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3. Comparative Toxicity of Trinitrotoluene and Aminodinitrotoluenes to *Daphnia magna*, *Dugesia dorotocephala*, and Sheep Erythrocytes

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2,4,6-Trinitrotoluene (TNT) and compounds associated with its production are toxic and phototoxic to a wide range of biota. The planarian *Dugesia dorotocephala*, but not *Daphnia magna*, metabolized TNT (1 mg/liter) to 4-amino-2,6-dinitrotoluene (4A; 0.4 mg/liter) and 2-amino-4,6-dinitrotoluene (2A; 0.2 mg/liter). Coexposure to near-ultraviolet (nuv) light enhanced the toxicity of 2A more than that of TNT and 4A. The toxicities of TNT, 4A, and 2A to *Du. dorotocephala* were all decreased by glutathione (GSH) conjugation. This suggests that all had mechanisms of toxic action involving formation of quinone–GSH conjugates. Dark and light mechanisms for TNT and 2A depended on GSH conjugation, but the specific mechanisms may be different for each compound. The dark and light mechanisms of toxic action for 4A appeared to be fundamentally different in that the dark toxic mechanism of action was less dependent on GSH conjugation. Hemolysis studies using sheep erythrocytes showed that the light-enhanced toxic mechanism of action for TNT, 2A, and/or 4A did not involve cellular membrane damage in response to nuv-induced anions. © 1994 Academic Press, Inc.

2,4,6-Trinitrotoluene (TNT) has been produced as an explosive for over 100 years (Zitting *et al.*, 1982). TNT and compounds associated with its production are toxic to a wide range of biota (Johnson *et al.*, 1994). TNT and related compounds are poorly soluble in water (95 mg/liter) and persistent in the environment, where they tend to concentrate in soil/sediments (Hoffsommer and Rosen, 1973). Environmental contamination with TNT wastes has been associated with the loss of entire biological communities (Palazzo and Leggett, 1986; Putnam *et al.*, 1981).

One may anticipate that the actual risk associated with the poorly water-soluble TNT/analogues to aquatic species to be minimal. However, recent studies have demonstrated that low concentrations (1 μg/ml) of TNT/analogues were toxic to *Daphnia magna* (water flea) and embryos of *Lytechinus variagatus* (sea urchin) (Davenport *et al.*, 1994), and to *Photobacterium phosphoreum* (Johnson *et al.*, 1994) when coexposed to near-ultraviolet light (nuv; 336–400 nm). nuv has been used for phototoxicity studies since it is the component of sunlight which penetrates up to a depth of 12 m in clear lake water (Holst and Giesy, 1989). The release from lake sediments of trace amounts

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of poorly water-soluble hydrocarbons, initiated by dredging operations or ship traffic, into the water column and concomitant exposure to sunlight or artificial exposure to nuv light is toxic to aquatic life (Allred and Giesy, 1985; Bowling *et al.*, 1983; Davenport and Spacie, 1991; Foran *et al.*, 1991; Holst and Giesy, 1989; Kagan *et al.*, 1985; Morgan and Warshawsky, 1977a,b; Newsted and Giesy, 1987; Oris and Giesy, 1985, 1986, 1987).

The nuv enhancement of TNT toxicity was anticipated since the photolysis of TNT initiated by wavelengths of light >280 nm produces an anion intermediate (Burlinson *et al.*, 1979; Suryanarayanan and Capellos, 1974). nuv coexposure is expected to have a similar effect on the toxicities of the major metabolites of TNT, 2-amino-4,6-dinitrotoluene (2A), and 4-amino-2,6-dinitrotoluene (4A), since their chemical structures and absorption maxima are very similar to those of TNT.

The authors postulated that the light-enhanced toxic mechanism of action for TNT, 4A, and/or 2A could be explained by cellular membrane damage in response to nuv-induced anions. The primary objective was to compare the dark and nuv coexposure toxicities of TNT, 2A, and 4A to *Da. magna* (daphnia), the asexual planarian *Dugesia dorotocephala*, and sheep red blood cells (SRBC). Additional objectives were to establish if planaria and/or daphnia metabolized TNT, determine if TNT, 2A, or 4A produced tumors in intact or regenerating planaria or developmental abnormalities in regenerating animals, and determine the role of glutathione conjugation in the toxicity of TNT, 2A, and 4A to planaria. α-Terthienyl, a compound which is known to induce cellular membrane damage with nuv coexposure (Downum *et al.*, 1982), was selected as a positive control for all bioassays. An understanding of the light and dark toxicity of TNT and related compounds in different phylla can enhance the assessment of the environmental hazard posed by these compounds.

EXPERIMENTAL METHODS

Organisms and Test Solutions

Du. dorotocephala (Carolina Biological Supply, Gladstone, OR) were held at 19°C with a 12:12 light:dark cycle and maintained/tested in a synthetic medium (Schaeffer *et al.*, 1991). Groups of 20 planaria (weighing between 10 and 15 mg each; 2.5 to 3.0 cm in length) were weighed as a group. The immature *Da. magna* used for testing were a gift from the laboratory of Dr. Ann Spacie (Purdue University). Well water from Dr. Spacie's laboratory was saturated with oxygen by aeration prior to being used for testing or maintenance of the daphnia. The daphnia were stored in the laboratory under light and temperature conditions similar to those for the planaria.

TNT, 2A, and 4A are poorly soluble in water so stock solutions containing 10,000 and 1000 mg/liter of compound were prepared in absolute ethanol (EtOH). Test solutions and controls were prepared by adding an appropriate volume of the stock solution to medium (planaria) or well water (daphnia). Final solutions were <0.5% EtOH, and those with <50 mg/liter compound did not contain EtOH. Planaria were exposed in 400-ml beakers containing 200 ml of solution and the daphnia were exposed in 100-ml beakers containing 50 ml of solution. A black line placed on each beaker demarcated the original liquid level, and distilled water was added during the test period to maintain this level. The pH of each solution was adjusted to 7.4 with 0.1 *N* HCl or 0.1 *N* NaOH prior to placing the planaria or daphnia into the beakers.

