FINAL REPORT

05-06-2012

DEVELOPMENT OF ENVIRONMENTAL HEALTH CRITERIA FOR INSENSITIVE MUNITIONS (IMX-101-104)

SERDP Project ER-2223

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
18-01-2018	SERDP Final Report	05-06-2012 to 18-01-2018

4. TITLE AND SUBTITLE
DEVELOPMENT OF ENVIRONMENTAL HEALTH CRITERIA FOR INSENSITIVE MUNITIONS (IMX-101-104)

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER
ER-2223

5e. TASK NUMBER
6

5f. WORK UNIT NUMBER
ER-2223

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
US Army Public Health Center
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Aberdeen Proving Ground, MD 21010-5403

8. PERFORMING ORGANIZATION REPORT NUMBER
N/A

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Strategic Environmental Research and Development Program (SERDP)
4800 Mark Center Drive, Suite 17D08, Alexandria, VA 22350

10. SPONSOR/MONITOR'S ACRONYM(S)
SERDP

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
ER-2223

12. DISTRIBUTION/AVAILABILITY STATEMENT
Unclassified, Unlimited Distribution - Approved for Public Release

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The objective of this work was to provide focused toxicity information for constituents of IMX-101 (NTO and DNAN) that will allow for more accurate benchmark derivation consistent with existing regulatory guidance and frameworks and allow for optimal operational flexibility at ranges while ensuring environmental health. Toxicity tests were conducted with NTO in mammals, birds, and an amphibian species; DNAN in aquatic species. Study results were also published in the peer-reviewed literature and provisional environmental criteria were developed to protect human health. These data will also be used to develop benchmarks for wildlife, used to develop waste water discharge allowances, and other criteria.

15. SUBJECT TERMS
Nitrotriazelone (NTO), dinitroanisole (DNAN), toxicity, mammals, birds, fish, invertebrates, amphibian

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Mark S. Johnson
UU	UU	UU	none	56	19b. TELEPHONE NUMBER *(Include area code)*: 410-436-3980

Sponsors

Department of Defense
Strategic Environmental Research and Development Program
4800 Mark Center Drive, Suite 17D08, Alexandria, VA 22350

Study Title

DEVELOPMENT OF ENVIRONMENTAL HEALTH CRITERIA FOR INSENSITIVE MUNITIONS (IMX-101-104)

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January 2018

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ABSTRACT

Objective – The objective of this work was to provide focused toxicity information for constituents of IMX-101 (NTO and DNAN) that will allow for more accurate benchmark derivation consistent with existing regulatory guidance and frameworks and allow for optimal operational flexibility at ranges while ensuring environmental health.

Technical Approach - Toxicity tests were conducted with NTO in mammals, birds, and an amphibian species; studies conducted with DNAN were done using conventional aquatic species. Study results were also published in the peer-reviewed literature and provisional environmental criteria were developed to protect human and environmental health.

Results – Acute and chronic DNAN aquatic toxicity data suggest that DNAN is slightly less toxic than other nitroaromatics such as TNT. Studies conducted using frog embryos (*Lithobates pipiens*) suggest low toxicity of NTO when buffered. Extended generation reproductive studies conducted in rats showed results suggestive of male reproductive effects (hypospermia), but no evidence of developmental effects or changes to number of offspring born. Acute effects in birds were marked by neurotoxic signs such as ataxia supported by adverse brain histology (cerebellar vacuoles); no reproductive effects were observed at lower exposures.

Benefits – Together, these data will allow development of toxicity-based benchmarks and science-based environmental criteria for environmental releases such as waste water discharge allowances, and clean-up criteria. Based on these and other data, media-based concentrations were developed for soil and drinking water.

LIST OF ACRONYMS

ACR – Acute-to-chronic ratio
ADD – Aceptable daily dose
AIC - Akaike Information Criterion
AGD - ano-genitial distance
BMDL – Benchmark dose – low
CD – Coefficient of differentation
CONUS – Continential United States
DNAN – 2,4 Dinitroanisole
DN – Double negative
DP – Double positive
ED - embryonic day
EMH – Extra medullary hematopoesis
FER – Food efficiency rate
IC – Incapacitating concentration
IV - Intravenous
LC_{50} – Median lethal concentration
LD_{50} – Median lethal dose
LOEC – Lowest observed effect concentration
LOAEC – Lowest adverse effect concentration
LOEL – Lowest observed effect level
LOAEL – Lowest adverse effect level
MATC - maximum allowable concentration
NOEC – No observed effect concentration
NOAEC – No adverse effect concentration
NOEL – No observed effect level
NOAEL – No observed adverse effect level
NQ – Nitroguanidine
NTO – Nitrotriazelone (3-nitro-1,2,4-triazol-5-one)
OCONUS – Outside continental United States
OECD – Organisation for the Economic Cooperation and Development
OPTEMPO – Operational tempo
PAS-H - Periodic acid–Schiff stain
PBS – Phosp[hate buffered saline
PPS - Preputial separation

PND – Post natal day
PPD – Post partum day
RBC – Red blood cell
RFD – Reference dose
SVL – Snout-to-vent length
UF – Uncertainty factor
VO – Vaginal opening

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1.0 BACKGROUND

OPTEMPO and sustained use of our ranges has been adversely affected from the use of energetic compounds that migrate off-range primarily through groundwater infiltration. New energetic formulations are being developed to provide for insensitive munitions to improve soldier survivability. IMX-101, a mixture of Dinitroanisole (DNAN), Nitroguanidine (NQ) and Nitrotriazolone (NTO) has been qualified as a TNT replacement while IMX-104, a mixture of DNAN, NTO and RDX, is nearing completion as a qualified replacement of Composition B. Components of these new IM formulations have a significant potential for migration from soil to surface and ground water sources when incomplete or unexploded detonations occur. Furthermore, recent toxicity data suggest that one component, NTO, is a male reproductive toxicant that may act through endocrine disruption. These data suggest that use of NTO in formulations could result in environmental conditions similar to that of RDX and perchlorate. Additionally, due to its IM design, blow-in-place may result in higher environmental residues exacerbating the magnitude of environmental releases at ranges at CONUS and OCONUS theater operations.

OBJECTIVE

The objective of this work was to provide focused toxicity information for NTO and DNAN that will allow for more accurate benchmark derivation consistent with existing regulatory guidance and frameworks and allow for optimal operational flexibility at ranges while ensuring environmental health. Through acquisition of needed toxicity data in this work and through the publication of these data in the scientific peer-reviewed literature, information obtained from this work will be provided to regulatory agencies to derive health-based criteria, enable wastewater permit actions, determine degree of personal protective equipment when exposed, and enable range managers to protect the environment while sustaining range operations.

2.0 TECHNICAL APPROACH

This effort includes several different study designs that address separate data gaps.

2.1 TASK 1 – EXTENDED ONE-GENERATION STUDY OF NTO IN RATS

The reproductive and developmental toxicity of NTO, an insensitive, energetic material used in explosive formulations, was assessed using a modified extended one-generation reproductive toxicity test (Cooper et al. 2006). This study evaluated the effects of NTO on male and female reproductive systems including: gonadal function, the estrous cycle, epididymal sperm maturation, mating behavior, conception, pregnancy, parturition, and lactation. Pre- and postnatal effects of NTO on development as well as systemic toxicity in pregnant and lactating females and young and adult offspring were also evaluated. In this study, rats were given ad libitum access to NTO in drinking water at four concentrations (0, 144, 720, or 3600 mg/l NTO) from pre-mating of the parental (P) generation through puberty of the offspring. The P generation was comprised of four groups of 25 sexually-mature rats per sex. In addition, the male P generation included two recovery groups (control and high dose) of 10

males per group. The recovery males were dosed concurrently with the main study animals and held for a period of 10 weeks following cessation of dosing. The purpose of the recovery groups is to evaluate the reversibility or persistence of the testicular toxicity and reduced sperm count associated with NTO exposure.

NTO was provided via drinking water to the P males for four weeks pre-mating and the P females for two weeks pre-mating and to both males and females during a two-week mating period. Treatment of the P generation males was continued for a complete spermatogenic cycle (i.e., 10 weeks). Treatment of P generation females was continued during pregnancy and lactation until euthanasia after weaning of the litters (*i.e.,* 10 weeks of treatment).

At weaning, F1 pups were selected for use on study (20 pups/sex/group; one male and one female/ litter/group). The F1 animals were given ad libitum access to NTO in drinking water from weaning through puberty (post-natal day (PND) 42±1 and PND 53±1 for females and males, respectively). Pups not selected for placement in treatment groups were bled, euthanized and submitted for gross necropsy (minimum 10/sex/group). The remaining pups not selected for placement in treatment groups were euthanized or transferred to another protocol (control animals).

All rats were monitored throughout the study for body weight changes and clinical signs of toxicity. The number and sex of pups, stillbirths, live births, and the presence of gross anomalies in each litter were determined on PND 0/1. The ano-genitial distance (AGD) of each pup was measured on PND 4 and male pups were examined for the presence of nipples on PND 13 to assess masculinization/feminization. F1 females and males were examined daily for attainment of sexual maturity. Females were examined daily (starting on PND 22) for vaginal opening (VO) and males were examined daily (starting on PND 30) for preputial separation (PPS).

Blood samples were collected at termination from ten randomly selected males and females per dose group for P and F1 animals and subjected to clinical chemistry and hematology assessments. Blood from F1 animals was analyzed for thyroid hormones (T4 and TSH). At the time of termination, a detailed gross necropsy was conducted on all P and F1 animals; weights were recorded for reproductive tissues, accessory reproductive tissues, thyroid, pituitary, adrenals, liver, kidneys, brain, heart, spleen, and thymus. Sperm was collected and sperm parameters measured in all P males using a computer assister sperm analyzer (TOX IVOS-CASA, Hamilton-Thorne Research). Selected tissues were processed for histopathology. Thymic and splenic lymphocyte subpopulations were also analyzed via cell surface markers and using flow cytometry.

Additional information can be found in:

US Army Public Health Center (Provisional). 2016. Extended On-Generation Reproductive Toxicity in Rats Exposed to 3-Nitro-1,2,4-Triazol-5-One (NTO). Toxicity Report No. S.0022062, Toxicology Directorate, Army Public Health Center, Aberdeen Proving Ground, MD (prepared by Emily May Lent).

Lent EM, Crouse LCB, Jackovitz AM, Carroll EE, Johnson MS. 2016. An extended one-generation reproductive toxicity test of 1,2,4-triazol-5-one (NTO) in rats. *Journal of Toxicology and Environmental Health*, Part A, DOI: 10.1080/15287394.2016.1219893.

2.2 TASK 2 – AVIAN EXTENDED ONE-GENERATION

The objective of this study was to evaluate the toxicity of 3-nitro-1,2,4-triazol-5-one (NTO in the environment and as a potential reproductive toxicant in birds using Japanese quail (*Corturnix japonica*) as a model species.

2.2.1 – Acute Oral Test in Birds

Acute toxicity testing of NTO demonstrated that NTO has low toxicity (LD_{50} > 5000 mg/kg) in rats and mice, therefore, only a Limit Dose test was conducted in quail as opposed to more extensive, sequential tests [6]. Quail were mature in plumage, but not in breeding condition, in accordance with OECD guidelines; therefore, birds were 5 weeks of age at the start of the Limit Dose test. Five animals were tested at the Limit Dose (2000 mg/kg), in addition to a control group consisting of 5 animals. This is the recommended strategy for testing materials that are unlikely to present a significant hazard. Mortality was the primary endpoint in this study and background mortality was presumed to be negligible [8].

Three females and 2 males were randomly assigned to the control group. Two females and 3 males were randomly assigned to the Limit Dose group. Females weighed 148.0 ± 7.3 grams (g), while males weighed 137.5 ± 6.1 g. All vehicle control and NTO doses were administered according to the body mass measured on the day of dosing. Oral dosing was performed using a stainless steel 16 gauge x 2 inch gavage needle. After receiving a single dose, all animals were observed for 14 days for signs of toxicity, morbidity, and mortality. Animals were euthanized at the conclusion of the test. If no mortality is observed for 14 days after dosing, it can be concluded with 95% confidence that the LD_{50} is above the Limit Dose.

During the Avian Acute Oral Toxicity Test, birds were observed continuously during the first 2 hours after dosing for regurgitation and for the onset of clinical signs on at least three evenly spaced additional occasions during the day. Following the initial dosing day, birds were observed for clinical signs at least once daily for 14 days. Observations on each individual included: regurgitation, signs of intoxication and remission, abnormal behavior, mortality, and time to death. Body weights were only collected on the day of dosing. Birds were assessed for morbidity based on loss of righting response, an inability to feed or drink independently, and signs indicative of dehydration or shock (e.g., lethargy, depression, wing droop, ruffled feathers, panting).

2.2.2 - One-Generation Study in Birds

A one-generation reproductive toxicity test was performed with NTO. Half the Limit Dose (1000 mg/kg-day) was selected for the high dose. The medium-high dose was set at 500 mg/kg-day, with the medium-low, and low doses set at five-fold intervals (e.g., 100, and 20 mg/kg-day,

respectively). All NTO doses and the control were administered based on body mass and volume of solution at rates of 10 milliliter per kilogram (ml/kg). Dosages were adjusted weekly for changes in body mass for the F0 generation and more frequently between hatch and week 4 for the F1 generation, to ensure that rapidly growing chicks received the appropriate dose. Oral dosing was performed using a stainless steel 16 gauge x 1-2 inch gavage needle.

To produce the F0 generation, 300 eggs were incubated. Between embryonic day (ED) 17 and 19, 264 chicks hatched. At day 10 of age, 260 birds were randomly sorted into 5 treatment groups. At this age, sex of the birds cannot be determined, but the sex ratio was assumed to be 1:1. Treatment began at week 2 and continued through termination. At week 4, when sex can be determined in quail, as many as 16 birds from each sex and dose group were moved to adult caging. Excess birds were culled. Once birds demonstrated an understanding of the automatic watering system, treatment groups were trimmed down to as many as 12 birds per sex and dose group, and excess birds were culled. Beginning at week 5, sexual development (including daily egg production and weekly cloaca gland measurements) was assessed. At week 6, immune function was assessed, as described in 5.6.3. At week 7, male copulatory behavior was assessed. After behavioral assessment and mating to produce eggs for the F1 generation had occurred (i.e., week 12), F0 birds were terminated and necropsied.

