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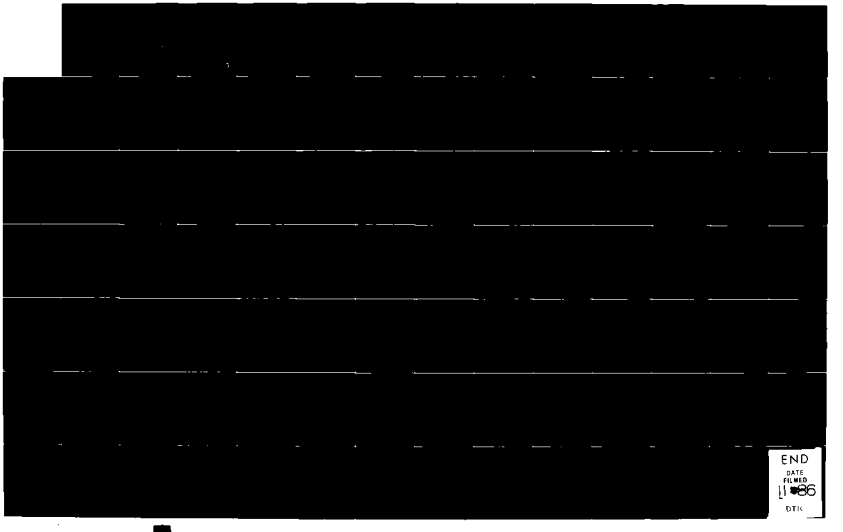

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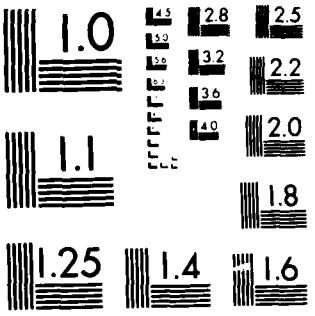

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DETERMINATION OF THE TOXICITY TO AQUATIC ORGANISMS OF HMX AND RELATED WASTEWATER CONSTITUENTS: Part 3 Toxicity of HMX, TAX and SEX to Aquatic Organisms

FINAL REPORT

by

R.E. Bentley
D.C. Surprenant
S.R. Petrocelli, Principal Investigator

October 1984

Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-80-C-0011

Springborn Bionomics, Inc.
790 Main Street
Wareham, Massachusetts 02571

Project Officer: William van der Schalie, Ph.D.
Health Effects Research Division
U.S. Army Medical Bioengineering Research and Development Laboratory
Fort Detrick, Frederick, Maryland 21701-5010

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The chronic toxicity of HMX was studied utilizing Daphnia magna in a 28-day chronic and fathead minnow in an embryo-larval study. No adverse effects of exposure to 3.9 or 3.3 mg/L, respectively, were observed. These concentrations closely approximate the limit of aqueous solubility. The acute toxicity of TAX was studied utilizing aquatic organisms representing several different trophic levels in aquatic systems. Generally, no adverse effects of exposure were observed among any of the algae, fish or invertebrate species tested. Acute toxicity was observed only for 24-hour-old fathead minnow fry.

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ABSTRACT continued -

This effect level closely approximated the limit of aqueous solubility of TAX at 600 mg/L. The acute toxicity of SEX was studied utilizing aquatic organisms representing several different trophic levels in aquatic systems. Generally, no adverse effects of exposure were observed among any of the algae, fish or invertebrate species tested up to the limit of aqueous solubility of ca. 12 mg/L. SEX was acutely toxic only to 7-day-old fathead minnow fry at a concentration of 10 mg/L which is close to the aqueous solubility limit.

FOREWARD

Citation of commercial organizations and trade names in this report does not constitute an official Department of the Army endorsement or approval of the products and services of these organizations.

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INTRODUCTION

Since the early 1970's, the U.S. Army Medical Research and Development Command has been supporting research on various munitions compounds to ascertain their effects when discharged into the aquatic environment. The chemical HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) is manufactured and processed at U.S. Army-owned ammunition plants for use in military high explosive compositions. SEX (1-acetyloctahydro-3,5,7-trinitro-1,3,5,7-tetrazocine) and TAX (1-acetylhexahydro-3,5-dinitro-1,3,5-triazine) are unavoidable co-products of the HMX manufacturing process. These compounds are discharged into the aquatic environment during the manufacturing and blending process and from the load, assemble and pack plants where these materials are loaded into bombs (Sullivan et al., 1979).

The objective of these studies was to provide the data base required to perform a hazard evaluation relative to the occurrence of HMX, SEX and TAX in the aquatic environment, and to recommend a criterion for the protection of freshwater aquatic life. To achieve this, a variety of representative freshwater fish and invertebrates were tested to determine the potential toxicity of these compounds.

All data generated during these studies are stored at Springborn Bionomics, Inc. (formerly EG&G Bionomics), 790 Main Street, Wareham, MA.

MATERIALS AND METHODS

Test Materials

The HMX used in these studies was received from the Holston Army Ammunition Plant in Kingsport, Tennessee, on 18 June 1980. The HMX (drum #1, batch #6HBC 10-13, reg. #17202) in the form of white crystals, was tested on a product basis, and HMX concentrations are reported as milligrams per liter in the test solutions (mg/L).

The TAX used in these studies was received from SRI International, Menlo Park, California, on 16 July and 14 October 1980, and 29 January and 2 September 1981. TAX, in the form of white crystals, was tested on a product basis, and concentrations are reported as milligrams of TAX per liter in the test solutions.

The SEX (labeled 99 + % pure) used in these studies was received from SRI International, Menlo Park, California, on 22 July 1981 and 29 June 1982. SEX, a whitish powder, was tested on a product basis, and concentrations are reported as milligrams of SEX per liter in the test solutions.

Test Organisms

The algae test was performed with the chlorophyte (green) Selenastrum capricornutum. The culture was obtained from the collection at the University of Indiana,

Bloomington, Indiana, and subsequently maintained in a stock culture at Bionomics Marine Research Laboratory, Pensacola, Florida. Culture medium was the standard algal assay procedure (AAP) growth medium (USEPA, 1978).

Macroinvertebrates tested were the water flea (Daphnia magna) and scud (Gammarus sp.). Daphnids used in testing were obtained from laboratory cultures maintained at Springborn Bionomics, Inc., and were less than 24 hours old. Daphnids, during culture, were fed a mixture of unicellular green algae and a fish food suspension ad libitum. The Gammarus sp. were collected from Horseshoe Pond in Wareham, Massachusetts, and cultured at Springborn Bionomics for 5 weeks prior to testing. The Gammarus sp. were fed vegetation obtained from the collection site. The mean (standard deviation) length, N=10, of the scud was 4.6(1.1) millimeters (mm).

The culture water for the macroinvertebrates was prepared by reconstituting deionized water (USEPA, 1975) and filtering it through an Amberlite XAD-7 resin column to remove any potential organic contaminants. This water had a total hardness and alkalinity as calcium carbonate ($CaCO_3$) of 165 $\pm$ 15 mg/L and 120 $\pm$ 10 mg/L, respectively, a pH range of 7.9-8.3, a temperature of 22 $\pm$ 1^{o}C, a dissolved oxygen (DO) concentration of greater than 5.3 mg/L (60% of saturation) and a specific conductance of 400-600 micromhos

per centimeter (umhos/cm). The specific conductance was measured with a YSI Model #33 salinity-conductivity-temperature meter and probe; the pH was measured with an Instrumentation Laboratory Model #175 pH meter and combination electrode; the DO was measured with a YSI Model #57 or #54 dissolved oxygen meter and probe and the temperature was measured with a Brooklyn alcohol thermometer. Total hardness and alkalinity were measured according to APHA et al. (1975).

Fathead minnow embryos, ≤48 hours old, were obtained from the brood culture unit at Springborn Bionomics, Inc., for use in the HMX early life-stage study. The early life-stage static acute toxicity tests with TAX and SEX utilized fathead minnow (Pimephales promelas) and rainbow trout (Salmo gairdneri) embryos, newly hatched larvae (less than 24 hours old) and 7-day-old larvae. The fathead minnow embryos and larvae were obtained from the brood culture unit at Springborn Bionomics, Inc. Fathead minnow embryos used in static testing were less than 24 hours old. Eyed rainbow trout embryos and subsequent larvae used in testing were obtained from the U.S. Fish and Wildlife Service, Beltsville, Maryland. Rainbow trout embryo tests were conducted with 21-23 day-old embryos. The well water which flowed into the holding tanks was characterized as having total hardness and alkalinity ranges as $CaCO_3$ of 23-32 mg/L and 18-24 mg/L, respectively, and a specific conductance range of 90-130 umhos/cm (Weekly Gravity Feed

Tank Water Quality Analysis Logbook). Fathead minnow fry and swim-up rainbow trout fry were fed concentrated live brine shrimp twice daily.

Fish utilized in the static acute toxicity tests were fathead minnow (Pimephales promelas), bluegill (Lepomis macrochirus), rainbow trout (Salmo gairdneri) and channel catfish (Ictalurus punctatus). Table 1 summarizes lot numbers, sizes and sources of the fish used in the static acute toxicity tests. The fathead minnow were cultured at Springborn Bionomics, Inc., Wareham, Massachusetts, while all other fish were obtained from commercial fish suppliers. All fish were kept under a photoperiod of 16 hours light and 8 hours darkness. Fish were fed a dry pelleted food, ad libitum, daily except during the 48 hours prior to testing. There was <1.0% mortality in all test populations during this 2-day period (Daily Record of Fish Holding Conditions). The well water which flowed into the holding tanks was characterized as having total hardness and alkalinity ranges as $CaCO_3$ of 20-32 mg/L and 20-28 mg/L, respectively, and a specific conductance range of 75-130 umhos/cm (Weekly Gravity Feed Tank Water Quality Analysis Logbook). Other parameters monitored in the holding tanks were a pH range of 6.6-7.2 and a DO range of 80->100% of saturation (Weekly Record of Fish Holding Water Characteristics). All test fish were maintained under these conditions for a minimum of 14 days. The temperature in the fathead minnow, bluegill and channel catfish holding

tanks was 22 ± 2°C and 12 ± 2°C in the rainbow trout holding tanks during the 48-hour period prior to each test.

Test Methods

Procedures used in the phytotoxicity tests followed those described in "The Selenastrum capricornutum Printz algal assay bottle test" (USEPA, 1978). Culturing and testing was done using synthetic Algal Assay Procedure growth medium. Test solutions were prepared by mixing the SEX and TAX in a solution with sterile distilled water at concentrations exceeding the limits of aqueous solubility for 48 hours prior to testing. A fraction of the water soluble portion was then siphoned from below any existing surface layer. Algal assay nutrients (USEPA, 1978) were then added to the sterile test flasks. The appropriate amount of an algal cell inoculum solution was added to each test flask to produce approximately 2×10^4 cells/mL in each of the three replicate flasks of each test concentration. The test flasks were rotated (100 rpm) on a gyrotory shaker table and provided with a controlled temperature of 24°C and continuous illumination of approximately 4000 lux. Each algal assay was continued until there was less than a 5% change in daily in vivo chlorophyll a measurements determined by fluorometer, or up to a maximum of 14 days. Cell counts were made at the end of the test using a hemacytometer.

Procedures used in all static acute toxicity tests followed those described in "Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians" (USEPA, 1975) and the appropriate Springborn Bionomics, Inc., protocol. Acute toxicity was evaluated for macroinvertebrates in 48-hour toxicity tests conducted under static conditions. TAX toxicity tests were conducted with Gammarus sp. and D. magna, while the SEX and HMX tests were conducted with D. magna only. The TAX toxicity tests were conducted in 250-milliliter (mL) beakers, each of which contained 150 mL of test solution, and SEX and HMX tests were conducted in 2-L battery jars, each of which contained 500 mL of test solution. The dilution water used was reconstituted from deionized water and had the same quality as the macroinvertebrate culture water previously mentioned. Each test concentration of TAX, SEX and HMX was prepared by weighing out the desired quantity of the appropriate test material and adding it directly to 1000 mL of diluent water. The solution was then vigorously mixed on a magnetic stirrer. Due to their limited solubilities, it was necessary to mix the TAX and SEX solutions for 5 days and the HMX solutions for 4 days. After mixing, 450 mL of each TAX solution was divided into three beakers to provide replicate exposure treatments, each containing 150 mL, while the SEX and HMX solutions were not divided. A set of control beakers or a control jar containing the same dilution water and maintained under the same conditions as

the exposure concentrations, but containing no test material, was established for each test. The ambient air temperature in the laboratory was controlled in order to maintain test solution temperatures at 22 $\pm$ 1°C. Test solutions were not aerated. The test area was illuminated with Durotest (Optima) fluorescent lights at an intensity of 4.3-7.6 hectolux. The photoperiod during testing was 16 hours light and 8 hours darkness.

Fifteen test organisms were impartially distributed to each concentration (or 5 per beaker) after the test solutions had been prepared. Mortalities in each test solution were recorded at 24- and 48-hour exposures. Biological observations and observations of the physical characteristics of each test solution were also made and recorded at 0, 24 and 48 hours. The pH and DO concentrations were measured at 0 and 48 hours in the control and the high, middle and low test concentrations. The temperature was measured at 0 and 48 hours in the control.

Water samples (10 mL) were collected with a volumetric pipet from each test vessel at 0 and 48 hours. Since toxicant-related effects were not observed in all cases, decisions on which samples for each test would be analyzed were made jointly by the COTR and Springborn Bionomics personnel.

The 96-hour acute toxicity tests with fish were conducted in 19.6-L glass jars which contained 15 L of test solution. The dilution water used was soft water reconstituted from deionized water according to recommended procedures (USEPA, 1975). This water had total hardness and alkalinity ranges as $CaCO_3$ of 40-44 mg/L and 29-36 mg/L, respectively, a pH range of 7.5-7.6 and a specific conductance range of 130-150 umhos/cm.