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Exposure Conditions

nuv coexposure experiments used planaria and daphnia which had been incubated with the compound for 12 hr in the dark to allow the parent compound to pass into the organisms. A dark preincubation period had previously been found to be required to observe the light-enhanced toxicity of TNT-related compounds in daphnia (Davenport *et al.*, 1994) and planaria (Arfsten and Schaeffer, unpublished).

Tests were carried out under normal laboratory lighting conditions (dark) and with nuv coexposure (light). L-Buthionine-*S,R*-sulfoximine (BSO; Schweitzer–Hall, South Plainfield, NJ) is a glutathione (GSH) synthesis inhibitor. Planarian groups included those not exposed to BSO, preexposed to 1 m*M* BSO for 48 hr, and preexposed to 1 m*M* BSO for 48 hr and then continuously coexposed to 1 m*M* BSO and the compound for 96 hr. Daphnia (which lack GSH) were not exposed to BSO. Planarian medium ± BSO ± nuv or well water ± nuv controls were concurrently tested. nuv was produced using a General Electric F20T12-BLB, 20-W light bulb (emission maximum, 354 nm); wavelengths <312 nm were filtered out by Mylar film (5 mil). The intensity of light reaching the surface of the fluid in the beakers was measured by an uv intensity meter (Model J-221, Longwave UV Meter, BLAK-RAY) and the distance between the light and beaker was adjusted to yield a constant intensity of 400 $\mu W/cm^2$. All groups were maintained on a 12:12 dark:light (incandescent light) photoperiod but the nuv coexposure groups were also irradiated with nuv light during their 12-hr incandescent light cycle. All medium and test solutions were replaced with fresh solutions every 24 hr.

nuv Stability of TNT, 4A, and 2A

Duplicate samples of TNT, 4A, or 2A (5 mg/liter; pH 7.4) in well water were protected from light for 48 hr, or exposed to 400 $\mu W/cm^2$ nuv for 12 hr, or exposed to nuv for 48 hr. The solution was then extracted with toluene (Almog *et al.*, 1983) and quantitated using a capillary gas chromatography (GC) (Model Sigma 2B, Perkin–Elmer, Norwalk, CT) fitted with a J&W Scientific (Folsom, CA) DB-5, 30 m × 0.25 mm i.d. capillary column and a nitrogen phosphorus detector (NPD) operating at 275°C injection temperature, 300°C oven temperature, an isothermal column temperature of 200°C, helium gas carrier with a 37 cm/sec flow rate, and a run time of 30 min. Well water spiked with each compound at 20, 50, 75, or 100 mg/liter and extracted with toluene was used to establish a standard curve for each compound based on peak area. The well water used to prepare the solutions was also extracted and assayed by GC to ensure the absence of organic contaminants.

Lethality Studies

The LC_{50} was determined from at least two separate experiments consisting of four concentrations, with duplicate beakers of 20 planaria or 50 daphnia for each concentration. Planarian death was determined by the visual observation of a prolonged lack of movement, severe shape changes, and/or physical breakdown. Daphnid death was determined by the observation of a prolonged lack of movement and the absence of a heartbeat. Planaria and daphnia presumed to be dead were removed from their respective test or control media, placed in fresh media, and observed for recovery. The planaria and daphnia were observed frequently during the first 12 hr of exposure and dead animals were removed and counted. All groups were monitored frequently

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Survival Time Studies

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Tumorigenicity to Pla

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until the end of the 96-hr exposure period. The endpoint was the number of dead daphnia or planaria at 96 hr. The trimmed logit method (Sanathanan *et al.*, 1987) was used to estimate the LC_{50} and confidence interval (CI). The slopes and intercepts of corresponding light and dark response curves were compared by analysis of covariance (ANCOVA).

Survival Time Studies

Experimental conditions were similar to those used for the 96-hr mortality tests. Groups of 20 daphnia or planaria were exposed for 48 hr and dead animals were counted and removed every 4 hr. Planarian tests used 5 mg/liter of TNT or 4A, or 10 mg/liter of 2A in medium. Daphnia tests used 5 mg/liter of TNT, 4A, or 2A in well water. Three groups were tested at each concentration: 48-hr dark exposure, 12-hr nuv preexposure of the test solution followed by a 48-hr incubation of planaria in the dark to the irradiated solutions, and 48-hr nuv coexposure with replacement of the test solution at 24 hr. Control groups of planaria and daphnia corresponding to each lighting condition were tested in medium or well water without compound. The survival curves for each compound were tested for significant differences due to nuv pre- and coexposure using ANCOVA following log transformation of the survival proportions.

Tumorigenicity to Planaria

Experimental conditions were similar to those for the LC_{50} tests. This experiment was conducted once, using 20 *Du. dorotocephala* in each experimental and control group. Animals were exposed to concentrations of TNT, 2A, or 4A at $LC_{50}/5$ and $LC_{50}/15$ for 2 weeks. All test solutions were replaced daily, and control group solutions were replaced every third day. Freshly fed planaria were preexposed to BSO for 48 hr to inhibit GSH synthesis. A 48-hr preexposure plus continuous BSO exposure during the entire exposure period group (per the acute studies) was not included. Control groups (± BSO, ± nuv, ± BSO + nuv) were run concurrently.

At the end of the 2-week exposure period, the planaria were placed in clean medium, fed beef liver, and then placed into fresh medium. The animals were maintained at 19°C with a 12:12-hr light:dark photoperiod (incandescent light) and fed beef liver weekly. They were monitored daily for the initial 30 days from the start of exposure for lesions, proliferative growths, morphological abnormalities, fissioning, and mortality. The animals were subsequently monitored for tumors on a weekly basis for an additional 3 months. Observations were made with the aid of 2× and 10× optical magnification. The time to development of abnormal growths, the growth site, and gross and histological characteristics were monitored. These observations were the endpoints used to determine the tumorigenic potential of each compound resulting from concentration of exposure, nuv, and BSO.