To generate the F1 generation, 170 eggs produced by the F0 generation were incubated. Between ED 17 and 19, 127 chicks hatched. Unlike the F0 generation where exposure began at week 2, F1 birds were exposed in ovo via material deposition. As such, hatched birds were banded immediately upon hatch to designate in which dose group the parental generation (and thus the F1 generation) – belonged. At day 2, oral exposure began and was continued until termination. At week 4 (when sex could be determined) as many as 18 birds/sex/dose were moved to adult caging. To improve the statistical power of the study, and unlike the F0 generation, sample size was not reduced after the quail demonstrated competent use of the automatic watering system. Beginning at week 5, sexual development (including daily egg production and weekly cloaca gland measurements) was assessed. At week 6, immune function was assessed. At week 7, male copulatory behavior was assessed. After behavioral assessment and mating to produce eggs to incubate to determine fertility (i.e., week 10), F1 birds were terminated and necropsied.

Beginning at week 5, males were observed daily for reproductive maturity, which is determined by the presence or absence of foam dispensed from the cloaca gland. Each immature male bird's foam production was observed daily until it was reproductively mature. Male cloaca glands were measured weekly beginning at week 6. In females, reproductive maturity was determined by the presence of the first egg. Egg production was monitored daily and classified as hard, soft, or broken.

Humoral response as an indicator of immunotoxicity was evaluated via a foreign red blood cell (RBC) challenge. Each animal received one 0.1 ml injection of a 5% pig RBC suspension in Phosphate Buffer Solution (PBS). Injections were performed intravenously (IV). For IV injections, a referenced safe maximum volume is 5 ml/kg body weight. IV injections were administered into the jugular vein found along the neck using a 1 ml syringe fitted with a 25 gauge needle. Immunotoxicity testing was done at week 6 in both generations.

Ten days post injection, up to 1 ml of blood was collected from the jugular vein of anaesthetized animals. Blood was placed in Sarstedt lithium heparin microtubes and refrigerated overnight. The next day, plasma was isolated by centrifugation (approximately 5 minutes at 2570 x g). Plasma was stored in 1 ml cryovials in a -30ºC freezer until analysis.

To measure the RBC antibody response, a hemaglutinnation assay was performed in which plasma was thawed, serially diluted in PBS, and re-exposed to the foreign antigen. To simulate re-exposure, 50 µl of the 5% RBCs were added to each well and the plates were gently agitated and then incubated at approximately 37°C for 3 hours. Agglutination appears as a diffuse red disc across the entire bottom of the well, whereas a lack of agglutination appears as a "button" at the bottom of the V-shaped well [9]. Titers were determined as the log 2 of the reciprocal of the highest dilution showing agglutination, which measures the activity of total hemagglutinating antibodies.

More information can be found in:

US Army Public Health Center. 2017. One-generation reproductive toxicity test in Japanese quail (*Coturnix japonica*) exposed orally to 3-nitro-1,2,4-triazol-5-one (NTO). Toxicity Study No. S.0027395-15. US Army Public Health Center, Toxicology Directorate, Aberdeen Proving Ground, MD (prepared by Jackovitz AM, Rice SW).

2.3 TASK 3 - AMPHIBIAN METAMORPHOSIS TOXICITY TESTS

A study was conducted to determine the effects of 3-nitro-1,2,4-triazol-5-one (NTO) on the northern leopard frog (*Lithobates pipiens*) during a one-generation study. Newly-hatched larvae were exposed to six treatments containing increasing concentrations of NTO (190 – 11,350 mg/L) in a definitive test; a negative control was also included. Prior to initiation of the definitive test, range-finding studies were conducted using both ambient NTO (pH ~3.7 in the highest test treatment) and neutralized (pH-adjusted) NTO with a pH of ~7.5. The range-finding studies were approximately one week long. The definitive study was terminated on day 70 when most of the surviving organisms had developed front limbs and completed metamorphosis into frogs.

The definitive test included six replicates per treatment (with five organisms in each replicate). One replicate chamber from each treatment was terminated on day 14, and a second replicate on day 28, to measure tadpole growth using the metrics snout-to-vent length (SVL) and body width. Exposure in the remaining four replicates continued until the end of the definitive study (70 d). The tests were conducted in an isolated environmental chamber with controlled lighting and temperature. On day 40 of the test larval anurans began presenting front limbs. Organisms with front limbs were transferred from exposure chambers to emergence chambers containing the same concentrations of NTO. Complete metamorphosis (Gosner Stage 46) occurred when the tail was completely or near-completely absorbed. Frogs were examined for internal and external abnormalities; growth was evaluated using a variety of parameters. Histopathological examination was conducted on a limited number of internal organs.

More information can be found in:

US Army Public Health Center. 2016. Short-Term Toxicity and Developmental Effects of 3-Nitro-1,2,4-Triazol-5-One (NTO) on the Northern Leopard Frog (*Lithobates pipiens*) in a One-

Generation Study. Study/contract no. W91ZLK-14P-0372. US Army Public Health Center, Toxicology Directorate, Aberdeen Proving Ground, MD (prepared by Pillard DA, TRE Environmental Strategies, LLC, Fort Collins, CO).

Pillard DA, Eck WS, Johnson MS, Packard S. 2017. Effects of 3-nitro-1,2,4-triazol-5-one on survival, growth and metamorphosis in the northern leopard frog, *Lithobates pipiens*). *Ecotoxicology* 26:1170-1180.

2.3 TASKS 4 & 5 – AQUATIC TOXICITY OF DNAN

Acute and chronic assays were conducted in fish (*Pimephales promelas*) and a cladoceran (*Ceriodaphnia dubia*). Testing followed the USEPA (2002a,b) methods described as in Kennedy et al. (2013, 2015). The assays were conducted using a minimum of 5 test concentrations of DNAN plus controls. Acute assays were conducted for 48 hours while chronic bioassays were conducted for 6 (*C. dubia*) or 7 days (*P. promelas*) with daily static renewals using fresh test solution. The water quality, light cycle (16 h light/8 h darkness) and temperature 25 ºC were controlled and were used in both assays.

2.3.1 ACUTE AQUATIC TOXICITY

For the acute assay using *C. dubia* , the study design involved 20 organisms per test concentration, with each test concentration consisting of 4 replicate chambers containing 5 individuals per replicate (15 mL). The *C. dubia* were cultured in laboratory prepared hard constituted "synthetic" water (reverse osmosis water with added essential minerals). The endpoint for the acute study was mortality. The LC_{50} and associated 95 % confidence interval were determined as in Kennedy et al. (2013,2015).

For the acute assay using *P. promelas*, the study design involved 40 organisms per test concentration, with each test concentration consisting of 4 replicate chambers containing 10 individuals per replicate (200 mL). The conditions of the water (e.g. temperature, dissolved oxygen, pH, conductivity) were monitored and controlled. The water temperature was maintained at 25 ˚C and chambers were kept on a 16 h light/8 h darkness schedule. For the acute study the endpoint was mortality. The LC_{50} and associated 95 % confidence interval were determined.

Acute Test Conditions

Parameter	**Freshwater Invertebrate (USEPA Method 2002.0)**	**Freshwater Fish (USEPA Method 2000.0)**
Species	*Ceriodaphnia dubia*	*Pimephales promelas*
Age	< 24 hours old	1-14 days old
Feeding	None	None
Concentrations	Control (0) + min of 5 concn., based on range-finding tests	Control (0) + min of 5 concn., based on range-finding tests

Renewal frequency	None	None
Replicates	4	4
Organisms/treatment	5	10
Control of bias (randomization)	Randomized	Randomized
Containers	30 to 50 ml glass beakers	250 ml glass beakers
Test solution volumes	15 ml	200 ml
Temperature	25 ± 1°C	25 ± 1°C
Duration	48 h	48 h
Dilution water	USEPA Mod Hard Recon Water	USEPA Mod Hard Recon Water
Endpoints	Survival NOAEC and LC_{50}	Survival NOAEC and LC_{50}

2.3.2 CHRONIC AQUATIC TOXICITY -

Chronic Assay *C. dubia* survival and reproduction test measures the chronic toxicity of DNAN to *C. dubia* using less than 24 h old neonates during a three-brood (usually six or seven day) static renewal test. The test followed the methods and guidance put forth in EPA 821-R-02-013. Briefly, individual *C. dubia* (time/age matched and less than 24 h old) were assigned to test chambers for a total of 10 chambers per concentration (5 DNAN + controls) and incubated at 25 ˚C. *Ceriodaphnia dubia* were fed and observed daily. Static renewal of the solution was performed daily. The DNAN concentrations were measured in the unused solutions prior to the renewal step to quantify the actual exposure concentrations. The solutions that were replaced were tested for DNAN after the exposure period to quantify test media concentrations. Analytical chemistries were completed using methods established by USAPHC/AIPH DLS Method Development Branch. Water quality parameters were measured as recommended in the EPA methods (e.g. dissolved oxygen, conductivity, pH, etc). *Ceriodaphnia dubia* were observed daily for the number of young produced. Potential effects from DNAN exposure included reduction in number of young produced and mortality. Tests were considered acceptable if 80% or greater survival occurs in the negative control and 15 or more young per surviving female are produced in the negative control test. The chronic LC_{50} and reproductive effects (IC_{25}, IC_{50} and EC_{50}) were calculated using point estimation techniques. The LOEC and NOEC for survival and reproduction were obtained.

P.p. larvae (newly hatched- less than 24 h old) were exposed in a static renewal system for seven days to different concentrations of DNAN. The results were based on the survival and weight of the larvae. Fish were transferred to fresh DNAN solutions daily. Daily and end- of study observations included mortality/survival and growth (LC_{50}, IC_{25} and IC_{50}). DNAN concentrations were measured as described in the *C. dubia* chronic methods.

Chronic Test Conditions

Parameter	Freshwater Invertebrate (USEPA Method 1002.0)	Freshwater Fish (USEPA Method 1000.0)
Species	*Ceriodaphnia dubia*	*Pimephales promelas*
Age	< 24 h (within 8h of same age)	< 24 h
Feeding	Daily (0.2 ml of YTC/algae mixture per USEPA)	0.1 ml brine shrimp nauplii, 2X daily; no food within 12 h of test termination
Concentrations	Control (0) + min of 5 concn., based on range-finding tests	Control (0) + min of 5 concn., based on range-finding tests
Renewal frequency	Daily	Daily
Replicates	10	4
Organisms/treatment	1	10
Control of bias (randomization)	Block randomization by parentage	Randomized
Containers	30 to 50 ml glass beakers	~250 ml glass beakers
Test solution volume	15 ml	200 ml
Temperature	25 ± 1°C	25 ± 1°C
Duration	7 d (or less contingent on 3 broods)	7 d
Dilution water	USEPA Mod Hard Recon Water	USEPA Mod Hard Recon Water
Endpoints	Survival NOEC and IC_{25}, Reproduction NOEC and IC_{25}	Survival NOEC and IC_{25}, Growth NOEC and IC_{25}

More information on Tasks 4 & 5 can be can be found in:

Kennedy AJ, Laird JG, Lounds C, Gong P, Barker ND, Brasfield SM, Russell AL, Johnson MS. 2015. Inter- and intraspecies chemical sensitivity: a case study using 2,4-dinitroanisole. *Environmental Toxicology and Chemistry* 34:402-411.

3.0 RESULTS AND DISCUSSION

3.1 TASK 1 – EXTENDED ONE-GENERATION STUDY IN RODENTS[1]

Three mortalities occurred in the P-generation, one male and two females. The male (high dose recovery group) died after four days of dosing and was found at necropsy to have undescended testes and abnormal penile anatomy. These findings were not considered to be treatment related. One control female died during parturition. One female in the 3600 mg/l group was found dead at post-partum day

[1]All 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 the Association for Assessment and Accreditation of Laboratory Animal Care International.

(PPD) 14 (i.e., 55 days of dosing). No clinical signs of toxicity were observed prior to death. Necropsy revealed enlarged adrenals, spleen, kidneys, and heart and a focal pale pink lesion on the heart.

The male and female mating indices (# of males or females with confirmed mating/total # males or females cohabitated) were 100, 96, 100 and 100% in the control, 144, 720, and 3600 mg/l groups. The male fertility indices (# males impregnating females/total # males cohabitated) were 96, 92, 100, and 88% while the female fertility indices (# of pregnant females/# of sperm positive females) were 96, 96, 100, and 88% in the control, 144, 720, and 3600 mg/l groups (Figure 3.1.1). The mean precoital interval was lowest in the control group (2.5 days), highest in the 144 mg/l group (3.3 days) and decreased with increasing dose (3.0 and 2.6 days in the 720 and 3600 mg/l groups, respectively).

All females determined to be pregnant gave birth to at least one live pup, resulting in gestation indices of 100% for all dose groups. The mean gestation interval was approximately 22 days for all dose groups. A total of 1,376 pups were born (1320 live, 41 stillborn, 15 found dead). The mean litter size was not affected by NTO treatment and ranged from 14.41 pups per litter in the 3600 mg/l group to 14.91 pups per litter in the 144 mg/l group. The number of live births and still births per litter did not differ among treated and control groups. Live births ranged from 13.63 to 14.44 per litter in the control and 720 mg/l dose groups, respectively. The number of stillbirths per litter was lowest in the control group (0.25) and highest in the 3600 mg/l group (0.68) (Figure 3.1.2). The percentage of pups that were male was not affected by NTO treatment, ranging from 45.3% in the 144 mg/l group to 53.5% in the control. Mortalities in the pups and F1 generation were largely limited to stillbirths and mortalities occurring by PND 4. Of the 29 pups that were found dead between PND 2 and PND 6, 4 were controls, 5 were from the 144 mg/l group, 4 were from the 720 mg/l group, and 16 were from the 3600 mg/l group. One control pup was found dead on PND 18 and one pup in the 720 mg/l group was found dead on PND 13; neither demonstrated clinical signs of toxicity prior to death (Figure 3.1.3).

