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TOXICITY OF THE EXPLOSIVES 2,4,6-TRINITROTOLUENE, HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE, AND OCTAHYDRO-1,3,5,7-TETRANITRO-1,3,5,7-TETRAZOCINE IN SEDIMENTS TO *CHIRONOMUS TENTANS* AND *HYALELLA AZTECA*: LOW-DOSE HORMESIS AND HIGH-DOSE MORTALITY

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Abstract—The toxicity of the explosives 2,4,6-trinitrotoluene (TNT); hexahydro-1,3,5-trinitro-1,3,5-triazine (royal demolition explosive [RDX]); and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (high-melting explosive [HMX]), was evaluated in spiked sediment with two freshwater invertebrates. The midge *Chironomus tentans* and the amphipod *Hyalella azteca* demonstrated significant toxic effects after exposure to TNT and its degradation products, 1,3,5-trinitrobenzene (TNB) and 2,4-diamino-6-nitrotoluene (2,4-DANT). Significant reductions in survival of *C. tentans* exposed to TNT, TNB, and 2,4-DANT were observed at nominal sediment concentrations as low as 200 mg/kg. *Hyalella azteca* was more sensitive to TNT, TNB, and 2,4-DANT than the midge, where significant reductions in survival were observed at nominal concentrations of 50, 100, and 200 mg/kg, respectively. Survival of the midge and the amphipod was unaffected after exposure to RDX or HMX at the highest concentrations of 1,000 and 400 mg/kg, respectively. Growth of the midge, measured as total weight, was significantly reduced by 2,4-DANT. However, significantly increased growth was observed after exposure to sublethal concentrations of RDX and HMX. Although significant reductions in amphipod survival were observed at high concentrations of TNB, growth was significantly increased at sublethal concentrations. The results of the current investigation suggest that organisms exposed to explosives at contaminated sites may be affected at concentrations less than 25 mg/kg through hormetic growth enhancement and at higher concentrations through increased mortality.

Keywords—Explosives Midge Amphipod Sediments Hormesis

INTRODUCTION

Contamination of soils, sediment, groundwater, and surface water with explosives is associated with military activities at ammunition production sites and military training facilities. At ammunition plants, this contamination occurs mainly as a result of contaminated runoff, effluent from the facilities, liquid waste lagoons, and spills [1]. The presence of explosives in environmental media at several production sites, such as the Louisiana Army Ammunition Plant (LA, USA) and the Cornhusker Army Ammunition Plant (NE, USA), has been well characterized. These sites have concentrations of explosives up to 87,000 mg/kg in soil, 711,000 mg/kg in sediment, and 3,375 mg/L in surface water [2–4]. Several military training facilities, including existing active bases and those undergoing closure (base realignment and closure) have contamination resulting from incomplete detonation of rockets, artillery shells, and bombs. Unexploded ordnance can leak material, resulting in elevated concentrations directly around the projectile, whereas incomplete detonation of a projectile results in dispersal of any undetonated explosive around the impact site. Currently, many of these production and use sites are the focus of federally mandated ecological risk assessments and environmental restoration and cleanup activities [5].

The main explosives found as environmental contaminants include 2,4,6-trinitrotoluene (TNT); hexahydro-1,3,5-trinitro-1,3,5-triazine (royal demolition explosive [RDX]); and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (high-melting explosive [HMX]), as well as several degradation products. Degradation products of TNT occur through reduction and oxidation pathways. Reduction of TNT occurs through amination of the nitro groups on the ring, initially forming amino-dinitrotoluene (ADNT), diamino-nitrotoluene (DANT), and lastly triaminotoluene. Photooxidation of TNT forms trinitrobenzaldehyde [6]. Subsequent decarboxylation occurs under aerobic conditions and results in the formation of trinitrobenzene. Most 1,3,5-trinitrobenzene (TNB) contamination occurs through the TNT degradation pathway, although TNB was used historically as a component of explosives and for the vulcanization of rubber [7].

Most explosives contamination occurs and originates in terrestrial systems. However, the high water solubility (6–130 mg/L) and low sorptive properties (TNT K_{OC} = 525) of these compounds result in migration of these contaminants into aquatic systems, including aquatic sediments and surface water during rainfall events [8]. Risk assessments and cleanup activities of contaminated manufacturing and training facilities require extensive exposure and effects data to make accurate and realistic cleanup decisions. For ecological risk assessments, most of the toxicological data used to characterize the effects (reduced survival, growth, or reproduction) of these compounds are for terrestrial organisms and aquatic organisms exposed through water only [1,9,10]. A paucity of toxicity data exists for aquatic organisms exposed to explosive-con-

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taminated sediment. Sediment spiked with ^{14}C-TNT resulted in significant TNT bioaccumulation in a marine polychaete (*Neanthes areneceodentata*) and amphipod (*Leptocheirus plumulosus*) [11]. In addition, Lotufo et al. [12] evaluated the toxicological effects of TNB, RDX, and HMX to marine organisms and observed adverse biological effects on a marine amphipod (*L. plumulosus*) at concentrations as low as 228 mg/kg (TNB) in sediment. Although these studies focused on the toxicity of explosives to marine organisms, no studies have focused on the toxicity of explosives in freshwater sediment systems.

The objective of the current study was to evaluate the toxicological effects of explosives (TNT, RDX, and HMX) to two freshwater organisms, *Chironomus tentans* and *Hyalella azteca*, through a sediment exposure. The two degradation products, TNB and 2,4-diamino-6-nitrotoluene (2,4-DANT), were selected for use in the current investigation because they have been reported to be more toxic than the other degradation products [13]. The current investigation was conducted by spiking sediment and exposing experimental organisms for 10-d, following standard protocols for these species. The toxicological endpoints evaluated were survival and growth. *Chironomus tentans*, a freshwater midge, and *H. azteca*, a freshwater amphipod, were selected for use in this study because of their ecological relevance and wide use for assessing contaminated freshwater sediments [14].