Test fish for the TAX static acutes were fathead minnow, bluegill, rainbow trout and channel catfish. The fathead minnow and rainbow trout test solutions were prepared by weighing out the desired quantity of TAX and adding it directly to 15 L of diluent water. The solutions were then vigorously mixed for 2 days with an Eberbach lab stirrer. For the bluegill and channel catfish tests, a TAX stock was prepared at a concentration equivalent to the desired high test concentration (580 mg/L) by adding 145 g of TAX to 250 L of diluent water and mixing with a Teel magnetic drive pump for 4 days. Test concentrations were prepared by adding the desired volume of this stock directly into a sufficient quantity of diluent water to total 15 L. The fathead minnow and rainbow trout tests were not replicated while the bluegill test was conducted with two replicates and the channel catfish test with four replicates.

The SEX acute tests were conducted with fathead minnow, bluegill and rainbow trout. A SEX stock was prepared at a concentration equivalent to the desired high test concentration (12 mg/L) by adding 2.52 g of SEX to 210 L of diluent water and mixing with a magnetic drive Teel pump for four days. Test concentrations were prepared in replicate by adding the desired volume of this stock directly into a sufficient quantity of diluent water to total 15 L.

A control or a set of control jars containing the same dilution water as used in the exposure jars, but containing no test material, was maintained for each test. Test solution temperatures were controlled by a system designed to maintain temperatures at 12 $\pm$ 1^{o}C for the rainbow trout tests and 22 $\pm$ 1^{o}C for all other tests. Test solutions were not aerated. The photoperiod during testing was the same as that provided during acclimation.

Ten fish were randomly distributed to each test concentration and were not fed during exposure. Mortalities were recorded and removed from each test jar and biological observations of the fish and observations of the physical characteristics of the test solutions were made and recorded at 0, 24, 48, 72 and 96 hours of exposure. The pH and DO concentrations were measured at 0, 24, 48 and 96 hours in one jar of the controls and the high, middle and low test concentrations. The temperature

was measured in the control jars at 0, 24, 48, 72 and 96 hours of exposure. Water samples (10 mL) were collected with a volumetric pipet from each test vessel at 0, 24, 48, 72 and 96 hours of exposure.

TAX and SEX static acute tests were conducted with several early life stages of fathead minnow and rainbow trout. The embryo tests were conducted in 250-mL glass beakers, each of which contained 150 mL of test solution. The larvae tests were conducted in 2-L glass battery jars, each of which contained 1 L of test solution. The dilution water used was soft water reconstituted from deionized water according to recommended procedures (USEPA, 1975). This water had total hardness and alkalinity ranges as $CaCO_3$ of 46-49 mg/L and 32-36 mg/L, respectively, a pH range of 7.4-7.8 and a specific conductance of 150-170 umhos/cm.

TAX and SEX stock solutions were prepared at concentrations equivalent to the high test concentration. The TAX stock (580 mg/L) was prepared by adding 29 g of TAX to 50 L of diluent water and mixing for four days. The SEX stock (12 mg/L) was prepared by adding 0.18 g of SEX to 15 L of diluent water and mixing for three days. Stocks were mixed with magnetic drive Teel pumps.

The embryo test concentrations were prepared by adding the desired volume of the appropriate stock directly into a sufficient quantity of diluent water to total 500 mL. For the fathead minnow embryo tests, 300 mL of each test

solution was divided into two beakers and for the rainbow trout embryo tests, 450 mL of each test solution was divided into three beakers to provide replicate exposure treatments, each containing 150 mL. A set of control beakers containing the same dilution water and maintained under the same conditions as the exposure conc ntrations, but containing no test material, was established for each test.

Ten fathead minnow embryos and five rainbow trout embryos were impartially distributed to the appropriate test beakers after the test solutions had been prepared. Mortalities were recorded and removed from each test beaker and biological observations of the embryos and observations of the physical characteristics of the test solutions were made and recorded at 0, 24, 48, 72 and 96 hours and each additional 24-hour interval of exposure until all embryos hatched. The pH and DO concentrations were measured at 0, 24, 48 and 96 hours and each additional 24-hour interval in the controls and high, middle and low test concentrations at the minimum. The temperature was measured in the control jars at the above-mentioned intervals.

Water samples (5 mL) were collected with a volumetric pipet from the A replicate of all test treatments in the fathead minnow and rainbow trout tests with TAX and the rainbow trout test with SEX, while the A and B replicates were sampled in the fathead minnow test with SEX. Samples

were collected at 0 hour and each 24-hour interval for the duration of the tests. Since toxicant-related effects were not observed in all cases, decisions on which samples for each test would be analyzed were made jointly by the COTR and Springborn Bionomics personnel.

Newly hatched (≤24 hours old) fathead minnow and rainbow trout larvae tests with TAX and SEX were conducted in 2-L glass battery jars, each of which contained 1 L of test solution. The dilution water and stock solutions were prepared as previously described for the embryo tests. The test concentrations were prepared in duplicate by adding the desired volume of the appropriate stock directly into a sufficient quantity of diluent water to total 1000 mL. A set of control jars containing the same dilution water and maintained under the same conditions as the exposure concentrations, but containing no test material, was established for each test.

To initiate the 96-hour larvae exposure, ten larvae (≤24 hours old) were impartially distributed to each test jar after the test solutions had been prepared with the exception of the TAX test with fathead minnow. In this test ten embryos were impartially distributed to each jar and the 96 hours of larvae exposure began when embryos had hatched which was after 72 hours of embryo exposure. Fathead minnow larvae exposure was extended to 120 hours in the SEX test due to sporadic mortality observed after 96

hours of exposure. Mortalities were recorded and removed from each test jar and biological observations of the larvae and observations of the physical characteristics of the test solutions were also made and recorded at 0, 24, 48, 72 and 96 hours and each additional 24-hour interval. The pH and DO concentrations were measured at 0, 24, 48 and 96 hours and each additional 24-hour interval in the controls and high, middle and low test concentrations. The temperature was measured in the control jars at the above-mentioned time intervals.

Water samples (5 mL) were collected with a volumetric pipet from both replicates of test solutions. Samples were collected at 0 hour and each 24-hour interval of the tests. Since toxicant-related effects were not observed in all cases, decisions on which samples for each test would be analyzed were made jointly by the COTR and Springborn Bionomics personnel.

TAX and SEX static acutes were also conducted with 7-day-old fathead minnow larvae and a TAX static was conducted with swim-up rainbow trout larvae, while the SEX static was conducted with 7-day-old rainbow trout larvae. These tests were conducted in 2-L glass battery jars, each of which contained 1 L of test solution. The dilution water, stock solutions and test concentrations were prepared as previously described for the newly hatched larvae tests. A set of control jars containing the same

dilution water and maintained under the same conditions as the exposure concentrations, but containing no test material, was established for each test.

To initiate the 96-hour larvae exposure, ten larvae were impartially distributed to each test jar after the test solutions had been prepared. Mortalities were recorded and removed from each test jar and biological observations of the larvae and observations of the physical characteristics of the test solutions were also made at 24-hour intervals. The pH and DO concentrations were measured at 0, 24, 48, and 96 hours in the controls and the high, middle and low test concentrations. The temperature was measured in the control jars at the above-mentioned time intervals. Water samples (5 mL) were collected as previously described for the newly hatched larvae tests.

Test solution temperatures for the early life stage tests were controlled by a system designed to maintain temperatures at 12 $\pm$ 1^{o}C for the rainbow trout and 22 $\pm$ 1^{o}C for the fathead minnow tests. Test solutions were not aerated. The photoperiod during the fathead minnow testing was 16 hours light and 8 hours darkness, while the rainbow trout were shielded from fluorescent light and sunlight. Larvae were fed 1-2 drops of concentrated brine shrimp daily during exposure.

The HMX embryo and larvae test was conducted according to the protocol entitled "Methods for conducting early life

stage toxicity tests with fathead minnow (Pimephales promelas)," prepared by Springborn Bionomics, Inc.

A modified, proportional diluter with a 0.50 dilution factor similar to that described by Mount and Brungs (1967) was used to deliver the desired concentration of HMX to the aquaria during the embryo and larvae exposure. The dilution water was well water which was pumped into an epoxy-coated concrete reservoir where it was supplemented with Town of Wareham untreated and unchlorinated well water and aerated. The water was heated to 25 $\pm$ 1°C in a gas-fired glass coil heater and passed through a calcite bed prior to delivery to the test system. The calcite increased the total hardness and pH of the water to approximately 35 mg/L as calcium carbonate ($CaCO_3$) and 7.7, respectively.

The diluter delivered five nominal concentrations of HMX and a diluent water control to duplicate test aquaria. Each glass test aquarium measured 39 x 20 x 25 centimeters (cm) with 19.5 cm high side drains which maintained constant test water volumes of 15 L. The diluter delivered 0.5 L of test water to each aquarium at an approximate rate of 193 times per day. This is equivalent to 6.4 aquarium volume replacements per 24-hour period. Illumination was provided by Cool White^R and Grow Lux^R fluorescent lights centrally located above the test aquaria. Twelve hours of light at 2.2-11 hectolux at the

water surface were provided each day. The aquaria rested in a water bath containing circulating water heated by immersion coil heaters and regulated by a mercury column thermoregulator designed to maintain the test water temperature at 25°C.

An FMI lab pump was used to deliver the HMX stock solution from a primary holding tank to the pre-dilution chamber of the diluter in preparation for each diluter cycle. The HMX stock solutions were prepared with dilution water at a concentration equal to the high test concentration. Due to the relatively low water solubility of HMX, the stock solutions were prepared by saturating the dilution water with HMX. Preliminary solubility work established that saturation was attained by adding 20 g of HMX to 1800 L of dilution water, mixing the stock for 4 days with a 1/2 HP Marlow swimming pool pump and then passing the stock solution through a cellulose acetate filter to remove the undissolved HMX.

The exposure of fathead minnow embryos to HMX was initiated with embryos obtained within 48 hours after fertilization from the fathead minnow culture unit at Springborn Bionomics, Inc., Wareham, Massachusetts. Sixty embryos were impartially distributed to each of 12 embryo cups, one of which was suspended in each of the test aquaria. Embryo incubation cups were glass jars (5 cm O.D., 8 cm high) with 40-mesh Nitex® screen bottoms. A

rocker arm apparatus, as described by Mount (1968), was used to gently oscillate the incubation cups in the test water. Dead embryos were counted and removed daily until hatching was complete. Percentage hatch calculations were based on the number of live larvae per incubation cup after hatching was completed compared to the number of embryos per cup (60) at the initiation of exposure.

To initiate the 32-day larvae exposure, all live larvae were transferred to the respective aquaria upon completion of hatching. Larvae were fed live brine shrimp (Artemia salina) nauplii three times daily on week days and twice daily on weekends and holidays. Aquaria were brushed and siphoned at least twice each week or as necessary to remove excess food and fecal matter. Behavior and appearance of larvae were observed daily and larvae were counted twice weekly. At 32 days post-hatch, the larvae from each aquarium were anesthetized with MS-222 (tricaine methanesulfonate), and percentage survival, mean total length, and average wet weight were determined. The larvae were measured individually to calculate mean and standard deviation total length while the average body weight of larvae in each aquarium was calculated from the total wet weight of all individuals in the aquarium. The study was initiated on 16 March and ended on 21 April 1982.

Dissolved oxygen concentration, pH and temperature were measured in every aquarium on day 0. Subsequently,

these parameters were measured daily in both replicate aquaria of one test treatment. Measurements were alternated among treatments such that each aquarium was measured once each week. Total hardness as $CaCO_3$ was measured on day 0 and weekly thereafter in alternating replicates of the high and low test concentrations and controls.

The control, high, middle and low test concentrations (A and B replicates) were sampled on two days prior to initiating the exposure. Results of the analysis of these samples were used to judge whether sufficient quantities of HMX were being delivered to the aquaria to initiate the test. All test solutions and the control were sampled on test day 0, day of hatch and weekly thereafter. In addition, two quality assurance samples were prepared at each sampling interval and remained with the set of samples through analysis. These samples were prepared in dilution water at a concentration unknown to the analyst. Each test solution sample (25 mL) was collected from midpoint (approximately equidistant from aquaria glass surfaces at half the aquaria water level (depth)) of the aquarium with a volumetric pipet and placed in a 150-mL glass culture tube.

Percentage hatch of embryos and survival, total length and average wet weight of larvae after 32 days exposure, were subjected to analysis of variance (Steel and Torrie,

1960, complete randomized block design, P=0.05). Data for percentage hatch and percentage survival were transformed to arc sin $\sqrt{\text{percentage}}$ prior to analysis. If treatment effects were indicated, the means of these parameters were compared to those from the control using Dunnett's procedure (Steel and Torrie, 1960). When a treatment mean was significantly different from the control mean (P=0.05), that treatment was considered to be an effect level. Based on these data, the MATC of HMX to fathead minnow embryos and larvae was estimated. The MATC is defined as the maximum concentration of test material which would not elicit an adverse response from the exposed organisms which was significantly different from that of the control organisms.

The HMX Daphnia magna chronic toxicity test was conducted according to the protocol entitled "Protocol for conducting chronic toxicity tests with the water flea (Daphnia magna)," prepared by Springborn Bionomics Inc. The chronic toxicity test was conducted from 13 January to 10 February 1982.

A 200-milliliter (mL) Mount and Brungs (1967) proportional diluter, calibrated to provide 50 percent dilutions, delivered the diluent water and HMX to the test aquaria during the chronic toxicity test. The dilution water was the same as previously described for invertebrate tests. The diluter was constructed entirely of glass,

Tygon^{R} tubing, silicone stoppers and sealant. Five-centimeter (cm) lengths of 2-millimeter (inside diameter) glass tubing were inserted between the mixing-splitting chambers of the diluter and test solution delivery tubes. This served to restrict the flow of the test solutions, minimizing turbulence in the aquaria. An FMI lab pump was used to deliver 390 mL of the HMX stock solution from a stainless steel primary holding tank to the pre-dilution chamber of the diluter in preparation for each diluter cycle. The HMX stock solutions were prepared in 200-L volumes with dilution water at a concentration equal to the highest test concentration. The 200 L of dilution water was saturated with HMX by adding 2.2 g of HMX and mixing for 3 days with two Little Giant pumps. The stock solution passed through filter floss to remove undissolved HMX prior to delivery to the predilution chamber.