Body mass did not differ among treated and control groups at any time for P1 generation males. In P1 females, the effect of NTO on body mass differed with time (interaction effect $p=0.001$). Body mass was generally unaffected by NTO treatment until the post-partum/lactation phase. During the lactation phase, body mass was reduced in females in the 3600 mg/l group compared to the remaining groups. This reduction in body mass was statistically significant at PPD 14 ($p<0.001$) and PPD 21 ($p<0.001$) (3% and 4%, respectively).

Food consumption for P1 generation females was unaffected by NTO treatment. In P1 males, the effect of NTO treatment on food consumption differed with time (interaction effect $p<0.001$). The only differences between control and treated groups were increases in food consumption in the 144 mg/l group during days 18-21 (7.4%, $p=0.027$) and 21-24 (8.8%, $p=0.031$). All remaining differences were due to consistently lower food consumption in the 3600 mg/l compared to the 114 mg/l group, resulting in an overall 8% lower food consumption rate ($p=0.027$). Food conversion efficiency (FER) was not affected by NTO treatment in P1 generation males, but was reduced in the P1 generation females in the 3600 mg/l group compared to the 144 mg/l group ($p=0.031$).

In F1 pups, the effect of NTO on body mass varied over time, with no effects being evident until PND 21 (interaction effect $p<0.001$). On PND 21, body mass of pups in the 3600 mg/l group (46.4 grams) was reduced relative to the other NTO treatment groups (50.7 g and 49.8 g for 144 and 720

mg/l groups, respectively) but not the control group (49.3 g) (p=0.004). Body mass of the F1 females was unaffected by NTO treatment. In the F1 males, body mass did not differ between control and NTO treated groups at PND 21, but was reduced by approximately 8% in the 3600 mg/l group from PND 28 through 52 (interaction effect p=0.011; p=0.007, p=0.003, p=0.016, p=0.019, and p=0.011, for PND 28, 35, 42, 49, and 52, respectively; Fig. 3.1.4).

Food consumption for F1 females was unaffected by NTO treatment. In F1 males, total food consumption was 10% lower in the 3600 mg/l group compared to the control and 144 mg/l group (p = 0.014). NTO treatment did not affect FER in F1 males or females (data not shown). Body mass at PPS did not differ among NTO treated rats and the control group. The mean number of nipples per pup per litter retained by male pups at PND 13 was lowest in the control group (0.4) and highest in the 144 and 3600 mg/L groups (1.1 and 1.0) (Figure 3.1.6).

Treatment with NTO increased both the percentage of pups per litter with retained nipples (>1 nipple) and the number of nipples retained per pup (p=0.012 and p=0.028, respectively). Percent of male pups with retained nipples at PND 13 was increased in all NTO dose groups (35%, 24%, and 30%) compared to controls (8%) (p=0.027, p=0.035, and p=0.017, respectively). Pups in the 144, 720 and 3600 mg/l NTO groups retained 1.1, 0.9, and 1.0 nipples per pup, respectively, compared to 0.4 nipples retained per pup in the control group. This difference was only statistically significant for the 144 and 3600 mg/l groups (p=0.041 and p=0.049, respectively).

NTO treated P generation males exhibited histologic changes consistent with seminiferous tubule degeneration or atrophy. Vacuoles within Sertoli cell cytoplasm were observed in 44% and 68% of animals in the 144 and 3600 mg/l groups (p =0.003 and p <0.001, respectively). Germ cell-free gaps were observed in 24% and 88% of animals in the 144 and 3600 mg/l groups (p = 0.022 and p <0 001, respectively). Animals in the 3600 mg/l group also exhibited retained spermatids in Stage IX-X (28%; p = 0.048), apoptotic cells (36% ; p=0.016), Sertoli-only tubules (28%), multinucleate giant cells (12%), sloughed germ cells (16%), and lack of elongating spermatids (8%). Animals in the 720 mg/l group did not exhibit similar signs of seminiferous tubule degeneration and the incidence of testicular interstitial proteinaceous fluid was lower in this group than in controls (p = 0.021). Reduction in sperm count and inappropriate cell types in the lumen of the epididymides were also noted for 20% of males in the 3600 mg/l group; however, the frequency of these lesions was not statistically different from control. No changes were noted in the epididymides in lower dose groups.

No treatment-related changes were noted in the accessory sex glands in P generation males. Control animals, however, had more intraluminal round cells in the seminal vesicles than did animals in the 144 and 720 mg/l groups (p = 0.0016 and p = 0.004, respectively).
Histologic findings observed in somatic tissues in the P generation male 3600 mg/l NTO group more frequently than controls included alveolar septal congestion in the lung (p = 0.023), mast cell infiltrate in lymph node (p = 0.002), changes in the parietal epithelium of the glomerular capsule in the kidney (p = 0.001), and minimally more extramedullary hematopoiesis (EMH) and pigment in the spleen ($p \leq 0.001$ and $p \leq 0.001$, respectively). Although seen more often in high dose rats, the scores were all ‘minimal’ or, rarely, ‘mild.’ These changes are commonly reported background lesions and were determined to be unrelated to the effect of the test article.

In P generation females, histologic findings in the kidneys, adrenal glands, and lymph nodes were observed more frequently in the 3600 mg/l group than controls. Pale eosinophilic proteinaceous fluid was noted in the renal tubules of 75% of females in the 3600 mg/l group ($p \leq 0.001$). Adrenocortical vacuolation was noted more frequently in females in the 3600 mg/l group (63%) than controls (17%; $p = 0.003$). The incidence of phagocytosed erythrocytes in the thymus-associated lymph node was greater in females in the 3600 mg/l group (43%) than controls (0%; $p = 0.043$). P generation females had a lower incidence of splenic EMH (4%) than controls (33%) ($p=0.023$). The presence of minimal splenic EMH is a normal background finding. The scores were all '0' (normal) or '1' (minimal), are by nature subjective, and were not considered exposure-related.
Histologic examination of the female (14-0210) from the 3600 mg/l group that died on PPD 14 revealed septicemia characterized by subacute, severe neutrophilic inflammation in multiple organs (lungs, heart, kidneys, with marked thymic involution) that was unrelated to test article administration. The data collected from this animal was excluded from all statistical evaluations of histopathologic lesions in P generation females. No significant lesions were noted in other tissues examined in P generation males and females.

Only reproductive tissues were evaluated in weanling animals. Apoptotic cells that were either condensed, pyknotic and shrunken, or appeared to have 'ropy' heterochromatin as if entering mitosis except the cytoplasm was pink (on PAS-H stain), separate from neighboring cells and usually bordering on luminal were noted in both control (20%) and 3600 mg/l (30%) weanling males. The frequency of this finding was not statistically significant. No histopathological changes, compared to control, were observed in the epididymides of weanling males given 3600 mg/l NTO. No histopathological changes, compared to control, were observed in the ovaries and uterus (including cervix, vagina) of weanling females given 3600 mg/l NTO.

NTO treated F1 generation males exhibited histologic changes consistent with seminiferous tubule hypoplasia or degeneration/atrophy. Testes of males in the 3600 mg/l group demonstrated apoptotic cells (100%; p <0 001), sloughed germ cells (100%; p <0 001), multinucleate giant cells (95%; p <0 001), lack of elongating spermatids (100%; p <0 001), germ cell-free gaps (95%; p <0 001), Sertoli cell vacuoles (85%; p <0.001); dilatation of seminiferous tubules (55%; p=0.012), Sertoli-only tubules (30%; p=0.031), and reduced diameter of the testis (100%; p<0.001). Males in the 720 mg/l group demonstrated reduction in testis diameter (p=0.028), but did not exhibit signs of seminiferous tubule degeneration.

Corresponding increases in the frequency of epididymal hypospermia (95, and 100%, respectively) were observed in the 720 and 3600 mg/l groups (p<0.001, and p<0.001, respectively). Inappropriate cell types in the lumen (90%) and cribriform change in cauda (85%) of the epididymides were also observed in males in the 3600 mg/l group (p<0.001 and p<0.001).
No treatment-related changes were noted in the accessory sex glands in F1 generation males.
In somatic (i.e., non-reproductive) tissues, differences between F1 males in the 3600 mg/l group and control rats included a slight increase in pulmonary alveolar hemorrhage (60%; p=0.019), minimal pyknosis of the inner stripe of the kidneys (55%; p=0.001), and minimal hepatic congestion (40%; p=0.003).

For F1 females in the 3600 mg/l group, minimal increased incidence of pale eosinophilic proteinaceous fluid in renal tubules (60%; p=0.023) and hepatic congestion (50%; p=0.041) relative

to controls were noted. This slight renal change was also noted in 20% of F1 females in the 720 mg/l group; however, this incidence was not different from controls.

No significant lesions were noted in other tissues examined in F1 generation males and females.

Only reproductive tissues were evaluated for recovery males. Animals in the 3600 mg/l recovery group exhibited protein between tubules (44%), Sertoli-only tubules (22%), vacuoles within Sertoli cell cytoplasm (22%), and germ cell-free gaps (11%). The incidence of these findings was not significantly different between treated recovery males and control recovery males.

There were no treatment-related effects on clinical chemistry parameters in P1 females or F1 pubertal animals. Glucose levels were elevated in P1 males exposed to NTO compared to controls (20 - 40%) and reported normal values. This increase was statistically significant only for the 144 mg/l group compared to the controls ($p=0.020$).

Red blood cell counts (RBC) were reduced (8%) in P1 males in the 3600 mg/l group compared to those from the control ($p=0.042$), but were within published normal ranges. Mean cell hemoglobin (MCH) was elevated (6%) in P1 males in the 3600 mg/l group; both compared to control ($p=0.002$) and normal ranges. Mean cell volume (MCV) was slightly increased (6% and 4%, respectively) in both P1 males and females ($p=0.002$ and $p=0.044$, respectively). There were no treatment-related effects on hematology parameters in F1 pubertal animals.

There was no consistent pattern of effects on thyroid hormones between sexes or across study phases. There were no treatment-related effects on thyroid hormones in P1 or F1 females or weanlings of both sexes. In P1 males, TSH levels demonstrated a non-significant dose response and were reduced (35%) in the 3600 mg/l group. In F1 males, T4 levels had a non-significant dose response and were reduced (15%) in the 3600 mg/l group. All thyroid hormone values were within previously reported control values for the species (Chang et al. 2008, Christian and Trenton 2003).
6.10 Thymic and Splenic Lymphocyte Subpopulation Analysis Thymocyte cellularity in F1 male and female rats did not differ between treatment groups. Thymus cellularity for one male in the 720 mg/l group (14-0276) was approximately double (14 X109) the average cellularity for that group and was dropped from further analysis. The distributions of DN/DP/CD4+/CD8+ thymocytes in female rats were not affected by treatment with NTO. In male rats, the percent of double negative cells (DN) was 33% lower in the 3600 mg/l group compared to the control ($p=0.036$). There were no differences between the control and NTO treated males for the remaining thymic cell types.
NTO had no effect on splenic cellularity or the proportion of B, T, and NK cells in F1 male and female spleens.

All measures of sperm count were reduced in P1 males in the 3600 mg/l group compared to controls. Total sperm concentration was reduced by 20% ($p=0.024$) while motile ($p=0.009$) and progressively motile sperm concentrations ($p=0.016$) were reduced by 27% and 28%, respectively. The percent motile sperm did not differ between NTO treated groups and the control. Although total (44%) and motile sperm (27%) concentrations were also reduced in the 3600 mg/l recovery males compared to control recovery males, these reductions were not statistically significant.

Across study phases NTO showed no evidence of adverse effects on somatic tissues at the highest concentration tested. Thus, benchmark dose modeling was not conducted for systemic toxicity. The unbounded NOAEL for systemic toxicity was 3600 mg/l in P1 males (160 mg/kg-day), P1 females (250-800 mg/kg-day),F1 generation (335 mg/kg-day). F1 males in the 3600 mg/l did demonstrate a reduction in body mass associated with reduced food consumption at PND 42-52. This effect was not likely due to systemic toxicity but rather taste aversion leading to decreased water consumption and in turn decreased food consumption and body mass gain. Fertility endpoints were unaffected by NTO; however, mass of male reproductive organs and histopathology of the testes demonstrated effects in all phases of the study, and nipple retention and PPS were altered in the F1 pubertal males. Delayed PPS and nipple retention in male rats are considered indicators of altered androgen status. Retention of nipples has been shown to be a permanent effect for some chemicals (McIntire et al. 2001, 2002) and is therefore considered a malformation and an adverse developmental effect (Foster and McIntyre 2002).

Histopathology endpoints were not modeled as they did not demonstrate a dose-response and the effects were only evident in highest dose group, with all other dose groups having a near zero percent response rate (Barnes and Dourson 1988). To establish a point of departure, dose response modeling was conducted for all other potential critical endpoints using EPA's Benchmark Dose Software (BMDS 2.6.0.1). The reproductive organ mass, sperm count, and PPS responses were modeled using all available BMDS continuous models. Nipple retention was modeled using the BMDS nested models with and without litter size as a litter specific covariate. The goodness of fit statistic, minimal Akaike Information Criterion (AIC), and scaled residuals near the benchmark response (BMR) were used to select among models for each potential critical effect. For P1 male epididymal mass, four models: Exponential3, polynomial2, polynomial3, and power had suitable model fit and the lowest combined AIC and scaled residuals and were selected. Five models, exponential2, exponential3, linear, polynomial2, polynomial3, and power met the criteria for selection for P1 male sperm count. For PPS, exponential2 and linear models were selected. For F1 testes mass, only two models had acceptable model fit, polynomial2 and polynomial3. Three models, exponential3, polynomial2, and polynomial3 were retained for F1 epididymal mass based on combined AIC and scaled residuals. No models could be selected for SVCG mass based on the goodness of fit statistic. For nipple retention, the initial run was conducted with default parameters (i.e., including litter specific covariate (LSC) and intralitter correlation (ILC)). However, the theta parameter was zero so the litter specific covariate was dropped in subsequent runs. Comparison of the AIC values between runs with and without the ILC indicated that the AIC and goodness of fit statistic were improved with the inclusion of ILC. NLogistic was selected based on lowest AIC values and residuals.