MATERIALS AND METHODS

Chemicals and chemical analysis

Analytical grade TNT (98% pure) was obtained from Chem Service (Westchester, PA, USA). Analytical grade TNB (99% pure) was obtained from Supelco (Bellefonte, PA, USA). The 2,4-DANT (>98% pure) was synthesized by SRI International (Menlo Park, CA, USA). Purified RDX (>98% pure) was obtained from the Naval Surface Warfare Center (Indian Head, MD, USA). The HMX (>98% pure) was obtained from the Holston Army Ammunition Plant (Kingsport, TN, USA). Chemical analysis for TNT (including degradation products), RDX, and HMX in sediment and tissue was conducted by high-performance liquid chromatography by following U.S. Environmental Protection Agency method 8330 [15]. Method detection limits for the analysis of solid samples with this method are 0.1 mg/kg for TNT, TNB, 2,4-DANT, and RDX, and 0.2 mg/kg for HMX. Recoveries for TNT, TNB, and 2,4-DANT in the sediment matrix were 98, 90, and 90%, respectively. Recoveries for RDX and HMX in sediment were 99 and 115%, respectively.

Experimental organisms

The amphipods, *H. azteca*, were cultured at the Engineering Research and Development Center (Vicksburg, MS, USA) with organisms originally obtained from the U.S. Geological Survey Biological Resources Division, Environmental and Contaminants Research Center (Columbia, MO, USA). Species identity has been verified by a genetic differentiation study [16]. Organisms were cultured in flow-through aged dechlorinated tap water and fed flake food (Aquatic Ecosystems, Apopka, FL, USA) and hard maple tree leaves. Juvenile test organisms were collected by methods described by sieving with stacked sieves; organisms passing through a 600-μm sieve and retained on a 425-μm sieve were collected for experimentation [17]. Juvenile organisms collected were estimated to be 10 to 12 d of age based on a length of 2.1 ± 0.2 mm [14]. Test organisms were acclimated to experimental conditions for 24 h before initiation of all experiments.

The midges, *C. tentans*, were obtained as egg masses from Environmental Consulting and Testing (Superior, WI, USA). Upon arrival, eggs were placed in aged dechlorinated tap water and hatched. The *C. tentans* were provided clean sand and fed flake food (Aquatic Ecosystems) daily. Organisms were reared to the second instar based on a head-capsule width of 0.2 mm [14].

Sediment exposures

Sediment exposures (10 d) were conducted following methods outlined by the U.S. Environmental Protection Agency [14]. Sediments used for the experiments were obtained from Brown's Lake at the Engineering Research and Development Center, and were characterized and analyzed for potential contaminants. The sediment was composed of 99.3% silt and clay and 0.7% sand, and contained 0.65% total organic carbon. Chemical analysis indicated that no polycyclic aromatic hydrocarbons (<250 μg/kg) and polychlorinated biphenyls (<12 μg/kg) were detectable. Chlorinated pesticides were less than 4.0 μg/kg. Metals (Ag, As, Cd, Cr, Cu, Pb, and Hg) were at background concentrations. Sediments were spiked with a single toxicant by adding toxicant in 5 ml of acetone to 750 g of whole sediment (42.3% water) in a 4-L beaker and stirring vigorously for 30 min on a stirplate in the dark. Treatments were prepared separately with dilutions of the acetone stock solution. To avoid excessive degradation of the toxicant, samples were collected for analysis and frozen at −80°C and sediment was added to exposure chambers immediately after mixing. Exposure chambers consisted of 300-ml lipless glass beakers containing 50 g of spiked sediment and 175 ml of overlying water. Water was gently added to the surface of the sediment and the spiked sediment was allowed to equilibrate for 24 h before addition of organisms. Treatments were based on previous range-finding experiments (J.A. Steevens, unpublished data). Concentrations were selected based on a geometric progression and each treatment was replicated eight times. After equilibration of the sediment, 10 known-age organisms were added to each beaker. Overlying water in the sediment chamber was renewed with toxicant-free water every 12 h with an Enviro Tox® flow-through bioassay diluter (Easley, SC, USA). Temperature and dissolved oxygen were monitored throughout the 10-d exposure period. Water-quality parameters including hardness, alkalinity, and ammonia were measured in each treatment with a commercially available kit from Lamotte (Chestertown, MD, USA) at the initiation and completion of the exposures. Water-quality parameters were within acceptable limits throughout the experiment: dissolved oxygen, 5.4 to 8.2 mg/L; pH, 7.8 to 8.0; ammonia, <1 ppm; hardness, 100 to 108 ppm $CaCO_3$; and alkalinity, 104 to 116 ppm $CaCO_3$. At the termination of each exposure, surviving organisms were recovered from the sediment by sieving and were saved for growth measures and chemical analysis. In addition, sediments were collected at the end of each exposure for chemical analysis.

Growth measures

Growth was determined for *H. azteca* and *C. tentans* by measuring length and wet weight, respectively. Length of *H. azteca* was measured with a Leica MZ12 dissecting microscope equipped with a digital image analysis system (Sony

Table 1. Concentration of 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitrobenzene (TNB), and 2,4-diamino-6-nitrotoluene (2,4-DANT) in spiked sediments (mg/kg dry wt)[a]

Chemical	Nominal	Measured (mg/kg) Initial	Final
TNT[b]	400	153.3	0.8
	200	66.5	0.0
	100	4.3	0.0
	50	0.1	0.0
	25	0.0	0.0
	12.5	0.0	0.0
TNB[c]	400	7.6	0.0
	200	7.7	0.0
	100	1.1	0.0
	50	0.0	0.0
	25	0.0	0.0
	12.5	0.0	0.0
2,4-DANT[d]	400	110.3	0.0
	200	32.9	0.0
	100	5.8	0.0
	50	0.3	0.0
	25	0.0	0.0
	12.5	0.0	0.0

[a] The method detection limit for TNT, TNB, and 2,4-DANT was 0.1 mg/kg. Recovery for TNT, TNB, and 2,4-DANT was 98, 90, and 90%, respectively.
[b] TNT is the sum of degradation products including 2,4-diamino-6-nitrotoluene, 2,6-diamino-4-nitrotoluene, 2-amino-4,6-ditrotoluene, and 4-amino-2,6-dinitrotoluene.
[c] TNB was determined from an unknown peak closest to 2,4-diamino-6-nitrotoluene and quantified with the 2,4-DANT standard curve.
[d] 2,4-DANT was measured as 2,4-DANT and included no other measurable degradation products.

