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Research Paper

# Ecotoxicity of the insensitive munitions compound 3-nitro-1,2,4-triazol-5-one (NTO) and its reduced metabolite 3-amino-1,2,4-triazol-5-one (ATO)

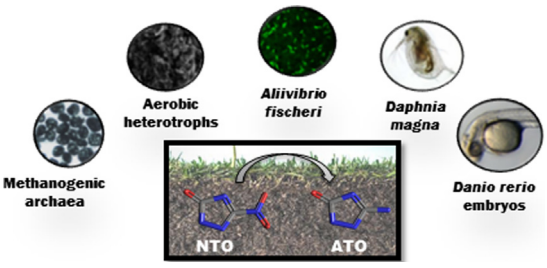
Camila L. Madeira[a], Jim A. Field[a], Michael T. Simonich[b], Robert L. Tanguay[b], Jon Chorover[c], Reyes Sierra-Alvarez[a,*]

[a] Department of Chemical and Environmental Engineering, University of Arizona, Tucson, AZ, USA
[b] Department of Environmental and Molecular Toxicology, Sinnhuber Aquatic Research Laboratory and the Environmental Health Sciences Center at Oregon State University, Corvallis, OR, USA
[c] Department of Soil, Water & Environmental Science, University of Arizona, Tucson, AZ, USA

## HIGHLIGHTS

- The toxicity of NTO and ATO was strongly dependent on the bioassay target.
- NTO was highly toxic to methanogenic archaea ($IC_{50}$ = 1 mM), whereas ATO was not.
- ATO and NTO showed low toxicity to aerobic heterotrophic bacteria and *A. fischeri*.
- Exposure of *Daphnia magna* to ATO (0.4 mM) was lethal to 50% of test organisms.
- No lethal/developmental effects were observed in zebrafish embryos (NTO/ATO = < 0.75 mM).

## GRAPHICAL ABSTRACT

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## ABSTRACT

The insensitive munitions compound 3-nitro-1,2,4-triazol-5-one (NTO) was recently approved by the U.S. Army to replace cyclotrimethylene trinitramine (RDX) in conventional explosives. As its use becomes widespread, concern about the potential toxicity of NTO increases. NTO can undergo microbial reduction to 3-amino-1,2,4-triazol-5-one (ATO), which is recalcitrant in waterlogged soils. In this study, the acute toxicity of NTO and ATO towards various organisms, including microorganisms (*i.e.*, methanogenic archaea, aerobic heterotrophs, and *Aliivibrio fischeri* (Microtox assay)), the microcrustacean *Daphnia magna* (ATO only), and zebrafish embryos (*Danio rerio*), was assessed. NTO was notably more inhibitory to methanogens than ATO ($IC_{50}$ = 1.2 mM, > 62.8 mM, respectively). NTO and ATO did not cause noteworthy inhibition on aerobic heterotrophs even at the highest concentrations tested (32.0 mM). High concentrations of both NTO and ATO were required to inhibit *A. fischeri* ($IC_{20}$ = 19.2, 22.4 mM, respectively). *D. magna* was sensitive to ATO ($LC_{50}$ = 0.27 mM). Exposure of zebrafish embryos to NTO or ATO (750 μM) did not cause lethal or developmental effects (22 endpoints tested). However, both compounds led to swimming behavior abnormalities at low concentrations (7.5 μM). The results indicate that the reductive biotransformation of NTO could enhance or lower its toxicity according to the target organism.



* Corresponding author.
*E-mail address:* rsierra@email.arizona.edu (R. Sierra-Alvarez).

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## 1. Introduction

The nitro heterocyclic compound 3-nitro-1,2,4-triazol-5-one (NTO) is one of the insensitive munitions constituents of important explosive formulations, such as IMX-101, IMX-104, and PAX48. NTO was recently approved by the U.S. Army as a safer and effective alternative to replace cyclotrimethylene trinitramine (RDX) in large caliber munitions [1]. The constituents of the insensitive formulations are more stable and safer for troops due to their lower likelihood for unintended explosions. During U.S. military training activities, approximately 3% of the insensitive munitions fired result in unexploded ordnance [2]. Pieces of undetonated explosives were found to be the primary source of contamination in numerous training ranges [3]. Due to its high solubility in water ($12.8\,g\,L^{-1}$ at 19 °C) [4], NTO has high mobility in the environment, thus becoming a source of soil and water contamination. As the production and usage of NTO increases, assessment of the toxicity and fate of this compound in the environment is needed.

NTO is known for readily undergoing microbial reduction under anaerobic conditions in soils. Its nitro group is reduced to an amino group, yielding 3-amino-1,2,4-triazol-5-one (ATO, Fig. 1), which was found to be persistent in the soil in the absence of oxygen [5–7]. The reductive transformation of NTO to ATO could alter the molecule's toxicity.

