



## **U.S. ARMY PUBLIC HEALTH CENTER**

8252 Blackhawk Road, Aberdeen Proving Ground, Maryland 21010-5403

**Toxicity Report No. S.0049418-17, July 2016 - August 2017**  
**Toxicology Directorate**

**Chronic Toxicity Test in Rats Exposed to 3-Nitro-1,2,4-Triazol-5-One (NTO) in  
Drinking Water, July 2016-August 2017**

**Prepared by Emily May Lent and Allison M. Narizzano**

**Toxicology Directorate  
Toxicity Evaluation Division  
Army Public Health Center**

**Approved for public release; distribution unlimited.**

**ARIMS designation: 500C Toxicity Test**

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| 14. ABSTRACT<br><p>To evaluate the effects of chronic exposure to NTO, male and female rats were given ad libitum access to NTO in drinking water at concentrations of 0, 36, 110, 360, 1100, and 3600 mg/l for one year. NTO was generally non-toxic in females at the doses tested. Toxicity in males was limited to testicular toxicity as demonstrated in previous studies. Chronic exposure did not result in testicular toxicity at lower doses and the toxicity observed only in the high dose group in this study is less severe than that observed in previous studies. A BMD of 1604 mg/l (76 mg/kg-d) and a BMDL10 of 921 mg/l (44 mg/kg-d) were determined for chronic effects of NTO in male rats.</p>                                                                                                                                                                                                                                                        |             |                                    |                               |                                                             |                                                           |  |
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Toxicology Study No. S.0049418-17  
Protocol No. 48-16-04-01  
Chronic Toxicity Test in Rats Exposed to 3-Nitro-1,2,4-Triazol-5-One (NTO) in Drinking  
Water, July 2016-August 2017

**Data Requirement**

OECD Guidelines for the Testing of Chemicals, Section 4, Test No. 452: Chronic  
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**Authors**

Emily May Lent and Allison M. Narizzano

**Study Completed On**

September 16, 2019

**Performing Laboratory**

Army Public Health Center  
Toxicology Directorate (MCHB-PH-TEV)  
8252 Blackhawk Road, Building 5158  
Aberdeen Proving Ground, MD 21010-5403

**Laboratory Project ID**

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### **Good Laboratory Practice Compliance Statement**

The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice Standards, except for the following:

1. Manufacturer reported purity of the neat compound (99%) was not verified analytically by the performing laboratory.

Submitted By: Emily May Lent

Study Director: Emily May Lent

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EMILY MAY LENT  
Toxicologist  
Toxicity Evaluation Division (TEV)

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Date

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**PROTOCOL NO. 48-16-04-01**  
**CHRONIC TOXICITY TEST IN RATS EXPOSED TO 3-NITRO-1,2,4-TRIAZOL-5-ONE**  
**(NTO) IN DRINKING WATER,**  
**JULY 2016–AUGUST 2017**

## **1 Summary**

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### **1.1 Purpose**

The main objective of the chronic toxicity study is to evaluate the toxicity of compounds following exposures that represent significant portions of the animals' lifespan (i.e., one year). The purpose of this study is to identify target organs affected by chronic exposure to NTO, characterize a dose-response relationship, identify a point of departure, and determine a benchmark dose which can be used for establishing safety criteria for human exposure.

### **1.2 Conclusions**

To evaluate the effects of chronic exposure to NTO, male and female rats were given *ad libitum* access to NTO in drinking water at nominal concentrations of 0, 36, 110, 360, 1100, and 3600 mg/l for one year. NTO did not affect body weight, body weight gain, or food consumption in either sex. No treatment-related effects were observed in clinical chemistry and hematology parameters at the 6 month or one year sampling. At both the interim and final sampling, both males and females from the 3600 mg/l group produced smaller volumes of urine that was darker, more concentrated, and contained more bilirubin than the controls. Total and motile sperm counts were not affected by NTO treatment. Absolute and relative organ weights did not differ between control and NTO treated groups for either sex. Spontaneous age-related neoplasms occurred in controls and NTO groups at rates consistent with historic controls. NTO was generally non-toxic in females at the doses tested. Toxicity in males was limited to testicular toxicity as demonstrated in previous studies. Chronic exposure did not result in testicular toxicity at lower doses and the toxicity observed only in the high dose group in this study is less severe than that observed in previous studies. A BMD of 1604 mg/l (76 mg/kg-d) and a BMDL<sub>10</sub> of 921 mg/l (44 mg/kg-d) were determined for chronic testicular toxicity of NTO in male rats.

## **2 References**

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See Appendix A for a listing of references.

## **3 Authority**

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This study was conducted with funding from the U.S. Army Environmental Command (AEC; MIPR# 10925996). This toxicology study addresses, in part, the environmental safety and occupational health requirements outlined in Army Regulations (AR) 200-1, AR 40-5, and AR 70-1; Department of Defense Instruction 4715.4; and Army Environmental



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Requirements and Technology Assessments (AERTA) (DA 2003, 2007a, 2007b, 2008; DOD 1996; USAEC 2009). It was performed as part of an on-going effort by the U.S. Army Environmental Quality Technology (EQT), Ordnance Environmental Program Pollution Prevention Team, to produce safer ordnance. This program is under the direction of the U.S. Army Research, Development, and Engineering Command Environmental Acquisition Logistics & Sustainment Program and EQT Pollution Prevention.

### 4 Background

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Nitrotriazolone (3-nitro-1,2,4-triazol-5-one; NTO) is a component of several insensitive munitions (IM) formulations such as IMX-101 and IMX 104. NTO and other IMs are being pursued as replacements for conventional energetics such as TNT (2,4,6 trinitrotoluene) and RDX (1,3,5-trinitro-1,3,5-triazine). Insensitive munitions are less sensitive to inadvertent detonation due to mechanical shock, gunfire, and impact by shrapnel, making IMs more stable during transport and handling (DSTO 1999, 1989; LANL 1985). The properties of IMs provide obvious advantages for enhancing warfighter safety. They do not, however, necessarily confer improved toxicological safety. Because NTO is highly water soluble (>2 g/L), soil contamination following incomplete detonations can result in ground water contamination and potential environmental and human exposures.

Recent *in vitro* and *in vivo* studies have demonstrated limited toxicity of NTO. NTO has an LD<sub>50</sub> >5g/kg in rats and mice, is negative in eye irritation and dermal sensitization tests, and produces mild, temporary skin irritation in the rabbit primary skin irritation test (DOE 1985). In a subchronic oral study, a lower 95% confidence interval on the Benchmark Dose (BMDL<sub>10</sub>) of 40 mg/kg-d was determined based on testicular and epididymal toxicity and hypospermia (Crouse et al. 2015). A battery of *in vitro* and *in vivo* endocrine disruptor screening assays did not demonstrate anti-androgenic or estrogenic activity for NTO at doses up to 1000 mg/kg-day (Quinn et al. 2014; APHC 2012). Effects of NTO on pubertal development were limited to reduced male reproductive organ weights, an effect that was likely secondary to direct testicular toxicity (Lent et al. 2015). Maternal and developmental toxicity were not observed at doses up to 3600 mg/l NTO in an extended one generation reproductive test. Although male reproductive toxicity (i.e., testicular hypoplasia, reduced sperm count) was observed at 3600 mg/l NTO, fertility was not affected (Lent et al. 2016).

The present study, a chronic toxicity study, was conducted to evaluate the safety of long-term exposures to NTO. The following table identifies the dates of critical study events.

