 or T4 levels were observed, and thyroid organ weights and histopathology were not changed from control.

Fetal litter parameters and malformation data are presented in Table 2. No treatment-related effects were observed on the number of pregnant females per group, the numbers of corpora lutea and implantation sites, early and late resorptions, pre- and post-implantation loss, the numbers of live and dead fetuses per litter, fetal sex ratio, fetal weights or fetal AGD measures.

Table 2. Litter parameters and fetal anomalies data from the prenatal developmental toxicity study of Dehyton® DC (Bressers, 2019)

Dose (mg/kg/day):	0	100	300	1000
# Females on study	22	22	22	22
# Euthanized or died on study	0	0	1	0
# Pregnant at scheduled necropsy (%)	22 (100)	22 (100)	20 (90.9)	22 (100)
Litter parameters				
Mean # corpora lutea per litter (± SD)	11.8 ± 2.34	12.4 ± 2.11	11.8 ± 2.170	12.2 ± 1.84
Mean # implantations per litter (± SD)	10.7 ± 2.98	10.7 ± 2.51	11.2 ± 1.63	10.7 ± 1.75
Mean # dead fetuses per litter (± SD)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Mean # viable fetuses per litter (± SD)	10.4 ± 2.91	10.4 ± 2.63	10.7 ± 1.75	10.3 ± 2.12
Mean # early resorptions per litter (± SD)	0.3 ± 0.65	0.3 ± 0.57	0.5 ± 0.69	0.4 ± 0.67
Mean # late resorptions per litter (± SD)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Mean % pre-implantation loss (± SD)	11.0 ± 18.42	13.7 ± 17.70	5.1 ± 9.87	10.8 ± 14.43
Mean % post-implantation loss (± SD)	2.7 ± 5.41	3.2 ± 6.29	4.1 ± 6.52	4.5 ± 8.27
% Males per litter	50.4	46.6	53.8	48.3
Mean male fetal weights per litter (± SD)	5.5 ± 0.25	5.4 ± 0.38	5.5 ± 0.29	5.4 ± 0.37
Mean female fetal weights per litter (± SD)	5.1 ± 0.45	5.2 ± 0.30	5.3 ± 0.26	5.2 ± 0.30
Mean male corrected AGD (± SD)	1.49 ± 0.113	1.48 ± 0.087	1.50 ± 0.096	1.47 ± 0.142
Mean female corrected AGD (± SD)	0.66 ± 0.100	0.67 ± 0.093	0.68 ± 0.091	0.67 ± 0.107

Dose (mg/kg/day):	0	100	300	1000
Fetal anomalies data				
# Fetuses (litters) examined externally	229 (22)	229 (22)	214 (20)	227 (22)
# Fetuses (litters) with external malformations	0 (0)	2 (2)	0 (0)	0 (0)
# Fetuses (litters) examined viscerally	115 (22)	117 (22)	107 (20)	114 (22)
# Fetuses (litters) with visceral malformations	0 (0)	2 (2)	1 (1)	1 (1)
# Fetuses (litters) examined skeletally	114 (22)	114 (22)	107 (20)	114 (22)
# Fetuses (litters) with skeletal malformations	3 (3)	3 (3)	1 (1)	1 (1)
Total # fetuses (litters) with anomalies	3 (3)	7 (4)	1 (1)	1 (1)

The individual fetuses with malformations are shown in Table 3. No treatment related external or skeletal malformations were observed. With regard to visceral anomalies, cardiovascular findings were noted in 0, 2, 1, and 1 fetuses in the control, low, mid, and high dose groups, respectively. The laboratory concluded that, although a dose-response could not be shown, because right-sided aortic arch occurred in the 1000 mg/kg/day dose group at an incidence above the HCD range (reported as a summary percent incidence) and other findings (transposition of the great vessels, interrupted aortic arch, retro-esophageal ductus arteriosus, and absent ductus arteriosus) were not reported in the HCD, a relation to treatment could not be excluded. We note, however, that right-sided aortic arch has been previously reported in the HCD that was provided in the study report, meaning that it has been seen in at least one control fetus in at least one past study – thus, at the same rate as in the current study. Significance of the cardiovascular findings reported in this study is discussed in greater detail in the assessment section found below.

Table 3. Malformation data by individual fetuses for the prenatal developmental toxicity study of Dehyton® DC (Bressers, 2019)

Dose (mg/kg/day):	Finding
0	Fetus A001-11 – bent limb bones (S) Fetus A005-05 – bent limb bones (S) Fetus A008-06 – vertebral anomaly with associated rib anomaly (S)
100	Fetus A029-02 – omphalocele (E) Fetus A029-07 – vertebral anomaly with associated rib anomaly (S) Fetus A039-04 – abnormal lung lobation (V), transposition of the great vessels (V) Fetus A041-07 – bent limb bones (S) Fetus A043-03 – abnormal lung lobation (V), situs inversus (V), ventricular septum defect (V), interrupted aortic arch (V), retro-esophageal ductus arteriosus (V) Fetus A043-04 – absent lower jaw (E) and cleft palate (E) *(findings confirmed at skeletal)* Fetus A043-05 – sternoschisis (S)
300	Fetus A058-03 – abnormal lung lobation (V), ventricular septal defect (V), absent ductus arteriosus (V), situs inversus (V)
1000	Fetus A068-10 – absent eyes (V), right-sided artic arch (V), ventricular septal defect (V), bent limb bones (S), skull bones fused (S), vertebral anomaly without associated rib anomaly (S)

E = external finding; S = skeletal finding; V = visceral finding

No external variations were noted. Small supernumerary liver lobes were noted as a visceral variation in the study, but the incidences were considered unrelated to treatment. At the skeletal examination, an increased incidence of 7th cervical vertebra ossification site presence was observed at 1000 mg/kg/day.

Based on these data, the laboratory concluded correctly that the NOAEL for maternal toxicity was 1000 mg/kg/day based on the absence of any observed effects. The laboratory could not come to a conclusion regarding the developmental NOAEL. As detailed further in the assessment that follows, it is our opinion that the fetal findings are not treatment-related and the NOAEL for developmental toxicity is 1000 mg/kg/day, the highest dose tested.

90-Day Study (Wagenaar, 2019)

This study was conducted in compliance with GLP and according to OECD TG No. 408 (1998); this OECD guideline has since been superseded with an updated version that includes additional assessment of various endocrine-sensitive endpoints. Wistar Han rats (10/sex per group) were dosed for 90 days by oral gavage with 0, 100, 300 or 1000 mg/kg/day of Dehyton® DC in water at dosing volumes of 1.895 mL/kg (to Day 34) and 1.796 mL/kg (beginning Day 35). These formulations were adjusted to account for purity of the test article (47.2% and 48%) and the top dose administered (1000 mg/kg/day) is the limit dose for this test.

Methods in Brief. Rats were checked twice daily for mortality/morbidity, once daily for clinical signs and once weekly for arena observations. Body weights and food consumption were measured weekly. Estrus cyclicity was assessed in female rats in weeks 11-13. Functional tests were conducted on 5 rats/sex per group in week 12. An ophthalmic examination was conducted on control and high dose rats in week 13. At sacrifice, blood was collected for assessment of hematology, clinical chemistry, and thyroid hormones (T3, T4, and TSH). Select organs were weighed and tissues collected for histopathologic examination of the control and high dose groups.

Results. No mortality and no treatment-related arena observations were observed. Salivation was noted in all animals at ≥300 mg/kg/day and incidental ploughing in all animals at 1000 mg/kg/day. The laboratory did not consider these findings to be toxicologically relevant, but rather, to be a physiological response to the taste of the test material. There were no effects of treatment on body weights and body weight gains in females at ≤1000 mg/kg/day and in males at ≤300 mg/kg/day. The terminal mean body weight for males at 1000 mg/kg/day was 88% of control; this difference was statistically significant. No significant differences from control were observed with regard to food consumption. Functional observations, including motor activity, were similar across treatment groups including control, and no effects of treatment were observed in the ophthalmic examinations or with regard to estrous cyclicity. In males, platelets were significantly increased at ≥300 mg/kg/day and alkaline phosphatase was significantly increased at 1000 mg/kg/day compared to concurrent control values, but the values were reported to be within the HCD range and considered to be not toxicologically relevant. Other statistical differences from control in

hematologic and clinical chemistry parameters were considered incidental based on their minimal degree of change and/or lack of dose-response. TSH concentrations were significantly lower than control in all groups of treated males, but without dose response. T4 was reduced in males at 1000 mg/kg/day. No changes in organ weights were considered direct effects of treatment. There were no treatment related histopathologic findings, including in the thyroid. Qualitative assessment of spermatogenesis revealed normal progression of the spermatogenic cycle.

Based on the absence of any treatment-related toxicity, the NOAEL for systemic toxicity was determined by the laboratory to be 1000 mg/kg/day, the highest dose tested. The NOAEL for females is 1000 mg/kg/day.

Miranol Ultra C32

Combined 28-Day Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (De Raat-Beekhuijzen, 2018)

This study was conducted in compliance with GLP and according to OECD TG No. 422 (2016). Male and female Wistar Han rats (10/sex per group) were dosed by oral gavage with 0, 100, 300, or 1000 mg/kg/day of Miranol Ultra C32 in water at a dosing volume of 5 mL/kg. These formulations were adjusted to account for purity of the test article (39.15%) and the top dose administered (1000 mg/kg/day) is the limit dose for this test. Males were dosed for a minimum of 29 days beginning 14 days prior to mating. Females were dosed beginning 14 days prior to mating, through mating, gestation, and 13 or 15 days of the lactation period.