To date, there is limited data regarding the toxicity of NTO, and ATO toxicity has never been assessed. Toxicological effects of insensitive munitions compounds to *Rana pipiens* tadpoles were assessed by Stanley et al. [8]. A chronic 28 days exposure study with NTO indicated that the survival LOEC (lowest observed effect concentration) was $5.0\,mg\,L^{-1}$. In addition, NTO was not found to bioconcentrate in the test organisms. A battery of genotoxicity assays performed with NTO, including *in vivo* and *in vitro* test, suggested that there is a low risk of genetic hazards associated to the exposure to this compound [9]. NTO was ranked as practically non-toxic in aquatic toxicity tests conducted with *Ceriodaphnia dubia* and the unicellular green algae *Selenastrum capricornutum* [10].

The objectives of this study were to evaluate the inhibitory effects of NTO and ATO to microorganisms and aquatic toxicity indicator organisms. The targets chosen to assess the microbial toxicity of these compounds were environmentally relevant microorganisms, such as aerobic heterotrophs and anaerobic methanogens. Furthermore, the Microtox assay was performed to assess the inhibition of bioluminescence activity of the marine bacterium *Aliivibrio fischeri* when exposed to NTO and ATO. Due to their ubiquitous presence and diverse functions, microorganisms can act as indicators of environmental pollution [11].

Tests were also conducted using representative species of freshwater environments, namely the microcrustacean, *Daphnia magna,* and *Danio rerio* (zebrafish) embryos. *D. magna* has been extensively used as a test animal for aquatic toxicology [12].

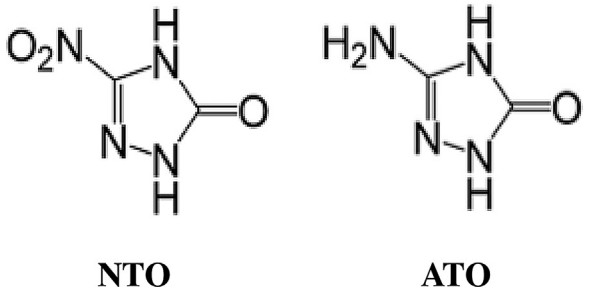


**Fig. 1.** Molecular structure of 3-nitro-1,2,4-triazol-5-one (NTO) and 3-amino-1,2,4-triazol-5-one (ATO).

Zebrafish is a highly prolific, small, complex organism that shares a highly conserved anatomy and physiology with all vertebrates [13]. Importantly, the critical processes of zebrafish neurodevelopment are homologous to those in humans [14]. Neuronal effects of developmental chemical exposure on embryo and larval photomotor activity can be easily measured in zebrafish, widening the potential field of bioactivity that can be detected [15,16]. It was found that developmental mortality/morphology endpoints, combined with the larval photomotor response, serve as a robust biological sensor for chemical hazard potential. In this study, zebrafish embryos were used as biological sensors to evaluate a comprehensive battery of developmental endpoints for chemical hazard via multiple mechanisms of action [17,18].

The results obtained in this research will contribute to a better understanding of the impacts of NTO release to the environment at different stages of its degradation pathway.

## 2. Materials & methods

### 2.1. Chemicals

NTO (3-nitro-1,2,4-triazol-5-one, CAS# 932-64-9, purity > 95%) was purchased from Interchem (San Pedro, CA, USA) and ATO (3-amino-1,2,4-triazol-5-one, CAS# 1003-35-6, purity > 95%) from Princeton BioMolecular Research (Monmouth Junction, NJ, USA).

### 2.2. Microbial inocula

The inoculum used in the methanogenic toxicity assay was anaerobic sludge obtained from a full-scale upflow anaerobic sludge blanket (UASB) bioreactor treating brewery wastewater (Mahou, Guadalajara, Spain). The inoculum for the aerobic heterotrophic inhibition assay was aerobic return activated sludge (RAS) collected from the Agua Nueva Water Reclamation Facility (Tucson, AZ, USA). The sludge samples were kept at 4° C. The anaerobic sludge was sieved to remove the excess of water before use. The aerobic sludge was centrifuged for 10 min at 4000 rpm, the supernatant was discarded and the pellet was resuspended in mineral medium for use in the bioassays. The volatile suspended solids (VSS) content was 7.92% wet weight for the anaerobic sludge, and 0.95% wet weight for the RAS.

### 2.3. Zebrafish embryo

Tropical 5D wild-type adult zebrafish were housed at an approximate density of 1000 per 100 gallons. Spawning funnels were placed into the tanks the previous night, and embryos were collected and staged [19]. To increase bioavailability, the chorion was enzymatically removed using pronase ($63.6\,mg\,mL^{-1}$, $\geq 3.5\,U\,mg^{-1}$) at 4 h post fertilization (hpf) using a custom automated dechorionator [20]. The zebrafish egg chorion remains an incompletely characterized barrier with respect to permeability toward diverse chemicals. Thus, to minimize its potential impact on bioavailability, the chorion is routinely removed.

