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EVALUATION OF AN AUTOMATED FISH VENTILATORY MONITORING SYSTEM IN A SHORT-TERM SCREENING TEST FOR CHRONIC TOXICITY

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A continuous automated fish ventilatory monitoring system was evaluated for use in short-term screening tests to determine the chronic toxicity of chemicals to fish. The ~~multi-parameter ventilatory~~ system monitors bluegills (Lepomis macrochirus) for four parameters: ventilatory rate, average ventilatory depth, cough rate, and percent movement. ~~The system~~ It was evaluated in tests with technical chlordane, 1,3,5-trinitrobenzene (TNB) and a solvent mixture (triton X-100 and acetone) that was used as a carrier solvent in the

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chlordane test. The lowest concentrations of chlordane and TNB causing changes in the ventilatory parameters during a 6-day exposure period were compared to reported chronic toxicity values.

Linear regression analysis of ventilatory signals monitored both visually and by the computer indicated high computer accuracy for ventilatory rate (R^2 = 0.976, slope = 0.972). The accuracy of the ventilatory rate led to the conclusion that the computer was also accurate for average ventilatory depth. Cough rate accuracy was poor in the solvent and chlordane tests (R^2 <0.300) but was better in the TNB test (R^2 = 0.766, slope = 1.177). Percent movement accuracy was fair for all three tests (R^2 = 0.586, slope = 0.764). The solvent mixture ventilatory test revealed no significant differences between control and solvent-exposed fish for any of the four parameters monitored. In the chlordane ventilatory test, no significant responses in any of the four parameters monitored were found at concentrations up to seven times the chlordane concentration reported to cause major chronic toxicity. The sensitivity of the ventilatory system to chlordane-induced effects may have been reduced either by poor cough accuracy, (possibly related to the small size of the bluegills tested) or by an insufficiently long period of exposure. TNB (using larger bluegills) caused significant ventilatory responses at concentrations as low as 0.128 mg/L, which compares favorably to the lowest concentrations of TNB that caused toxicant effects in early life stage tests with fathead minnows (0.12 mg/L) and rainbow trout (0.17 mg/L).

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INTRODUCTION

The US Army Medical Bioengineering Research and Development Laboratory (USAMBRDL) is investigating environmental effects of Army-relevant materials. The Aquatic Toxicology Section of USAMBRDL has, as one of its tasks, the development of faster and less expensive means of measuring the toxicity of such materials to aquatic organisms. One such project in this area is to evaluate an automated continuous biological monitoring system using the bluegill (Lepomis macrochirus). The system, developed by van der Schalie,[1] monitors three ventilation parameters and whole body movement. The parameters mentioned might be used to detect chronic effects of toxicants on fish in a short period of time.

There are data in the literature to support the use of ventilatory parameters as a predictor of chronic toxicity to fish. A summary is given in Table 1, showing the response of ventilatory signals to toxicants relative to the lowest concentration causing chronic effects. Differences between test systems, such as fish species, ventilatory parameter monitored, length of exposure, and dilution water quality contribute to the variation in ventilatory response to similar compounds and make comparisons with the results of other investigations difficult. An in-depth review of the literature related to ventilatory responses of fish prior to 1977 was reported by Drummond and Carlson.[2] Their review demonstrated that changes in gill purge (cough) rates were rapid and sensitive indicators of the potentials of toxicants to produce chronic effects. Fackelmann[3] reported that increases in cough rates were more sensitive predictors of the concentrations likely to produce chronic toxicity, as compared to changes in blood composition. Changes in the cough rate of bluegills were observed for copper, cadmium, chromium, and zinc near the chronic levels for some of these metals.

Several experiments were designed by Bishop and McIntosh[4] to determine the effects of sublethal cadmium exposure on the ventilation rate and the cough response of the bluegill. Exposure to cadmium produced significant increases in both the ventilatory rate and the cough rate. There was a similar relationship between known cadmium chronic toxicity and cadmium concentrations producing significant increases in cough rate. Maki[5] compared several surfactants and found close relationships between the chronic toxicity to fathead minnows and the concentrations of surfactants that elicit statistically significant changes in the diurnal ventilation frequencies of exposed bluegills.

Ventilatory responses of fish to sublethal concentrations of copper and zinc were examined by Sellers et al.[6] One or more of the ventilatory parameters measured on rainbow trout, Salmo gairdneri, (opercular pressure amplitude, ventilation frequency, and cough frequency) were found to change under toxicant stress for concentrations at or below the LC50. Majewski et al.[7] reported that acetone and ethanol at about 0.48 and 0.26 of the fingerling trout LC50, respectively, affected respiratory parameters in adult rainbow trout. Acetone produced an increase in ventilation rate as well as an increase in buccal pressure amplitude. Ethanol-exposed trout exhibited a slight depression in ventilation rate and buccal pressure amplitude.

Sloof[8] looked at the respiration frequency response of 13 chemicals to rainbow trout. The results showed that the ventilatory frequency response was

TABLE 1. COMPARISON OF VENTILATORY RESPONSE CONCENTRATIONS WITH CHRONIC TOXICITY

Compound	Fish	Ventilatory Test			Lowest Chronic Effect Concentration[a] (µg/L)	Ref.
		Parameter Monitored	Length of Exposure (days)	Lowest Effect Concentration[a] (µg/L)		
Cadmium chloride	Brook trout	Cough	4	5	3	2
Cadmium sulfate	Bluegill	Cough	1	<1,045	80	3
Cadmium chloride	Bluegill	Cough and ventilatory rate	3	<50	80	4
Sodium dichromate	Brook trout	Cough	4	860	350	2
Potassium dichiomate	Bluegill	Cough	1	<931	350[b]	3
Copper sulfate	Brook trout	Cough	4	10	17	2
Copper sulfate	Bluegill	Cough	1	185	40	3
Zinc sulfate	Brook trout	Cough	4	1,392	1,368	2
Zinc sulfate	Bluegill	Cough	1	<4,140	235	3
Zinc chloride	Steelhead	Ventilatory rate	4	144	260[c]	24
Methymercuric chloride	Brook trout	Cough	4	3	0.9	2

Lead nitrate	Brook trout	Cough	4	80	119	2
Diazinon	Brook trout	Cough	4	25	<0.8[d]	2
Malathion	Brook trout	Cough	4	7	<16	2
Lindane	Brook trout	Cough	4	6	17	2
$C_{11.8}$-LAS[e]	Bluegill	Ventilatory rate	2	2,190	1,000[f]	5
C_{14-15}-Alkyl ethoxylate	Bluegill	Ventilatory rate	2	<390	240[f]	5
C_{13} LAS[e] alkyl ethoxylate sulfate	Bluegill	Ventilatory rate	2	390	200[f]	5
C_{12-13}-Alkyl ethoxylate	Bluegill	Ventilatory rate	2	>1,560[b]	280[f]	5
C_{11-12}-Alkyl dimethylamine oxide	Bluegill	Ventilatory rate	2	2,990	1,000[f]	5
NTA	Bluegill	Ventilatory rate	2	>181,000	>54,000[f]	5

a. Lowest concentration tested to cause a significant change from the controls in any parameter monitored. Metal concentrations are reported as the metal, not the compound.
b. Lowest chronic effect concentration for brook trout.
c. Lowest chronic effect concentration for rainbow trout.[25]
d. < = significant effect at lowest concentration tested, > = no effect at highest concentration tested.
e. LAS - linear alkyl benzene sulfonate.
f. Lowest chronic effect concentration for fathead minnows.

between 1 and 33 percent of the 48 hr LC50. Morgan,[9] using Micropterus salmoides, Sarotherodon mossambicus, and Barbus holubi reported a ventilatory frequency response of 5 to 10 percent of the 48 hr LC50.

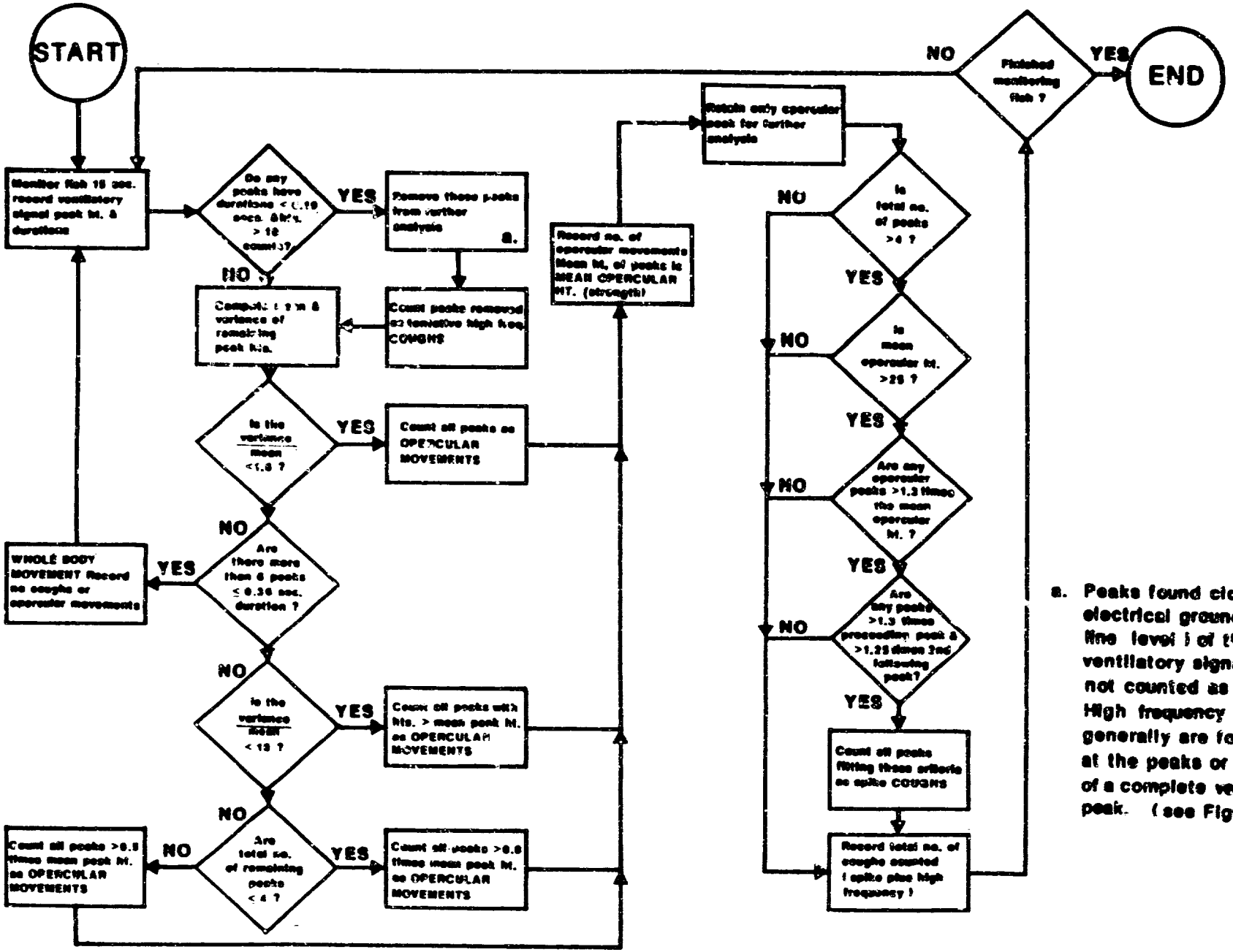
The unique characteristics of the monitoring system developed by van der Schalie[1] allow four parameters to be analyzed continuously from the ventilatory signal of the fish. These parameters are: ventilatory rate, mean peak height (ventilatory depth), gill purges (coughs), and whole body movement. Figure 1 is the decision chart for the microcomputer and summarizes the analysis of the ventilatory signal into the various parameters. The system was designed to monitor 30 fish simultaneously. The ability to monitor 30 fish allows flexibility in the number of treatments and the number of replicates per treatment. Toxicants may change any of the four ventilatory parameters. A system that monitors all four parameters may detect changes that would be missed if only one or two parameters were being monitored. Continuous monitoring and analysis by a computer gives a large data base to analyze for changes in ventilatory signal in response to toxicant stress. Most prior ventilatory monitoring systems[2-9] used visual data collection and therefore required a great expenditure of resources to generate a relatively small data base. Another benefit of using computer analysis of the ventilatory signal is consistency. Visual analysis of the ventilatory signal is subject to interpretation by the person analyzing the signal. Maki[5] did not attempt to obtain cough data from bluegills because of the poor replicability in counts made by different technicians.