Aquaria were glass battery jars having a volume capacity of 1.75 liters. Test solutions drained from aquaria through a 3 x 8 cm notch cut on the upper edge of the jars. Notches were covered with a Nitex^{R} 40-mesh screen to prevent loss of the daphnids. Five concentrations of HMX were tested in addition to a control. Four replicate aquaria were established at each treatment level. Test solutions were delivered to the aquaria at a rate of four to five aquarium volumes per day.

Twenty D. magna (≤24 hours old) were impartially assigned to each test aquarium (80 daphnids per treatment) at the initiation of the test. Adult survival was determined weekly and determinations of offspring production were made on every weekday from day 7 through 21. The offspring were removed, counted with a Fisher Count-A1 Model 600 particle counter (LeBlanc, 1979) and discarded.

Test organisms were fed a combination of Rangen Salmon Starter fish food suspension (5 mg/L) and a unicellular green algae (Selenastrum capricornutum) (2.0×10^4 cells/mL). The food was introduced at a rate of 2.5 mL of fish food suspension and 1 mL of algal suspension three times daily on weekdays and once daily on weekends. Test aquaria were rinsed with diluent water, followed by the replacement of the original test solution, whenever survival and reproduction were assessed.

The dissolved oxygen concentration and temperature of the test solutions were monitored on every weekday within one replicate aquarium of each treatment level and the control. Total hardness, alkalinity, specific conductance and pH of the test solutions were monitored weekly in one aquarium from each treatment and control.

Ten-milliliter water samples were removed from two replicate test vessels of the high, middle and low HMX concentrations and the control on two days prior to

initiating the exposure. Results of these analyses were used to judge whether sufficient quantities of HMX were being delivered to the aquaria to initiate the test. During the exposure, 10-mL water samples were removed and analyzed weekly from two replicate test vessels of each treatment level and the control. Alternate aquaria were sampled each week. Two quality assurance samples were prepared at each sampling interval and remained with the set of samples through extraction and analysis. These samples were prepared in dilution water at an HMX concentration unknown to the analyst. Each test solution sample was collected from the midpoint of the aquarium with a volumetric pipet and placed in a 150-mL glass culture tube.

Weekly survival data, transformed to arc sin $\sqrt{\text{percentage}}$, and the determinations of cumulative production of offspring per female derived during the chronic toxicity test, were subjected to analysis of variance according to Steel and Torrie (1960). If significant (P=0.05) differences between treatments were observed, the Dunnett's procedure (Steel and Torrie, 1960) was used to determine which treatments, if any, varied from the control. Results of the statistical analyses were used to estimate the maximum acceptable toxicant concentration (MATC). The MATC is defined as the maximum concentration of HMX, in water, which would not elicit an adverse response by D. magna during chronic exposure.

The development of basic analytical techniques for the monitoring of HMX, TAX and SEX in water was accomplished by our subcontractor, Monsanto Research Corporation. The detection limits for these compounds were established at 0.1 mg/L with no sample concentration step. All test concentrations which required chemical analysis were analyzed according to these methods (Appendices A and B).

RESULTS AND DISCUSSION

The results of the Monsanto Research Corporation determinations of water solubilities at various temperatures as well as the octanol/water partition coefficients for TAX and SEX are presented in Table 2. The results of precision and accuracy studies for these compounds performed at Springborn Bionomics, Inc., are presented in Tables 3 and 4.

Results on the water solubility of HMX were in conflict with those obtained by Barkley (personal communication) of 6.6 mg/L after stirring at 20°C for 24 hours in distilled water and 15.0 mg/L after stirring at 20°C for 24 hours in distilled water with 2% acetone. A study was therefore conducted at Bionomics utilizing both vertebrate and invertebrate reconstituted water at concentrations of 10.0, 5.0 and 2.5 mg/L of HMX. No solvents were utilized. Solutions were analyzed immediately, after 24 hours, 48 hours and 1 week of continuous stirring at 25°C. These results indicated that the HMX was most completely solubilized between 48 hours and 1 week of mixing and that the expected concentration at saturation should be ca. 2.8 mg/L (Table 5). Test solutions for the embryo-larvae exposure and the daphnid chronic test were therefore formulated in large batches utilizing an excess of HMX and were allowed to mix for a period of 4 days prior to use.

Bentley et al. (1977) previously performed toxicity tests with HMX and a wide range of organisms. The lack of toxicity observed during this testing at nominal concentrations <32 mg/L of HMX was used as the rationale for not conducting additional static, acute toxicity tests during the present study. The one test during which apparent adverse effects were observed was the 96-hour static exposure of 7-day-old fathead minnow fry. Based upon this potential toxicity and the need to accurately define the MATC, the Army chose to test the fathead minnow in an embryo-larval study and Daphnia magna in a chronic study.

The results of preliminary static acute toxicity tests with D. magna and fathead minnow at nominal concentrations ranging from 1.9 mg/L to 15 mg/L are presented in Tables 6 and 7. Since no adverse effects were observed in either the daphnid or fathead minnow tests, the daphnid chronic and fathead minnow embryo-larval studies were initiated at the limit of HMX aqueous solubility. The results of fortified quality assurance blind samples for each study are presented in Tables 8 and 9. The results of water quality measurements made during each study are presented in Tables 10 and 11. The concentrations of HMX measured in each test solution during the daphnid chronic and fathead minnow embryo-larval exposure are presented in Tables 12 and 13 for D. magna and 14 for fathead minnow. While the average measured concentrations ranged from 11-69% of

nominal in these tests, the high degree of similarity between almost all corresponding concentrations indicates that the limit of aqueous solubility was nearly attained. The survival of daphnids exposed to all test solutions containing HMX was comparable to the survival of control daphnids through the 28-day exposure (Table 15). The number of offspring produced by daphnids exposed to all concentrations of HMX was comparable to control daphnids throughout the chronic exposure (Table 16). Percentage hatch, percentage survival and mean weight and length in all concentrations were comparable to the control for fathead minnows exposed to HMX for 32 days post-hatch (Table 17).

The results of TAX testing with the alga (Selenastrum capricornutum) are presented in Tables 18 and 19. These suggest that TAX is not acutely toxic to S. capricornutum up to the limit of aqueous solubility of 455 mg/L. Results of the D. magna and Gammarus sp. testing similarly indicate a lack of acute toxicity of TAX at concentrations equal to the material's aqueous solubility limit (Table 20). Despite some recrystallization problems experienced in the water samples of the channel catfish and bluegill acute toxicity tests, the results of the analyses of all acute test solutions are substantially in agreement (Table 21). No mortality was observed during tests with bluegill or channel catfish, while sporadic mortality was observed in the fathead minnow and rainbow trout testing. Due to

this observed mortality, a decision to test various life stages of the fathead minnow and rainbow trout was made. The stages selected corresponded to those used by Bentley et al. (1977) in the previous HMX testing and represented the potentially most sensitive life stages of these species. The results of the chemical analyses of the test solutions taken during these tests are presented in Table 22. As can be seen, all measured concentrations closely approximated nominal and the limit of aqueous solubility. The increased concentration of TAX measured in most concentrations at 72, 96 and 120 hours was unable to be explained but was confirmed by re-analysis of the high concentration at 72 and 96 hours. The levels of TAX measured in the controls were slightly above the detection limit. The results of this testing suggest that the fathead minnow 24-hour-old fry was adversely affected with a maximum mortality at 168 hours of 35% (Table 23) at a concentration of 600 mg/L. The rainbow trout embryo exposure, begun with 21-23-day-old embryos, yielded substantial control mortality. It is believed that this was due to the addition of nonviable eggs at test initiation. The mortality observed in all concentrations is attributed to the same problem and is not considered to be toxicant related. The suggestion of acute effects was observed in the rainbow trout swim-up stage with all fry exhibiting lethargic behavior at measured concentrations of

370 and 550 mg/L. These concentrations approximate the limit of aqueous solubility of TAX.

The results of SEX testing with the alga (Selenastrum capricornutum) are presented in Tables 24 and 25. These data clearly reveal that SEX is not acutely toxic to S. capricronutum at levels closely approximating the limits of aqueous solubility. Due to extremely limited quantities of SEX, testing was conducted on one invertebrate species (Daphnia magna), three fish species (bluegill, fathead minnow and rainbow trout) and the life stages of fathead minnow and rainbow trout as used in the TAX testing. Results of the testing with daphnids indicate a lack of acutely toxic effects up to the limits of aqueous solubility (Table 26). Similarly, the testing with bluegill, fathead minnow and rainbow trout indicate a paucity of toxicant-related effects (Table 27). The results of the chemical analysis of the test solutions taken during the life stage testing of fathead minnow and rainbow trout are presented in Table 28. The results of this testing indicate that virtually no significant mortality occurred in this testing (Table 29). The 50% mortality observed at 144-hour in the fathead minnow embryo test at a measured concentration of 6.4 mg/L resulted from embryos dying in one replicate and 10% of the embryos dying in the other replicate of that concentration. This is therefore considered an anomaly.

It is apparent that HMX is not acutely or chronically toxic to the organisms tested at concentrations equal to the material's limit of aqueous solubility. While no solvents were utilized in these studies, extraordinary means were undertaken to solubilize the material, yielding a mean solubility after 1 week of mixing at ca. 25°C of 2.8 ± 0.86 mg/L (range = 1.4-4.2 mg/L).

Based upon the absence of observed toxicity to D. magna in the chronic toxicity test, the MATC of this compound for D. magna was estimated to be >3.9 mg/L. Similarly, based on the lack of observed toxicity to fathead minnows in an embryo-larval exposure, the MATC of this compound for fathead minnows was estimated to be >3.3 mg/L. While these data are not in agreement with the results reported in Bentley et al. (1977), suggesting toxicity to 7-day-old fry, these data represent the approximate aqueous solubility of HMX without the use of solvents to solubilize these materials. We therefore feel that the data from the embryo-larval testing, when compared with the daphnid results, are more clearly indicative of the true environmental concentration and should have the most credence.

The results of the TAX testing suggests there may be an adverse effect on two life stages tested - fathead minnow 24-hour-old fry and rainbow trout swim-up stage.

The rainbow trout effect is a more subjective observation while the mortality in the fathead minnow test, even if corrected for the spurious control mortality, appears to be a real effect. However, based upon the significant amount of material which would have been required to conduct an embryo-larval study and the fact that the concentration tested approached the limit of aqueous solubility, a decision was made to not pursue further testing with this material. We would therefore suggest that, based upon the acute toxicity observed to fathead minnow 24-hour-old fry, the effect level is ca. 600 mg/L.

The results of the SEX testing suggests there may be an adverse effect on the fathead minnow 7-day-old fry at the highest concentration tested (10 mg/L measured concentration). This mortality was evident at 24 hours and was present in both replicates and is considered to be a real effect. However, as in the TAX testing, the limited availability of this material and the fact that the concentration tested approached the limit of aqueous solubility, led to a decision not to pursue further testing with the SEX. Based upon the acute toxicity observed to the fathead minnow 7-day-old fry, the effect level is ca. 10 mg/L.

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Fish Lengths and Weights Log, Volumes III and IV

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[a]All documents are stored at Springborn Bionomics, Inc.

Table 1. Fish used in static acute toxicity tests with HMX, TAX and SEX.

Compound	Species	Bionomics lot #	Wet weight[a] (g)	Length[a] (mm)	Source
HMX	Fathead minnow	80A11	0.79(0.32-1.54)	45(40-55)	Springborn Bionomics, Inc.
TAX	Fathead minnow	81A13	0.27(0.18-0.42)	32(26-36)	Springborn Bionomics, Inc.
	Bluegill	81A25	0.54(0.23-1.2)	37(31-44)	Commercial Hatchery W. Willington, Connecticut
	Rainbow trout	81A12	0.31(0.17-0.44)	33(29-38)	Commercial Hatchery Lewiston, Montana
	Channel catfish	81A14	1.8(1.0-2.5)	61(52-68)	Commercial Hatchery Osage Beach, Missouri
SEX	Fathead minnow	82A6	0.29(0.16-0.62)	33(26-41)	Springborn Bionomics, Inc.
	Bluegill	81A31	0.34(0.22-0.62)	33(26-40)	Commercial Hatchery Brady, Nebraska
	Rainbow trout	82A3	0.28(0.18-0.42)	35(30-38)	U.S. Fish & Wildlife Beltsville, Maryland

[a]Mean (range, N=30), Fish Weights and Lengths Log.

Table 2. Results of solubility and octanol/water partition coefficient analyses for TAX and SEX.

TAX: Solubility in Water[a,b]

Temperature (°C)	Solubility (mg/L) (± std. dev.)	Relative std. dev. (%)	Sample size
10	339 ± 14	4.1	n = 9
20	576 ± 9	1.6	n = 9
30	918 ± 16	1.6	n = 9

SEX: Solubility in Water[a,b]

Temperature (°C)	Solubility (mg/L) (± std. dev.)	Relative std. dev. (%)	Sample size
10	8 ± 0.7	9	n = 9
20	12 ± 2	18	n = 9
30	25 ± 6	23	n = 9

TAX: Octanol/Water Partition Coefficient (25°C)[a,c]

	P	log P	Relative std. dev. (%)	Sample size
Method I	1.3	0.114	±4.8	n = 18
Method II	1.3	0.114	±6.9	n = 18

SEX: Octanol/Water Partition Coefficient (25°C)[a,b]

P	log P	Relative Std. Dev. (%)	Sample size
0.56 ± 0.03	-0.25	±6	n = 18

Table 2 (continued)

[a] Column conditions. Lichrosorb RP 18, 10 μm particle dia., packed in a 250 mm (L) x 4.6 mm (I.D.) stainless steel column with 2 mℓ/min flow rate of mobile phase.

[b] HPLC Parameters - Solubility determination: 15% methanol, 85% deionized water isocratic, 2 mℓ/min, range: 0.04 AUFS, wavelength: 240 nm. A calibration curve was determined in the range of 10 to 1,000 ppm TAX and was used to quantify each injection.