**Table 1. Critical study events**

| Critical Event               | Date of Event |
|------------------------------|---------------|
| Animal Use Protocol Approved | 04/06/2016    |
| In-Life Initiation           | 07/19/2016    |
| In-Life Completion           | 08/03/2017    |
| Experimental Completion      | 08/13/2019    |
| Study Completion             | 09/16/2019    |

## 5 Materials and Methods

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### 5.1 Test Article

Neat NTO (CAS # 932-64-9; lot BAE15J300-012; purity: 99%) was obtained from BAE Systems, Inc. Dosing solutions were prepared by weighing the required amount of NTO, transferring to a 4 L volumetric flask, adding approximately 3.5 L of water from the animal room, stirring using a magnetic stir bar and stir plate until dissolved, and adding water to the 4 L mark. Solutions were prepared as needed according to the consumption rate of the rats. Water bottles (polycarbonate 80706PC; Lab Products Inc.) were refilled as needed, changed weekly, and solutions replaced completely every 2 weeks. A one milliliter sample was taken from each batch of dosing solution prepared and analyzed by Army Public Health Center (APHC) Laboratory Sciences via high performance liquid chromatography with ultra violet detection to verify the concentration. NTO was previously determined to be stable in water for at least six weeks (USAPHC 2010); therefore a stability study was not conducted.

### 5.2 Animals and Housing Conditions\*

Young adult male (n=144; 185.4 ± 10.6 g) and female (n=144; 161.8 ± 8.2 g) Sprague Dawley (CrI:CD(SD) CD<sup>®</sup>; Charles River Laboratories, Wilmington, Massachusetts) rats, approximately 8 weeks of age, were acclimated to the animal facility for 5 days before initiation of dosing. An additional group of sentinel animals (4 animals per sex) were also included for monitoring of disease status during the study. Sentinel animals (1 per sex) were tested at approximately the 3, 6, 9, and 12 month time points. Each rat was uniquely identified by number via cage card and tail marking.

All animals were housed in temperature-, relative humidity-, and light-controlled rooms. The target conditions of the rooms were 68-79 °F and 30-70 percent humidity. An automatically controlled 12:12-hour light/dark cycle was maintained, with the dark period beginning at 1800 hours. A certified pesticide-free rodent chow (Harlan Teklad<sup>®</sup>, 2016C Certified Rodent Diet) was available *ad libitum*. Control animals were provided filtered tap water *ad libitum* whereas treated rats were provided solutions of NTO in filtered tap water *ad libitum*. Drinking water was tested semi-annually for pesticides, metals, and organic contaminants. All rats were individually housed in suspended polycarbonate cages with Sani-Chip<sup>®</sup> bedding. Rats with open wounds or ulcerated masses were housed on Diamond Dry<sup>®</sup> bedding.

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\* Animal use procedures were approved by the Army Public Health Center (APHC) Institutional Animal Care and Use Committee. Animal care and use was conducted in accordance with *The Guide for the Care and Use of Laboratory Animals* and all applicable Federal and DOD regulations. The APHC Animal Care and Use Program is fully accredited by AAALAC.

### **5.3 Quality Assurance**

The APHC Quality Systems and Regulatory Compliance Office audited critical study phases. Appendix B provides the dates of these audits, the phases audited, and the dates the results were reported to the Study Director (SD) and Management.

### **5.4 Study Personnel**

Appendix C lists the names of individuals contributing to the study performance.

### **5.5 Dose Selection and Test Substance Administration**

Dose selection was based on the ultimate objective that the highest dose level be chosen to identify principal target organs and toxic effects while avoiding suffering, severe toxicity, morbidity, or death, unless limited by the physical-chemical nature of the test substance. NTO has demonstrated minimal systemic toxicity in subacute and subchronic studies, with effects being generally limited to testicular toxicity at 315 mg/kg-d (Crouse et al. 2015). Rats exposed to NTO in drinking water at concentrations (3600 mg/l) that were selected to approximate doses (mg/kg-day) which previously caused testicular toxicity demonstrated taste aversion and reduced intake. The 3600 mg/l concentration therefore represents a maximal useable concentration. Four subsequent dose groups were set at half log intervals to aid in bench mark dose modeling and in determining a dose-response at concentrations applicable to potential human exposures. Six drinking water dosing solutions of 0, 36, 110, 360, 1100, 3600 mg NTO/L water were administered 7-days per week via drinking water at a constant dietary concentration (mg/l).

### **5.6 Study Design**

NTO was administered orally via drinking water to 6 groups of 24 male and 24 female Sprague Dawley rats for 1 year (five NTO doses and a control). Assignment to dose groups was accomplished using a stratified random procedure, with animals stratified according to pre-study body weight and dose groups assigned by random draw. Body weight did not differ among dose groups prior to initiation of dosing. Cages were arranged in racks in a randomized complete block design to minimize possible effects due to cage placement. Males and females were each divided into six time-separated necropsy groups, with animals from each dose group approximately evenly distributed across necropsy days.

### **5.7 Clinical Observations, Body Weight, Food Consumption**

All animals were observed at least twice daily during the week, once daily during weekends and holidays, for signs of toxicity, morbidity, and mortality. All animals were given a thorough physical examination at least once per week.

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All animals were weighed on the first day of dosing, weekly thereafter, and at termination. Food consumption was monitored weekly. Frequency of water consumption monitoring varied based on consumption rate.

### **5.8 Clinical Pathology**

Fasted blood samples for clinical pathology were collected at 6 months and at termination. Interim samples were collected from the lateral saphenous vein. Due to sample volume requirements, half of the rats were randomly selected for hematology analyses and the remaining rats for clinical chemistry analyses. At termination, blood samples were collected via intracardiac puncture and subjected to clinical chemistry and hematology analyses.

Blood for clinical chemistry analyses was transferred to collection tubes free of additives, was allowed to clot at room temperature for 30 to 40 minutes, and was centrifuged for approximately 2.5 minutes at 12,000 x g. Serum was removed and immediately analyzed for clinical chemistry parameters. The following clinical chemistry parameters were evaluated using the Idexx VetTest® 8008 Chemistry Analyzer: alanine aminotransferase (ALT), albumin (ALB), alkaline phosphatase (ALKP), aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium (CA), cholesterol (CHOL), creatinine (CREA), globulins (GLOB), glucose (GLU), total bilirubin (TBIL), total protein (TP), and triglycerides (TRIG). Electrolytes consisting of sodium, potassium, and chloride were also analyzed if sufficient sample volume was available.

Blood for hematology analyses was transferred to tubes containing tripotassium ethylenediamine-tetraacetic acid (K<sub>3</sub>EDTA). The following hematology parameters were evaluated using the Cell-Dyn 3700 Hematology Analyzer (Abbott Laboratories, Abbott Park, IL 60064): erythrocyte count (RBC), hematocrit (HCT), hemoglobin concentration (HGB), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), red blood cell distribution width (RDW), total white blood cell count (WBC), differential leukocyte count (neutrophils: NEU, lymphocytes: LYM, monocytes: MONO, eosinophils: EOS, basophils: BASO), platelet count (PLT), and mean platelet volume (MPV). To determine average activated prothrombin time (APTT) and prothrombin time (PT), blood was transferred to a tube containing sodium citrate and centrifuged for approximately 2.5 minutes at 12,000 x g. The plasma was analyzed using the BFT II Analyzer (Siemens Health Care Diagnostics, Tarrytown, NY 10591).

### **5.9 Ophthalmology**

All study animals were examined pre-study and animals from the control and high dose (3600 mg/l) groups were examined within one week of the conclusion of the study. The fundus and anterior chamber of the eye were examined using a Welch Allyn ophthalmoscope after instillation of 1% tropicamide ophthalmic solution.

### 5.10 Urinalysis

At the interim sampling (approximately test week 26) and the night prior to terminal sacrifice, each animal was placed in a metabolism cage capable of separating urine and feces for an overnight period of approximately 12 hours during which free-catch urine was collected. Animals were fasted during this period, but water/NTO was available *ad libitum*. Urine samples were transferred to clear, graduated conical centrifuge tubes and the volume, color, and appearance of each sample were recorded. The color of each sample was determined based on comparison with a urine color chart with nine colors ranging from pale yellow/straw to dark amber. Specific gravity was tested using a refractometer. Multistix 7 Reagent strips were used to perform analysis of pH, protein, glucose, ketones, bilirubin, blood, urobilinogen, nitrites, and leukocytes.

