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Toxic Effects of Hydroxylamino Intermediates from Microbial Transformation of Trinitrotoluene and Dinitrotoluenes on Algae *Selenastrum capricornutum*

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Trinitrotoluene (TNT) has been used extensively as an explosive since 1902. As a result of the production, purification, and loading at the ammunition plants, explosives contamination of soil and groundwater has become an enormous problem in the U.S. and worldwide. Among the various types of explosives, TNT and its major by-products, 2,4-dinitrotoluene (24DNT) and 2,6-dinitrotoluene (26DNT), are the most important contaminants in terms of volume and impact on the aquatic environment. They are all listed as the priority pollutants by the U.S. Environmental Protection Agency (Keith and Telliard 1979).

The aquatic toxicity of TNT, DNTs and other related nitroaromatic explosives has been a subject of many acute studies utilizing fish and invertebrates (Rosenblatt et al. 1991). Several studies have been reported on algal toxicity, however, data are limited primarily in TNT and its amino degradation products (Hudock and Gring 1970; Smock et al. 1976; Won et al. 1976). As an increasing number of microbial transformation products become identified, research are needed to establish the toxicity profiles of these new compounds. One of the ideal host models in aquatic toxicology is unicellular algae, since they are subjected to a variety of pollutants from various sources, and they have been shown to be good indicators in response to a variety of toxicants (Tadros et al. 1990; 1994; 1995).

In our recent investigations, we demonstrated the presence of hydroxylamino intermediates during the transformation of TNT, 24DNT, and 26DNT by *Clostridium acetobutylicum* (Hughes et al. 1998a; 1998b; 1999). The toxicity profiles of these metabolites are unknown, and are of importance in designing bioremediation systems. Such information is critical to best monitor the treatment process and to determine the time needed for cost-effective treatment of explosives. With this ultimate goal, the work reported herein examines the toxicity of hydroxylamines relative to their parent compounds and other known metabolites using a fresh water green algae *Selenastrum capricornutum* as the host model.

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MATERIALS AND METHODS

The culture species of unicellular green algae *Selenastrum capricornutum* was obtained from the Algal Culture Collection, University of Texas, Austin. Algal toxicity tests were performed in triplicate test tubes, each contained 25 mL of growth medium consisting of (per liter) the following: $NaNO_3$, 0.17 g; $NaH_2PO_4 \cdot H_2O$, 0.01 g; Na_2CO_3, 0.02 g; $NaSiO_3 \cdot H_2O$, 0.015 g; H_3BO_3, 0.568 g; $ZnCl_2$, 0.268 g; $CuCl_2 \cdot 2H_2O$, 0.252 g; $Na_2MO_4 \cdot 2H_2O$, 0.252 g; $CoCl_2.H_2O$, 0.042 g; $FeSO_4$ 1.36 g; $MnCl_2 \cdot H_2O$, 0.36 g. The pH of the growth medium was adjusted to 8.0. Each test tube was then inoculated with exponential growing cells at an initial density of approximately 4 x 10^3 cells/mL. After the addition of the test compounds, cultures were incubated on a shaker at 25°C under a continuous cycle of cool-white light producing 100 µE/m^2s irradiation. After 96 hours of incubation, samples were withdrawn and subjected to cell counts. The growth was measured spectrophotometrically at 525 nm with a Fisher electrophotometer (Pittsburgh, PA, USA). All experiments were conducted in a controlled environmental chamber.

A total of twelve compounds used in this toxicity study are listed in Table 1 according to the degradation order for each category of the compounds (i.e., TNT, 24DNT, or 26DNT). The mono- and di-hydroxylamines were isolated and purified according to the procedures described previously (Hughes et al. 1998a; 1998b; 1999). Their toxicity was compared to their respective parent compounds including TNT (Chem Services, West Chester, PA), 24DNT (Aldrich, Milwaukee, WI), and 26DNT (Aldrich), and other commercially available and structurally related test standards (all from Aldrich) such as 2A4NT, 24DAT, 2A6NT, and 26DAT.