Methods in Brief. Parental animals were checked twice daily for mortality/morbidity and clinical signs, and once weekly for arena observations. Females were screened for estrus cyclicity during the first 14 days prior to mating, after which mating was conducted on a 1:1 basis until mating was confirmed (designated GD 0). Females were allowed to litter normally. Body weights and food consumption were measured weekly in males and in females prior to mating; after mating, female body weights and food consumption were measured on GDs 0, 4, 7, 11, 14, 17, and 20 and during lactation on PNDs 1, 4, 7, and 13. Functional assessments were conducted on 5 rats/sex per group in their respective last weeks of treatment. At sacrifice, blood was collected from

parental animals for assessment of hematology, clinical chemistry and thyroid hormone (T4 and TSH). Pups were checked daily for mortality/morbidity and clinical signs, and weighed on PND 1, 4, 7, and 13. The numbers of live and dead pups were recorded on PND 1, sex was assessed on PNDs 1 and 4, AGD (normalized to the cube root of body weight) was measured on PND 1, and nipple retention was evaluated in males on PND 13. On PND 4, litters were culled to 8 pups each (4 of each sex), when possible. Thyroid hormone (T4) was assessed in 2 pups/litter per group from culled animals on PND 4 and in remaining pups at the end of study. At necropsy, select organs from parental animals were weighed in all dose groups, and tissues were evaluated for histopathology in the control and high dose groups.

Results. No treatment-related mortalities were observed. At 1000 mg/kg/day, 1 male and 2 females died due to gavage error; the primary findings for each of these animals are shown in Table 4. It should be noted, however, that the macroscopic and microscopic findings for Female #78 are not consistent with gavage error. No other deaths were reported on study.

Table 4. Morbidity/mortality at 1000 mg/kg/day in the combined repeat-dose/DART screening test of Miranol Ultra C32 (De Raat-Beekhuijzen, 2018).

Animal	Reported findings
Male 33 Euthanized *in extremis* on Day 17	Clinical signs: lethargy, flat posture, gasping, piloerection, squeaking, chromodacryorrhea Macroscopic findings: mucous contents in trachea; gas distension of parts of gastrointestinal tract Microscopic findings: acute inflammation of the trachea
Female 74 Found dead on GD 1	Clinical signs: gasping, dyspnea Macroscopic findings: swollen lungs with dark red foci and containing watery fluid Microscopic findings: amorphous alveolar contents and congestion of the lungs, marked bronchial mucosal erosion of the lungs, marked ulceration/erosion of the trachea
Female 78 Euthanized *in extremis* on PND 1	Clinical signs: rough fur, lunched posture, ptosis, pale appearance Macroscopic findings: gelatinous contents of the stomach, discoloration of the liver (pale), discoloration of the kidneys (greenish) Microscopic findings: marked ulceration of forestomach, moderate lymphogranulocytic inflammation of the forestomach

Salivation was noted in the animals treated at 1000 mg/kg/day and occasionally in some animals treated at 300 mg/kg/day. This finding was interpreted as a physiological response to dosing rather than a sign of systemic toxicity. Rales were noted in a few animals at 1000 mg/kg/day; however, because this finding was observed only on one or a few days, it was not considered toxicologically relevant. No statistically significant effects of treatment on male or female body weights and body weight gains were observed. However, in high dose females, body weight gain was slightly reduced at the end of gestation (~15%) and 3 females were noted as showing substantially reduced weight gain or weight loss at the end of lactation. In line with these findings, female food consumption at 1000 mg/kg/day was significantly reduced at the end of gestation (~12.5-13%) and in the last week of lactation (~20%). Male food consumption was unaffected. Functional observational and hematologic parameters were not changed by treatment. Clotting time was significantly reduced in males at 1000 mg/kg/day compared to concurrent controls, but all values were within the expected range. Alanine aminotransferase levels and bile acids were increased in males at 1000 mg/kg/day, but these differences from control were not statistically significant and the individual values were within their respective HCD ranges as reported in the study. Thus, these differences were not considered toxicologically relevant. Serum T4 concentrations were unaffected by treatment in males.

Absolute brain weight was significantly lower in high dose females (~7%) compared to controls. However, individual values at 1000 mg/kg/day were similar to the HCD mean value as reported in the study, while those of the concurrent control were above the HCD range and brain weight relative to body weight was unaffected. No treatment-related histopathology was seen in the high dose group males and females.

Reproductive and litter parameters are presented in Table 5. Estrous cyclicity was unaffected by treatment. No significant effect of treatment was observed on mating, fertility or gestation indices, precoital time or the mean number of implantations per female. No effects of treatment were observed on gestation duration and no indications of prolonged parturition were noted. Litter size was not significantly affected by treatment but was generally lower in the treated groups compared to control. Live birth and lactation indices were unaffected by treatment.

At the first litter check, 7 pups from 4 litters in the 1000 mg/kg/day dose group were found dead. One of these litters (from which 3 pups were found dead) was for a dam (No. 78) that was euthanized *in extremis* on PND 1. The number of dead pups at 1000 mg/kg/day was considered by the laboratory to be within the normal limits and unrelated to treatment. The viability index at 1000 mg/kg/day was lower than in the control group; this index was substantially affected by euthanization of the full litter (10 pups) from dam No. 78. The laboratory considered the lower viability index to be unrelated to treatment. It is noted that the viability index should have been calculated without including the litter from Dam #78, as she was euthanized, and the loss of her pups were an indication of maternal toxicity rather than a lack of maternal care or impaired pup health.

Table 5. Reproductive and litter parameters from the combined repeat-dose/DART screening test of Miranol Ultra C32 (De Raat-Beekhuijzen, 2018)

Dose (mg/kg/day):	0	100	300	1000
Mating index (%)	100	90	100	100
Fertility index (%)	80	89	90	89
Gestation index (%)	100	100	89	100
Mean precoital time (days)	2.1	2.2	2.9	2.6
Mean # implantations (± SD)	13.6 ± 1.8	13.5 ± 2.1	11.1 ± 3.9	13.1 ± 2.9
Gestation duration (days ± SD)	21.3 ± 0.5	21.5 ± 0.5	21.5 ± 0.5	21.8 ± 0.5
# dead pups at 1st litter check (# affected litters)	0 (0)	1 (1)	0 (0)	7 (4)
Mean # live pups at 1st litter check (± SD)	13.3± 2.0	11.4 ± 1.6	11.5 ± 1.1	11.0 ± 2.6
Mean postnatal loss (± SD)	0.0 ± 0.0	0.1 ± 0.4	0.3 ± 0.5	1.9 ± 3.4
Post-implantation survival index (%)	97	85	92	90
Live birth index(%)	100	99	100	93
Viability index (%)	100	99	98	83
Lactation index (%)	100	100	100	100

Clinical signs were observed in those pups that did not survive until scheduled sacrifice. Otherwise, no effects of treatment were observed on pup clinical signs, body weights, sex ratio,

anogenital distance, nipple retention, or serum T4 levels in male or female pups and no macroscopic findings related to treatment were observed at necropsy.

Based on these data, the laboratory concluded that the parental, reproductive and developmental NOAELs were all 1000 mg/kg/day, the highest dose tested. We agree with these calls.

Fish Early-Life Stage (FELS) Toxicity Test (Tobor-Kaplon, 2019)

This study was conducted in compliance with GLP and according to OECD TG No. 210 (2013). Fathead minnow (*Pimephales promelas*) were exposed to target concentrations of Miranol Ultra C32 (39.6%) of 0, 0.050, 0.13, 0.31, 0.78 and 2.0 mg solids/L in a flow-through system for 33 days. In preparing the test concentrations, a correction factor of 2.525 was applied to account for the water content of the test substance and concentrations are expressed based on solids. The test concentrations were selected based on results of a range-finding assay in which levels of embryonic mortality of 5%, 10%, and 40% were recorded at Miranol Ultra C32 test concentrations of 0.05, 0.5, and 5.0 mg solids/L, respectively; hatching was delayed by one day at the highest test concentration, and larval mortality rates of 5.3% and 75% were recorded at 0.5 and 5.0 mg solids/L, respectively. The definitive test was performed with 4 replicates of 20 eggs per group.

Methods in brief. The stages of embryonic development, hatching, survival and any abnormalities in appearance were assessed daily. At the end of the study, all surviving fish were weighed (blotted dry weights) and lengths were measured.

Results. At the target concentrations of 0.050, 0.13, 0.31, 0.78 and 2.0 mg solids/L, actual mean concentrations of 0.035, 0.090, 0.22, 0.61, and 1.6 mg solids/L were measured. The reason for the lower measured concentrations was not clear, but likely due to (a)biotic loss processes within the test system such as biodegradation and adsorption.

Parameters measured in the fish early life-stage test of Miranol Ultra C32 are shown in Table 6. Treatment had no effect on embryonic survival (i.e., the percent of embryos that hatched) or on larval survival (i.e., post-hatch mortality). However, exposure affected both larval body weight and length, with a significant effect on both parameters at the highest test concentration of 1.6 mg

solids/L. At this concentration, malformation of the caudal fin was observed in all larvae; at lower test concentrations, various abnormalities of the skeleton, eyes, swim bladder or other systems were recorded. Specific to the cardiac system, cardiac edema was reported for one larva of replicate A at 1.6 mg solids/L on Days 17 & 18 only.

Table 6. Measured parameters in the fish early life-stage test of Miranol Ultra C32 (Tobor-Kaplon, 2019)

Measured mg solids/L:	0	0.035	0.090	0.22	0.61	1.6
% Embryos hatched (Day 8)	99	100	94	100	95	95
% Post-hatch mortality (Day 33)	6	16	5	10	12	13
Mean body weight (± SD; Day 33)	74.450 ± 5.7356	73.850 ± 4.9776	76.550 ± 2.0469	69.150 ± 4.6658	74.475 ± 8.9481	46.025 ± 4.9026
% Body weight reduction (Day 33)	---	0.81	-2.8	7.1	-0.034	38*
Mean body length (± SD; Day 33)	21.21 ± 0.395	21.29 ± 0.335	21.43 ± 0.326	20.48 ± 0.268	21.26 ± 0.826	17.50 ± 0.709
% Body length reduction (Day 33)	---	-0.38	-1.0	3.5	-0.26	18*

* statistically significant

Based on these data, the laboratory considered the no observed effect concentration (NOEC) to be 0.61 mg solids/L for effects on larval growth and caudal fin malformation. The NOEC for embryonic hatching success and larval survival was 1.6 mg solids/L, the highest concentration tested. We agree with these calls.