### 5.11 Pathology

At the end of the one year dosing period, all surviving animals were anesthetized with CO<sub>2</sub>, blood was collected by intracardiac puncture, and rats were euthanized by CO<sub>2</sub> asphyxiation followed by thoracotomy. A complete necropsy was performed on all animals. Adrenals, brain, heart, kidneys, epididymides, liver, ovaries (without oviducts), prostate, seminal vesicles with coagulating glands (weighed with fluid), spleen, testes, thymus, thyroid (trimmed and weighed post-fixation), and uterus (with oviducts and cervix) were collected, weighed, and preserved. Tissues collected for histopathology but not weighed included: all gross lesions, eye (including retina), lacrimal gland (exorbital), salivary gland, esophagus, trachea, lung, lymph nodes (both superficial and deep), pancreas, stomach (forestomach, glandular stomach), ileum, duodenum, jejunum, caecum, colon, rectum, vagina, urinary bladder, mammary gland (obligatory for females and, if visibly dissectible, from males), skin, skeletal muscle, peripheral nerve, spinal cord, fresh bone marrow aspirate, and Harderian gland

All tissues were preserved in 10% buffered formalin except the testes and epididymides which were placed in Davidson's fixative no longer than 24 hours. After fixation, all tissues were rinsed and stored in 70% ethanol.

A complete necropsy was performed on 16 males and 10 females that died or were euthanized prior to scheduled termination.

### 5.12 Histopathology

Tissues were fixed in formalin, trimmed into cassettes, processed, embedded in paraffin, and sectioned to a thickness of 4-5 µm and stained with hematoxylin and eosin using a routine automatic stainer. Testis and epididymis were fixed in modified Davidson's solution and additionally stained with periodic acid-Schiff (PAS) stain. Sections of the brain were trimmed according to OECD and INHAND trimming guidelines (Kittel et al. 2004; Morawietz et al. 2004; OECD 1997; Ruehl-Fehlert et al. 2003) and included five sections of the nasal cavity and turbinates and seven sections of the brain. Tissues were examined

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by a board certified veterinary pathologist via light microscopy. All available tissues/slides from the high dose group, control group, and animals that died or were euthanized as moribund were processed and examined. Tissues from lower dose groups were examined if exposure-related effects were seen in the high-dose group, gross lesions were present, or other signs of organ toxicity were noted in the dose group (e.g., changes in organ mass). Only the liver, pituitary, thyroid, and testes were examined in the 36, 110, 360, and 1100 mg/l dose groups. Histopathologic findings were subjectively graded across a 6-point scale, where Grade 0 (essentially normal) referred to tissue that is essentially normal or tissues with changes observed in <1% of the sampled tissue; Grade 1 (minimum) referred to a change which affected <5% of the presented tissue area, and Grade 2 (mild) referred to a change which affected 6 to 20% of the tissue area. Grade 3 (moderate) was scaled to refer to a change of which affected 21 to 40% of the tissue area, Grade 4 (marked) was scaled for lesions affecting 41 to 80% of the tissue area, and Grade 5 (severe) indicated that >80% of the tissue was affected. A notation of absent indicates that the tissue was not present for microscopic examination due to sampling error, out of the plane of section, or other similar reason.

### **5.13 Sperm Analysis**

Cauda epididymal sperm counts were determined using a computer assisted sperm analyzer (TOX IVOS II-CASA; Hamilton-Thorne Research, Beverly, Massachusetts). The cauda was weighed and placed in a well of a petri dish containing 10 ml M199 medium at 34-37 °C and the surface minced using a scalpel to release sperm. The cauda was incubated for 10 minutes at 34-37 °C, gently mixed to uniformly suspend the sperm, and 0.5 ml of the suspension transferred to another well containing 5 ml of medium. The number of sperm, number of motile sperm, and number of progressive sperm was determined for 8-12 frames for each animal.

### **5.14 Thyroid Morphometric Analysis**

In order to quantify or objectively evaluate the variability in size of thyroid follicles, a morphometric assessment was performed on thyroid glands from a randomly selected sub-set of animals in selected dose groups. Two follicles, localized on to 2 a priori selected fixed coordinates on the slide, were selected from each of 5 non-adjacent fields from each lobe of the thyroid for each animal (at 20X). If no follicles were located at the fixed coordinates, no data were collected for that point. This resulted in up to 20 follicles being analyzed per animal. The luminal area and epithelial area were measured using Image J (Rasband 2018) by outlining the perimeter of the lumen and the perimeter of the follicle. Epithelial area was calculated by subtracting the luminal area from the follicular area and the luminal to epithelia (L/E) ratio was calculated. Pixels were converted to square micrometers using a stage micrometer.

### 5.15 Data Collection, Calculations, and Statistical Analyses

Experimental data generated during the course of this study were recorded by hand and tabulated, summarized, and/or statistically analyzed using Microsoft® Excel and SPSS® 21.0. Environmental data were automatically recorded using MetaSys® Building Management System.

Organ mass ratios were calculated relative to final body mass and brain mass. Urine parameters with mixed numerical and categorical data were coded prior to analysis. Rats noted to continuously play with the water bottle and make it drip, or remove (and not eat) food from the feeder were eliminated from water and food consumption analyses. All analyses were performed separately by sex. Survival was analyzed using the Kaplan-Meier method. Continuous data were analyzed using a one-way ANOVA with dose group as the main effect. Absolute organ mass was analyzed by Analysis of Covariance (ANCOVA), with dose group as the main effect and body mass at the end of the study as the covariate (Bailey et al. 2004). Interpretation of changes in absolute organ mass, organ-to-body mass ratio, and organ-to-brain mass ratio in the evaluation of compound related effects was based on published analysis of control animal data (Bailey et al. 2004). Weekly body weight and food and water consumption data were analyzed using repeated measures ANOVA to determine dose effect. If the interaction between the within and between subject factors was significant, the effect of dose group on the parameter was determined within each sampling week using a one-factor ANOVA. Due the occurrence of background chronic infections (e.g., pododermatitis and infections in mammary masses) and the likelihood that they would alter hematology and clinical chemistry parameters and hinder detection of NTO effects, presence of pododermatitis and/or mammary masses were included in the model for these analyses. When significant main effects were observed ( $p < 0.05$ ), appropriate post hoc tests were used to compare dose groups to the control group [e.g., Tukey's multiple comparison test, Dunnett's T3 (if variances were unequal), or Sidak (for ANCOVA)]. Variance equality was determined by Levene's test. If the data were not normally distributed, the data were either log transformed or arcsine transformed prior to ANOVA/ANCOVA.

Fisher's exact test was used to determine significant differences between treated and control groups for nominal or count data (e.g., histology and mass incidence). Histopathology severity categories were consolidated to present or absent for statistical analyses. Significant overall effects were followed with comparisons between the control group and each NTO treatment group.

Animals that died or were euthanized as moribund with less than 9 months of exposure were excluded from statistical analyses beyond survival analyses.

## 6 Results

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### 6.1 Analytical Chemistry

The analytical chemistry results are summarized in Appendix D. Mean analytical concentrations were  $35.8 \pm 1.4$ ,  $114.1 \pm 11.3$ ,  $379.5 \pm 33.2$ ,  $1135.8 \pm 50.1$ , and  $3716.1 \pm 152.2$  mg/l for the 36, 110, 360, 1100, and 3600 mg/l NTO solutions, respectively. All results were within the 70-130% recovery limits for the analysis. As such, all results were reported using the nominal concentrations.

### 6.2 Water Intake and Calculated Doses

Individual animal and summarized water consumption data are presented in Appendix E. In male rats, water consumption and relative water consumption were 22-27% lower in the 3600 mg/l group than the control for the first seven weeks of the study ( $p < 0.001$ ,  $p = 0.031$ ,  $p < 0.001$ ,  $p = 0.001$ ,  $p < 0.001$ ,  $p < 0.001$ , and  $p < 0.001$ ). Thereafter, weekly and yearly mean consumption did not differ between NTO groups and the control for male rats. In females, water consumption and relative water consumption were 11-23% lower in the 3600 mg/l group than in the control for the first five weeks of the study ( $p = 0.014$ ,  $p = 0.001$ ,  $p < 0.001$ ,  $p = 0.039$ , and  $p = 0.003$ ). From week 31 through week 49, water consumption was reduced by 18-29% in both the 3600 and 1100 mg/l groups compared to the control group. Mean consumption over the year was reduced ( $p = 0.004$ ) by 12% in the 3600 mg/l group compared to the control.