### 2.4. Assay to assess inhibition of methanogens

The bioassay was conducted in glass serum bottles (160 mL) containing methanogenic sludge ($1.5\,g\,VSS\,L^{-1}$) suspended in mineral medium (25 mL) supplemented with sodium acetate ($3.26\,g\,L^{-1}$). The medium recipe is described in the supplementary material. The bottles were sealed with rubber stoppers and aluminum seals and flushed with a gas mixture containing 80% $N_2$ and 20% $CO_2$. The bottles were incubated overnight for adaptation of the microorganisms. On the following day, NTO or ATO were added to the bottles through concentrated stock solutions (NTO or

ATO powder dissolved in DI water), and the bottles were flushed again. The final concentrations were 0.10, 0.25, 0.50, 0.75, 1.00, 2.00, 5.00, 10.00, 25.00 mM for the test with NTO, and 3.10, 25.1, 31.4, 50.2, 62.8 mM for the test with ATO. The bottles were incubated in the dark at 30 °C in an orbital shaker (130 rpm). Samples of the headspace were taken and injected in a gas chromatograph for methane production analysis.

### 2.5. Assay to assess respiration inhibition of aerobic heterotrophic bacteria

The bioassay was conducted in serum bottles (160 mL) containing aerobic sludge (0.5 g VSS $L^{-1}$) suspended in mineral medium (25 mL) supplemented with sodium acetate (1.08 g $L^{-1}$). The composition of the mineral medium was the same as the one described in the methanogenic toxicity assay. The bottles were sealed with rubber stoppers and aluminum seals and flushed with a gas mixture containing 60% He, 20% $CO_2$ and 20% $O_2$. The bottles were incubated overnight. On the following day, NTO or ATO were added through concentrated stock solutions, and the bottles were flushed with the gas mixture again. The final concentrations were 1.0, 2.0, 4.0, 16.0, 32.0 mM for the tests with NTO or ATO. The bottles were incubated in the dark at 30 °C in an orbital shaker (130 rpm). Samples of the headspace were taken periodically using a chromatographic syringe and analyzed for oxygen content. Samples for HPLC analysis were taken using syringes to preserve the composition of the gas phase into the bottles.

### 2.6. Assay to assess bioluminescence inhibition in Aliivibrio fischeri

The Microtox assay [21] was used to assess the inhibition of the bioluminescence activity of the marine bacterium *A. fischeri* (NRRL number B-11177). The bacterial culture, the reconstitution solution and the diluent were purchased from AZUR Environmental (Carlsbad, CA, USA). A Microtox Model 500 analyzer (Strategic Diagnostics, Inc. SDIX, Newark, DE, USA) was used to measure the intensity of the bioluminescence produced by the bacteria when exposed to different concentrations of NTO or ATO for 30 min compared to the toxicant-free control. The pH of the stock solutions of NTO and ATO were adjusted to 7.0. The final concentrations were 0.12, 0.24, 0.48, 0.95, 1.90, 7.60, 15.20, 30.40 mM for the test with NTO, and 0.38, 0.76, 1.53, 3.05, 6.10, 12.20, 24.40, 48.80, 97.60 mM for the test with ATO. The experiment was performed in duplicate.

### 2.7. Daphnia lethality bioassay

*Daphnia magna* are small planktonic crustaceans (0.2–5 mm in length) that are members of the order Cladocera. The acute toxicity of ATO towards *D. magna* was assessed using the standardized test procedure recommended by USEPA [22]. The tests were performed by the Laboratory of Bioassays, Faculty of Natural Sciences and Oceanography, University of Concepcion, Chile. In this assay, *D. magna* was exposed to ATO for 48 h, and the test was performed with 4 replicates (5 organisms per replicate) for each concentration of ATO. The tested concentrations were 0.003, 0.016, 0.08, 0.4, 2.0 and 10.0 mM. In one of the treatments, the organisms were not exposed to ATO (toxicant-free control). The pH was kept within 7.8 and 8.0 along the assay. NTO was not tested due to U.S. International Traffic in Arms Regulations restrictions in transporting and shipping to a service laboratory.

### 2.8. Statistical analysis

The statistical analysis performed for the microbial toxicity and the Daphnia bioassays are described in the supplementary material.