Overall, the resulting BMDL10 values ranged from 2335 to 2775 mg/l (140-160 mg/kg-day) for reproductive effects in P1 males and 1048 to 2794 mg/l (120-310 mg/kg-day) for reproductive/ developmental effects in F1 males, depending on the response variable (Table 3.1.1). These results are consistent with the study findings for these endpoints, generally falling between the LOAEL of 3600 mg/l and the NOAEL of 720 mg/l.

Figure 3.1.1

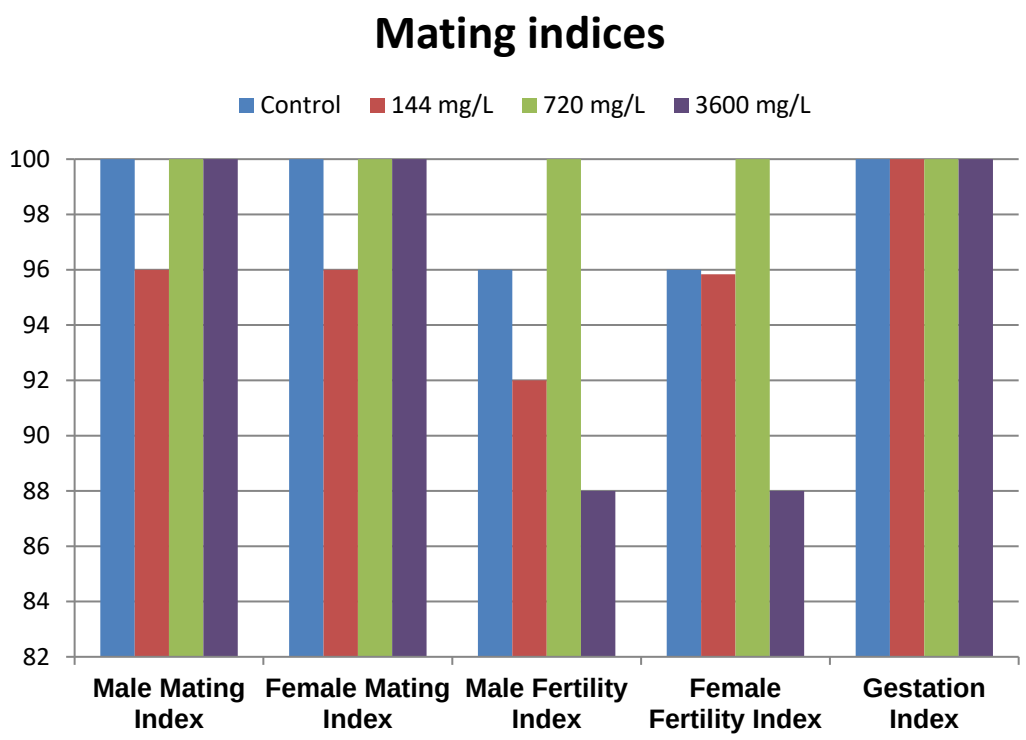


Figure 3.1.2

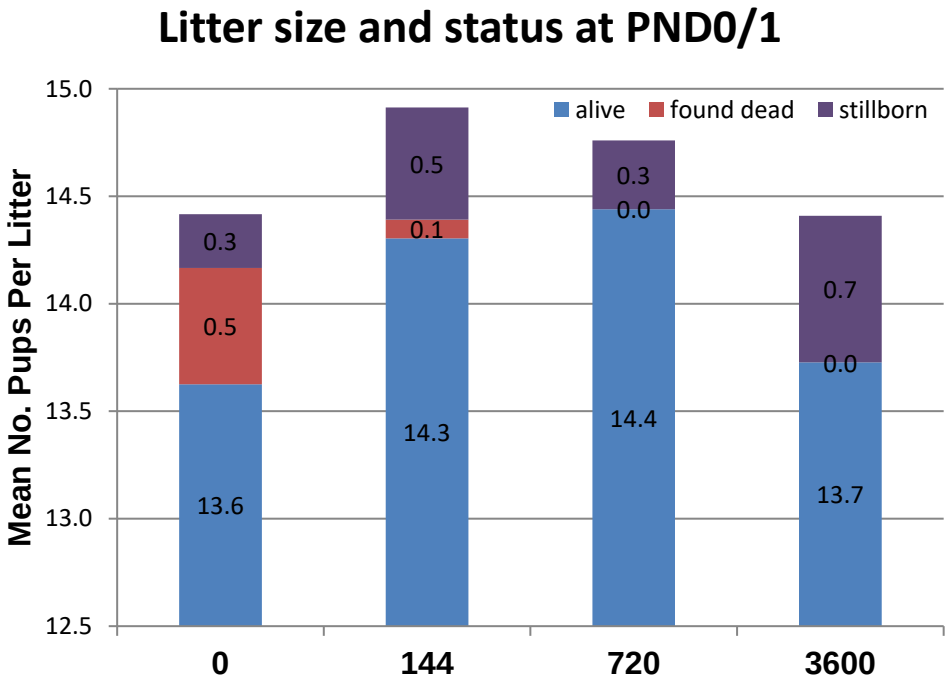


Figure 3.1.3

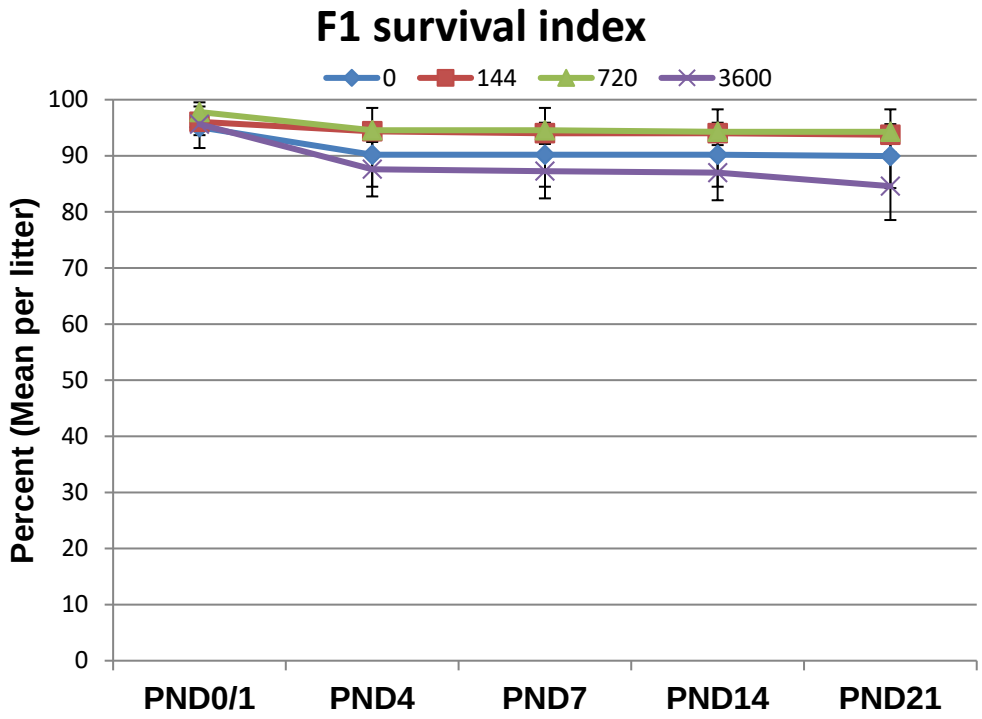


Figure 3.1.4

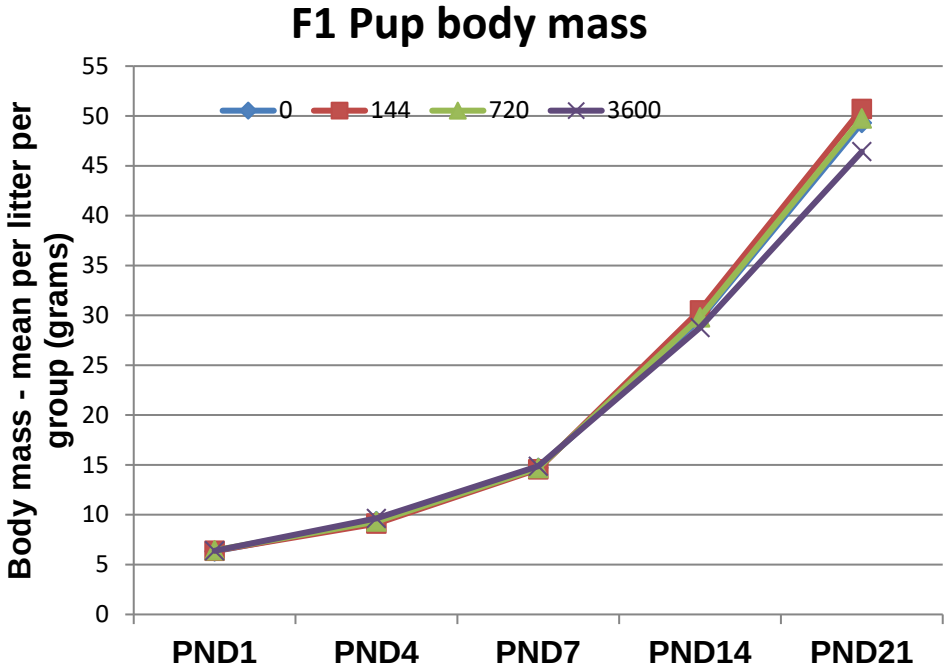


Figure 3.1.5

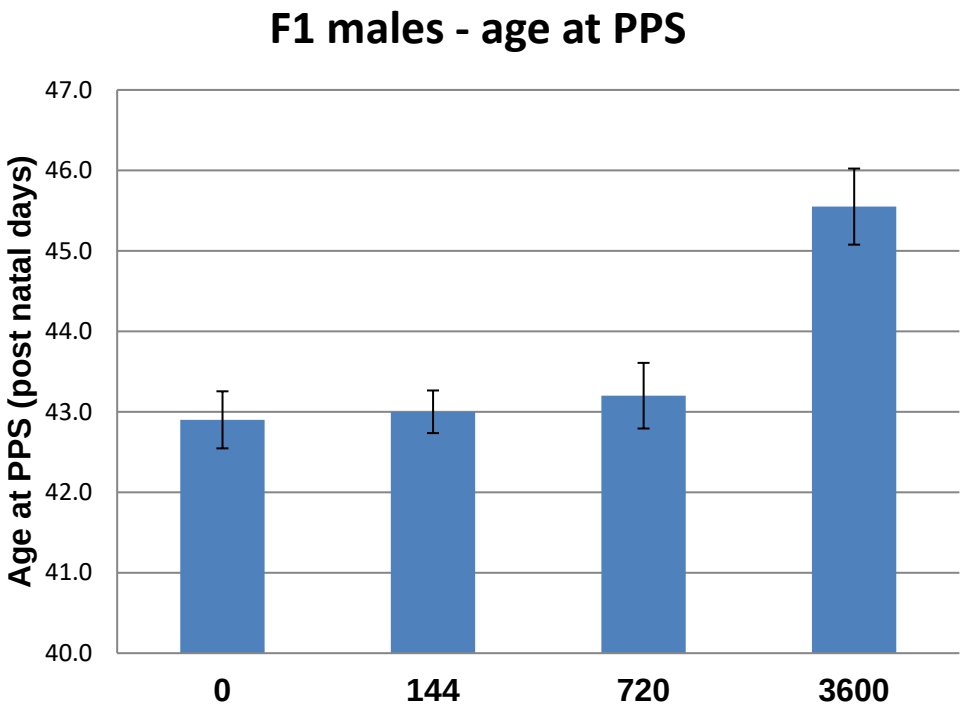


Figure 3.1.6

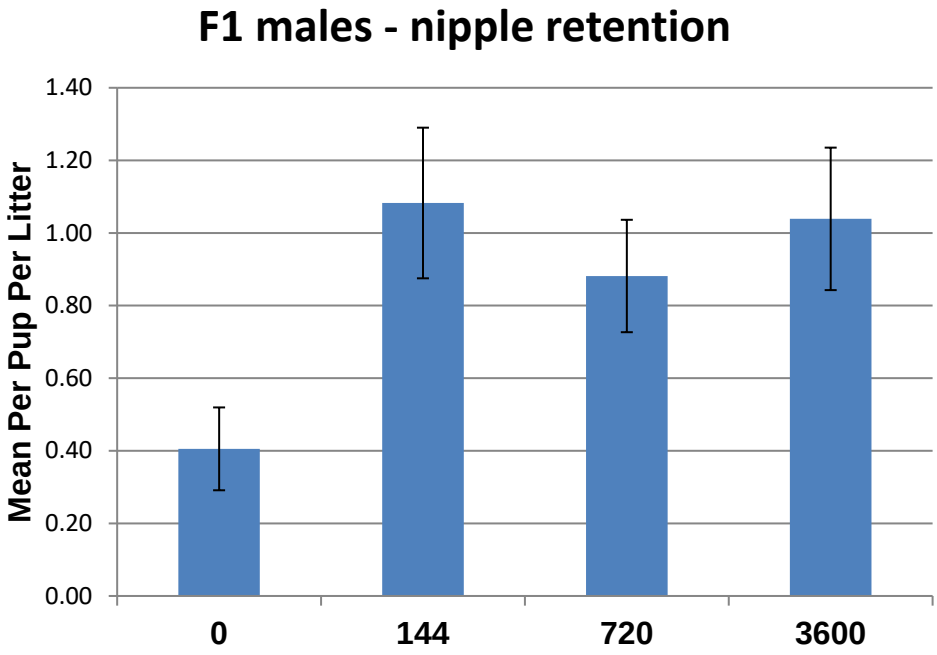


Table 3.1.1 BMD modeling summary

Critical Effect	BMD	$BMDL_{10}$
P1 Males		
epididymal mass	3465	2335
sperm count	4520	2775
F1 Pubertal Males		
nipple retention	3304	1048
PPS	1970	1420
testes mass	2839	2443
epididymal mass	1896	1149
SVCG mass	no acceptable model fit	

3.2 TASK 2 – AVIAN TOXICITY TO NTO[2]

3.2.1 – Avian Acute Oral Test

Approximately 24 hours after oral dosing in an acute toxicity study, one female displayed neuromuscular signs including loss of balance and an inability to stand (ataxia), and exhibited tremors, suggestive of neurological toxicity. All other treated birds appeared normal. Therefore, the LD50 for male and female Japanese quail was greater than the limit dose of 2000 mg/kg.