PC-2020-926

Dose Range-finding Prenatal Study (Viends, 2022a)

In preparation for a definitive prenatal developmental toxicity study, a dose range-finding (DRF) study was conducted. The study was not GLP compliant but generally followed OECD TG No. 414 (2018) with exceptions for the number of animals per group, fetal visceral examinations limited to the heart and great vessels only, and no fetal skeletal examinations. Time-mated female Wistar Han rats (6/group) were dosed by oral gavage with 0, 300, 600 or 1000 mg/kg/day of PC-2020-926 in water at a dosing volume of 1.695 mL/kg. These formulations were adjusted to

account for water content of the test material using a correction factor of 2. Dosing was from GD 6 though GD 20.

Methods in Brief. Dams were checked twice daily for mortality/morbidity and at least once daily for clinical signs; detailed clinical observations were conducted on GD 2, 6, 15 and 21. Body weights and food consumption were measured on GDs 2, 6, 9, 12, 15, 18, and 21. On GD 21, dams were subjected to examination of the thoracic and abdominal cavities. Data regarding litter indices were collected. Uteri of non-pregnant rats were stained for identification of implantation sites. Livers were collected and weighed in the control and high dose group. All live fetuses were sexed, weighed, and examined for external anomalies. All live fetuses in each litter were also examined for visceral anomalies of the heart and great vessels. Fetal data were appropriately assessed based on litter means.

Results. No treatment-related mortality or clinical signs of toxicity were observed. Maternal body weight in the treated groups was comparable to control; mean body weight gain corrected for gravid uterine weight, however, was slightly lower at 1000 mg/kg/day compared to control. Absolute and relative liver weights at the high dose were comparable to control. Fetal litter parameters and malformation data are presented in Table 7. Compared to control, post-implantation loss was higher in the low dose group and pre-implantation loss was higher in the mid-dose group; no differences in these parameters were observed between the control and high dose group. No treatment-related effects were observed on the number of pregnant females per group, the numbers of corpora lutea and implantation sites, early and late resorptions, the numbers of live and dead fetuses per litter, fetal sex ratio, or fetal weights.

Table 7. Litter parameters and fetal anomalies data from the DRF prenatal developmental toxicity study of PC-2020-926 (Vriends, 2022a)

Dose (mg/kg/day):	0	300	600	1000
# Females on study	6	6	6	6
# Euthanized or died on study	0	0	0	0
# Pregnant at scheduled necropsy (%)	6 (100)	5 (83.3)	4 (66.7)	6 (100)
Litter parameters				
Mean # corpora lutea per litter (± SD)	12.3 ± 0.8	12.8 ± 1.3	12.5 ± 1.3	11.7 ± 2.0
Mean # implantations per litter (± SD)	11.2 ± 2.4	11.6 ± 3.3	9.5 ± 4.7	11.0 ± 1.7
Mean # dead fetuses per litter (± SD)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Mean # live fetuses per litter (± SD)	10.7 ± 2.3	10.4 ± 3.0	9.3 ± 4.5	10.8 ± 1.5
Mean # early resorptions per litter (± SD)	0.5 ± 0.5	1.2 ± 2.2	0.3 ± 0.5	0.2 ± 0.4
Mean # late resorptions per litter (± SD)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Mean % pre-implantation loss (± SD)	10.23 ± 14.44	10.63 ± 19.75	26.33 ± 32.74	5.46 ± 6.19
Mean % post-implantation loss (± SD)	4.23 ± 4.71	8.81 ± 15.47	1.92 ± 3.85	1.28 ± 3.14
% Males per litter	60.49	48.92	41.24	60.04
Mean male fetal weights per litter (± SD)	5.303 ± 0.215	5.203 ± 0.150	5.557 ± 0.218	5.220 ± 0.330
Mean female fetal weights per litter (± SD)	5.029 ± 0.253	4.971 ± 0.217	5.210 ± 0.080	4.917 ± 0.316

Results of the fetal external and visceral heart/great vessel examinations were reported in the individual animal data. No external or visceral malformations were observed.

Prenatal Developmental Toxicity Study (Vriends, 2022b)

This study was conducted in compliance with GLP and according to OECD TG No. 414 (2018). Time-mated female Wistar Han rats (22/group) were dosed by oral gavage with 0, 100, 300 or 1000 mg/kg/day of PC-2020-926 in water at a dosing volume of 1.695 mL/kg. These formulations were adjusted to account for water content of the test material using a correction factor of 2 and the top dose administered (1000 mg/kg/day) is the limit dose for this test. Dosing was from GD 6 though GD 20.

Methods in Brief. Dams were checked twice daily for mortality/morbidity and at least once daily for clinical signs; detailed clinical observations were conducted on GD 2, 6, 15 and 21. Body weights and food consumption were measured on GDs 2, 6, 9, 12, 15, 18, and 21. At sacrifice on

GD 21, blood was collected from the dams for assessment of thyroid hormones (T3, T4, and TSH). Dams were subjected to examination of the thoracic and abdominal cavities. Uterus and thyroid glands were weighed, and the thyroid collected for histopathologic examination. Data regarding litter indices were collected. Uteri of non-pregnant rats were stained for identification of implantation sites. All live fetuses were sexed, weighed and examined for external anomalies; AGD (normalized to the cube root of fetal body weight) was also measured. One half of the fetuses in each litter was examined by fresh dissection for visceral anomalies of the body and by Wilson's technique for visceral anomalies of the head. The other half of the fetuses in each litter was subjected to skeletal examination using Alizarin Red S staining. Fetal data were appropriately assessed based on litter means.

Results. No treatment-related mortality or clinical signs of toxicity were observed. At 300 mg/kg/day, a single dam was euthanized *in extremis* on GD 9 after exhibiting clinical signs, reduced food consumption and body weight loss; at necropsy, the intestines were found to be filled with gas. Because similar findings were not seen in other animals in this or the highest dose group, the death was considered to be unrelated to treatment. Increased salivation after dosing was observed in all dams at 1000 mg/kg/day; this finding was considered by the laboratory to be a physiological response to the test article rather than a clinical sign of toxicity. No treatment-related effects on body weight or food consumption were observed. Serum levels of T3, T4 and TSH were similar across treatment groups, and thyroid organ weights and histopathology were not changed from control.

Fetal litter parameters and malformation data are presented in Table 8. No treatment-related effects were observed on the number of pregnant females per group, the numbers of corpora lutea and implantation sites, early and late resorptions, pre- and post-implantation loss, the numbers of live and dead fetuses per litter, fetal sex ratio, fetal weights or fetal AGD measures.

Table 8. Litter parameters and fetal anomalies data from the prenatal developmental toxicity study of PC-2020-926 (Vriends, 2022b)

Dose (mg/kg/day):	0	100	300	1000
# Females on study	22	22	22	22
# Euthanized or died on study	0	0	1	0
# Pregnant at scheduled necropsy (%)	21 (95.5)	22 (100)	20 (95.2)	22 (100)
Litter parameters				
Mean # corpora lutea per litter (± SD)	12.6 ± 1.8	13.5 ± 1.7	13.6 ± 1.7	12.7 ± 3.0
Mean # implantations per litter (± SD)	12.0 ± 1.9	12.4 ± 1.7	12.9 ± 1.4	11.8 ± 2.3
Mean # dead fetuses per litter (± SD)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Mean # viable fetuses per litter (± SD)	11.4 ± 2.0	11.6 ± 2.0	12.2 ± 2.0	11.3 ± 2.3
Mean # early resorptions per litter (± SD)	0.6 ± 0.9	0.7 ± 0.9	0.8 ± 1.0	0.5 ± 0.7
Mean # late resorptions per litter (± SD)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Mean % pre-implantation loss (± SD)	4.88 ± 10.91	7.96 ± 7.40	4.84 ± 6.90	6.55 ± 7.65
Mean % post-implantation loss (± SD)	4.64 ± 7.23	6.03 ± 7.80	6.12 ± 7.96	3.66 ± 5.83
% Males per litter	50.82	46.36	50.00	50.89
Mean male fetal weights per litter (± SD)	5.207 ± 0.203	5.289± 0.253	5.297 ± 0.265	5.308 ± 0.348
Mean female fetal weights per litter (± SD)	4.960 ± 0.194	5.044 ± 0.205	4.998 ± 0.256	5.105 ± 0.265
Mean male corrected AGD (± SD)	1.794 ± 0.126	1.805 ± 0.076	1.782 ± 0.093	1.751 ± 0.103
Mean female corrected AGD (± SD)	0.828 ± 0.083	0.796 ± 0.068	0.822 ± 0.081	0.813 ± 0.104
Fetal anomalies data				
# Fetuses (litters) examined externally	239 (21)	256 (22)	243 (20)	249 (22)
# Fetuses (litters) with external malformations	0 (0)	1 (1)	0 (0)	0 (0)
# Fetuses (litters) examined viscerally	119 (21)	128 (22)	122 (20)	125 (22)
# Fetuses (litters) with visceral malformations	0 (0)	2 (2)	0 (0)	0 (0)
# Fetuses (litters) examined skeletally	120 (21)	128 (22)	121 (20)	124 (22)
# Fetuses (litters) with skeletal malformations	1 (1)	0 (0)	0 (0)	1 (1)
Total # fetuses (litters) with anomalies	1 (1)	2 (2)	0 (0)	1 (1)

The individual fetuses with malformations are shown in Table 9. No treatment related external, visceral, or skeletal malformations were observed. No visceral findings of the head were reported.