Two toxicants were used in the present study: 1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene (chlordane) and 1,3,5-trinitrobenzene (TNB). Technical grade chlordane was used as a test compound because there was a substantial amount of literature concerning its toxicity to bluegills. The dominant constituents are trans-chlordane, cis-chlordane, heptachlor, chlordenes, trans-nonachlor and cis-nanachlor. Cardwell et al.[10] reviewed the complex chemistry of technical grade chlordane as well as its toxicity to aquatic organisms. They determined the 96-hr LC50 of technical grade chlordane to the bluegill to be 59 µg/L, while the lowest concentration of chlordane having chronic toxic effects on bluegills was 1.22 µg/L. No chronic effects were observed at 0.54 µg/L. TNB was selected because of the large acute and chronic toxicity data base available for this compound in water from the same water source as was used in ventilatory testing. The 96-hr LC50 of TNB to the bluegill was reported by van der Schalie[11] to be 0.85 mg/L. Also reported was the toxicity of TNB in two early life stage (ELS) tests. The no-effect - effect ranges for TNB obtained for the rainbow trout and the fathead minnow in the ELS tests were 0.08 to 0.17 mg/L and 0.08 to 0.12 mg/L, respectively.

OBJECTIVES

1. Improve test chamber design and reduce external noise to provide an optimal ventilatory signal for computer analysis.

2. Establish the accuracy of ventilatory monitoring by comparing the computer generated data with simultaneously generated stripchart (visual) records and looking for consistent trends between them.



a. Peaks found close to electrical ground (base line level) of the ventilatory signal were not counted as coughs. High frequency coughs generally are found only at the peaks or troughs of a complete ventilatory peak. (see Figure 3)

Figure 1. Computer Decision Chart.

3. Develop procedures to evaluate the effects of chronic concentrations of toxicants on the ventilatory signals of the bluegill and to establish the lowest toxicant concentrations causing statistically significant changes in the ventilatory signal.

4. Compare the lowest concentrations of TNB and chlordane causing short-term change in bluegill ventilatory patterns with the lowest concentrations of the same materials known to cause chronic toxicity to fish.

MATERIALS AND METHODS

TEST COMPOUNDS AND STOCK SOLUTION PREPARATION

Technical grade chlordane ("99 percent purity," as given on the reagent bottle) was acquired from Chem Service, West Chester, PA, and is identified as PS-75, PRD-EPA #174. The stock solution for the static acute test was prepared by weighing out 54 mg of technical chlordane and placing it into 250 mL of acetone. The solution was poured slowly into 2 liters of constantly stirred glass-distilled water. The volume was then raised to 2.5 liters with distilled water to give a final technical grade chlordane concentration equal to 21.6 mg/L. The stock solution was sealed and mixed continuously until the test solutions were prepared. The technical grade chlordane stock (2 liters) for the ventilation study was prepared in pesticide-free acetone at a nominal concentration of 40 mg/L technical grade chlordane and 20 mg/L triton X 100 (surfactant). Stock preparation for the ventilatory test followed procedures described by Cardwell et al.[10] to maintain a similar chlordane exposure for later comparisons.

The 1,3,5-trinitrobenzene (TNB) used for evaluation with the ventilatory system was synthesized by USAMBRDL chemists. The compound had a measured purity of 99.97 percent, determined by gas chromatography.

The TNB stock solution for the dynamic acute toxicity test was prepared by placing 3.001 g of TNB into 10 liters of deionized distilled water. The solution was mixed with a stainless steel mechanical stirrer for 72 hours giving a nominal concentration of 300 mg/L TNB. A sample of the stock solution was analyzed by gas chromatography to verify TNB concentration.

The stock solution for the ventilatory study was prepared similarly to the dynamic acute stock. Thirteen liters were prepared at 242 mg/L TNB with well water for dilution. The stock solution was analyzed to verify the concentration of TNB.

SAMPLE ANALYSIS

Chlordane test solution concentrations for the static acute test were measured by gas chromatography. The method is described in Appendix A. Owing to resource limitations, all the test solutions could not be measured and the intermediate concentrations were extrapolated from the results of the measured test solutions. For the ventilatory test chlordane was analyzed by a different method; the detection limit was 0.4 µg/L. The method of analysis was taken from the USFDA pesticide analytical manual.[12] A spiked sample was

included with each sample set and measured concentrations adjusted according to percent recovery of spiked sample.

Test solution concentrations of TNB for the dynamic acute test were measured by high pressure liquid chromatography.[13] The detection limit was 0.10 mg/L. A spiked sample was sent with each batch of samples to establish a percent recovery.

TNB in the ventilatory test was analyzed by gas chromatography.[13] The detection limit for GC analysis was 0.02 mg/L. Low- and high-spike samples were included with each sample set to establish percent recovery in analysis.

Dilution Water Quality

The dilution water used for testing was taken from a 62-meter well. The water was filtered, sterilized with ultraviolet light, and temperature-adjusted before being used in toxicity tests. A water softener was placed in line to reduce precipitation of calcium carbonate on the glassware and equipment. A mixture of 60 percent unsoftened and 40 percent softened water was used in testing, except for the chlordane static acute test, which was completed prior to addition of the water softener to the system. A summary of dilution water quality 2 weeks before, during, and 2 weeks after testing is given in Table 2. For all testing, unionized ammonia was less than 10 µg/L and total suspended solids were less than 2.0 mg/L. A comprehensive dilution water analysis for the 4-year testing period is provided in Table 3. The only contaminant during this study was 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane (DDD), found in 1982 to be at a concentration near the detection limit of 0.02 µg/L.

Test Bluegills

Specific information on the bluegills used in the report is given in Table 4. During acclimation and testing, wide-spectrum fluorescent bulbs with a color rendering index of 91 were used. The diurnal photoperiod for the acute tests was 16 hours of light and 8 hours of darkness. Bluegills used for the ventilatory toxicity tests were held under continuous light for at least 2 weeks prior to testing. Testing was done under continuous light to eliminate diurnal changes in bluegill ventilatory patterns. All fish were fed Rangen's (#3) trout food plus frozen brine shrimp (Living World). Fish were not fed during tests. The acclimation and test temperature was 22°C (±2).

TEST DESIGN

Three toxicity tests were conducted with chlordane. A 96-hr static acute test was done to establish the sensitivity of the bluegills on hand to the technical chlordane available. The results were compared to the results of a published acute test done in conjunction with a chronic toxicity test.[10] A carrier solvent ventilatory test was run with the carrier solvent (Triton X-100 and acetone) to be used in the chlordane study to determine whether changes in the ventilatory signal would be caused by the addition of a solvent. Since no solvent effects on ventilation were found, the chlordane ventilatory test was conducted with five toxicant concentrations but no solvent control. The top concentration in the chlordane test was set at 25 percent of the 96-hour LC50.