3.2.2 - Avian One-Generation Study

Repeated oral exposure to 500 and 1000 mg/kg-day-NTO induced neuromuscular signs and compound-related pre-term mortality in male and female Japanese quail. Following five days of oral exposure, parental generation (F0) birds from the 1000 mg/kg-day group began displaying ataxia. In addition, birds exhibited convulsions, circling on the floor of the cage, and backward arching of the neck (opisthotonos), and alternated between prostrate inactivity and ataxic wing activity beginning 3-4 hours after dosing. In conjunction with neuromuscular signs, decreased body mass gain occurred in birds as early as one week into exposure. After 17 days of exposure, birds from the 500 mg/kg-day group began displaying neuromuscular signs. Ultimately, all of the 1000 mg/kg-day birds and all but one of the 500 mg/kg-day birds met euthanasia criteria and were sacrificed. No NTO-related mortality occurred in the 100 or 20 mg/kg-day groups.

Histopathology could not determine the cause of death in F0 generation birds from the 500 and 1000 mg/kg-day groups. However, vacuolization of the cerebellum and/or the brainstem was observed

[2]All 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 the Association for Assessment and Accreditation of Laboratory Animal Care International.

and these changes were present in a dose dependent manner. Neither brain lesions nor convulsions have been seen in previous toxicology studies in rodents exposed to NTO.

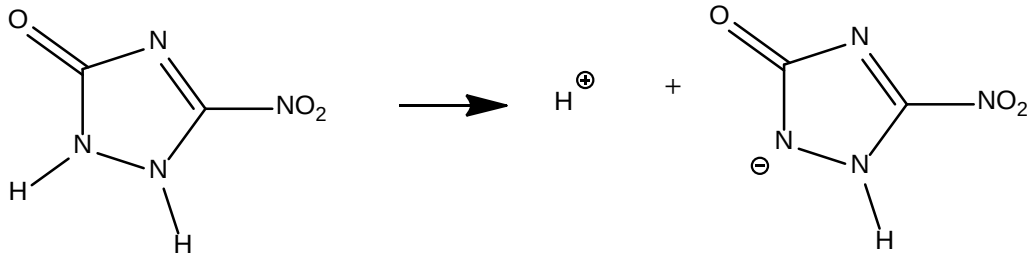
Mild neuromuscular signs occurred in 10% of first generation (F1) birds from the 100 mg/kg-day group, but not in birds from the 20 mg/kg-day group or control birds in either generation. No other sublethal adverse effects were observed. Tissues from animals exposed to daily doses of 100 and 20 mg/kg-day NTO were generally unaffected. Therefore, mortality was identified as the critical endpoint in this study. A mean BMD of 348 mg/kg-day was calculated for male and female F0 generation quail based on the results of the 5 BMDL models. This corresponded to a BMDL10 of 151 mg/kg-day for male and female F0 generation quail.

3.3 TASK 3 – AMPHIBIAN TOXICITY

Amphibians are the preferred test system for evaluating the effects of compounds on organism development and effects to the thyroid. Amphibian development has been extremely well characterized so that by determining the time required for parameters such as front limb eruption, tail resorption, and overall length and width of the tadpoles, quantitative impacts on development may be determined. These endpoints are sensitive to substances that inhibit thyroid function.

NTO is acidic in water due to dissociation of a ring hydrogen (Fig. 3.3.1). Untreated water can develop pHs as low as 2-3, a factor that in itself can inhibit development or even be toxic to aquatic species.

Figure 3.3.1 - Acid dissociation of NTO



Range Finding Tests:

Frog embryos were exposed for a period of 7 days beginning 24-hours after hatching to concentration of NTO up to 11,350 mg/L. Solutions were adjusted for pH so that effects of NTO could be separated from pH effects. In these range finding tests, tadpoles exposed to non-pH-adjusted NTO solutions died within 24-hours at a concentration of 383 mg NTO/L, which had a pH of 3.7. No mortality was observed at a lesser concentration where the pH was 4.3. In short-term range finding tests employing pH-adjusted NTO solutions, no significant mortality was observed up to 3710 mg NTO/L, indicating an LC50 > 3710 mg/L.

Figure 3.3.2 - Northern leopard frog (*Lithobates pipiens*)—immature and adult phases.

Definitive Tests: These tests were conducted for a period of up to 70 days. Development of *L. pipiens* tadpoles was delayed by exposure to NTO; development of front and hind limbs was greatly retarded or resulted in abnormally small limbs (Table 3.3.1). Early tadpole growth was inhibited by NTO, with snout-vent-length (SVL) lower than the control in all NTO treatments (as low as 201 mg/L). However, these differences disappeared by Day 28 of the test, except in the 7775 mg/L treatment. The mean time required for front limb emergence in the 3338 mg/L treatment group was 59.2 days, compared to 46.3 days in the controls. Time to complete metamorphosis in the same treatment group was 61.9 days compared to 51.0 days in controls. The median lethal concentration for NTO over the 70-day exposure was 3670 mg/L. Some tadpoles in the 8382 mg/L treatment survived until day 63, but never grew and never developed limbs. Photgraphic examples of *L. pipiens* at advanced emergence and complete metamorphosis is presented in Fig. 3.3.2. Proportion of response for survival front limb emergence and complete metamorphosis is shown in Fig. 3.3.3.

Few external physical abnormalities were noted in surviving frogs, and snout-vent length was generally not affected by NTO. However, liver and heart weights of frogs increased with NTO exposure, especially at concentrations at or above 517 mg/L. If the increases in liver and heart weight are taken as the most sensitive endpoints, the no observed effect concentration was 190 mg/L.

The absolute weight of various organs increased with increasing NTO concentrations. When normalized to the mass of the whole frog, however, there were few significant differences (data not shown). Only kidney biomass in the 3338 mg /L NTO treatment was statistically greater than the control. Crouse et al. (2015) reported that kidney mass in rats exposed by gavage to 1500 mg/kg-d of NTO was significantly elevated, relative to the control group. Crouse et al. (2015) also reported a statistically significant increase in the brain mass of female rats exposed to NTO at doses of 100 and

1000 mg/kg-d. Females in an intermittent treatment group (315 mg/kg-d) also had larger, but not significantly larger, brain mass than females in the control group.

The mass of testes and epididymides was significantly reduced in male rats at NTO doses as low as 30 mg NTO/kg-d in a 90-d study (Crouse et al. 2015). Microscopic examination of reproductive tissues from male rats in the 315 and 1000 mg/kg/d dose groups demonstrated moderate to severe degeneration and atrophy of the seminiferous tubules. Interestingly, the tubules were more severely atrophied in the 315 mg/kg-d group than in the 1000 mg/kg-d group, with no spermatic precursor cells or adult sperm (Crouse et al. 2015). In the current study, there were fewer, possibly less robust spermatogonia in some specimens from the 3338 mg/L NTO-exposure group, although data were inadequate to draw definitive conclusions regarding the impact of NTO on the *L. pipiens* male reproductive system.

Pathology was absent from the liver sections. Pathology was recognized in the frog renal tissue, in four of five tissues from the control and five of five tissues from the 3338 mg/L treatment. The number of granulomas ("multiple aggregates of flattened macrophages encircling necrotic cellular debris and granulocytes" see pathology report in USAPHC 2017) was higher in the kidney samples from the 3338 mg/L NTO group, relative to the control. Also, those in the 3338 mg/L NTO exposure group were scored somewhat higher (greater effect), with four "2s" (6 to 20% affected cell type) and one "3" (21 to 40% affected cell type). Of the four control renal tissue samples that demonstrated granulomas, three were scored "1" (up to 5% affected cell type) and one was scored "2."

In both of the control testes samples, spermatogonia were numerous. Two of the three testes collected from 3338 mg/L NTO treatment frogs contained possibly fewer spermatogonia than in the control. In one specimen from the 3338 mg/L treatment, the seminiferous tubules appeared smaller than in the control tissues. The pathology results did not provide a clear, definitive picture of the effects of NTO on testicular tissue.

Table 3.3.1 Summary of Time to Front Limb Eruption and Metamorphosis in the 70-d Definitive Study[a]

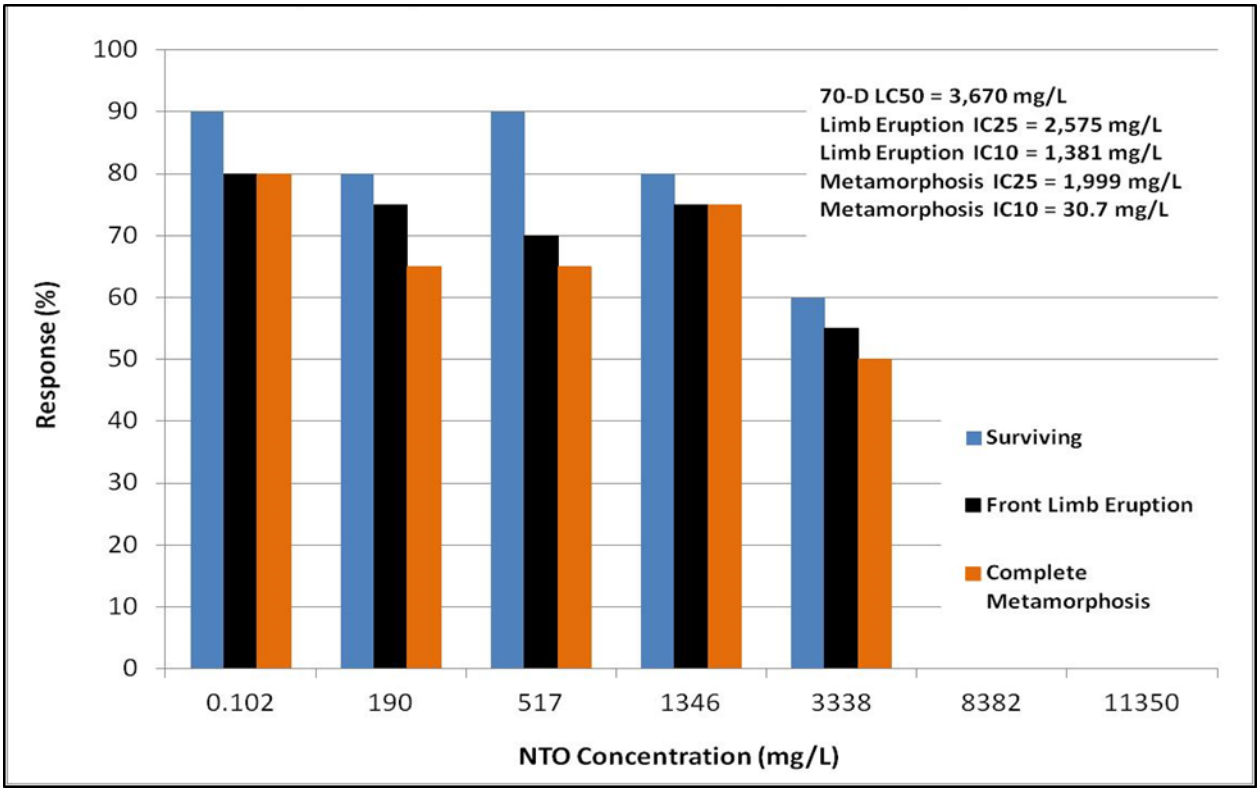
NTO T-W Avg. Concn. (mg/L)	Time to Front Limb Erupt. (Days)	Min. Time to Front Limb Erupt. (Days)[b]	Time to Metamorphosis (Days)	Min. Time to Metamorphosis (Days)[b]
0.04	46.3 (1.8)	40.8 (0.5)	51.0 (2.0)	45.2 (0.8)
190	53.2 (0.8)[c]	44.8 (1.3)	55.8 (0.6)	49.2 (1.8)
517	50.4 (1.7)	43.0 (1.3)	53.5 (0.7)	47.8 (1.8)
1346	49.7 (2.0)	43.5 (1.6)	54.6 (2.2)	48.0 (2.0)
3338	59.2 (2.3)[c]	52.0 (2.7)[c]	61.9 (2.5)[c]	58.0 (2.5)[c]

[a] Mean (S.E.) values are shown.

[b] Minimum time to front limb eruption and to metamorphosis represents the time it took the first organism in the treatment to reach the respective endpoint.

[c] Significantly (α=0.05) higher than the control.

Figure 3.3.3 - 70-Day survival, front limb eruption and complete metamorphosis.



In the absence of pH adjustment, NTO was toxic to test animals at about 250 mg/L for a 7-day exposure period. Adjustment of pH to 7.4-7.8, common for natural waters, increased the LC50 to 3670 mg/L. Time for front limb eruption and completion of metamorphosis were increased by the presence of NTO, but few external physical abnormalities were noted. The no observed effect concentration for the most sensitive endpoints (including time to front limb eruption, time to metamorphosis and kidney biomass) was 1346 mg/L.

TASKS 4-5 AQUATIC TOXICITY – Specific data for the following can be found in Kennedy et al. (2013).

