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**TOXICITY OF TEREPHTHALIC ACID (TPA) SMOKE MIX
TO ALGAE, DAPHNIA, FATHEAD MINNOWS, AND EARTHWORMS**



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13. ABSTRACT (Maximum 200 words) Terephthalic Acid (TPA) is being considered as a replacement smoke for Hexachloroethane (HC). The TPA smoke mix was subjected to toxicity studies to test its effects on <i>Daphnia magna</i> (water flea), <i>Selenastrum capricornutum</i> (unicellular green algae), <i>Pimephales promelas</i> (fathead minnow), and <i>Eisenia foetida</i> (earthworm). Toxicity screening was conducted using neat TPA smoke mix in pressed (to simulate grenade packaging) and loose forms, and as burned residues. The toxicity of neat TPA was less toxic than neat HC (burned HC residues were not available). When added to water, the burned TPA residues caused the pH to drop to 4.5, which is not tolerated by the test organisms. However, when the pH was adjusted, the toxicity was greatly reduced. The severity of environmental impact from burned TPA mix will be inversely proportional to the buffering capacity of the ecosystem.				
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PREFACE

The work described in this report was authorized under Project No. 10162622A552, Smoke and Obscurants. This work was started in May 1992 and completed in May 1993.

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QUALITY ASSURANCE

This study, conducted as described by Protocols 22093000X041, 22093000X042, 22093000X043, and 22093000X058, was examined for compliance with Good Laboratory Practices as published by the U. S. Environmental Protection Agency in 40 CFR Part 792 (effective 17 Aug 1989). The dates of all inspections and the dates the results of those inspections were reported to the Study Director and management were as follows:

<u>Phase inspected</u>	<u>Date</u>	<u>Date reported</u>
Collection of residue	5 Apr 93	7 Apr 93
Daphnia dosing	27 Apr 93	28 Apr 93
Data & Final Report	17 Mar 95	17 Mar 95

To the best of my knowledge, the methods described were the methods followed during the study. The report was determined to be an accurate reflection of the raw data obtained.


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TOXICITY OF TEREPHTHALIC ACID (TPA) SMOKE MIX
TO ALGAE, DAPHNIA, FATHEAD MINNOWS, AND EARTHWORMS

1. INTRODUCTION

Terephthalic Acid (TPA) is being considered as a training replacement smoke for Hexachloroethane (HC). The combustion of HC smoke produces zinc oxychloride and zinc chloride. Low concentrations of phosgene and carbon tetrachloride have also been detected as part of the combustion by-products.¹ Zinc chloride is a very corrosive irritant and has been reported to cause either lung damage² or death to personnel accidentally exposed.³

With the intention of meeting the obscurant requirements for white smoke, the TPA smoke was developed to be less toxic. This smoke mix was prepared and provided by the Pyrotechnics Group, U.S. Army Edgewood Research, Development and Engineering Center. The processes used in preparing the smoke and starter mix are listed in Table 1.

Table 1. Formulation of the TPA Smoke Mixture

TPA smoke mix formula by Glatt process (batch #2066-1)	
	<u>parts by wt</u>
Terephthalic Acid	57
Sugar	14
Magnesium Carbonate	3
Potassium Chlorate	23
Steric Acid	3
Polyvinyl Alcohol (binder)	1
Starter mix formula by Hobart process (batch #2029-1)	
	<u>parts by wt</u>
Silicon	16
Potassium Nitrate	51
Charcoal	17
Steric Acid	10
Nitrocellulose	4

This study investigated the toxic effects of the TPA smoke mix on several aquatic organisms (*Daphnia magna*, the water flea; *Selenastrum capricornutum*; a unicellular green algae; and *Pimephales promelas*; the fathead minnow) and one terrestrial organism (*Eisenia foetida*, the earthworm). The toxicity of HC to daphnia was also investigated using the same methods used in the TPA studies. The TPA smoke mix contains both smoke and starter ingredients and will be referred to as pellet and loose mix in the following text.