Approximate administered dose levels were estimated based on drinking water consumption data, corresponding body weight data, and nominal concentrations (Table 2). Target dose levels were 5, 15, 50, 150, and 500 mg/kg-day. Calculated administered dose levels, averaged across the year, were generally lower than target dose levels. This was due to a combination of reduced water consumption associated with taste aversion and the body weight of study rats exceeding the standard average rat weight used to estimate experiment-wide dose levels. Although the calculated administered doses differ from the target doses, the remaining results will be reported based on drinking water concentration for ease of reading.



**Table 2. Drinking Water/NTO Consumption and Approximate Dose**

|             | ml/kg BW-day | mg NTO/kg BW-day | % Target |
|-------------|--------------|------------------|----------|
| <b>0</b>    |              |                  |          |
| Males       | 50           |                  |          |
| Females     | 70           |                  |          |
| <b>36</b>   |              |                  |          |
| Males       | 50           | 2                | 35       |
| Females     | 80           | 3                | 60       |
| <b>110</b>  |              |                  |          |
| Males       | 50           | 6                | 38       |
| Females     | 80           | 9                | 58       |
| <b>360</b>  |              |                  |          |
| Males       | 50           | 20               | 34       |
| Females     | 80           | 30               | 54       |
| <b>1100</b> |              |                  |          |
| Males       | 50           | 50               | 35       |
| Females     | 70           | 80               | 53       |
| <b>3600</b> |              |                  |          |
| Males       | 50           | 170              | 33       |
| Females     | 70           | 260              | 53       |

### 6.3 Survival and Clinical Observations

No treatment-related clinical signs were noted in males. In females, axillary and inguinal masses along the mammary chain were noted in 34 females, with similar distribution among controls and NTO treated groups (7, 4, 6, 4, 7, and 6 in controls and 36, 110, 360, 1100, and 3600 mg/l groups, respectively). Masses were first noted at 24 weeks of dosing and ranged in maximal size from 4 mm to 80 mm in diameter (Figure 1). Several masses were noted to shrink and/or disappear over time. The incidence of these masses was not treatment-related. Other clinical observations were limited to abrasions/scabs, barbering, alopecia, dirty hair coat, chromodacryorrhea, and swollen/lacerated plantar surface.

Survival did not differ among treatment groups for male or female rats or across both sexes (Figure 2). Sixteen male rats were found dead or euthanized prior to scheduled termination; 3 controls and 2, 4, 4, and 3 males from the 36, 110, 1100, and 3600 mg/l NTO treatment groups, respectively. Except chromodacryorrhea in one 1100 mg/l animal (#16-0245), no clinical signs of toxicity were noted in any male rats that were found dead prior to necropsy. Two male rats (#16-0191 and #16-0212; 110 and 1100 mg/l groups) exhibited extreme lethargy and weakness of hind limbs and were euthanized; both rats had enlarged spleens and pathology indicative of leukemia. One rat in the 110 mg/l (#16-0229) was found with a 1.5 cm mass on the ventral pharyngeal region and was euthanized due to labored breathing. One control rat (#16-0213) exhibited unexplained weight loss and became moribund. One rat in the 3600 mg/l group (#16-0220) had a laceration on the right shoulder that failed to heal after treatment with antibiotics and steroids and was euthanized due to severe weight loss.

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Eight female rats (1 control and 2, 2, and 3 from the 36, 1100, and 3600 mg/l NTO groups, respectively) were euthanized prior to termination of the study due to the size and/or condition of axillary and inguinal masses. Two females (#16-0406 and #16-0328; control and 1100 mg/l NTO) were euthanized due to weight loss and moribund condition and were found to have an enlarged pituitary gland.

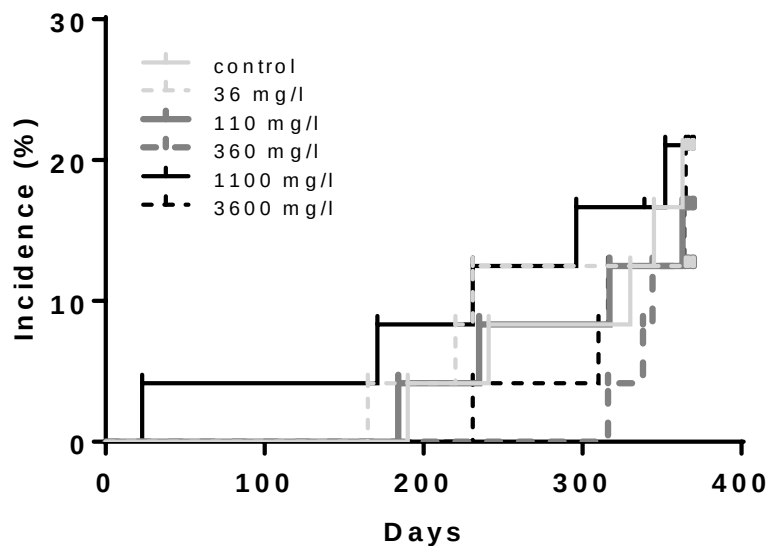


Figure 1. Incidence (%) over time of non-regressive externally palpable tumors in the mammary chain of female rats given *ad libitum* access to NTO in water at 0, 36, 110, 360, 1100, and 3600 mg/l for one year.

