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AQUATIC TOXICITY OF 3-NITRO-1,2,4-TRIAZOL-5-ONE

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14. ABSTRACT:
3-Nitro-1,2,4-triazol-5-one (NTO) is an explosive developed at the Los Alamos National Laboratory in 1984. The thermal decomposition characteristics of NTO, including calculated detonation velocity and pressure, are similar to those of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX); however, NTO is less sensitive and more stable. Little or no open literature is available about the environmental toxicity of NTO and understanding of potential ecological impacts of its accidental release into aquatic ecosystems is necessary prior to its large-scale production. We conducted aquatic toxicity testing of NTO using the freshwater organism *Ceriodaphnia dubia* in a 7 day survival and reproduction assay and the unicellular green algae *Selenastrum capricornutum* in a 96 hr growth inhibition assay. The studies were conducted under pH-adjusted conditions due to a concentration-dependent increase in acidification of aqueous media by the addition of NTO. The resulting inhibitory concentration (IC) IC_{20} and IC_{50} values for reproduction of *C. dubia* were 51 and 57 mg/L, respectively. The estimated IC_{20} and IC_{50} values for *S. capricornutum* growth inhibition were 2195 and 3465 mg/L. Applying these results to the Chemical Scoring System for Hazard and Exposure Identification, NTO was ranked as practically nontoxic.

15. SUBJECT TERMS

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Selenastrium capricornutum	Chronic toxicity	Explosive

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PREFACE

The work described in this report was authorized under Project No. 8RLAX1, U.S. Army Center for Health Promotion and Preventive Medicine, Directorate of Toxicology. The work was started in November 2008 and completed in March 2009.

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AQUATIC TOXICITY OF 3-NITRO-1,2,4-TRIAZOL-5-ONE

1. INTRODUCTION

The U. S. Army Center for Health Promotion and Preventive Medicine (USACHPPM), Directorate of Toxicology is investigating energetic material 3-nitro-1,2,4-triazol-5-one (NTO) for its potential ecological impact in case of accidental release into the environment. 3-nitro-1,2,4-triazol-5-one is a relatively new explosive developed at the Los Alamos National Laboratory in 1984.[1] The explosive power of NTO is similar to hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX); however, NTO is less sensitive and more stable.[2] 3-Nitro-1,2,4-triazol-5-one ($C_2H_2N_4O_3$) has a molecular weight of 130 g/mole and aqueous solubility of 12.8 g/L at 19 °C[3] and 49 g/L at an undefined temperature.[2] Figure 1 shows the chemical structure of NTO.

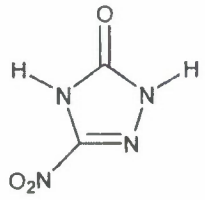

Figure 1. Chemical structure of NTO

The Environmental Toxicology Branch, U.S. Army Edgewood Chemical Biological Center (ECBC), was tasked to conduct aquatic toxicity screening of NTO using the freshwater organism *Ceriodaphnia dubia* in a 7 day survival and reproduction assay and the unicellular green algae *Selenastrum capricornutum* in a 96 h growth inhibition assay. The stability of NTO dissolved in Ceriodaphnia media was also investigated for 7 days. Due to the acidic nature of NTO, the toxicological studies presented in this report were conducted under pH-adjusted conditions (unless otherwise stated).

2. METHODS

2.1 Analytical Procedures

2.1.1 Preparation of Standard Solutions

Crystalline NTO (99.6% pure, Lot No. BAE07B305-001) was obtained from BAE Systems through USACHPPM. A primary stock solution of NTO was prepared by dissolving crystalline NTO in acetonitrile with 0.1% trifluoroacetic acid. This stock solution was then serially diluted using acetonitrile with 0.1% trifluoroacetic acid to create NTO concentrations in analytical standards of 0, 19.0, 38.1, 76.1, 152.2, 304.4, and 608.8 mg/L (r^2 values for NTO standards ranged from 0.9998 to 0.9999). The standards were stored in darkness at 4 °C.

2.1.2 Ultrapure Distilled Water

The ultrapure distilled water (distilled water) used in this study was generated from municipal water at ECBC. Municipal water was passed through a carbon filter, a water softener, a Hero 205 reverse osmosis (R.O.) system, stored in a pressurized holding tank, and passed through a Barnstead Nanopure System containing pretreatment, high capacity deionization, ultrapure mixed bed deionization, and organic removal cartridges. This water was then distilled using a Corning MP-6A glass still and stored in a glass holding tank until needed.

2.1.3 Analytical Methods

The analytical determinations of NTO in water were conducted using High Performance Liquid Chromatography (HPLC; 1100 Series, Agilent Corp., Wilmington, DE) with UV Diode Array Detection (DAD). Separation was achieved using a 150 x 4.6 mm Hypercarb column (Thermo Scientific, Waltham, MA). The isocratic mobile phase consisted of 25:75 ratio of acetonitrile containing 0.1 % Trifluoroacetic acid and distilled water. Acetonitrile was filtered using 0.2 μm, 47 mm nylon filter disks (Fisher Scientific, Pittsburgh, PA), while the ultrapure distilled water was filtered through 0.45 μm, 47 mm cellulose acetate filter disks before use. The mobile phase flow rate was set at 1.5 mL/min and the injection volume was 50 μL. Detection and subsequent concentration determination of NTO was performed at 315 nm wavelength. The Limits of Detection (LOD) under the experimental conditions were 0.002 mg/L (peak to peak).