Both the pellet and loose mix used in these toxicity studies were prepared at a ratio of 5.0 g of TPA smoke mix to 90.0 mg of starter mix. This ratio was consistent with the ratio of materials packed into an XM83 grenade. The smoke materials were received as a pressed pellet (simulating material packed into a grenade) and loose (unpressed). Residue samples of burned TPA were also collected and subjected to toxicity studies. Burned HC smoke residues were not available for comparison studies.

This study investigated the toxicity of the TPA / starter mix in loose, pellet, and burned forms. Because the TPA smoke is being developed as a training replacement for HC smoke, the HC loose and pellet materials were also subjected to similar toxicity studies for comparison.

2. METHODS AND MATERIALS

All testing conformed to U.S. Environmental Protection Agency (EPA)^{4,5} and American Society for Testing and Material (ASTM)⁶ guidelines. These studies were conducted under good laboratory practices and conformed to all interagency standard operating procedures.

The smoke materials (5.9 g) were placed in 1 L of the media (daphnia or algae) and gently agitated for 24 hr. The undissolved particulate was filtered using a single layer of cheesecloth to remove the largest particles, and the remaining supernatant was then diluted for toxicity studies. Filtration was conducted to reduce possible variation in sample transfer and eliminate mechanical stress to the test organisms. The concentration of dissolved TPA in the supernatant was determined using high performance liquid chromatography (HPLC) (discussed in Section 3).

The burned TPA smoke residue was collected in stainless steel pans located on the bottom of a 20,000-L combustion chamber. After test burns, the residue was removed from the pans, weighed, and stored under desiccation. Stocks of the smoke residues were prepared as described above. However, the cheesecloth filtration methods only removed large pieces of material, which were not suitable for algal studies. The media must be clear enough to allow the algal cells to be counted under a microscope. Therefore, stock preparation for use with the algae studies was passed through ashless paper #41 (particle retention size 20-25 μm) to remove all particles, allowing for more accurate counting of algae cells during growth studies.

3. ANALYTICAL ANALYSIS

Due to the complexity of the TPA smoke mixture, the concentration of all the dissolved components could not be determined; therefore, only the concentration of dissolved TPA was measured.

Water samples were analyzed for TPA by HPLC. After thorough mixing, a 10-mL aliquot was withdrawn and filtered through a 0.45- μ filter. The samples were injected onto a Unisphere column for peak separation and quantitation. Quantitation was performed by comparing the area counts of the samples to a regression line produced from the injection of TPA standards. The TPA standards were prepared by adding known

amounts of TPA and 0.01 N NaOH into a volumetric flask containing distilled water. The HPLC parameters for TPA analysis are given in Table 2.

Table 2. HPLC Parameters for Analyzing TPA

HPLC Model	Perkin Elmer, Series 4
Column	Unisphere, -C18, 4.6 x 250 mm (Biotage, Incorporated, Charlottesville, VA)
Mobile Phase	50% Acetonitrile / 50% water with 0.1% NH ₄ OH
Detection	Diode Array UV, @ 235 nm \pm 15 bandwidth
Flow Rate	2.0 mL/min

4. ALGAE ASSAYS

Algae cultures of *Selenastrum capricornutum* were obtained from Dr. Freida Taub, University of Washington, Seattle, WA. Stock cultures of algae were maintained on 1.5% Difco-Bacto agar slants. Test algae were grown in a semiflow through culture apparatus using T82MV⁷ media and taken during log phase growth for inoculation into the test flasks. Erlenmeyer flasks (500 mL) with ground glass stoppers were used as test chambers. The TPA supernatant was added to the test chambers and diluted with media to the proper concentration. The total volume in each chamber did not exceed 100 mL. The test chambers were inoculated with approximately 4.0×10^4 algal cells per milliliter and placed in an incubator at 20 °C, with a light-dark cycle of 16:8 hr with 315 ft-c of light. Using a Newbauer Counting Chamber, cell densities were determined every 24 hr for 5 consecutive days. The area under the growth curve (A) was calculated using the following equation:

$$A = \frac{(N_0 + N_1) - 2N_0}{2} X (t_1) + \frac{(N_1 + N_2) - 2N_0}{2} X^{(t_2 - t_1)} + \frac{(N_{n-1} + N_n) - 2N_0}{2} X^{(t_n - t_{n-1})} \quad (1)$$

where

N_0 = number of cells at t_0

N_1 = number of cells at t_1

N_n = number of cells at t_n

t_1 = time of first measurement

t_n = time of the nth measurement

The percentage of inhibition was calculated using the area under the growth curve. The following equation was used to calculate the percentage of inhibition (%In):

$$\%In = \frac{A_c - A_t}{A_c} \times 100 \quad (2)$$

where

A_c = area of control growth curve

A_t = area of treatment growth curve

The %In values were plotted against the concentrations. A least square regression line was calculated, and the IC_{50} (concentration at which algal growth was reduced to 50% of the control) was determined. Analysis of variance (ANOVA) was run on the replicates to determine if any of the groups were significantly different ($p \leq 0.05$). The Dunnett's test was conducted to determine significant growth differences from the control.

5. DAPHNIA ASSAYS

The *Daphnia magna* were obtained from Dr. Freida Taub, (University of Washington) and reared in this laboratory since 1985 using methods described by Goulden et al.⁸ *Daphnia* stock cultures were fed a mixture of vitamin enriched *Ankistrodesmus falcatus*, *Selenastrum capricornutum*, and *Chlamydomonas reinhardtii*. *Daphnia* culture media was prepared from well water, which was passed through a treatment system containing limestone pH adjustment, iron removal, carbon filtration, and UV sterilization. The well water was monitored semiannually for 92 commonly found ground water pollutants by Watercheck National Testing Laboratories, Incorporated, (Ypsilanti, MI).

The test beakers were placed in a temperature-controlled room at 20 °C with a light-dark cycle of 16:8 hr with 315 ft-c of light. Two replicates per concentration were used for each study. Each replicate contained 10 daphnia (< 24 hr old) per 100 mL of test solution. The pH and dissolved oxygen measurements were taken at the beginning of each test. *Daphnia* were checked for mortality at 24- and 48-hr intervals. If the daphnia were not actively swimming, they were manually touched with a pasture pipette. If there was no response or if the daphnia could not swim actively for 15 s, they were considered immobilized. The EC_{50} (concentration at which 50% of the organisms were immobilized) values were computed using the probit analysis prepared by Kessler.⁹ The EC_{50} s were also tabulated graphically using a least square regression analysis, verifying all probit results.

6. FISH ASSAYS

Adult fish were originally obtained from Kurtz's Fish Hatchery (Elverson, PA). Fish were maintained in 40-gal glass aquaria equipped with under gravel biofilters. The culture water was the same as that described in Section 5. Adult fish were fed Tetramin flake food in the mornings and *Lumbriculus variegatus* (black worms) in the afternoon. This feeding regime encouraged the fish to breed continuously. Adult breeding fish were replaced annually to maintain a healthy gene pool.

After the adult fish deposited eggs on the under sides of clay pots, the fertilized eggs were transferred to hatchery tanks. Upon hatching, the fry were fed newly hatched brine shrimp twice a day and were used in toxicity tests when they became 14 days old. The loading did not exceed 0.8 g of fish per liter of solution. If they appeared stressed or 5% of them died within 48 hr before testing, the fry were discarded. Water temperature was maintained at 20 ± 1 °C with a light-dark cycle of 16:8 hr.

The test chambers consisted of 1-gal glass jars. The test chambers and glassware were cleaned with phosphate-free soap, rinsed with tap water to remove the soap, and then rinsed with distilled water.

