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THE TOXICITY OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX) TO THE FRESHWATER GREEN ALGA *SELENASTRUM CAPRICORNUTUM*

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Abstract. RDX was not acutely toxic to the green alga *Selenastrum capricornutum* when tested at the solubility limit of the compound in algal assay media. A maximum reduction of 38% in cell density occurred after a 96-h exposure to 36.7 mg L^{-1}; thus, an EC50 could not be determined. The lowest-observed-effect concentration (LOEC) and no-observed-effect concentration (NOEC) for the green alga were 4.8 and 0.5 mg L^{-1} (reduction in cell density), respectively, when chronic end points were used to analyze the data. A comparison of the data in this study with RDX toxicity data from the literature for the diatom *Navicula pelliculosa*, and the blue-green algae (cyanobacteria) *Microcystis aeruginosa* and *Anabeana flos-aquae* shows that *S. capricornutum* is the most sensitive to RDX. A U.S. Environmental Protection Agency (EPA) numerical water quality criterion Final Plant Value based on the green alga data should protect the above algal groups.

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is an explosive used extensively by the military for various applications. The compound may be released to the aquatic environment at munitions plants during manufacturing and blending operations. RDX may also be released to the aquatic environmental at load, assembly, and pack (LAP) plants where the compounds is loaded into bombs and projectiles (Etnier, 1986).

One laboratory study has been conducted on the toxicity of RDX to aquatic algae (Bentley *et al.*, 1977). No EC50s (cell density and chlorophyll *a*) were obtained for four species exposed to nominal RDX concentrations up to 32 mg L^{-1} for 96-h. Sullivan *et al.* (1979) re-analyzed the data obtained by Bentley *et al.* (1977) for the four algal species and concluded that RDX inhibited the growth of the algal species. A field survey at an Army ammunition plant has been conducted to evaluate the inpact of munitions waste effluent on freshwater biotic communities. The survey showed that RDX was present in the discharge from the munitions plant; however, it was not clearly established whether or not RDX caused a detrimental effect to periphyton and phytoplankton communities (Sullivan *et al.*, 1977).

The acute and chronic toxicity of RDX to invertebrates have been studied by Peters *et al.* (1991). They found that RDX was not acutely toxic to several

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species of invertebrates at concentrations near the solubility limit of the compound in water. Chronic toxicity was observed in one of two invertebrates studied by Peters *et al.* (1991). The LOEC and NOEC for a reduction in neonate production in the cladoceran *Ceriodaphnia dubia* were 6.0 and 3.6 mg L^{-1}, respectively. No chronic toxicity was found in a life cycle study of the midge *Paratanytarsus parthenogeneticus*.

In contrast to algae and invertebrates, RDX has been shown to be both acutely and chronically toxic to fish. Acute toxicity 96-h LC50s range from 3.6 mg L^{-1} for juvenile bluegill (*Lepomis macrochirus*) to > 100 mg L^{-1} for fathead minnow (*Pimephales promelas*) embryos (Bentley *et al.*, 1977). Burton *et al.* (1992) conducted a 28-day early life stage test with the fathead minnow and obtained a LOEC and NOEC based on both wet and dry weight of 2.4 and 1.4 mg L^{-1}, repectively.

An interim EPA numerical water quality criterion Final Acute Value of 5.18 mg L^{-1} was established for RDX by Etnier (1986). Appropriate data were not available to establish a Final Plant Value (Stephen *et al.*, 1985).

The objectives of the present study were to (a) verify the potential toxicity of RDX to freshwater algae since the only data available for algae are based on nominal test concentrations and (b) provide quantitative data to establish an EPA numerical water quality criterion Final Plant Value. The green alga *S. capricornutum* was selected for study because it is a 'standard' algal test species and was used in the Bentley *et al.* (1977) study as one of four algal species exposed to RDX.

Materials and Methods

EXPERIMENTAL PROCEDURES

A green alga (*S. capricornutum*) starter culture was obtained from the culture collection at the University of North Texas, Denton, TX. Stock algal cultures were reared in 2.5 L Pyrex culture flasks containing 1 L of sterilized double strength 'AAP' algal assay medium, with sufficient P added to achieve a 20:1 N:P ratio as described in Miller *et al.* (1978). Cultures were maintained in a constant temperature incubator under constant cool-white fluorescent lights ($\approx$ 4.75 W m^{-2}; $\approx$ 300 foot candles) at a temperature of 20 ($\pm$ 1) °C on a shaker table oscillating at 100 ($\pm$ 10%) rpm.