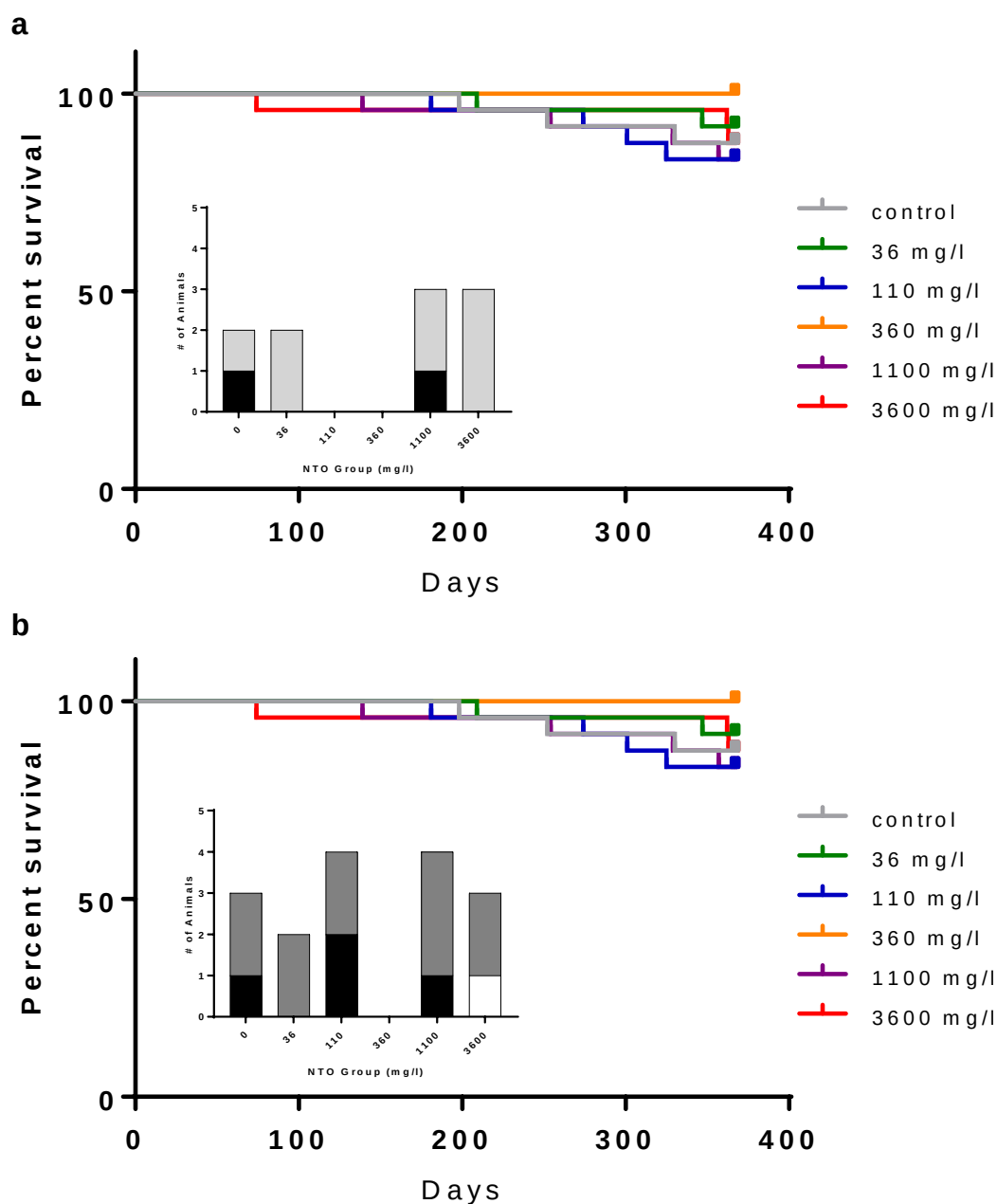


Figure 2. Cumulative percent survival over time of a) female and b) male rats given *ad libitum* access to NTO in water at 0, 36, 110, 360, 1100, and 3600 mg/l for one year. Inset bar histograms indicate causes of mortality. Black: euthanasia due to moribund condition; light grey: euthanasia due to size/condition of mammary tumor(s); white: euthanasia due to injury; dark grey: spontaneous death.

#### **6.4 Body Weight and Food Consumption**

Individual and summarized body weight and food consumption data are presented in Appendices F and G. Body weight increased over time in all dose groups and did not differ between NTO treated and control groups for either males or females (irrespective of inclusion/exclusion of females with inguinal/axillary masses). Body weight gain decreased over the first 12 weeks of the study in all dose groups and did not differ between NTO treated and control groups. Food consumption and food efficiency ratios were unaffected by NTO treatment in both males and females.

#### **6.5 Organ Weight**

Individual and summarized organ weight data are presented in Appendix H. An apparent reduction in absolute and relative thyroid weights was noted in both males and females in the intermediate dose groups. However, this apparent effect resulted from systematic bias introduced during histopathology processing due to inter-technician differences in trimming that was not evenly distributed among groups. When analyzed controlling for technician and date prepared, absolute and relative thyroid weights did not differ among control and NTO treatment groups for either males or females. Although not statistically significant, spleen weight appeared to be reduced in all female dose groups relative to the control group. These apparent reductions were due to two extreme outliers in the control group (#16-0319 and #16-0330) which were noted to have grossly enlarged spleens and neoplastic infiltrates and/or neoplastic histiocytic sarcoma. If these two animals are excluded, the apparent reduction in spleen weight in NTO treatment groups is eliminated. Pituitary gland weight was increased by approximately 33% in the 110 mg/l group relative to the control group, however this increase was not statistically significant. Once again, this increase was due to outliers in the group, specifically animals with grossly enlarged pituitary glands associated with pituitary adenoma. No effects of NTO treatment were apparent after exclusion of animals with enlarged pituitary glands, a common background lesion in aged-rats (see section 5.9 Histopathology). No other organs demonstrated statistically significant or biologically meaningful effects of NTO in either males or females.

#### **6.6 Gross Pathology**

The full pathology report can be found in Appendix I. The incidence of macroscopic findings including hepatic pallor, pituitary enlargement, masses associated with the mammary gland or concretions of mammary secretions (galactoceles), reduced testis size (unilateral), and proliferative lesions on the plantar aspect of the metatarsals did not differ among dose groups.

#### **6.7 Histopathology**

Table 3 summarizes the most frequently occurring anatomical pathologies (noted in 5 or more animals in either sex). Treatment-related microscopic effects were limited to the

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testes. Testicular tubular atrophy was observed in 14% of control rats and 4-48% of rats in NTO-treated groups. The incidence of testicular tubular atrophy was higher only in the 3600 mg/L dose group (48% of rats) compared to the control group ( $p=0.023$ ). The incidence in lower dose groups (4-5%) did not differ from the control group. In high dose animals that had spermatid tubular atrophy, there was complete loss of germ cells within approximately 5% or less of the individual tubules scattered throughout the cross section of the testis examined. This was accompanied by a reduction in sperm count and intratubular cellular debris in epididymides in the 3600 mg/L group (11% of rats); however, the frequency of these lesions was not statistically different from control (0% of rats). Epididymides were not examined in lower dose groups. No treatment-related changes were noted in the male accessory sex glands or female reproductive tissues.

The incidence of hepatic lesions including lipid-type vacuolar degeneration of hepatocytes, hepatocellular necrosis, neutrophilic/lymphocytic infiltrates, and biliary hyperplasia differed among control and NTO-treated groups. Lipid-type vacuolar degeneration of hepatocytes, primarily in periportal hepatocytes was observed in both controls (2% and 77% in females and males, respectively) and the NTO-treatment groups (23-65%). Only the reduced incidence observed in males in the 1100 mg/l group (23%) differed from the control group ( $p=0.001$ ). Mild multifocal hepatocellular necrosis, characterized by loss of normal arrangement of hepatocytes and mild shrinking of the hepatic lobule, occurred more frequently in males in the 36 and 3600 mg/l dose groups (26 and 48%) than in the controls (0%;  $p=0.022$  and  $p<0.001$ , respectively). In NTO-treated females, the incidence of multifocal hepatocellular necrosis did not differ among groups; however, the incidence of necrosis without a specific distribution was higher only in the 110 mg/l group (50%) than the control (17%;  $p=0.030$ ). Infiltrates of neutrophilic and lymphocytic cells were commonly observed in both controls and NTO-treated animals. Neutrophilic infiltrates were observed more frequently in females in the 3600 mg/l group (29% of rats) relative to the controls (0% of rats) ( $p=0.009$ ). Lymphocytic infiltrates were observed less frequently in females in the 1100 mg/l group (21% of rats) compared to controls (54% of rats;  $p=0.036$ ). Ductular reaction, or biliary hyperplasia, was observed in nearly all groups and did not differ among groups for either females or males. Although these hepatic lesions can result from or be exacerbated by exposure to xenobiotics, they may also result from chronic hepatic injury, dietary factors, metabolic and hormonal status, restraint or debilitation of animals, age, and fasting before necropsy (Thoolen et al. 2010). Therefore, these changes, occurring in individual sexes and dose groups, were not considered biologically meaningful and not test-article related (Thoolen et al. 2010).

Common changes observed in all dose groups examined, including adenoma of the pituitary gland, angiectasis of the adrenal cortex, spongiosis hepatis, biliary hyperplasia, hyaline casts, and mammary gland fibroma/adenoma/fibroadenoma, were determined to be common spontaneous age-related lesions in this strain (Chandra et al. 1992; Dinse et al. 2010; Son 2004; Hoenerhoff et al. 2014; Thoolen et al. 2010; Seely et al. 2014).

Incidental findings included an adrenal gland pheochromocytoma, thyroid gland adenoma, leiomyosarcoma within the small intestine, esophageal skeletal muscle neoplasm (rhabdomyosarcoma), submucosal rectal lymphoma, histiocytic sarcoma in multiple

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organs/tissues including the liver, spleen, lymph nodes, thymus, and lung, cerebral astrocytoma, lymphoid neoplasm within the bone marrow, sarcoma within the lung, and a renal epithelial neoplasm. The incidence of neoplastic lesions was not considered to be treatment-related for either sex.

All other tissues examined (see section 5.8 Pathology) from the control and 3600 mg/l groups were determined to be essentially normal.