A stock solution of the toxicant was prepared and dispensed directly into the test chambers, then diluted to obtain the desired concentrations. The dissolved oxygen and pH were measured before fish were transferred to the test chambers. After the fish were added to the test chambers, a random number table was used to assign each chamber (including controls) to one of two blocks. Next, the chambers were assigned a location number for each of the treatments within that block. The EC_{50} values (the concentration that caused 50% of the fish to die, the endpoint for mortality was no gill movement) and the 95% confidence intervals were computed by the probit analysis method and checked graphically using the same procedure described in Section 5.

7. EARTHWORM ASSAYS

Earthworm toxicity testing used *Eisenia foetida* as the test organism. Survival rates and weight changes were used as indices of toxicity. Test methods used for these toxicity studies were adapted from Karnak and Hamelink¹⁰ and Neuhauser et al.¹¹

Earthworms were originally purchased from Bert's Bait Farm (Irvine, KY) and were cultured in a 50/50 mixture of peat and potting soil. The worms were maintained on a diet of fermented alfalfa pellets and were housed in styrofoam coolers under laboratory conditions. The contents of each cooler were mixed once per week to loosen and aerate the soil medium and evenly distribute water throughout the container. Any food remaining on top of the medium was discarded before mixing. After mixing, fresh food was added to the container.

The earthworm toxicity test consisted of placing 200 g of substrate and five earthworms into a 600-mL glass beaker. Earthworm media used in testing consisted of a nonsterile artificial soil mixture and distilled water.¹¹ The use of artificial soil provides a reproducible soil mixture that reduces the variability that could occur between tests if field soil were used. The components of the artificial soil are listed in Table 3.

Table 3. Components of the Artificial Soil Used in the Earth Worm Toxicity Test

Stock Components	% by wt
Lime	1
Finely-ground sphagnum peat moss	10
Kaolinite clay	20
Fine sand	69

The test soil was prepared by mixing (in a food blender) the artificial soil with the TPA residue. Distilled water was slowly added and mixed until a uniform texture was established (25% soil moisture). The test soil was then divided into replicates and placed into 600-mL beakers.

After the beakers were prepared with soil, 75-100 earthworms were removed from one of the styrofoam coolers and put into a plastic pan. The earthworms were quickly rinsed in tap water and excess water drained from the pan. Five earthworms were randomly selected, quickly blotted with a paper towel, and weighed as a group ($n = 5$). The group of five earthworms was then placed into a beaker, which was covered with nylon screen and cheesecloth secured with a rubber band. The beakers were randomly placed in plastic trays within an incubator. Water was added to the trays so the increased humidity would reduce water loss from the soil in the beakers. The incubator lights were set for continuous operation. Since the earthworms are photophobic, the light encouraged them to burrow into the soil and helped prevent them from crawling out of the beakers.

The worms remained in the incubator for a 2-wk exposure period. Beakers were re-randomized in the trays at the end of the first week. On day 14, the earthworms were removed from each beaker and reweighed. They were examined for changes in color, texture, motility, and general physical condition. The statistical methods used to evaluate earthworm data were the Analysis of Covariance (ANCOVA) and the Newman Keuls pairwise comparison of means.¹²

8. RESULTS/DISCUSSION

When added to daphnia media, the neat TPA smoke material (loose and pellet forms) settled to the bottom of the test chamber. The concentration of dissolved TPA produced from the loose smoke material was 286.5 mg/L and from the pellet smoke material 83 mg/L. The water remained clear; however, there was a pH change. The loose TPA material lowered the pH from 7.8 to 4.9 (not tolerated by daphnia), while the TPA pellet reduced the pH to 6.7 (Figure 1).

The burned TPA material consisted of smoke residue, bits of paper, and other fibrous material used in packaging the grenade. The residue was very fine. When it was suspended in water, it caused the entire water column to become cloudy. After approximately 1 hr, the particulate settled to the bottom, leaving the water column clear. The burned TPA residues also reduced the pH to below tolerable limits for daphnia ($\text{pH} = 4.7$). When the daphnia were exposed to the burned residue, they were able to ingest enough of the suspension to pack the entire gut. This was most evident in the higher concentrations.