The toxicity (96-h EC50 for cell density) of RDX to the green alga was determined by the procedures given in EPA Toxic Control Substance Act (TSCA) Method 797.1060 (U.S. EPA, 1985 and 1986). Deviations from the test method are discussed where appropriate. The 96-h EC50 algal test for growth is considered to be an acute test by EPA's TSCA Office. EPA's Office of Research and Development considers the test to be a chronic test for determining the toxicity of effluents (Horning and Weber, 1985; Weber *et al.*, 1989) as do other investigators for evaluating single chemicals (for ex., see Hughes *et al.*, 1988). We analyzed the data using both acute and chronic test end points so acute and chronic comparisons could be made with the limited RDX data base.

The nutrient medium used for the bioassay was the same medium used for culturing the organism. Algal test solutions were prepared by dilution of the stock solutions with filter sterilized assay media within a sterile transfer room. Test solutions (100 mL total volume) were dispensed into 250 mL Delong flasks and inoculated with *S. capricornutum* cells in log growth to achieve a density of ≈ 10 000 cells mL^{-1}. Triplicates were prepared for each treatment. The flasks were placed on a shaker table in an incubator set at the culturing conditions described above; the mean temperature during the bioassay was 20.0 (Range = 19.9 to 20.1) °C. Growth measurements (cell density) were made from all replicates in each treatment at 0, 24, 48, 72, and 96-h. Algal cell density was determined from a 1 mL sample with a Model ZBI Coulter Counter (Coulter Electronics Inc., Hialeah, FL). The instrument was calibrated with each use via hemocytometer counts.

TOXICANT

RDX (CAS No. 121-82-4) was obtained from the Health Effects Research Division of U.S. Army Biomedical Research and Development Laboratory (USABRDL), Ft. Detrick, Frederick, MD. The compound was recrystallized to a purity of > 99% as measured by HPLC and stored in amber bottles in the dark at room temperature. A saturated stock solution of RDX was prepared for the algal test by adding RDX to algal assay medium, stirred at room temperature for 24-h, and filter sterilized prior to test initiation.

ANALYTICAL CHEMISTRY

Aqueous concentrations of RDX were determined by the isocratic HPLC method of Brueggeman (1983) with minor exceptions in the operating conditions as described below. A Waters HPLC system (Waters Associates, Milford, MA) was used which consisted of the following components: dual M45 pumps with Model 680 gradient controller, Model 780 data module (integrator), U6K injector, Model 481 variable wavelength UV detector, Z-Module radial compression column system, and Model 712 Waters intelligent sample processor (WISP). A Waters μBondapak C_{18} reverse phase column was used. The mobile phase, delivered at a rate of 1.0 mL min^{-1}, was 55% methanol: 45% water. The effluent was monitored at 240 nm, 0.1 absorbance units full scale (a.u.f.s.). The injection volume was 2–200 μL depending on the concentration of material in solution.

HPLC grade methanol was used; the water was deionized glass distilled. Standard solutions of RDX were prepared by dilution of a stock solution freshly prepared each day of analysis. Precision and accuracy analyses of the HPLC method were conducted prior to the start of the bioassay. Precision was evaluated by injecting a sample three times on three separate days. Mean relative standard deviations for the low and high concentrations of RDX routinely used to generate standard curves for evaluating test concentrations were 2.01 and 0.43%, respectively. The accuracy of the method was evaluated by quantifying the concentration of RDX in

an unknown sample prepared by an independent chemist. Accuracy, expressed as percent error, was 0.93%.

The aqueous samples were injected directly into the HPLC after filtration to romove particles > 0.45 μm. RDX concentrations were measured at the beginning and end of the test (i.e., 0 and 96-h) in all replicates at each treatment. The analytical measurements were made the day the samples were taken from the sample flasks. RDX concentrations decreased slightly from the start of the test to the end of the test at 96 hours. The average decrease for all treatments was 4.7%; the range was 2.6 to 8.0%.

Statistical Analyses

All concentration data used in the statistical analyses were mean measured concentrations. The test end point for the toxicity of RDX to the green alga was growth, measured as cell density (cells mL^{-1}). An acute 96-h EC50 for reduction in algal growth could not be calculated because a maximum reduction of 38% occurred in cell density at the solubility limit of the compound in algal assay media (see below).

The LOEC and NOEC were determined by a t-test with Bonferroni adjustment of error rate because the number of replicates was not constant among treatments. One of the three control replicates was statistically eliminated as an outlier (decision beased on a regression analysis and comparison of residuals) and was not included in the growth calculations and subsequent statistical analyses. The Bonferroni t-test consists of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison test for comparing each of the treatment means with the control mean. The assumptions upon which the use of the Bonferroni t-test are contingent are that the observations within treatments are independent and nomally distributed, with homogeneity of variance. The chi-square test for normality and Bartlett's test for homogeneity of variances were performed before the Bonferroni t-test was used. The above statistical tests were performed using Toxstat (Gulley *et al.*, 1989). A minimum probability level of 0.05 was used.