DXC CCD video camera, Sony, Tokyo, Japan, and Flashpoint frame grabber and Optimas 6.1 image analysis software, Media Cybernetics, Silver Spring, MD, USA). Digital images of live organisms were captured within 24 h of sample collection to avoid use of sample preservatives that could distort the size and shape of the organism. Total body length of *H. azteca* was determined by measuring along the dorsal side from the base

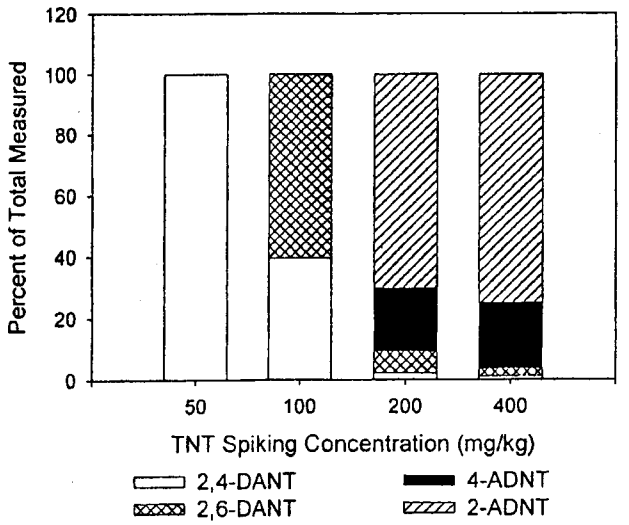

Fig. 1. Percent of total composition by mass of 2,4,6-trinitrotoluene (TNT) degradation products in sediments spiked with TNT. Degradation products measured included 2,4-diamino-6-nitrotoluene (2,4-DANT), 2,6-diamino-4-nitrotoluene (2,6-DANT), 2-amino-4,6-dinitrotoluene (2-ADNT), and 4-amino-2,6-dinitrotoluene (4-ADNT).

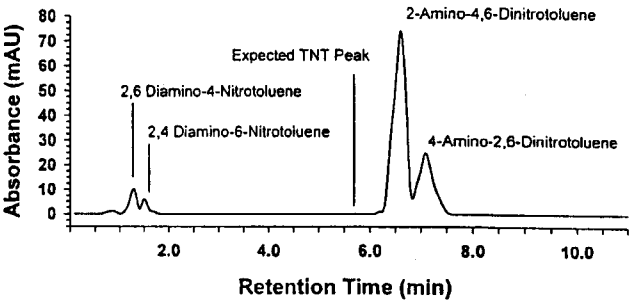

Fig. 2. High-performance liquid chromatography chromatogram from a sample taken from sediment spiked with 2,4,6-trinitrotoluene (TNT) at 400 mg/kg. Where TNT was not detected, degradation products (2,6-diamino-4-nitrotoluene, 2,4-diamino-6-nitrotoluene, 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene) were summed to calculate the amount of 2,4,6-trinitrotoluene equivalent to the degradation products.

of the first antenna to the end of the third uropod [17]. Wet weight of surviving *C. tentans* collected at the termination of the exposures was measured with a Mettler AC245 analytical balance (Mettler-Toledo, Columbus, OH, USA). Organisms within replicates were pooled and weight normalized per organism.

Statistical analysis

Statistical analysis was conducted with Sigma Stat® Ver 2.03 (Jandel Scientific, SPSS®, Chicago, IL, USA). Statistical analysis was conducted with nominal chemical concentration values because of difficulties associated with measuring explosives in sediment. Significant differences in survival and growth of organisms were detected with one-way analysis of variance (ANOVA) and comparisons between treatments were determined with Bonferroni's *t* test. Where results did not have a normal distribution, ANOVA on ranks was used, followed by Dunn's method for multiple comparisons. For the purposes of these experiments a value of $p < 0.05$ was used for testing of significance for all statistical tests. Median lethal concentration (LC50) values were calculated with the trimmed Spearman–Karber method and are represented as mg/kg or mmole/kg (95% confidence interval).

RESULTS

Sediment chemistry

Sediment concentrations of the nitroaromatic compounds and their degradation products were determined from samples collected before addition of the sediment to exposure chambers. Sediment concentrations of TNT, TNB, and 2,4-DANT, shown in Table 1, were consistently less than the nominal concentration. Chemical analysis of sediment samples indicated that TNT parent compound was not present; therefore, breakdown compounds (monoamino- and diamino-nitrotoluenes) were summed to represent total TNT and equivalents (Figs. 1 and 2). Similarly, TNB was not observed in any of the sediment samples. However, an unidentified peak near the 2,4-DANT standard was quantified with the 2,4-DANT standard. Dinitrobenzene was detected in the TNB treatments at low concentrations. Sediments spiked with 2,4-DANT were determined to have 5 to 25% of the nominal concentration. All three nitroaromatic compounds, TNT, TNB, and 2,4-DANT, were not detected in any sediment samples at the end of the 10-d exposure period. Percent recovery of TNT, TNB, and 2,4-DANT was greater than 90% for analytical laboratory