[c] HPLC Parameters - Octanol/Water Partition Coefficient Determination: 50% methanol, 50% water isocratic, 2 mℓ/minute, range: 0.01 AUFS, wavelength: 240 nm. An external standard method was used with a HP 3350 data system so that the results were reported directly in units of ppm.

Table 3. Results of precision and accuracy studies conducted with TAX.

DISTILLED WATER - PRECISION

	Nominal Concentration (mg/L)			
	0.56	1.1	2.2	5.6
Measured Concentration (mg/L)		1.2		
		1.2		
		1.2		
		1.2		
		1.1		
		1.2		
		1.2		
		1.2		
		1.1		
		1.2		
$\bar{X}$ ± S.D.		1.2±0.04		
(meas. as % of nom.)		(110)		

DISTILLED WATER - ACCURACY

	Nominal Concentration (mg/L)			
	0.56	1.1	2.2	5.6
Measured Concentration (mg/L)	0.54	1.1	2.2	5.5
	0.55	1.1	2.1	5.4
	0.56	1.1	2.2	5.5
	0.58	1.1	2.2	5.7
	0.58	1.1	2.2	5.7
	0.56	1.1	2.2	5.6
	0.62	1.1	2.2	5.7
	0.58	1.1	2.2	5.6
	0.59	1.1	2.2	5.7
	0.58	1.1	2.2	5.7
$\bar{X}$ ± S.D.	0.57±0.02	1.1±0	2.2±0.03	5.6±0.11
(meas. as % of nom.)	(102)	(100)	(100)	(100)

Table 3 (continued)

VERTEBRATE WATER - ACCURACY

	Nominal Concentration (mg/L)			
	0.56	1.1	2.2	5.6
Measured Concentration (mg/L)	0.59	1.2	2.2	5.6
	0.57	1.1	2.3	5.5
	0.60	1.1	2.2	5.6
	0.54	1.1	2.3	5.5
	0.54	1.1	2.3	5.6
	0.54	1.1	2.3	5.5
	0.53	1.1	2.2	5.3
	0.53	1.0	2.2	5.5
	0.59	1.2	2.3	5.3
	0.51	1.1	2.2	5.4
$\bar{X}$ ± S.D.	0.55±0.03	1.1±0.06	2.3±0.05	5.5±0.11
(meas. as % of nom.)	(98)	(100)	(105)	(98)

INVERTEBRATE WATER - ACCURACY

	Nominal Concentration (mg/L)			
	0.56	1.1	2.2	5.6
Measured Concentration (mg/L)	0.59	1.1	2.2	5.5
	0.58	1.1	2.2	5.5
	0.57	1.1	2.1	6.6
	0.59	1.1	2.1	5.4
	0.55	1.1	2.2	5.6
	0.57	1.1	2.2	5.5
	0.57	1.1	2.2	5.4
	0.56	1.1	2.2	5.5
	0.57	1.0	2.2	5.4
	0.55	1.1	2.2	5.6
$\bar{X}$ ± S.D.	0.57±0.01	1.1±0.03	2.2±0.04	5.5±0.08
(meas. as % of nom.)	(102)	(100)	(100)	(98)

Table 4. Results of precision and accuracy studies conducted with SEX.

DISTILLED WATER - PRECISION

	Nominal Concentration (mg/L)			
	0.5	1.0	2.0	5.0
Measured Concentration (mg/L)		0.98		
		1.0		
		1.0		
		1.0		
		1.1		
		0.94		
		1.1		
		1.1		
		0.99		
		0.99		
$\bar{X}$ ± S.D.		1.0 ± 0.058		
(meas. as % of nom.)		(100)		

DISTILLED WATER - ACCURACY

	Nominal Concentration (mg/L)			
	0.5	1.0	2.0	5.0
Measured Concentration (mg/L)	0.47	0.94	1.9	5.0
	0.48	0.95	2.1	5.0
	0.49	0.99	2.0	5.0
	0.48	0.95	2.0	5.0
	0.44	0.98	2.1	5.0
	0.48	0.99	2.0	5.0
	0.44	1.1	2.0	5.0
	0.46	1.0	2.1	5.1
	0.43	0.95	2.0	5.1
	0.49	1.0	2.0	5.0
$\bar{X}$ ± S.D.	0.47±0.022	0.98±0.046	2.0±0.063	5.0±0.042
(meas. as % of nom.)	(94)	(98)	(100)	(100)

Table 4 (continued)

VERTEBRATE WATER - ACCURACY

	Nominal Concentration (mg/L)			
	0.5	1.0	2.0	5.0
Measured Concentration (mg/L)	0.54	1.0	2.0	5.0
	0.54	1.1	2.0	5.0
	0.55	0.97	2.0	5.1
	0.55	1.0	2.0	5.1
	0.57	1.0	2.0	5.2
	0.46	1.0	2.1	5.1
	0.51	0.98	2.0	5.0
	0.51	1.0	2.0	5.0
	0.52	1.0	2.0	5.2
	0.50	1.0	2.1	5.0
	0.49			
$\bar{X}$ ± S.D.	0.52±0.032	1.0±0.035	2.0±0.042	5.1±0.082
(meas. as % of nom.)	(104)	(100)	(100)	(102)

INVERTEBRATE WATER - ACCURACY

	Nominal Concentration (mg/L)			
	0.5	1.0	2.0	5.0
Measured Concentration (mg/L)	0.47	1.0	2.0	5.0
	0.49	1.0	2.1	5.0
	0.49	0.99	2.0	5.0
	0.49	0.98	2.0	5.1
	0.50	0.96	2.0	5.2
	0.50	1.0	2.2	5.3
	0.46	0.95	2.0	5.1
	0.53	0.96	2.2	5.1
	0.51	0.96	2.0	5.0
	0.50	0.95	2.0	5.0
$\bar{X}$ ± S.D.	0.49±0.020	0.98±0.021	2.0±0.085	5.1±0.10
(meas. as % of nom.)	(98)	(98)	(100)	(102)

Table 5.

SOLUBILITY OF HMX IN INVERTEBRATE AND VERTEBRATE DILUTION WATER

SAMPLE CONCENTRATION	ANALYTICAL RESULTS (mg/L)				REMARKS
	0 HOURS[a]	24 HOURS[a]	48 HOURS[a]	1 WEEK[a]	
2.5 mg/L INVERT.	0.02 0.04 0.04 $\bar{x}$ = 0.04	0.85 0.86 0.83 $\bar{x}$ = 0.85	1.7 2.3 $\bar{x}$ = 2.0	2.6 2.5 $\bar{x}$ = 2.6	@ 48h, temp. was 27°C due to heat from magnetic stir plate and remained warmer than others throughout the study.
5.0 mg/L INVERT.	0.06 0.07 0.07 $\bar{x}$ = 0.07	0.36 0.40 0.38 $\bar{x}$ = 0.38	0.52 0.63 $\bar{x}$ = 0.58	1.4 1.4 $\bar{x}$ = 1.4	@ 48h, temp. 25.8°
10 mg/L INVERT.	0.19 0.20 0.25 $\bar{x}$ = 0.21	0.63 0.63 0.62 $\bar{x}$ = 0.63	1.8 1.8 $\bar{x}$ = 1.8	3.3 3.4 $\bar{x}$ = 3.4	@ 48h, temp. 25.5°
2.5 mg/L VERT.	0.09 0.11 0.12 $\bar{x}$ = 0.11	0.58 0.59 0.57 $\bar{x}$ = 0.57	1.3 1.3 $\bar{x}$ = 1.3	2.5 2.5 $\bar{x}$ = 2.5	@ 48h, temp. 27° also remained warm.
5.0 mg/L VERT.	0.16 0.14 0.20 $\bar{x}$ = 0.17	2.8 2.7 2.8 $\bar{x}$ = 2.8	4.0 4.1 $\bar{x}$ = 4.0	4.2 4.2 $\bar{x}$ = 4.2	@ 48h, temp. 25.5°
10 mg/L VERT.	0.23 0.24 0.23 $\bar{x}$ = 0.23	1.1 1.2 1.2 $\bar{x}$ = 1.2	1.8 1.8 $\bar{x}$ = 1.8	2.9 2.9 $\bar{x}$ = 2.9	@ 48h, temp. 26°

[a]Represents amount of time mixed prior to analysis.

Table 6. Concentrations tested and corresponding percentage mortalities of water flea (Daphnia magna) exposed to HMX for 24 and 48 hours.

Nominal concentration (mg/L)	% Mortality 24-hour	48-hour
15[a]	0	0
9.0[a]	0	0
5.4[a]	0	0
3.2[a]	0	0
1.9[a]	0	0
control	0	0

[a] A white crystalline material was observed on the bottom of all test vessels.

Table 7. Concentrations tested and corresponding percentage mortalities of fathead minnow (Pimephales promelas) exposed to HMX for 24, 48, 72 and 96 hours.

Nominal concentration (mg/L)	% Mortality			
	24-hour	48-hour	72-hour	96-hour
15[a]	0	0	0	0
9.0[a]	0	0	0	0
5.4[a]	0	0	0	0
3.2	0	0	0	0
1.9	0	0	0	0
control	0	0	0	0

[a]Undissolved chemical was present on the bottom of these test vessels.

Table 8. Results of HMX fortified quality assurance samples analyzed during the HMX daphnid chronic.

Test day	Theoretical concentration (mg/L)	Measured concentration (mg/L)	% Recovery
0	2.6	2.2	84
	2.6	2.7	108
7	2.6	3.0	115
	2.6	3.0	115
14	2.6	2.6	100
	2.6	2.5	96
21	2.6	2.6	100
	2.6	2.6	100
28	2.6	2.5	96
	2.6	2.3	88
			$\bar{x}$ = 100 ± 9.7[a]

[a] Mean and standard deviation

Table 9. Results of fortified quality assurance blind samples analyzed during the HMX embryo-larval exposure.

Test Day	Prepared Concentration (mg/L)	Measured Concentration (mg/L)	% Recovery
0	2.4	2.4	100
	4.8	0.5	94
4	2.4	2.3	96
	2.4	2.4	100
11	2.0	1.8	90
	4.0	3.4	85
18	2.0	2.0	100
	4.0	0.92	23[a]
25	4.0	3.9	98
	2.0	1.9	95
32	2.0	1.8	90
	8.0	6.0	75
			$\bar{x}$ = 93 ± 7.7%[b]

[a]This value is considered an outlier based on Chauvenet's criterion and was not used in the calculation of the mean.

[b]Mean and standard deviation.

Table 10. Water quality analysis of test solutions during the chronic exposure of Daphnia magna to HMX.

Nominal concentration (mg/L)	Mean (standard deviation)					
	Dissolved oxygen[a] (mg/L)	Temperature[a] (°C)	Total hardness[b] (mg/L $CaCO_3$)	Total alkalinity[b] (mg/L $CaCO_3$)	Specific conductance[b] (µmhos/cm)	pH[b] range
11	7.5(0.5)	22(1)	200(10)	140(10)	600 (0)	7.8-8.5
5.5	8.0(0.5)	22(1)	190(10)	140(10)	580 (40)	7.9-8.5
2.8	8.2(0.4)	22(1)	180)20)	130(10)	540 (50)	7.9-8.4
1.4	8.3(0.5)	22(1)	180(20)	130(10)	540 (50)	7.9-8.4
0.69	8.3(0.5)	22(1)	180(20)	130(10)	540 (50)	7.9-8.3
control	8.4(0.5)	22(1)	170(20)	130(10)	540 (50)	8.0-8.3

[a] n = 21

[b] n = 5

Table 11. Water quality analysis of test solutions during the embryo-larval exposure of fathead minnows to HMX.

Nominal concentration (mg/L)	Mean (standard deviation)			
	Dissolved oxygen (mg/L)	Temperature (°C)	Total hardness (mg/L $CaCO_3$)	pH range
5.0	8.4(0.5)[a]	25(1)[a]	34(1.8)[c]	7.2-7.8[a]
2.5	8.5(0.4)[a]	25(1)[a]	—[d]	7.3-7.8[a]
1.2	8.5(0.4)[b]	24(1)[b]	—[d]	7.5-8.0[b]
0.62	8.4(0.5)[b]	25(1)[b]	—[d]	7.6-8.3[b]
0.31	8.4(0.4)[b]	25(1)[b]	35(0.98)[c]	7.7-8.3[b]
control	8.4(0.4)[b]	25(1)[b]	35(0.98)[c]	7.7-8.2[b]

[a] n = 12

[b] n = 10

[c] n = 5

[d] Measurement not taken.

Table 12. Mean (standard deviation) HMX concentrations measured before initiating the chronic exposure of D. magna.

Nominal concentration (mg/L)	Mean measured concentration[a] (mg/L)
11	3.3(0.7)
2.8	0.66(0.01)
0.69	0.21(0.02)
control	<0.062

[a] $n = 2$

Table 13. Results of analyses of HMX solutions sampled during a 28-day chronic toxicity study with Daphnia magna.

Nominal concentration (mg/L)		Day 0	Day 7	Day 14	Day 21	Day 28	$\bar{X}$(S.D.)[a]
11	A		4.0		3.9		
	B		3.9		4.1		
	C	2.7		5.7		2.7	3.9(1.1)
	D	2.8		6.0		2.9	
5.5	A		1.7		2.1		
	B		1.8		1.9		
	C	1.4		2.9		1.6	1.9(0.50)
	D	1.4		2.8		1.6	
2.8	A		0.83		0.90		
	B		0.86		0.94		
	C	0.57		1.4		0.56	0.85(0.26)
	D	0.65		1.2		0.61	
1.4	A		0.54		0.51		
	B		0.52		0.51		
	C	0.48		0.82		0.50	0.55(0.14)
	D	0.40		0.80		0.42	
0.69	A		0.16		0.27		
	B		0.20		0.24		
	C	0.23		0.35		0.19	0.23(0.07)
	D	0.15		0.36		0.18	
control	A		<0.0064		<0.044		
	B		<0.0064		<0.044		
	C	<0.050		<0.056		<0.049	
	D	<0.050		<0.056		<0.049	
QA	A		3.0(115)		2.6(100)		
	B		3.0(115)		2.6(100)		
	C	2.2(85)[b]		2.6(100)		2.5(96)	
	D	2.8(108)		2.5(96)		2.3(88)	

[a]Mean (standard deviation). [b]Percent of nominal.