2.2 NTO Stability Testing

3-Nitro-1,2,4-triazol-5-one stock solution was prepared by placing 1000 mg NTO into a 1000 mL volumetric flask and then adding 500 mL of Ceriodaphnia media (Section 2.3). The flask was then sealed and immersed in a sonic water bath for 1 min, removed, and the contents swirled to resuspend the NTO particles, and then placed back into the sonic water bath for an additional minute. This was repeated up to 10 times until the NTO was dissolved. The flask was then filled using Ceriodaphnia media and the pH of the NTO stock was adjusted to 7.5 using 0.1 M NaOH. The NTO stock was serially diluted using Ceriodaphnia media to yield nominal concentrations of 0 (control; stock solution without NTO), 31.2, 62.5, 125, 250, and 500 mg/L. Triplicate samples of each concentration were placed into amber crimper vials, capped, then stored under Ceriodaphnia 7 day Survival and Reproduction test conditions (Section 2.3), and sampled for HPLC analyses every 24 h for 7 days. Stock solutions of NTO used in toxicity testing were prepared as described above.

2.3 Ceriodaphnia 7 Day Survival and Reproduction Assay

Ceriodaphnia 7 day Survival and Reproduction Assays were conducted with *C. dubia* in accordance with the U.S. Environmental Protection Agency (USEPA) standard methods.[4] The media for Ceriodaphnia cultures consisted of 20% Well water (see Appendix A for Well water analysis) and 80% R.O. water. Ceriodaphnia were fed a mixture of green algae *S. capricornutum* (6×10^5 cells/mL) and cerophyl extract (20 μL/mL). Cerophyl extract was prepared by blending 3.75 g of Cerophyl (Cereal Grass Media, Scholar Chemistry) with 500 mL of distilled water for 5 min at high speed. The cerophyl mixture was placed in a 1000 mL

Erlenmeyer flask, capped, and stored in a refrigerator overnight (19 h). Next the extract was decanted slowly into a clean beaker to prevent the transfer of detritus sediment, and stored frozen in 40 mL aliquots until thawed for use. Test chambers consisted of 30 mL plastic beakers containing 15 mL of solution. Ten replicates of each treatment group and control were prepared, with each replicate containing one Ceriodaphnia. The test media were renewed and fresh food added daily, for 7 days. Mortality, reproduction, pH, and dissolved oxygen measurements were recorded at 24 h intervals. A diurnal photoperiod cycle was maintained at the ratio of 16 h light/ 8 h dark. The ambient light intensity was ~90 ft candles. The temperature was maintained at 25 °C. An NTO stock solution of 1000 mg/L was prepared using pH adjusted and serially diluted Ceriodaphnia media to obtain nominal treatment concentrations of 0 (control), 31.2, 62.5, 125, 250, and 500 mg/L. The respective daily measured NTO concentrations are listed in Table 1.

2.4 Algal Growth Inhibition Assay

Algal Growth Inhibition Assays were conducted in accordance with the USEPA standard methods.[5] The algal media used in these assays was a modification of the Bristol's media[6] (see Appendix B). The algal media was prepared and adjusted to pH 7.1 before use. An NTO stock solution of 2800 mg/L was prepared using algal media, pH adjusted, then serially diluted in order to obtain the nominal treatment concentrations of 0 (control), 175, 350, 700, 1400, and 2800 mg/L. Stock cultures of the unicellular green algae *S. capricornutum* were grown in 3 L batches and taken during log phase growth for inoculation of test flasks. The initial algal concentration was 1 x 10^4 cell/mL per test chamber. The total volume for each treatment and control replicate was 100 mL. The test design consisted of four replicates of each treatment and control group, using 250 mL Erlenmeyer flasks as test chambers. The treatment groups and controls were subjected to test conditions for 96 h (static non-renewal). Following inoculation with algae, individual test chambers were randomly placed in an incubator at 25 °C, and exposed to an average of 235 ft candles (n = 9, SD = 31 ft candles) of continuous light. The test chambers were shaken by hand twice-daily. Cell density determinations were accomplished via the manual microscope counting method (hemocytometer) at 0, 24, 48, 72, and 96 h. The NTO concentrations were analytically determined at t = 0 and again at the end of the 96 h exposure.

2.5 Determination of Toxicity Parameter Values and Statistics

Toxicity data were analyzed using statistical regression models described in the Environment Canada Guidance Document.[7] The nonlinear logistic (Gompertz) model shown in the equation given below had the best fit for the data. The best fits for the curves generated by the model were closest to the data points, the variances of the residuals were the smallest, and the residuals had the best appearance (i.e., most random scattering). This model was

$$Y = a \times \exp[\log(1 - p)] \times (C \div ICp)^b] \qquad \text{(eq}$$

where

Y	= dependent variable (e.g., quantity of offspring)
a	= the y-intercept (i.e., the control response)
exp	= the exponent of the base of the natural logarithm
p	= desired value for 'p' percent inhibition or mortality (e.g., 0.50 for IC_{50})
C	= exposure concentration in test media
ICp/LCp	= estimate of inhibitory/lethal concentration for a specified percent effect
b	= a scale parameter that defines the shape of the equation

The ICp (inhibitory concentration) parameters and 95% confidence intervals (CI) associated with the point estimates included the NTO concentration producing a 20% (IC_{20}) or 50% (IC_{50}) reduction in *S. capricornutum* cell growth. The LCp (lethal concentration) used in this study included the NTO concentration producing a 20% (IC_{20}) or 50% (IC_{50}) reduction in the production of offspring by *C. dubia* and 50% (LC_{50}) reduction in the survival of adult *C. dubia*.