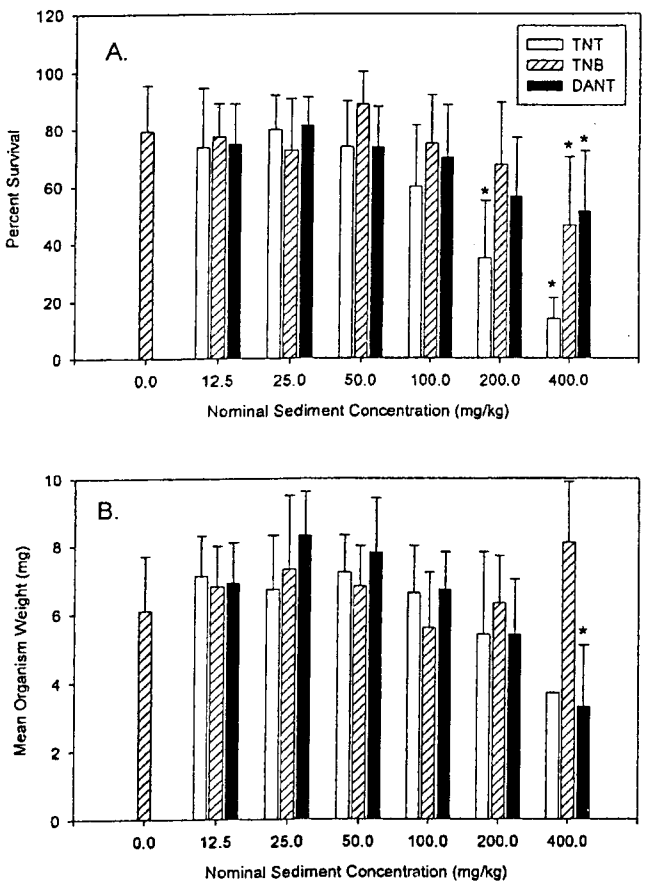

Fig. 3. Percent survival (A) and mean organism weight (B) of *Chironomus tentans* after exposure to 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitrobenzene (TNB), and 2,4-diamino-6-nitrotoluene (DANT). Asterisks indicate treatments that were statistically different from the control response (analysis of variance, Bonferroni's pairwise comparison). Error bars indicate variation as the standard deviation of the mean.

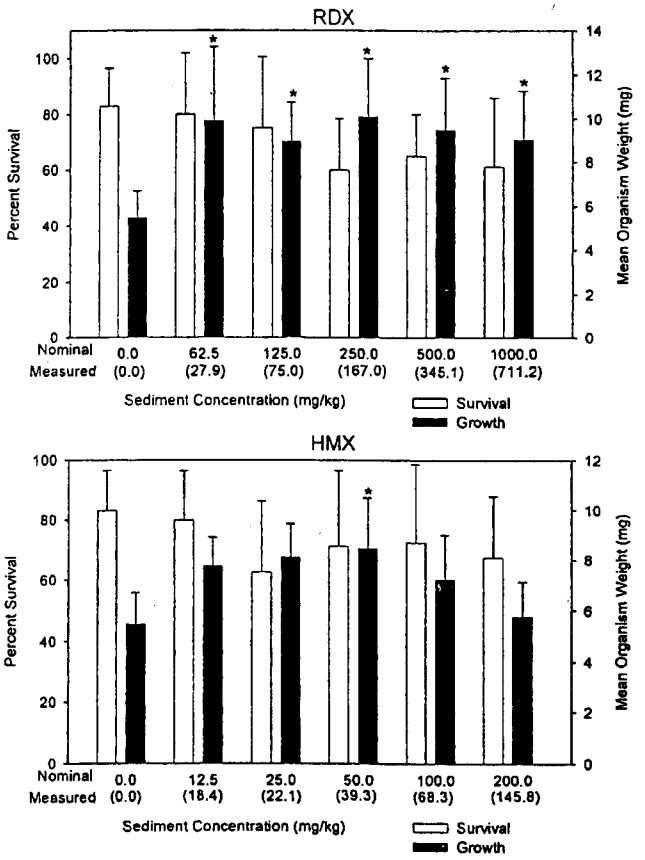

Fig. 4. Percent survival and mean organism weight of *Chironomus tentans* after exposure to hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). Asterisks indicate treatments that were statistically different from the control response (analysis of variance, Bonferroni's pairwise comparison). Error bars indicate variation as the standard deviation of the mean.

sediment–matrix spikes. The change in measured TNT immediately after spiking may be a result of the 30-min mixing period. Furthermore, these data for TNT and its breakdown products do not absolutely demonstrate the absence of the nitroaromatic compounds, but rather the potential for these compounds to degrade rapidly or bind strongly to sediment material and resist solvent extraction. All three nitroaromatic compounds, TNT, TNB, and 2,4-DANT, were not detected in any of the tissue samples, suggesting that in vivo metabolism was rapid, the parent compounds were degraded in the sediment, or the explosives were not bioaccumulated to detectable levels.

Sediment concentrations of the nitro-heterocyclic compounds were determined from samples collected before addition of the sediment to exposure chambers. Sediment concentrations of RDX and HMX corresponding to the nominal concentration were consistently less than expected. Sediment concentrations of RDX were 45 to 71% of nominal concentrations. Measured HMX concentrations were 68 to 79% of nominal concentrations. During the 10-d exposure, RDX and HMX concentrations decreased to 60 and 42% of the nominal concentrations, respectively. Similar to the nitroaromatic compounds, RDX and HMX were not detected in any of the tissue samples, suggesting in vivo metabolism, degradation of RDX and HMX in the sediment, or that the compounds were not accumulated to measurable concentrations.

Midge survival and growth

Survival of midges was significantly reduced in sediments spiked with TNT, TNB, and 2,4-DANT during the 10-d exposure. Midge survival (Fig. 3A) was significantly decreased as compared to the control treatment after exposure to 200 and 400 mg/kg TNT, 400 mg/kg TNB, and 400 mg/kg 2,4-DANT ($p < 0.05$). The LC50 value calculated for TNT was 176 mg/kg (95% confidence interval [CI] 151–205) or 0.78 mmoles/kg sediment. The LC50 values could not be calculated for TNB or 2,4-DANT. Mean organism weight (Fig. 3B) was significantly reduced by 12% after exposure to 400 mg/kg 2,4-DANT as compared to the control treatment. However, TNT and TNB did not result in any significant effects on organism weight. Solvent control treatments were conducted for all midge exposures and weight was not significantly different (within 2.8–9.3%) from control treatments by ANOVA and pairwise comparisons by Bonferroni's *t* test.

