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Wildlife Toxicity Assessment for 3-Nitro-1,2,4-Triazol-5-One (NTO)

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14. ABSTRACT
3-Nitro-1,2,4-Triazol-5-One, also known as Nitrotriazolone (or NTO), is a less sensitive explosive than many conventional formulations and is considered a potential replacement for cyclotrimethylene trinitramine (or RDX) and other energetics in conventional explosives. This Wildlife Toxicity Assessment (WTA) summarizes current knowledge of the toxicological effect of NTO on wildlife. Evaluating the toxicity of NTO will contribute to the derivation of toxicity reference values (TRVs) for use as screening-level benchmarks for wildlife near contaminated sites. The protocol for the performance of this WTA is available in detail in Technical Guide No. 254 (Standard Practice for Wildlife Toxicity Reference Values).

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Wildlife Toxicity Assessment for NTO

CAS No. 932-64-9 January 2025

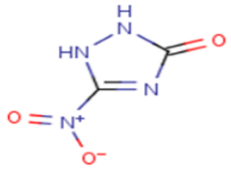
1. INTRODUCTION

3-Nitro-1,2,4-triazol-5-one, also known by its synonym Nitrotriazolone (NTO) is a less sensitive explosive than many conventional formulations and has been prepared as a potential replacement for cyclotrimethylene trinitramine (RDX) and other energetics in conventional military munitions. NTO is a crystalline powder exhibiting no odor and decomposes before melting. NTO is a key component of the formulation of insensitive munition explosive (IMX); IMX-101 represents one of those formulations and is comprised of NTO, 2,4-dinitro anisole (DNAN) and nitroguanidine (NQ).

NTO was first prepared in 1905 by nitration of 1,2,4-triazole-5-one (TO) (Manchot and Noll, 1905, and the first report on the explosive nature of NTO was published by Lee and Coburn in 1985. In 1999, the Defense Science and Technology Organisation (DSTO) reported further development of NTO as a potential replacement for RDX and other energetic substances in military munitions and explosives. The use of this class of insensitive munition has the potential to save lives on the battlefield due to its ability of preventing inadvertent or accidental explosions by more conventional munitions.

IMX-101 and other insensitive formulations exhibit reduced potential for detonation that might result from accidental impacts and fires in military combat vehicles and aircraft. NTO is now used in several weapon system formulations. The U.S. Army is currently evaluating novel explosive formulations containing NTO for use in additional weapon systems that have been developed and fielded. Table 1 compiles and summarizes the chemical/physical properties of NTO.

Table 1. Summary of the Physical-Chemical Properties of NTO

Structure	
CAS No.[1-4]	932-64-9
Chemical formula	$C_2H_2N_4O_3$
Molecular weight	130
Color [2]	White to pale yellow
Physical state[2]	Crystalline powder
Melting point	268–271°C
Boiling point (760 mmHg)[2]	Thermal degradation above its melting point
Odor	Odorless
Solubility in water[1,4]	12,800mg/L at 19°C; 17,200 mg/L at 25°C
Solubility in other solvents[4]	Solubility in acetone, ethyl acetate and dichloromethane reported as 16.8, 2.8, and <0.20 g/L, respectively at 19°C
Partition coefficients:	
Log K_{OW}	0.858
Log K_{OC}[5,6]	< 1.0 (as tested in 11 different soils)
Vapor pressure at 25°C [2]	No data available
Specific gravity	1.93 g/cm^3
Henry's Law constant at 25°C	4.1x10^{-13} atm-m^3/mole
Vapor density	No data available
Conversion factors	1 ppm = 5.31 mg/m^3; 1 mg/m^3 = 0.188 ppm

Legend:
atm-m^3/mole = air to moles per cubic meter for water
degrees Celsius = °C
g/cm = grams per centimeter
Log Know = octanol-water partition coefficient
Log Koc = organic carbon partition coefficient
mg/L = milligrams per liter
mm Hg = millimeters of mercury
mg/m^3 = milligrams per cubic meter
ppm = parts per million
Notes:
[1]DSTO, 1999
[2]HSDB, 2018
[3]USAPHC, 2010
[4]ECHA, 2020
[5]Mark et al., 2016
[6]Swann et al., 1983

2 TOXICITY PROFILE

2.1 Literature Review

Electronic searches of relevant biomedical, toxicological, and ecological databases (e.g., PubMed® (Public/Publications MEDLINE), BIOSIS Previews®, (Biosciences Information Service); DTIC® (Defense Technical Information Center); Online Multisearch, Scopus®, Web of Science and TOXNET® – an aggregated tool for simultaneously searching the following databases: HSDB (Hazardous Substance Data Bank); TOXLINE® (National Library of Medicine); ChemIDplus® (PubChem); IRIS (Integrated Risk Information System); LactMed®; DART® (Developmental and Reproductive Toxicology); TOXMAP®; TRI (Toxic Release Inventory); CTD (Comparative Toxicogenomics Database); Household Products Database; HazMap®; ITER (International Toxicity Estimates of Risk); ALTBIB (Alternative to Animal Testing Bibliography; CCRIS (Chemical Carcinogenesis Research Information System); CPDB (Carcinogenic Potency Database); and GENE-TOX (Genetic Toxicology) were conducted on August 10–12, 2018, with the aim of identifying primary reported studies and reviews on the toxicology of NTO. Separate searches were conducted for general toxicology and specific searches for birds, reptiles, amphibians, and wildlife. Each database was searched using keywords to include 3-Nitro-1,2,4-triazol-5-one; its Chemical Abstract Service (CAS) number (932-64-9); its synonym Nitrotriazolone; or its acronym NTO, plus toxicity, ecotoxicology, wild, wildlife, avian, bird, frog, amphibian, reptile, or environment. Appendix B documents the details and results of the search strategies. The article titles identified in each search were reviewed for relevance. Potentially relevant articles focused on the toxicological effects of NTO on terrestrial vertebrates or its environmental fate and transport. All potentially relevant articles were acquired as electronic files. Review articles provided additional technical resources not identified during the initial database searches. Appendix B describes the specific details of the literature search strategy.

2.2 Environmental Fate and Transport

The environmental fate of NTO is limited to a few reports. Due to its high solubility and ionic nature, NTO readily dissolves in water (DSTO Materials Research Laboratory, 2011; Table 1). Other sources have reported the low absorption and high mobility of NTO in soils and groundwater (Mark et al., 2016), and, therefore, is an important source of soil and water contamination. NTO was found to adsorb to soils and exhibits a low affinity for clay soils (Linker et al., 2015). NTO was also shown to adsorb to positively-charged organic carbon (OC) in the soil.

Studies of the adsorption and attenuation behavior of NTO in 11 soils showed that the measured adsorption coefficient was less than 1 $cm^3 g^{-1}$ for all the soils investigated (Mark et al., 2016). There was also a highly significant inverse relationship between the measured NTO adsorption coefficient and the soil pH (Mark et al., 2016). The attenuation of NTO was higher in untreated versus sterilized soil samples, which an observation suggested NTO was biodegraded by microbial metabolic pathways and in soils with a high organic content (Mark et al, 2016; Temple et al., 2018).

Similar studies of the environmental fate of NTO (Cardenas-Hernandez et al., 2020) have focused on NTO reduction by the hematite -Fe2+ redox couple with the intent of understanding the attenuation and remediation of NTO. This group found that both hematite and Fe^{2+}(aq) contributed to the quantitative reduction of NTO to 3-amino-1,2,4-triazol-5-one (ATO) following first-order kinetics and showing a strong pH dependency between 5.5 and 7.0. This work

revealed the importance of iron oxide-Fe^{2+} in controlling NTO transformation (Cardenas-Hernandez et al., 2020). NTO is rapidly reduced to ATO by iron (II) monosulfide (FeS) minerals that are usually abundant in freshwater and marine sediments, marshes, and hydrothermal environments under anoxic subsurface environments (Menezes et al., 2021).