With regard to cardiovascular findings, we note that these were observed in two fetuses in the low dose groups only.

Table 9. Malformation data by individual fetuses for the prenatal developmental toxicity study of PC-2020-926 (Vriends, 2022b)

Dose (mg/kg/day):	Finding
0	Fetus 11-L1 – lumbar centrum, 1 or more absent (S)
100	Fetus 28-R7 – omphalocele (E), aortic arch interrupted (V), ventricular septal defect (V), trachea cartilage rings absent (V) Fetus 31-L6 – transposition of the great vessels (V), ventricular septal defect (V),
300	---
1000	Fetus 88-R10 – sternoschisis (S)

E = external finding; S = skeletal finding; V = visceral finding

No external variations were noted. Visceral variations were limited to supernumerary liver lobes, convoluted and dilated ureters and absent renal papilla, the incidences of which were unrelated to treatment. Skeletal examination revealed a diverse array of variations, none of which showed a relationship with treatment.

Based on these data, the laboratory concluded that, in the absence of any observed effects, the NOAELs for maternal toxicity and developmental toxicity were both 1000 mg/kg/day, the highest dose tested. We agree with these calls.

90-Day Study (Gerding, 2022)

This study was conducted in compliance with GLP and according to OECD TG No. 408; the exact version of the OECD guideline followed is not indicated, but based on when the study was completed, it is assumed to be the most recent (2018) version. Wistar Han rats (10/sex per group) were dosed for 90 days by oral gavage with 0, 100, 300 or 1000 mg/kg/day of PC-2020-926 in water at a dosing volume of 1.695 mL/kg. The dosing formulations were adjusted to account for water content of the test material using a correction factor of 2 and the top dose administered (1000 mg/kg/day) is the limit dose for this test. An additional 5/sex per group in the control and high dose groups were dosed as described and maintained for a 28-day recovery period post-dosing to address reversibility of any observed effects.

Methods in Brief. The rats were checked twice daily for mortality/morbidity, at least once daily for clinical signs and once weekly for arena observations. Body weights and food consumption were measured weekly. Estrus cyclicity was assessed in female rats at the end of treatment and at the end of the recovery period. Functional tests were conducted on 5 rats/sex per group in week 13. An ophthalmic examination was conducted on control and high dose rats in week 13. At sacrifice of both main study and recovery animals, blood was collected for assessment of hematology, clinical chemistry, and thyroid hormones (T3, T4, and TSH). At the end of both the main study and the recovery period, select organs were weighed (all groups) and tissues collected for histopathologic examination (control and high dose groups).

Results. No mortality was observed. Abnormal breathing sounds were noted in all treated groups, with a dose-dependent increase in incidence; deep, labored, or shallow breathing was also seen incidentally in all treated groups. Another noted clinical sign was retching in all female treated groups and in males at 100 and 1000 mg/kg/day. At ≥300 mg/kg/day, salivation and ploughing were noted, which the laboratory considered to be a physiological response to the taste of the test material and not toxicologically relevant. There were no effects of treatment on body weights, body weight gains or food consumption. With regard to functional observations, motor activity was reduced in females at 1000 mg/kg/day, but mean values were reported by the testing laboratory to be within the HCD range. No effects of treatment were observed in the ophthalmic examinations or with regard to estrous cyclicity. In males, red blood cell counts, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) were significantly increased and triglycerides non-significantly in males at 1000 mg/kg/day; these values were again reported by the laboratory to be within the HCD range, and at the end of the recovery period, comparable to control. T4 concentrations were increased in females at 100 and 1000 mg/kg/day but were considered by the laboratory to be within HCD range; no effects were seen on T3 or TSH concentrations. Compared to control, absolute weights of the kidneys and liver were significantly increased in females at 1000 mg/kg/day by 18% and 20%, respectively. The weights of these organs relative to body weight were also increased 12-14%, but these differences from control disappeared during the recovery period. At necropsy, 3 of 10 males at 1000 mg/kg/day were observed with minimal hyperplasia of the goblet cells of the rectum; no such findings were noted at the end of the recovery period. We are unaware of the significance of goblet cell hyperplasia.

Squamous cell hyperplasia of the non-glandular stomach (accompanied by hyperkeratosis) was seen in 1 of 10 and 3 of 10 females at 300 and 1000 mg/kg/day, respectively; this finding was not reported in high dose animals at the end of the recovery period. At 1000 mg/kg/day, focal erosion of the non-glandular and glandular portions of the stomach were also noted in 1 of 10 females at the end of the dosing period and in 1 of 5 females at the end of the recovery period; this finding was interpreted by the laboratory to be indicative of a dosing procedure-related event rather than an effect of treatment. We disagree with this interpretation in that the findings do not seem consistent with gavage error. Thus, we cannot discount that they may possibly be treatment related.

Based on these data, the NOAEL for systemic toxicity was determined by the laboratory to be 1000 mg/kg/day, the highest dose tested. In our opinion, the NOAEL in females may be 100 mg/kg/day based on squamous cell hyperplasia with hyperkeratosis of the non-glandular stomach in 1 and 3 females at 300 and 1000 mg/kg/day, respectively. The NOAEL for males may be 300 mg/kg/day based on goblet cell hyperplasia of the rectum in 3 males at 1000 mg/kg/day. It is noted, however, that the results of this study generally support the NOAEL for maternal toxicity determined in the prenatal developmental toxicity study of PC-2020-926 (1000 mg/kg/day), as histopathologic examination is not a part of the prenatal study design, and therefore, the findings of concern from the 90-day study would not have been observed. Further, the prenatal study involves dosing for a shorter duration; thus, it is very possible that the findings from the 90-day study would not have yet developed by the end of dosing in the prenatal study.

Sodium lauroamphoacetate

Dose Range-finding Prenatal Study (Langedijk, 2022)

In preparation for a definitive prenatal developmental toxicity study, a DRF study was conducted. The study was not GLP compliant but generally followed OECD TG No. 414 (2018) with exceptions for the number of animals per group, fetal visceral examinations limited to the heart and great vessels only, and no fetal skeletal examinations. Time-mated female Wistar Han rats (6/group) were dosed by oral gavage with 0, 300, 600 or 1000 mg/kg/day of sodium lauroamphoacetate in water at a dosing volume of 2.596 mL/kg. These formulations were adjusted

to account for the water content of the test material using a correction factor of 2.83. Dosing was from GD 6 though GD 20.

Methods in Brief. Dams were checked twice daily for mortality/morbidity and at least once daily for clinical signs; detailed clinical observations were conducted on GD 2, 6, 15 and 21. Body weights and food consumption were measured on GDs 2, 6, 9, 12, 15, 18, and 21. On GD 21, dams were subjected to examination of the thoracic and abdominal cavities. Data regarding litter indices were collected. Uteri of non-pregnant rats were stained for identification of implantation sites. All live fetuses were sexed, weighed, and examined for external anomalies. All live fetuses in each litter were also examined for visceral anomalies of the heart and great vessels. Fetal data were appropriately assessed based on litter means.

Results. A single female at the mid-dose was found dead on GD 15; this death was attributed to gavage error. No treatment-related mortality was observed. Clinical signs of abnormal breathing sounds were noted for individual animals in all treated groups. Maternal body weight in the treated groups was comparable to control. Fetal litter parameters and malformation data are presented in Table 10. Compared to control, the mean number of implantations and the mean number of live fetuses was significantly increased and mean fetal weights were significantly reduced in the high dose group. The reduced fetal weight at the high dose, however, appears to be a function of the increased number of fetuses per litter (13.3 vs 9.5 in the control group) as the mean total litter weight in the high dose group was much greater than that of the control group (66.1 g compared to 50.7 g in the control group).[1] No treatment-related effects were observed on the number of pregnant females per group, the numbers of corpora lutea, early and late resorptions, the number of dead fetuses per litter, or fetal sex ratio.

[1] Calculated based on reported mean number of fetuses per litter and mean fetal weights.

Table 10. Litter parameters and fetal anomalies data from the DRF prenatal developmental toxicity study of sodium lauroamphoacetate (Langedijk, 2022)

Dose (mg/kg/day):	0	300	600	1000
# Females on study	6	6	6	6
# Euthanized or died on study	0	0	1	0
# Pregnant at scheduled necropsy (%)	5 (83.3)	6 (100)	5 (83.3)	6 (100)
Litter parameters				
Mean # corpora lutea per litter (± SD)	10.8 ± 5.5	12.8 ± 0.4	12.2 ± 2.0	14.8 ± 1.0
Mean # implantations per litter (± SD)	10.2 ± 4.8	12.7 ± 0.8	11.4 ± 1.3	13.8* ± 1.2
Mean # dead fetuses per litter (± SD)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Mean # live fetuses per litter (± SD)	9.5 ± 4.8	11.3 ± 2.0	11.0 ± 1.2	13.3* ± 1.0
Mean # early resorptions per litter (± SD)	0.7 ± 0.8	1.3 ± 1.4	0.4 ± 0.5	0.3 ± 0.5
Mean # late resorptions per litter (± SD)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.2 ± 0.4
Mean % pre-implantation loss (± SD)	4.17 ± 7.57	1.39 ± 3.40	5.71 ± 7.82	6.69 ± 5.79
Mean % post-implantation loss (± SD)	20.40 ± 39.40	10.96 ± 11.61	3.33 ± 4.56	3.51 ± 3.88
% Males per litter	51.83	36.51	47.37	50.05
Mean male fetal weights per litter (± SD)	5.500 ± 0.350	5.617 ± 0.258	5.296 ± 0.272	5.111 ± 0.126
Mean female fetal weights per litter (± SD)	5.139 ± 0.225	5.192 ± 0.175	5.102 ± 0.172	4.815§ ± 0.126

* p≤0.05 by Kruskal-Wallis followed by Dunn test
§ p≤0.05 by ANOVA followed by Dunnet's test

Results of the fetal external and visceral heart/great vessel examinations were reported in the individual animal data. No external or visceral malformations were observed.