TABLE 2. DILUTION WATER QUALITY SUMMARY DURING ACCLIMATION AND TESTING

Test Type	Toxicant		pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)
Static Acute	Chlordane	mean	--	219	295	658
		range	7.6	216-223	274-312	640-670
Ventilatory	Solvent (Triton X-100 acetone)	mean	--	205	180	812
		range	8.2-8.4	177-227	163-206	720-875
Ventilatory	Chlordane	mean	--	209	176	805
		range	8.2-8.3	177-229	163-189	720-875
Dynamic Acute	1,3,5-Trinitrobenzene	mean	--	241	173	622
		range	8.2-8.3	227-250	170-174	602-644
Ventilatory	1,3,5-Trinitrobenzene	mean	8.2	217	164	662
		range	--	205-233	154-174	598-706

TABLE 3. ANNUAL COMPREHENSIVE DILUTION WATER ANALYSES, 1980-1983

Parameter	Concentration (mg/L)			
	1980	1981	1982[a]	1983
Ammonia (as N)	0.33	0.49	0.03	<0.05
Nitrite (as N)	<0.01	—[c]	<0.02	—
Nitrate (as N)	0.02	—	0.45	0.16
Chloride	—	—	91.5	70.5
Fluoride	2.0	0.18	0.33	0.19
Sulfate	39.2	37.6	46.8	39.3
Aluminum	0.047	<0.005	<0.002	<0.002
Barium	0.140	0.097	0.113	0.089
Boron	<1.5	—	—	<0.05
Cadmium	0.0004	<0.001	<0.001	<0.0005
Calcium	73.8	58.0	45.7	47.5
Cobalt	<0.003	<0.005	<0.004	<0.002
Copper	<0.02	<0.005	<0.003	<0.002
Iron	<0.03	<0.005	<0.003	<0.1
Lead	<0.001	<0.002	<0.002	<0.002
Magnesium	23.2	17.3	15.5	14.7
Manganese	<0.02	<0.005	—	<0.002
Mercury	<0.001	<0.005	<0.0005	<0.0005
Potassium	0.60	3.3	1.2	1.25
Silicon	6.10	5.6	5.4	2.7
Silver	<0.05	—	—	<0.0005
Sodium	30.2	52.0	119.0	93.5
Tin	<0.002	—	—	—
Zinc	0.282	<0.005	<0.02	<0.02
Cyanide	—	—	—	<0.002
Arsenic	—	—	—	<0.002
Molybdenum	—	—	—	<0.003
Nickel	—	—	—	<0.002
Phosphorus	—	—	—	<0.01
Sulfide	—	—	—	<0.05
Selenium	—	—	—	<0.002

Parameter	Chlorinated Hydrocarbons Concentration				Detection Limit (µg/L)	
	1980	1981	1982	1983	1980-1982	1983
Aldrin	X[b]	X	X	X	0.02	0.05
p,p'-DDT	X	X	X	X	0.02	0.05
o,p-DDT	X	X	X	X	0.02	0.05
DDD	0.04	X	0.026	X	0.02	0.05
Dieldrin	T	X	X	X	0.02	0.05
Endrin	X	X	X	X	0.02	0.05
Heptachlor	X	X	X	X	0.02	0.05
Heptachlor Epoxide	X	X	X	X	0.02	0.05
Lindane	X	X	X	X	0.01	0.05
Chlordane	X	X	X	X	0.20	0.20
Alpha-BHC	X	X	X	X	0.01	0.05
Beta-BHC	T	-	X	X	0.02	0.05
Delta-BHC	X	-	X	X	0.01	0.05
Toxaphene	X	X	X	X	1.0	1.0
Methoxychlor	X	X	X	X	0.20[d]	0.05
Polychlorinated Biphenyls	-	<0.1	<0.001	X	—	1.0
2,4-D	-	-	-	X	—	0.05
2,4,5-T	-	-	-	X	—	0.05
Silvex	-	-	-	X	—	0.05
Diazinon	-	-	-	X	—	0.20
Malathion	-	-	-	X	—	0.20
Parathion	-	-	-	X	—	0.20

a. Sample taken in 1982 was after switch from well water to 60% well water plus 40% softened well water.
b. X = below detection limit; T = trace (detectable peak, but below detection limit and not quantifiable). Concentrations reported in µg/L.
c. Not measured.
d. Detection limit 0.02 µg/L in 1982.

TABLE 4. INFORMATION ON BLUEGILLS USED IN TESTING

Test Type	Test Material	Source	Mean Size Used in Testing		Age (weeks)	Acclimation (weeks)
			Length, mm (SD)	Weight, g (SD)		
Static acute	Chlordane	Harrison Lake National Fish Hatchery, Harrison Lake, VA	32 (3.52)	0.74 (0.302)	20	10
Ventilatory	Triton X-100 and Acetone (solvent test)	Kurtz Fish Hatchery, Elverson, PA	38 (2.75)	1.15 (0.237)	30	6
Ventilatory	Chlordane	Kurtz Fish Hatchery, Elverson, PA	42 (2.17)	1.78 (0.315)	34	10
Dynamic acute	1,3,5-Trinitrobenzene	Kurtz Fish Hatchery, Elverson, PA	54 (5.00)	3.59 (0.897)	56	3[a]
Ventilatory	1,3,5-Trinitrobenzene	Kurtz Fish Hatchery, Elverson, PA	57 (3.37)	4.26 (0.321)	58	5[b]

a. Temperature was at test temperature for 1 week. The first 2 weeks of acclimation were used to raise temperature from 10°C to 22°C.
b. The first 2 weeks of acclimation were used to raise the temperature to 22°C.

Two toxicity tests were performed with TNB. A 96-hour dynamic (flow-through) acute toxicity test was conducted to demonstrate the sensitivity of the bluegill to TNB under flow-through conditions. The results of this test were also compared to a 96-hour static acute test done previously in the same dilution water.[11] The comparison was done to link the batch of TNB and bluegill stock used in previous testing with the batch of TNB and bluegill stock used for testing in the ventilatory study. The TNB ventilatory test was conducted at the established 96 hr LC50 value as a top toxicant concentration.

Static Acute Toxicity

The static acute methods generally followed those recommended by the American Society for Testing and Materials.[14] Test chambers were 19-liter glass jars containing 14 liters of test solution. The chlordane stock was added to the test jars with 13 liters of well water in them. The volume was raised to 14 liters and stirred vigorously for 1 minute with a hand-held stainless steel mixer. Five toxicant concentrations, a solvent control, and a well water control were used. There were two glass jars of 10 fish at each treatment level. Concentrations were set in a logarithmic series (100, 56, 32, 18, and 10 µg/L). A chlordane control test jar (56 µg/L nominal) was used to monitor chlordane loss from the test jars. The chlordane control jar contained no fish.

Feeding of bluegills was stopped 48 hours before the start of each test to reduce fecal material in the test tanks. The bluegills were randomized in the test jars and the test jars were randomized in the test rack by means of a randomization table generated by a computer program.[15] The dissolved oxygen and pH readings were taken from one replicate tank of the control, low, medium, and high treatments. Mortality and water quality readings were taken at time 0, 48, and 96 hours into the test. Temperature was checked daily with a calibrated thermometer and was continuously monitored with a thermometer connected to a chart recorder. Length and weight of control fish were measured at the end of the test. Samples of chlordane test solutions were taken initially and at 72 hours after exposure was begun from one of the low, medium, and high treatment test chambers. The 96-hour LC50 was derived from measured concentrations by the Trimmed Spearman Karber Method.[16,17]

Dynamic Acute Toxicity

The bluegills were exposed in 9.5-liter aquaria containing 7.6 liters of test solution. Ten fish were randomized to each of the test aquaria as was described in the static acute test. Fish were not fed beginning 48 hours prior to the start of the test.

The toxicant diluter was similar to that used by van der Schalie[11] to deliver test solutions to the tanks. The toxicant was introduced to the diluter by a peristaltic pump controlled by a Chrontrol® timer. The cycle time was regulated with a 60-minute Tork time controller. The Tork time controller opened a solenoid valve that allowed dilution water to enter the diluter system every 10 minutes (six cycles per hour). The cycle time provided about five tank volume exchanges per day per tank.

Dissolved oxygen, pH, and temperature were measured from at least one replicate tank of each treatment daily. Temperatures were monitored continuously with a Cole Parmer temperature recorder. Sample sets were sent for analysis on day 1 and day 4 of the exposure. Test tanks did not have to be aerated because the dissolved oxygen concentration never fell below 60 percent of saturation.

LC50s were calculated from measured concentrations. The 96-hour LC50 was estimated by the Trimmed Spearman Karber method.[16,17]

Ventilatory Toxicity

The test apparatus described by van der Schalie[1] was modified for these tests. Caputo[18] demonstrated the advantages of the dorsal and ventral electrode chamber over the anterior and posterior electrode chamber. The electrode chambers were rebuilt to accommodate electrodes on the top and bottom of each test chamber (Fig. 2). The electrodes and holding screws were made of stainless steel. A chamber was constructed for each fish from 3 mm glass plates cemented with clear silicone sealer. A piece of black plastic was attached to the outside of each tank to prevent any fish from being disturbed by fish in the other chambers. Thirty-one test chambers were enclosed in a flat black plywood box with fluorescent light fixtures mounted above the chambers. Light intensity at the top of the test chamber ranged from 50 to 100 footcandles. Water flow was continuous through the test chambers. One of the 31 chambers was used only for continuous temperature monitoring.

The electrodes from each chamber were connected to individual amplifier/filter boards[19] with a two-conductor shielded cable. The ventilatory signal from each amplifier was sent to a Cromemco model Z-80 microcomputer equipped with a multichannel multiplexer and an analogue-to-digital converter. All connections were made with shielded cable to reduce background electrical interference. The microcomputer was connected to a Cromemco model PFD disk drive (for program storage) and to a Texas Instruments 780 series data terminal (used to load the ventilatory signal analysis program and generate a hard copy of the ventilatory data). Ventilatory data were also collected on magnetic tape by a Columbia Data Products data logger. The tape permitted quick transfer of data to other computer systems for statistical analysis.

The toxicant diluter operation resembled that described by van der Schalie.[11] A 30-minute Tork time controller was set to cycle the diluter every 3 minutes. This 3-minute cycle time resulted in a continuous flow of test solution to each test chamber, but the flow rate did vary slightly during each cycle. The mean flow rate per diluter cycle into each test chamber was 28 mL/min. There were six flow-splitting chambers, thus allowing five treatments (50% dilution series) and a dilution water experimental control. From each flow-splitting chamber, five glass tubes led to the test chambers. An overflow tube was set up in each flow-splitting chamber to channel excess test solution to drain. All test chambers drained into a common drain and the drain led to a large plastic container filled with activated charcoal. The effluent was monitored to ensure that the filtration was effective. Diluter operation was monitored daily during testing through a record of the number of diluter cycles and toxicant stock solution used. The toxicant stock solution