3.4.1 Acute toxicity testing

Test method acceptability criteria for control survival (≥ 90%) and water quality were met for all bioassays. Acute reference toxicity tests (KCl) for *P. promelas* and *C. dubia* resulted in 48-hr LC50 values of 0.78 (95% confidence limits: 0.69 – 0.87) and 0.68 (0.62 – 0.74) g KCl/L, respectively. This indicates comparable sensitivity to the historic ranges in control charts (± 2 S.D. from the mean) for *P. promelas* (0.56 – 1.01 g KCl/L) and *C. dubia* (0.20–0.76 g KCl/L). Range finding tests at 100 mg/L DNAN (nominal) was used to select the concentration series used for the definitive tests below. Complete mortality was observed at 100 mg/L (nominal) DNAN (Tables 3.4.1 and 3.4.2).

In the definitive acute (48-hr) toxicity testing, the concentration of DNAN during the exposure period remained relatively stable (Table 3.4.2), and all DNAN acute toxicity endpoints were based on measured concentrations (Table 3.4.3). Survival response curves for *P. promelas* and *C. dubia* were within the same dose range (Figure 3.4.1). The 48-hr LC50 trended lower for *P. promelas* (33) relative to *C. dubia* (42; Haley et al. 2009), suggesting slightly greater acute sensitivity to DNAN. Further, mortality in the fish was observed relatively sooner (within 2hr of exposure) compared to *C. dubia* (within 24 to 48 hr). After 48hr, however, the 95% confidence intervals for the two test species overlapped, indicating statistically similar acute sensitivity to DNAN.

3.4.2 Chronic toxicity testing

Test method acceptability criteria for control survival (≥ 80%; Table 3.4.4) and water quality were met for both species. The sublethal endpoints for *P. promelas* (> 0.25 mg dry mass) and *C. dubia* (three broods of ≥ 15 neonates) also met control acceptability criteria. Chronic reference toxicity testing results were consistent for both species during the testing period. The concentration of DNAN during the chronic exposures remained relatively stable (Table 3.4.5), and all DNAN chronic toxicity endpoints were based on measured concentrations averaged over the duration of the testing (Table 3.4.3). The *C. dubia* test was terminated after 6 days since all individuals in the control achieved third brood (Table 3.4.4). As expected, the chronic bioassays provided more sensitive endpoints relative to the acute tests (Tables 3.4.3 and 3.4.4). The chronic *C. dubia* survival (LC50 > 24.2 mg/L) endpoint was less sensitive than the *P. promelas* survival (LC50 = 10.0 [8.8 – 11.2] mg/L) endpoint (Table 3.4.3). However, the sublethal endpoints for both organisms were more similar in sensitivity, with chronic DNAN toxicity falling in the range of 8 – 15 mg/L (Table 3.4.3).

Table 3.4.1 Nominal 2,4 dinitroanisole (DNAN) concentrations and survival for the acute (48-hr) range-finding *Pimephales promelas* and *Ceriodaphnia dubia* bioassays. Concentrations were not measured, as range-finding bioassays served only to determine appropriate exposure concentrations for definitive acute testing. Asterisks denote statistically significant reductions relative to the control.

(a)

Nominal (mg/L)	48-hr *Pimephales promelas* survival	48-hr *Ceriodaphnia dubia* survival
Control	100 ± 0	100 ± 0
0.01	100 ± 0	87 ± 23
0.1	100 ± 0	93 ± 12
1	100 ± 0	100 ± 0
10	100 ± 0	100 ± 0
100	0 ± 0*	0 ± 0*

Table 3.4.2 Nominal and measured 2,4 dinitroanisole (DNAN) concentrations and survival for the definitive acute (48-hr) *Pimephales promelas* and *Ceriodaphnia dubia* bioassays. Asterisks denote statistically significant reductions relative to the control.

(a)

Nominal (mg/L)	Measured Test Initiation (mg/L)	Measured Test Termination (mg/L)	Mean Survival (± 1 S.D.)
0	<0.01	<0.01	100 ± 0
6	7.1	6.6	100 ± 0
12	13	14	100 ± 0
25	28	28	75 ± 6*
50	74	74	0 ± 0*
100	120	120	0 ± 0*

(b)

Nominal (mg/L)	Measured Test Initiation (mg/L)	Measured Test Termination (mg/L)	Mean Survival (± 1 S.D.)
0	<0.01	<0.01	90 ± 20
6	7.1	7.2	95 ± 10
12	13	14	80 ± 0
25	28	28	95 ± 10
50	74	68	0 ± 0*
100	120	120	0 ± 0*

Figure 3.4.1 48-hr dose response curves for *Pimephales promelas* and *Ceriodaphnia dubia* exposed to DNAN in definitive acute bioassay testing. Note that concentration data are plotted on a log10 scale.

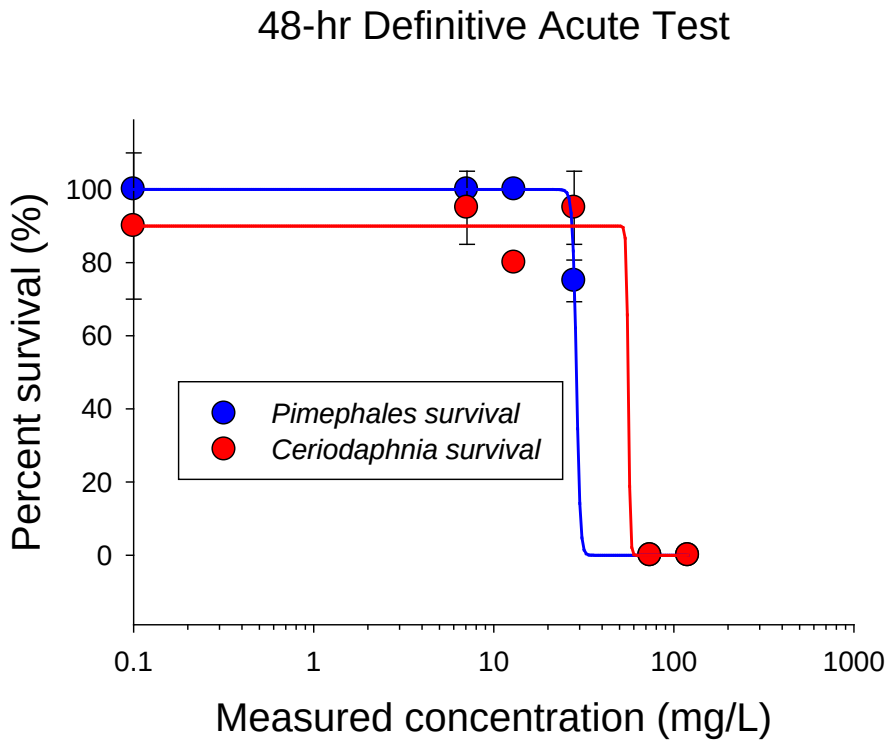


Table 3.4.3 Toxicity reference values for *Pimephales promelas* and *Ceriodaphnia dubia* exposed to 2,4-dinitroanisole. The no observable effect concentration (NOEC), lowest observable effect concentration (LOEC), maximum allowable concentration (MATC)[3], median lethal concentration (LC50) and acute-to-chronic ratio (ACR) are provided. Ninety-five percent confidence intervals for LC50 values are indicated in parentheses. The combined measure provides the most sensitive value among the tested endpoints (e.g., survival vs. growth/reproduction).

Species	Exposure Duration	Measure	Endpoint	DNAN (mg/L)
Pimephales promelas	24-h	Survival	NOEC	13
		Survival	LOEC	28
		Survival	LC50	41 (37 – 45)
	48-h	Survival	NOEC	13
		Survival	LOEC	28
		Survival	LC50	37 (33 – 41)
	7-d	Survival	NOEC	5.8
		Survival	LOEC	11.6
		Survival	LC50	10.0 (8.8 – 11.2)
		Growth	NOEC	11.6
		Growth	LOEC	24.6
		Combined	MATC / ChV	8.2
		Combined	IC25	10.4 (8.2 – 14.3)
		Combined	IC50	15.1 (12.3 – 17.7)
		Combined	ACR	4.5
Ceriodaphnia dubia	24	Survival	NOEC	74
		Survival	LOEC	120
		Survival	LC50	82 (72 – 93)
	48	Survival	NOEC	28
		Survival	LOEC	74
		Survival	LC50	42 (37 – 47)
	6-d	Survival	NOEC	24.2
		Survival	LOEC	>24.2
		Survival	LC50	>24.2

[3] Also known as chronic value (ChV).

Species	Exposure Duration	Measure	Endpoint	DNAN (mg/L)
		Reproduction	NOEC	6.2
		Reproduction	LOEC	12.2
		Combined	MATC	8.7
		Combined	IC25	8.2 (7.4 – 8.7)
		Combined	IC50	10.6 (10.0 – 11.2)
		Combined	ACR	4.8

Table 3.4.4 Results from the 7-day *Pimephales promelas* chronic toxicity test (a), and the three-brood *Ceriodaphnia dubia* chronic toxicity test (b)**.** NA = Not available due to complete mortality.

(a)

Mean Measured Concentration mg/L (± 1 S.D.)	Mean Survival (± 1 S.D.)	Mean Biomass (± 1 S.D.)	Mean Growth (± 1 S.D.)
Control (0.2 ± 0.7)	98 ± 4%	0.421 ± 0.044	0.291 ± 0.044
0.7 ± 0.2	100 ± 0%	0.418 ± 0.034	0.288 ± 0.034
1.4 ± 0.5	96 ± 5%	0.454 ± 0.059	0.324 ± 0.059
2.5 ± 1.0	98 ± 4%	0.436 ± 0.074	0.306 ± 0.074
5.8 ± 1.7	90 ± 12%	0.452 ± 0.064	0.322 ± 0.064
11.6 ± 3.6	38 ± 4%*	0.300 ± 0.077*	0.170 ± 0.077*
24.6 ± 1.8	0 ± 0%*	NA	NA

(b)

Mean Measured Concentration mg/L (± 1 S.D.)	Mean Survival (± 1 S.D.)	Mean Total Reproduction (± 1 S.D.)	Mean Total Neonates/survivor (± 1 S.D.)
Control (0.0 ± 0.0)	90 ± 32%	34.8 ± 13.0	38.7 ± 4.7
0.7 ± 0.1	100 ± 0%	38.4 ± 4.6	38.4 ± 4.6
1.5 ± 0.3	100 ± 0%	38.3 ± 3.9	38.3 ± 3.9
3.1 ± 1.8	100 ± 0%	39.7 ± 5.5	39.7 ± 5.5
6.2 ± 0.5	100 ± 0%	36.2 ± 2.9	36.2 ± 2.9

Mean Measured Concentration mg/L (± 1 S.D.)	Mean Survival (± 1 S.D.)	Mean Total Reproduction (± 1 S.D.)	Mean Total Neonates/survivor (± 1 S.D.)
12.2 ± 1.2	100 ± 0%	12.8 ± 4.6*	12.8 ± 4.6*
24.2 ± 2.0	90 ± 32%	0.2 ± 0.4*	0.1 ± 0.3*

Table 3.4.5 Nominal and measured 2,4 dinitroanisole (DNAN) concentrations provided as means (± one standard deviation from the mean) for the chronic *Pimephales promelas* (a), and *Ceriodaphnia dubia* (b), bioassays. In-water is defined as the freshly prepared DNAN water used in water exchanges while out-water is defined as the 24-hrold water sampled prior to water renewal.

(a)

Nominal (mg/L)	Measured In-water Mean (mg/L)	Measured Out-water Mean (mg/L)	Overall Mean (in- and out-water) (mg/L)
0	0.2 ± 0.7	0.4 ± 1.1	0.2 ± 0.7
0.8	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.2
1.6	1.3 ± 0.6	1.4 ± 0.2	1.4 ± 0.5
3.1	2.3 ± 1.4	2.8 ± 0.2	2.5 ± 1.0
6.3	5.6 ± 2.0	5.9 ± 0.6	5.8 ± 1.7
12.5	11.0 ± 3.6	12.2 ± 2.4	11.6 ± 3.6
25.0	24.0 ± 1.9	25.3 ± 1.7	24.6 ± 1.8

(b)

Nominal (mg/L)	Measured In-water Mean (mg/L)	Measured Out-water Mean (mg/L)	Overall Mean (in- and out-water) (mg/L)
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.8	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
1.6	1.5 ± 0.4	1.5 ± 0.2	1.5 ± 0.3
3.1	2.6 ± 1.3	3.6 ± 2.1	3.1 ± 1.8
6.3	6.4 ± 0.4	6.1 ± 0.6	6.2 ± 0.5
12.5	12.3 ± 0.8	12.0 ± 1.5	12.2 ± 1.2
25.0	24.0 ± 1.9	24.4 ± 2.3	24.2 ± 2.0

4.0 CONCLUSIONS

4.1. EXTENDED ONE-GENERATION STUDY IN RATS (NTO) –

Trends from recent toxicity studies suggest kinetics to oral exposures to NTO are important. Specifically, data collected from oral gavage data should be treated with caution, as those bolus exposures resulted in more pronounced effects (at lower nominal concentrations) than those observed during the present study where animals were exposed to concentrations of NTO in drinking water ad libitum. This suggests that absorption and elimination is relatively rapid when rats are exposed in this manner, which is more similar to a potential environmental application using such data for a drinking water standard. However, taste aversion may have resulted in reduced consumption of the higher doses of NTO, resulting in lower delivered doses. Approximate doses calculated based on measured consumption and body mass indicate that the dose delivered to the P generation males was likely below those at which pronounced effects were observed in gavage studies. Caution is recommended in the extrapolation of data from fertility indices, as humans are known to be more sensitive to changes in fertility than rodents. Delays in PPS and increased rate of nipple retention in male pups are suggestive of effects on retarded male reproductive development. Combined with the testicular toxicity previously documented in adult and pubertal males, these data are consistent with these effects on Sertoli cell reported previously.