Exposure to RDX and HMX produced a significant increase (5–54% increase for HMX; 64–84% increase for RDX) in mean organism weight (Fig. 4). Mean weight of *C. tentans* exposed to RDX ranged from 8.9 to 10.0 mg, as compared to the mean organism weight for control organisms (5.4 mg). In

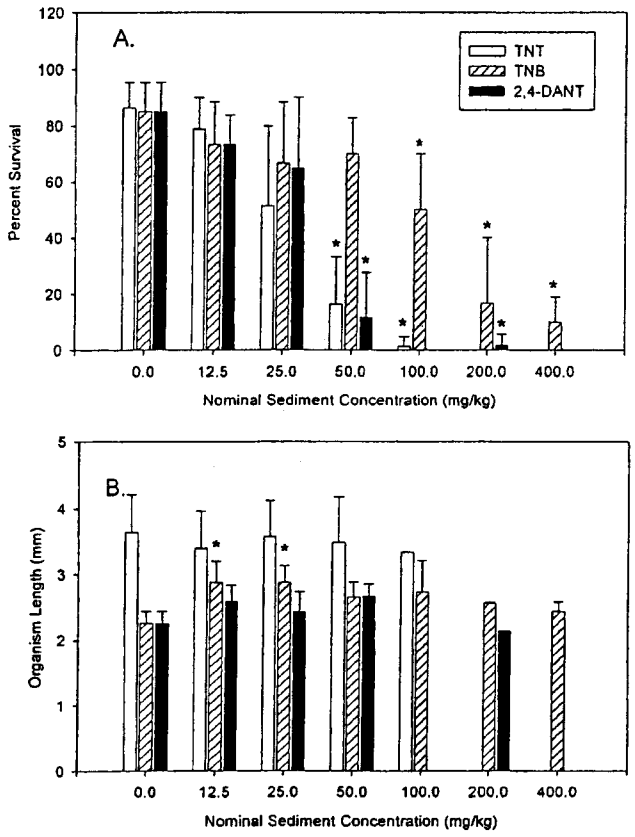

Fig. 5. Percent survival (A) and mean organism length (B) of *Hyalella azteca* after exposure to 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitrobenzene (TNB), and 2,4-diamino-6-nitrotoluene (2,4-DANT). Data are from two experiments with TNT treatments compared to a control, and TNB and 2,4-DANT treatments compared to a control. Asterisks indicate treatments that were statistically different from the respective control response (analysis of variance, Bonferroni's pairwise comparison). Error bars indicate variation as the standard deviation of the mean.

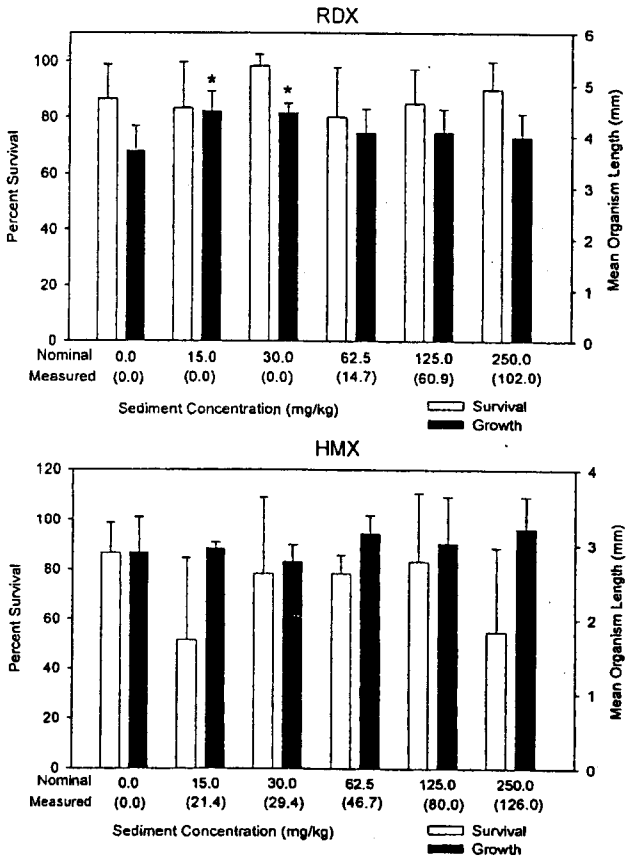

Fig. 6. Percent survival and mean organism length of *Hyalella azteca* after exposure to hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). Asterisks indicate treatments that were statistically different from the control response (analysis of variance, Bonferroni's pairwise comparison). Error bars indicate variation as the standard deviation of the mean.

the same way, mean organism weight was significantly increased at the intermediate treatment concentration of 50 mg/kg HMX (39.3 mg/kg measured); the overall concentration–response curve was bell shaped. Although RDX and HMX had an effect on growth, the explosives did not have a significant effect on survival at concentrations up to 1,000 mg/kg (711.2 mg/kg measured) or 200 mg/kg (145.8 mg/kg measured), respectively.

Amphipod survival and growth

Amphipods were more sensitive to TNT and its degradation products, TNB and 2,4-DANT, than were midges (Fig. 5). Exposure to TNT resulted in a significant concentration-dependent effect on amphipod survival at concentrations at and exceeding 50 mg/kg. Similar to TNT, sediments spiked with 2,4-DANT resulted in a significant decrease in survival at 50 mg/kg and greater. Comparatively, TNB (LC50 100.8 mg/kg, 95% CI 84–121; or 0.47 mmoles/kg) was less toxic than TNT (LC50 28.9 mg/kg, 95% CI 25.8–32.5; or 0.13 mmoles/kg) or 2,4-DANT (LC50 32.1 mg/kg, 95% CI 29.0–35.6; or 19 mmoles/kg). Significantly reduced survival was observed at concentrations of 100 mg/kg and greater for TNB. Sediments spiked with TNT and 2,4-DANT did not affect the length of juvenile organisms at the concentrations examined in this study. However, a significant increase in growth (27%) was observed for organisms exposed to 12.5 and 25 mg/kg of TNB. Solvent control treatments were conducted for all amphipod exposures and length was not significantly different (within 0.8–6.5%) from control treatments by ANOVA and pairwise comparisons by Bonferroni's *t* test.