Results and Discussion

The toxicity data for *S. capricornutum* exposed to RDX are given in Table I. A maximum reduction of 38% in cell density occurred after a 96-h exposure to 36.7 mg L^{-1}; thus, an EC50 could not be determined. The maximum reduction in cell density occurred at the solubility limit of RDX in algal assay media at the assay temperature of 20 °C. The saturation concentration of 36.7 mg L^{-1} obtained in this study in algal assay media is slightly lower than the values reported in the literature for aqueous solutions. Sikka *et al.* (1980) and the U.S. Army (1984) reported saturated concentration values at 20 °C of 42.3 and 50 mg L^{-1}, respectively.

Bentley *et al.* (1977) studied the acute toxicity of RDX to the green alga *S. capricornutum*, the diatom *N. pelliculosa*, and the blue-green algae (cyanobacteria)

TABLE I

Green alga mean cell density (cells mL^{-1}) after 96 hours of exposure to RDX

Conc. ($mg\ L^{-1}$)	Rep	Mean cell density				
		0-h	24-h	48-h	72-h	96-h
control	1	10 699	16 256	26 597	83 213	269 507[a]
	2	9 093	13 395	50 195	220 883	1 044 928
	3	9 568	14 571	63 989	261 008	1 186 997
0.47	1	8 477	11 656	91 688	220 040	989 500
	2	9 523	13 419	48 744	215 171	1 003 753
	3	8 848	12 037	47 064	237 661	1 033 027
4.81	1	10 600	13 269	56 651	259 448	901 000[b]
	2	10 317	15 328	46 224	176 947	802 913
	3	10 203	16 043	46 181	160 723	787 680
7.95	1	10 227	22 896	46 475	215 797	928 147[b]
	2	10 531	17 216	59 595	270 280	1 017 213
	3	10 219	13 389	49 837	232 611	915 880
13.02	1	10 707	16 339	56 029	229 739	895 780[b]
	2	11 493	12 736	53 909	252 917	847 627
	3	9 643	13 104	49 304	232 821	840 587
21.80	1	12 144	12 024	55 877	189 949	670 067[b]
	2	11 163	15 091	48 888	181 339	683 847
	3	10 651	17 109	46 363	204 784	769 940
36.69[c]	1	13 624	14 709	52 251	214 413	767 220[b]
	2	10 693	15 664	49 152	187 171	638 587
	3	9 520	14 197	48 056	183 629	662 040

[a] Replicate 1 of the control was treated as an outlier (decision based on a regression analysis and comparison of residuals) and was not included in the growth calculations and subsequent statistical analyses.

[b] Mean cell density of the three replicates was significantly different from the mean cell density of the control at alpha = 0.05.

[c] Saturation concentration at 20 °C.

M. aeruginosa, A. flos-aquae. No 96-h EC50s for cell density or chlorophyll *a* could be determined for the four species exposed to nomial concentrations up to 32 mg L^{-1}. Thus, Bentley *et al.* (1977) concluded that RDX had no effect on the four species exposed for 96-h to 32 mg L^{-1} (nominal). Harvey *et al.* (1991) found that concentrations of RDX up to 10 mg L^{-1} (highest concentration tested) did not appear to affect the growth of the dicotyledon *Phaseolus vulgaris* (bush bean)

when grown for 7 days under hydroponic conditions; a 10-fold uptake of RDX did occur in the leaves.

Statistically significant ($\alpha = 0.05$) reductions in cell density were observed when chronic end points were used in the analysis of the data in this study. The LOEC and NOEC for reduction in growth were 4.8 and 0.5 mg L^{-1}, respectively. The NOEC is approximately an order of magnitude lower than the LOEC. It should be noted that in chronic studies, the NOEC is always dependent on the choice of exposure concentrations (usually a dilution factor of 50-60%) since the calculations involved are not based on a point estimate from a response curve but on 'hypothesis' tests of specific test concentrations. The actual 'safe' or 'no effect concentration' falls somewhere between the LOEC and NOEC determined in any given study. The further the test concentrations are apert, the less exact is the estimate. Thus, it is possible that the NOEC may not be as low as that determined in this study if a smaller concentration interval had been used in the experimental concentration series.