Prenatal Developmental Toxicity Study (van Otterdijk, 2022)

This study was conducted in compliance with GLP and according to OECD TG No. 414 (2018). Time-mated female Wistar Han rats (22/group) were dosed by oral gavage with 0, 100, 300 or 1000 mg/kg/day of the sodium lauroamphoacetate in water at a dosing volume of 2.596 mL/kg. These formulations were adjusted to account for the water content of the test material using a correction factor of 2.83 and the top dose administered (1000 mg/kg/day) is the limit dose for this test. Dosing was from GD 6 though GD 20.

Methods in Brief. Dams were checked twice daily for mortality/morbidity and at least once daily for clinical signs; detailed clinical observations were conducted on GD 2, 6, 15 and 21. Body

weights and food consumption were measured on GDs 2, 6, 9, 12, 15, 18, and 21. At sacrifice on GD 21, blood was collected from the dams for assessment of thyroid hormones (T3, T4, and TSH). Dams were subjected to examination of the thoracic and abdominal cavities. Uterus and thyroid glands were weights and the thyroid collected for histopathologic examination. Data regarding litter indices were collected. Uteri of non-pregnant rats were stained for identification of implantation sites. All live fetuses were sexed, weighed and examined for external anomalies; AGD (normalized to the cube root of fetal body weight) was also measured. One half of the fetuses in each litter was examined by fresh dissection for visceral anomalies of the body and by Wilson's technique for visceral anomalies of the head. The other half of the fetuses in each litter was subjected to skeletal examination using Alizarin Red S staining. Fetal data were appropriately assessed based on litter means.

Results. No treatment-related mortality or clinical signs of toxicity were observed. At 300 mg/kg/day, a single dam was found dead on GD 12 due to gavage error. At ≥300 mg/kg/day, rats exhibited increased salivation after dosing, which was considered by the laboratory to be a physiological response to the test article rather than a clinical sign of toxicity. Also, 2 and 4 rats at 300 and 1000 mg/kg/day, respectively, exhibited abnormal breathing sounds between GD 8 and GD 18, typically on a single day of treatment. A transient body weight loss upon initiation of treatment (GD 6-9 interval) was reported for 2 and 3 dams at 300 and 1000 mg/kg/day, respectively. Otherwise, no significant differences from control were observed for body weight or body weight gains. Compared to the control group, food consumption was significantly reduced for the dosing intervals of GD 6-9 and GD 9-12 at doses of 300 mg/kg/day (~10%) and 1000 mg/kg/day (~18-20%). Food consumption from GD 6 to GD 21, was significantly reduced at 1000 mg/kg/day by ~10% compared to control. At 1000 mg/kg/day, mean serum concentrations of total T3 were ~77% of control values, although the individual values were within the HCD range. No treatment related effects were noted on serum concentrations of T4 or TSH, and thyroid organ weights and histopathology were not changed from control. At necropsy, 12 of 22 dams at 1000 mg/kg/day exhibited irregular surface of the glandular stomach.

Fetal litter parameters and malformation data are presented in Table 11. No treatment-related effects were observed on the number of pregnant females per group, the numbers of corpora lutea

and implantation sites, early and late resorptions, pre- and post-implantation loss, the numbers of live and dead fetuses per litter, fetal sex ratio, fetal weights or fetal AGD measures.

Table 11. Litter parameters and fetal anomalies data from the prenatal developmental toxicity study of sodium lauroamphoacetate (van Otterdijk, 2022)

Dose (mg/kg/day):	0	100	300	1000
# Females on study	22	22	22	22
# Euthanized or died on study	0	0	1	0
# Pregnant at scheduled necropsy (%)	21 (95.5)	21 (95.5)	20 (95.2)	22 (100)
Litter parameters				
Mean # corpora lutea per litter (± SD)	13.0 ± 1.6	12.7 ± 2.1	12.9 ± 1.4	13.0 ± 2.1
Mean # implantations per litter (± SD)	12.2 ± 1.8	11.2 ± 2.31	12.2 ± 1.5	12.4 ± 2.4
Mean # dead fetuses per litter (± SD)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Mean # viable fetuses per litter (± SD)	11.9 ± 1.6	10.9 ± 2.5	11.8 ± 1.6	12.0 ± 2.6
Mean # early resorptions per litter (± SD)	0.3 ± 0.7	0.4 ± 0.8	0.4 ± 0.5	0.4 ± 1.1
Mean # late resorptions per litter (± SD)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Mean % pre-implantation loss (± SD)	5.71 ± 8.38	11.69 ± 11.69	5.29 ± 7.21	4.78 ± 9.22
Mean % post-implantation loss (± SD)	2.46 ± 4.75	3.73 ± 8.24	3.29 ± 4.15	3.39 ± 9.85
% Males per litter	48.28	46.24	51.93	48.74
Mean male fetal weights per litter (± SD)	5.275 ± 0.290	5.334 ± 0.323	5.378 ± 0.179	5.246 ± 0.272
Mean female fetal weights per litter (± SD)	4.987 ± 0.237	5.074 ± 0.260	5.065 ± 0.192	4.980 ± 0.338
Mean male corrected AGD (± SD)	1.774 ± 0.093	1.755 ± 0.113	1.730 ± 0.099	1.759 ± 0.109
Mean female corrected AGD (± SD)	0.804 ± 0.090	0.798 ± 0.089	0.778 ± 0.096	0.783 ± 0.080
Fetal anomalies data				
# Fetuses (litters) examined externally	249 (21)	228 (21)	235 (20)	263 (22)
# Fetuses (litters) w/ external malformations	0 (0)	0 (0)	0 (0)	1 (1)
# Fetuses (litters) examined viscerally	124 (21)	115 (21)	119 (20)	132 (22)
# Fetuses (litters) w/ visceral malformations	0 (0)	1 (1)	0 (0)	0 (0)
# Fetuses (litters) w/ head malformations	2 (2)	0 (0)	1 (1)	0 (0)
# Fetuses (litters) examined skeletally	125 (21)	113 (21)	116 (20)	131 (22)
# Fetuses (litters) w/ skeletal malformations	0 (0)	0 (0)	0 (0)	0 (0)
Total # fetuses (litters) with anomalies	2 (2)	1 (1)	1 (1)	1 (1)

The individual fetuses with malformations are shown in Table 12. No treatment related external, visceral, or skeletal malformations were observed. Further, no cardiovascular findings were noted.

Table 12. Malformation data by individual fetuses for the prenatal developmental toxicity study of sodium lauroamphoacetate (van Otterdijk, 2022)

Dose (mg/kg/day):	Finding
0	Fetus 05-L2 – small eye lens, right (V) Fetus 16-R7 – small eye, right (V)
100	Fetus 39-L5 – situs inversus (V)
300	Fetus 48-L9 – large eye lens, left (V)
1000	Fetus 84-L3 – subcutaneous edema (E)

E = external finding; S = skeletal finding; V = visceral finding

No external variations were noted. Visceral variations of supernumerary liver lobes, convoluted ureters, and a fluid-filled thorax were seen, but the incidences were considered unrelated to treatment. A diverse array of skeletal variations was noted across all groups without relation to treatment.

Based on these data, the laboratory concluded that, in the absence of any observed effects, the NOAELs for maternal toxicity and developmental toxicity were both 1000 mg/kg/day, the highest dose tested. We agree with these calls.

3. Assessment

A total of four commercial amphoacetate surfactant products have been evaluated in combined repeat-dose reproduction and developmental toxicity screening tests or prenatal developmental toxicity studies conducted in Wistar Han rats. In all definitive studies, mated rats received the test article by oral gavage at doses of 0 (control), 100, 300 or 1000 mg/kg/day. In the combined repeat-dose and reproduction/developmental toxicity screening tests, dosing occurred for two weeks prior to mating and throughout gestation. In the prenatal developmental toxicity studies, the treatment period occurred during presumed GDs 6-19.

In the five reproductive and developmental studies under consideration, the parental and developmental NOAELs were the highest dose tested (1000 mg/kg/day) for all test articles except Dehyton® DC. It is additionally noted that the developmental NOAEL for Miranol Ultra C32 was not based on a prenatal development toxicity study, but rather, on results from the 28-day repeat-dose reproductive and developmental toxicity screening test.

Parental NOAELs

Although Dehyton® DC was tested in a prenatal development toxicity study, it was also the subject of a 28-day combined repeat-dose and reproductive and developmental toxicity screen test wherein four of ten high-dose females died or exhibited signs of toxicity, resulting in premature euthanasia of the entire group on GD 14 due to humanitarian concerns. The premature euthanasia of pregnant dams, in turn, meant that there were no high dose (1000 mg/kg/day) fetuses to examine at term and the mid dose (300 mg/kg/day) became the *de facto* NOAEL for both maternal and developmental toxicity. The study director considered the maternal deaths to be test article related, which contributed to the determination of the mid-dose as the NOAEL. However, the data surrounding this call are complex and require further discussion.