### 2.9. Zebrafish embryo assay

#### 2.9.1. Chemical exposures

ATO and NTO solutions were prepared as 10 x concentrated stock solutions in ultrapure water with phosphate buffer added (buffer strength of 2.5 mM) due to the acidic character of NTO. Both were tested as 1 x final concentrations (750, 75, 7.5, 0.75, 0.075, 0 μM). The final test concentrations were achieved by adding 10 μL of the appropriate 10 × stock to 90 μL of embryo medium [23] containing a single 6 hpf embryo in plate wells. No changes in the pH were observed after the addition of the NTO stock solution. Plates were run in duplicate to obtain N = 32 animals per concentration; 16 embryos exposed per concentration per plate.

#### 2.9.2. Larval photomotor response (LPR)

At 120 hpf, zebrafish are free swimming larvae, and the larval photomotor response was measured by determining total movement (swim distance) in response to multiple light –> dark transitions. Briefly, a Zebrabox behavior chamber (ViewPoint Life Sciences, Montreal, CA) with an infrared backlit stage was used to track total movement in 96 wells during a 24-min assay. HD video was captured at 30 frames $s^{-1}$ and processed in real time by the manufacturer's software. The assay consisted of 3 cycles of 3 min visible light (1050 lx), 3 min dark (IR light). Additional animals dead or malformed at the 120 hpf time point were excluded from the larval behavior data analysis.

#### 2.9.3. Mortality and morphology responses

Embryos were statically exposed until 120 hpf. At 24 hpf, embryos were assessed for four developmental toxicity endpoints (MO24: mortality at 24 hpf, DP: developmental progression, SM: spontaneous movement, and NC: notochord distortion) [24]. At 120 hpf, 17 developmental endpoints were assessed (Table 1) [24]. The zebrafish acquisition and analysis program (ZAAP), a custom program designed to inventory, acquire, and manage zebrafish data, was used to collect developmental endpoints as either present or absent (*i.e.*, binary responses were recorded).

#### 2.9.4. Statistical analysis for zebrafish assays

The statistical analyses performed for the zebrafish embryo assay are described in the supplementary material.

### 2.10. Analytical methods

The concentrations of methane and oxygen in the gas phase were determined by gas chromatography, as described in the supplementary material. The concentrations of NTO and ATO were verified via HPLC-DAD analysis. The method was previously described by Madeira et al. [5].

## 3. Results and discussion

### 3.1. Microbial toxicity

Methanogenic archaea present in granular sludge from an anaerobic reactor were exposed to different concentrations of NTO and ATO. Under ideal conditions, methanogens are able to utilize the acetate present in the medium and produce methane as a metabolic byproduct. However, in the presence of toxicants, the rate of methane production can decrease, indicating reduction of the methanogenic activity. Figs. S1 and S2 (supplementary

**Table 1**
Zebrafish embryo toxicity endpoints assessed for NTO and ATO exposure.

| Time | Endpoints |
|---|---|
| 24 hpf | Mortality, developmental delay, spontaneous movement, notochord |
| 120 hpf | Mortality, notochord, yolk sac edema, body axis, eye defect, snout, jaw, otic vesicle, pericardial edema, brain somite, pectoral fin, caudal fin, pigment, circulation, truncated body, swim bladder, touch response |

hpf = hours post fertilization.

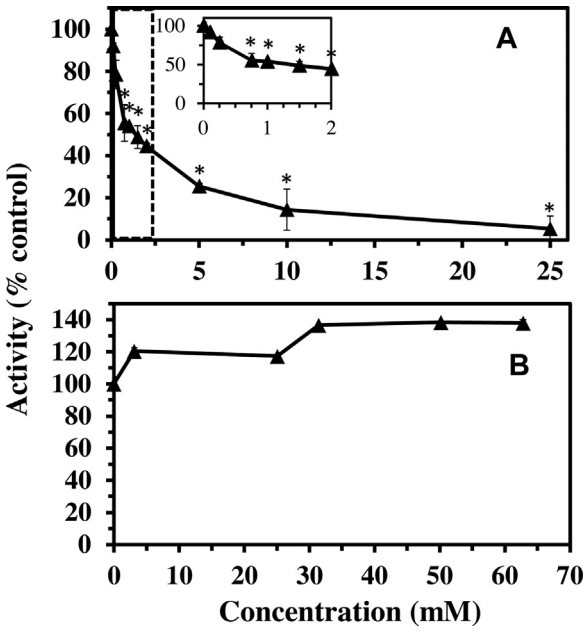


**Fig. 2.** Inhibition of the acetoclastic methanogenic archaea of anaerobic sludge by NTO (panel A) and ATO (panel B) (expressed as a percentage of the control activity). The treatments statistically different from the controls (p value = < 0.05) are represented by (*).

material) illustrate the production of methane over time for cultures exposed to NTO and ATO, respectively. The methanogenic activity was calculated based on the slope of the curve methane concentration versus time. The time interval was chosen as the period in which the highest methane production rate was observed: 0–29 h for NTO and 0–58 h for ATO, and then normalized by the methane production rate displayed by the control free of toxicant. Fig. 2 illustrates the normalized activity of methanogenic archaea exposed to NTO and ATO. In all the treatments containing NTO, the methanogenic activity was lower compared to the control. The decreased activity was dependent on increasing concentrations (Fig. 2A), indicating that NTO caused a concentration dependent inhibition of the methanogens. At a NTO concentration of 1.15 mM, the methane production rate was only half of the rate observed in the control, demonstrating 50% of inhibition ($IC_{50}$ = 1.15 mM). At a concentration of 25 mM, there was no significant methane production.