Developmental Toxicity to Planaria

The experimental conditions were similar to those used for the tumorigenicity testing using groups of $N = 20$ *Du. dorotocephala* with an average weight of 15 mg/animal. Animals were sectioned sagitally with a single razor blade cut just cranial to their pharngeal area. Head and tail sections were exposed in separate beakers containing

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medium or compound (TNT, 2A, or 4A ± nuv ± BSO) at $LC_{50}/8$, except that tails exposed to 2A were in $LC_{50}/15$ due to toxicity. For positive controls, groups of 20 whole planaria were exposed to 0.2 mg/liter Cd (Hall *et al.*, 1986a,b; Johnson *et al.*, 1991; Schaeffer *et al.*, 1991) or *p*-nitrophenol (NP; 4 mg/liter) (Villar *et al.*, 1993). Test solutions were replaced daily in all groups exposed to nuv and every third day in the remaining control and test groups. Two independent experiments, designated E1 and E2, were carried out.

Head and tail sections were monitored daily with the aid of 10× optical magnification during the 2-week exposure period. The toxicologic endpoints of interest in addition to mortality were the incidence and prevalence of blastemia cone and acephalic, anopthalmic, teratomorphic, and normal head development for the tail sections. The head sections were monitored for mortality and the incidence and prevalence of normal tails. The criteria developed by Nentwig (1978) were used to determine the morphological characteristics of the blastemia cone and normal head formation. Mortality was determined by the criteria given above. Morphological characteristics given by Child (1941) were used to determine abnormal tail and head development. A normal planarian head is triangular with two symmetrically placed eyes and auricles at lateral margins. The teratophthalmic head is similar in shape to the normal head but the eyes indicate all degrees of approximation to the median plane from two distinct eyes with pigment connected to complete cyclopia. The teratomorphic head is more round in shape compared to the normal head with a single median eye and with anteriorly placed auricles which demonstrate all degrees of approximation to the median plane to a single median auricle. Anophthalmic heads are rudimentary heads without eyes. Sections without any sign of head development are termed acephalic. The head frequency index (HFI; Child and Watanabe, 1935),

$$\text{HFI} = (100\,(\text{No. normal}) + 80\,(\text{No. teratophthalmic}) + 60\,(\text{No. teratomorphic}) + 40\,(\text{No. anophthalmic}) + 20\,(\text{No. acephalic}))/N,$$

was calculated for all experimental and control groups on the ninth day of exposure, by which time all control animals had developed fully.

Metabolism of TNT by Planaria or Daphnia

Two beakers (500 ml) containing 100 *Du. dorotocephala* (average weight, 15 mg), and a similar beaker without planaria, were exposed to TNT (1 mg/liter) and maintained for 48 hr at 19°C under normal laboratory lighting conditions. Two beakers (250 ml) containing 50 *Da. magna* (immature), and a similar control beaker without daphnia, were exposed to TNT (5 mg/liter) and maintained for 48 hr at 19°C under laboratory lighting. Aliquots (100 ml) from each beaker were extracted with toluene and the amounts of TNT, 4A, and 2A were quantified by gas chromatography.

SRBC's Mechanism of Action Study

Freshly harvested sheep blood was diluted with 30 equal volumes of phosphate-buffered saline (PBS) and centrifuged at approximately 900*g* for 15 min. The erythrocyte pellet was washed twice with PBS and a 0.1% erythrocyte–PBS suspension was prepared. Five test solutions (3 ml) which contained no compound, 20 mg/liter of TNT, 2A, or 4A, or 1 μg/ml of α-terthienyl in the 0.1% erythrocyte–PBS suspension

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nuv Stability of TNT, 2A

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Planaria LC_{50}

Table 1 gives 96-hr LC
errors (SE), for the plan
groups are in Fig. 1.

Agent	BSO conditions
TNT	NBSO
TNT	NBSO
TNT	PBSO
TNT	PBSO
TNT	CBSO
TNT	CBSO
4A	NBSO
4A	NBSO
4A	PBSO
4A	PBSO
4A	CBSO
4A	CBSO
2A	NBSO
2A	NBSO
2A	PBSO
2A	PBSO
2A	CBSO
2A	CBSO

Note. NBSO, no BSO expo
and continuous exposure to

were prepared in six duplicate sets in nuv-transparent glass test tubes. One tube of each duplicate pair was exposed to a 1200 $\mu W/cm^2$ intensity of nuv. The other tube of each pair was protected from nuv light. A duplicate set was removed from nuv exposure and dark storage every 10 min until 1 hr had elapsed. Once the tubes were removed, the erythrocytes were pelleted by centrifugation and the percentage of hemolysis was estimated from the hemoglobin absorbance maximum at 542 nm.

RESULTS

nuv Stability of TNT, 2A, and 4A

The 12- and 48-hr exposures of TNT to nuv (400 $\mu W/cm^2$) resulted in 40 and 75% decreases in recovery, respectively, compared to the samples held in the dark for the same periods. The 12-hr nuv exposure did not reduce the recovery of 4A or 2A. The 48-hr exposures reduced the amount of 4A recovered by 60% and 2A by 42% compared to the dark samples. The dark and nuv light-exposed well water contained trace amounts of organic compounds which eluted off the column at very different times than TNT, 4A, or 2A.

Planaria LC_{50}

Table 1 gives 96-hr LC_{50}'s and 95% confidence intervals, and the slopes their standard errors (SE), for the planaria response curves. The significance of differences between groups are in Fig. 1.