Table 14. Concentrations of HMX measured in test aquaria during the fathead minnow embryo-larval exposure.

Nominal Conc. (mg/L)		Test Day: Pretest 1	Pretest 2	0	4	11	18	25	32	Mean	Standard Deviation	n
		Concentration (mg/L)										
5.0	A	4.7	4.8	3.5	2.9	2.3	2.3	2.6	2.6	3.3	0.99	16
	B	4.9	4.8	3.5	2.9	2.4	2.4	3.0	2.6			
2.5	A	—	—	1.7	1.4	1.1	1.1	1.3	1.3	1.3	0.21	12
	B	—	—	1.7	1.4	1.1	1.1	1.3	1.3			
1.3	A	1.2	1.2	0.89	0.70	0.55	0.58	0.65	0.66	0.78	0.24	16
	B	1.1	1.1	0.82	0.69	0.54	0.57	0.68	0.62			
0.65	A	—	—	0.42	0.29	0.27	0.36	0.27	0.31	0.32	0.053	12
	B	—	—	0.40	0.31	0.27	0.29	0.26	0.34			
0.32	A	0.31	0.31	0.28	0.17	0.21	0.21	0.12	0.18	0.22	0.063	16
	B	0.26	0.33	0.26	0.16	0.16	0.22	0.17	0.20			
Control	A	<0.019	<0.023	<0.070	<0.029	<0.040	<0.074	<0.05	<0.032			
	B	<0.019	<0.023	<0.070	<0.029	<0.040	<0.074	<0.05	<0.032			

Table 15. Weekly mean (standard deviation) percentage survival of the water flea (Daphnia magna) during chronic exposure to concentrations of HMX.

Mean measured concentration (mg/L)	Percentage survival			
DAY/	7	14	21	28
3.9	96(5)	94(2)	94(2)	90(0)
1.9	98(5)	94(6)	90(4)	90(4)
0.85	98(5)	93(5)	91(5)	91(5)
0.55	98(5)	94(6)	91(5)	91(5)
0.23	98(3)	94(2)	92(3)	90(0)
control	95(4)	95(4)	94(2)	94(2)

Table 16. Cumulative offspring produced per female D. magna during continuous exposure to concentrations of HMX.

Mean measured concentrations (mg/L)	Average cumulative number offspring per female D. magna															
DAY/	7	8	9	12	13	14	15	16	19	20	21	22	23	26	27	28
3.9	2(1)	6(0)	9(1)	18(2)	21(4)	26(4)	29(4)	33(4)	50(5)	53(5)	56(6)	62(5)	64(5)	80(8)	84(8)	90(9)
1.9	2(0)	4(2)	9(1)	16(0)	20(2)	24(2)	28(2)	32(5)	46(6)	50(6)	55(8)	60(8)	66(8)	86(8)	92(10)	102(8)
0.85	1(0)	4(2)	7(2)	18(4)	22(5)	27(5)	30(4)	34(4)	50(5)	55(6)	59(7)	64(6)	68(6)	88(7)	94(7)	102(7)
0.55	0(0)	2(1)	4(0)	14(4)	18(4)	21(4)	24(5)	27(5)	42(4)	48(4)	53(6)	59(5)	62(6)	82(8)	86(8)	94(9)
0.23	2(2)	4(3)	8(4)	23(12)	27(12)	32(12)	35(13)	38(12)	57(12)	63(12)	68(14)	72(14)	76(15)	94(14)	99(16)	106(16)
control	2(2)	3(2)	5(2)	17(5)	24(7)	28(8)	32(8)	35(8)	52(8)	58(9)	63(10)	68(10)	71(10)	91(8)	95(8)	102(9)

Table 17. Percentage hatch, survival, and mean length and weight of fathead minnow fry (Pimephales promelas) exposed to HMX for 32 days post-hatch.

Mean measured HMX concentration (mg/L)		% Hatch	% Survival	Mean Length (mm)	Mean Weight (g)
3.3	A	93	98	21 (2.6)[a]	0.066
	B	95	98	21 (2.2)	0.072
1.3	A	93	96	21 (2.3)	0.068
	B	95	100	21 (2.5)	0.063
0.78	A	100	95	21 (1.9)	0.064
	B	98	97	20 (2.4)	0.058
0.32	A	97	95	21 (2.0)	0.064
	B	97	95	19 (2.7)	0.054
0.22	A	98	100	20 (2.5)	0.060
	B	93	100	20 (2.7)	0.058
Control	A	95	96	20 (2.1)	0.062
	B	97	95	20 (2.5)	0.060

[a]Standard Deviation

Table 18. In vivo chlorophyll a (expressed in relative fluorescence units determined with a Turner Model 111 fluorometer) during a 5-day continuous exposure of Selenastrum capricornutum to TAX. Values are means of three flasks. Percentage change is increase of relative fluorescence units in exposed cultures as compared to the control at day 5.

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Relative fluorescence units			Percentage change Day 5
		Day 1	Day 3	Day 5	
Control	<0.12	26 (1)[c]	780 (60)	1,980 (300)	---
36	—[a]	26 (2)	840 (50)	2,160 (360)	+9
72	—[a]	26 (3)	860 (60)	2,100 (360)	+6
143	—[a]	24 (2)	760 (60)	1,860 (360)	+6
286	—[a]	22 (3)	780 (80)	2,280 (360)	+15
572	455[b]	21 (3)	770 (80)	2,280 (240)	+15

[a]Not measured.

[b]N=2.

[c]Standard deviation.

Table 19. Cell numbers x 10^4/milliliter (determined by hemacytometer) during a 5-day continuous exposure of Selenastrum capricornutum to TAX. Values are means of three flasks. Percentage change is increase or decrease of cell numbers in exposed cultures as compared to the control at day 5.

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Cell numbers x 10^4/mL		Percentage change Day 5
		Day 3	Day 5	
Control	<0.12	68.3 (3.8)[c]	243 (14)	---
36	—[a]	73.0 (5.3)	244 (17)	0
72	—[a]	72.8 (2.9)	255 (16)	+5
143	—[a]	63.2 (5.5)	224 (12)	+9
286	—[a]	65.5 (5.8)	242 (23)	0
572	455[b]	65.8 (5.8)	235 (15)	-3

[a]Not measured.

[b]N=2.

[c]Standard deviation.

Table 20. Concentrations tested and corresponding percentage mortalities for water flea (Daphnia magna) and scud (Gammarus sp.) exposed to TAX during 48-hour static acute toxicity tests.

Species	Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	% mortality 24-hour	% mortality 48-hour
Daphnia magna	580	490[a]	0	0
	350	—[b]	0	0
	210	—[b]	0	0
	130	—[b]	0	0
	79	—[b]	0	0
	Control	<0.072	0	0
Gammarus sp.				
	580	467[a]	0	7
	350	—[b]	0	0
	210	—[b]	14	14
	130	—[b]	7	7
	79	—[b]	7	7
	Control	<0.079	0	0

[a] N=6

[b] Not measured.

Table 21. Concentrations tested and corresponding observed percentage mortalities for bluegill (Lepomis macrochirus), channel catfish (Ictalurus punctatus), fathead minnow (Pimephales promelas) and rainbow trout (Salmo gairdneri) exposed to TAX during static acute toxicity tests.

Species	Nominal concentration (mg/L)	Mean measured concentration (mg/L)	Percentage Mortality			
			24-hour	48-hour	72-hour	96-hour
Bluegill	580	645	0	0	0[a]	0[ab]
	350	384	0	0	0	0
	210	—[c]	0	0	0	0
	120	—	0	0	0	0
	75	81	0	0	0	0
	control	<0.24	0	0	0	0
Channel catfish	580	661	0	0	0[d]	0[d]
	350	393	0	0	0	0
	210	—	0	0	0	0
	120	—	0	0	0	0
	75	86	0	0	0	0
	control	<0.24	0	0	0	0

Table 21 (continued)

Species	Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Percentage Mortality 24-hour	48-hour	72-hour	96-hour
Fathead minnow	580	507	10[b]	20[f]	20[f]	20[f]
	350	–[c]	0	0[f]	0	0
	210	203	0	0[f]	0	0
	130	–[c]	0	0[f]	0	0
	75	72	0	0[f]	0	0
	Control	<0.086	0	0	0	0
Rainbow trout	580	380	0[ae]	10[ae]	20[e]	40[ef]
	350	337	0	0[b]	0[a]	10[f]
	210	200	0	0	0	0[f]
	130	120	0	0	0	0[f]
	75	70	0	0	0	0
	Control	0.094	0	0	0	0

[a]Fish were dark in coloration and lethargic.

[b]Some fish exhibited a partial loss of equilibrium.

[c]Not analyzed.

[d]Fish were lethargic.

[e]Some fish exhibited either a partial or complete loss of equilibrium.

[f]Test solution was slightly cloudy.

Table 22. Results of analyses of TAX solutions measured during static, acute toxicity tests with various life stages of *fathead minnow* and *rainbow trout*.

Species/stage	Nominal conc. (mg/L)	Measured Concentration (mg/L)								$\bar{X}$(S.D.)[a]
		0-hour	24-hour	48-hour	72-hour	96-hour	120-hour	144-hour	168-hour	
					800[b]	1200[b]				
fathead minnow/ embryo	580	590	630	540	1000	1300	880			870(260)
	350	360	380	410	360	490	540			420(68)
	75	74	78	96	120	110	110			98(17)
	control	0.35	0.34	<0.42	0.82	0.93	0.50			—
	QA	2.2(110)[c]	5.6(112)	1.8(90)	5.2(104)	1.9(95)	4.9(98)			
fathead minnow/ 24-hour-old fry	580 A	610	600	620	540	620	630	620	580	600(37)
	B	660	610	620	500	600	610	600	640	
	350 A	420	360	370	380	400	380	380	380	380(22)
	B	410	380	320	390	380	380	350	380	
	75 A	82	78	78	80	76	78	79	76	79(2.1)
	B	77	81	76	81	78	81	82	81	
	control A	<0.57	<0.43	<0.47	<0.47	<0.43	<0.50	<0.50	<0.43	—
	B	<0.57	<0.43	<0.47	<0.43	<0.43	<0.50	<0.43	<0.43	
	QA A	5.1(102)	5.1(102)	5.2(104)	5.2(104)	5.0(100)	2.0(100)	5.0(100)	5.0(100)	—
	B	2.2(110)	1.9(95)	2.0(100)	2.0(100)	2.1(105)	5.1(102)	2.0(100)	2.0(100)	
fathead minnow/ 7-day-old fry	580 A	610	600	630	500	620				580(55)
	B	620	600	620	460	560				
	350 A	400	370	370	380	360				380(10)
	B	380	370	380	380	380				
	75 A	80	79	79	77	77				80(2.1)
	B	83	83	80	81	82				
	control A	<0.48	<0.48	<0.47	<0.49	<0.49				—
	B	<0.48	<0.47	<0.47	<0.49	<0.49				
	QA A	5.1(102)	5.2(104)	2.0(100)	2.0(100)	5.2(104)				—
	B	2.0(100)	2.1(105)	5.2(104)	5.1(102)	2.0(100)				

Table 22(continued)

Species/stage	Nominal conc. (mg/L)	Measured Concentration (mg/L) 0-hour	24-hour	48-hour	72-hour	96-hour	120-hour	144-hour	168-hour	$\bar{X}$(S.D.)[a]
rainbow trout/ embryo	580	560	550	510	580	570	520			550(25)
	350	380	420	370	410	450	350			400(33)
	75	80	85	70	83	75	79			79(5.0)
	control	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25			—
	QA	2.0(100)[c]	4.9(98)	1.8(90)	4.8(96)	2.0(100)	5.2(104)			—
rainbow trout/ sac fry	580 A	630	590	620	550	520				590(40)
	B	650	630	600	550	580				
	350 A	360	360	320	440	340				390(46)
	B	420	430	400	470	360				
	75 A	87	76	78	82	83				81(4.3)
	B	87	77	74	83	79				
	control A	<0.44	<0.38	<1.0	<0.50	<0.37				—
	B	<0.44	<0.38	<0.44	<0.50	<0.37				
	QA A	2.1(105)	2.1(105)	2.1(105)	4.8(96)	5.0(100)				—
	B	5.2(104)	5.1(102)	5.0(100)	2.1(105)	2.1(105)				

Table 22 (continued).

	Nominal conc. (mg/L)		Measured concentration (mg/L)							
			0-hour	24-hour	48-hour	72-hour	96-hour	120-hour	144-hour	$\bar{X}$(S.D)[a]
Rainbow trout/ swim-up stage	580	A	490	590	510	600	580	560	460	550(50)
		B	620	460	560	580	580	530	510	
	350	A	360	370	380	360	370	380	370	370(11)
		B	360	370	390	390	360	370	350	
	75	A	77	81	77	74	76	76	71	77(2.7)
		B	81	77	78	74	80	76	76	
	Control	A	1.7	<0.43	<0.51	<0.49	<0.49	<0.48	<0.48	-
		B	<0.43	0.89	<0.51	<0.49	<0.48	<0.48	<0.48	-
	QA	A	2.1(105)[c]	1.9(95)	5.0(100)	2.1(105)	5.0(100)	5.2(104)	1.9(95)	-
		B	5.1(102)	5.2(104)	1.9(95)	4.9(98)	2.1(105)	2.1(105)	4.8(96)	-

[a]Mean (standard deviation).

[b]Due to the high values obtained, the samples at these time periods were re-analyzed for confirmation.

[c]Percent of nominal.

Table 23. Concentrations tested and corresponding observed percentage mortalities of various life stages of fathead minnow and rainbow trout exposed to TAX during a static acute exposure.