During the final stage of the experiment with NTO (from 217 to 338 h), an increase in the methanogenic activity was observed, which can be attributed to the reduction of NTO to ATO. The acetate present in the culture medium or endogenous substrates in inoculum may serve as electron donor for NTO reduction. After 28 h, 20% of NTO was reduced to ATO, achieving 100% conversion after 200 h (data not shown), leading to detoxification. Compounds containing nitro functional groups have been reported to present higher toxicity to anaerobic microorganisms than corresponding compounds containing amino functional groups. These results were also observed by Johnson and Young [25] in a study of inhibition of anaerobic digestion by nitrobenzene. When nitrobenzene was reduced to aniline, recovery of methane production occurred.

In stark contrast to NTO, exposure to ATO did not lower the methanogenic activity. On the contrary, the presence of ATO even provided a slight stimulation of the methanogenic activity (Fig. 2B). The time course data in Fig. S2 shows that the methane production was similar to that of the control even at the highest concentration tested (62.8 mM), which is 3-fold higher than the concentration of NTO causing near 100% inhibition.

Organic compounds containing nitro groups, such as nitrobenzenes and nitrophenols, have been reported to be toxic to microbial processes in anaerobic digesters [26]. A comprehensive study including several N-substituted aromatic compounds demonstrated that nitroaromatics are up to 500-fold more toxic to acetoclastic methanogens than their respective aromatic amines [27]. Anaerobic granular sludge was exposed to concentrations ranging from 13 to 130 μM of the nitroaromatic, 2,4-dinitroanisole (DNAN), which is another constituent of insensitive explosive formulations [28]. DNAN was found to cause 50% methanogenic inhibition at a concentration of 41 μM. Similar to NTO, DNAN metabolites containing amino groups decreased the toxicity to methanogenic microorganisms. The 50% inhibiting concentration of the reduced DNAN metabolites were 175 and 176 μM for 2-methoxy-5-nitroaniline (MENA) and 2,4-diaminoanisole (DAAN), respectively. This suggests a greater than 4-fold reduction in methanogenic toxicity if one or more of nitro-groups in DNAN were reduced. As a conclusion, the reduction of nitro functional groups in munition compounds to their correspondent amines causes significant lowering of methanogenic toxicity.

The toxicity of NTO and ATO was also assessed using aerobic heterotrophic bacteria as target organisms. The activity of these microorganisms was assessed by measuring $O_2$ consumption in the gas phase. The time course of $O_2$ consumption due to acetate metabolism in RAS is shown in Fig. S3 for different concentrations of NTO. The raw data show that none of the concentrations of NTO tested reduced the rate of $O_2$ uptake. The slopes from 2 to 6.3 h were used to normalize the data to a control (without added toxicant). The normalized activity as a function of NTO concentration is shown in Fig. 3A. The graph clearly shows that there is no significant decrease in the normalized activity due to NTO even up to a concentration of 32 mM, at which concentration the normalized respiratory activity was still as high as 87%. Thus, NTO does not cause any noteworthy toxicity to $O_2$ uptake by RAS.

The time course of $O_2$ consumption in RAS is shown in Fig. S4 for different concentrations of ATO. The raw data show that the higher tested concentrations of ATO lowered the slope of the $O_2$ uptake. The slopes from 4.3 to 10 h were used to normalize the data to a control (without added toxicant). The normalized $O_2$ uptake activity in response to increasing ATO concentration is illustrated in Fig. 3B. Exposure to ATO decreased the normalized activity at high concentrations. However, the activity of heterotrophic microorganisms was not statistically lower than the activity of control even at the higher concentration tested, which can be attributed to the large error bars of the data. No noteworthy toxicity was observed when the aerobic heterotrophs were exposed to the same concentration of NTO. Thus far, only a few literature studies have reported the toxicity of compounds containing nitro or amino groups to