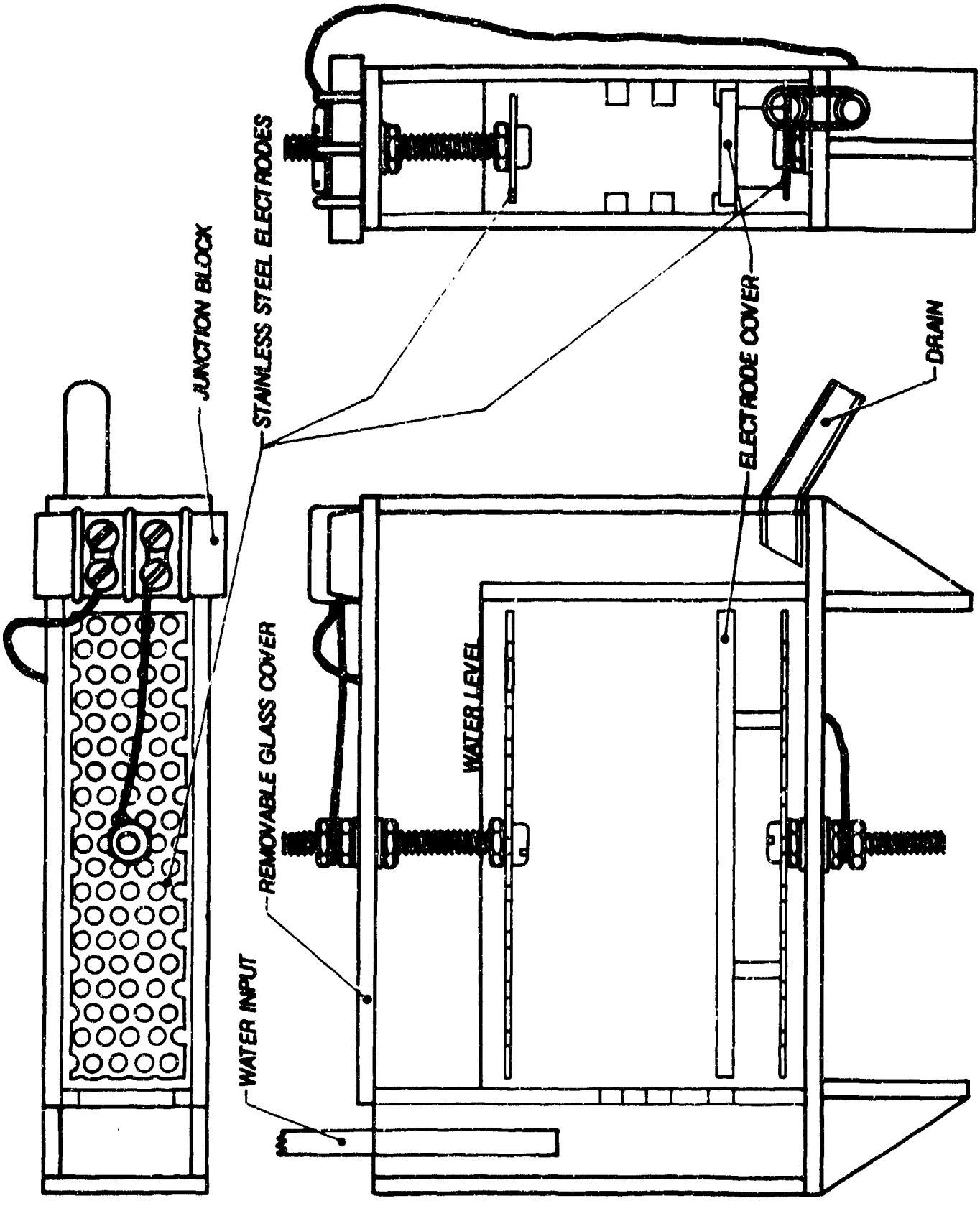


Figure 2. Test Chamber, Full Scale.

for the solvent and chlordane tests was introduced with a 1-mL Lab Industries Repipette®. For the TNB ventilatory test, the toxicant stock was introduced to the diluter by a peristaltic pump controlled by a Chrontrol® timer. The solvent ventilatory test consisted of two treatment levels and a dilution water control.

Chlordane ventilatory sample sets were taken from one replicate tank of all treatments and sent for analysis on day 1 and day 6 of the exposure period. TNB ventilatory sample sets were taken 3 hours after exposure to TNB had begun and on days 2, 4, and 6 of the exposure period from one replicate tank of each treatment.

Ventilatory Data Collection and Analysis

The four ventilatory parameters from each test fish were monitored continuously, calculated at 15-second intervals, and compiled every 15 minutes. The 15-minute data records were stored on a magnetic tape data logger and printed on a Texas Instrument data terminal. Data stored on the data logger were transmitted to an AMDAHL 470-V7 computer for further analysis with the Statistical Analysis Systems (SAS).[20] Each test was divided into three periods: a 3-day acclimation period (data not recorded); a 4-day pre-exposure period; and a 6-day exposure period.

The four ventilatory parameters were calculated as follows from each 15-minute data summary: ventilatory rate was equal to the number of ventilatory peaks per minute; cough rate was equal to the number of coughs per minute; ventilatory depth was calculated by dividing the total peak height by the total number of ventilatory peaks counted in the 15-minute interval; percent movement was calculated by dividing the number of 15-second intervals of movement within the 15-minute interval by 60 (the total number of 15-second records in 15 minutes). Ventilatory rate, ventilatory depth, and cough rate computations excluded any 15-second periods during which movement was detected.

Graphs were generated for each fish and ventilatory parameter by use of the 15-minute data points through the entire test (excluding the acclimation period). Because of the occurrence of sporadic, very high, or very low readings from the fish under constant control water quality conditions, a moving average technique was used to smooth the data. The moving average period was selected based on an initial review of the data and on cough response patterns described by Drummond and Carlson.[2] Comparisons of overall mean values for the pre-exposure and exposure periods masked significant signal changes during the test, since some fish had a definite but transient response to toxicant exposure. Use of a 1-hour moving average technique did not smooth the data sufficiently to remove short-term events not related to toxicant exposure. A 4-hour moving average of the ventilatory parameters was found to be the best compromise for separating toxicant and non-toxicant related ventilatory events.

To calculate the 4-hour moving average, 16 consecutive data points representing 4 hours of ventilatory signal were averaged. With each new 15-minute data point, the first data point was dropped and the next data point was added. A new mean value was then generated. This process continued

through the last data point of the pre-exposure period and started again with the first 16 data points of the exposure period.

By means of the 4-hour moving average data points, maximum and minimum values for each ventilatory parameter were established for the pre-exposure period and subtracted from the corresponding maximum and minimum values of the exposure period. The differences were averaged for all the fish at each treatment level. The SAS General Linear Models Procedure (GLM) was used.[20] Analysis of variance (ANOVA) was performed to determine treatment effects. Where significant effects were indicated ($P < 0.05$), pairwise comparisons between each treatment level and the control were made using Student's t-tests with Bonferroni's correction for simultaneous comparisons, as described by Feder.[21]

Periodically during ventilatory testing, a multichannel stripchart recorder was used to simultaneously record 15 minutes of several ventilatory signals monitored by the computer. Data output from the computer was then directly compared to manual counts of the ventilatory parameters. Over all three ventilatory tests, 292 15-minute stripchart records were manually counted. The information gathered from each visual record included the number of ventilatory peaks, the number of coughs, and the number of 15-second blocks of irregular signal corresponding to fish body movement. The ventilatory peak height was not manually measured.

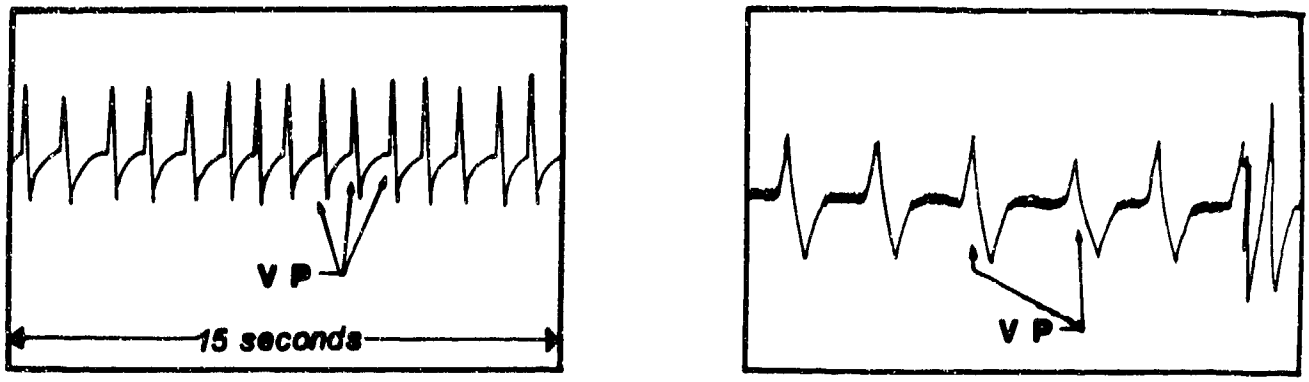
Manual collection of the ventilatory parameters from strip chart recordings introduces an element of subjectivity in signal interpretation. To reduce inconsistency, manual signal analysis followed that described by Carlson.[22] Typical ventilatory peaks, coughs, and movement that were counted visually are shown in Figure 3.

The visual and computer data were compared by using the SAS GLM program.[20] Simple linear regression analysis was performed for each ventilatory parameter. Regressions were done both on individual test data sets and on the combined data sets, and plots of the computer versus the visual data were generated.