4.2 EXTENDED ONE-GENERATION IN QUAIL (NTO) –

Effects observed in *C. japonica* from oral exposures to NTO were largely unexpected given the lack of neuromuscular effects reported from relatively high oral exposures of NTO to mammalian species. Levels of oral NTO exposures in mammals that caused decreases in testicular mass and sperm production coincided with levels that caused neuromuscular effects in quail. Investigations into effects of NTO causing reductions in sperm production in quail were equivocal and likely of small importance relative to the neuromuscular effects from oral exposures. This effect was also observed in birds of the F1 generation and no reductions in offspring production or developmental effects were observed. The mean BMD of 348 mg/kg-day was calculated for male and female F0 generation quail based on the results of the 5 BMDL models. This corresponded to a BMDL10 of 151 mg/kg-day for male and female F0 generation quail which can be used as a point of departure to develop a Toxicity Reference Value for birds.

4.3 AMPHIBIAN METAMORPHOSIS AND TOXICITY (NTO) –

NTO in neutralized (pH-adjusted) solutions caused statistically significant mortality to *L. pipiens* at concentrations of 8382 mg/L and greater in a 70-d exposure. While mortality at 3338 mg/L was not shown to be statistically different from the control, reduced survival at test termination (60%) in this concentration may be ecologically important, and suggests that statistically significant impacts might be identifiable if a larger sample size is used in future testing.

NTO exposure caused no obvious abnormalities or malformations in the test organisms exposed up to, and including, 3338 mg NTO/L. Exposure to NTO increased the amount of time required for

front limb eruption and completion of metamorphosis, although the effects on limb eruption and metamorphosis were only statistically significant at 3338 mg/L. Delay in metamorphosis and emergence from a lentic waterbody could increase the likelihood of death and/or reduced adult fitness with implications for long-term survival and breeding success. The apparent effects on growth in young (14 d) *L. pipiens* tadpoles may be reflected in the increased time required for larval organisms to present front limbs and complete metamorphosis (see Table 3.3.1). The increasing amount of time needed for larval frogs to complete metamorphosis has ecological implications, where survival to maturity might be significantly decreased if the time spent in an ephemeral pool is increased by only a few days. Larval anurans developing in shrinking ponds may die if all the water evaporates or is drained from the basin (in the case of irrigation supply reservoirs) before animals have a chance to emerge as adults. In addition, extended time in juvenile form might increase the likelihood of predation from fish, birds, reptiles or other aquatic predators since escape out of the water is not an option. Reduced adult fitness may also result from less time to feed in the terrestrial environment and build much-needed fat reserves before winter sets in (James et al. 2005). A reduced pool of breeding adults could lead to lower reproduction and emergence the following year.

Weight of internal organs increased with NTO exposure. When normalized to body mass, only kidney biomass from frogs in the 3338 mg/L treatment was statistically higher than the control.

The lowest NOEC calculated in the current study was 1346 mg NTO/L for the following parameters: Time to Front Limb Eruption, Minimum Time to Front Limb Eruption, Time to Metamorphosis, Minimum Time to Metamorphosis and Kidney Biomass.

No pathology was evident in hepatic tissues but pathology was identified in the renal tissues, with a higher number of granulomas present in animals from the 3338 mg/L NTO treatment group.

The effect of NTO on the *L. pipiens* reproductive system in unclear. Although some NTO-exposed animals demonstrated fewer, less robust spermatogonia, there are insufficient observations to draw definitive conclusions.

4.4 AQUATIC TOXICITY (DNAN) -

4.2.1 Acute Toxicity Testing

The 48-hour acute toxicity of DNAN ranged from 37 to 42 mg/L for the two species tested. These results place DNAN into the toxicity category of slightly toxic (Figure 4.4.1a). The cited toxicity categories were distributed by the U.S. Fish and Wildlife Service (USFWS 1984) to serve as general guidance to compare the toxicity of different various chemicals and are non-regulatory. In comparison, data acquired from Talmage et al. (1999) and the USEPA's Environmental Protection Agency's ECOTOX database (http://cfpub.epa.gov/ecotox/; queried May 2012) for other more traditional munitions fall into the more toxic categories of highly toxic and moderately toxic. Also, 2,4-dinitrophenol and royal demolition explosive (RDX) are classified as slightly toxic; the acute

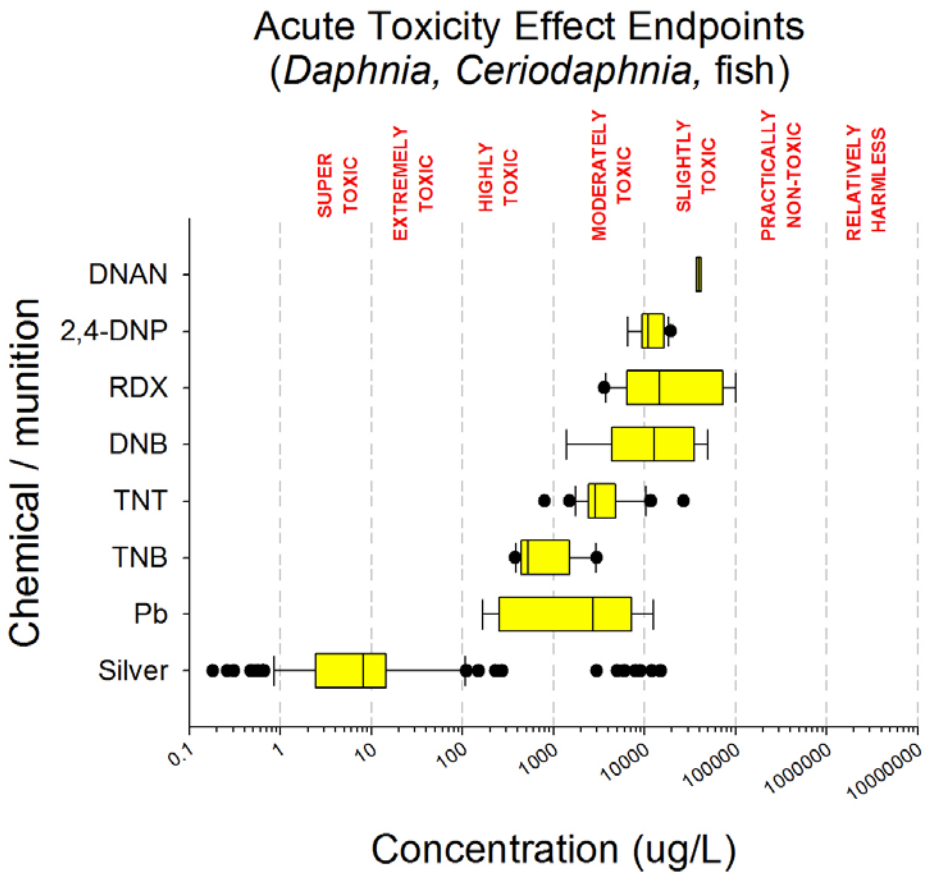
toxicity of DNAN was less than literature- reported toxicity ranges for 2,4-dinitrophenol, RDX, dinitrobenzene, trinitrotoluene (TNT), and lead (Figure 4.4.1a).

4.2.2 Chronic Toxicity Testing

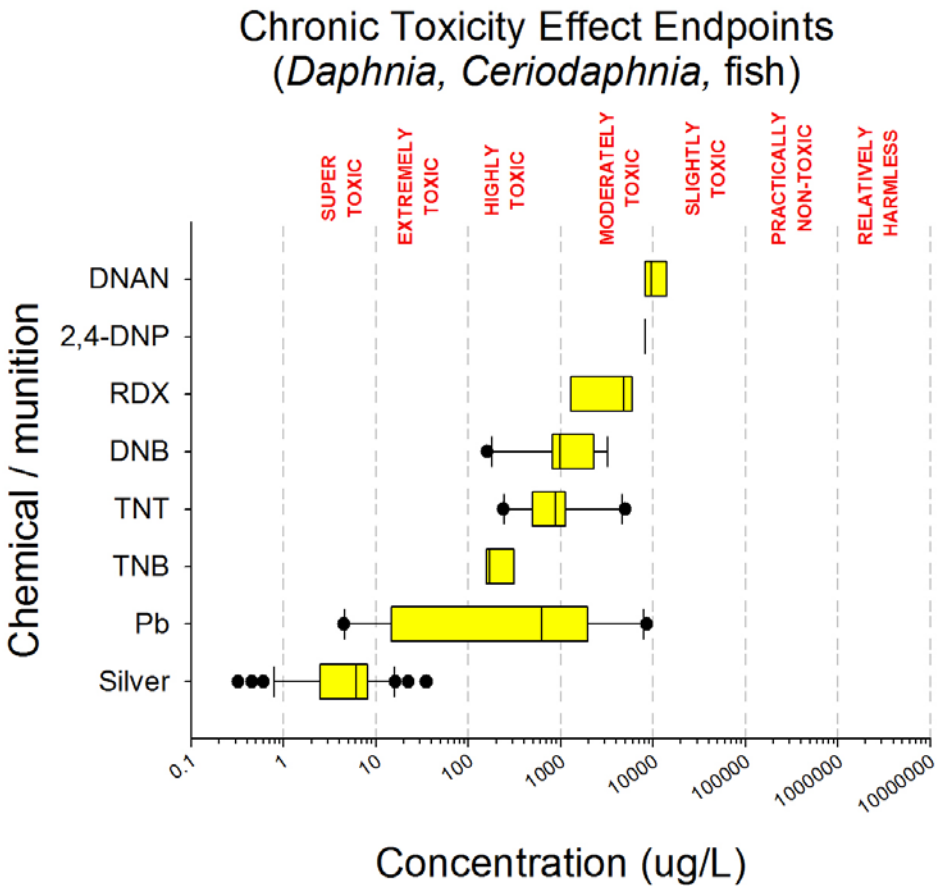
The chronic toxicity of DNAN ranged from 8.2 to 10.0 mg/L, using the most sensitive effect endpoint obtained for each of the two species tested. The median value of all chronic toxicity effect endpoints places DNAN into the toxicity category of moderately toxic (USFWS 1984; Figure 4.4.1b), although most of the DNAN data distribution is in the slightly toxic category. In comparison, data acquired from Talmage et al. (1999) and the USEPA's Environmental Protection Agency's ECOTOX database (http://cfpub.epa.gov/ecotox/; queried May 2012) for other more traditional munitions fall into the more toxic categories of super toxic and highly toxic, while 2,4-dinitrophenol and RDX are also classified as moderately toxic. The chronic toxicity of DNAN was less than the literature- reported toxicity ranges for 2,4-dinitrophenol, RDX, dinitrobenzene, trinitrotoluene (TNT) and lead (Figure 4.4.1b).

Figure 4.4.1 Comparison of toxicity reference values for 2,4-dinitoanisole (DNAN) relative to those of other munition constituents. Acute toxicity is provided in panel (a) while chronic toxicity is provided in panel (b). All summarized toxicity reference values are effect endpoints (e.g., LC50, IC50, LOEC, etc.) for fish, *Daphnia* and *Ceriodaphnia* species obtained from Talmage et al. (1999) and the USEPA ECOTOX database (http://cfpub.epa.gov/ecotox/). Box margins represent the 25th and 75th percentiles of the data distribution, error bars represent 10th and 90th percentiles of the data distribution (single points represent outlier data in the top and bottom 10% of the data distribution), and lines within the boxes represent the median toxicity reference value. General toxicity severity ranges (USFWS 1984) are indicated in a red font across the top of the figure. Note that the x-axis is plotted on a log10 scale. 2,4-DNP = 2,4 dinitrophenyl, RDX = royal demolition explosive, DNB = dinitrobenzene, TNT = trinitrotoluene, TNB = dinitrobenzene, Pub = lead.

(a)



(b)



5.0 ENVIRONMENTAL CRITERIA

There are no state or Federal regulations governing the allowable concentrations of the IMX-101 constituents in soil or water. There are multiple approaches available to derive environmental criteria; the values presented here were based on methods used by the Environmental Protection Agency (EPA). These criteria are intended to protect humans against the toxic effects of the IMX-101 components: 3-nitro-1, 2, 4-triazol-5-one (NTO), dinitroanisole (DNAN) and nitroguanidine (NQ) and to make risk assessment decisions (USAPHC 2015). A summary of the recommended criteria are presented in the enclosed table. None of these compounds are thought to be carcinogenic, and the recommendations are based on systemic toxicity endpoints. Values for ecological risk assessment purposes are on-going and will follow guidance found in TG-254 (USACHPPM 2000).

The Reference Dose (RfD) is a key value used in risk assessments and derivations of environmental criteria. An RfD is an EPA-published value and represents an estimate of a daily oral exposure to a chemical that is not likely to cause deleterious effects during a lifetime of exposure (USEPA 1993). RfDs are frequently based on animal data extrapolated to humans; they are estimates and are believed to be accurate to within one order of magnitude. RfDs are typically conservative since

they are intended to be protective of the general population including sensitive subgroups. For the IMX-101 components, the EPA has published an RfD for nitroquanidine (NQ) but not for NTO or DNAN. In the exposure recommendations presented here, an RfD surrogate was used and referred to as an Acceptable Daily Dose (ADD). The Workplace Environmental Exposure Level (WEEL) committee recently completed an extensive review of the toxicity information for NTO and DNAN and recommended exposure criteria protective for occupational exposures (OARS 2014a,b). In order to provide consistency between the occupational criteria and the current environmental criteria, key toxicity values from the WEEL documents were used as a basis for deriving the ADDs.

The WEEL committee used the testicular effects seen in a 90-day, sub-chronic study as the critical effect for estimating the occupational exposure level (OEL) (OARS 2000a). Using EPA Benchmark Dose (BMD) software (USEPA 2000), a dose response curve was extrapolated from the data. The lower 95% confidence interval on the 10% response ($BMDL_{10}$) was chosen as the critical toxic effect or point of departure (POD). The $BMDL_{10}$ of 40 mg/kg-day represents a composite of several models from the BMD suite for non-cancer effects. To derive the ADD, the WEEL $BMDL_{10}$ was modified by applying uncertainty factors (UFs) to adjust for extrapolation from animals to humans and for variations in response within the human population. The UFs chosen were 10 for interspecies and intraspecies extrapolation and 3 to estimate chronic effects from sub-chronic exposure data. Also included was a factor of 3 for uncertainties in the database. In risk assessments, the factor of 3 represents the square root of 10; therefore, for NTO the total composite UF was 1000.