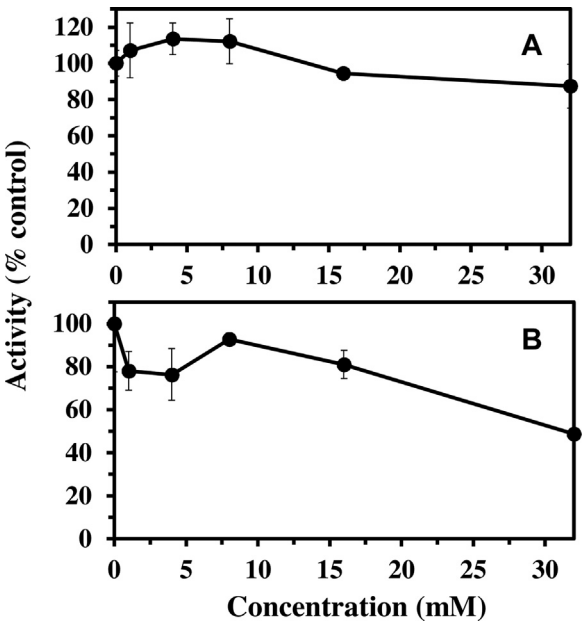


**Fig. 3.** Inhibition of oxygen respiration in return activated sludge exposed to increasing concentrations of NTO (panel A) and ATO (panel B), (expressed as a percentage of the control activity). The treatments statistically different from the controls (p value = < 0.05) are represented by (*).

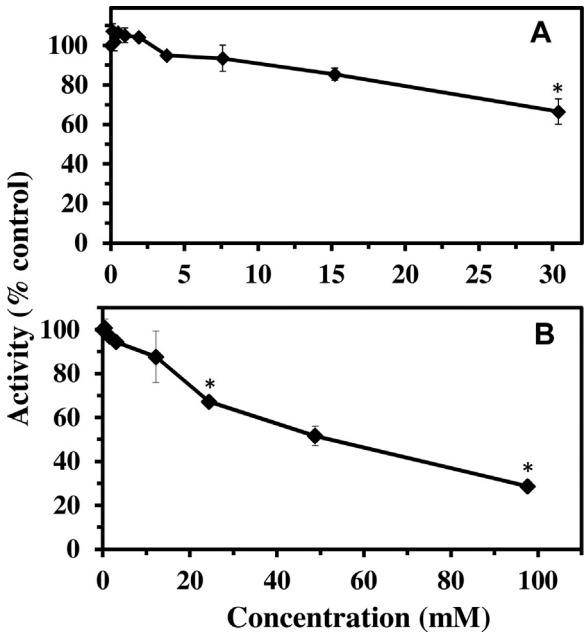


**Fig. 4.** Inhibition of the bioluminescence activity of *A. fischeri* exposed to NTO (panel A) and ATO (panel B) for 30 min (expressed as a percentage of the control activity). The treatments statistically different from the controls (p value = < 0.05) are represented by (*).

heterotrophic bacteria. 2-Aminophenol was found to cause higher inhibition than 2-nitrophenol to a mixed culture of aerobic heterotrophic bacteria [29]. While the $IC_{50}$ for 2-nitrophenol was determined as 79.1 μM, the $IC_{50}$ for 2-aminophenol was 0.37 μM, showing that the reduction of the nitro group to amino group increased the toxicity of the compound. Another evidence of inhibition of aerobic heterotrophic activity by a compound containing functional amino groups was found by Gundersen et al. [30]. The rate of $O_2$ consumption by a lake-water bacterial community decreased when these microorganisms were exposed to concentrations of monoethanolnitramine higher than 4 mg $L^{-1}$.

Another test consisted in the exposure of bioluminescent bacteria to NTO and ATO. When *A. fischeri* is exposed to toxic compounds that inhibit respiration, its light output is reduced, leading to a decrease of bioluminescence activity. Fig. 4 illustrates how the exposure to different concentrations of NTO and ATO for 30 min impacted the bioluminescence activity of *A. fischeri*. Both NTO and ATO had a similar effect on *A. fischeri* bioluminescence activity, which was dependent on NTO and ATO concentration. Both compounds decreased the normalized activity by 20% at similar concentrations (19.2 and 22.4 mM, respectively). At the highest concentration of NTO tested (30 mM), the decrease in the normalized activity was 44%. At the highest concentration of ATO tested (98 mM) the decrease in the normalized activity was 71%.