Species/stage	Nominal conc. (mg/L)	Mean meas. conc. (mg/L)	Percentage Mortality						
			24-h	48-h	72-h	96-h	120-h	144-h	168-h
fathead minnow/ embryo	580	870	0	15	15	15	15		
	350	420	0	5	5	5	5		
	210	—[a]	0	5	10	10	10		
	130	—	0	0	0	0	0		
	75	98	0	0	0	0	0		
	control		0	10	10	10	10		
fathead minnow/ 24-hour-old fry	580	600	5	5	10	10	25	25	35
	350	380	5	5	5	5	5	5	5
	210	—	5	5	5	5	5	5	5
	130	—	0	0	0	5	5	5	5
	75	79	0	0	0	5	5	5	5
	control		0	5	5	5	5	5	5
fathead minnow/ 7-day-old fry	580	580	0	0	0	5[b]			
	350	380	0	0	0	10			
	210	—	0	0	0	0			
	130	—	0	0	0	0			
	75	80	0	0	0	0			
	control		0	0	0	0			
rainbow trout/ embryo	580	550	0	0	14	21	35	49	
	350	400	0	0	14	14	21	21	
	210	—	0	7	7	7[c]	14	21[c]	
	130	—	0	0	0	7	14	35[c]	
	75	79	0	0	14	21[c]	35	35[d]	
	control		0	0	33	49	49	49	
rainbow trout/ sac fry	580	590	5	5	5	5			
	350	390	5	5	5	5			
	210	—	0[e]	0[d]	0[d]	0[d]			
	130	—	0	0[c]	0[c]	0[c]			
	75	81	0	0	0[c]	0[c]			
	control		0	0	0	0			
rainbow trout/ swim-up stage	580	550	0	0	0[e]	0[fh]	0[fh]	0[fh]	
	350	370	0	0	0[g]	0[gh]	0[fh]	0[fh]	
	210	—	0	5	5	5	5	5	
	130	—	0	0	0	0	0	0	
	75	77	0	0	0	0	0	0	
	control		0	0	0	0	0	0	

[a] Not analyzed.
[b] One fry was lethargic.
[c] One fry had scoliosis.
[d] Two fry had scoliosis.
[e] Nearly all fry lethargic.
[f] All fry lethargic.
[g] Several fry lethargic.
[h] Precipitate observed on the surface of the water, on bottom of the tank and adhered to fry.

Table 24. In vivo chlorophyll α (expressed in relative fluorescence units determined with a Turner Model 111 fluorometer) during a 5-day continuous exposure of Selenastrum capricornutum to SEX. Values are means of three flasks. Percentage change is increase or decrease of relative fluorescence units in exposed cultures as compared to the control at day 5.

Nominal concentration (mg/L;ppm)	Mean measured conc. (mg/L)	Relative fluorescence units			Percentage Change Day 5
		Day 1	Day 3	Day 5	
control	—	26(2)[a]	883(32)	1,007(61)	---
0.625	0.54	25(2)	747(203)	840(87)	-17
1.25	1.0	25(1)	657(31)	953(64)	-5
2.5	2.0	27(5)	663(15)	1,060(80)	+5
5.0	4.4	27(4)	680(30)	1,247(46)	+24
10.0	8.1	27(3)	660(30)	1,333(42)	+32

[a]Standard deviation.

Table 25. Cell numbers x 10^4 per milliliter (determined by hemacytometer) during a 5-day continuous exposure of Selenastrum capricornutum to SEX. Values are means of three flasks. Percentage change is increase or decrease of cell numbers in exposed cultures as compared to the control at day 5.

Nominal concentration (mg/ℓ;ppm)	Mean measured concentration (mg/ℓ)	Cell numbers x 10^4/mℓ Day 3	Cell numbers x 10^4/mℓ Day 5	Percentage change Day 5
control	——	315(17)[a]	466(57)	---
0.625	0.54	294(30)	407(41)	-13
1.25	1.0	301(52)	448(47)	-4
2.5	2.0	275(24)	422(48)	-9
5.0	4.4	274(17)	421(28)	-10
10.0	8.1	262(21)	484(24)	+4

[a]Standard deviation.

Table 26. Concentrations tested and corresponding observed percentage mortality of water flea exposed to SEX in a static, acute toxicity test.

Nominal concentration (mg/L)	Mean measured concentration (mg/L)	Percentage Mortality	
		24-hour	48-hour
12	12	0	0
7.2	6.7	0	0
4.3	4.3	0	0
2.6	2.2	0	0
1.6	1.4	0	0
control	control	0	0

Table 27. Concentrations tested and corresponding observed percentage mortality of various species exposed to SEX in static, acute toxicity tests.

Species	Nominal conc. (mg/L)	Mean meas. conc. (mg/L)	Cumulative % Mortality											
			24-hour			48-hour			72-hour			96-hour		
			A	B	$\bar{x}$	A	B	$\bar{x}$	A	B	$\bar{x}$	A	B	$\bar{x}$
Bluegill	12[a]	10	0	10	5	0	20	10	0	20	10	0	20	10
	7.2[a]	—[b]	20	0	10[b]	20	0	10	20	0	10	30	0	15[c]
	4.3[a]	3.6	0	10	5	0	10	5	0	10	5	0	10	5
	2.6[a]	—	0	0	0	0	0	0	20	10	15	20	10	15
	1.6[a]	1.3	0	10	5	0	10	5	0	10	5	0	10	5
	control	<0.27	0	0	0	0	0	0	0	0	0	0	0	0
Fathead minnow	12	10	0	0	0	0	0	0	0	0	0	0	0	0
	7.2	—	0	0	0	0	0	0	0	0	0	0	0	0
	4.3	3.4	0	0	0	0	0	0	0	0	0	0	0	0
	2.6	—	0	0	0	0	0	0	0	0	0	0	0	0
	1.6	1.2	0	0	0	0	0	0	0	0	0	0	0	0
	control	<0.26	0	0	0	0	0	0	0	0	0	0	0	0
Rainbow trout	12	10	0	0	0	0	0	0	0	0	0	0	0	0
	7.2	—	0	0	0	0	0	0	0	0	0	0	0	0
	4.3	3.7	0	0	0	0	0	0	0	0	0	0	0	0
	2.6	—	0	0	0	0	0	0	0	0	0	0	0	0
	1.6	1.3	0	0	0	0	0	0	0	0	0	0	0	0
	control	<0.27	0	0	0	0	0	0	0	0	0	0	0	0

[a]All fish were respiring rapidly throughout the exposure.

[b]Not analyzed.

[c]Some of the fish were at the surface.

Table 28. Results of analyses of SEX solutions measured during static, acute toxicity tests with various life stages of fathead minnow and rainbow trout.

Species/Stage	Nominal conc. (mg/L)		Measured Concentration (mg/L) 0-hour	24-hour	48-hour	72-hour	96-hour	120-hour	144-hour	168-hour	$\bar{X}$(S.D.)[a]
fathead minnow/ embryo	12	A	9.6	9.9	11	9.8	11	12	12	13[c]	(n = 16) 11(1.2)
		B	9.7	9.7	11	10	10	12	11	8.2[c]	
	7.2	A	5.7	5.8	6.0	5.7	6.3	6.5	7.1	7.4	6.4(0.59)
		B	5.9	6.2	6.2	5.9	6.5	6.5	7.0	7.5	
	1.6	A	1.2	1.2	1.3	1.3	1.3	1.4	1.4	1.4	1.3(0.070)
		B	1.3	1.3	1.4	1.3	1.3	1.4	1.4	1.4	
	control	A	<0.24	<0.24	<0.24	<0.23	<0.23	<0.23	<0.17	<0.17	
		B	<0.24	<0.24	<0.24	<0.23	<0.23	<0.23	<0.17	<0.17	
	QA	1	2.0(100)[b]	2.0(100)	2.1(105)	2.0(100)	2.0(100)	2.3(115)	1.9(95)	2.0(100)	
		2	3.9(98)	3.9(98)	3.9(98)	3.6(90)	3.8(95)	4.0(100)	4.1(102)	4.2(105)	
fathead minnow/ 24-hour-old fry	12	A	9.4	9.7	9.8	10	11	11			(n = 12) 10(0.55)
		B	10	9.8	10	10	10	11			
	7.2	A	5.9	5.9	6.1	6.2	6.4	6.5			6.2(0.34)
		B	5.8	5.9	6.1	6.2	6.5	7.0			
	1.6	A	1.2	1.3	1.3	1.3	1.4	1.4			1.3(0.075)
		B	1.3	1.2	1.3	1.4	1.4	1.4			
	control	A	<0.056	<0.056	<0.056	<0.071	<0.071	<0.19			
		B	<0.056	<0.056	<0.056	<0.071	<0.071	<0.19			
	QA	1	20(100)[b]	2.0(100)	2.0(100)	2.0(100)	2.0(100)	2.0(100)			
		2	3.9(98)	4.0(100)	3.9(98)	4.0(100)	4.0(100)	4.2(105)			
fathead minnow/ 7-day-old fry	12	A	9.5	6.2[c]	10	11	11				(n = 10) 10(0.70)
		B	9.2	9.9[c]	9.6	11	10				
	7.2	A	5.8	6.1[c]	6.1	6.7	6.9				6.3(0.38)
		B	5.9	9.6[c]	6.2	6.5	6.6				
	1.6	A	1.3	1.2	1.2	1.3	1.4				1.3(0.082)
		B	1.2	1.3	1.3	1.4	1.4				
	control	A	<0.23	<0.23	<0.23	<0.23	<0.18				
		B	<0.23	<0.23	<0.23	<0.23	<0.18				
	QA	A	2.0(100)[b]	2.0(100)	2.0(100)	2.2(110)	1.9(95)				
		B	3.8(95)	3.7(92)	4.1(102)	4.1(102)	4.1(102)				

Table 28 (continued)

	Nominal conc. (mg/L)		Measured Concentration (mg/L) 0-hour	24-hour	48-hour	72-hour	96-hour	120-hour	$\bar{X}$(S.D.)[a]
rainbow trout/ embryo	12		9.6	9.9	9.1	9.9	9.4	9.6	(n =6) 9.6(0.31)
	7.2		5.7	5.8	5.8	6.1	5.5	5.7	5.8(0.20)
	1.6		1.3	1.3	1.3	1.3	1.2	1.3	1.3(0.04)
	control		<0.22	<0.22	<0.22	<0.22	<0.26	<0.22	
	QA		4.9(98)[b]	4.8(96)	5.0(100)	5.1(102)	4.8(96)	4.9(9.8)	
rainbow trout/ sac fry	12	A	8.5	9.2	9.6	10	10		(n = 10) 9.5(0.55)
		B	9.2	8.8	9.8	10	10		
	7.2	A	5.5	5.4	5.6	5.9	5.7		5.6(0.20)
		B	5.4	5.6	5.6	5.9	5.9		
	1.6	A	1.3	1.3	1.2	1.3	1.3		1.3(0.057)
		B	1.3	1.3	1.3	1.4	1.4		
	control	A	<0.40	<0.40	<0.26	<0.26	<0.26		
		B	<0.40	<0.40	<0.26	<0.26	<0.26		
	Q		4.9(98)[c]	4.4(88)	4.8(96)	4.5(90)	5.0(100)		
			4.4(88)	4.9(98)	4.7(94)	4.9(98)	4.7(94)		
rainbow trout/ swim-up stage (7-day-old fry)	12	A	9.7	10	8.4	9.8	11		(n = 10) 9.8(0.81)
		B	9.0	11	9.2	10	9.8		
	7.2	A	5.4	6.0	6.5	6.4	5.8		6.0(0.44)
		B	5.3	5.8	6.3	6.5	5.8		
	1.6	A	1.3	1.4	1.5	1.2	1.2		1.3(0.13)
		B	1.2	1.4	1.4	1.1	1.2		
	control	A	<0.29	<0.29	<0.29	<0.28	<0.28		
		B	<0.29	<0.29	<0.29	<0.28	<0.28		
	QA	A	1.0(100)	1.2(120)	1.1(110)	1.0(100)	1.1(110)		
		B	10(100)	11(110)	9.6(96)	9.9(99)	9.0(90)		

[a] Mean (standard deviation).

[b] Percent of nominal.

[c] Numbers reported are mean of two analyses.

Table 29. Concentrations tested and corresponding observed percentage mortalities of various life stages of fathead minnow and rainbow trout exposed to SEX during a static acute exposure.

Species/stage	Nominal conc. (mg/L)	Mean meas. conc. (mg/L)	Percentage Mortality						
			24-h	48-h	72-h	96-h	120-h	144-h	168-h
fathead minnow/ embryo	12	11	0	5	5	5	5	5	5
	7.2	6.4	5	5	5	5	10	50	50
	4.3	—[a]	5	5	5	5	5	5	5
	2.6	—	10	15	15	15	15	25	25
	1.6	1.3	0	0	0	0	0	5	5
	control	<0.24	0	0	0	0	5	10	10
fathead minnow/ 24-hour-old fry	12	10	0	0	0	5	10		
	7.2	6.2	0	5	5	10	10		
	4.3	—	0	0	0	20	20		
	2.6	—	0	0	0	15	15		
	1.6	1.3	0	10	10	15	15		
	control	<0.19	0	0	10	15	15		
fathead minnow/ 7-day-old fry	12	10	60	70	70	75			
	7.2	6.3	5	5	5	5			
	4.3	—	0	5	5	5			
	2.6	—	0	0	0	0			
	1.6	1.3	0	5	5	5			
	control	<0.23	5	5	5	5			
rainbow trout/ embryo	12	9.6	0	0	0	0	0		
	7.2	5.8	0	0	0	0	0		
	4.3	—	0	0	0	0	20		
	2.6	—	0	0	0	10	10		
	1.6	1.3	0	0	0	0	0		
	control	<0.26	0	0	10	10	10		
rainbow trout/ sac fry	12	9.5	0	0	0	0	0		
	7.2	5.6	0	0	0	0	0		
	4.3	—	0	0	0	0	0		
	2.6	—	0	5	5	5	5		
	1.6	1.3	0	0	0	0	0		
	control	<0.40	0	0	0	0	0		
rainbow trout/ swim-up stage	12	9.8	0	0	0	0	0		
	7.2	6.0	0	0	0	0	0		
	4.3	—	0	0	0	0	0		
	2.6	—	0	0	0	0	0		
	1.6	1.3	0	0	0	0	0		
	control	<0.29	0	0	0	0	0		

[a] Not analyzed.