Table 1. Chemical structure of test compounds

Compound	Synonym	Structure
2,4,6-trinitrotoluene	TNT	R_2,R_4,R_6=NO_2
2-hydroxylamino-4,6-dinitrotoluene	2HA46DNT	R_2=NHOH; R_4,R_6=NO_2
2,4-dihydroxylamino-6-nitrotoluene	24DHA6NT	R_2,R_4=NHOH; R_6=NO_2
2,6-diamino-4-nitrotoluene	26DA4NT	R_2,R_6=NH_2; R_4=NO_2
2,4-dinitrotoluene	24DNT	R_2,R_4=NO_2
4-hydroxylamino-2-nitrotoluene	4HA2NT	R_4=NHOH; R_2=NO_2
2-amino-4-nitrotoluene	2A4NT	R_2=NH_2; R_4=NO_2
2,4-diaminotoluene	24DAT	R_2,R_4=NH_2
2,6-dinitrotoluene	26DNT	R_2,R_6=NO_2
2-hydroxylamino-6-nitrotoluene	2HA6NT	R_2=NHOH; R_6=NO_2
2-amino-6-nitrotoluene	2A6NT	R_2=NH_2; R_6=NO_2
2,6-diaminotoluene	26DAT	R_2,R_6=NH_2

Toxicity was tested at various concentrations (0.14, 0.7, 1.4, 7.0, 14, 28 mg/L) within the solubility limits of individual compound. Generally, a stock solution of 1 mg/mL in acetonitrile was prepared. Each stock was then diluted to 10 mL with culture media, and different volumes were added to vials to obtain various concentrations. Several higher concentrations tested (i.e., TNT: 7.0, 14, 28 mg/L; 24DHA6NT: 14, 28 mg/L; other compounds: 28 mg/L) caused bleaching of the tested organism and therefore they were not considered in the assay. The responses in the treated groups were compared to the control group without the addition of test compounds. Results of cell counts were converted to the percentage of the control. Data are expressed as the means and standard deviations from three independent experiments. Significance in the mean growth inhibition were tested using standard one-way analysis of variance (ANOVA) followed by Bonferroni's method in GraphPad Prism 2 (San Diego, CA). Statistical significance are considered at $p < 0.05$.

RESULTS AND DISCUSSION

Data on the mean growth inhibition were analyzed for each category of the nitroaromatic compounds using two-way ANOVA. Results indicated that toxicities differ significantly ($p < 0.05$) among the metabolites and its corresponding parent compound (TNT, 24DNT, or 26DNT). The concentration effects and the interaction effects between concentrations and compounds in each group (i.e., TNT, 24DNT, or 26DNT) were highly significant as well ($p < 0.001$), indicating that the differences in mean growth inhibition depend on the concentration. Mean growth inhibition was further tested by one-way ANOVA, and the results are shown in Table 2.

For TNT and its metabolites, Table 2 shows that 26DA4NT is significantly less toxic than TNT at a concentration range of 0.14 .~ 28 mg/L. Monohydroxylamino intermediate (2HA46DNT) is significantly less toxic than TNT at a concentration range of 0.7 ~ 14 mg/L. Dihydroxylammino intermediate (24DHA6NT) is less toxic than TNT at lower concentrations (0.7 -1.4 mg/L), but is as toxic as TNT at higher concentrations (7 ~ 28 mg/L). For 24DNT and its metabolites, however, significant difference in algal toxicity was noted only for 4HA2NT and 24DAT at 1.4 mg/L, and 2A4NT at 14 mg/L when compared to 24DNT at the same concentration. In the case of 26DNT and its metabolites, 2A6NT showed significantly lower toxicity than 26DNT when the concentrations are between 0.14 and 1.4 mg/L. However, hydroxylamino intermediate (2HA6NT) has significantly higher toxicity than 26DNT when the concentrations are in the range of1.4~14mg/L.

The algal toxicity data were also employed to quantify the relative toxicity of various compounds by estimating the 96-hr median effective concentration

(EC50). EC50 is the calculated concentration that results in a 50% growth inhibition of the control. It was estimated by graphical interpolation of a semi-logarithmic plot (Figure 1). The values of EC50s are summarized in Table 3 according to the degradation order for each category of the compounds (i.e., TNT, 24DNT, or 26DNT).