Inhibition of *A. fischeri* by other nitro-heterocyclic explosives compounds, RDX (1,3,5-trinitroperhydro-1,3,5-triazine) and HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine), has been reported [31,32]. Compared to NTO, RDX was found to inhibit the bioluminescence of *A. fischeri* at a much lower concentration ($IC_{50}$ = 0.34 mM [31] and $IC_{20}$ = 0.18 mM [32]). For HMX, no toxicity was observed at 0.08 mM, a value close to its aqueous solubility. The $IC_{50}$ of the conventional explosive 2,4,6-trinitrotoluene (TNT) was reported as 4.2 μM [32]. In a study by Xu et al. [33], the toxicity of N-containing heterocyclic compounds was measured by Microtox. The toxicity of these compounds was shown to display a direct correlation with hydrophobicity. Hence, the lower hydrophobicity of NTO (octanol-water partition coefficient ($K_{ow} = 10^{0.86}$) can justify its lower toxicity compared to the more hydrophobic N-heterocyclics (RDX and HMX). NTO was also found to be less toxic than DNAN ($IC_{50}$ = 57 μM) and its reduced metabolites [28], which are more hydrophobic compounds.

A summary of the microbial toxicity assays containing the $IC_{20}$ and $IC_{50}$ values for each test organism is shown in Table 2.

### 3.2. Aquatic toxicity

#### 3.2.1. *Acute toxicity to* Daphnia magna

In this study, the microcrustacean *D. magna* was exposed to several concentrations of ATO for 48 h. Exposure to 0.28 mM (confidence interval: 0.16–0.47) was lethal to 50% of the test organisms (Fig. 5). At the highest concentration tested (10 mM), the lethal effect was 100%. The results of this experiment indicate that ATO is slightly toxic according to the acute toxicity rating scale from the United States Fish and Wildlife Service [34]. Haley and coworkers reported that the concentration of NTO at pH 7 causing 50% lethal effect after 48 h of exposure (48-h $LC_{50}$) towards *Ceriodaphnia dubia* was 3.5 mM [10]. Thus, ATO was approximately 10-fold more toxic to *D. magna* than NTO was to *C. dubia*. Although different

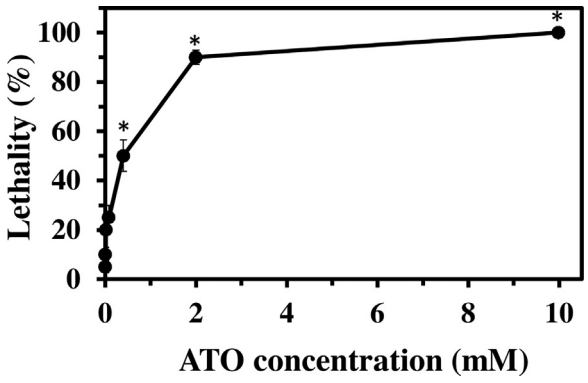


**Fig. 5.** Lethal effect of ATO to *Daphnia magna* after 48 h of exposure. The treatments statistically different from the controls (p value = < 0.05) are represented by (*).

**Table 2**
Inhibitory concentrations of NTO and ATO (in mM) determined in methanogenic activity, aerobic heterotrophs activity, and Microtox (*A. fischeri*) assays.

| Compound | Methanogens | | | Aerobic heterotrophs | | | *A. fischeri* | | |
|---|---|---|---|---|---|---|---|---|---|
| | Conc. (mM) | $IC_{20}$[b] | $IC_{50}$[b] | Conc. (mM) | $IC_{20}$ | $IC_{50}$ | Conc. (mM) | $IC_{20}$ | $IC_{50}$ |
| NTO | 0.1–25.0 | 0.19 (0.12–0.25) | 1.15 (0.95–1.40) | 1.0–32.0 | NT[a] | NT[a] | 0.1–30.4 | 19.16 (14.88–23.46 | NT[a] |
| ATO | 3.1–62.8 | NT[a] | NT[a] | 1.0–32.0 | NT[a] | NT[a] | 1.5–97.6 | 22.43 (18.66–26.38) | 82.00 (71.51–96.82) |

[a] Compound was not toxic at the highest concentration tested (NT = non-toxic).
[b] The values of the 95% confidence intervals are shown in the parenthesis below the mean IC20 and IC50 values.

**Table 3**
Significant larval photomotor responses (LPR) determined in assays with zebrafish at 120 hpf.

| Treatmentgroup | Concentration(μM) | Interval[a] | N[b] | P value | Activity[c] |
|---|---|---|---|---|---|
| ATO | 7.5 | LIGHT | 25 | <0.0001 | HYPER |
| ATO | 75 | LIGHT | 29 | <0.0001 | HYPER |
| ATO | 750 | LIGHT | 28 | <0.0001 | HYPO |
| NTO | 7.5 | LIGHT | 27 | <0.0001 | HYPO |
| NTO | 75 | BOTH | 28 | <0.0001 | HYPO |

[a] Light: interval in which embryos were exposed to visible light; Both: behavior outcome was present in both light and dark exposure intervals.
[b] 32 larvae per concentration initially. Animals dead or malformed at 5 hpf were excluded from the LPR assay.
[c] Hyper: hyperactivity comparing to the toxicant-free control; Hypo: hypoactivity comparing to toxicant-free control.

test organisms were used in NTO and ATO bioassays, comparisons between the sensitivity of *D. magna* and *C. dubia* demonstrated that both organisms have very similar response when exposed to several organic and inorganic compounds [35]. Therefore, *C. dubia* can be used to predict acute effects on the other Cladoceran species. The results of the present study indicate that the reduction of the nitro group in NTO leads to an increase in the toxicity to microcrustaceans.