Analysis of Variance (ANOVA) was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values for Ceriodaphnia reproduction and algal cell growth data. Mean separations were determined using Fisher's Least Significant Difference pair-wise comparison tests. A significance level of $p \leq 0.05$ was accepted for determining the NOEC and LOEC values. Statistical analyses were performed using SYSTAT 11.0 (Systat Software Inc., Chicago IL, USA).[8]

3. RESULTS

When NTO was dissolved into aqueous media, the media pH shifted from 7.5 to as low as 2.7 (in algal media stock solutions of 3000 mg/L NTO). Therefore, all the stock solutions were pH-adjusted using 0.1 M NaOH. Figure 2 shows the shift in pH of Ceriodaphnia media after the addition of NTO up to 1000 mg/L. The initial Ceriodaphnia media had pH 7.5. The pH of aqueous media decreased to 3.1 at an NTO concentration of 1000 mg/L.

The stability of NTO in Ceriodaphnia media was determined using an NTO stock solution of 500 mg/L. The pH of the media was adjusted to 7.5 using 0.1 M NaOH. This pH-adjusted media was then serially diluted with Ceriodaphnia media to prepare nominal NTO concentrations of 31.2, 62.5, 125, 250, and 500 mg/L. The concentration of NTO in each of the three treatment replicates was monitored for 7 days. Table 2 shows the results of the 7 day stability test. Overall, there was ~10% loss of NTO in each treatment group during the 7 day experiment.

Acute 24 and 48 h Ceriodaphnia range-finding studies were conducted using the NTO stock solutions that were either pH-adjusted or non-adjusted to discern the toxicity that could be attributed to the acidity of NTO, and to select between the pH treatment alternatives for exposure media in the definitive test. These range-finding studies were also used to bracket the

concentration range for the definitive test. Ceriodaphnia were exposed to pH-adjusted and non-pH-adjusted concentrations of NTO for up to 48 h. The analytically determined concentrations of NTO were 0 (control), 62, 124, 246, 479, and 881 mg/L for the pH-adjusted treatment groups, and 0 (control), 33, 64, 127, 248, 489, and 904 mg/L for the non-pH-adjusted treatment groups. The results showed that adjusting the pH of NTO treatment groups decreased toxicity by 92% in the 24 h exposure test, and by 87% in the 48 h exposure test (Table 3). The respective LC_{50} values determined in the 24 and 48 h acute Ceriodaphnia studies differed between the two pH treatments of the exposure media by approximately an order of magnitude. The respective LC_{50} values for the 24 and 48 h acute Ceriodaphnia studies were 830 and 460 mg/L in pH-adjusted media, and 66 and 62 mg/L in non-pH-adjusted media (Table 3).

Based on the results of range-finding studies, the definitive chronic 7 day Ceriodaphnia study was conducted using pH-adjusted NTO treatments only. During the chronic Ceriodaphnia study, the media and food were renewed and the NTO concentrations were analytically determined daily. The range of NTO concentrations selected for the definitive chronic study was sufficient to establish toxicity benchmarks on the basis of concentration-response relationship (Figure 3). The resulting 7 day average NTO concentrations in treatment groups were 0 (control), 34, 66, 133, 262, and 523 mg/L [(the highest NTO treatment concentration was analytically determined and the average concentration calculated through 4 days only for the 500 mg/L nominal treatment because the *C. dubia* mortality was 100% after 4 days; therefore, this treatment group was terminated) (Tables 3 and 4)]. The 7 day IC_{20} and IC_{50} values for NTO were 51 and 57 mg/L, respectively. The NOEC and LOEC values for NTO were 34 and 66 mg/L, respectively (Table 3). The cumulative totals of Ceriodaphnia offspring produced in treatment groups are reported in Table 4. There was no mortality at NTO concentrations below 523 mg/L; however, no eggs were produced at the NTO concentration of 262 mg/L. At the lower NTO concentration of 133 mg/L, eggs were produced but they did not develop.

The definitive 96 h algal growth inhibition study was conducted using pH-adjusted NTO treatment groups. The NTO concentrations were analytically determined at the start of testing and after the 96 h exposure concluded. At the commencement of testing, the respective analytically determined NTO concentrations and corresponding standard deviations (±SD) [t = 0] were 0 (control), 175.6 (1.0), 347.0 (0.7), 692.2 (7.7), 1375.2 (11.7), and 2679.7 (3.8) mg/L. The NTO concentrations remained relatively stable in all treatment groups over the 96 h period (Table 5). The range of NTO concentrations selected for the definitive study was sufficient to establish the IC_{20} benchmark on the basis of a concentration-response relationship (Figure 4). The 96 h IC_{20} value for the inhibition of cell growth by NTO was 2195 mg/L. The estimated IC_{50} value 3465 mg/L was outside the tested range of NTO treatment concentrations (spanning 175.6 to 2679.7 mg/L); however the lower limit value of the 95% CI for the IC50 value was 2304.8 mg/L, and well-within the bracketing range of NTO treatment concentrations. Because the lower limit value for the IC50 fell within the range of the NTO treatments, this result imparts reasonable confidence in the IC_{50} estimate determined in this study (Table 6).