Necropsies of the four deceased high-dose dams revealed morphologic findings in the respiratory tracts including erosions and/or ulceration of epithelium of the trachea and bronchi. There were no reports of irritation or ulceration in the esophagi of these animals. The study director ascribed

the findings to "regurgitation" and considered the findings to be test article related; however, necropsies of the remaining six high-dose dams found no lesions in the esophagus, trachea or bronchi. The absence of respiratory tract findings in any of the other female rats in this group, the absence of findings in the esophagus (which must be traversed to reach the respiratory tract in cases of regurgitation), the inability of the rat to vomit, and the lack of similar findings among the male rats suggest that the limited number of lesions may have been due to the gavage procedure. Importantly, neither the prenatal development toxicity study nor the 90-day oral gavage study of Dehyton DC in rats conducted at the same dose levels reported any lesions of the esophagus or respiratory tract. The absence of respiratory effects is of interest because of the much longer dosing periods (~50 days versus 90 days). There is, however, a difference between the dose volumes administered in the repeat-dose 28-day study (5 mL/kg) versus the other two studies 1.796 mL/kg and 1.895 mL/kg). It is likely that the reduced dosing volume reduced the likelihood of the dosing fluid escaping from the stomach. Notably, due to the differences in dosing volume, the concentration of test article at the high dose of 28-day study was 200 mg/mL whereas the concentration in the 90-day study was 557 mg/mL. In the 90-day study of PC-2020-926 (high-dose volume of 1.695 mL; concentration of test article: 590 mg/mL), the stomachs of females in the high (3/10) and mid-dose (1/10) groups exhibited epithelial hyperplasia/hyperkeratosis of the non-glandular stomach. These findings were not present at the end of the recovery period. No macroscopic changes of the viscera were reported in the prenatal development toxicity study of PC-2020-926; however, no histopathology of the stomach was conducted. Taken together, it is not possible to exclude that the lesions in the respiratory tracts of high-dose Dehyton DC treated dams were the result of the gavage procedure; consequently, these findings in the respiratory tract should not be considered as the basis for maternal toxicity. Taken in combination with the determination that 1000 mg/kg/day is the NOAEL for maternal/adult female toxicity in the other two studies, 1000 mg/kg/day might more appropriately be considered the maternal NOAEL for Dehyton® DC in the 28-day combined repeat-dose and reproductive and developmental toxicity screen test.

Developmental NOAELs

The developmental toxicity potential of the amphoacetates as a group appears to be low. With the exception of Dehyton® DC, the study directors determined the developmental toxicity NOAEL

for each of the amphoacetates to be 1000 mg/kg/day. However, due to the occurrence of several cardiovascular malformations in all Dehyton® DC treated groups in the prenatal development toxicity study, the study director did not identify a developmental NOAEL.

The collated data for this assessment were obtained from investigations of four commercial amphoacetate surfactant products with remarkably similar chemical structures (varying only in C-chain length and the proportion/ratio of monoacetate and diacetate forms). Their structural and compositional similarity allows for the findings across these compounds to be grouped, provided that the study designs are comparable.

Among the study reports supplied to us were two DRF prenatal developmental toxicity studies and three prenatal development toxicity studies conducted using Wistar Han rats. Visceral malformations of the cardiovascular system were observed in two of the three definitive studies; no cardiovascular malformations were seen in the two DRF studies, despite specific examination of the fetal hearts and great vessels in these studies . Because cardiac and other cardiovascular malformations can only be detected in prenatal development toxicity studies, the following assessment is based on visceral data for the three amphoacetates (Dehyton® DC, PC-2020-926, and sodium lauroamphoacetate) that were tested in definitive prenatal development toxicity studies. Low incidences of cardiac and great vessel malformations were observed in treated groups of two of the three studies. Consequently, it was deemed important to determine whether these compounds as a group might alter cardiovascular development. Since the test articles share similar chemical structures and all definitive studies were conducted using the same dose levels (0 [control], 100, 300, or 1000 mg/kg/day), it is instructive to display the cardiovascular malformations, as presented in Table 13 below.

Table 13. Cardiovascular Malformations Reported in Embryofetal Definitive Prenatal Development Toxicity Studies of Amphoacetates[a]

Dose (mg/kg/d)	Test Article	VSD	Trans of Great Vessels	Inter-rupted Aortic Arch	Right Sided Aortic Arch	Ductus Arteriosus Absent	Ductus Arteriosus Retro-Esophageal	# Affected Fetuses	Total # Fetuses Examined Viscerally
0	D	--	--	--	--	--	--	--	115
	P	--	--	--	--	--	--	--	119
	L	--	--	--	--	--	--	--	124
100	D	1	1	1			1	2	117
	P	2	1	1	--	--	--	2	128
	L	--	--	--	--	--	--	--	115
300	D	1				1	--	1	107
	P	--	--	--	--	--	--	--	122
	L	--	--	--	--	--	--	--	119
1000	D	1	--	--	1	--	--	1	114
	P	--	--	--	--	--	--	--	125
	L	--	--	--	--	--	--	--	132

D = Dehyton DC; P = PC-2020-926; L = sodium lauroamphoacetate; VSD = Ventricular Septal Defect

[a] The heart and great vessels were evaluated in the DRF studies for PC-2020-926 and sodium lauroamphoacetate and no malformations were found; however, because these studies were conducted with only 6 dams per group and used different doses, they were not included in this table.

Further support for the absence of compound-related cardiovascular defects for PC-20-926 and sodium lauroamphoacetate is available from the preliminary dose range finding studies (DRFs) for these chemicals. In both cases, the test agents were administered at doses of 0, 300, 600 or 1000 mg/kg bw/day via oral gavage from GD 6 – GD 19 to groups of 6 mated rats. Test agents did not increase the resorption rates or decrease the mean number of pups per litter. Mean fetal weights were not adversely affected. Fetuses were subjected to gross examination and a modified visceral examination that included the great vessels and the heart. No visceral malformations were reported in either DRF study.

Assessment by type of malformation

The overall incidence of malformations reported across the studies, both within individual studies and in all studies in total, were low. However, most malformations identified during the visceral examinations were related to the cardiovascular system (heart and great vessels). Among the three definitive prenatal development toxicity studies, the majority of the cardiovascular findings (8 of 12) were in the low dose groups and the incidences were not dose responsive. Thus, the individual malformations data do not support there being an effect due to treatment with the amphoacetates.

Further, it does not appear that these findings are indicative of a low dose effect. The condition when adverse effects occur at low doses, but not at higher doses, has been termed a "non-monotonic" dose response relationship (Vandenberg et al, 2012). Much of the data to support this concept comes from cell-based systems and involves hormones or endocrine disrupting chemicals (Vandenberg, 2012); however, data in whole animals is scant (Rhomberg and Goodman, 2012). In the case of developmental data, what may seem to be a non-monotonic effect (i.e., malformations observed in low dose animals but not in the higher dose groups) occasionally occurs because the higher doses either kill the offspring or cause severe toxicity in the pregnant dam such that she either resorbs her litter or dies. In either case, there would be fewer (or no) exposed near-term fetuses to examine in the higher dose groups, and those that remain represent a less sensitive population. In the current data set, however, there were no significant incidences of severe maternal toxicity or total litter losses at the higher doses.

Assessment by malformed fetus

The embryologic development of the heart and the aortic arch system that gives rise to the great vessels is complex. Perturbations of the morphogenetic processes involved in development of this region underlie multiple malformations that often occur together. Merely counting the number of malformations often overstates the significance of the problem. Among the three prenatal development toxicity studies considered in the present report, there were a total of 12 cardiovascular findings that occurred in a total of 6 fetuses. The distribution of malformed fetuses included 4 fetuses in the combined low dose groups and one fetus in each of the combined mid- and high-dose groups. Again, there was no dose-response when considered on a malformed fetus basis.

Note that cardiovascular malformations can be detected only during the visceral examinations that are conducted as part of a prenatal development toxicity study. As a result, roughly one half of the fetuses were examined viscerally. The grand totals of fetuses that underwent visceral examinations in the combined dose groups of the three prenatal development toxicity studies were:

0 mg/kg bw/day:	358 fetuses
100 mg/kg bw/day:	360 fetuses
300 mg/kg bw/day:	348 fetuses
1000 mg/kg bw/day:	371 fetuses

Inspection of the table reveals that a total of 12 cardiovascular malformations were reported in the treated groups; however, the malformations occurred in a total of 6 fetuses. Notably, 8 of the cardiovascular malformations (in 4 fetuses) occurred in the low dose group; two cardiovascular malformations in a single fetus were reported in each of the mid- and high dose groups. Thus, there is no dose-response when the data are considered on either a malformation basis or on the basis of malformed fetuses.

Assessment by perturbed morphogenetic process

Despite there being no clear dose-response for the observed cardiovascular findings, it is important to understand if there is a link between amphoacetate exposure and congenital heart defects. First, it should be recognized that congenital heart malformations are rarely reported in rats, perhaps due to the small size of their hearts. This means that a finding could be missed if one relies on only a single study. Among these three studies, there were low incidences of cardiac and great vessel malformations in two of the three studies.

Second, the anatomy of the heart and its embryology are complex. Because different individual cardiac defects can be caused by perturbation of a single morphogenetic process, it is possible to combine the findings of defects that result from the same morphogenetic process. As an analogy, consider for example the effect an earthquake might have on a town. The major road passes multiple buildings (e.g., the church, the school, the town hall, etc.). Because the road is destroyed, the entrances to each of these buildings may also be ruined. In a damage report, the entrances to

each of the affected buildings may be all reported separately; however, the underlying event that caused the damage (the earthquake-induced damage to the major road) is the same in all cases. The changes at each building are not independent events. In another town, the set of affected buildings due to the earthquake-induced damaged major road may be different (e.g., the department store, the firehouse, the police station), but the underlying problem is the same. By looking only at individual buildings, one can miss the bigger issue, which is that all findings were caused by the damaged major road. The situation is similar with regard to teratogen-induced malformations. The rationale for grouping defects by the embryologic process that might be perturbed is based on the well-accepted tenet that teratogens interact with embryos via specific mechanisms to cause malformation (Wilson, 1959; 1973). With regard to cardiac embryology, a set of morphogenetic processes has been proposed to underlie most of the major anatomic features of the heart and great vessels (Clark, 1986). Using an approach that allows for the grouping of various cardiac defects by a common perturbed morphogenetic process has been used to assess the potential teratogenicity of other substances (e.g., Watson et al, 2006). Additionally, it must be recognized that a given teratogen may cause malformations by means of multiple mechanisms (DeSesso and Goeringer, 1990).