The photocatalytic degradation of NTO in an aqueous suspension of TiO_2 was found to be mediated via degraded ring cleavage and subsequent elimination of the two carbon atoms of NTO as CO_2. No significant photodegradation of NTO was detected in the absence of a catalyst. Long-term irradiation over 1 week led to complete degradation to nitrites, nitrates, and CO_2 (Le Campion et al., 1999).

The metabolism of NTO was investigated *in vitro* using rat liver microsomes and a bacterial model system (Le Campion et al., 1997 and 1998). Rat liver microsome catalysis of NTO under an atmosphere of nitrogen produced primarily amines and 5-amino-1,2,4-triazol-3-one; however, in the presences of oxygen, microsome catalysis of NTO produced a major product, which was identified as 5-hydroxy-1,2,4-triazol-3-one urazol, and a minor product, which was identified as an amine (Le Campion et al., 1997 and 1998). The addition of NTO to an aqueous medium releases protons and lowers the pH. The microbial metabolism of NTO is pH-dependent in aqueous systems. Optimal microbial reduction of NTO was found at pH 6.0 in the presence of sucrose, while by contrast, optimal ring cleavage was found at pH 8.0 (Le Campion et al., 1998). Microbial degradation studies of NTO in industrial waste and soil enrichment cultures showed efficient nitro-reduction with formation of the primary amine, ATO, which was determined to be persistent in the soil under anaerobic conditions (Le Campion et al., 1999; Krzmarzick et al., 2015; Madeira et al., 2017). The end-products of the biodegradation of NTO were CO_2, urea and a polar compound, which was assumed to be hydroxyl urea (Le Campion et al., 1999; Krzmarzick et al., 2015).

2.3 Bioaccumulation and Elimination

NTO is readily absorbed and distributed in the body and is rapidly eliminated when dosed orally from studies conducted in monkeys. When dosed at 50 milligrams/kilogram (mg/kg) the blood concentrations peaked at about 5 hours and eliminated in 24 hours (Hoyt et al., 2013). No reports on the metabolism of NTO in avian, reptilian, and amphibian species were located in the current contents of the available literature.

NTO was taken up by the roots and shoots of a mixture of big bluestem grass (*Andropogon gerardii*), Indian grass (*Sorghastrum nutans*), and switch grass (*Panicum vigatum*) during phytoremediation of soils that were contaminated by up to 50 mg/kg of IMX -101. Complete degradation to below detection limits occurred over 225 days (Richard and Weidhaas, 2014).

2.4 Summary of Mammalian Toxicity

2.4.1 Mammalian Oral and Inhalation Toxicity

2.4.1.1 Mammalian Oral Toxicity – Acute and Subacute Toxicity

The acute oral LD_{50} for NTO is reported to be greater than 5 grams/kilogram (g/kg) in both rats and mice. Further, NTO did not induce dermal sensitization when tested in guinea pigs and was not found to be an eye irritant; however, it did elicit mild skin irritation in a rabbit (LANL, 1985).

In subacute toxicity studies of NTO, investigators sought to select suitable doses of this test article for subsequent subchronic toxicity studies as described in the section below (Crouse et al., 2015). In these subacute toxicity studies, male and female Sprague-Dawley rats were dosed orally in an aqueous medium that was suspended in polyethylene glycol (PEG)-200 to minimize palatability challenges often associated with an acidic dosing regimen. Six groups of male and female rats were orally exposed to NTO via gavage in aqueous media that was suspended in PEG and subsequently administered at doses of 0, (PEG-200 control), 250, 500, 1,000, 1,500, and 2,000 mg/kg/day of NTO (Crouse et al., 2015). Body mass and food consumption were measured on days 0, 1, 3, 7, and 14 following the exposure. Clinical signs of toxicity and mortality were observed twice daily. No compound-related lethality was observed throughout the 14-day dosing period, and the majority of the observed changes were found in male reproductive organ weights and weight ratios. Results from this subacute oral toxicity study showed significantly lower testicular weights in the high-dose (≥ 500 mg/kg) groups (Crouse et al., 2015). This group also showed that Sertoli cells were the target site of testicular injury in Sprague Dawley rats that were orally dosed with NTO (500 mg/kg/day) in corn oil for 1, 3, 7, or 14 days (Lent et al., 2018).

2.4.1.2 Mammalian Oral Toxicity - Subchronic

A subchronic (90-day) oral gavage study was conducted in male and female rats where NTO was administered 7 days a week for a total of 13 weeks (Crouse et al., 2015). Selected NTO doses were derived from the 14-day subacute repeated dose study and were set at 0 (PEG-200 control), 30, 100, 315, and 1,000 mg/kg/day for both male and female rats. No compound-related mortality was observed in any of the dose groups throughout the 90-day dosing period. All surviving animals were euthanized on day 91, at which time a complete necropsy was performed, organs were removed and weighed, and blood was collected for clinical and hematological analyses.

Statistically significant decreases in organ weights as compared the control group of animals were explored at dose levels equal to, or exceeding 315 mg/kg/day, and were predominantly limited to male reproductive organ effects and altered organ weight ratios. Sporadic but significant changes in many clinical and biological parameters were found when comparing observations between the NTO dosed groups and on comparison to the controls. These included: body weight, body weight gain, various organ weights and weight ratios, hematological parameters, and clinical chemistry (Crouse et al., 2015).

Rats that were treated with NTO at doses of 315 and 1,000 mg/kg/day had moderate to severe degeneration and atrophy of the seminiferous tubules. These changes were statistically significant as compared to the control group. The incidence of testicular changes in this rat model at doses of 30 and 100 mg/kg/day was similar to the control animals. Testicular effects were considered the critical effect for deriving various exposure criteria.

A benchmark dose (BMD) analysis of the re-evaluated subchronic study data was also performed (Crouse et al., 2015). The incidence of seminiferous tubular degeneration and atrophy observed in male rats was the most sensitive adverse outcome seen from this study. Thus, the incidence of adverse findings was used in the BMD model (Crouse et al., 2015). This analysis modeled the lower 95% confidence interval on the 10% response rate for the critical effect (Crouse et al., 2015). The output from nine models was evaluated for acceptable P-values, Akaike’s Information Criterion values, and curve fitting of the input data. The following three models were selected: logistical, probit, and multistage-2 modeling. From this analysis of

the three selected models, it was possible to derive an estimated BMD of 70 mg/kg/day (TRV_{High}, Figure 1) and an estimated benchmark dose lower confidence limit ($BMDL_{10}$) of 44 mg/kg/day (TRV_{Low}, Figure 1) for testicular changes in rats, i.e., hypoplasia lENT (Crouse et al., 2015).