In comparison to traditional explosives, NTO and ATO had lower toxicity effects. In a test with *D. magna* exposed to TNT for 48 h, The $IC_{50}$ was determined as 52 μM [36]. RDX did not cause acute toxicity to *C. dubia* at its solubility limit (77 μM) [37].

### 3.2.2. Toxicity to zebrafish embryos

Of the zebrafish assays conducted in this study to evaluate the impacts of NTO and ATO, the most important impact was observed with larval photomotor response (LPR). The significant behavioral outcomes of the LPR assay are summarized in Table 3. We note, by way of background information, that the dark phases of the LPR are invariably where control fish demonstrate at least 2× more swimming activity than in the lighted phases. The general LPR activity patterns reported here were consistent with the light/dark differential (Figs. S5 and S6). Developmental exposure to the higher concentrations of ATO was associated with modest but significant hyperactivity in the lighted phases. However, the highest concentration of ATO depressed swimming activity in the lighted phases. During the dark phases, ATO was consistently associated with apparent but not significant hyperactivity. The association of a broad concentration range of ATO exposures with significant hyperactivity during the normally quiescent lighted phases and apparent hyperactivity during the normally active dark phases suggests that ATO may be anxiogenic in the developmental zebrafish. NTO was associated with significantly altered LPR at 7.5 and 75 μM where the only effect was hypoactivity in the lighted phases, or both for the 75 μM group.

With regard to other zebrafish assays conducted, the responses observed in the 22-endpoint battery that encompasses zebrafish development from 24 to 120 hpf are summarized in Figs. S7 and S8 . No significant incidences of the endpoints were observed for ATO or NTO, even when the incidences were pooled by dose into the 'any effect' metric.

The zebrafish model has been used to characterize the toxicity of other energetic materials. The behavioral effects of acute exposure to RDX on adult zebrafish were assessed by Williams et al. [38]. After 30 min of exposure to 0.1 mM RDX, hyperactivity and decreased freezing behavior were observed. At 1.0 mM RDX, spasms and hyperactive bursts (seizure-like activity) were found in the zebrafishes. For larval zebrafish, mortality and vertebral column deformities were used as the end-points to characterize the acute toxicity of RDX [39]. The lethal concentration for 50% of the organisms ($LC_{50}$) was estimated at 0.10 mM, and the concentration causing vertebral column deformities ($EC_{50}$) was estimated at 0.09 mM. The same protocol described in this work was used to evaluate the toxicity of DNAN transformation products [40]. The aromatic amines MENA and DAAN did not cause mortality or developmental toxicity to the test-organisms at concentrations as high as 640 and 64 μM, respectively. However, 640 μM DAAN caused the reduction of motion in the dark phase. Since no malformations were observed, the impairment of the locomotor behavior in the zebrafish embryos could be related to neurotoxic effects. Despite a lack of overt toxicity, the aberrant larval photomotor responses (LPR) associated with exposure to either ATO or NTO suggested that these compounds may be bioactive having significantly altered some aspect(s) zebrafish development. Aberrant larval behavioral outcomes point to developmental hazard potential, but beyond that, the environmental relevance of the larval zebrafish behavior data is unknown. The larval photomotor response outcomes are bona fide manifestations of chemical toxicity, not merely markers of future toxicity. However, the extent to which abnormal larval photomotor responses predict abnormal behavior in adults is still unknown. The aberrant LPRs cannot yet be directly attributed to perturbation of a specific target(s) as a multitude of possibilities exists. Whether these LPR deficits would manifest as more complex central nervous system deficits is not readily predictable, but should be investigated as a logical next step.

## 4. Conclusions

NTO and ATO were found to cause different toxic effects on each group of organisms tested in the present study. NTO caused significant inhibition of methanogenic activity at concentrations as low as 0.2 mM. The microbial reductive transformation of NTO to ATO decreased its toxicity to methanogens. High ATO concentrations led to partial inhibition towards aerobic heterotrophs and in the partial mortality of the microcrustacean *D. magna*. Exposure of zebrafish embryos to NTO and ATO led to aberrant larval photomotor responses. No significant incidences of the developmental toxicity endpoints were observed for ATO or NTO. Further studies are needed to understand how NTO and ATO affect the behavior of zebrafish embryos and to predict whether the impairment of the locomotor behavior would persist into adulthood.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhazmat.2017.09.052.

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