TABLE 1

PLANARIAN 96-HR ACUTE TOXICITY TEST RESULTS

Agent	BSO conditions	nuv (+/−)	LC_{50} (mg/liter)	95% CI (mg/liter)	Slope	SE of slope
TNT	NBSO	+	1.56	1.28–1.91	2.79	0.68
TNT	NBSO	−	1.29	0.98–1.70	1.62	0.39
TNT	PBSO	+	1.69	1.40–2.05	2.97	0.68
TNT	PBSO	−	3.45	2.56–4.66	1.71	0.42
TNT	CBSO	+	10.89	8.65–13.71	2.96	0.89
TNT	CBSO	−	10.99	8.73–13.85	1.84	0.44
4A	NBSO	+	1.56	1.28–1.92	1.78	0.48
4A	NBSO	−	1.80	1.12–2.91	1.06	0.29
4A	PBSO	+	0.30	0.19–0.46	1.46	0.49
4A	PBSO	−	1.83	1.14–2.94	1.00	0.31
4A	CBSO	+	0.86	0.75–0.97	3.81	0.84
4A	CBSO	−	1.96	1.63–2.37	2.12	0.37
2A	NBSO	+	2.57	1.72–3.85	1.47	0.45
2A	NBSO	−	25.23	19.88–32.02	2.02	0.38
2A	PBSO	+	3.97	2.01–7.85	1.18	0.45
2A	PBSO	−	21.86	17.84–26.80	1.89	0.37
2A	CBSO	+	6.84	5.24–8.92	1.91	0.51
2A	CBSO	−	31.10	30.37–31.86	16.25	2.72

Note. NBSO, no BSO exposure; PBSO, 48-hr preexposure of planaria to BSO; CBSO, 48-hr preexposure and continuous exposure to BSO plus the compound for 96 hr.

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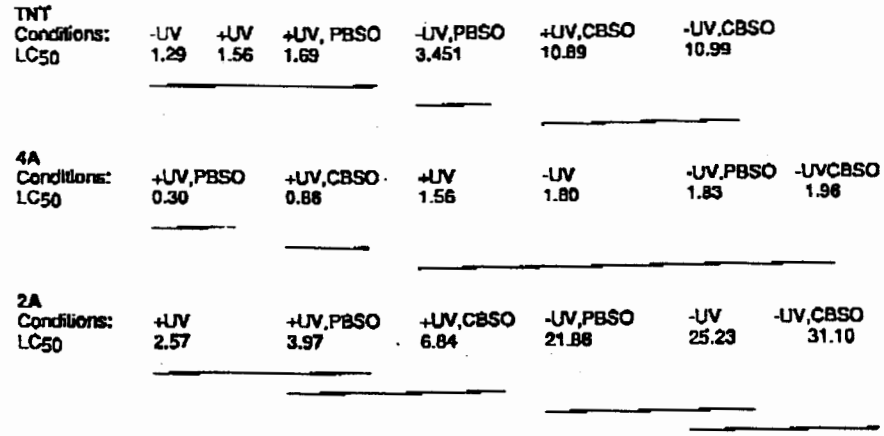

FIG. 1. Significance of differences in LC_{50} for TNT, 2A, and 4A. Underlined values are not significantly different ($P > 0.05$).

In the absence of any BSO exposure (NBSO), coexposure of *Du. dorotocephala* to nuv did not significantly ($P > 0.05$) change the LC_{50} for TNT. However, the LC_{50}'s for TNT dark groups preexposed (PBSO) and continuously exposed (CBSO) to BSO were significantly ($P < 0.05$) increased by three- and ninefold, respectively. The TNT dark and light toxic response curves were not significantly different ($P > 0.05$) for the following comparisons: [NBSO, dark | NBSO, light], [NBSO, light | PBSO, light], [NBSO, dark | PBSO, dark], [NBSO, dark | CBSO, light], [PBSO, dark | PBSO light], [PBSO, light | CBSO, light], [CBSO, dark | CBSO, light], [CBSO, dark | NBSO, dark], and [CBSO, light | NBSO, light].

Coexposure to either nuv or BSO did not significantly ($P > 0.05$) enhance the toxicity of 4A. However, coexposure to nuv + BSO significantly ($P < 0.05$) enhanced the toxicity of 4A. Coexposure to nuv significantly ($P < 0.05$) enhanced the toxicity (10×) of 2A, without regard to BSO exposure. Coexposure to nuv + BSO significantly decreased the light toxicity of 2A when compared to nuv coexposure alone. Continuous BSO coexposure significantly decreased the dark toxicity of 2A. Preexposure to BSO alone did not significantly change the toxicity of 2A, either in the dark or with coexposure to nuv. 4A and 2A dark and light response curves were significantly different ($P < 0.05$) for [PBSO, dark | CBSO, dark], [PBSO, light | CBSO, light], [CBSO, dark | CBSO, light], [CBSO, dark | NBSO, dark], and [CBSO, light | NBSO light].

Daphnia LC_{50}

Table 2 gives 96-hr LC_{50}'s and 95% confidence intervals, slopes and SE, and significance of differences for daphnia. nuv significantly ($P < 0.05$) increased the toxicity of TNT and 4A. The toxicity of 2A was also increased, but the effect was not statistically significant ($P > 0.05$) due to large variances.

Survival Time Studies

Mortality rates for *Du. dorotocephala* exposed to TNT (5 mg/liter), 4A (5 mg/liter), or 2A (10 mg/liter) for groups preexposed to the test solution prior to nuv light,

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Agent	nuv (+/−)
TNT	+
TNT	−
4A	+
4A	−
2A	+
2A	−

* Significantly different (P

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Metabolism of TNT b

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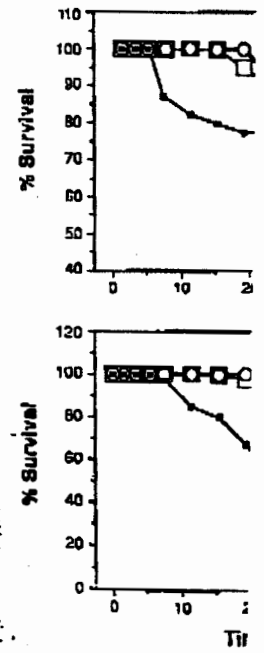

FIG. 2. Survival (%) of p
light to: (A) 5 mg/liter TN
4,6-dinitrotoluene (2A). (I
near-ultraviolet light.

TABLE 2

DAPHNIA 96-HR ACUTE TOXICITY TEST RESULTS

Agent	nuv (+/−)	LC_{50} (mg/liter)	95% CI (mg/liter)	Slope	SE of slope
TNT	+	0.98	0.57–1.67*	1.02	0.182
TNT	−	5.10	1.45–17.87	0.70	0.138
4A	+	1.31	0.54–3.18*	1.82	0.280
4A	−	5.11	1.99–13.10	1.07	0.185
2A	+	0.20	0.04–0.98	0.64	0.117
2A	−	1.06	0.54–2.07	0.82	0.130

* Significantly different ($P < 0.05$) from the corresponding dark group.

coexposed to nuv. or exposed in the dark are displayed in Fig. 2. Mortality rates for *D. magna* exposed to 5 mg/liter of TNT, 4A, or 2A are in Fig. 3.