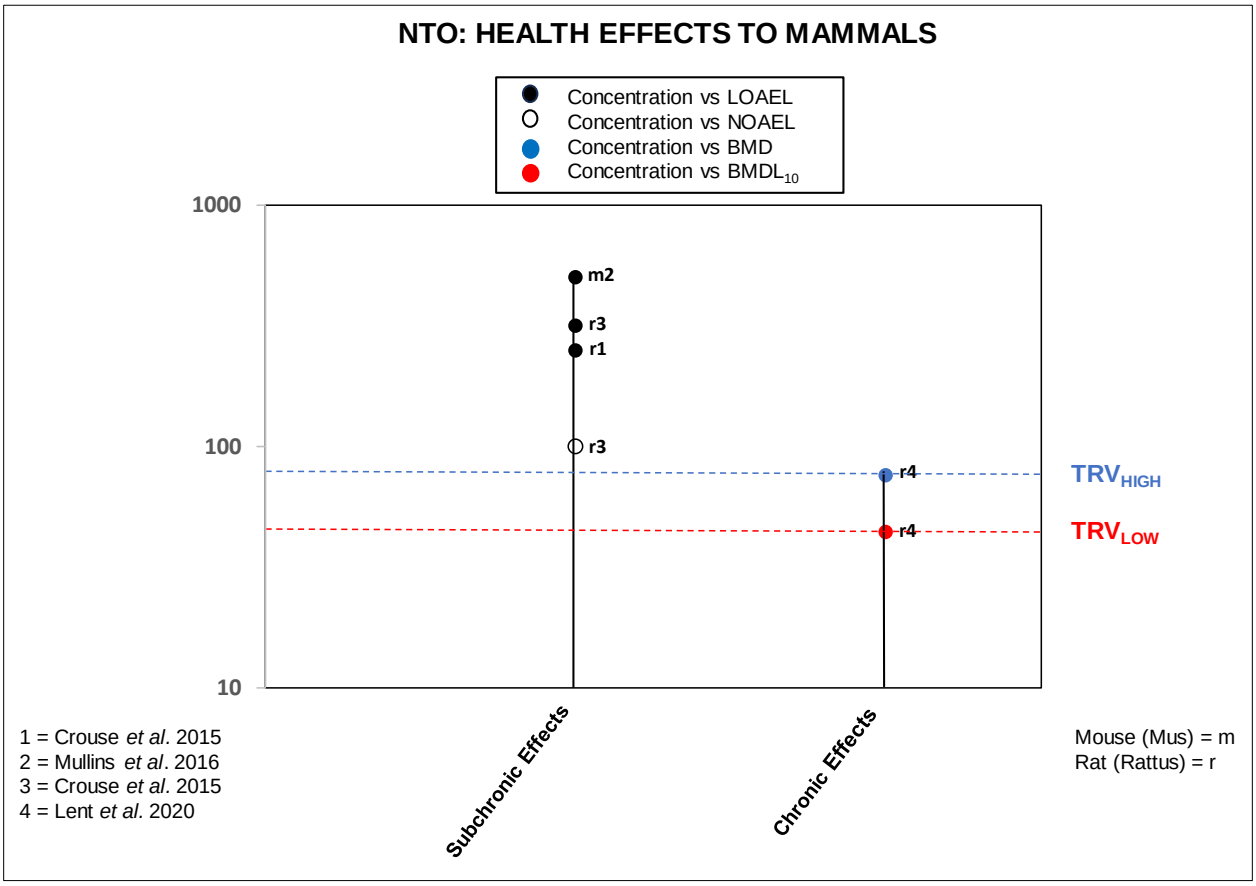


Figure 1. 3-Nitro-1,2,4-Triazol-5-One: Ingestion Health Effects of Mammals

Others have studied the effects of NTO in adult male BALB/c mice (Mullins et al., 2016). Initially, pilot study data was analyzed to verify the selected doses of NTO from the earlier rat study (Crouse et al., 2015) and planned for the subsequent 14-day mouse model study (Mullins et al., 2016). In the pilot study, NTO was administered as a corn oil suspension at doses of 0, 250, 500, and 1,000 mg/kg/day in a total of 12 animals (n = 3 per dose level), with the study being terminated at day 14. There were no adverse clinical effects at any dose of NTO administered to the mice in the pilot arm to the study, and mice treated with NTO did not show any changes in body weight at any time in this 14-day study. Additionally, absolute and relative testicular and epididymal masses were similar between the control group and the NTO treatment groups (Mullins et al., 2016). However, in this pilot arm of the study, preliminary histopathology suggested partial germ cell degeneration in animals that were dosed at 500 and 1,000 mg/kg/day following 14 days of NTO treatment. Differences in testicular histopathology between the control and 250 mg/kg/day-treated animals were unremarkable.

Considering the above studies, the main component to the study was undertaken where mice (at n = 5 mice per group) were exposed to NTO as a suspension in corn oil by oral gavage at 0, 500, and 1,000 mg/kg/day for 1, 3, 7, and 14 days; subsequent testicular effects were evaluated (Mullins et al., 2016). Treatment with NTO-induced testicular effects at day 7 in animals treated at 1,000 mg/kg/day and at day 14 in animals treated at 500 mg/kg/day. In these groups, animals showed the degeneration of bi- and multi-nucleated giant cells. Animals dosed with 1,000 mg/kg/day NTO showed significantly decreased testicular absolute weights when compared with the controls. These observations showed that NTO is a testicular toxicant and confirmed the previous observations from rat studies (Mullins et al., 2016; Crouse et al., 2015).

2.4.1.3 Mammalian Oral Toxicity – Subchronic: Reproductive and Developmental Toxicity

Due to the observed effects of NTO on testes, the potential for endocrine effects of NTO in Sprague-Dawley rats (n = 15 per sex per group) was studied by administering this test article to male rats at doses of 0, 250, and 500 mg/kg/day and female rats at 0, 500, and 1,000 mg/kg/day (Lent et al., 2015). Dosing commenced at the time of weaning through post-natal day 54 and 43, respectively. Body mass measurements and age at vaginal opening and preputial separation, and measures of estrous cycling were unaffected by treating with NTO.

Male rats that were treated with NTO exhibited reduction in testis mass, tubular degeneration and atrophy, which was less pronounced at the lower administered dose of 500 mg/kg/day (Lent et al., 2015). NTO did not affect thyroid or testosterone hormone levels. Collectively, these results suggested that NTO was not serving as an estrogenic or thyroid active compound. However, it might indicate an overt effect on testicular toxicity (Lent et al., 2016; Table 2).

The reproductive and developmental oral toxicity of NTO was studied by exposing rats via the drinking water at 0, 144, 720, or 3,600 milligrams per liter (mg/L) in a modified extended one-generational study (Lent et al., 2016). Treatment of the parental generation began with two female and four male rats from premating and continued until weaning of the litters. Direct dosing of offspring (F1) occurred from weaning through puberty. Pups were counted and weighed on post-natal day (PND) 1.

Anogenital distance was measured on PND 4, and males were examined for nipple characteristics on PND 13. The F1 offspring were examined daily for attainment of puberty. The NTO did not markedly affect measures of fertility, including mating indices or gestation indices. Following exposure to NTO at 3,600 mg/L changes in litter size and sex ratio were observed in the F1 offspring (Lent et al., 2016). Additionally, seminiferous tubule degeneration or atrophy were observed in both the P1 and F1 males only in the 3,600 mg/L group. F1 males also exhibited reduced testicular organ mass, and retention of the nipples was also increased following exposure to NTO at 3,600 mg/L.

This study showed that NTO is a testicular toxicant with developmental effects that may be secondary to its observed testicular injury in male rats. This study did not measure the daily volume of water consumed by treated rats, which prevented estimation of the actual dose of NTO consumed (Lent et al., 2016).

Table 2. Summary of Subacute, Subchronic, and Chronic Oral NTO Toxicity in Mammals

Test Organism	Test Duration	Test Results					
		NOAEL (mg/kg/day)	LOAEL (mg/kg/day)	$BMDL_{10}$	BMD	Effects Observed at the LOAEL	Study
Sprague-Dawley Rats	14-day oral	250	500	ND	ND	No mortality or clinical signs, reduced testicular weights at 500 mg/kg/day	Crouse et al., 2015.
Mice, BALB/c	14-day oral	NA	500	ND	ND	No adverse clinical effects. No effects on body weights. Testicular effects seen at 500 (day 14) and 1,000 (day 7) mg/kg/day	Mullins et al., 2016
Sprague – Dawley rats	90-day oral	100	315	40	70	No effect on food consumption, body mass or neurobehavioral tests. Testicular effects above 315 mg/kg/day	Crouse et al., 2015
Sprague – Dawley rats	Post-natal day 21, dosed orally, 250 or 500 mg/kg for males and 500 or 1,000 mg/kg for females	NA	250	ND	ND	Testicular effects seen in males by histopathology. No effects on testosterone or estrogen hormone levels	Lent et al., 2015
Sprague – Dawley rats (Pubertal observations)	Extended one generation reproductive toxicity, dosed orally in water at 144, 720, 3600 mg/L/day	80	340	360–378	120–310	No treatment related effects on fertility, mating indices, gestation index, or liter size in parental (P1) and the F1 generation. Reduced testicular organ mass in F1 (pubertal) generation	Lent et al., 2016
Sprague – Dawley rats	12 months oral	50	166	44	76	Chronic exposure did not affect food consumption, body weight/weight gain clinical chemistry or hematology in males or females. Male reproductive effects observed (testicular atrophy only) by testicular histopathology	Lent et al., 2020

Legend: NA = not available; ND = not determined; NOAEL = no observed adverse effect level; LOAEL = lowest observed adverse effect level; mg/kg/day = milligrams per kilogram per day; mg/L/day = milligrams per liter per day

2.4.1.4 Mammalian Oral Toxicity – Chronic: Reproductive and Developmental Toxicity

In one identified study from the available literature (Lent et al., 2020), the effects of chronic exposure to NTO were evaluated in male and female Sprague-Dawley rats at dose-dependent concentrations in drinking water of 0, 36, 110, 360, 1100, and 3600 mg/L for a 1 year. No effects of NTO exposure were observed on body weight, body weight gain, or food consumption in either male or female rats (Lent et al., 2020). Additionally, treatment-associated effects were not observed on clinical chemistry or hematology parameters at either 6- or 12-months of exposure when determining effects in either male or female rats, nor were there any changes in the context of absolute and relative organ weights for either sex when comparing control and NTO-treated animals (see Table 2).