4. DISCUSSION

Solubilizing NTO in the Ceriodaphnia media considerably decreased the pH of the media. The pH of the media decreased in an NTO concentration-dependent manner (Figure 2) and ranged from pH 6.8 at the NTO concentration 7.8 mg/L to pH 3.1 at the NTO concentration 998 mg/L. This finding is consequential because it suggests the release of NTO into an aqueous environment can affect its pH, and that the extent of pH change will depend on the buffering capacity of the specific aquatic ecosystem.

These studies were conducted using pH-adjusted NTO treatment groups (except for Ceriodaphnia acute range-finding tests). This pH adjustment was done to approximate the effects of NTO that are representative of downstream exposure conditions, as well as for reasonably well-buffered aquatic systems. The results of our studies showed that if NTO is released into the environment, the pH can be the primary toxicity factor for directly exposed organisms in the immediate vicinity of the release. This effect would be especially dominant in aquatic systems having little or no pH buffering capacity. As NTO is transported away from the direct exposure area and becomes diluted, pH will no longer remain the primary toxicity factor.

We ranked the toxicity benchmarks determined in our studies using the Chemical Scoring System for Hazard and Exposure Identification.[9] This system is typically used in the preliminary screening process and is not intended to be a substitute for risk assessment. This system assigns a score from 0 to 9 (9 being the most toxic) based on the acute and chronic toxicity data. The scoring system developed by O'Bryan and Ross[9] does not rank the scores using common terms typically used in mammalian toxicity rankings; however, the U.S. Fish and Wildlife Service (USFWS) published a Research Information Bulletin, Acute Toxicity Rating Scales[10] suggesting relative aquatic toxicity terms (ranks) and we utilized this rating system on the basis of our EC_{50} data. The ranking system considers the EC_{50} values greater than 1000 mg/L to be "Relatively Harmless," and values less than 0.01 mg/L as "Super Toxic." Similar descriptive rankings for mammalian toxicity have been used by Kamrin.[11]

The scoring protocols by O'Bryan and Ross[9] stipulate that when multiple scores are assigned in the acute and chronic category, the highest score (the most toxic) should be selected as the overall aquatic toxicity score. Using the Chemical Scoring System for Hazard and Exposure Identification, the highest score received was 3 (Table 7), which ranks NTO as practically nontoxic to aquatic organisms in situations where acidification effects of NTO are not a primary factor. For comparison, toxicity data for the new energetic material 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (CL-20) were included in Tables 2, 6, and 7. The toxicity data for Ceriodaphnia and algae show that CL-20 was ~53 and 77 times more toxic than NTO, respectively, and CL-20 received a score of 7 which ranked it as moderately toxic.[12] Acetone and the organophosphorus pesticide malathion were also ranked using data from Ceriodaphnia assays. Acetone scored 0, which was ranked as Relatively Harmless,[13] and malathion scored 9, which ranked it as Super Toxic.[14,15]

A Programmatic Environmental Safety and Occupational Health Evaluation (PESHE) is required by the U.S. Army (AR 70-1, Army Acquisition Policy) before new energetic compounds can become fielded.[16] The PESHE is an acquisition policy requirement

(Department of Defense Instruetion, DoDI 5000.02) as part of the systems engineering process for all acquisition category programs.[17] Within a PESHE, ecotoxicity evaluations are required to assess potential environmental toxicity. In this report, we have provided aquatic toxicity data and evaluation of acute and sub-chronic effects that NTO has on ceriodaphnia survival and reproduction, and algal cell growth. These data and the associated evaluation of potential aquatic toxicity of NTO should be incorporated into the PESHE. Additionally, these initial test results, utilized for screening and identifying potential toxicity issues, justify proceeding with more refined ecotoxicity testing that is required to successfully complete a PESHE. To increase thc reliability of environmental toxicity assessment, the American Standards for Testing Materials (ASTM) recommends that additional ecotoxicity testing be completed in higher levels of ecotoxicity assessment, including an aquatic 7 day Fathead Minnow Growth Study, plus terrestrial ecotoxicity studies that include soil invertebrate toxicity bioassays [e.g., ISO 11268-2:*1998 Soil Quality – Effects of Pollutants on Earthworms (Eisenia fetida) – Part 2: Determination of Effects on Reproduction*; ISO 11267:*1998(E) Soil Quality – Inhibition of Reproduction of Collembola (Folsomia candida) by Soil Pollutants*; ISO DIS 16387:*2003 Soil Quality: Effects of Pollutants on Enchytraeidae (Enchytraeus sp) – Determination of Effects on Reproduction and Survival*], and plant bioaeeumulation and phytotoxieity studies (e.g., ASTM Standard E1963-02, 2002, *Standard Guide for Conducting Terrestrial Plant Toxicity Tests,* ASTM Intcrnational: West Conshohocken, PA, 2002, **DOI:** 10.1520/E1963-02, www.astm.org; USEPA. *Ecological Effects Test Guidelines, Early Seedling Growth Toxicity Test*; EPA 712–C–96–347, OPPTS 850.4230; U.S. Environmental Protection Ageney, U.S. Government Printing Offiee: Washington, DC, 1996).

5. CONCLUSIONS

- Solubilizing 3-Nitro-1,2,4-triazol-5-one (NTO) led to a eoneentration-dependent increase in acidification of aqueous media.