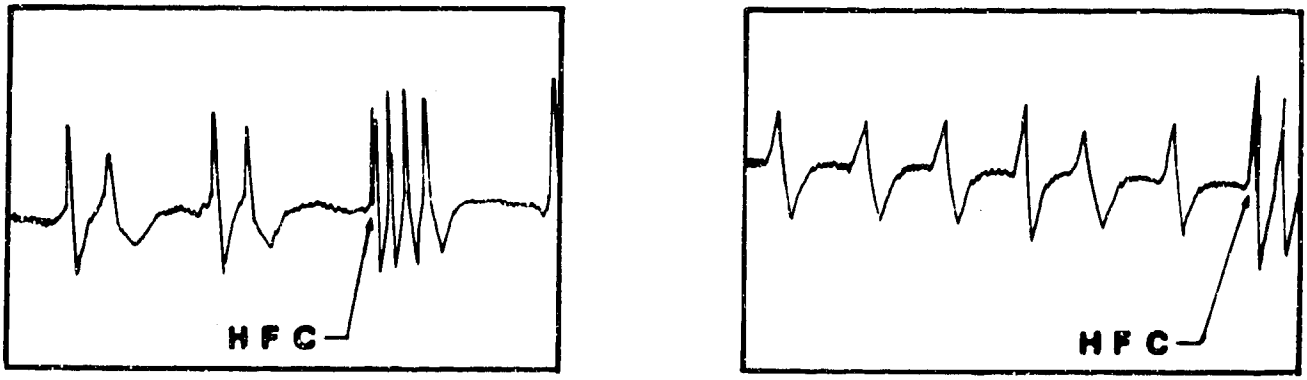
RESULTS AND DISCUSSION

VENTILATORY MONITORING SYSTEM ACCURACY

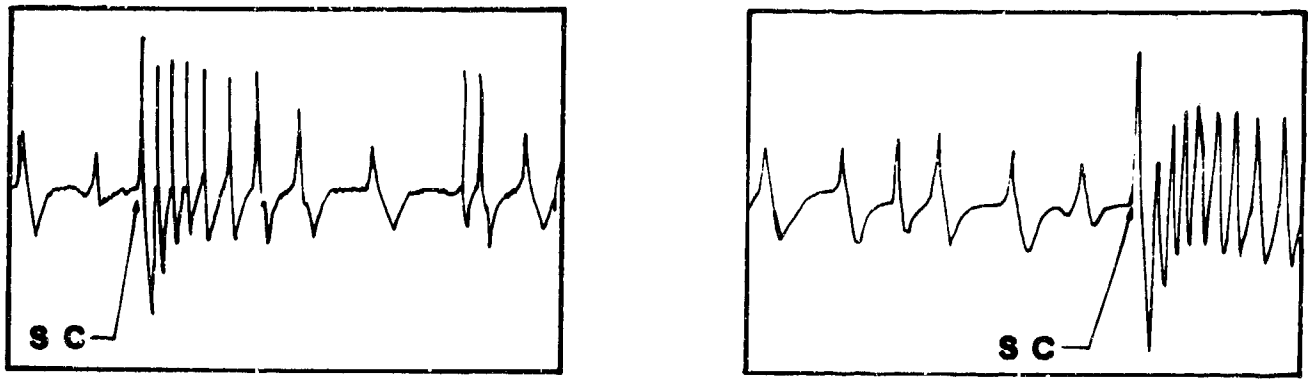
Regression analysis revealed statistically significant relationships ($P < 0.05$) between visual and computer counts for all ventilatory parameters. Visual and computer counts for ventilatory peaks were highly correlated. Linear regression analysis of visual and computer ventilatory peak data for all three ventilatory tests (solvent, chlordane, and TNB) produced an R^2 value of 0.976 and a slope of 0.972 (see Fig. 4). The relationship demonstrates that ventilatory peak analysis by the computer was accurate. Although visual measurements of the ventilatory peak depth were not made, it follows that if the ventilatory peak counts are accurate, the average depths of the ventilatory signals are also accurate.



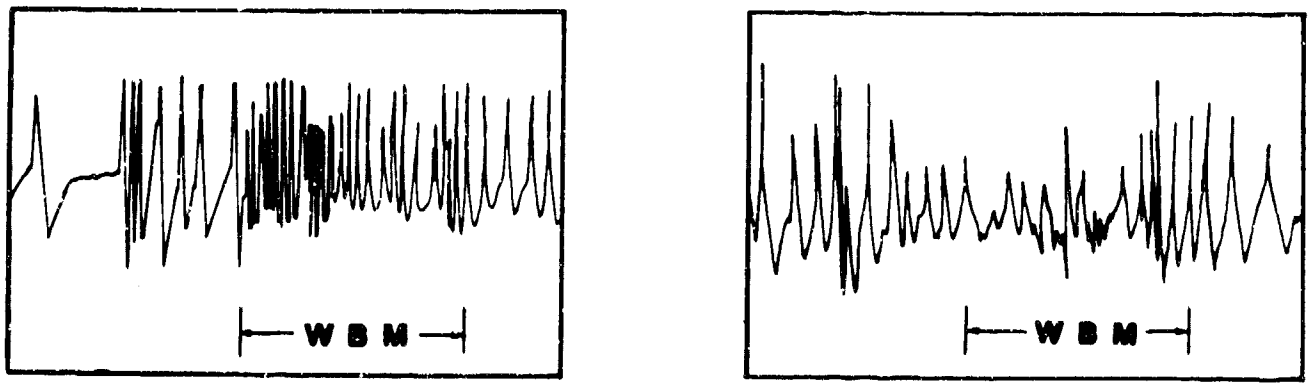
V P - VENTILATORY PEAK



H F C - HIGH FREQUENCY COUGH



S C - SPIKE COUGH



W B M - WHOLE BODY MOVEMENT

Figure 3. Typical Ventilatory Signal Recordings.

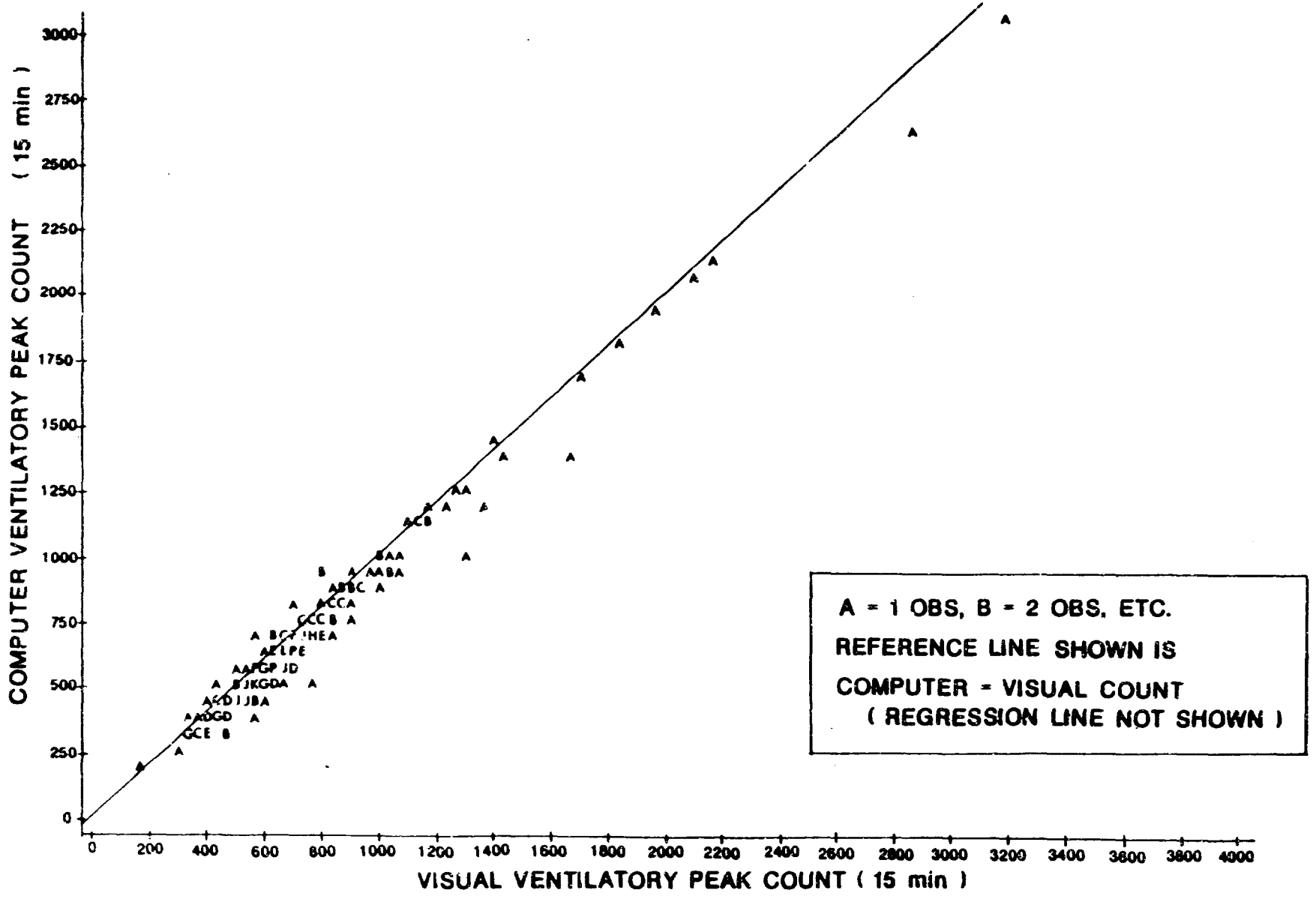


Figure 4. Visual vs. Computer Ventilatory Peak Counts, All Tests.

Coughs were very difficult to count visually with consistency due to signal variability. The visual-computer count relationship for the cough was weak over all three tests combined. The R^2 value was 0.358 and the slope was 0.658 (Fig. 5). Low R^2 values were also found for two ventilatory tests analyzed individually: the solvent test had an R^2 value of 0.216 and a slope of 0.658 (Fig. 6), while the chlordane test had an R^2 value of 0.213 and a slope of 0.464 (Fig. 7). These weak relationships are in sharp contrast to the results from analysis of the TNB ventilatory test, which had an R^2 value of 0.766 and a slope of 1.177 (Fig. 8). One difference that may have contributed to the differences in cough analysis accuracy was the size of the test fish. The bluegills used in the TNB ventilatory test were 3.7 and 2.4 times larger by weight than those used in the solvent and chlordane ventilatory tests, respectively (Table 4). The larger fish had a stronger ventilatory signal initially, and their signal amplitude did not decrease during the test as much as did the signals of the smaller fish. Lower signal amplitudes increased the amount of error associated with the computer's analysis of the ventilatory signal. The lack of cough response in the chlordane test (described below) could be assigned, in part, to the loss of computer accuracy resulting from low ventilatory signal amplitudes.

The number of 15-second movement intervals counted was very low. Many of the 15-minute records had zero or one 15 second movement interval for both the computer and visual count (Figure 9). The correlation for movement was fair, with an R^2 value of 0.586 and a slope of 0.764. Analyzing the data for each test separately had no apparent effect on the visual-computer movement relationship.

Based on the observed visual-computer relationships, the computer cough analysis in the chlordane test may not have been sufficiently accurate to use the parameter as a predictor of chlordane toxicity. However, computer analysis of ventilatory rate and average depth were very accurate and would probably have picked up a response to the toxicant. Although the incidence of movement was very low in all tests, a major increase would probably have been detected. The TNB ventilatory test had good computer accuracy for all four ventilatory parameters and therefore would have had the greatest sensitivity to changes in one or all parameters caused by TNB.