APPENDIX A

Method For The Determination of HMX and TAX in

Aquatic Test Water

1. Scope and Application

1.1 This method covers the determination of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and hexahydro-1-acetyl-3,5-dinitro-1,3,5-triazine (TAX).

1.2 This method is applicable to the measurement of these compounds in aquatic test waters. It presupposed a high expectation of finding the specific compounds of interest. If the user is attempting to screen samples for any or all of the compounds above, he must develop independent protocols for the verification of identifications implied with the use of these techniques.

1.3 The sensitivity of this method is usually dependent on the level of interferences rather than instrumental limitations. The limits of detection listed in Table I represent sensitivities that can be achieved in aquatic test waters in the absence of interferences.

1.4 The method is recommended for use only by experienced analysts or under the close supervision of such qualified persons.

2. Summary of Method

2.1 100 mg/L stock solutions of HMX and TAX were prepared in acetonitrile. Subsequent dilutions provided standard solutions of 10 mg/L, 1 mg/L, and 0.1 mg/L HMX and 10 mg/L, 1 mg/L, 0.5 mg/L and 0.1 mg/L TAX in aquatic test water obtained from fish tanks with continuous water turnover. Calibration data for these two compounds was obtained by direct injections of 100 µL aliquots of the standard solutions in aquatic test water into a high performance liquid chromatography system (HPLC) with U.V. detection.

3. Interferences

3.1 Although the detection system is highly selective, solvents, reagents, glassware, and other sample processing hardware

may yield discrete artifacts and/or elevated baselines causing misinterpretation of chromatograms. All of these materials must be demonstrated to be free from interferences under the conditions of the analysis. Specific selection of reagents and purification of solvents by distillation in all-glass systems may be required.

3.2 Interferences contained in the samples will vary considerably from source to source. Unique samples may require additional cleanup approaches to achieve the sensitivities stated in Table I.

3.3 The aquatic test water used for dilutions was found to contain no interferences to HMX and TAX. Blank samples of aquatic test water were analyzed by HPLC to confirm this.

4. Apparatus and Materials

4.1 Volumetric flasks - 100 mL, with stoppers.

4.2 Sample bottles with septums.

4.3 Pipettes - 10 mL and 25 mL and bulb.

4.4 Pipettes - Disposable, pasteur with bulb.

4.5 Beakers - 100 mL.

4.6 Filter funnel, filter flask, and filter paper (Millipore®).

4.7 High performance liquid chromatograph - analytical system complete with column supplies, recorder, syringes, and the following components:

4.7.1 Solvent Programmer - (Waters Associates, Model 660)
4.7.2 Chromatography Pump - (Waters Associates Model M-6000A)
4.7.3 Sample Injection Value - (Waters Associates Model U6K)
4.7.4 Column - (EM, Hibar, Lichrosorb RP18, 10 µm; 250 mm(L) x 4.6 mm(ID))
4.7.5 Variable wavelength U.V. detector - (Shoeffel Instrument Corp SF770)
4.7.6 Recorder - (Hewlett-Packard 7130A)

5. Reagents

5.1 Acetonitrile (U.V.) - distilled in glass (Burdick & Jackson).

5.2 Methyl Alcohol (U.V.) - distilled in glass (Burdick & Jackson).

5.3 Water - purified (distilled and deionized).

5.4 Aquatic Test Water - From fish tanks with continuous water turn over.

5.5 HPLC Mobile Phase - Prepare a 15% methanol solution in deionized water. Mix well and filter (through a Millipore® type LS filter, or equivalent). Refilter and prepare fresh daily.

5.6 Stock Standards - Prepare stock standard solutions at a concentration of 100 mg/L by dissolving 0.0100 gram of assayed reference material in U.V.(distilled in glass) quality acetonitrile and diluting to volume in a 100 mL ground glass stoppered volumetric flask. The stock solutions are stored in a refrigerator and the ground glass joints wrapped with Parafilm® (or equivalent). They are checked frequently for signs of degradation or evaporation, especially just prior to preparing working standards from them.

6. Calibration

6.1. Prepare calibration standards that contain the compounds of interest, singly. The standards should be prepared from the stock standards at the following concentrations that will bracket the working range of the chromatographic system:

HMX (mg/L)	TAX (mg/L)
10	10
1.0	1.0
0.1	0.5
0.01	0.1
	0.01

6.2 Assemble the necessary liquid chromatographic apparatus and establish operating parameters equivalent to those indicated in Table I.

6.3 A constant injection volume of 100 microliters should be employed for all subsequent measurements.

6.4 In order to determine the precision of the HPLC system, a series of 6 replicate injections of a 100 mg/L solution of HMX in acetonitrile and TAX in acetonitrile should be made. A precision of ±4% should be achieved for the peak areas of both HMX and TAX. This measurement should be made every few weeks or whenever instrument related problems are apparent. Sample chromatograms are shown in Figures A-1 and A-2.

6.5 Retention times should remain relatively constant (within ±5% day to day) with HMX being 7.04 minutes and TAX being 9.77 minutes under the specified conditions. These values should be checked daily when the calibration injections are made.

6.6 U.V. scans from 190 nm to 300 nm were carried out for both HMX and TAX to determine λmax for the wavelength setting on the detector. HMX was run at 230 nm, TAX was run at 240 nm.

6.7 When leaving the instrument idle, it is advisable to maintain a flow of 0.1 mL/min of mobile phase through the HPLC column in order to prolong column life.

7. Quality Control

7.1 Before processing any samples, the analyst should demonstrate through the analysis of a distilled water blank, and an aquatic test water blank, that all glassware and reagents are interference-free. Each time there is a change in reagents, a method blank should be processed as a safeguard against laboratory contamination.

7.2 Standard quality assurance practices should be used with this method. Standard replicates should be collected to validate the precision of the technique. Laboratory replicates should be analyzed to validate the precision of the analysis. Fortified samples should be analyzed to validate the accuracy of the analysis.

8. Liquid Chromatography (HPLC).

8.1 Table I summarizes the recommended liquid chromatographic column material and operating conditions for the instrument. Included in this table are estimated retention times and sensitivies that should be achieved by this method. Example of the separation achieved by this column are shown in Figures A-1 and A-2. Calibrate the system daily with a minimum of three injections of calibration standards.

9. Calculations

9.1 Correlation coefficients and calibration factors for calibration factors for calibration curves were determined by linear regression.

9.2 Determine the concentration of HMX or TAX in a sample aquatic test water of unknown concentration according to the equation:

$$\text{concentration mg/L} = \frac{(A)\ (B)}{(V)}$$

A = Calibration factor for chromatographic system in mg material per unit peak area
B = Peak size of an injection of sample in peak area units
V = Volume of injection (L) or ($\mu L \times 10^6\ \mu/L$)

9.3 Report Results in mg/L (or ppm)

TABLE A-1

Compound [a]	Retention Time (min.)	Detection limit (mg/L) [b]
HMX	7.04	0.1
TAX	9.77	0.5

[a] Column conditions. Lichrosorb RP 18, 10 µm particle dia., packed in a 250 mm (L) x 4.6 mm (I.D.) stainless steel column with 2 mL/min flow rate of mobile phase (15% Methanol: 85% deionized water).

[b] Detection limit is calculated from the minimum detectable response of the U.V. detector (λHMX = 230 nm) (λTAX = 240) at 0.01 AUFS being equal to five times the background noise, assuming an injection volume of 100 µL.

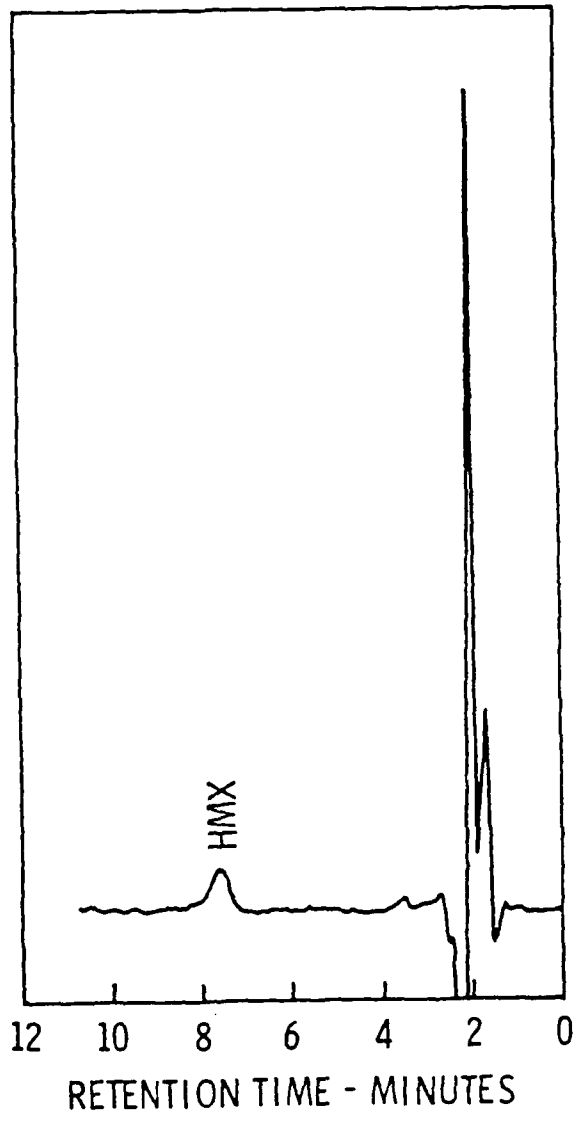

Figure A-1. Liquid chromatogram of 0.1 mg/L HMX in aquatic test water.

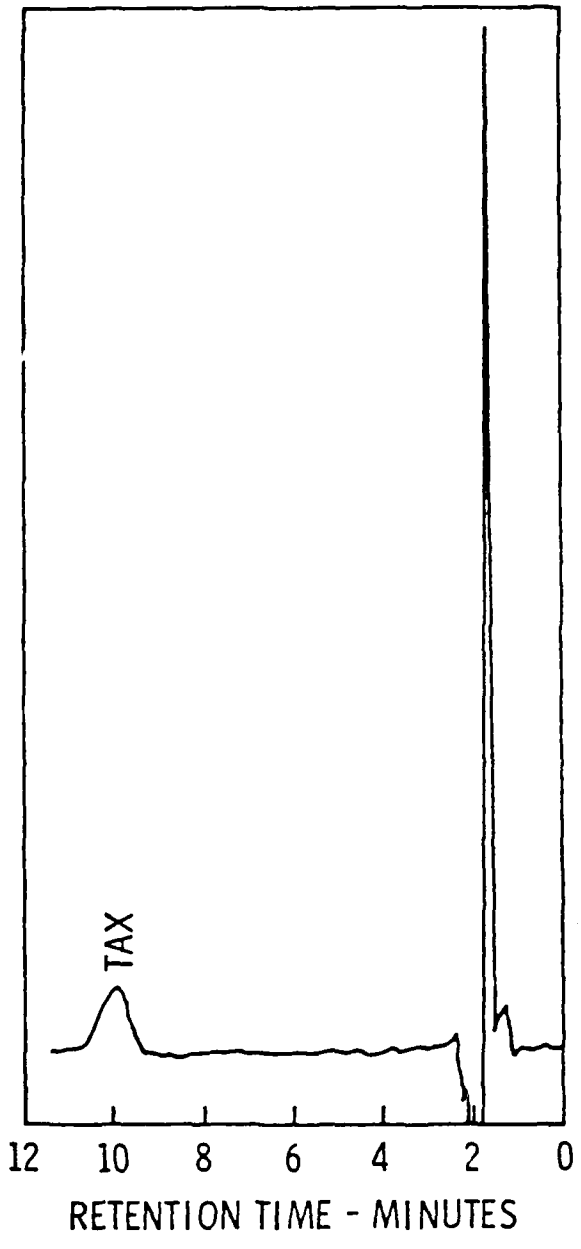

Figure A-2. Liquid chromatogram of 0.5 mg/L TAX in aquatic test water.