Table 2. Growth inhibition (%) of algae *Selenastrum capricornutum*

Conc. (mg/L)	0.14	0.7	1.4	7.0	14	28
TNT	100.0±2.7a	32.3±3.2c	5.0±1.7c	0c	0c	0b
2HA46DNT	82.7±3.1a	75.7±5.0b	50.0±1.0b	39.7±4.5b	32.7±3.5b	2.3±1.5b
24DHA6NT	102.7±2.1a	93.3±3.5a	75.0±5.0a	5.3±2.3c	0c	0b
26DA4NT	82.0±2.7b	77.7±3.1b	53.7±4.7b	51.3±2.1a	43.3±5.1a	6.0±1.0a
24DNT	82.3±2.5a	78.7±1.2a	75.3±0.6a	29.3±2.1ab	7.0±1.0b	
4HA2NT	85.3±4.7a	81.3±3.2a	59.3±4.0c	21.7±1.5b	9.0±1.7ab	
2A4NT	74.3±3.2a	78.0±2.0a	67.7±2.5ab	31.7±3.8a	11.3±1.5a	
24DAT	82.3±4.9a	79.0±1.0a	62.7±3.8bc	24.0±3.0b	5.3.0±0.6b	
26DNT	85.3±3.1c	71.0±1.0b	63.3±2.5b	29.3±2.1a	8.3±1.5ab	
2HA6NT	53.7±1.5d	77.7±0.6ab	52.0±2.0c	19.3±1.2b	7.3±1.5c	
2A6NT	104.7±1.5a	84.3±4.5a	75.7±1.2a	36.0±5.3a	12.7±1.5a	
26DAT	94.7±4.1b	76.3±3.2ab	67.7±2.3b	29.3±0.6a	7.7±1.5bc	

Data are presented in mean ± standard deviation. At the same concentration of each category of compounds (TNT, 24DNT or 26DNT), means bearing the same letter(s) do not differ significantly ($p < 0.05$).

Table 3. EC50s for various compounds tested with *Selenastrum capricornutum*

TNT and products	EC50 (mg/L)	24DNT and products	EC50 (mg/L)	26DNT and products	EC50 (mg/)
TNT	0.48	24DNT	3.0	26DNT	2.3
2HA46DNT	2.9	4HA2NT	2.2	2HA6NT	1.9
24DHA6NT	2.4	2A4NT	2.8	2A6NT	3.6
26DA4NT	6.0	24DAT	2.3	26DAT	2.7

As can be seen from Figure 1 and Table 3, dose response relationship and toxicity differs significantly among TNT and its transformation products. TNT is the most toxic to the test algae (EC50 = 0.48 mg/L). While it is transformed to 2HA46DNT, toxicity decreased initially, followed by an increase in toxicity due to the formation of dihydroxylamino- intermediate (24DHA6NT). As compared to TNT, the toxicity among dinitrotoluenes and their metabolites only varies