In order to document significant differences due to toxicant exposure, variability in the ventilatory parameters within each fish as well as between fish within treatment had to be overcome. The vastly different signals between individual fish forced each fish to be used as its own control. The 4 day pre-exposure (control) period was established to generate the normal range for each ventilatory parameter for each individual fish. The subsequent 6-day exposure was a compromise between the 96-hour exposure period recommended by Drummond and Carlson[2] as being adequate to detect changes in cough rates induced by most chemicals and the 10-day period cited by them as necessary for detecting responses to low levels of certain chemicals.

Plots of ventilatory depth versus time revealed several important points about variability in the data (see Figs. 10 and 11). Unexplained increases and decreases in average depth occurred throughout both the pre-exposure and exposure periods. Examples (Fig. 10) include events in the pre-exposure period at 18 hr (decrease), 27 hr (increase), 36 hr (decrease), 45 hr (increase), and at 48 hr (decrease). In addition, at 27 hr, the response to a

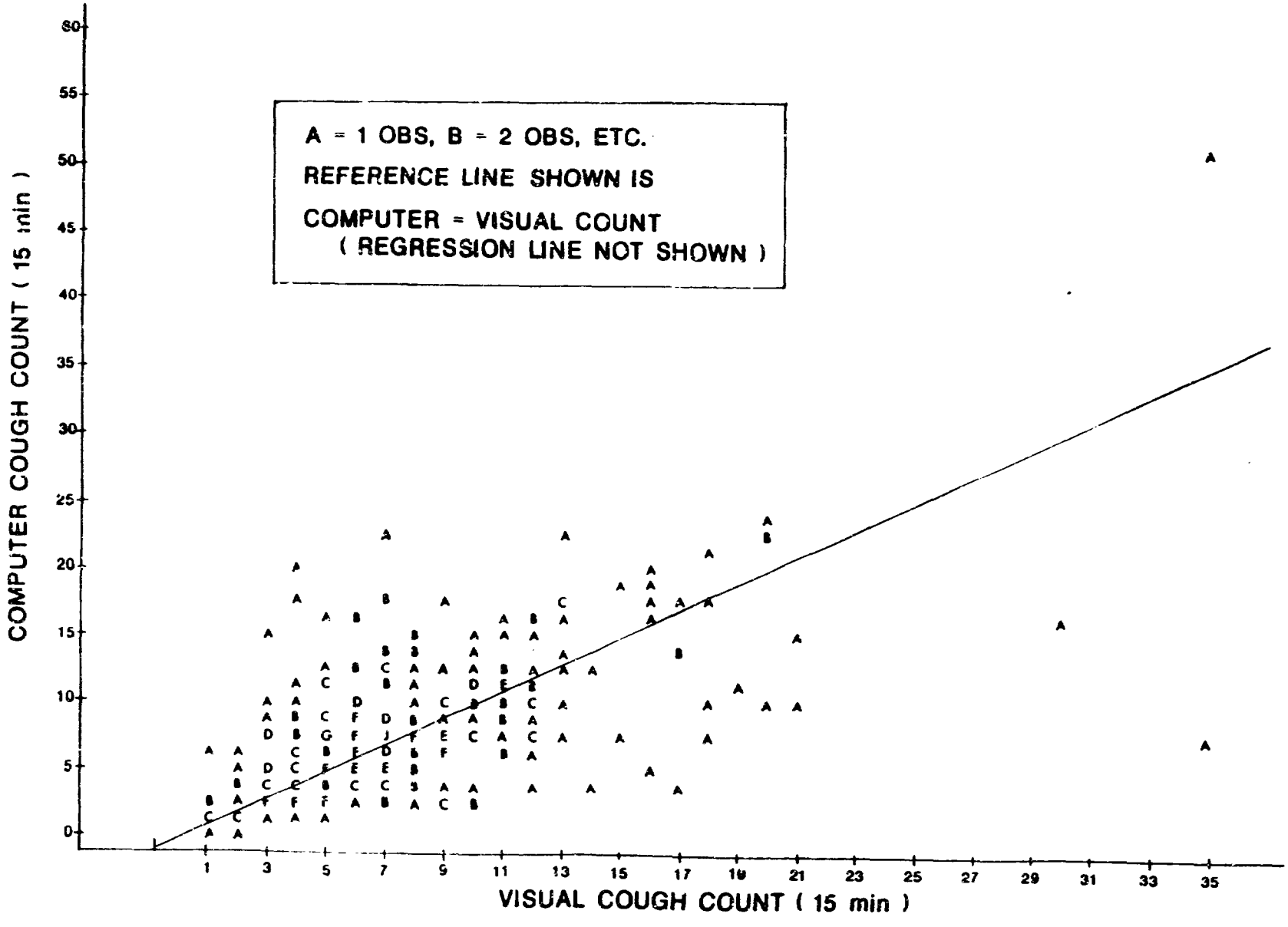


Figure 5. Visual vs. Computer Cough Counts, All Tests.

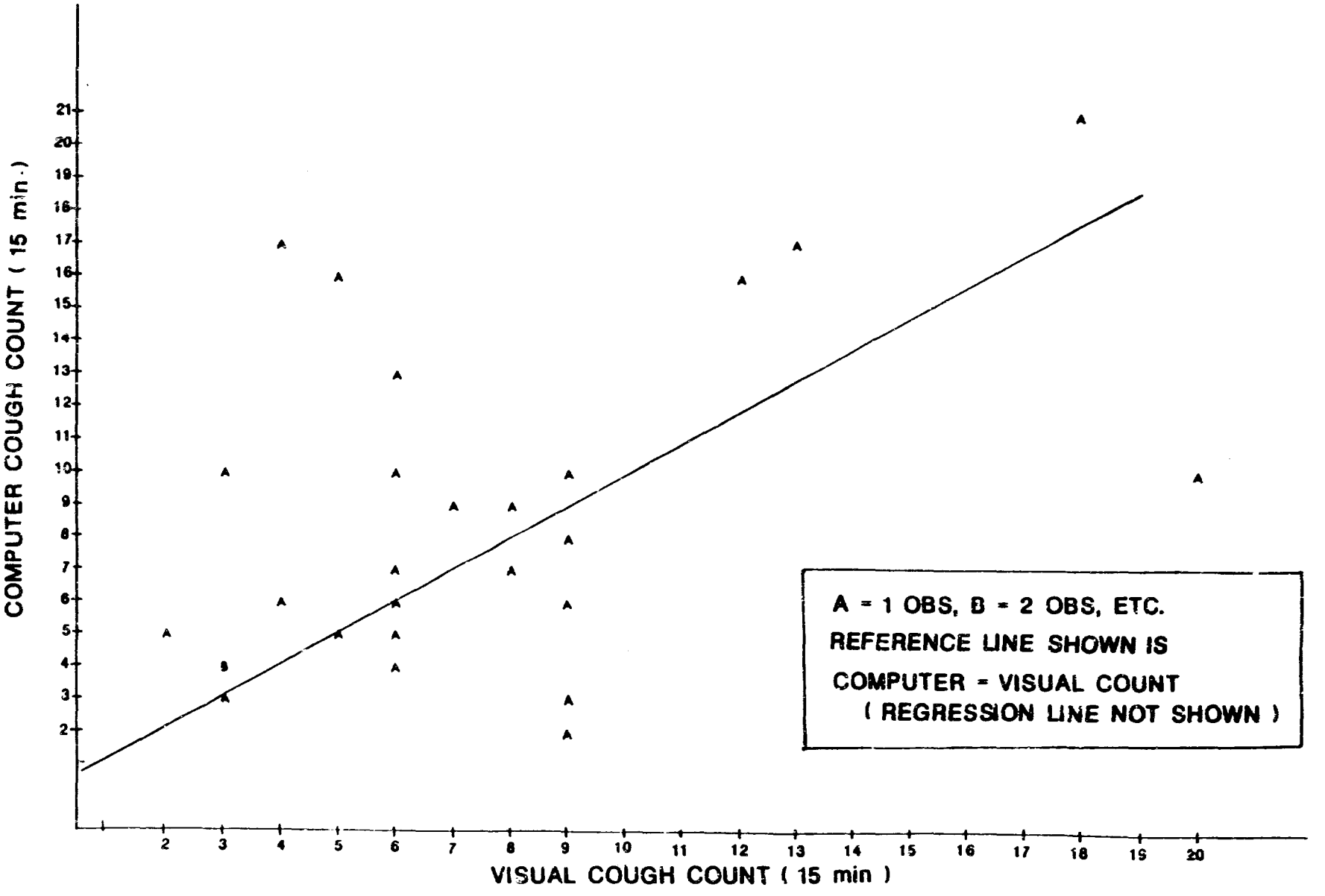


Figure 6. Visual vs. Computer Cough Counts, Solvent Test.