Metabolism of TNT by Planaria and Daphnia

The 48-hr dark exposure of planaria to 1 mg/liter TNT resulted in the recovery of 4A (0.4 mg/liter), 2A (0.2 mg/liter), and TNT (0.03 mg/liter) from the culture medium. This indicated that approximately 40% of the TNT was reduced to 4A and 20% was

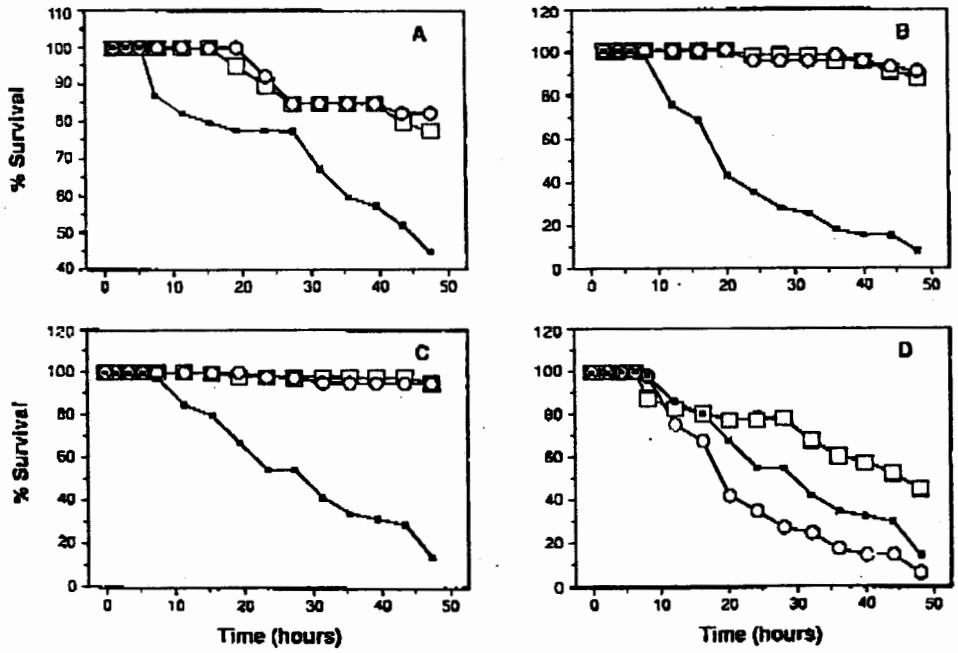

FIG. 2. Survival (%) of planaria in the dark (□) and during pre (○)- and coexposure (■) to near-ultraviolet light to: (A) 5 mg/liter TNT, (B) 5 mg/liter 4-amino-2,6-dinitrotoluene (4A), and (C) 10 mg/liter 2-amino-4,6-dinitrotoluene (2A). (D) Comparison of survival to TNT (□), 2A (■), and 4A (○) during coexposure to near-ultraviolet light.

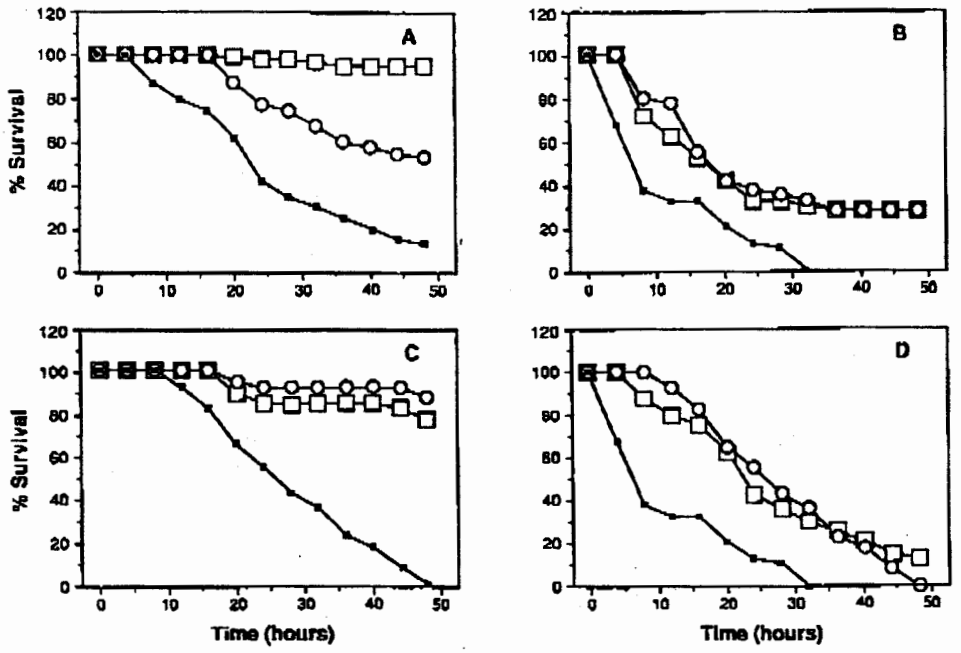

FIG. 3. Survival (%) of daphnia in the dark (□) and during pre (○)- and coexposure (■) to near-ultraviolet light to: (A) 5 mg/liter TNT, (B) 5 mg/liter 2-amino-4,6-dinitrotoluene (2A), and (C) 5 mg/liter 4-amino-2,6-dinitrotoluene (4A). (D) Comparison of survival to TNT (□), 2A (■), and 4A (○) during coexposure to near-ultraviolet light.

reduced to 2A. A substantial fraction was not accounted for perhaps due to absorption onto, and uptake into, the planarian. 4A or 2A were not recovered from the daphnia culture water after a 48-hr exposure to 1 mg/liter TNT. The detection limit for 2A and 4A averaged 0.01 mg/liter; approximately 80% of the TNT (0.80 mg/liter) was recovered from the well water in which the daphnia had been exposed. Johnson and Schaeffer (in preparation) found that male Fischer 344 rats metabolized 50%, and females 90%, of TNT (gavage, 50 mg/kg) into equal proportions of 2A and 4A within 48 hr, as determined by GC analysis of urine.