The burned TPA residues were slightly more toxic than the loose materials (Table 4). However, adjusting the pH eliminated the toxicity to algae (Figures 2 and 3) and reduced the toxicity to daphnia by 34%. The fathead minnows were exposed to loose TPA mix and showed no apparent effects at 70%. The earthworms showed no lethal or sublethal effects (weight loss) when exposed to TPA residues up to 5,000 mg/Kg.

pH CHANGE AFTER TPA ADDITIONS

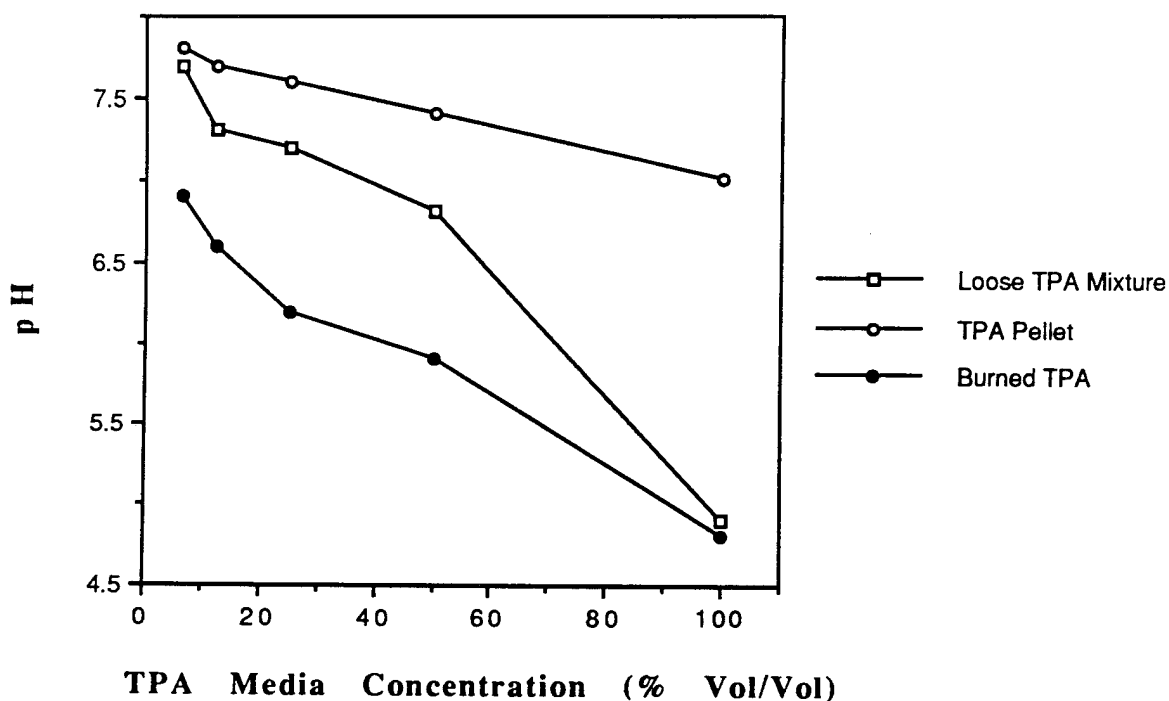


Figure 1. The Loose and Burned TPA Smoke Materials Reduced pH to Below Tolerable Limits for Daphnia

Comparison studies using HC smoke mix¹³ were also run on loose and pellet material. The loose HC smoke material was approximately four times more toxic than loose TPA. The pellet HC smoke material was 25 times more toxic to daphnia than the TPA pellet. Studies using HC residues were not conducted because HC residues were not available. Originally, the loose smoke material was expected to be more toxic than the pellet due to having a much larger surface area, allowing more material to dissolve. In both the TPA and the HC formulations, the pellet form was more toxic than the loose materials. This toxicity also occurred in a study by Chester et al.,¹³ where a slightly different formulation of TPA smoke mix (without starter) was used. The authors cannot explain why this has occurred. However, there are some possible theories that may explain why the pellet is more toxic than the loose material. When the pellet is formed, the loose material is placed into a brass press that is coated with silicone lubricant. The material is pressed into a pellet using 10,000 lb dead load. A silicone lubricant is used so the pellet can be removed from the press easily. The silicone lubricant may contain materials that are toxic to aquatic organisms. Also, the brass material the press is