In an attempt to increase the likelihood of discerning a potential class effect of amphoacetate exposure, we combined the results of all three definitive prenatal development toxicity studies and grouped the reported cardiac defects according to the underlying morphogenetic process that would have been perturbed. Thus, the reported malformations can be sorted as follows.

> Cellular migration and targeted growth. Cardiac neural crest cells and cells from the pharynx migrate into the heart and great vessels to form a population of cells that grow into the lumen of the truncus arteriosus, where they underlie successful development of the aorticopulmonary septum and the membranous portion of the interventricular septum. Perturbation of these processes can result in transposition of the great vessels and membranous ventricular septal defect. Thus, the incidences of these two malformations can be combined for analysis.
>
> Hemodynamics and cellular death. Remodeling of the aortic arch system depends upon differential blood flow strength and patterns (hemodynamics) and removal of unnecessary

potions of the vessels (controlled cellular death). Perturbations of these processes can result in aberrant great vessel patterns, including interrupted aortic arch, right sided aortic arch, and absence/misplacement of the ductus arteriosus. Thus, the incidence of these malformations can be pooled for analysis.

The merged malformation data categorized by morphogenetic process are displayed in Table 14 below. Most incidences of perturbed morphogenetic processes occurred in the low dose group, with only single occurrences in each of the mid- and high dose groups. Thus, there is no indication of a dose-response for either of the morphogenetic processes that underlie the cardiovascular malformations reported in the prenatal development toxicity studies.

Table 14. Incidence of Cardiovascular Malformations Grouped by Perturbed Morphogenetic Process in Combined Embryofetal Development Studies of Amphoacetates

Dose (mg/kg/day)	Cellular Migration & Targeted Growth	Hemodynamics and Targeted Cellular Death
0	0	0
100	5	3
300	1	1
1000	1	1

Taken together, the combined data do not support a causal relationship between the amphoacetates tested in the prenatal development toxicity studies and malformations of the heart and great vessels.

Non-mammalian Data

The results of the Fish Early-life-Stage (FELS) Toxicity Test using Miranol Ultra C32 (Tobor-Kaplon, 2019) are consistent with the mammalian data in that no gross alterations in cardiac development were reported. However, it must be noted fish hearts differ substantially from mammalian hearts in that fish hearts have only 2 chambers, undergo limited cardiac looping, and have a single circuit circulatory system making cardiovascular development much simpler than in mammals (Tang et al, 2018; Barresi and Gilbert, 2020). Further, the observations in the FELS

test were limited to gross changes in body form and/or concurrent aberrant behavioral during development rather than detailed observation of cardiac development. Observations of the heart included verification of beating; however, the rate of cardiac rhythmicity was not measured and estimates of stroke volume and cardiac output were not made. The latter data, although not typically collected in a FELS test, would have provided more critical indications of normal development (Burggren and Blank, 2009). Nevertheless, the absence of significant cardiac findings at any dose and the overall survival of fish throughout the study provide no reason to suggest that the cardiovascular system is a specific target organ for toxic effects from exposure to Miranol Ultra C32 and, by extrapolation, to the other amphoacetates reviewed herein.

There were, however, post-hatching malformations of the caudal (tail) fin observed among developing larvae in the high exposure (1.6 g/L) group. In the lower exposure groups, some larvae developed non-dose dependent, minor abnormalities that were considered not treatment related. None of these findings were associated with cardiac development.

Potential Role for Impurity

One of the ingredients used in the synthesis of amphoacetates is AEEA and residual amounts may be found in the finished products as an impurity (Foti et al., 2001). To test whether AEEA could cause adverse effects on reproduction and development, Schneider et al. (2012) performed a repeated dose and reproductive and developmental toxicity screening test (OECD 421) with AEEA in rats by oral gavage doses at doses of 0, 50, 250, or 1000 mg/kg/day. The results of that initial experiment and a follow-on experiment in the same study conducted at 0, 0.2, 1, 5, or 50 mg/kg/day found malformations of the great vessels at doses of ≥50 mg/kg/day. The NOAEL for these findings was thus 5 mg/kg/day. These malformations consisted of high aortic arch, aberrant course of the carotid arteries, and aneurysms in the walls of the aorta. Moore et al. (2012) confirmed the great vessel findings and determined that prenatal exposure was sufficient to cause the great vessel anomalies. Importantly, the findings produced by AEEA were all in the great vessels (although considerably different from the defects reported with the amphoacetates) and did not include the cardiac malformations (VSD) reported in the amphoacetate studies under consideration here.

Foti et al. (2001) measured the amounts of AEEA in a variety of preparations of all four amphoacetates used in cosmetics, the AEEA amounts were small and ranged between 4.9-15.3 ppm in the test samples.[2] AEEA analyses were conducted for all amphoacetates discussed in this report and the highest level (14 ppm) was measured in the C8-C18 amphoacetates (Appendix Table). Based on the maximum estimated concentration of residual AEEA (15.3 ppm, Foti et al, 2001), the highest dose of amphoacetates administered in the studies discussed herein (1000 mg/kg/day) would result in a potential AEEA exposure that is 2 orders of magnitude below the NOAEL of 5 mg/kg/day determined by Schneider et al. (2012).[3] It can be thus concluded that residual AEEA in the amphoacetates preparations did not cause the cardiac defects observed in the studies under consideration in this report.

[2] One measurement of Miranol HM Special (1130 ± 30 ppm) was much higher than all others and was stated to have been likely due to faulty purification of the sample.

[3] At 15.3 ppm (15.3 µg/1000 mg), an amphoacetates dose of 1000 mg/kg/day would translate to 15.3 µg/kg/day of AEEA, which is 327x below the AEEA NOAEL of 5 mg/kg/day or 5000 µg/kg/day.

4 Conclusions

The developmental and reproductive toxicity (DART) properties of four commercial amphoacetate surfactant products (Dehyton® DC, Miranol Ultra C32, PC-2020-265, and sodium lauroamphoacetate) were evaluated at doses of 0 (control), 100, 300 or 1000 mg/kg/day. Because there were few adverse fetal cardiovascular findings, the available data from all of the amphoacetates were combined to maximize the ability to discern the presence of adverse reproductive or developmental effects. The parental and developmental NOAELs in the three prenatal developmental toxicity studies (for Dehyton® DC, PC-2020-265, and sodium lauroamphoacetate,) were the highest dose tested (1000 mg/kg/day). The maternal NOAELs for the prenatal developmental toxicity studies of Dehyton® DC and PC-2020-926 are generally supported by results from their respective 90-day repeat-dose studies. It is noted, however, that for Dehyton® DC due to perceived maternally toxic effects at the high dose in the combined 28-day repeat-dose and reproduction/developmental toxicity screening test (OECD 422), the high dose dams were euthanized at GD 14, which precluded examination of fetuses at term and necessitated the call of NOAELs at the next lower dose (300 mg/kg/day). The developmental NOAEL for the fourth amphoacetate (Miranol Ultra C32) was also determined to be 1000 mg/kg/day, but that assessment was based on an OECD 422, which does not include visceral examination.

A low incidence of cardiac / great vessel malformations occurred in each of the three prenatal developmental toxicity studies. None of the malformations was significantly increased and, within each study, the greatest number of malformations occurred in the low dose group. Increased maternal toxicity and/or resorptions/post-implantation loss did not occur at higher doses; thus, there is no evidence to support this being a low-dose effect. In order to discern if there might be a trend for production of cardiovascular malformations, the data for all three definitive prenatal developmental toxicity studies were combined. Whether the combined data were assessed based on the incidences of malformations, number of malformed fetuses, or underlying perturbed morphogenetic processes, there was neither statistical significance nor a dose responsive increase.

Residual amounts of a starting material used to synthesize amphoacetates (AEEA) was also evaluated as a potential causative agent. While AEEA has been reported to cause aneurysms of the great vessels and alterations in the pattern of distribution of the vessels, it did not cause heart defects or any of types of the vessel defects observed in the subject amphoacetate studies. Additionally, the NOAEL for AEEA developmental toxicity is two orders of magnitude above the highest potential AEEA exposure that might occur due to amphoacetate exposure in the studies reviewed herein. Thus, AEEA is not likely to be a factor in any of the defects observed in the subject studies.

Taken together, in-depth analyses of the available developmental and reproductive data for the four subject amphoacetates do not support the classification of these substances as reproductive or developmental hazard. Likewise, in-depth analysis of the cardiac and great vessel systems of fetuses exposed to Dehyton® DC, PC-2020-265, and sodium lauroamphoacetate at doses as high as the limit dose does not support that these substances cause malformation of the target area. This conclusion is also supported by the absence of any treatment-related cardiac abnormalities in both the FELS toxicity test of Miranol Ultra C32 and the dose range-finding studies for PC-2020-265, and sodium lauroamphoacetate (which included visceral examinations of fetal hearts).

5 Limitations

The purpose of this analysis is limited to a review the results from the available DART studies of amphoacetates. This assessment is based on review of the individual study reports, and the authors' combined expertise in developmental and reproductive toxicology. The opinions presented herein are made to a reasonable degree of scientific certainty. Exponent reserves the right to supplement this report and to expand or modify the conclusions and findings based on the review of additional materials as they become available through additional work, or through the review of additional work performed by others. The scope of services performed during this investigation may not adequately address the needs of other users of this report, and any re-use of this report or its findings, conclusions or recommendations as presented herein are at the sole risk of the user.