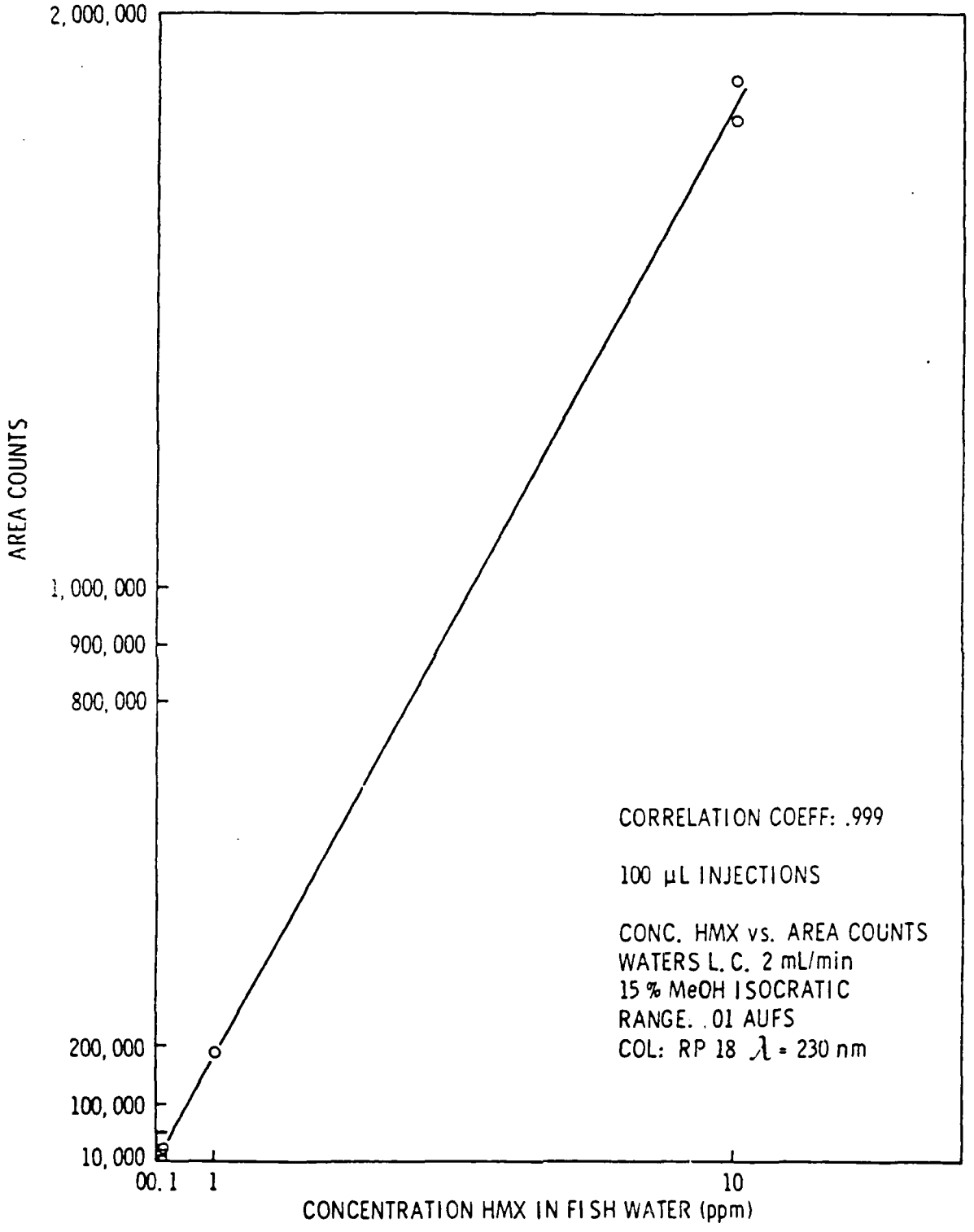
2,000,000
1,000,000
900,000
800,000
200,000
100,000
10,000
AREA COUNTS
00.1
1
10
CONCENTRATION HMX IN FISH WATER (ppm)
CORRELATION COEFF: .999
100 µL INJECTIONS
CONC. HMX vs. AREA COUNTS
WATERS L.C. 2 mL/min
15 % MeOH ISOCRATIC
RANGE. .01 AUFS
COL: RP 18 λ = 230 nm

Figure A-3.

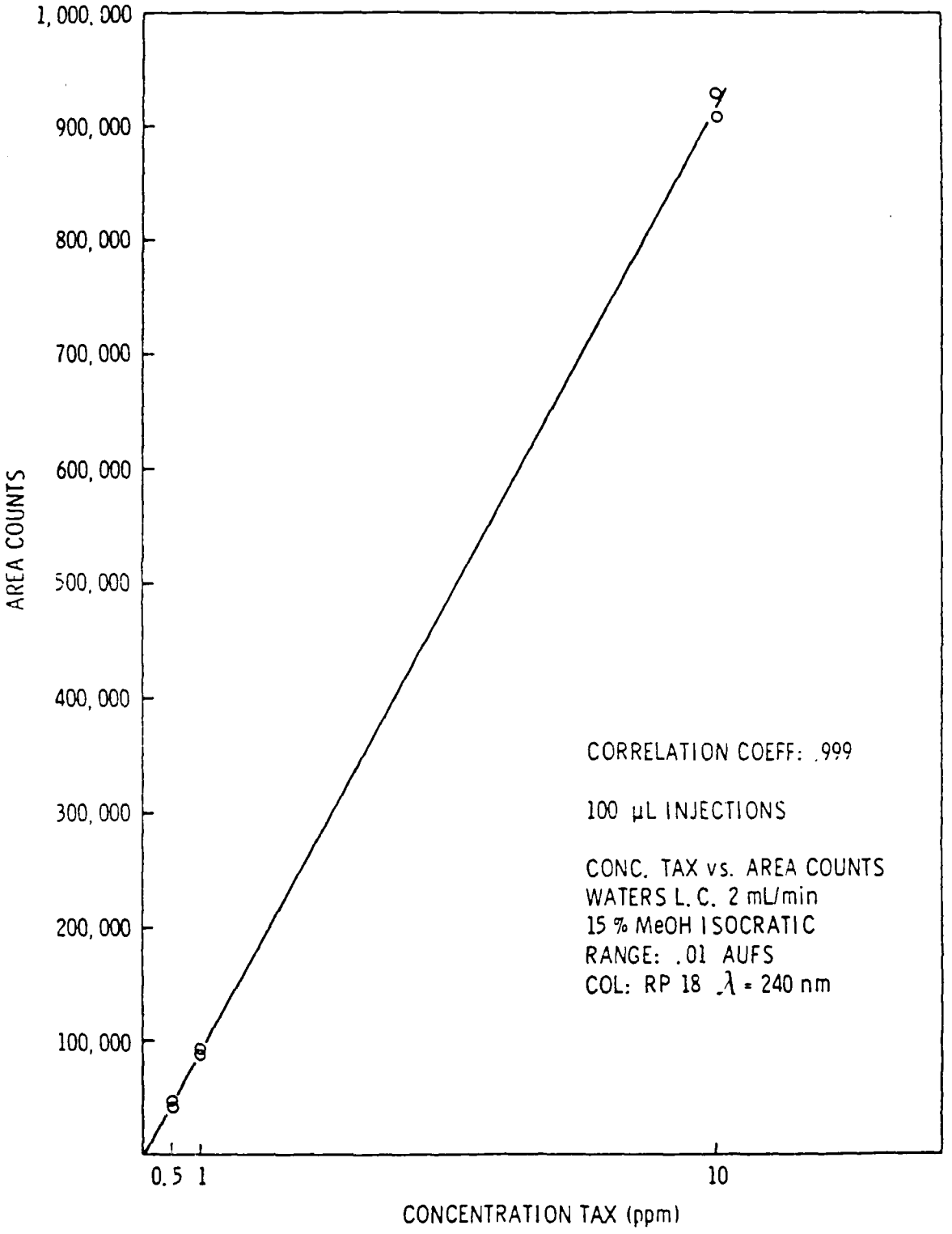
1,000,000
900,000
800,000
700,000
600,000
500,000
400,000
300,000
200,000
100,000
AREA COUNTS
0.5
1
10
CONCENTRATION TAX (ppm)
CORRELATION COEFF: .999
100 µL INJECTIONS
CONC. TAX vs. AREA COUNTS
WATERS L.C. 2 mL/min
15 % MeOH ISOCRATIC
RANGE: .01 AUFS
COL: RP 18 λ = 240 nm

Figure A-4.

causing misinterpretation of chromatograms. All of these materials must be demonstrated to be free from interferences under the conditions of the analysis. Specific selection of reagents and purification of solvents by distillation in all-glass systems may be required.

3.2 Interferences contained in the samples will vary considerably from source to source. Unique samples may require additional cleanup approaches to achieve the sensitivities stated in Table I.

3.3 The aquatic test water used for dilutions was found to contain no interferences to SEX. Blank samples of aquatic test water were analyzed by HPLC to confirm this.

4. Apparatus and Materials

4.1 Volumetric flasks - 100 mL, with stoppers.

4.2 Sample bottles with septums.

4.3 Pipettes - 10 mL and 25 mL and bulb.

4.4 Pipettes - Disposable, pasteur with bulb.

4.5 Beakers - 100 mL.

4.6 Filter funnel, filter flask, and filter paper (Millipore®).

4.7 Hewlett Packard 1084B - High performance liquid chromatograph - analytical system complete with column supplies, recorder, syringes, microprocessor.

4.7.1 Column - (EM, Hibar, Lichrosorb RP18, 10 µm; 250 mm(L) x 4.6 mm(ID))

5. Reagents

5.1 Acetonitrile (U.V.) - distilled in glass (Burdick & Jackson).

5.2 Methyl Alcohol (U.V.) - distilled in glass (Burdick & Jackson).

5.3 Water - purified (distilled and deionized).

5.4 Aquatic Test Water - From fish tanks with continuous water turn over.

5.5 HPLC Mobile Phase - Prepare a 15% methanol solution in deionized water. Mix well and filter (through a Millipore® type LS filter, or equivalent). Refilter and prepare fresh daily.

APPENDIX B

Method for the Determination of SEX in
Aquatic Test Water

1. Scope and Application

1.1 This method covers the determination of 1-acetyloctahydro-3,5,7-trinitro-1,3,5,7-tetrazocine (SEX).

1.2 This method is applicable to the measurement of this compound in aquatic test waters. It presupposes a high expectation of finding the specific compound of interest. If the user is attempting to screen samples for the compound above, he must develop independent protocols for the verification of identifications implied with the use of these techniques.

1.3 The sensitivity of this method is usually dependent on the level of interferences rather than instrumental limitations. The limits of detection listed in Table I represent sensitivities that can be achieved in aquatic test waters in the absence of interferences.

1.4 The method is recommended for use only by experienced analysts or under the close supervision of such qualified persons.

2. Summary of Method

2.1 10 mg/L stock solution of SEX was prepared in 20% acetonitrile (aq). Subsequent dilutions provided standard solutions of 10 mg/L, 1 mg/L, and 0.1 mg/L SEX in aquatic test water obtained from fish tanks with continuous water turnover. Calibration data for this compound was obtained by direct injections of 100 µL aliquots of the standard solutions in aquatic test water into a high performance liquid chromatography system (HPLC) with U.V. detection.

3. Interferences

3.1 Although the detection system is highly selective, solvents, reagents, glassware, and other sample processing hardware may yield discrete artifacts and/or elevated baselines

5.6 Stock Standards - Prepare stock standard solutions at a concentration of 10 mg/L by dissolving 0.0010 gram of assayed reference material in 20 mL U.V.(distilled in glass) quality acetonitrile and diluting to volume with aquatic test water in a 100 mL ground glass stoppered volumetric flask. The stock solutions are stored in a refrigerator and the ground glass joints wrapped with Parafilm® (or equivalent). They are checked frequently for signs of degradation or evaporation, especially just prior to preparing working standards from them.

6. Calibration

6.1. Prepare calibration standards that contain the compound of interest. The standards should be prepared from the stock standards at the following concentrations that will bracket the working range of the chromatographic system:

SEX (mg/L)
10
1.0
0.1

6.2 Assemble the necessary liquid chromatographic apparatus and establish operating parameters equivalent to those indicated in Table B-1.

6.3 A constant injection volume of 100 microliters should be employed for all subsequent measurements.

6.4 In order to determine the precision of the HPLC system, a series of 6 replicate injections of a 10 mg/L solution of SEX should be made. A precision of ±4% should be achieved for the peak areas of SEX. This measurement should be made every few weeks or whenever instrument related problems are apparent.

6.5 Retention times should remain relatively constant (within ±5% day to day) with HMX being 5.02 minutes, TAX being 6.23 minutes, and SEX being 3.95 minutes under the specified conditions. These values should be checked daily when the calibration injections are made.

6.6 A U.V. scan from 190 nm to 300 nm was carried out for SEX to determine λmax for the wavelength setting on the detector. SEX was run at 226 nm.

6.7 When leaving the instrument idle, it is advisable to maintain a flow of 0.1 mL/min of mobile phase through the HPLC column in order to prolong column life.

7. Quality Control

7.1 Before processing any samples, the analyst should demonstrate through the analysis of a distilled water blank, and an aquatic test water blank, that all glassware and reagents are interference-free. Each time there is a change in reagents, a method blank should be processed as a safeguard against laboratory contamination.

7.2 Standard quality assurance practices should be used with this method. Standard replicates should be collected to validate the precision of the technique. Laboratory replicates should be analyzed to validate the precision of the analysis. Fortified samples should be analyzed to validate the accuracy of the analysis.

8. Liquid Chromatography (HPLC).

8.1 Table I summarizes the recommended liquid chromatographic column material and operating conditions for the instrument. Included in this table are estimated retention times and sensitivies that should be achieved by this method. Calibrate the system daily with a minimum of three injections of calibration standards.

9. Calculations

9.1 Correlation coefficients and calibration factors for calibration curves were determined by linear regression.

9.2 Determine the concentration of SEX in a sample of aquatic test water of unknown concentration according to the equation:

$$\text{concentration mg/L} = \frac{(A)\ (B)}{(V)}$$

A = Calibration factor for chromatographic system in mg material per unit peak area
B = Peak size of an injection of sample in peak area units
V = Volume of injection (L) or (μL x 10^6 μL/L)

9.3 Report Results in mg/L (or ppm)

TABLE B-1

Compound[a]	Retention Time (min.)	Detection limit (mg/L)[b]
SEX	3.95	0.1

[a]Column conditions. Lichrosorb RP 18, 10 μm particle dia., packed in a 250 mm (L) x 4.6 mm (I.D.) stainless steel column with 2 mL/min flow rate of mobile phase (15% Methanol: 85% deionized water).

[b]Detection limit is calculated from the minimum detectable response of the U.V. detector (λSEX = 226nm) being equal to five times the background noise, assuming an injection volume of 100 μL.

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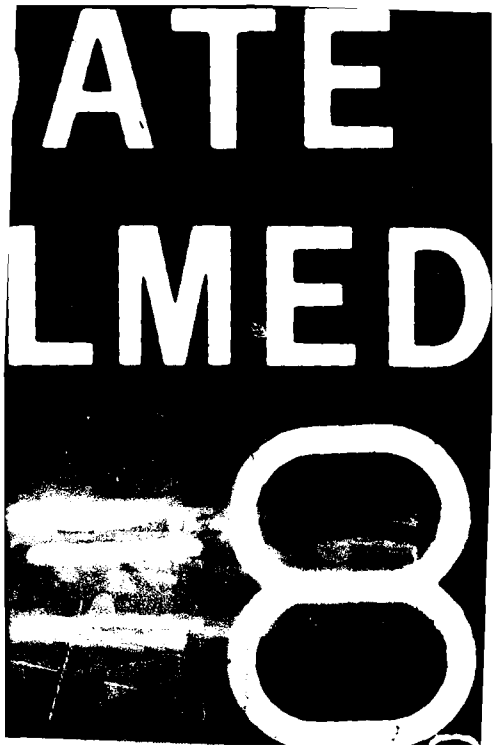
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