SRBC's Mechanism of Action Study

The positive control α-terthienyl (1 mg/liter) produced 100% lysis of the SRBC with a 10-min nuv coexposure. TNT, 4A, or 2A (20 mg/liter) did not produce lysis of the SRBC after 60 min of exposure. Binary mixtures of TNT, 4A, or 2A (40 mg/liter) did not produce lysis of the SRBC after 60 min of exposure. Changes in tubes used to expose the cells, increasing the nuv intensity, preincubation of the cells with the toxicants, and increasing the concentration of compounds up to 100 mg/liter all failed to produce cell lysis with TNT, 2A, or 4A.

Tumorigenicity to Planaria

No proliferating lesions ("tumors": Schaeffer *et al.*, 1991) were seen during a 4-month examination period for intact planaria exposed to TNT, 2A, or 4A. No lesions

PHOTOTO

HEAD FREQUENCY INDE
EXPOSED TO

nuv
BSO
Experiment 1 (head frequency)
Experiment 2 (head frequency)
Average (head frequency)

were observed during a 3-r
opmental studies. A total
exposed to 0.2 mg/liter Cd
like growths were seen in pc
significant ($P < 0.05$, pow
sectioned control groups (C
0.2 mg/liter Cd. A minimu
exposed to 96-hr LC_{50}/5 c
was noted in groups expose
(14 days) was noted in all

Developmental Toxicity to

The control groups (no t
sections all had fully devel
dicated normal head devel
all showed some degree of a
(Table 3). Anophthalmic, t
opment was observed only i
Acephalic heads developed

Planarian LC_{50}

There were no consistent
to BSO (NBSO) but expose

NUMBER OF ABNORMAL H
PLANARIAN SECTIONS T

	PN
nuv	–
BSO	–
Acephalic	9
Anophthalmic	2
Teratomorphic	1
Teratophthalmic	9

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TABLE 3

HEAD FREQUENCY INDEX VALUES CALCULATED FOR POSTERIOR PLANARIAN SECTIONS EXPOSED TO *PARA*-NITROPHENOL (PN), 4A, AND 2A FOR 9 DAYS

	PN	4A	4A	4A	4A	2A	2A	2A	2A
nuv	−	−	−	+	+	−	−	+	+
BSO	−	+	−	+	−	+	−	+	−
Experiment 1 (head frequency)	63	57	49	13	90	79	99	23	27
Experiment 2 (head frequency)	24	12	16	18	48	68	92	32	80
Average (head frequency)	44	34	32	16	69	74	96	28	54

were observed during a 3-month period for the sectioned planaria used in the developmental studies. A total of 6/40 tumor-like growths were seen in intact planaria exposed to 0.2 mg/liter Cd (following BSO preexposure) for 14 days and 17/40 tumor-like growths were seen in posterior sections; the difference in proportions is statistically significant ($P < 0.05$, power >0.7). No tumor-like growths were seen in any of the sectioned control groups (0 toxicants, ± BSO, ± nuv) or anterior sections exposed to 0.2 mg/liter Cd. A minimum of 50% survival (14 days) was noted in all intact groups exposed to 96-hr $LC_{50}/5$ concentrations, and a minimum of 90% survival (14 days) was noted in groups exposed to $LC_{50}/15$ concentrations. A minimum of 50% survival (14 days) was noted in all sectioned groups exposed to $LC_{50}/8$ concentrations.

Developmental Toxicity to Planaria

The control groups (no toxicant, ± BSO, ± nuv) and TNT groups of planarian tail sections all had fully developed heads by the ninth experimental day; HFI > 95 indicated normal head development. The groups exposed to 2A, 4A, or *p*-nitrophenol all showed some degree of arrested or abnormal developmental during this same period (Table 3). Anophthalmic, teratomorphic, and teratophthalmic abnormal head development was observed only in posterior sections exposed to 2A or 4A + nuv coexposure. Acephalic heads developed in 2A or 4A sections ± nuv coexposure (Table 4).

DISCUSSION

Planarian LC_{50}

There were no consistent differences between comparable TNT groups not exposed to BSO (NBSO) but exposed or not exposed to nuv. These results are consistent with

TABLE 4

NUMBER OF ABNORMAL HEADS OBSERVED DURING A 9-DAY EXPOSURE OF 40 POSTERIOR PLANARIAN SECTIONS TO *PARA*-NITROPHENOL (PN), 4A, OR 2A ± NUV COEXPOSURE

	PN	4A	4A	4A	4A	2A	2A	2A	2A
nuv	−	−	−	+	+	−	−	+	+
BSO	−	+	−	+	−	+	−	+	−
Acephalic	9	19	11	31	1	12	2	6	6
Anophthalmic	2	0	0	0	0	0	0	3	4
Teratomorphic	1	0	0	0	0	0	0	2	1
Teratophthalmic	9	0	0	0	3	0	0	8	0

similar dark and light toxic mechanisms of action for TNT. The dark TNT + BSO coexposure (CBSO) LC_{50} indicates that the dark toxicity was enhanced by GSH conjugation. Glutathione conjugation is usually a protective process. However, studies have shown that quinone–glutathione conjugates can be more toxic than the parent quinone molecule (Koob and Dekant, 1991).

Similarly, the 2A + BSO (PBSO) ± nuv results indicate that the dark and light 2A toxic mechanisms are similar to the extent that both are enhanced by 2A–glutathione conjugation. In contrast, the 4A + BSO (PBSO) ± nuv results indicate that the light-enhanced toxicity of 4A is enhanced by the GSH conjugation pathway, but the dark toxicity of 4A is not; i.e., the mechanisms of 4A light and dark toxicity are fundamentally different.