The nitro-heterocyclic compounds, RDX and HMX, had no significant effects on amphipod survival after a 10-d exposure at concentrations up to 250 mg/kg (102 mg/kg RDX and 126 mg/kg HMX measured; Fig. 6). However, similar to the increase in weight observed in the midge experiments, increased growth was observed for the RDX treatment. Amphipods exposed to 15 and 30 mg/kg RDX (0.0 mg/kg measured) in the sediment were significantly larger than the control treatments (20–21%). Higher concentrations of RDX did not have a significant effect on organism length.

DISCUSSION

Trinitrotoluene and its degradation products, TNB and 2,4-DANT, resulted in adverse effects on survival after a 10-d exposure period. Several studies have demonstrated differential toxicity to TNT and its degradation products [13,18–21]. In this investigation, we observed TNT to be the most potent toxicant to *C. tentans*, whereas TNT and 2,4-DANT were equally potent toxicants to *H. azteca* either expressed in terms of mass or molar quantities. These results are in contrast

to those reported by Liu et al. [18] for water-only exposures to fathead minnows and *Daphnia magna*. In that study, TNB was more toxic to both fathead minnows and *D. magna* as compared to TNT by 2.7- and 4.3-fold, respectively. Griest et al. [13] evaluated the toxicity of TNT and nine degradation products to *Ceriodaphnia dubia*. In water-only exposures of fish and invertebrates to TNT, the degradation products were generally found to be more toxic than TNT itself. However, all of these studies were conducted through water only, which may explain the differences in observed toxicity with the current sediment toxicity study. Furthermore, the differences between the current study and previously reported results could be due to species-specific differences in sensitivity or as a result of differences in bioavailability in the sediment matrix.

In the current study, RDX and HMX did not result in adverse effects at relatively high concentrations (>200 mg/kg) in spiked sediment. Previous studies focusing on RDX and HMX have observed that these nitro-heterocyclics have little effect on survival or other toxicological endpoints such as growth. Bentley et al. [22] conducted an extensive toxicological study with *D. magna*, *Gamarus fasciatus* (amphipod), *Asellus militaris*, and *C. tentans* and found RDX to be nontoxic at concentrations up to 100 mg/L in water during acute exposures and 20 mg/L during chronic exposures. In the same study, HMX was found to be nontoxic to these organisms at concentrations up to 32 mg/L in acute studies and 3.9 mg/L in chronic studies. The results of the current study indicate that RDX and HMX are relatively nontoxic to aquatic invertebrates at concentrations approaching water solubility (RDX water solubility is 42.3 mg/L at 20°C [23]; HMX water solubility is 2.6 mg/L at 20°C [24]) and above the concentrations typically observed in sediment at field-contaminated sites.

Exposure to TNB, RDX, and HMX resulted in nontraditional responses for growth in *C. tentans* and *H. azteca*. Increased growth was observed for *C. tentans* exposed to RDX and HMX and *H. azteca* exposed to RDX and TNB. *Chironomus tentans* exhibited a statistically significant, nearly twofold, increase in growth at all exposure concentrations of RDX (Fig. 4). Likewise, *H. azteca* responded in terms of increased length at the two lowest concentrations of RDX (Fig. 6). A dose-dependent trend of increased growth was observed for midges exposed to HMX. Lastly, amphipods exposed to the lowest concentrations of TNB attained significantly larger size over the 10-d exposure period. Although these results initially appeared to be abnormal and biologically insignificant, the manifestation of this hormetic type of response was observed for three of the explosive compounds in both organisms. Hormetic responses, defined by Calabrese [25], refer to the stimulation of an organism's performance at low concentrations of a compound, when at higher concentrations adverse effects are observed. These responses are suggested to be indicative of adaptation, alter the interpretation of typical dose–response data, and have been associated with population changes in response to the presence of a contaminant [26].

To date, several studies have reported hormetic responses in microbes, algae, daphnids, and fish after exposure to explosives. In a study by Gong et al. [27], an inexplicable increase in microbial nitrogen fixation activity was observed in soils containing 200 and 400 mg/kg TNT. Bailey et al. [28] reported a significant increase in the number of offspring produced by *D. magna* at a sublethal concentration (0.08 mg/L) during a 28-d exposure to TNT. Bentley et al. [22] demonstrated that fathead minnow spawns and the number of eggs released per female were significantly greater than in control treatments during a 240-d exposure to RDX. Eggs produced per female fathead minnow exposed to RDX increased with increasing concentration of RDX and minnows exposed to RDX at 6.3 mg/L produced nearly 3.5 times the number of eggs compared to control treatments. In another study by Bentley et al. [29], the density of *Selanastrum capricornutum* cells increased (6–15%), based on total chlorophyll measures, after exposure to HMX at concentrations ranging from 36 to 572 mg/L.

The mechanism responsible for the positive responses observed in these studies is unknown. However, increased growth or reproduction may be related to mineralization of the explosives, which may provide a nutritional source of nitrogen for bacteria, fungi, or algae. These microorganisms, in turn, provide a food source for the midge or amphipod. To date no studies have been published that have been conducted with explosives to evaluate the plausibility of this mechanism.

A second explanation for the hormetic effects observed with explosives is that the presence and activity of metabolic products of TNT, RDX, and HMX may have a specific direct effect on growth and reproduction. A study evaluating the carcinogenicity of dimethylhydrazine, a metabolic product of RDX and HMX, observed hormetic responses when hepatic DNA damage was measured in rats [30]. This study suggested that a potential compensatory mechanism in the organism may be induced by exposure to this compound. Lotufo et al. [12] found that apoptosis, or programmed cell death, was significantly reduced in two aquatic invertebrates after exposure to TNB, 2,4-DANT, HMX, and RDX. Apoptosis is a genetically controlled mechanism involved in normal cell cycling and cell death. Through an unknown mechanism, these compounds may inhibit apopotosis, resulting in higher level responses (i.e., growth and reproduction). These results suggest a gene-mediated link between low-level exposure to these explosives and the hormetic responses that were observed in the current study.