Histologic examination of the eight females that were euthanized prior to schedule due to the size and/or condition of masses in the mammary chain revealed no other significant pathology and indicated that these animals were otherwise similar to the remaining animals. With the exception of rat #16-0282 which was euthanized prior to nine months of exposure, females with externally observed masses in the mammary chain were included in all statistical analyses. The two females euthanized due to weight loss and moribund condition were found to have enlarged pituitary glands compressing surrounding tissue; both were included in further analyses.

Histologic examination of the 11 males that died on study revealed no specific apparent cause of death. Four of these animals (#16-145, 16-188, 16-205, and 16-255) were eliminated from further analyses due to inadequate exposure time. In the five males that were euthanized as moribund, two male rats (#16-0191 and #16-0212; 110 and 1100 mg/l groups) had neoplastic infiltrates of the liver, spleen, lymph nodes, lung, and bone marrow. One rat in the 110 mg/l group (#16-0229) had rhabdomyosarcoma in the ventral tracheal region and metastatic sarcoma in the lung. No pathology was noted in the control rat (#16-0213) that exhibited unexplained weight loss and became moribund or the 3600 mg/l group rat (#16-0220) that had a laceration on the right shoulder that was euthanized due to severe weight loss. Three of these animals (#16-0191, 16-0212, and 16-0220) were removed from further analyses because their total exposure period was less than nine months.

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**Table 3. Summary of the most frequently observed anatomical pathologies**

| NTO Dose Group (mg/l)                    | Female |       |      |      |      |      | Male |      |      |      |      |      |
|------------------------------------------|--------|-------|------|------|------|------|------|------|------|------|------|------|
|                                          | 0      | 36    | 110  | 360  | 1100 | 3600 | 0    | 36   | 110  | 360  | 1100 | 3600 |
| <b>Adrenal</b>                           | (24)   | (1)   | (0)  | (0)  | (3)  | (24) | (21) | (0)  | (3)  | (0)  | (2)  | (22) |
| Angiectasis                              | 19     | 0     |      |      | 2    | 13   | 2    |      | 0    |      | 1    | 0    |
| Fibrin thrombi within angiectasis        | 4      | 0     |      |      | 0    | 3    | 0    |      | 0    |      | 0    | 0    |
| <b>Bone Marrow</b>                       | (22)   | (1)   | (0)  | (0)  | (2)  | (24) | (21) | (0)  | (1)  | (0)  | (1)  | (22) |
| Increased number of mast cells           | 7      | 0     |      |      | 0    | 3    | 2    |      | 0    |      | 0    | 0    |
| <b>Heart</b>                             | (21)   | (1)   | (0)  | (0)  | (1)  | (23) | (21) | (0)  | (3)  | (0)  | (2)  | (23) |
| Myocardial degeneration                  | 3      | 0     |      |      | 1    | 0    | 3    |      | 0    |      | 1    | 1    |
| <b>Kidney</b>                            | (22)   | (1)   | (0)  | (0)  | (3)  | (22) | (22) | (0)  | (3)  | (0)  | (2)  | (23) |
| Interstitial lymphocytic infiltrates     | 0      | 0     |      |      | 0    | 0    | 1    |      | 0    |      | 0    | 5    |
| Intratubular protein accumulation        | 4      | 0     |      |      | 1    | 7    | 0    |      | 1    |      | 0    | 0    |
| <b>Liver</b>                             | (24)   | (23)  | (24) | (24) | (24) | (24) | (22) | (23) | (23) | (24) | (22) | (23) |
| Vacuolar degen lipid-centrilobular multi | 5      | 2     | 0    | 0    | 1    | 1    | 0    | 0    | 3    | 4    | 6*   | 1    |
| Vacuolar degen lipid-periportal multi    | 4      | 6     | 11   | 9    | 10   | 11   | 17   | 15   | 13   | 15   | 5*   | 12   |
| Infiltrates neutrophilic                 | 0      | 0     | 0    | 1    | 0    | 7**  | 3    | 4    | 1    | 3    | 2    | 2    |
| Infiltrates lymphocytic                  | 13     | 6     | 15   | 13   | 5*   | 16   | 20   | 13   | 17   | 17   | 15   | 16   |
| Necrosis hepatocellular                  | 4      | 3     | 12*  | 8    | 10   | 11   | 12   | 12   | 16   | 16   | 9    | 12   |
| Necrosis hepatocellular multifocal       | 3      | 1     | 3    | 3    | 4    | 5    | 0    | 6*   | 3    | 5    | 3    | 11** |
| Biliary hyperplasia                      | 0      | 2     | 4    | 2    | 1    | 1    | 1    | 3    | 3    | 8    | 3    | 3    |
| Spongiosis hepatitis                     | 0      | 0     | 1    | 2    | 2    | 0    | 2    | 3    | 1    | 1    | 3    | 0    |
| <b>Mammary Gland</b>                     | (24)   | (2)   | (5)  | (4)  | (6)  | (24) |      |      |      |      |      |      |
| Intraductular secretory product          | 19     | 1     | 0    | 1    | 0    | 12   |      |      |      |      |      |      |
| Galactoceles cyst                        | 4      | 0     | 1    | 0    | 1    | 2    |      |      |      |      |      |      |
| Fibroadenoma                             | 9      | 2     | 5    | 3    | 5    | 10   |      |      |      |      |      |      |
| <b>Pituitary</b>                         | (24)   | (220) | (24) | (24) | (22) | (23) | (22) | (23) | (22) | (24) | (20) | (23) |
| Adenoma - pars distalis                  | 1      | 4     | 7    | 2    | 4    | 5    | 1    | 4    | 3    | 2    | 3    | 0    |
| Hyperplasia - pars distalis              | 3      | 7     | 4    | 7    | 2    | 5    | 3    | 6    | 3    | 2    | 1    | 0    |
| <b>Skin</b>                              | (20)   | (1)   | (0)  | (0)  | (3)  | (18) | (7)  | (0)  | (3)  | (0)  | (1)  | (5)  |
| Epithelial hyperplasia rear legs         | 6      | 0     |      |      | 0    | 3    | 0    |      | 1    |      | 0    | 0    |
| Fibrosis and fibroplasia                 | 5      | 0     |      |      | 0    | 3    | 0    |      | 0    |      | 0    | 0    |
| Granulation tissue                       | 6      | 0     |      |      | 0    | 2    | 0    |      | 0    |      | 0    | 0    |
| Sub-mucosa necrosis                      | 5      | 0     |      |      | 0    | 1    | 0    |      | 0    |      | 0    | 0    |
| <b>Spleen</b>                            | (24)   | (1)   | (0)  | (0)  | (3)  | (24) | (22) | (1)  | (3)  | (0)  | (2)  | (21) |
| Increased intrahistiocytic pigment       | 19     | 1     |      |      | 3    | 19   | 15   | 0    | 0    |      | 1    | 9    |
| <b>Testis</b>                            |        |       |      |      |      |      | (22) | (23) | (23) | (24) | (21) | (23) |
| Atrophy few tubules at periphery         |        |       |      |      |      |      | 5    | 4    | 4    | 4    | 6    | 9    |
| Spermatic tubules atrophy/degen          |        |       |      |      |      |      | 3    | 1    | 1    | 1    | 1    | 11*  |
| <b>Thyroid</b>                           | (24)   | (23)  | (24) | (24) | (23) | (23) | (22) | (22) | (23) | (24) | (21) | (21) |
| Cyst ultimobranchial                     | 11     | 9     | 4    | 8    | 9    | 3    | 7    | 9    | 10   | 13   | 11   | 2    |
| Hyperplasia follicular cell              | 0      | 1     | 1    | 0    | 1    | 0    | 2    | 2    | 0    | 1    | 3    | 0    |
| C cell focal hyperplasia                 | 1      | 1     | 0    | 3    | 1    | 0    | 4    | 1    | 1    | 2    | 5    | 1    |

Numbers in parentheses indicate number of tissues examined. \*significantly different from control, p<0.05;

\*\*significantly different from control, p<0.01 based on Fisher's Exact test

## **6.8 Thyroid Morphometry**

Follicular cell area, colloid area, and the ratio of colloid to follicular cell area did not differ between the control and NTO treated groups for either males or females.