- NTO was relatively stablc in Ceriodaphnia media adjusted to pH 7.5 and maintained at 25 °C. Analytieal determinations showed that after the 7 day experiment, ~90% of thc initial NTO concentrations remained in the NTO media treatments, which ranged from 34.9 (31.2 nominal) to 523.7 (500 nominal) mg/L.

- Increasing the pH of Ceriodaphnia media containing NTO (to pH 7.5; the pH of Ceriodaphnia media without NTO) decreased the toxicity in acute Ceriodaphnia tests. Thc LC_{50} values differed by approximately an order of magnitude between the two pH treatments of the exposure media containing NTO (pH unadjusted, or pH adjusted to accommodate the respeetive NTO content).

- Toxieity results, generated from pH adjusted, aquatic exposure media that eontained NTO, were ranked using thc Chemical Scoring System for Hazard and Exposure Identification, and scored using the U.S. Fish and Wildlife Service Acute Toxicity Rating Scales. NTO rceeived a score of 3, ranking it as Practically Nontoxic to aquatic organisms. Sueh rankings are useful for comparisons of relative toxicities, but are not a substitute for risk assessment of specific site conditions.

- Screening results from these initial aquatic ecotoxicity tests justify proceeding with more refined ecotoxicity testing, as is required to successfully complete a Programmatic Environmental Safety and Occupational Health Evaluation (PESHE) that is mandated by the U.S. Army before new energetic compounds can become fielded

- Initial aquatic toxicity test results justify proceeding with more refined ecotoxicity testing that is required in a PESHE, including an aquatic 7-day Fathead Minnow Growth Study, terrestrial ecotoxicity studies that incorporate appropriate soil invertebrate toxicity bioassays, and plant bioaccumulation and phytotoxicity studies.

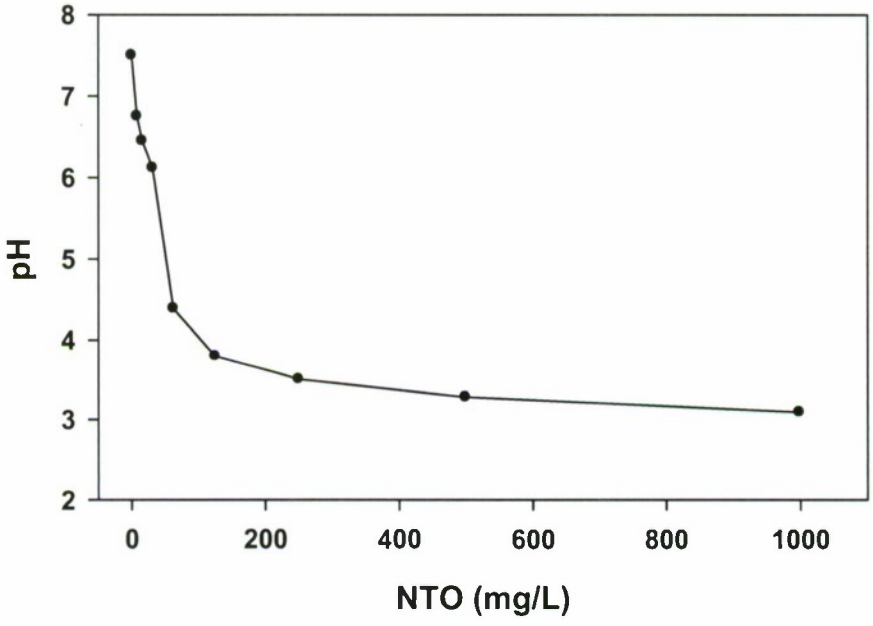

Figure 2. pH of Ceriodaphnia media after addition of NTO. The initial Ceriodaphnia media pH = 7.5.

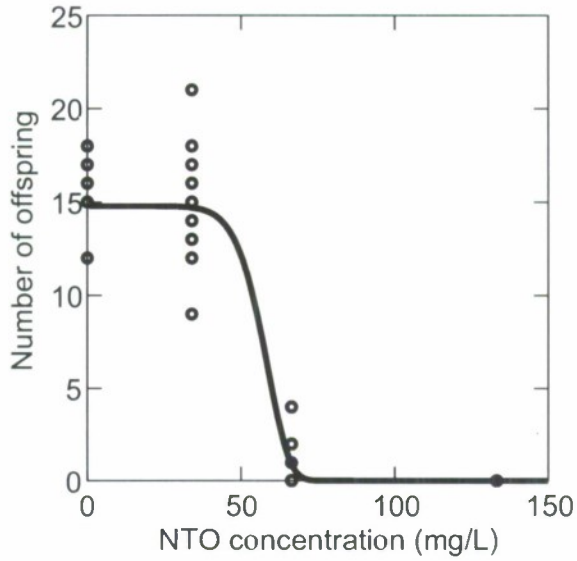

Figure 3. Effects of NTO on offspring production by *C. dubia* established in definitive 7 day study using pH-adjusted media.