In summary, coexposure to nuv enhanced the toxicity of 2A more than it did for TNT and 4A. The toxicity of TNT, 4A, and 2A were all decreased, to some extent, by GSH conjugation. This suggests that all had mechanisms of toxic action which depend on formation of GSH conjugates. This outcome was anticipated since these compounds can form quinone–glutathione conjugates which studies with other compounds have shown to often be more toxic than the parent molecule (Koob and Dekant, 1991). Dark and light mechanisms for TNT and 2A both depended on glutathione conjugation, but the specific mechanisms may be different for each compound. The dark and light mechanisms of toxic action for 4A appeared to be fundamentally different in that the dark toxic mechanism of action was less dependent on GSH conjugation.

Daphnia LC_{50}

nuv coexposure decreased the LC_{50}'s of 4A, 2A, and TNT by a factor of 5. The 4A and TNT light LC_{50}'s differed significantly ($P < 0.05$) from the dark values. The light enhancement of 2A was probably significant, but larger variances for 2A (probably due to its poor solubility) resulted in an inability to prove a difference (Type 2 error). The slopes of the light and dark response curves were significantly different for 4A but not for TNT or 2A. These results are compatible with different light and dark toxic mechanisms for 4A, but not for TNT or 2A.

Survival Time Studies

The daphnia and planaria results indicated that nuv coexposure enhanced the toxicity of TNT, 4A, and 2A on a time of exposure to time of death basis. Toxicity enhancement resulted from a compound–nuv–planarian/daphnid interaction. One explanation for these coexposure requirements is that in the dark, the uncharged compounds passed into planarian/daphnid cells where it subsequently was activated by nuv to an anion. This anion could be converted to a more toxic molecule or it could be an active toxicant operating by a different mechanism than the uncharged molecule. This explanation is consistent with the finding that nuv enhancement occurred only with planaria which had been preexposed for 12 hr to the compound but not with newly exposed animals (Arfsten and Schaeffer, unpublished observations).

Metabolism of TNT by Planaria and Daphnia

The planaria effectively reduced 60% of the TNT in the medium to 4A and 2A in 48 hr. A higher percentage (40%) of 4A was produced than 2A (20%). Planarian me-

tabolism of TNT is similar to mammalian metabolism of TNT in that the predominant metabolites formed were 2A and 4A. However, planaria produced a 2:1 ratio of 4A:2A, whereas Fischer 344 rats produced them in a 1:1 ratio.

The daphnia were not expected to metabolize TNT. Production of 2A and 4A was not observed in daphnia at concentrations above the detection limit of 0.01 mg/liter. The daphnia were sensitive to the dark and light-enhanced toxicity of TNT. Since daphnia did not metabolize TNT, the observed light-enhanced toxicity for TNT was not the result of light-enhanced toxicity of 2A or 4A.

Tumorigenicity

Sectioning and exposure of posterior sections to 0.2 mg/liter Cd + 1 m*M* BSO resulted in a greatly enhanced incidence of tumor-like lesions compared to similarly exposed intact animals. Anterior sections exposed to 0.2 mg/liter Cd + 1 m*M* BSO did *not* develop any tumor-like lesions. As we expected, the reconstituting head tissues of the posterior sections (having increased mitotic activity) were more sensitive to the complete tumorigenic potential of Cd than were the mature head tissues of intact planarians.

Coexposure to nuv did not enhance the tumorigenic potential of TNT, 2A, or 4A in the planarian. Inhibition of glutathione conjugation by inhibiting GSH synthesis with BSO did not increase the tumorigenicity of these compounds, irrespective of nuv coexposure. Exposing planarians to TNT, 2A, or 4A during a period of increased mitosis in response to sectioning (Nentwig, 1978) did not result in tumor formation. Sectioning of planaria and nuv coexposure did not promote tumor development by TNT, 2A, or 4A. The lack of tumorigenicity associated with exposure to TNT, 2A, or 4A confirms previous findings in our laboratory that TNT does *not* act as a complete tumorigen in planaria (Hankenson and Schaeffer, unpublished observations).

Tissues reconstituting a tail in anterior sections were not sensitive to Cd tumorigenicity, although intact animals exposed to 0.2 mg/liter Cd + 1 m*M* BSO in this and previous (Johnson *et al.*, 1991; Schaeffer *et al.*, 1991; Tchseen *et al.*, 1992) studies have developed tumor-like tail tip growths. It is possible that the development of tails in the anterior sections is the result of tissue remodeling rather than new growth as is observed postfissioning for head reconstitution (Nentwig, 1978). If this is the case, new tail tissues would not be sensitized toward genotoxic agents since they would not have an increased rate of mitotic activity during the exposure period. Other possible explanations for this finding are that the precursor cells responsible for the development of tumor-like growth are located mainly in the posterior section, these cells do not migrate to the cut, or separation of the head from the tail section reduced the inhibition of unrestricted cellular growth in the posterior sections.

Developmental Toxicity

Exposure of planarian sections to BSO + nuv, or BSO or nuv alone, did not delay head development or result in abnormal heads. In this study, *p*-nitrophenol (4 mg/liter) appreciably inhibited head development (HFI = 44), in support of findings by Hill *et al.* (1992).

In contrast, TNT did not delay or alter head development under any of the experimental conditions tested. The lack of TNT developmental toxicity in planaria, with

or without nuv coexposure, is consistent with similar findings for sea urchin embryos (Davenport *et al.*, 1994).

Exposure of posterior sections to 2A or 4A resulted in arrested head development and abnormal head forms. The developmental toxicities of 2A or 4A were enhanced by nuv coexposure. Inhibition of GSH synthesis by BSO appeared to enhance the developmental toxicity of both 2A and 4A, with and without nuv. In contrast, the acute toxicity of 2A and 4A were enhanced by GSH conjugation. The differences between the developmental and the acute toxicity studies with regard to the effects of GSH conjugation are consistent with a (postulated) fundamentally different process by which GSH modulates developmental and acute toxicity.