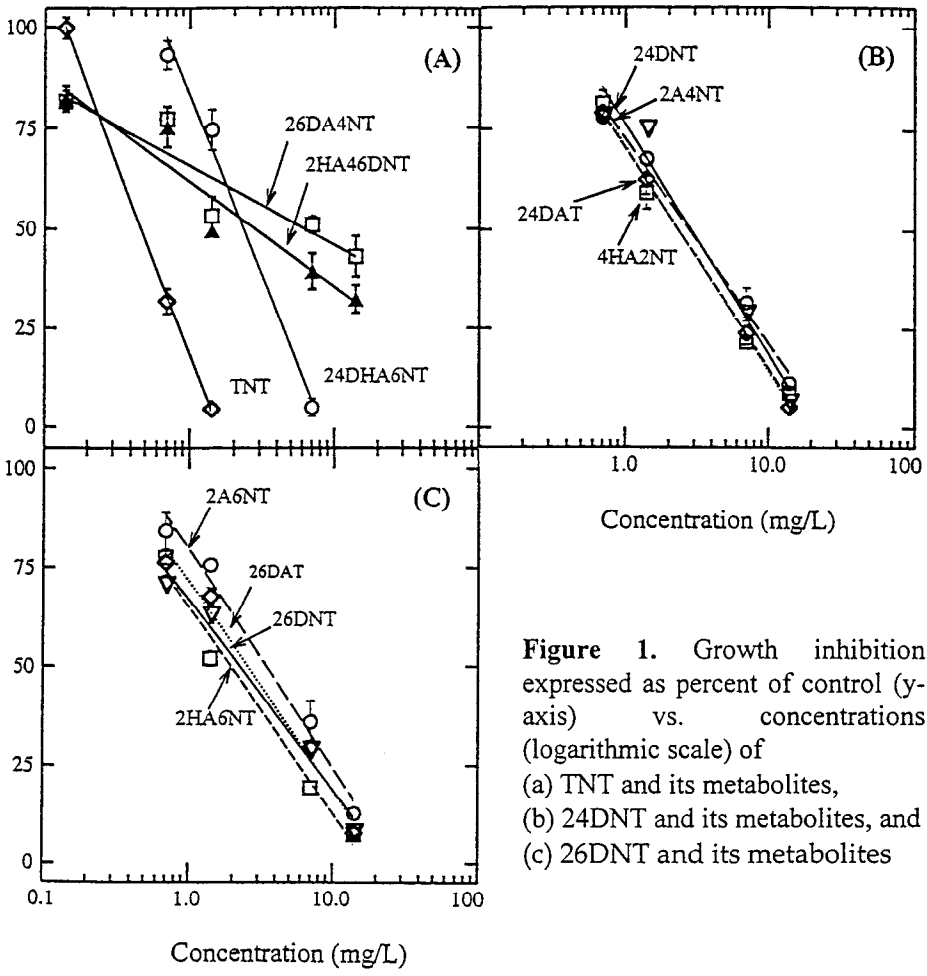

Figure 1. Growth inhibition expressed as percent of control (y-axis) vs. concentrations (logarithmic scale) of
(a) TNT and its metabolites,
(b) 24DNT and its metabolites, and
(c) 26DNT and its metabolites

slightly (Figure 1, Table 3). Despite this small difference, it is interesting to note that hydroxylamino intermediates (4HA2NT and 2HA6NT) have the lowest EC50s (thereby highest toxicity) among the parent compound and all the metabolites tested, an indication of increased toxicity after the microbial degradation of dinitrotoluenes.

It was noted that TNT at a concentration of 1.4 mg/L bleached cells and resulted in 95% of the growth inhibition as compared to the control group. In a similar study using the same algae species, Won et al. (1976) demonstrated that TNT at a concentration of 2.5 mg/L suppressed algal growth as well as some morphological changes, including evoked chlorosis, ballooned and granulated cells. 24DNT and 26DNT appear to be less toxic than TNT to the test algal species, since the EC50s for both dinitrotoluenes were much lower than 'TNT. The relative toxicities between two isomers of dinitrotoluenes are in agreement

with those earlier studies by Liu et al. (1984), who found that the 96-hr LC50s for fathead minnow were 32.8 mg/L for 24DNT and 18.5 mg/L for 26DNT.

The toxicity profile of hydroxylamino compounds is of importance in designing and implementing bioremediation systems for explosives compounds. In an early study by Won et al. (1976), two similar hydroxylamino compounds (2,6-dinitro-4-hydroxylaminotoluene and 2,4-dinitro-6-hydroxylaminotoluene) at a concentration of 5 mg/L did not show toxic and mutagenic effects. Results from this study, however, indicated that 2HA46DNT, 24DHA6NT, 4HA2NT and 2HA6NT at a concentration of 1.4 mg/L inhibited algal growth by 50 ~ 75% of the control. Unlike arylamines, both mono- and hydroxylamino- intermediates exhibit high toxicity to algae. It is therefore important to note that, even though their parent compounds have been degraded, the accumulation of these hydroxylamino intermediates in the environment or remediation systems may still represent a concern.

In summary of this work, the following major conclusions can be drawn: (1) The toxicity of three parent compounds to *the* test species *Selenastrum capricornutum* are in the order of TNT > 26DNT > 24DNT. (2) All TNT metabolites were less toxic than TNT, the relative algal toxicity of TNT and its major products appears to be: TNT > 24DHA6NT > 2HA26DNT > 26DA4NT (Table 3); An exception of this toxicity profile is when TNT at the lowest concentration tested (0.14 mg/L) and 24DHA6NT at the high concentrations (7 ~ 28 mg/L). (3) With regard to 24DNT and 26DNT, the toxicity difference between the parent compound and its metabolites are less apparent than TNT. Hydroxylamino- intermediates are slightly more toxic than amino- intermediates and the parent compounds.

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