The reported observations (Lent et al., 2020) were similar to those previously reported for sub-chronic studies on NTO exposure (Crouse et al., 2015; Lent et al., 2015; Lent et al., 2016). For male rats, reproductive effects were as similarly noted before (Crouse et al., 2015; Lent et al., 2015; Lent et al., 2016) and limited to testicular toxicity. Chronic exposure of rats to lower NTO doses did not yield any testicular toxicity, and the observed toxicity in the high-dose group was less severe than that observed in shorter exposure studies (Crouse et al., 2015; Lent et al., 2015), which formally suggested the possibility of the study design on physiological kinetics affecting the observed testicular toxicity in male rats (Lent et al., 2020). From this work, a BMD of 1604 mg/L (76 mg/kg/day) and a lower bound $BMDL_{10}$ of 921 mg/L (44 mg/kg/day) were determined for the chronic exposure effects of NTO in male rats (Lent et al., 2020; Table 2).

2.4.1.5 Mammalian Inhalation Toxicity – Acute

An acute inhalation study with NTO was conducted to estimate a 4-hr LC_{50} in a rat model (USAPHC, 2013). Since NTO is an explosive material, it was not possible to generate atmospheres of vapor or dry dust. Instead, NTO was solubilized in water that was subsequently aerosolized permitting a nose-only exposure of rats in this model system. At the highest concentration tested of 0.184 mg/L, there were no compound-related animal deaths, and the LC_{50} was estimated at greater than 0.184 mg/L. This study also included an investigation of the time course of NTO absorption following both oral and inhalational modes of exposure (USAPHC, 2013).

Information on the relative rates of compound absorption between the oral and inhalation routes of exposure was intended to aid in extrapolating the subchronic oral exposure data to the inhalation route, which could facilitate derivation of an occupational exposure level. Observations from this component of the study suggested that peak NTO blood levels were reached earlier in animals that were exposed by inhalation as compared to animals that were exposed by oral gavage. In both groups, blood levels of NTO dropped quickly and were near zero by 8 hours post-exposure (USAPHC, 2013).

In a study with orally dosed primates, similar doses of NTO were also eliminated by 8 hours post-exposure (Hoyt et al., 2013).

2.4.2 Mammalian Toxicity – Other

2.4.2.1 Mammalian Toxicity – Other: Carcinogenicity

No relevant toxicological data for the effects of NTO and development of carcinogenic effects were located in the primary peer-reviewed or grey literature.

2.4.2.2 Mammalian Toxicity – Other: Endocrine Effects

In addition to the study described in section 2.4.1.3 (Lent et al., 2015), the endocrine active potential of NTO was tested in a rat model using the Hershberger and uterotropic bioassays, which was conducted according to the U.S. Environmental Protection Agency's Tier 1 *in vivo* screen for endocrine disruption (Quinn et al., 2014). This bioassay measures differences in androgen and estrogen sensitive tissue when conducted in castrated and ovariectomized rats. The gonad-ectomized rats were administered NTO in corn oil by the oral route of exposure at doses of 250, 500, or 1,000 mg/kg for 10 or 3 days for the Hershberger and uterotropic bioassays, respectively. Male rats were subcutaneously administered testosterone at a dose of 0.2 mg/kg and orally dosed with anti-androgenic flutamide at 3 mg/kg/day as negative and positive controls, respectively. Female rats were subcutaneously administered 17 α-ethyl estradiol at a dose of 0.3 µg per day as a positive control. NTO failed to adversely affect androgen-sensitive male reproductive selected tissues (i.e., seminal vesicles with/without fluid, glans pens, Cowper gland, ventral prostate, and levator ani bulbocavernosus), animal weights, nor any changes in uterine weights. At the selected doses, the results of this study did not provide any evidence suggesting NTO behaves as an estrogenic or anti-androgenic endocrine disruptor in rats (Quinn et al., 2014).

2.4.2.3 Mammalian Toxicity – Other: Miscellaneous Toxicology

NTO was also subjected to a battery of five *in vitro* tests that included estrogen and androgen receptor binding, estrogen transactivation, aromatase, and steroidogenesis. Ultimately, these assays provided negative outcomes for NTO, and no significant effects were seen (USAPHC, 2012). Others have attempted to identify potential biomarkers of toxicological effect in Rhesus macaques following exposure to NTO (Hoyt et al., 2013). NTO was dissolved in deionized water and administered to anesthetized macaques through a gastric tube. Serial blood and urine specimens were taken for 5 hours. From this limited dataset, NTO appears to be readily detected in the blood and urine, even at the lowest (5 mg/kg) dose tested (Hoyt et al., 2013)

2.5 Summary of Avian Toxicology

The acute oral toxicity of NTO in corn oil was explored in a limit test at a dose of 2,000 mg/kg in 5-week-old Japanese quail (*Coturnix japonica*) comprising three male and two female quail (Jackovitz et al., 2018; Table 3). No mortality was seen during the 14-day observational period post-exposure. However, the quail showed signs of neurological toxicity (i.e., head tilt, ataxia, and tremors) at approximately 48-hours post-exposure. After dosing, no other adverse clinical signs were observed. The LD_{50} value was greater than 2,000 mg/kg. However, these values were derived from a single recently reported study (Jackovitz et al., 2018; Table 3).

This same group also conducted a one generational reproductive toxicity bioassay with NTO in Japanese quail (Jackovitz et al., 2018). Parental (F0) generation birds were exposed orally with NTO in corn oil at doses of 0, 20, 100, 500, and 1,000 mg/kg/day beginning when quail had

reached 2 weeks of age. The F0 generation was terminated at 12 weeks of age (i.e., after 10 weeks of dosing). Gross necropsy and histopathology were evaluated in selected organs for any toxicological effects of NTO (Jackovitz et al., 2018). Quail that were dosed with 1,000 mg/kg/day for 5 days displayed neuromuscular anomalies including loss of balance, convulsions, and mortality. Ultimately, all of the 1,000 mg/kg/day dosed quail and all but one of the 500 mg/kg/day dosed quail met euthanasia criteria; they were humanely euthanized prior to behavioral and reproductive evaluations. The remaining quail in the 0, 20, and 100 mg/kg/day dosed groups were evaluated for any toxicological or reproductive effects (Jackovitz et al., 2018; Table 3).

Reproductive effects were not seen for any of the parameters analyzed, which included time to reproductive maturity, cloaca gland size, caudal sperm concentration, egg fertility, eggshell thickness, and eggshell strength. No hematological parameters studied were significantly affected at 20 and 100 mg/kg dose groups. Histopathological analysis of the liver, kidneys, heart, spleen, and reproductive organs did not demonstrate any pathological manifestations. Quail that were exposed to 100, 500, and 1,000 mg/kg/day NTO had vacuoles in the brainstem and cerebellum, which were significant at the two highest doses. Due to pre-term deaths/euthanasia and a resulting lack of age-matched controls, it was not determined if daily NTO doses of 500 and 1,000 mg/kg/day affected testicular development in juvenile Japanese quail (Jackovitz et al., 2018). Testicular size and weight were unaffected at the lower doses of NTO exposure (20 or 100 mg/kg/day).