Table 4. Toxicity Comparison of Various Forms of the TPA Smoke and HC Smoke Mixtures

Species	TPA Burned Residue	TPA Loose Mix	TPA Pellet	HC Loose Mix	HC Pellet
<i>Daphnia magna</i> (Water Flea, 48 hr EC ₅₀)	25.5% (38.7%)*	43.5%	26.9%	11.1%	1.1%
<i>Selenastrum capricornutum</i> (Green Algae, 96 hr IC ₅₀)	22.0% (N.T.)*	24.1%	---	---	---
<i>Pimephales promelas</i> (Fathead Minnow, 96 hr EC ₅₀)	---	N.T.** (@ 70%)	---	---	---
<i>Eisenia foetida</i> (Earthworm, 14 day EC ₅₀)	N.T.** (@5,000 mg/Kg)	---	---	---	---

*pH adjusted results

**N.T. - not toxic

ALGAE EXPOSED TO TPA RESIDUE

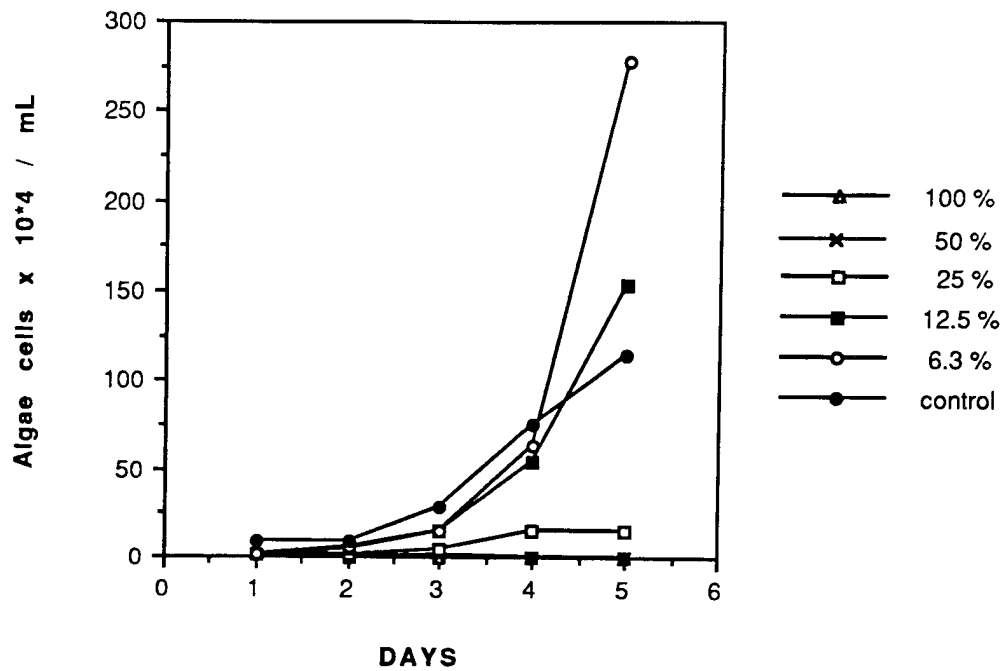


Figure 2. The Unicellular Green Algae *Selenastrum Capricornutum* was Exposed to Burned TPA Residue Produced by an XM83 Smoke Grenade (the pH was not adjusted)