Sections exposed to 2A or 4A + nuv developed all the types of the abnormal head forms, while in the dark the only abnormality was acephalia. Groups coexposed to 2A or 4A + nuv had HFI which were much lower than those from groups exposed in the dark. Development of all types of abnormal heads only in the nuv groups may indicate that nuv-enhanced developmental toxicity involved DNA damage by a mitogen or that nuv damaged the DNA, resulting in a decreased cell repair capacity which facilitated subsequent damage by the chemical.

SRBC's Mechanism of Action Study

The SRBC were a sensitive target for α-terthienyl, which is known to be an oxygen-dependent, nuv-enhanced destroyer of cellular membranes. TNT, 4A, 2A, and their binary combinations did not lyse the cells with excessive nuv light intensities (1200 $\mu W/cm^2$) at high (100 mg/liter) concentrations. The authors conclude that the light-enhanced toxic mechanisms of action for TNT, 2A, or 4A are not associated with frank cellular membrane damage.

CONCLUSIONS

Planaria were more sensitive to 2A with nuv coexposure, but nuv coexposure did not enhance the toxicity of TNT or 4A in this species. Daphnia were more sensitive to TNT, 2A, or 4A with nuv coexposure. We found *P. phosphoreum* to be more sensitive to TNT, 2A, and 4A with nuv coexposure (Johnson *et al.*, 1994). The light and dark LC_{50}'s were the lowest for *Da. magna*, somewhat higher for *Du. dorotocephala*, and highest for *P. phosphoreum*. These results indicate that the planaria and daphnia have similar sensitivities to the acute toxic effects of TNT, 2A, or 4A and are appreciably more sensitive than *P. phosphoreum*.

BSO-treated planaria were less sensitive to TNT or 2A with or without nuv coexposure. Apparently, GSH conjugates (presumably GSH–quinone conjugates) of TNT and 2A are more toxic than the parent compound. BSO-treated planaria were more sensitive to 4A (but not 2A or TNT) with nuv coexposure than in the dark. This suggests different light and dark toxic mechanisms of action for 4A, and different mechanisms for 4A relative to either 2A or TNT, in planaria (but not daphnia).

The daphnia and planaria survival time studies indicated that nuv coexposure, but not nuv preexposure of the test solutions, significantly enhanced the toxicity of all the compounds. This finding supports our interpretation of *P. phosphoreum* data (Johnson *et al.*, 1994) that the neutral TNT, 4A, or 2A compounds more readily diffuse through cell membranes than do nuv-induced charged anions (Suryanarayanan and Capellos, 1974). Further support for this interpretation is provided by our other studies (Dav-

enport *et al.*, 1994; Arfsten and Schaeffer, unpublished) showing that planaria and daphnia must be preincubated in the dark with TNT, 2A, or 4A in order to see the full effect of nuv enhancement. Apparently, anions produced by nuv photolysis of TNT, 2A, or 4A inside the cells are more toxic than the parent compounds.

Planaria, but not daphnia, effectively metabolized TNT to 4A and 2A in 48 hr. Evidently, the toxicity of TNT to daphnia in the light was not a response to 2A or 4A metabolites. However, some of the TNT toxicity to planaria could be due to 2A or 4A metabolites.

Mechanism of action studies exposed sheep erythrocytes to TNT, 4A, or 2A ± nuv light. nuv coexposure of the compounds to erythrocytes did not result in cell lysis. We conclude from this that the nuv enhancement of the toxicity of TNT, 2A, and 4A found in *P. phosphoreum* (Microtox), *Da. magna*, and *Du. dorotocephala* was not due to severe damage of cell membranes caused by initiation of cell membrane lipid peroxidation.

Planarians did not develop tumor-like growths in response to exposure to TNT, 2A, or 4A. This confirmed an earlier finding in our laboratory that TNT does not act as a complete tumorigen in planarians. The inhibition of GSH synthesis by preexposure to BSO, nuv coexposure, or sectioning of the planarians did not enhance the tumorigenic potential of TNT, 2A, or 4A. Planarian sectioning prior to Cd exposure did increase its tumorigenic potential towards the posterior planarian sections. The anterior planarian sections exposed to similar Cd concentrations failed to develop tumor-like growths; perhaps these sections did not contain the correct cell type required for tumor development or only a small number of the cells responsible for tail section reconstitution were undergoing mitosis.

TNT did not inhibit the development of planaria with or without nuv coexposure at the concentrations tested. This result was consistent with Davenport *et al.* (1994) who found that TNT in the dark did not cause developmental toxicity to *Da. magna* (5 μg/ml) and *Lytechinus variagatus* embryos (10 μg/ml). In the dark, the 2A and 4A metabolites of TNT were potent inhibitors of development in planaria (and *L. variagatus* embryos), but not daphnia, at the concentrations tested. nuv light enhanced the toxicity in all species tested. The concentrations of 2A and 4A tested are environmentally relevant and their developmental toxicity must be considered as an important component in a comprehensive environmental hazard assessment of TNT-related wastes. Inhibition of GSH synthesis by BSO enhanced the developmental toxicity of 2A and 4A. BSO inhibition of GSH synthesis decreased the acute toxicity of 2A and 4A.

The studies reported here demonstrate an enhancement of the toxicity of TNT, and its major metabolites 2A and 4A, by nuv irradiation corresponding to the longer nuv wavelengths of sunlight at relatively low fluxes. Mammals and aquatic species with glutathione activity rapidly and effectively metabolize TNT to nitrotoluidines (and other species: Rickert, 1987), but in different ratios. In these species, the toxicity of TNT solutions is due, in part, to the 2A and 4A metabolites. These metabolites have different mechanisms of toxicity in the presence of nuv, so the effect of mixtures is supraadditive (Johnson *et al.*, 1994). Our findings and those of others for bacteria (Kaplan and Kaplan, 1982; Klausmeier *et al.*, 1973; McCormick *et al.*, 1976, 1978; Naumova *et al.*, 1988; Traxler *et al.*, 1976), invertebrates, and mammals (Snyder, 1946), and the results of others for terrestrial plants (Palazzo and Leggett, 1986) and fungi (Parrish, 1977; Fernando *et al.*, 1990), support and help explain numerous ter-

restrial and aquatic field studies (Cairns and Dickson, 1973) which found severe ecosystem damage from TNT at all trophic levels.

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