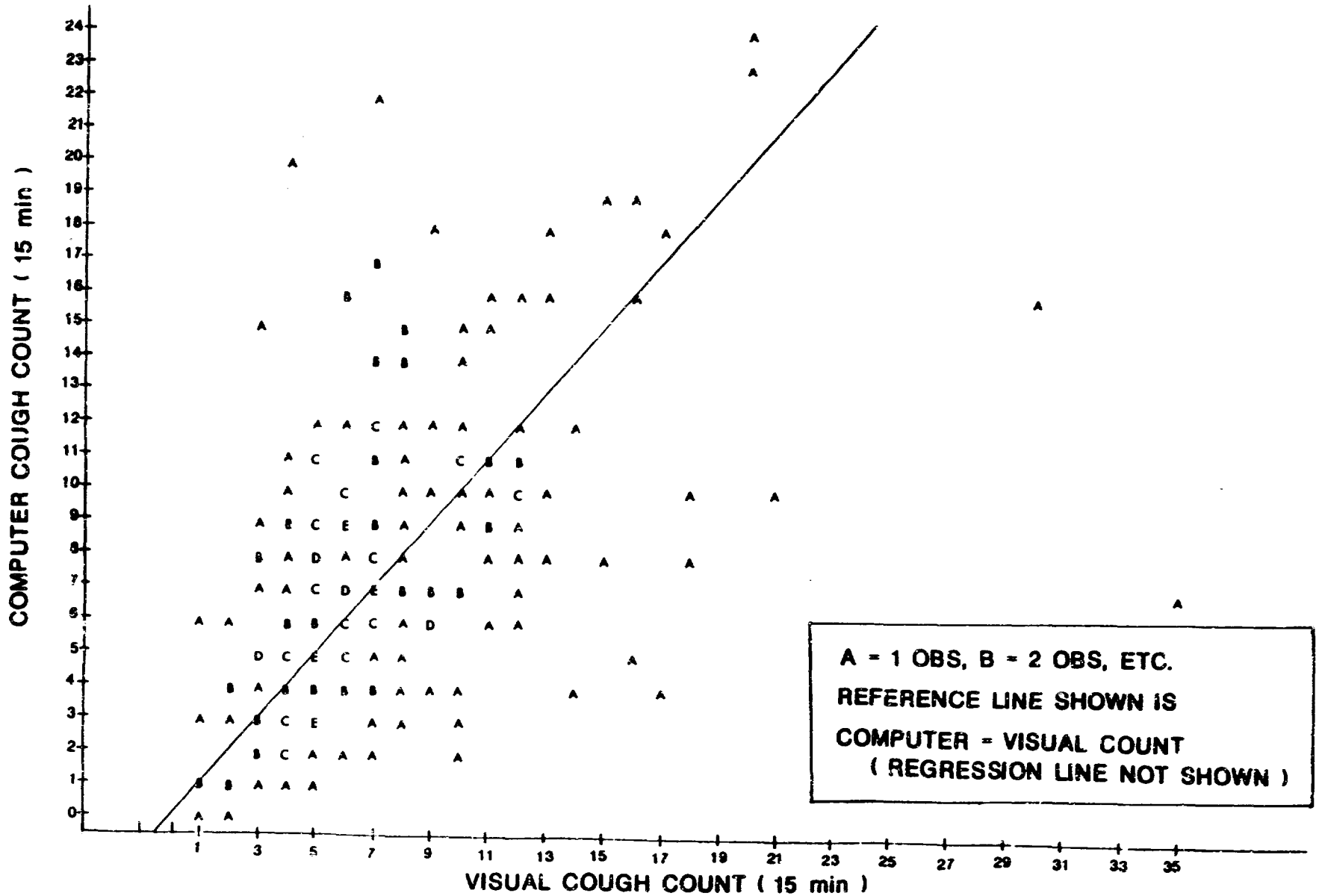


Figure 7. Visual vs. Computer Cough Counts, Chlordane Test.

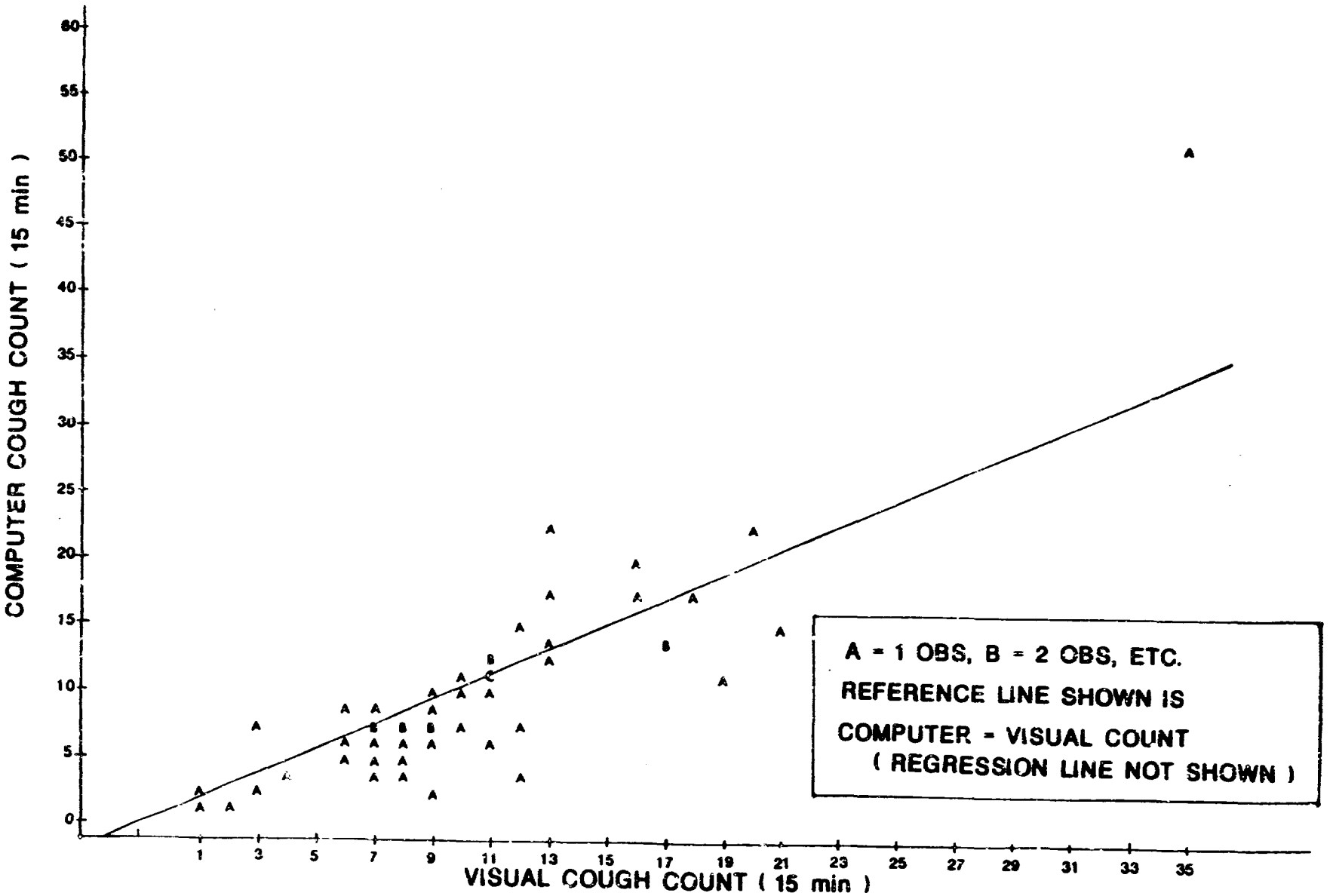


Figure 8. Visual vs. Computer Cough Counts, TNB Test.

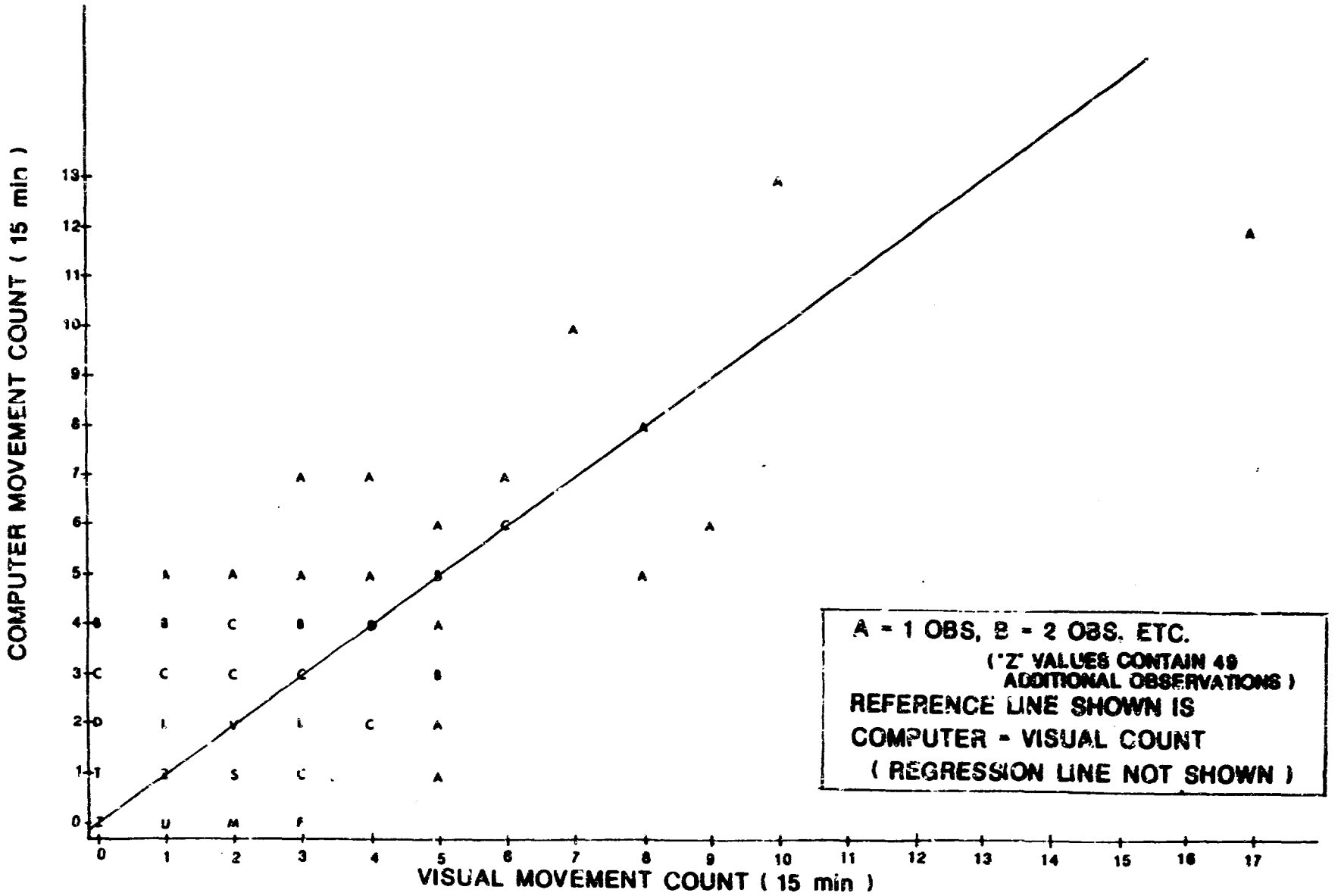


Figure 9. Visual vs. Computer Movement Counts, All Tests.