Calculations:

NTO: From WEEL document- $BMDL_{10}$ = 40 mg/kg-d

UF = 1000

ADD = (40 mg/kg-d)/1000 = 0.04 mg/kg-d

DNAN. For DNAN the WEEL committee again used toxicity data from a 90-day, sub-chronic study to derive the OEL (ref b). The POD used was extra-medullary hematopoiesis, and the $BMDL_{10}$ was 0.93 mg/kg-day. The UFs chosen for the DNAN ADD included 10 for interspecies and intraspecies extrapolations and 3 for the sub-chronic to chronic data extrapolation. Information on the toxicity of DNAN is not as extensive as available data for NTO, so a factor of 10 was applied to address these database uncertainties. This yields a composite UF of 3000.

Calculations:

DNAN: From WEEL document – $BMDL_{10}$ = 0.93mg/kg-d

UF = 3000

ADD = (0.93 mg/kg-d)/3000 = 0.31 µg/kg-d

Water Criteria

Health Advisories. Recommended criteria for water were adapted from the EPA method for Health Advisories (HA). The HAs are non-regulatory guidance for contaminant levels in drinking water (USEPA 2012). The EPA published a lifetime HA for NQ and that value is included in the Table 4.4.2. EPA methodology was used to derive lifetime HAs for NTO and DNAN, but the exposure criteria were updated to be consistent with current guidance (USEPA 2013). Exposures were based on an 80-kg human consuming 2.5L of water per day (vs. 70-kg body weight and 2-L/day water consumption used in the NQ derivation). The lifetime HA also assumes an individual only receives 20% of their total exposure to the material via drinking water with the remainder coming through soil ingestion and inhalation; this relative source contribution (RSC) factor reduces the acceptable water concentration by 80%. The 20% RSC is a default value, and for consistency it was included in the HA values presented here. The general equation for calculating the lifetime HA is:

LHA = (RfD or ADD mg/kg-day) x (body weight) x RSC/daily water consumption 2.5 l/day

NTO Calculation:

LHA = (0.04 mg/kd-day) x (80 kg) x 0.2/2.5 l/day = 0.26 mg/l

DNAN Calculation:

LHA = (0.3 µg/kg-day) x (80 kg) x 0.2/ 2.5 l/day = = 0.002 mg/l

Soil Criteria

The soil values presented are Regional Screening Levels (RSLs). The RSLs are non-regulatory, risk-based criteria, which are derived using standard default exposure assumptions and appropriate toxicity values. The EPA has published a list of RSL values for chemicals with toxicity criteria, and NQ is included in the list (USEPA 2015a). For NTO and DNAN, we estimated RSLs using an EPA published RSL calculator (USEPA 2015b). A number of exposure scenarios are available in the online calculator. For this exercise, a relatively conservative residential scenario and a less conservative construction worker scenario were chosen in an attempt to show a range of estimates. The previously derived ADD toxicity criteria were used in the RSL calculations. Additional chemical-specific parameters required for these estimates included the dermal absorption (ABS), gastrointestinal absorption (GIABS), and the relative availability fraction (RAF). Limited dermal absorption data are available for these compounds. For other explosive compounds shown in the RSL tables, ABS values range from 0.006 for HMX to 0.102 for 2,4-DNT. For the purpose of this exercise, we assumed a dermal ABS of 0.1, a GIABS of 1, and an RAF of 1. These represent conservative exposure values resulting in conservative estimates for recommended soil concentrations. With more complete toxicity information and site-specific exposure data, these criteria can be refined to reduce uncertainty in the recommendations.

Table 4.4.2: Environmental Criteria - IMX-101 Components

Environmental Criteria - IMX-101 Components			
	NTO	**DNAN**	**NQ**
ADD/RfD mg/kg-day	4.0E-02	3.0E-04	1.00E-01
Lifetime HA mg/l	2.6E-01	2.0E-03	7.0E-01
RSL Residential Soil mg/kg soil	2.5E+03	2.0E+01	6.3E+03
RSL Industrial Soil mg/kg soil	3.3E+04	2.5E+2	8.2E+04

6.0 SUMMARY AND IMPLICATIONS/FUTURE RESEARCH

Together, these data fill critical environmental toxicology data gaps for components of IMX-101. These data suggest that DNAN is less toxic than TNT to aquatic and terrestrial receptors, but is the most toxic of the three components of IMX-101. Water solubility issues associated with NTO suggested that aquatic toxicity and effects to mammals, birds and amphibians were important to characterize. As the methods and models used are consistent with regulatory requirements and convention, these data can be used to help obtain wastewater discharge permits, develop remedial concentrations in environmental media, and accurately characterize risk in areas where there are environmental releases.

The environmental criteria developed herein were developed from the data obtained through this work and other work to-date. As it has not been review by regulatory agencies, it can be used in contract requirements at GOCO ammunition plants when and where unexpected environmental releases occur.

That neurological effects occurred in birds and not mammals from oral exposures to NTO is noteworthy. Further studies investigating the mechanism for this anomaly in birds is suggested. Furthermore, there is still a lack of understanding for the mechanism of NTO-induced hypospermia. The evidence suggests that NTO exerts direct effects to either the developing germ cells or inhibits Sertoli cell function. How precisely occurs is not known, but would help in extrapolating these effects to other species including humans.

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APPENDIX A
PUBLICATIONS

Journal of Toxicology and Environmental Health, Part A
Current Issues

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: http://www.tandfonline.com/loi/uteh20

An extended one-generation reproductive toxicity test of 1,2,4-Triazol-5-one (NTO) in rats

Emily May Lent, Lee C. B. Crouse, Allison M. Jackovitz, Erica E. Carroll & Mark S. Johnson

To cite this article: Emily May Lent, Lee C. B. Crouse, Allison M. Jackovitz, Erica E. Carroll & Mark S. Johnson (2016): An extended one-generation reproductive toxicity test of 1,2,4-Triazol-5-one (NTO) in rats, Journal of Toxicology and Environmental Health, Part A, DOI: 10.1080/15287394.2016.1219893

To link to this article: http://dx.doi.org/10.1080/15287394.2016.1219893

Published online: 18 Oct 2016.

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MCHB-PH-TEV

14 AUGUST 2017

MEMORANDUM FOR Strategic Environmental Research and Development Program (SERDP) 4800 Mark Center Drive, Alexandria, VA 22350-3605

SUBJECT: Toxicology Study No. S.0027395-15, Protocol No. 80-14-07-02, Extended One-Generation Reproductive Toxicity Test in Japanese Quail *(Coturnix japonica)* Exposed Orally to 3-Nitro-1,2,4-Triazol-5-One (NTO), February 2015-August 2015

1. An electronic copy of the subject report has been provided.

2. The U.S. Army Public Health Center point of contact is Ms. Allison Jackovitz, Toxicity Evaluation Division (TEV), at DSN 584-3980, commercial 410-436-3980, or by e-mail: usarmy.apg.medcom-dcs-ph.mbx.tox-info@mail.mil.

FOR THE DIRECTOR:

Encl

MARK S. JOHNSON
Director, Toxicology

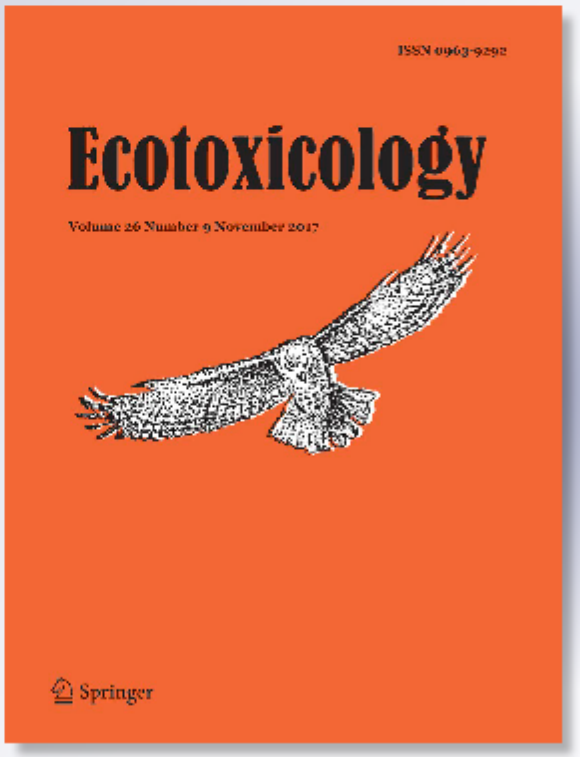
Effects of 3-Nitro-1,2,4-triazol-5-one on Survival, Growth and Metamorphosis in the Northern Leopard Frog, Lithobates pipiens

David A. Pillard, William S. Eck, Mark S. Johnson & Stephanie Packard

Ecotoxicology

ISSN 0963-9292
Volume 26
Number 9

Ecotoxicology (2017) 26:1170-1180
DOI 10.1007/s10646-017-1842-z



Springer

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 9999, No. 9999, pp. 1–10, 2014

Printed in the USA

Environmental Toxicology

INTER- AND INTRASPECIES CHEMICAL SENSITIVITY: A CASE STUDY USING 2,4-DINITROANISOLE

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(Submitted 2 July 2014; Returned for Revision 14 August 2014; Accepted 18 November 2014)

Abstract: Insensitive munitions offer increased safety because of their "insensitivity" to unintended detonation relative to historically used formulations such as 2,4,6-trinitrotoluene (TNT). Dinitroanisole (DNAN) is an insensitive munition constituent, and its solubility and stability warrant investigations of potential toxicological hazard related to manufacturing discharges and training ranges. Although ecotoxicology data are available for other insensitive munition constituents, few data are available for DNAN. In the present study, acute and chronic exposures of a fish (*Pimephales promelas*) and 2 cladocerans (*Ceriodaphnia dubia*, *Daphnia pulex*) were conducted. The 50% lethal concentration (LC50) values of DNAN ranged from 14.2 mg/L to 42.0 mg/L, depending on species. In chronic exposures, fish survival (LC50 = 10.0 mg/L) was more sensitive than cladoceran survival (LC50 = 13.7 to >24.2 mg/L). However, cladoceran reproduction was equally or more sensitive to DNAN (50% inhibition values 2.7–10.6 mg/L, depending on species) than fish endpoints. *Daphnia pulex* was the most sensitive species, with only slight differences between the 3 populations tested. Although the aquatic toxicity of DNAN was lower than previously reported in the literature for TNT, future research is needed to determine the potential synergistic toxicity of all the constituents in insensitive munition mixtures and the implications of photo-oxidation. *Environ Toxicol Chem* 2014;9999:1–10. © 2014 SETAC

Keywords: Dinitroanisole Dinitrophenol Toxicity Insensitive munition Species sensitivity

INTRODUCTION

The US military is replacing traditional munitions constituents, such as 2,4,6-trinitrotoluene (TNT), with insensitive munitions for some applications [1]. Relative to TNT, insensitive munitions have similar detonation velocity but are less sensitive to shock and inadvertent detonation [1–3]. A number of insensitive munition formulations have been developed (e.g., IMX-101, IMX-104, PAX-48, PAX-21, and PAX-41) for various applications such as artillery projectiles, and tank ammunitions [3]. The different insensitive munitions are composed of a variety of constituents, including 2,4-dinitroanisole (DNAN), 3-nitro-1,2,4-triazol-5-one (NTO), nitroguanidine (NQ), royal demolition explosive, and high–melting point explosive. These constituents are manufactured at ammunition facilities, some of which discharge treated wastewater [2]. The insensitive munition IMX-101 is a mixture of DNAN, NTO, and NQ [3–6].

Environmental regulations such as the Clean Water Act of 1972 (Section 402) [7] protect waterways by regulating the discharge of chemicals. Point source discharges to surface waters in the United States require authorization via a permit process, such as the National Pollution Discharge Elimination System. A state-established mixing zone is employed to determine if the effluent discharge is acceptably diluted by the receiving system, often considering worst-case (low-flow) conditions. Under such regulation, repeated monitoring, whole-effluent toxicity testing, and reporting often are required (40 CFR Part 122.44[d]) [8]. Determination of the total maximum daily load of the effluent and an effective management strategy for insensitive munitions and their constituents depend on accurate risk assessment and quality ecotoxicology data generated from testing multiple and appropriately sensitive aquatic species.

Although at least some aquatic ecotoxicology data are available for the other munitions constituents and insensitive munition constituents [9–15], there is a paucity of whole-animal, aquatic ecotoxicology data for DNAN in the peer-reviewed literature. Some human health [5,16,17], microbial [15,18], algal [2,15], and terrestrial [2,15] toxicity data exist for DNAN. Dinitroanisole is a nitroaromatic compound that is structurally similar to TNT, the TNT by-product 2,4-dinitrotoluene (DNT), and 2,4-dinitrophenol (DNP) [2,15,19]. The solubility [15,20] and degradation [4,21] of DNAN have been studied previously. Solubility ranges from 191 mg/L to 276 mg/L (25°C) in ultrapure water [15,20]. Provided its current production, use, and stability [15,22] and its potential to be released during munitions manufacturing, testing, training, and use [20], it is logical to determine the potential for DNAN to cause toxicity to surface water organisms. Insensitive munition wastewaters may also have a yellow coloration that impacts the aesthetics of the surface water that receives the effluent [4].

The objectives of the present study were to generate acute and chronic aquatic ecotoxicity data for DNAN using 1 fish and 2 cladoceran species; to determine inter- and intraspecific sensitivity using both multiple test species and populations of the same test species to contribute to a data set needed for deriving water-quality criteria; and to compare the resulting

All Supplemental Data may be found in the online version of this article.

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Published online 5 December 2014 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/etc.2819