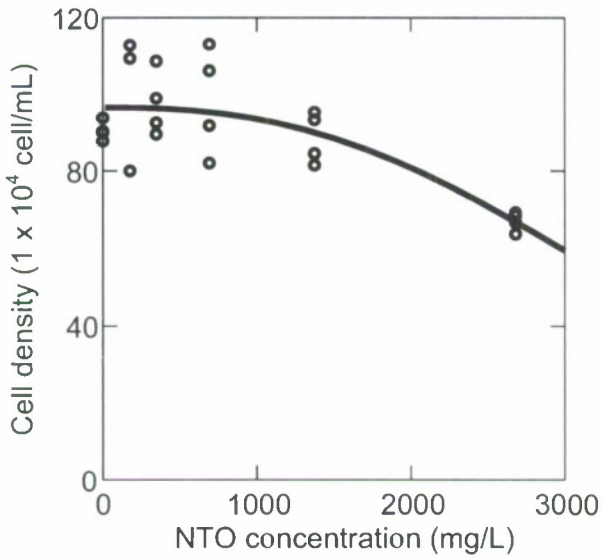

Figure 4. Algal Growth Inhibition by NTO determined in definitive 96 h study with *S. capricornutum* exposed in pH-adjusted media.

Table 1. Analytically determined daily exposure concentrations of NTO in Ceriodaphnia study.

Day	Nominal NTO Treatments, mg/L				
	31.2	62.5	125	250	500
	Analytically determined daily exposure concentrations of NTO, mg/L (SD)				
1	35.0 (0.1)	68.7 (0.1)	136.8 (0.1)	268.8 (0.1)	523.6 (0)
2	31.6 (0)	63.0 (0)	124.3 (0)	247.1 (0)	483.2 (0.6)
3	35.8 (0.1)	69.4 (0.2)	138.9 (0.3)	279.7 (0.1)	541.2 (0.3)
4	35.7 (0.1)	69.2 (0)	141.4 (0.1)	276.2 (0.1)	541.9 (0.1)
5	36.2 (0.1)	70.4 (0.1)	138.6 (0.2)	273.5 (0.2)	ND
6	32.7 (0.1)	63.9 (0)	128.6 (0.1)	249.6 (0.1)	ND
7	31.4 (0.1)	60.9 (0)	123.9 (0.2)	241.5 (0.1)	ND
7 Day Avg.	34.0 (2.0)	66.5 (3.6)	133.2 (7.1)	262.3 (15.1)	522.5 (25.5)*

SD – [(Standard Deviation) presented in parentheses] for analytically determined daily exposure concentrations, n = 2, for the 7 day average exposure concentration, n = 14.
ND - (Not Determined) Concentrations of NTO were not determined after day 4 of the study due to 100% mortality in the 500 mg/L treatment group.
*4 day average value.
The Ceriodaphnia media was changed daily and analyzed after the additions of food (algae and cerophyl extract).

Table 2. Analytically determined daily concentrations of NTO in five nominal treatments used to assess stability of NTO in Ceriodaphnia media.

Day	Nominal NTO Treatments, mg/L				
	31.2	62.5	125	250	500
	Analytically determined daily concentrations of NTO, mg/L (SD)				
1	34.9 (0.1)	68.6 (0.1)	136.7 (0.1)	268.6 (0.2)	523.7 (0.1)
2	32.3 (0.1)	63.4 (0.0)	126.1 (0.2)	248.0 (0.2)	481.8 (0.4)
3	32.2 (0.1)	63.3 (0.1)	125.6 (0.3)	247.3 (0.7)	482.9 (0.7)
4	32.2 (0.1)	63.2 (0.1)	125.8 (0.3)	247.4 (0.4)	483.1 (0.6)
5	32.1 (0.1)	63.2 (0.2)	125.7 (0.2)	247.3 (0.7)	482.1 (1.1)
6	32.1 (0.1)	62.8 (0.1)	124.9 (0.3)	245.8 (1.2)	479.2 (0.9)
7	31.5 (0.1)	61.7 (0.2)	122.5 (0.4)	241.9 (1.3)	470.0 (0.7)
% Loss	9.7	10.1	10.4	9.9	10.2

Data are presented as means [Standard Deviation (SD) presented in parentheses]; n = 3. The pH of NTO stock solution was adjusted to 7.5 before being serially diluted with Ceriodaphnia media and then stored at 25 °C.
Over 7 days, there was ~10% loss in NTO concentration.

Table 3. Summary of toxicity benchmarks for NTO and CL-20 determined in studies with *C. dubia*.

Toxicological benchmark	NTO		CL-20*
	pH-adjusted media	pH non-adjusted media	
(mg/L)	(pH = 7.5)	(pH 3.1-7.5)	
Acute 24 h LC_{50}	830	66	
(95% CI)	(807-854)	(CNBD)	NC
r^2	1.0	1.0	
Acute 48 h LC_{50}	460	62	
(95% CI)	(CNBD)	(CNBD)	NC
r^2	0.999	0.989	
7 Day IC_{20}	51		1.2
(95% CI)	(0-107)	NC	(0.9-1.5)
r^2	0.961		
7 Day IC_{50}	57		1.9
(95% CI)	(22-93)	NC	(1.6-2.2)
r^2	0.961		
NOEC	34	NC	0.4
p	0.939		0.714
LOEC	66	NC	0.7
p	<0.0001		0.025

Acute toxicity data were generated in the range-finding studies with NTO using either pH-adjusted or non-pH-adjusted media. Chronic toxicity data for NTO were established in the 7 day study using pH-adjusted media. Chronic toxicity data for explosive CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) were included in the table for comparison.

*CL-20 studies and data analyses were conducted by the Environmental Toxicology Branch, ECBC;[13] the results are provided here for comparison with NTO toxicity.