Chemical analysis of the sediments resulted in measured concentrations that were less than the target nominal concentration for all of the explosives. Similar observations have been reported in other studies with soil or sediment exposure media [12,31]. Two potential processes exist that may explain our inability to recover TNT and degradation products from sediment, including compound degradation and strong binding of the compound to sediment particles. Volatilization is not likely for any of these compounds because of their low Henry's law constant ($<10^{-4}$ atm/m^2/mole). Reductive degradation of TNT has been clearly demonstrated in soils and sediments and likely occurred during the process of sediment spiking and during the exposure period. Elovitz and Weber [32] demonstrated that TNT degrades rapidly in aerobic sediment. In the same study, the ^{14}C-labeled degradation products were sequestered from the aqueous to the sediment phase. The strength of the adsorption was associated with the increasing number of amino to nitro-group substitutions. Adsorption of nitroaromatic compounds occurs mainly to clay particles [33,34] with some covalent binding to humic acids [35]. Sediment used for the current study was 99.3% silt and clay and, therefore, the poor recovery with the standard extraction method is likely to be the result of covalent binding of the explosives to the humic acids and adsorption to clay particles. Although the extractability of the nitroaromatic compounds from the sediment was

low, a sufficient degree of bioavailable chemical was present to produce effects.

The difficulties observed for the sediment analysis were also observed for tissue analysis. Green et al. [11] and Lotufo et al. [12] observed the uptake of ^{14}C-labeled TNT, RDX, and HMX in marine invertebrates exposed to sediment. However, chemical analysis indicated that the parent explosives were not accumulated to a significant or measurable quantity. Studies with plant tissues have demonstrated degradation products of ^{14}C-RDX and ^{14}C-TNT to bind to biomolecules with a molecular weight ranging from 350 to 750 [36]. These studies demonstrated the ability of the organisms to accumulate the radiolabel from the parent compound and for the metabolite to form strong associations with cell molecules. In the current study, no explosives were detected in the tissues of either the midge or amphipod. However, based on the finding of Green et al. [11] and Lotufo et al. [12], these compounds and their products were likely bound to tissue constituents, resisted solvent extraction by acetonitrile, and were present in the extract at concentrations below the detection limits for analytical chemistry methods.

In the current study, adverse effects to freshwater midges and amphipods were observed after exposure to TNT, TNB, and 2,4-DANT. However, RDX and HMX were found to be relatively nontoxic at the concentrations that were evaluated. These results suggest that TNT and its degradation products may pose an ecological threat at sites where sediment contamination exists. In the current study, these effects were observed in treatments where no measurable compounds were present (<50 mg/kg TNB and 2,4-DANT). The hormetic growth responses observed after low-level exposure to TNB, RDX, and HMX would suggest that a population might be enhanced by the presence of these compounds. However, the mechanism responsible for the hormetic response needs to be clearly understood before any conclusions can be made regarding low-dose exposure to explosives. Furthermore, tissue and sediment chemistry did not correspond to expected concentrations and therefore would be poor indicators of exposure. Overall, these results indicate the difficulty associated with estimating the effects associated with a sediment exposure to explosives, particularly with the incidence of nontraditional concentration–response curves. Additional research should be conducted to address the interpretation of these types of responses and to further understand the mechanism and consequences of such a hormetic response.