Table 3. Summary of Acute and Subchronic NTO Toxicity in Avian Species

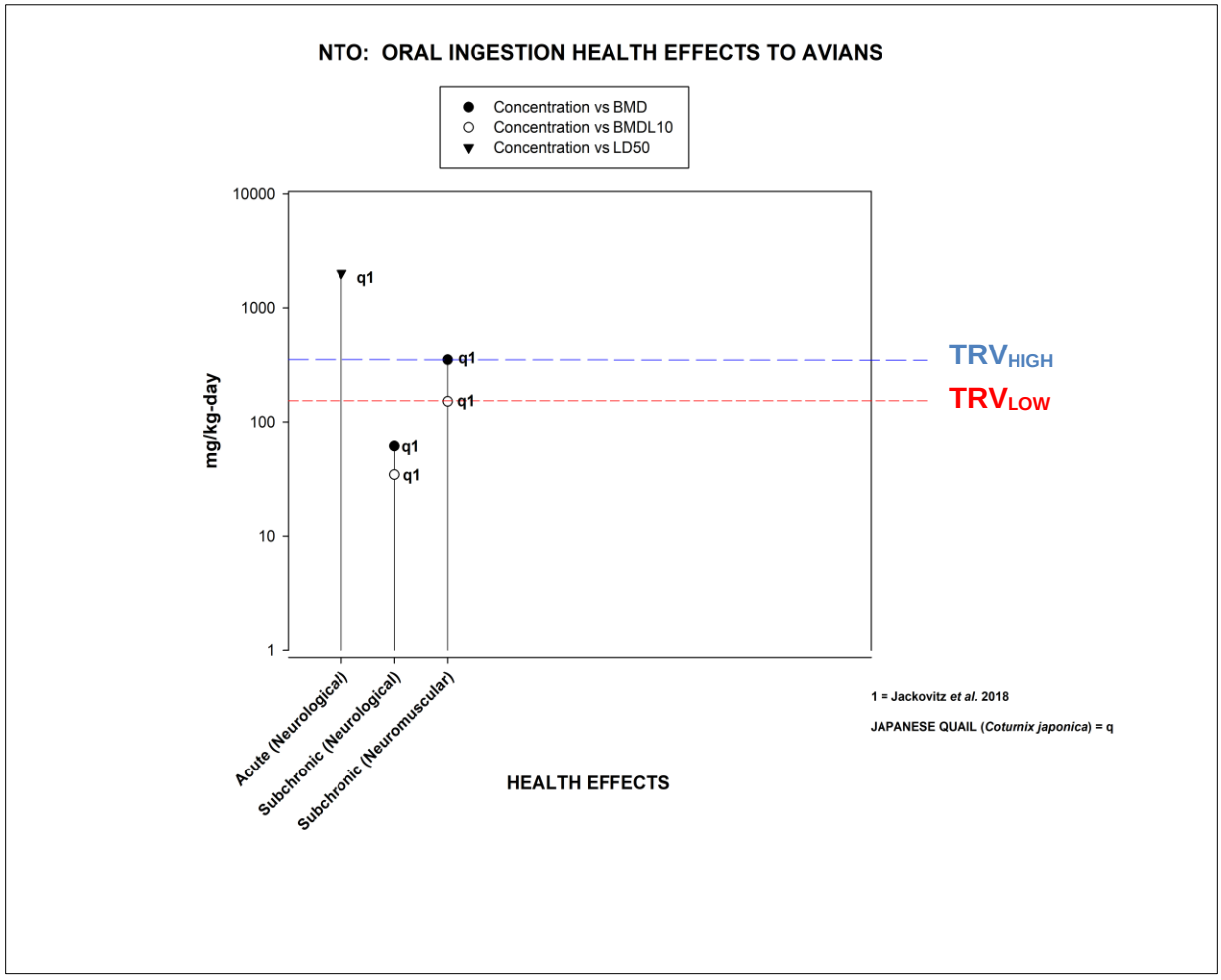
Test Organism	Duration	LD_{50} (mg/kg)	Test Results					
			NOAEL (mg/kg/day)	LOAEL (mg/kg/day)	BMD	$BMDL_{10}$	Effects Observed at the LOAEL	Study
Japanese quail (*Coturnix japonica*)	Acute oral	>2000	--	--			Neurological effects (head tilt, ataxia, and tremors)	Jackovitz et al., 2018
Japanese quail (*Coturnix japonica*)	One generation reproduction study F0 parents	---	20	100	62	35	No significant effects for any reproductive parameters studied brain and cerebellum vacuoles	Jackovitz et al., 2018
					348	151	Neurological effects	
Japanese quail (*Coturnix japonica*)	One generation reproduction study F1 generation	---	20	100			No significant effects on hematology, on sperm concentration at 20 mg/kg dose and at high dose brain lesions	Jackovitz et al., 2018

Legend: mg/kg = milligrams per kilogram; mg/kg/day = milligrams per kilogram per day;
NOAEL= no observed adverse effect level; LOAEL= lowest observed adverse effect level;
BMD = benchmark dose; $BMDL_{10}$ = benchmark dose lower confidence limit

The vacuolization of the cerebellum and/or brain stem and evidence of neuromuscular anomalies were considered critical end points, which demonstrated a dose-dependent response (Jackovitz et al., 2018). Using the vacuolization of the cerebellum and brainstem as critical end points, the BMD multistage model was selected to derive a BMD for F0 generation quail. The BMD for brain vacuoles for both male and female quail of 62 mg/kg/day was derived, which corresponded to a $BMDL_{10}$ of 35 mg/kg/day. Using neuromuscular effects as a critical end point, the Logistic, Log-Logistic, Probit, Log–Probit, and Weibull models were selected. Based on standard goodness-of-fit criteria and statistical parameters, a mean BMD for neuromuscular effects of 348 mg/kg/day (TRV_{High}, Figure 2) was derived for male and female F0 Japanese quail. This was based on the results of these five curve-fitting models, which corresponded to a $BMDL_{10}$ of 151 mg/kg/day (TRV_{Low}, Figure 2) in quail that had been exposed for 10 weeks (Jackovitz et al., 2018).

A reproductive study was also conducted in the F1 generation of quail that were exposed *in ovo* via maternal deposition (Jackovitz et al., 2018). Eggs that had hatched from the F0 birds were used in the preceding study. Individual birds that were selected and used for F1 dosing were aged 2 days old and were dosed orally at 0, 20, and 100 mg/kg/day for 10 weeks. The results showed that there were no significant effects in any of the reproductive parameters or in the

context of organ or body weights in F1 quail that had been orally dosed at 20 or 100 mg/kg as seen in their parental group (F0).



Note: Letters by symbols indicate test species, and the number represents the study.

Figure 2. NTO Health Effects to Avian Species

No biologically significant effects were seen for clinical chemistry or hematological parameters. Additionally, the caudal sperm in F1 birds were unaffected—an observation that was concordant with that seen in the F0 group. Vacuolation of the brainstem and cerebellum were seen in only a single dosed group (i.e., 100 mg/kg/day males—an outcome that precluded derivation of BMD values (Jackovitz et al., 2018).

2.6 Summary of Amphibian Toxicology

The toxicological data for amphibians is limited to a few studies currently. In one study (Stanley et al., 2015), the reported 28-day low observed effect concentration (LOEC) for survival in NTO exposed Leopard Frogs was 5 mg/L. During the 28-day study, the growth and developmental stage of the frog were unaffected, and there was no evidence of mortality at higher doses of

NTO (i.e., at 20 to 100 mg/L) in aqueous environmental media that were adjusted to pH 7.5 using NaOH (Stanley et al., 2015).