In the Bentley *et al.* (1977) study of RDX toxicity to four algal species, the investigators reported that there was no significant ($\alpha = 0.05$) difference in cell density or chlorophyll *a* for any of the species based on probit EC50 analyses. Bentley *et al.* (1977) did not analyze their data for any chronic end points. As was the case in the present study for cell density, reductions in cell density and chlorophyll *a* concentrations occurred for various species in the Bentley *et al.* (1977) study. Sullivan *et al.* (1979) reanalyzed the nominal concentration data of Bentley *et al.* (1977) and found that RDX significantly ($\alpha = 0.05$) inhibited growth in all species and to a lesser extent chlorophyll *a* concentrations in all four species. Based on the analyses of Sullivan *et al.* (1979) and the raw data of Bentley. *et al.* (1977), the LOEC (cell density) for *A. flos-aquae* and *N. pelliculosa* would be 10 mg L^{-1} (nominal) and 32 mg L^{-1} (nominal) for *M. aeruginosa*; no LOEC can be determined for *S. capricornutum*. The NOECs for reduction in growth as reported by Sullivan *et al.* (1979) were < 0.32 mg L^{-1} (nominal) for the green alga; 3.2 mg L^{-1} (nominal) for the diatom and *A. flos-aquae* and 10 mg L^{-1} (nominal) for *M. aeruginosa*.

Sullivan *et al.* (1979) determined that the NOEC for *S. capricornutum* was < 0.32 mg L^{-1} (nominal). They point out with regard to the green alga that the inhibition of growth and chlorophyll *a* at the concentration of 0.32 mg L^{-1} (nominal) in the Bentley *et al.* (1977) study were only 2 and 3%, respectively. Sullivan *et al.* (1979) state that the statistical methods they used in their analyses were extremely sensitive and that the small change of 2 to 3% which was statistically significant may not prove to be biologically significant. The statistical methods used in the present study are apparently more conservative than those used by Sullivan *et al.* (1979). We could not detect a 9.6% reduction in growth between the cells exposed to 0.5 mg L^{-1} and the control cells. Based on the LOEC (cell density) of 4.8 mg L^{-1} and NOEC of 0.5 mg L obtained in this study, the NOEC of < 0.32 mg

L^{-1} (nominal) reported by Sullivan *et al.* (1979) appears to be low for the green alga.

The data in the Bentley *et al.* (1977) study and subsequent reanalysis by Sullivan *et al.* (1979) are based on nominal RDX concentrations; therefore, the toxicity values should be viewed with some caution. Although the toxicity values are based on nominal concentrations, the data show that some species are more sensitive than others to RDX exposure. Based on the analysis of Sullivan *et al.* (1979), the green alga *S. capricornutum* is the most sensitive species; the blue-green alga *A. flos-aquae* and the diatom *N. pelliculosa* are intermediate in sensitivity followed by the blue-green alga *M. aeruginosa* which is the most tolerant. The bush bean (*P. vulgaris*), when grown under hydroponic conditions, also appears to be more tolerant to RDX relative to *S. capricornutum*; 10 mg L^{-1} (highest concentration studied) had no toxic effect on the bush bean after a 7-day exposure. The quantitative data obtained in this study for the green alga can be used for the EPA numerical water quality criterion Final Plant Value. The criterion based on the green alga data should protect the algal groups studied by Bentley *et al.* (1977).

The toxicity of RDX to naturally occurring aquatic periphyton and phytoplankton communities is not clear. Sullivan *et al.* (1977) conducted an aquatic impact study at the Holston Army Ammunition Plant (Kingsport, TN) and found effects on periphyton in water containing RDX at concentrations as low as 20 μg L^{-1} in the Holston River. However, they could not attribute the effects observed to RDX because other munitions and conventional contaminants were present in the effluent discharged by the plant. Concentrations of RDX as high as 1.9 and 1.7 mg L^{-1} were found in wastewaters from Areas A and B, respectively, of the Holston Army Ammunition Plant by Stilwell *et al.* (1977).

In summary, the lack of RDX acute toxicity to the green alga *S. capricornutum* was confirmed. RDX is toxic to the green alga when chronic end points are used to analyze the data. The LOEC and NOEC, based on cell density, were 4.8 and 0.5 mg L^{-1}, respectively. Of four algal species studied to data, *S. capricornutum* is the most sensitive species; the blue-green alga *A. flos-aquae* and the diatom *N. pelliculosa* are intermediate in sensitivity followed by the blue-green alga *M. aeruginosa* which is the least sensitive. The quantitative data obtained in this study for the green alga can be used for the EPA numerical water quality criterion Final Plant Value. A criterion based on the green alga data should protect the above algal groups. It should be pointed out, however, that a criterion based on the green alga may not necessarily protect periphyton.

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