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Appendix

Table. Selected Amphoacetates – Identification and Characteristics.

Identification: Amphoacetates C8-C18		
	Type of substance:	UVCB Monoacetate form (contains appr. 95% monoacetates and 5% diacetates) Diacetate form (contains appr. 40% monoacetates and 60% diacetates)
	IUPAC name:	Reaction products of 1H-Imidazole-1-ethanol, 4,5-dihydro-, 2-(C7-C17 odd-numbered, C17-unsatd. alkyl) derivs. and sodium hydroxide and chloroacetic acid
	Synonyms:	C8-18 Amphoacetates Sodium Cocoamphoacetate Dehyton® DC (Diacetate form) Miranol Ultra C32 (Monoacetate form)
	CAS Number:	-
	Alternative CAS numbers	68650-39-5; 68334-21-4; 68390-66-9; 61791-32-0; 90387-76-1; 68608-65-1
	EC/List Number:	931-291-0
	Molecular Weight (for the CSA):	446 g/mol
	Compositional information (as manufactured, w/w)	
	Water	47-64%
	Total solids:	36-53%
	Total alkylamphoacetate derivatives	27-43%
	NaCl	0-15%
	Sodium glycolate	0-6%
	Alkyl amidoamine	0-3%
	Sodium chloroacetate	0-600 ppm

<table>
<tr><th colspan="3">Identification: Amphoacetates C8-C18</th></tr>
<tr><td></td><td>2-(2-aminoethylamino)ethanol</td><td>0-6 ppm</td></tr>
<tr><td></td><td>Compositional information (solvent free condition, w/w)</td><td></td></tr>
<tr><td></td><td>Total alkylamphoacetate derivatives</td><td>65-86%[4]</td></tr>
<tr><td></td><td>Alkyl chain distribution, Cn</td><td>
<table>
<tr><th>Cn</th><th>Mono#</th><th>Di#</th><th>Total</th></tr>
<tr><td>C8</td><td>0-11%</td><td>0-2%</td><td>0-11%</td></tr>
<tr><td>C10</td><td>0.1-10%</td><td>0-2%</td><td>0-11%</td></tr>
<tr><td>C12</td><td>16-56%</td><td>0-36%</td><td>42-64%</td></tr>
<tr><td>C14</td><td>5-20%</td><td>0-15%</td><td>6-26%</td></tr>
<tr><td>C16</td><td>1-22%</td><td>0-8%</td><td>4-22%</td></tr>
<tr><td>C18</td><td>0.1-16%</td><td>0-7%</td><td>0.1-18%</td></tr>
<tr><td>C18:1 and/or C18:2 [5]</td><td>0-9%</td><td>0-12%</td><td>0-20%</td></tr>
</table>
</td></tr>
<tr><td></td><td>NaCl</td><td>0-26%</td></tr>
<tr><td></td><td>Sodium glycolate</td><td>0-12%[6]</td></tr>
<tr><td></td><td>Alkyl amidoamine</td><td>0-6%</td></tr>
<tr><td></td><td>Sodium chloroacetate</td><td>0-1500ppm</td></tr>
<tr><td></td><td>2-(2-aminoethylamino)ethanol</td><td>0-14ppm</td></tr>
</table>

Identification: Amphoacetates C12-C14		
	Type of substance:	UVCB Diacetate form only (contains appr. 40 to 45% monoacetate and 55 to 60 % diacetates)
	IUPAC name:	Reaction products of 1H-Imidazole-1-ethanol, 4,5-dihydro-, 2-(C11-C13 odd-numbered alkyl) derivs. and sodium hydroxide and chloroacetic acid
	Synonyms:	Acetic acid, 2-chloro-, reaction products with 2-C11-13-alkyl-4,5-dihydro-1H-imidazole-1-ethanol and sodium hydroxide

[4] The lower range figure for the surfactant fraction is due to the greater difficulty in drying the C8-18 substance and residual water

[5] Number of unsaturations per C18 alkyl chain: 0.001 - 0.15

[6] Analysed as glycolic acid and converted to sodium glycolate as this is the form more likely present in the UVCB substance. Compositional information in registration dossiers may be given as glycolic acid (due to the analytical method) and/or can be also converted to the sodium salt.

<table>
<tr><th colspan="3">Identification: Amphoacetates C12-C14</th></tr>
<tr><td></td><td></td><td>C12-14 Amphoacetates
PC-2020-926
Rewoteric AM2L</td></tr>
<tr><td></td><td>CAS Number:</td><td>1689515-39-6</td></tr>
<tr><td></td><td>Alternative CAS numbers</td><td>66161-62-4; 68608-66-2</td></tr>
<tr><td></td><td>EC/List Number:</td><td>938-645-3</td></tr>
<tr><td></td><td>Molecular Weight (for the CSA):</td><td>367 g/mol</td></tr>
<tr><td></td><td>Compositional information (as manufactured, w/w)</td><td></td></tr>
<tr><td></td><td>Water</td><td>50-51%</td></tr>
<tr><td></td><td>Total solids:</td><td>49-50%</td></tr>
<tr><td></td><td>Total alkylamphoacetate derivatives</td><td>≥39%</td></tr>
<tr><td></td><td>NaCl</td><td>0-10%</td></tr>
<tr><td></td><td>Sodium glycolate</td><td>2-4%</td></tr>
<tr><td></td><td>Alkyl amidoamine</td><td>0-2%</td></tr>
<tr><td></td><td>Sodium chloroacetate</td><td>0-65 ppm</td></tr>
<tr><td></td><td>2-(2-aminoethylamino)ethanol</td><td>0-5 ppm</td></tr>
<tr><td></td><td>Compositional information (solvent free condition, w/w)</td><td></td></tr>
<tr><td></td><td>Total alkylamphoacetate derivatives</td><td>≥78%</td></tr>
<tr><td></td><td>Alkyl chain distribution, Cn</td><td>
<table>
<tr><th>Cn</th><th>mono</th><th>di</th><th>total</th></tr>
<tr><td>C8</td><td>n.d.[7]</td><td>n.d.</td><td>n.d.</td></tr>
<tr><td>C10</td><td>≤2%</td><td>≤2%</td><td>≤4%</td></tr>
<tr><td>C12</td><td>26-37%</td><td>36-49%</td><td>67-80%</td></tr>
<tr><td>C14</td><td>7-16%</td><td>10-20%</td><td>20-32%</td></tr>
<tr><td>C16</td><td>≤2%</td><td>≤2%</td><td>≤4%</td></tr>
<tr><td>C18</td><td>n.d.</td><td>n.d.</td><td>n.d.</td></tr>
<tr><td>C18:1 and/or C18:2</td><td>n.d.</td><td>n.d.</td><td>n.d.</td></tr>
</table>
</td></tr>
<tr><td></td><td>NaCl</td><td>0-20%</td></tr>
<tr><td></td><td>Sodium glycolate[8]</td><td>6 -11%</td></tr>
</table>

[7] n.d – Not determined.

[8] Analysed as glycolic acid and converted to sodium glycolate as this is the form more likely present in the UVCB substance. Compositional information in registration dossiers may be given as glycolic acid (due to the analytical method) and/or can be also converted to the sodium salt.

Identification: Amphoacetates C12-C14		
	Alkyl amidoamine	0-6%
	Sodium chloroacetate	0-130ppm
	2-(2-aminoethylamino)ethanol	0-14ppm

Identification: Amphoacetates C12		
	Type of substance:	UVCB Monoacetate form only (contains appr. 75 to 100% monoacetate and 0 to 25% diacetates)
	IUPAC name:	Reaction products of 1H-Imidazole-1-ethanol, 4,5-dihydro-, 2-(C11 alkyl) derivs. and sodium hydroxide and chloroacetic acid
	Synonyms:	Acetic acid, chloro-, sodium salt, reaction products with 4,5-dihydro-2-undecyl-1Himidazole- 1-ethanol and sodium hydroxide Sodium Lauroamphoacetate C12 Amphoacetates EMPIGEN® CDL60/P
	CAS Number:	68608-66-2
	EC Number:	271-794-6
	Molecular Weight (for the CSA):	367 g/mol
	Compositional information (as manufactured, w/w)	
	Water	60-70%
	Total solids:	30-40%
	Total alkylamphoacetate derivatives	23-31%
	NaCl	5-8%
	Sodium glycolate	0.5-4%
	Alkyl amidoamine	0-0.3%
	Sodium chloroacetate	0-5000 ppm
	2-(2-aminoethylamino)ethanol	0-4 ppm

<table>
<tr><th colspan="3">Identification: Amphoacetates C12</th></tr>
<tr><td></td><td>Compositional information (solvent free condition, w/w)</td><td></td></tr>
<tr><td></td><td>Total alkylamphoacetate derivatives</td><td>76-80%</td></tr>
<tr><td></td><td>Alkyl chain distribution, Cn</td><td><table><tr><th>Cn</th><th>mono</th><th>di</th><th>total</th></tr><tr><td>C12</td><td>61-93%</td><td>0.1-21%</td><td>80-99.9%</td></tr><tr><td>Unknown</td><td>-</td><td>-</td><td>0.1-20%</td></tr></table></td></tr>
<tr><td></td><td>NaCl</td><td>16-20%</td></tr>
<tr><td></td><td>Sodium glycolate</td><td>4-8%</td></tr>
<tr><td></td><td>Alkyl amidoamine</td><td>0-0.5%</td></tr>
<tr><td></td><td>Sodium chloroacetate</td><td>0-9000ppm</td></tr>
<tr><td></td><td>2-(2-aminoethylamino)ethanol</td><td>0-10ppm</td></tr>
</table>