The acute toxicity of NTO was dose-dependently explored in the Northern Leopard Frog (*Lithbates pipiens*) that was exposed via ambient water (non-PH adjusted). Young tadpoles (i.e., at 72-hr of development.) and older (i.e., 18-day) tadpoles were evaluated in two separate studies at concentrations of 0, 24.5, 47.9, 95.8, 191.5, and 383 mg/L (Pillared et al., 2017). Within 24 hours of exposure, 100% mortality was demonstrated in frogs that were exposed at the highest concentration (383 mg/L) of NTO at pH 3.7. The 48-hour and 7-day LC_{50} values for NTO in 72-hour old larvae were 271 and 253 mg/L, respectively. The LC_{50} values for the same period for 18-day old tadpoles were 236 and 255 mg/L. No substantial difference in response was observed when contrasting younger and older frogs in this study (Pillared et al., 2017).

The toxicity of NTO in Leopard frog (*Rana pipiens*) tadpoles, demonstrated an LC_{50} for NTO of 24.3 mg/L at 96-hour post-exposure (Stanley et al., 2015). In a 28-day study from the same group, the effects of swimming distance were measured, from which the derived LOEC for NTO was 5mg/L. This 28-day study also showed that in an NTO dose of 25.6 mg/L, non-monotonic and non-significant effects were evident (Stanley et al., 2015; Table 4).

Table 4. Summary of Acute and Subacute of NTO Toxicity in Amphibian Species

Test Organism	Duration	EC_{50} (mg/L)	Test Results			
			NOEC (mg/L)	LOEC (mg/L)	Observed Effects	Study
Leopard Frog *Rana pipiens*	28 days	NA	NA	5.0	No developmental stage nor growth was significantly affected	Stanley et al., 2015
Northern Leopard Frog *Lithobates pipiens*	48 hr and 7 days (Frogs at age 72 hr)	253	NA	NA	No significant reduction in survival	Pillard et al., 2017
	70 days (Frogs at age 18 days)	255	NA	NA	Growth and metamorphosis delay increase in mortality.	
Northern Leopard Frog *Lithobates pipiens*	70 days for survival analysis	NA	3,338	8,382	Frog survival as the endpoint	Pillard et al., 2017

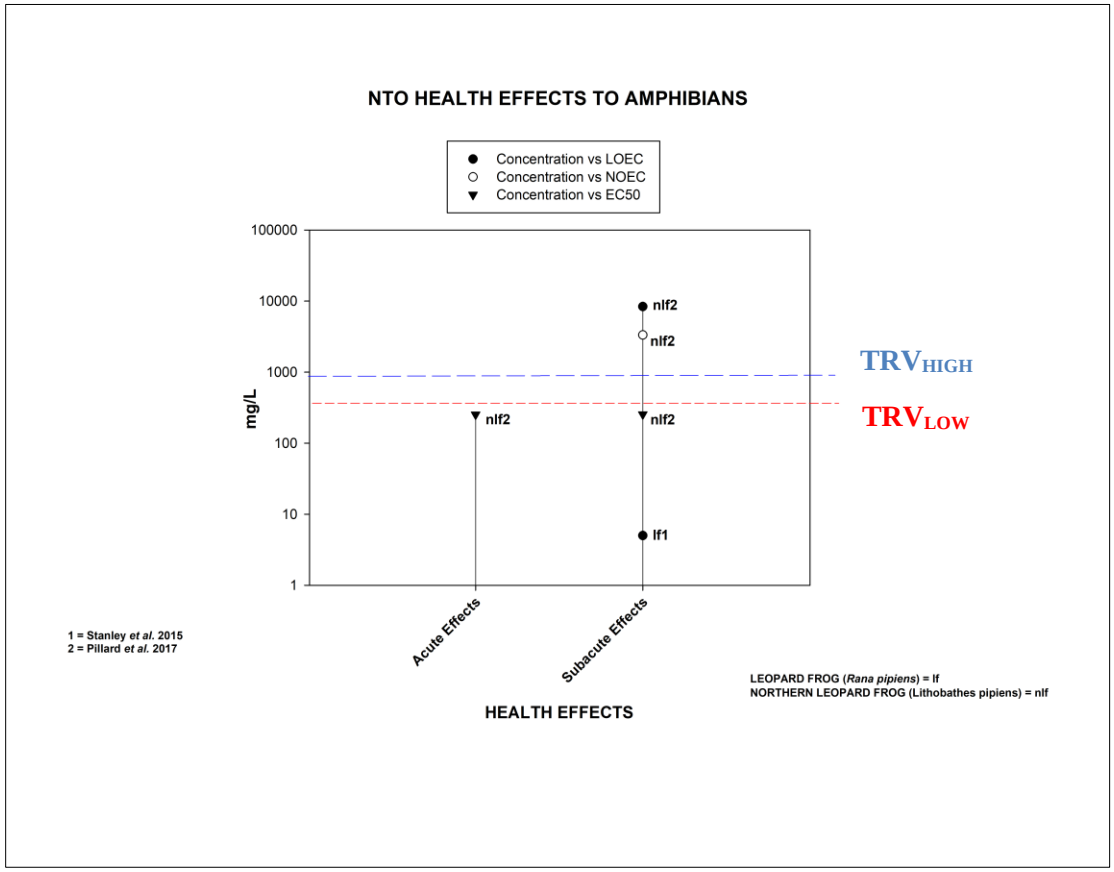
Legend: NA = not available; NOEC = No Observed Effect Concentration;
LOEC = low observed effect concentration; mg/L = milligrams per liter

A subacute and long-term toxicological study of NTO found that in pH-adjusted neutralized waters, the frog larvae were exposed to NTO for 14 and 28 days at various concentrations (1,000 to 9,000 mg/L (Pillard et al., 2017)). The LC_{50} values and growth inhibition concentration (IC) values were determined for their body width (BW) and snout-vent length (SVL). The results showed that all larvae exposed to 11,350 mg/L had died by day 2 of exposure. The BW and

SVL were determined. The IC_{25} and IC_{10} values for adverse effects on BW were respectively 6,059 and 1,307 mg/L for a 14-day exposure. The IC_{25} and IC_{10} values for adverse effects on SVL were respectively 6,295 and 61 mg/L SVL for a 14-day exposure. In the 28-day study, the IC_{10} and IC_{25} values for adverse effects on BW were respectively 28 and 4,350 mg/L, and for effects on SVL, the IC_{10} was 4,025 and the IC_{25} was 6,044, respectively (Pillard et al., 2017; Table 4).

A long-term study was conducted (i.e., 70-day exposure) to explore NTO effects in Northern Leopard Frog larvae by dose-dependently exposing the larvae to NTO (i.e., at 0.04, 190, 517, 1,346, 3,338, and 8,382 mg/L) (Pillard et al., 2017). The evaluated end points included survival, metamorphosis, limb eruption, and growth (i.e., SVL). The 14-, 28-, and 70-day LC_{50} values for survival following NTO exposure were respectively 5,040, 5,279, and 3,670 mg/L. From these data, a NOEC of 3,338 mg/L and a LOEC of 8,382 mg/L were derived following a 70-day exposure to NTO. No adverse histopathological effects were evident when studying liver sections at a 3,338 mg/L dose (Table 4).

It was also shown that there was 100% mortality at the high concentration (383 mg/L) of NTO at pH 3.7 within 24-hours of exposure. The 48-hour and 7-day LC_{50} for 72-hour old larvae were 271 (48-hour) and 253 mg/L NTO, respectively. The LC_{50} for the same period for 18-day old tadpoles were 236 (48-hour) and 255 mg/L NTO. No substantial difference in responsiveness was seen between the younger and older frogs (Pillard et al., 2017).



Note: Letters by symbols indicate test species, and the number represents the study.

Figure 3. NTO Health Effects to Amphibians

2.7 Summary of Reptilian Toxicology

No toxicological data for the effects of NTO on reptiles was in the literature.