## **6.9 Clinical Chemistry**

Individual and summarized clinical chemistry data are presented in Appendix J. There were no treatment-related effects on clinical chemistry in males or females at the interim sampling point.

At the terminal sampling, effects on clinical chemistry were limited to a slight (16%) increase in cholesterol level in females in the 36 mg/l group compared to the control group ( $p=0.026$ ). Cholesterol did not demonstrate a dose-response and the increase observed only in the low dose group is not considered to be treatment-related. No other clinical chemistry parameters differed between NTO treatment groups and controls at the terminal sampling.

## **6.10 Hematology**

Individual and summarized hematology data are presented in Appendix K. At the interim sampling, effects on hematology parameters were limited to reduced (44%) basophil count in males in the 1100 mg/l group compared to the control group ( $p=0.017$ ). Platelet count was increased by 66% and 39% in females in the 36 and 110 mg/l groups, respectively, compared to the control group, ( $p=0.025$  and  $p=0.011$ , respectively). Platelet count was within historical control ranges for the species (CRL 2006). These parameters did not demonstrate a dose response and are not considered treatment-related effects. No other hematology parameters differed between NTO treatment groups and controls at the interim sampling.

There were no treatment-related effects on hematology parameters in males or females at the terminal sampling.

## **6.11 Ophthalmology**

No abnormalities were noted in the pre-study or terminal ophthalmologic examinations.

## **6.12 Urinalysis**

Individual and summarized urinalysis data are presented in Appendix L. In males, higher levels of protein and blood were detected in urine from the 3600 mg/l NTO group than other groups at the interim sampling ( $p=0.014$  and  $p=0.034$ , respectively). These are considered spurious results, potentially due to sample contamination, as similar increases were not observed at the final sampling point. At both the interim and final sampling,



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females and males from the 3600 mg/l group produced approximately 41-57% less urine than controls (males:  $p=0.001$  and  $p=0.013$ , respectively; females:  $p=0.039$  and  $p=0.002$ , respectively). Urine from the 3600 mg/l group was darker (males:  $p<0.001$  and  $p<0.001$ , respectively; females:  $p<0.001$  and  $p<0.001$ , respectively) and more concentrated (i.e., higher specific gravity; males:  $p<0.001$  and  $p=0.002$ , respectively; females:  $p=0.001$  and  $p<0.001$ , respectively) than urine from the controls and other NTO groups at both the interim and final sampling points. Additionally, bilirubin was detected in greater amounts in the urine of males and females in the 3600 mg/l NTO group at both sampling points (males:  $p=0.002$  and  $p=0.003$ , respectively; females:  $p=0.019$  and  $p=0.002$ , respectively). Urine volumes and specific gravity levels were within historical background ranges for the species (Maeda et al. 1999; Matsuzawa et al. 1999). Water intake, glucose, ketones, pH, urobilinogen, nitrites, and leukocytes did not differ between controls and NTO groups at either sampling point for males or females.

### **6.13 Sperm Analyses**

Individual and summarized sperm analysis data are presented in Appendix M. Total sperm count and sperm count adjusted for cauda weight ranged from 114 to 130 million and 400 to 452 million/gram, respectively, and did not differ between controls and NTO treated groups. The proportion of motile and progressively motile sperm ranged from 26 to 33% and 1.8 to 2.1%, respectively, and did not differ between groups.

### **6.14 Determination of BMD and BMDL<sub>10</sub>**

Summarized results of the Benchmark Dose Modeling are presented in Appendix N. In both males and females, NTO showed no evidence of adverse effects on food consumption, body weight, hematology, clinical chemistry, and urinalysis parameters. Additionally, in females, there were no changes in organ weights or histopathology that were considered treatment-related and adverse. Therefore, BMD modeling was not conducted for effects in females and an unbounded NOAEL of 3600 mg/l (260 mg/kg-d) was determined for chronic effects of NTO in female rats. In males, only effects on histopathology of the testis were considered to be treatment-related and adverse. To establish a point of departure, dose response modeling was conducted using EPA's Benchmark Dose Software (BMDS 3.1). Bayesian Model averaging was used, giving equal weight to all available dichotomous models. This resulted in a BMD of 1604 mg/l (76 mg/kg-d) and a BMDL<sub>10</sub> of 921 mg/l (44 mg/kg-d) for chronic effects of NTO in male rats.

### **6.15 Standing Operating Procedure and Protocol Deviations**

The following deviations occurred during the study but were not considered to have compromised the integrity or validity of the study results:

Per the protocol, animal room relative humidity was to be maintained between 30 and 70%. The mean relative humidity in the room housing male rats was 47% and ranged from 13% to 97%. Relative humidity was low on 5 days and high 20 days. In the room housing

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female rats, mean relative humidity was 48% and ranged from 18% to 100%; being low on one day and high on 8 days. Humidity generally remained out of range for less than 2 hours on each occasion.

The SOP (VMO SOP 007 Animal Quality Assurance and Quality Control/Health Monitoring Procedures) states that sentinel animals will be placed on the lowest shelf of the center most rack in the room and a small scoop of dirty bedding will be collected from each dirty animal cage, mixed, and added to the clean sentinel cages. However, sentinel animals were initially placed in cages in the last column of the rack closest to the door in each animal room and dirty bedding was collected from a randomly selected subset of dirty cages. The protocol was modified to specify that dirty bedding would be collected from one randomly selected cage per row of each rack and sentinel cages were moved to be in compliance with the SOP.

Collection of thyroid morphometry data was not included in the protocol. The protocol was modified, however, this occurred after data collection had begun.

## 7 Discussion

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Consistent with previous repeat-dose studies with NTO, chronic exposure to NTO did not affect food consumption, body weight, body weight gain, clinical chemistry, or hematology in either males or females (Crouse et al. 2015; Lent et al. 2016; Lent et al. 2015). Survival was not affected by NTO treatment, with mortality being attributed to age-related spontaneous lesions. The most commonly observed lesions in this study, including pituitary adenoma, angiectasis, and mammary fibroadenoma are known to increase in incidence with age in Sprague-Dawley rats (Thoolen et al. 2010; NTP 2014). The incidence in the current study is within historical ranges for the species (CRL 2001, 2004, 2013) and is not considered to be related to NTO treatment. Other less common lesions included neoplastic histiocytic sarcoma, rhabdomyosarcoma, leiomyosarcoma within the small intestine, rhabdomyosarcoma in the ventral tracheal region, and submucosal rectal lymphoma. These lesions, though rare, are documented spontaneous lesions occurring in other long-term studies at rates similar to the current study (CRL 2001, 2004, 2013).

The common non-neoplastic lesions in this study, including protein accumulation in the kidneys (hyaline casts), multifocal hepatocellular necrosis, spongiosis hepatitis, and biliary hyperplasia, are spontaneous lesions common in aged rats (NTP 2014; Thoolen et al. 2010). Although individually several of the liver lesions (i.e., hepatocellular necrosis and biliary hyperplasia) can occur spontaneously in rodents, they may also be produced or exacerbated by xenobiotics (Thoolen et al. 2010). When taken together, the aggregate liver effect may appear to represent an adverse, treatment-related effect (Hailey et al. 2014). Rats given the same concentration of NTO in drinking water (3600 mg/l: 157 and 250 mg/kg-d in males and females, respectively) for approximately 10-12 weeks did not exhibit hepatotoxicity (Lent et al. 2016). While rats given higher doses of NTO (1000 mg/kg-d) via oral gavage for 90 days exhibited mild treatment-related centrilobular hyperplasia (Crouse et al. 2015). Other changes including inflammation, single cell necrosis, microvesiculation (midzonal and periportal), and bile duct hyperplasia were also

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noted but occurred in both control and treated animals. The absence of hepatocellular necrosis after sub-chronic exposure at higher doses suggests the liver effects in the current study are likely age-related rather than treatment-related.