CNBD - (Could Not Be Determined) range-finding test with reduced replication (n = 2) conducted to bracket concentration range for the definitive test and to select between the pH treatment alternatives for exposure media in the definitive test.

NC – Experiment not conducted.

CI – [Confidence Intervals (95%)] presented in parentheses.

Table 4. Cumulative total of Ceriodaphnia offspring in control and NTO treatment groups. The treatment concentrations listed in this table are the 7 day averages of the analytically determined values.

Replicate	0 (control) mg/L[c]	34 mg/L	66 mg/L	133 mg/L[a]	262 mg/L[b]	523
	Cumulative Total of Ceriodaphnia Offspring					
1	18	21	0	0	0	0
2	16	18	0	0	0	0
3	15	12	2	0	0	0
4	17	16	0	0	0	0
5	7[d]	12	0	0	0	0
6	12	13	4	0	0	0
7	12	15	1	0	0	0
8	12	9	0	0	0	0
9	15	17	2	0	0	0
10	16	14	0	0	0	0
Total Offspring	140	147	9	0	0	0

[a]Eggs were produced but did not develop.
[b]Eggs were not produced.
[c]100% mortality after 4 d.
[d]Outlier value (Studentized Residual = -3.382); excluded from the toxicity benchmark determinations.

Table 5. The initial (0 h) and final (96 h) analytically determined NTO concentrations in nominal treatments of algal media used in the algal growth inhibition studies with *S. capricornutum*.

Exposure duration (h)	Nominal NTO Treatments, mg/L: 175	350	700	1400	2800
	Analytically determined concentrations of NTO, mg/L (SD)				
0	175.6 (1.0)	347.0 (0.7)	692.2 (7.7)	1375.2 (11.7)	2679.7 (3.8)
96	174.8 (1.3)	345.4 (0.5)	690.3 (5.3)	1374.2 (11)	2680.9 (12.3)
Average	175.2 (1.2)	346.2 (1.0)	691.2 (6.5)	1374.7 (11)	2680.3 (8.8)

The media was adjusted to pH 7.1 and was maintained at 25 °C under constant light conditions.
The loss of NTO in algal media was less then 1% after 96 h.
SD – [(Standard Deviation) presented in parentheses] for 0 and 96 h n = 8; for the average, n = 16.

Table 6. Summary of toxicity benchmarks for NTO and CL-20 determined in separate 96 h algal growth inhibition studies with *S. capricornutum*.

Toxicological benchmark	NTO mg/L	Cl-20[a] mg/L
96 hr IC_{20}	2195	31
(95% CI)	(1569-2820)	(18-45)
r^2	0.990	
96 hr IC_{50}	3465[b]	116
(95% CI)	(2305-4625)	(88-143)
r^2	0.990	
NOEC	1375	10.5
p	0.865	0.815
LOEC	2680	21.5
p	0.039	<0.0001

The media was adjusted to pH 7.1 before exposing the algae.

[a]CL-20 studies and data analyses were conducted by the Environmental Toxicology Branch, ECBC;[13] toxicity data for explosive CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) were included in the table for comparison.

[b]Estimated IC_{50} value is outside the tested range of NTO concentrations (from 175 to 2680 mg/L); the lower 95% CI is within the tested range of NTO concentrations.

CI – [Confidence Intervals (95%)] presented in parentheses.

Table 7. Toxicity rankings for NTO, CL-20, acetone, and malathion.

Toxicity Benchmark	Concentration (mg/L)	Score*	Ranking
NTO			
C. dubia 24 h EC_{50}	830	1	Practically Nontoxic
C. dubia 48 h EC_{50}	460	2	Practically Nontoxic
C. dubia 7 Day IC_{50}	57	2	Practically Nontoxic
C. dubia NOEC	34	3	Practically Nontoxic
S. capricornutum NOEC	1375	0	Relatively Harmless
	CL-20		
C. dubia 7 Day IC_{50}	1.9	5	Slightly Toxic
C. dubia NOEC	0.4	7	Moderately Toxic
	Acetone		
C. dubia 96 h EC_{50}	8098[13]	0	Relatively Harmless
	Malathion		
C. dubia 96 h EC_{50}	0.002[14,15]	9	Super Toxic

Toxicity ranking determined according to O'Bryan and Ross Chemical Scoring System for Hazard and Exposure Identification,[9] and the U.S. Fish and Wildlife Service ranking.[10]
The NTO toxicity data listed in this table were generated from assays using pH-adjusted exposure media.
*Score values can range from 0 to 9 (9 being most toxic).