3 RECOMMENDED TOXICITY REFERENCE VALUES (TRVs)

3.1 Toxicity Reference Values for Mammals

3.1.1 TRVs for Ingestion Exposures for the Class Mammalia

The acute oral LD_{50} value for NTO was greater than 5,000 mg/kg in rats, which is a dose that is not considered to be acutely toxic in animals (LANL, 1985). The subacute 14-day oral toxicity study in rats, when dosed with NTO at 250, 500, 1,000, 1,500, and 2,000 mg/kg/day, showed testicular toxicity when NTO exceeded a dose of 500 mg/kg/day. No compound-related mortality or clinical signs were observed (Crouse et al., 2015). A subchronic study in rats that were administered NTO by oral gavage at doses of 30, 100, 315, and 1,000 mg/kg showed no treatment-related effects at the two lowest doses of 30 and 100 mg/kg. The two highest NTO doses of 315 and 1,000 mg/kg showed moderate to severe degeneration and atrophy of somniferous tubules—physiological changes that were both dose-dependent and statistically significant (Crouse et al., 2015). These data were used to derive BMD/$BMDL_{10}$ TRVs. For ingestion exposures to NTO in mammals, the BMD or ED_{10} of 70 mg/kg was derived and a lower bound $BMDL_{10}$ or LED_{10} value of 44 mg/kg/day was determined (Crouse et al., 2015; Table 5).

Table 5. Selected Ingestion TRVs for Class Mammalia

TRV	Dose	Confidence
TRV_{Low}	44 mg/kg/day	Medium-High
TRV_{High}	76 mg/kg/day	Medium-High

The TRVs derived from these data were determined according to DCPH-A Technical Guide (TG) 254 (DCPH-A, 2023) and were derived and confirmed from few animal model studies available for analysis (Crouse et al., 2015; Lent et al., 2016; Lent et al., 2018; Lent et al., 2020). Other limited rat model studies that were conducted in mice at NTO doses of 500 and 1,000 mg/kg in a 14-day study (Mullins et al., 2016), as well as reproductive and developmental studies at NTO dose levels of up to 3,600 mg/L in the drinking water, showed only evidence of testicular toxicity (Lent et al., 2016; Lent et al., 2018; Lent et al., 2020).

The chronic toxicity study reported by Lent et al. (2020) confirmed that NTO was relatively non-toxic in female Sprague-Dawley rats, and it confirmed that toxicity in male rats was limited to testicular toxicity. A Benchmark Dose (BMD) of 76 mg/kg/day (TRV-High) and a Benchmark Dose Lower Bound (BMDL10) of 44 mg/kg/day (TRV-Low) were determined for the chronic effects of NTO in male rats (Lent et al., 2020). A medium-high confidence level (Table 5) was determined because of the consistent and reproducible findings from high-quality *in vivo* dose-response modeling as reported above (Crouse et al., 2015; Lent et al., 2016; Lent et al., 2018; Lent et al., 2020).

3.1.2 TRVs for Inhalation Exposures for the Class Mammalia

Only single inhalation acute toxicity data were available that gave LC_{50} values that were greater than 0.184 m/L (USAPHC, 2013). However, these data were insufficient to derive an inhalational TRV for mammals.

3.2 Toxicity Reference Values for Avian Oral Toxicity

In the only study currently available to evaluate NTO toxicity and to derive a TRV for avian species (Jackovitz et al., 2018), Japanese quail (*Coturnix japonica*) were exposed to NTO in corn oil by oral gavage at doses of 20, 100, 500, and 1,000 mg/kg/day. Dosing commenced in 2-week-old birds and was terminated when birds reached 12 weeks of age (i.e., representing 10 weeks of dosing). The acute oral LD_{50} value of NTO was found to exceed 2,000 mg/kg.

No reproductive effects were observed by any of the parameters analyzed, which included time to reproductive maturity, cloaca gland size, caudal sperm concentration, egg fertility, eggshell thickness, and eggshell strength. No hematological parameters were significantly affected at doses of either 20 or 100 mg/kg/day (Jackovitz et al., 2018). However, quail that were dosed with NTO at 100, 500, and 1,000 mg/kg showed adverse neuromuscular effects that included balance loss, convulsions, and mortality.

Histopathological analysis showed vacuoles in the brain stem and cerebellum were significant at the two highest doses tested. Vacuolization of the cerebellum and brain stem were considered critical effects (Jackovitz et al., 2018).

The BMD for F0 generation quail was derived for both brain lesions (see Table 6) and neuromuscular effects (see Table 7). For brain lesions, a BMD (ED_{10}) value for male quail was estimated to be 62 mg/kg/day, which corresponded to a $BMDL_{10}$ (LED_{10}) of 35 mg/kg/day (Table 6). Using neuromuscular effects as the toxicological end point, a BMD (ED_{10}) value of 348 mg/kg/day was derived for male and female quail, which corresponded to a $BMDL_{10}$ (LED_{10}) of 151 mg/kg in quail that had been exposed to NTO for 10 weeks (Table 7; Jackovitz et al., 2018).

Table 6. Selected Oral TRVs for Avian Species: Brain Lesions

TRV	Dose	Confidence
TRV_{Low}	35 mg/kg/day	Low
TRV_{High}	62 mg/kg/day	Low

Table 7. Selected Oral TRVs for Avian Species: Neuromuscular Lesions

TRV	Dose	Confidence
TRV_{Low}	151 mg/kg/day	Low
TRV_{High}	348 mg/kg/day	Low

The TRV derived from these data were determined according to TG 254 (DCPH-A, 2023). Since the approach of benchmark doses were employed, no uncertainty factors were needed to derive the TRV as described in Tables 6 and 7 above. The derived BMD or ED_{10} was 62 mg/kg and lower bound of $BMDL_{10}$ was 35 mg/kg for brain vacuoles, as well as the derived ED_{10} was 348 mg/kg and LED_{10} was 151 mg/kg for birds. These values appear to be protective for avian species.

3.3 Toxicity Reference Values for Amphibian Toxicity

Two studies were identified that provided novel data on the toxicological assessment of NTO in amphibian species that included the Northern Leopard Frog (*Lithobates pipiens)* and tadpoles of *Rana pipiens* (Pillard et al., 2017; Stanley et al., 2015). The 96-hour LC_{50} value in *Rana pipiens* was 24.3 mg/L (Stanley et al., 2015) and for Leopard Frog larvae, the 48-hour and 7-day LC_{50} values were greater than 250 mg/L. The 70-day LC_{50} value for Northern Leopard Frogs challenged with NTO in pH-neutralized water was 3,670 mg/L.

In a 28-day exposure, the LOEC for survival was estimated as 5 mg/L in *Rana pipiens* (Stanley et al., 2015). The NOEC for the time duration to metamorphosis seen in Northern Leopard Frog larvae was 1,346 mg/L and the LOEC was 3,338 mg/L (Pillard et al., 2017). For other end points, a NOEC of 3,338 mg/L and a LOEC of 8,382 mg/L for survival in a 70-day exposure study were established and considered for TRV derivation (Pillard et al., 2017). Similarly, a NOEC of 3,338 mg/L and a LOEC of 8,382 mg/L were derived based on the number of Leopard Frogs completing metamorphosis. These values were also considered for TRV derivation since it included the entire developmental stage of this amphibian species and represented a chronic exposure study that was conducted in neutralized water (Pillard et al., 2017).

The TRV for amphibians was derived from LOEC and NOEC values according to TG 254 (DCPH-A, 2023; Table 8). The study conducted in frogs (Pillard et al., 2017) included the complete developmental stages of the frog with several end points. This study also included acute toxicity studies of NTO in an acidic environment without any pH adjustment. Therefore, the derived TRV values are considered protective for this amphibian species.

Table 8. Selected Ingestion TRVs for Class Amphibians

TRV	Dose	Confidence
TRV_{Low}	334 mg/kg/day	Low
TRV_{High}	838 mg/kg/day	Low

3.4 Toxicity Reference Values for Reptilian Toxicity

No data are available to develop toxicity reference value.