Reduction of water intake in the high dose group (3600 mg/l NTO) was consistent with that observed in the extended one-generation reproductive test, with intake being approximately 30% and 50% of target in males and females, respectively in both studies (Lent et al. 2016). Water intake did not differ among treatment groups during the urinalysis, likely due to acclimation to new cages, fasting, and animals playing with water bottles, and could not be directly correlated with urinalysis parameters. However, production by the high dose group of smaller volumes of urine that were darker, more concentrated, and had higher amounts of bilirubin is likely a result of reduced water intake and/or excretion of NTO. Urinalysis in the subchronic study similarly demonstrated an increase in bright yellow colored urine in animals given 315 and 1000 mg/kg-d NTO (Crouse et al. 2015). Other urinalysis parameters including urine volume and specific gravity did not differ among treatment groups in the subchronic study suggesting that the changes in urine volume and specific gravity in the current study were due to reduced water consumption. However, bilirubin in the urine (bilirubinuria) is considered an abnormal finding that indicates cholestasis (Blue et al. 2013). Although liver enzymes (ALKP) and serum bilirubin were not elevated suggesting that this may not reflect cholestatic or hepatocellular damage (Cuperus et al. 2017), liver histopathology (hepatocellular necrosis, biliary hyperplasia, cholangiofibrosis) indicates the bilirubinuria reflects hepatotoxicity.

Although previous studies have demonstrated reductions in male reproductive organ weights and reduced sperm counts (Crouse et al. 2015; Lent et al. 2016; Lent et al. 2015), similar effects were not observed in the current study. NTO-related reproductive toxicity in males was observed only in testes histopathology. Although testicular toxicity was observed, it was less severe than in previous studies. Atrophy, the only described testicular lesion, was categorized as mild because it only affected a small percentage of observed tubules. In contrast, in the subchronic and pubertal development and thyroid toxicity studies, in which rats were administered NTO via oral gavage, moderate to severe atrophy was observed in rats given 315 and 1000 mg/kg-day for 90-days (Crouse et al. 2015), while degeneration/atrophy was moderate in rats given 250 mg/kg-day and marked to severe in rats given 500 mg/kg-day for approximately 30 days (Lent et al. 2015). In animals dosed with 315, 500, or 1000 mg/kg-day NTO, the majority of tubules retained only supporting Sertoli cells. Spermatogonia and early spermatocytes were present in a limited number of tubules while spermatids and late spermatocyte stages were absent or necrotic (Crouse et al. 2015; Lent et al. 2015). In rats orally gavaged with 250 mg/kg-day, damage to germ cell layers was less pronounced as additional layers remained intact (i.e., round spermatids) or were present but degenerate/necrotic (i.e., late stage spermatocytes and elongating spermatids). In rats exposed via drinking water at the same maximal concentration as the current study, degeneration/atrophy of seminiferous tubules (Sertoli-only tubules) was generally moderate in the parental generation (exposed for 10 weeks) and mild in the F1 generation (exposed in utero then directly for approximately 30 days) (Lent et al. 2016). More subtle effects including germ cell-free gaps, vacuoles within

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Sertoli cells, lack of elongating spermatids, retained spermatids, and apoptotic cells were also noted in tubules not exhibiting severe atrophy (i.e., Sertoli-cell only).

Repetitive and prolonged exposure to toxic compounds results in damage to germ cells and successive loss of germ cell populations over time through a process termed maturation depletion (Creasy 1997; Vidal et al. 2014). Germ cells are lost, regardless of the mechanism of toxicity, due to their dependence on the function and processes of other cell types within the testis (i.e., Sertoli cells). Because Sertoli cells are extremely resistant to cell death, prolonged dosing often results in seminiferous tubules lined only with Sertoli cells (Creasy 1997). Although resistant to cell death, Sertoli cells are sensitive to alterations in function and are a target of testicular toxicants including 2,5-hexandione, tri-o-cresyl phosphate, 1,3-dinitrobenzene, cyclohexylamine, perfluorooctanesulfonic acid (PFOS), and phthalate esters (Blackburn et al. 1988; Boekelheide 1988; Chapin et al. 1983; Creasy et al. 1987; Creasy et al. 1990; Qiu et al. 2013; Somkuti et al. 1991). Time-course studies in rats and mice indicate that the Sertoli cell is also the initial target of testicular toxicity for NTO (Lent et al. 2018; Mullins et al. 2016). The varying degrees of degeneration/atrophy observed across NTO studies is consistent with Sertoli cell toxicity as longer term exposure results in variable germ cell loss, irregularly arrayed germ cell layers, unmasking of Sertoli cell cytoplasm, and, with more severe toxicity, diffuse atrophy (Vidal et al. 2014). Thus, the more severe (diffuse) toxicity observed in the subchronic and pubertal development and thyroid toxicity studies is likely due to the higher delivered doses (250 to 1000 mg/kg-d) in those studies compared to the delivered doses in the extended one-generation reproductive test (150 mg/kg-d in parental generation and 335 mg/kg-day in the F1 generation) and the current study (170 mg/kg-d). Toxicokinetic differences between oral gavage and drinking water routes of administration may also have contributed to differences in the severity of effects (Vandenberg et al. 2014).

Despite differences in delivery methods and duration of exposure, the BMDL and BMDL<sub>10</sub> for testicular atrophy in the current study (76 mg/kg-d and 44 mg/kg-d, respectively) are consistent with those reported for male reproductive toxicity in previous NTO studies. A BMD of 70 mg/kg-d and a BMDL<sub>10</sub> of 40 mg/kg-d were derived based on testicular effects (e.g., degeneration/atrophy) in the subchronic study (Crouse et al. 2015). BMDL<sub>10</sub> values for male reproductive toxicity (e.g., reduced testes and epididymal mass, sperm count, nipple retention) ranged from 140-160 mg/kg-d in parental males and 120-310 mg/kg-d in F1 offspring (Lent et al. 2016). This consistency indicates that chronic dosing does not increase the incidence/severity of observed testicular toxicity or reduce the dose at which it is observed.

## 8 Conclusions

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To evaluate the effects of chronic exposure to NTO, male and female rats were given *ad libitum* access to NTO in drinking water at concentrations of 0, 36, 110, 360, 1100, and 3600 mg/l for one year. NTO did not affect body weight, body weight gain, or food consumption in either sex. No treatment-related effects were observed in clinical chemistry and hematology parameters at the 6 month or one year sampling. At both the interim and final sampling, both males and females from the 3600 mg/l group produced smaller

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volumes of urine that was darker, more concentrated, and contained more bilirubin than the controls. Total and motile sperm counts were not affected by NTO treatment. Absolute and relative organ weights did not differ between control and NTO treated groups for either sex. Spontaneous age-related neoplasms occurred in controls and NTO groups at rates consistent with historic controls. NTO was generally non-toxic in females at the doses tested. Toxicity in males was limited to testicular toxicity as demonstrated in previous studies. Chronic exposure did not result in testicular toxicity at lower doses and the toxicity observed only in the high dose group in this study is less severe than that observed in previous studies. A BMD of 1604 mg/l (76 mg/kg-d) and a BMDL<sub>10</sub> of 921 mg/l (44 mg/kg-d) were determined for chronic effects of NTO in male rats.

### 9 Point of Contact

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Questions pertaining to this report should be referred to Emily May Lent at DSN 584-3980, commercial 410-436-3980, or by e-mail: [usarmy.apq.medcom-aphc.mbx.tox-info@mail.mil](mailto:usarmy.apq.medcom-aphc.mbx.tox-info@mail.mil).

Prepared By:

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EMILY MAY LENT  
Toxicologist  
Toxicity Evaluation Division (TEV)

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Date

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ALLISON M. NARIZZANO  
Biologist  
TEV

---

Date

Approved By:

---

ARTHUR J. O'NEILL  
Division Chief, TEV

---

Date

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MARK S. JOHNSON  
Director, Toxicology

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Date

## Appendix A

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