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APPENDIX A
WELL AND R.O. WATER ANALYSIS

Water analyses from the National Testing Laboratories, LTD, 556 South Mansfield, Ypsilanti, MI 48197-5166. ND = Not Detected,

Chemical	Detection Level	Well Water	R.O. Water
	(mg/L)	Level Detected (mg/L)	Level Detected (mg/L)
Metals:			
Aluminum	0.05	ND	ND
Antimony	0.001	ND	ND
Arsenic	0.002	ND	ND
Barium	0.10	ND	ND
Beryllium	0.001	ND	ND
Boron	0.10	ND	ND
Cadmium	0.001	ND	ND
Calcium	2.0	46	14
Chromium	0.001	ND	ND
Copper	0.002	ND	0.003
Iron	0.020	ND	ND
Lead	0.001	ND	ND
Magnesium	0.10	5.5	ND
Manganese	0.004	0.010	ND
Mercury	0.0002	ND	ND
Nickel	0.002	ND	ND
Potassium	1.0	3.2	ND
Selenium	0.002	ND	ND
Silver	0.002	ND	ND
Sodium	1	2	1
Thallium	0.001	ND	ND
Zinc	0.004	ND	ND
Alkalinity	20	130	20
Bromide	0.005	0.012	ND
Chloride	1.0	1.7	ND
Corrosivity	---	-1.1	-6.1
Fluoride	0.1	ND	ND
Sulfate	5.0	10	ND
Hardness (as $CaCO_3$)	10	140	ND
Total Dissolved Solids	5	150	ND
Nitrate	0.05	ND	0.22

Nitrite	0.05	ND	ND
Ortho Phosphate	2.0	ND	ND
pH	---	6.5	5.6
Turbidity	0.1	0.1	ND
Color	3.0	ND	ND
Foaming Agent	0.1	ND	ND
Odor Threshold	---	ND	ND
Trihalomethanes			
Bromoform	0.0005	ND	ND
Bromodichloromethane	0.0005	ND	0.0052
Chloroform	0.0005	ND	0.016
Dibromochloromethane	0.0005	ND	ND
Total THMs	0.0005	ND	0.021
Volatile Organics:			
Benzene	0.0005	ND	ND
Bromobenzene	0.0005	ND	ND
Bromochloromethane	0.0005	ND	ND
Bromomethane	0.0005	ND	ND
n-Butylbenzene	0.0005	ND	ND
sec-Butylbenzene	0.0005	ND	ND
tert-Butylbenzene	0.0005	ND	ND
Carbon Tetrachloride	0.0005	ND	ND
Chlorobenzene	0.0005	ND	ND
Chloroethane	0.0005	ND	ND
Chloromethane	0.0005	ND	ND
2-Chlorotoluene	0.0005	ND	ND
4-Chlorotoluene	0.0005	ND	ND
Dibromomethane	0.0005	ND	ND
1,2-Dichlorobenzene	0.0005	ND	ND
1,3-Dichlorobenzene	0.0005	ND	ND
1,4-Dichlorobenzene	0.0005	ND	ND
Dichlorodifluoromethane	0.0005	ND	ND
1,1-Dichloroethane	0.0005	ND	ND
1,2-Dichloroethane	0.0005	ND	ND
1,1-Dichloroethene	0.0005	ND	ND
cis-1,2-Dichloroethene	0.0005	ND	ND
trans-1,2-Dichloroethene	0.0005	ND	ND
1,2-Dichloropropane	0.0005	ND	ND
1,3-Dichloropropane	0.0005	ND	ND
2,2-Dichloropropane	0.0005	ND	ND
1,1-Dichloropropene	0.0005	ND	ND
cis-1,3-Dichloropropene	0.0005	ND	ND

APPENDIX A

trans-1,3-Dichloropropene	0.0005	ND	ND
Ethylbenzene	0.0005	ND	ND
Hexachlorobutadiene	0.0005	ND	ND
Isopropyltoluene	0.0005	ND	ND
Dichloromethane	0.0005	ND	ND
Naphthalene	0.0005	ND	ND
Propylbenzene	0.0005	ND	ND
Styrene	0.0005	ND	ND
1,1,1,2-Tetrachloroethane	0.0005	ND	ND
1,1,2,2,-Tetrachloroethane	0.0005	ND	ND
Tetrachloroethene	0.0005	ND	ND
Toluene	0.0005	ND	ND
1,2,3-Trichlorobcnzene	0.0005	ND	ND
1,2,4-Trichlorobenzene	0.0005	ND	ND
1,1,1-Trichloroethane	0.0005	ND	ND
1,1,2-Trichloroethane	0.0005	ND	ND
Trichloroethene	0.0005	ND	ND
Trichlorofluoromethane	0.0005	ND	ND
Trichlorotrifluoroethane	0.0005	ND	ND
1,2,3-Trichloropropane	0.0005	ND	ND
1,2,4-Trimethylbenzene	0.0005	ND	ND
1,3,5-Trimethylbenzene	0.0005	ND	ND
Vinyl Chloride	0.0005	ND	ND
Methyl-Tert-Butyl-Ether	0.0005	ND	ND
Methyl Ethyl Ketone	0.005	ND	ND
o-Xylene	0.0005	ND	ND
m-Xylene	0.0005	ND	ND
p-Xylene	0.0005	ND	ND

APPENDIX A

Blank

APPENDIX B
ALGAE MEDIA

To prepare 1 L of algal media:

To ~900 mL of ultrapure distilled water, add (in the order listed below) 10 mL of each of the stock solutions below. Bring the total volume to 1L with ultrapure distilled water. Cover and autoclave for 20 min. After the media has cooled, adjust the pH as needed to 7.1.

Component	Stock Concentration ($g\ L^{-1}\ dH_2O$)	Volume of Stock Used (mL)	Final Concentration (mM)
$NaNO_3$	25.00	10	2.94
$CaCl_2 \cdot 2H_2O$	2.50	10	0.17
$MgSO_4 \cdot 7H_2O$	7.50	10	0.3
K_2HPO_4	7.50	10	0.43
KH_2PO_4	17.50	10	1.29
NaCl	2.50	10	0.43