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REFERENCES

1. Talmage SS, Opresko DM, Maxwell CJ, Welsh CJE, Cretella FM, Reno PH, Daniel FB. 1999. Nitroaromatic munition compounds: Environmental effects and screening values. *Rev Environ Contam Toxicol* 161:1–156.
2. Simini M, Wentsel RS, Checkai RT, Phillips CT, Chester NA, Majors MA, Amos JC. 1995. Evaluation of soil toxicity at Joliet Army ammunition plant. *Environ Toxicol Chem* 14:623–630.
3. Bruns-Nagel D, Schmidt TC, Drzyzga O, von Low E, Steinbach K. 1999. Identification of oxidized TNT metabolites in soil samples of a former ammunition plant. *Environ Sci Pollut Res* 6:7–10.
4. Godejohann M, Preiss A, Levsen K, Wollin KM, Mugge C. 1998. Determination of polar organic pollutants in aqueous samples of former ammunition sites in Lower Saxony by means of HPLC/photodiode array detection (HPLC/PDA) and proton nuclear magnetic resonance spectroscopy (H-1-NMR). *Acta Hydrochim Hydrobiol* 26:330–337.
5. U.S. Department of Defense. 1997. Closed, transferred, and transferring ranges containing military munitions; range rule. Federal Register 32 CFR Part 178. U.S. Army Environmental Center, Washington, DC.
6. Burlinson NE. 1980. Fate of TNT in an aquatic environment: Photodecomposition vs. biotransformation. Technical Report 79-445. Naval Surface Weapons Center, Silver Springs, MD, USA.
7. Barnart RR. 1981. Rubber compounding. In Eller E, Frederick D, Grayson M, Eckroth D, eds, *Kirk–Othmer Encyclopedia of Chemical Technology*, 3rd ed. John Wiley, New York, NY, USA, pp 1031–1036.
8. McGrath CJ. 1995. Review of formulations for processes affecting the subsurface transport of explosives. Report IRRP-95-2. U.S. Army Corps of Engineers, Vicksburg, MS.
9. Yinon J. 1995. *Toxicity and Metabolism of Explosives*. CRC, Boca Raton, FL, USA.
10. Reddy G, Reddy TV, Choudhury H, Daniel FB, Leach GJ. 1997. Assessment of environmental hazards of 1,3,5-trinitrobenzene. *J Toxicol Environ Health* 52:447–460.
11. Green AS, Moore DW, Farrar D. 1999. Chronic toxicity of 2,4,6-trinitrotoluene to a marine polychaete and an estuarine amphipod. *Environ Toxicol Chem* 18:1783–1790.
12. Lotufo GR, Farrar JD, Inouye LS, Bridges TS, Ringelberg DB. 2001. Toxicity of nitroaromatic and cyclonitramine compounds to aquatic invertebrates in exposures to spiked sediments. *Environ Toxicol Chem* 20:1762–1771.
13. Griest WH, Vass AA, Stewart AJ, Ho CH. 1998. Chemical and toxicological characterization of slurry reactor biotreatment of explosives-contaminated soils. Technical Report SFIM-AEC-ET-CR-96186. U.S. Army Environmental Center, Aberdeen, MD.
14. U.S. Environmental Protection Agency. 2000. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. EPA/600/R-99/064. Office of Research and Development, Washington, DC.
15. U.S. Environmental Protection Agency. 1998. Method 8330: Nitroaromatics and nitramines by high performance liquid chromatography (HPLC). In *Test Methods for Evaluating Solid Wastes, Physical/Chemical Methods*. SW-846. Office of Solid Waste, Washington, DC.
16. Duan Y, Guttman SI, Oris JT. 1997. Genetic differentiation among laboratory populations of *Hyalella azteca*, implications for toxicology. *Environ Toxicol Chem* 16:691–695.
17. Ingersoll CG, Nelson MK. 1990. Testing sediment toxicity with *Hyalella azteca* (Amphipoda) and *Chironomus riparius* (Diptera). In Landis WG, van der Schalie WH, eds, *Aquatic Toxicology and Risk Assessment*. STP 1096. American Society for Testing and Materials, Philadelphia, PA, pp 93–109.
18. Liu DH, Bailey HC, Pearson JG. 1983. Toxicity of a complex munitions wastewater to aquatic organism. In Bishop WE, Cardwell RD, Heidolph BB, eds, *Aquatic Toxicology and Hazard Assessment*. STP 802. American Society for Testing Materials, Philadelphia, PA, pp 135–150.
19. Dodard SG, Renoux AY, Hawari J, Ampleman G, Thiboutot S, Sunahara GI. 1999. Ecotoxicity characterization of dinitrotoluenes and some of their reduced metabolites. *Chemosphere* 38: 2071–2079.
20. Smock LA, Stoneburner DL, Clark JR. 1976. The toxic effects of trinitrotoluene (TNT) and its primary degradation products on two species of algae and fathead minnow. *Water Res* 10:537–543.
21. Won WD, DiSalvo LH, Ng J. 1976. Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. *Appl Environ Microbiol* 31:576–580.
22. Bentley RE, Leblanc GA, Hollister TA, Sleight BH. 1977. Acute toxicity of octahydro-1,3,5,7-tetranitro-octahydro-1,3,5,7-tetrazocine (HMX) to aquatic organisms. Final Report. AD A061 730. EG & G Bionomics, Wareham, MA, USA.
23. Sikka HC, Banerjee S, Pack EJ, Appleton HT. 1980. Environmental fate of RDX and TNT. Technical Report 81538. U.S. Army Medical Research and Development Command, Ft. Detrick, Frederick, MD.
24. Spangord RJ. 1980. Environmental fate studies on certain munition wastewater constituents. Final Report SRI LSU-7934. SRI International, Menlo Park, CA, USA.
25. Calabrese EJ. 1994. Primer on BELLE. In Calabrese EJ, ed, *Bi-*

ological Effects of Low Level Exposures: Dose–Response Relationships. CRC, Boca Raton, FL, USA, pp 27–42.
26. Forbes VE. 2000. Is hormesis an evolutionary expectation? *Funct Ecol* 14:12–24.
27. Gong P, Wilke BM, Fleischmann S. 1999. Soil-based phytotoxicity of 2,4,6-trinitrotoluene (TNT) to terrestrial higher plants. *Arch Environ Contam Toxicol* 36:152–157.
28. Bailey HC, Spanggord RJ, Javitz HS, Liu DH. 1985. Toxicity of TNT wastewaters to aquatic organisms. Vol 3—Chronic toxicity of LAP wastewater and 2,4,6-trinitrotoluene. Final Report. AD-A164 282. SRI International, Menlo Park, CA, USA.
29. Bentley RE, Petrocelli SR, Suprenant DC. 1984. Determination of the toxicity to aquatic organisms of HMX and related wastewater constituents. Part III. Toxicity of HMX, TAX and SEX to aquatic organisms. Final Report. AD A172 385. Springborn Bionomics, Wareham, MD, USA.
30. Kitchin KT, Brown JL. 1996. Dose–response relationship for rat liver DNA damage caused by 1,2-dimethylhydrazine. *Toxicology* 114:113–124.
31. Robidoux PY, Hawari J, Thiboutot S, Ampleman G, Sunahara GI. 1999. Acute toxicity of 2,4,6-trinitrotoluene in earthworm (*Eisenia andrei*). *Ecotoxicol Environ Saf* 44:311–321.
32. Elovitz MS, Weber EJ. 1999. Sediment mediated reduction of 2,4,6-trinitrotoluene and fate of the resulting aromatic (poly) amines. *Environ Sci Technol* 33:2617–2625.
33. Pennington JC, Patrick WH. 1990. Adsorption and desorption of 2,4,6-trinitrotoluene by soils. *J Environ Qual* 19:559–567.
34. Haderlein SB, Weissmahr KW, Schwarzenbach RP. 1996. Specific adsorption of nitroaromatic explosives and pesticides to clay minerals. *Environ Sci Technol* 30:612–622.
35. Daun G, Lenke H, Reuss M, Knackmuss HJ. 1998. Biological treatment of TNT-contaminated soil. 1. Anaerobic cometabolic reduction and interaction of TNT and metabolites with soil components. *Environ Sci Technol* 32:1956–1963.
36. Larson SL, Jones RP, Escalon L, Parker D. 1999. Classification of explosives transformation products in plant tissue. *Environ Toxicol Chem* 18:1270–1276.