4 IMPORTANT RESEARCH NEEDS

Toxicological data for NTO across mammalian, avian, and amphibian species are minimal, and there are currently no data available for reptilian species to develop a TRV. Consequently, the lack of data on the toxicity of NTO to wildlife species weakens the development of a TRV in

many cases. More toxicological studies of the compound are recommended since confidence in many of the derived TRVs are low for avian and amphibian species. Despite some well-designed studies conducted in Japanese quail and amphibian species, corroborating data across species is severely lacking for wild species and for a comparative analysis to be made of the toxicological impacts of NTO exposure of birds, reptiles, and amphibians to that of mammalian species. Adequate dermal, inhalational, and reproductive/developmental toxicity data are lacking for all groups. In addition, future work should focus on greater understanding of the kinetics of NTO in diverse gastrointestinal physiologies. The toxicity literature is scant for all species described in this WTA and is completely lacking for reptiles. Studies that focus on both acute and chronic toxicity on wild mammals, as well as non-mammalian wildlife such as birds, reptiles, and amphibians, are urgently warranted.

APPENDIX A

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APPENDIX B

LITERATURE REVIEW

A very broad search on August 10–12, 2018, using DTIC's Multisearch function used the single search term, NTO and then 3-Nitro-1,2,4-Triazol-5-One. This search identified 8,259 documents for NTO, and 77 documents for 3-Nitro-1,2,4-Triazol-5-One for a total of 8,336 documents.

Focused searches on August 12, 2018, using DTIC's Multisearch function used the following terms:

3-Nitro-1,2,4-Triazol-5-One + quail*. This search identified 6 documents.
3-Nitro-1,2,4-Triazol-5-One + mallard*. This search identified 2 documents.
3-Nitro-1,2,4-Triazol-5-One + bird*. This search identified 15 documents.
3-Nitro-1,2,4-Triazol-5-One + avian. This search identified 11 documents.
3-Nitro-1,2,4-Triazol-5-One + mouse. This search identified 65 documents.
3-Nitro-1,2,4-Triazol-5-One + mice. This search identified 62 documents.
3-Nitro-1,2,4-Triazol-5-One + rat. This search identified 59 documents.
3-Nitro-1,2,4-Triazol-5-One + mammal*. This search identified 59 documents.
3-Nitro-1,2,4-Triazol-5-One + ecotox*. This search identified 61 documents.
3-Nitro-1,2,4-Triazol-5-One + toxic*. This search identified 61 documents.
3-Nitro-1,2,4-Triazol-5-One + amphib*. This search identified 3 documents.
3-Nitro-1,2,4-Triazol-5-One + frog. This search identified 11 documents.
3-Nitro-1,2,4-Triazol-5-One + *Xenopus*. This search identified 19 documents.
3-Nitro-1,2,4-Triazol-5-One + reptil*. This search identified 2 documents.

Additional focused searches on August 17, 2018, by DTIC's Multisearch function used the following terms:

NTO + quail*. This search identified no new documents.
NTO + mallard*. This search identified no new documents.
NTO + bird*. This search identified no new documents.
NTO + avian. This search identified no new documents.
NTO + mouse. This search identified one new document.
NTO + mice. This search identified one new document.
NTO + rat. This search identified two new documents.
NTO + mammal*. This search identified no new documents.
NTO + ecotox*. This search identified no new documents.
NTO + toxic*. This search identified no new documents.
NTO + amphib*. This search identified no new documents.
NTO + frog. This search identified no new documents.
NTO + *Xenopus*. This search identified no new documents.
NTO + reptil*. This search identified no new documents.

On August 12, 2018, a search of the U.S. EPA's online Ecotox database used the CAS No. 932-64-9 to identify any additional articles. No references were returned for amphibians, reptiles, birds or mammals. Thus, no new references were identified. A search of the TOXLINE database of the National Library of Medicine's TOXNET system (http://toxnet.nlm.nih.gov), on August 12,

2018, used the CAS No. 932-64-9 as the search term identified two articles. Additional refined searches were not needed.

On August 11–12, 2018, an additional date unrestricted literature search (all years) was conducted using the Johns Hopkins Welch Medical Library Multisearch Database. Using 3-Nitro-1,2,4-Triazol-5-One as a single search term in the title of the document, this search strategy identified 0 documents with 3-Nitro-1,2,4-Triazol-5-One in the title of the article in Web of Science; 184 documents in PubMed; 0 documents in CINAHL Plus; 0 documents in WorldCat Advanced Search (FirstSearch), of which 21 articles were reviewed.

For additional targeted searches, a standard search of PubMed (National Library of Medicine, NIH) of 3-Nitro-1,2,4-Triazol-5-One [TI] as the anchored word in the title with the following search strings were selected using the wild-card (*) function where appropriate for optimal returns on search terms and contexts. Species specific search strings yielded the following hits from PubMed:

3-Nitro-1,2,4-Triazol-5-One AND mammal returned 76 hits
3-Nitro-1,2,4-Triazol-5-One AND animal returned 75 hits
3-Nitro-1,2,4-Triazol-5-One AND quail returned 1 hit
3-Nitro-1,2,4-Triazol-5-One AND mallard returned 0 hits
3-Nitro-1,2,4-Triazol-5-One AND bird returned 1 hit
3-Nitro-1,2,4-Triazol-5-One AND avian returned 1 hit
3-Nitro-1,2,4-Triazol-5-One AND mouse returned 28 hits
3-Nitro-1,2,4-Triazol-5-One AND mice returned 26 hits
3-Nitro-1,2,4-Triazol-5-One AND rat returned 27 hits
3-Nitro-1,2,4-Triazol-5-One AND wildlife returned 1 hit
3-Nitro-1,2,4-Triazol-5-One AND ecotox* returned 3 hits
3-Nitro-1,2,4-Triazol-5-One AND amphib* returned 3 hits
3-Nitro-1,2,4-Triazol-5-One AND frog returned 3 hits
3-Nitro-1,2,4-Triazol-5-One AND *Xenopus* returned 0 hits
3-Nitro-1,2,4-Triazol-5-One AND reptile returned 0 hits

During the revision process, additional literature searches were conducted on October 12, 2020, using the above search strategies using the Johns Hopkins Welch Medical Library Multisearch Database. Using 3-Nitro-1,2,4-Triazol-5-One as a single search term in the title of the document, this search strategy identified an additional three articles, of which only one was aligned to the scope and content of this WTA (Lent et al., 2018).

During the final revision and preparation of this report, an additional literature search for the above search terms to include 3-Nitro-1,2,4-Triazol-5-One; NTO and its synonym Nitrotriazolone was conducted by targeted searches of the literature from November 18–20, 2024. First, a standard broad search of PubMed (National Library of Medicine, NIH) using the following search terms and Boolean operators was conducted for 3-Nitro-1,2,4-Triazol-5-One [TIAB] OR NTO [TIAB] OR Nitrotriazolone [TIAB] as the anchored keywords in the Title and Abstract [TIAB] was conducted for the searched dates of January 1, 2016 to November 20, 2024, to yield 296 hits. Preprint articles and those not in the English language were excluded.

Next, specific targeted searches in PubMed were conducted for NTO fate, by using the following search string: ‘3-Nitro-1,2,4-Triazol-5-One [TIAB] OR NTO [TIAB] OR Nitrotriazolone [TIAB]

AND Fate'. This yielded 20 hits, of which 4 articles were determined as providing relevant and quality information in our report (Cárdenas-Hernández et al., 2020; Menezes et al., 2021; Temple et al., 2018; Polyakov et al., 2023).

The PubMed database was further examined with the following search string '3-Nitro-1,2,4-Triazol-5-One [TIAB] OR NTO [TIAB] OR Nitrotriazolone [TIAB] AND TOXIC* [TIAB]' that was selected to explore articles published from 2016 through November 2024 for toxicological studies and using the wild-card (*) function for optimal returns on search terms and contexts for the phrase "TOXIC*). Search terms were restricted to their appearance in the Title or Abstract [TIAB] to yield 27 hits from PubMed. Of those 27 hits, there were two articles considered in scope and relevant to this WTA report and as indicated in the text (Lent et al., 2018; Lent et al., 2020).