Algae Growth in Burned TPA Residue After pH Adjustment

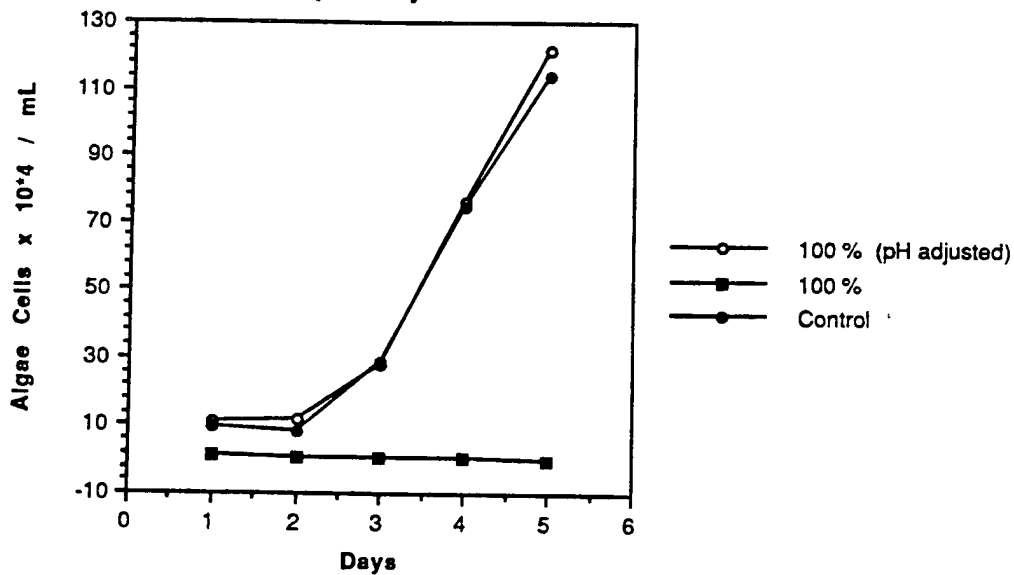


Figure 3. After Adjusting the pH of the Supernatant Produced from TPA Residue, the Toxicity to Algae was Eliminated. There is no significant difference between control growth and 100% pH adjusted supernatant.

constructed of contains copper and zinc, both of which are highly toxic to aquatic organisms at part per billion levels.¹⁴ It may be possible that the pellet is being contaminated by brass from the press. These theories need to be investigated in future research.

Potentially, the greatest impact to the environment will come from burning the TPA smoke mixture during training and testing exercises. Wind and rainwater runoff could carry residues great distances from the test site. The severity of impact to the environment will be directly related to the buffering capacity of the area effected. As seen above, adjusting the pH greatly reduced the toxicity to daphnia and eliminated the toxicity to algae.

In preparing the TPA residues for this study, 5.9 g of material was placed into 1 L of water to simulate the weight and volume used in preparing the neat material. However, due to the laws of mass balance, the mass of the residues remaining from a single burning of neat mix (5.9 g of material) would be much less. During combustion, gasses such as CO, CO₂, and NO₂ are emitted¹⁵ and are not available to aquatic or terrestrial organisms. Therefore, the concentrations used in these controlled laboratory studies most likely exceed the concentration that would be encountered in open environmental conditions. The residue ground deposition gradient (during various weather conditions) needs to be investigated to determine typical residue concentration at and around the detonation site. Gradient concentration data coupled with the toxicity data presented here could be used to yield more reliable estimates of environmental impact.

9. CONCLUSIONS

The following conclusions are provided as a result of the study conducted:

- The pellet material [83 mg/L of dissolved Terephthalic Acid (TPA)] was more toxic than the loose material (286 mg/L of dissolved TPA).
- The source of increased toxicity in the pellet material (apparently due to the pelletizing process) merits further investigation.
- When added to water, both loose TPA mix and burned TPA residue reduced the pH to below tolerable limits for daphnia. When the pH was adjusted, the toxicity was reduced in daphnia and eliminated in algae.
- Buffering capacity of the environment will greatly affect the toxicity of burned TPA residues.
- Concentration gradients of burned TPA residue during various field conditions should be determined to yield more accurate estimates of environmental impact.

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