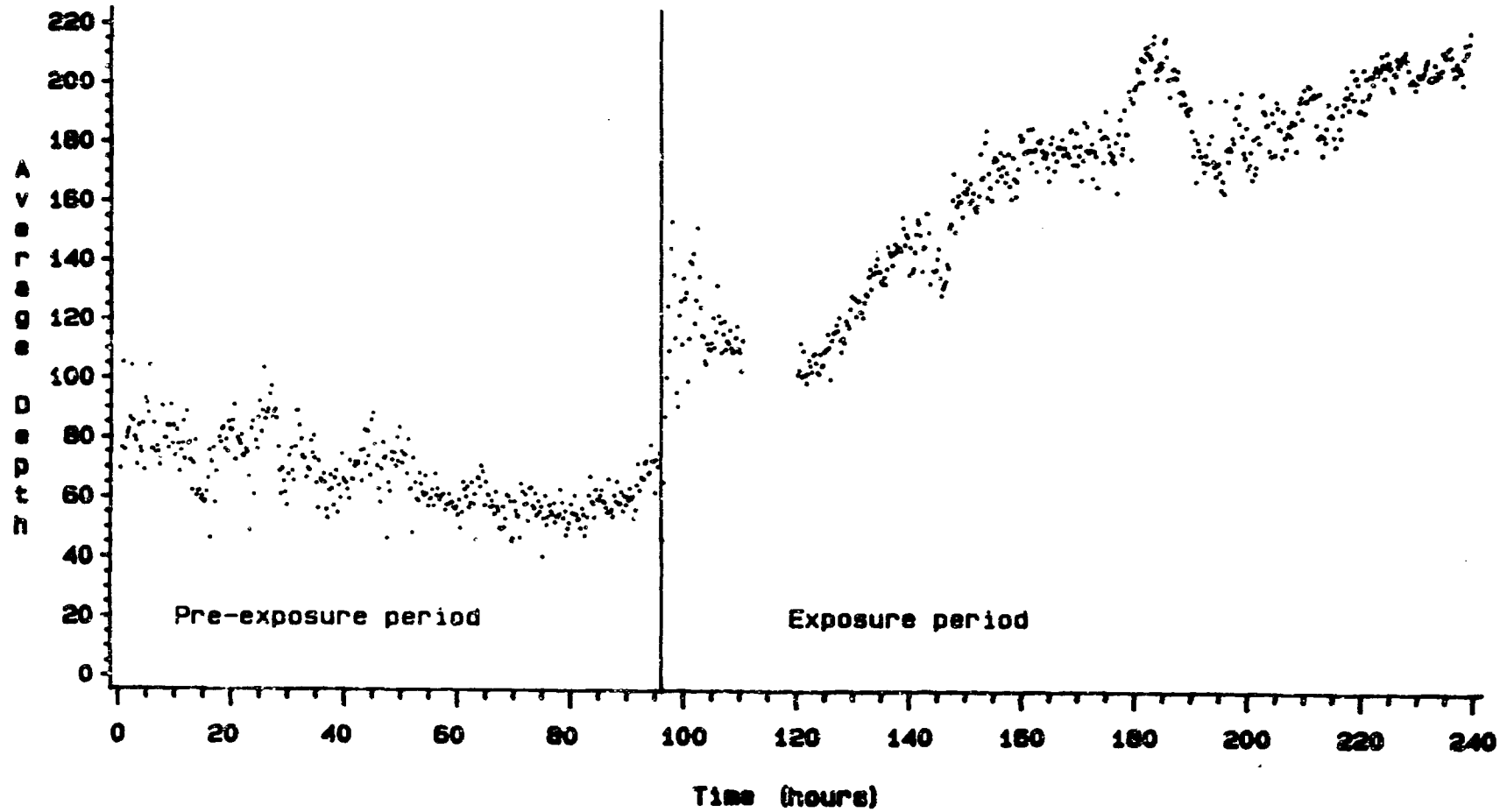


Figure 10. Plot of Average Depth vs. Time, Fish #1, 0.613 mg/L TNB.

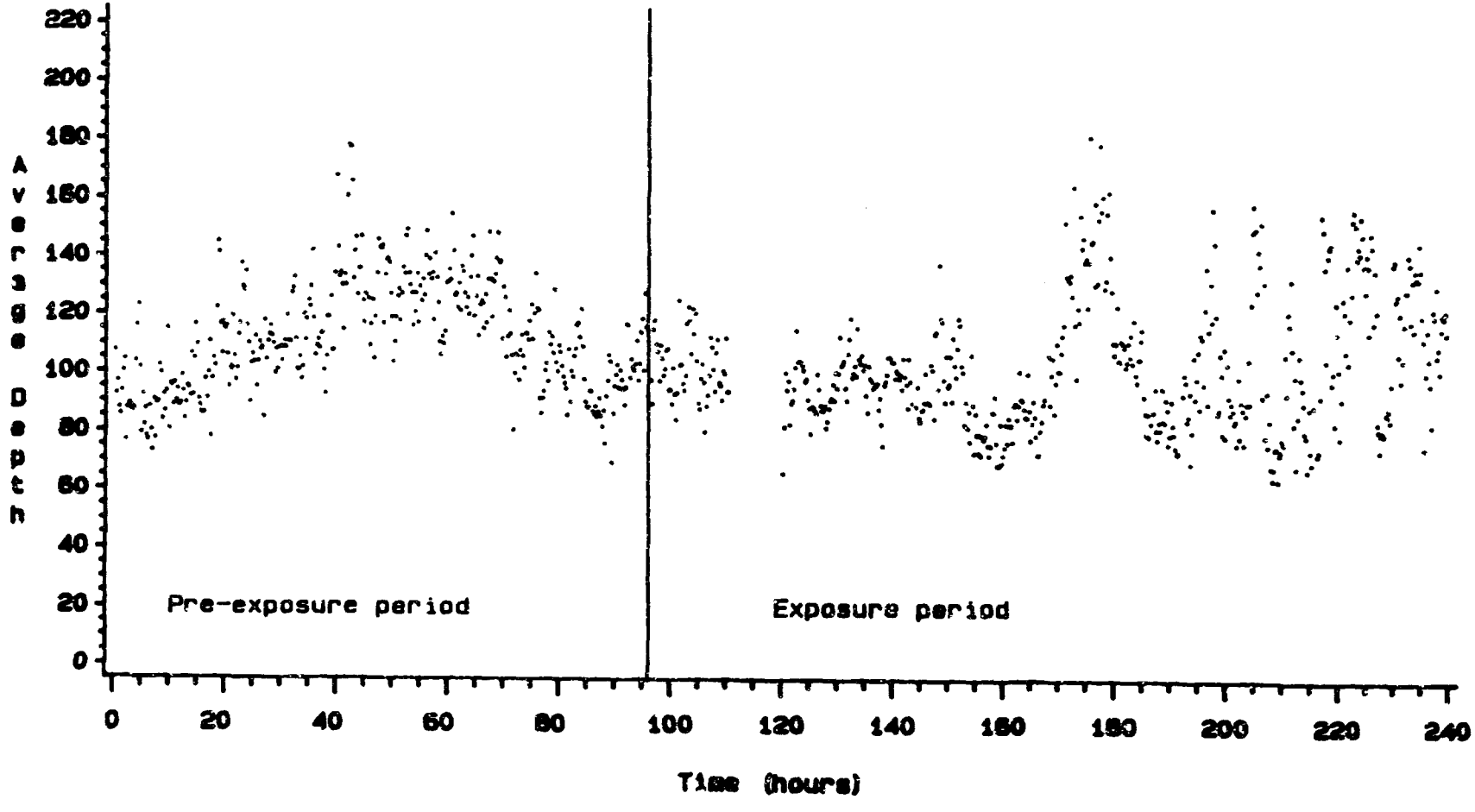


Figure 11. Plot of Average Depth vs. Time, Fish #25, Control, TNB.

loud noise can be observed in Figures 10 and 11. Fish number 1 (Fig. 10) responded by increasing ventilatory depth and fish number 25 (Fig. 11) responded by decreasing ventilatory depth. While the random variations, such as those described above, were adequately addressed through the use of the moving average data analysis, certain other events (with both known and unknown causes) were of sufficient magnitude and duration to show up even after the data were smoothed using the moving average technique (Figs. 12 and 13). A partially clogged flow splitter supplying dilution water to fish 25 (Fig. 13) was cleared at 45 hr, causing a rapid drop in ventilation depth. Most events of known cause such as this were for the most part of short duration, affecting the ventilatory parameters for about an hour or less. A major event occurred at about 180 hr (Figs. 12 and 13). This event occurred at about midnight, when variations in the external environment would be the least likely to cause such an event. The event was unexplained and had some effect on all fish in the TNB test. The gap in the data from 114-120 hr (Figs. 10 to 13) resulted from a computer breakdown. Due to the total volume of data collected, the 9.5 hours of missing data should not have impacted greatly on the test results. A change in ventilatory average depth from the pre-exposure to the exposure periods can be seen when Figures 12 and 13 are compared. Soon after TNB exposure had begun (99 hr), fish number 1 (Fig. 12, acutely toxic TNB exposure) responded with a rapid increase in average ventilatory depth. Fish number 25 (Fig. 13, no TNB exposure) had no such increase at that time. The TNB-exposed fish gradually increased ventilatory depth throughout the exposure period while the control fish did not. Although the TNB-related change in average depth was quite marked in this case, much of the apparent toxicant-related responses were much more subtle. The high variability in the ventilatory signals in general may have masked the more subtle toxicant-related effects.

In some cases, non-toxicant related transient events affected the maximum and minimum values used to establish the difference between pre-exposure and exposure periods. The clogged flow splitter (Fig. 13, 45 hr) and the unexplained event (Fig. 13, 180 hr) set the maximum ventilatory depth for the pre-exposure and the exposure periods, respectively. The maximum value in ventilatory depth for the exposure period for TNB-exposed fish 1 was established by the event at 180 hr or by the gradual increase to the maximum at 234 hr (Fig. 12). Although these apparent non-toxicant related events created a great amount of variability in the ventilatory signal, in both fish 1 and 25 the relative changes in ventilatory depth maxima would have been the same with or without the events. The TNB ventilatory test had many more apparent non-toxicant related events (explained and unexplained), when compared to the chlordane and solvent tests.

SOLVENT AND CHLORDANE RESULTS

The solvent test was done to ensure that the ventilatory response levels found in the chlordane test were not due to solvent effects. Test results are given in Tables 5 and 6. There were no statistically significant differences between the controls and solvent-exposed fish for any of the four ventilatory parameters. However, as stated earlier, the accuracy of computer cough rate analysis in this test was not good. Average depth maxima and minima do show a slight but non-significant tendency to decrease with increasing solvent concentration (Tables 5 and 6). The lowest solvent concentration tested was selected for use in conjunction with the chlordane ventilatory test.

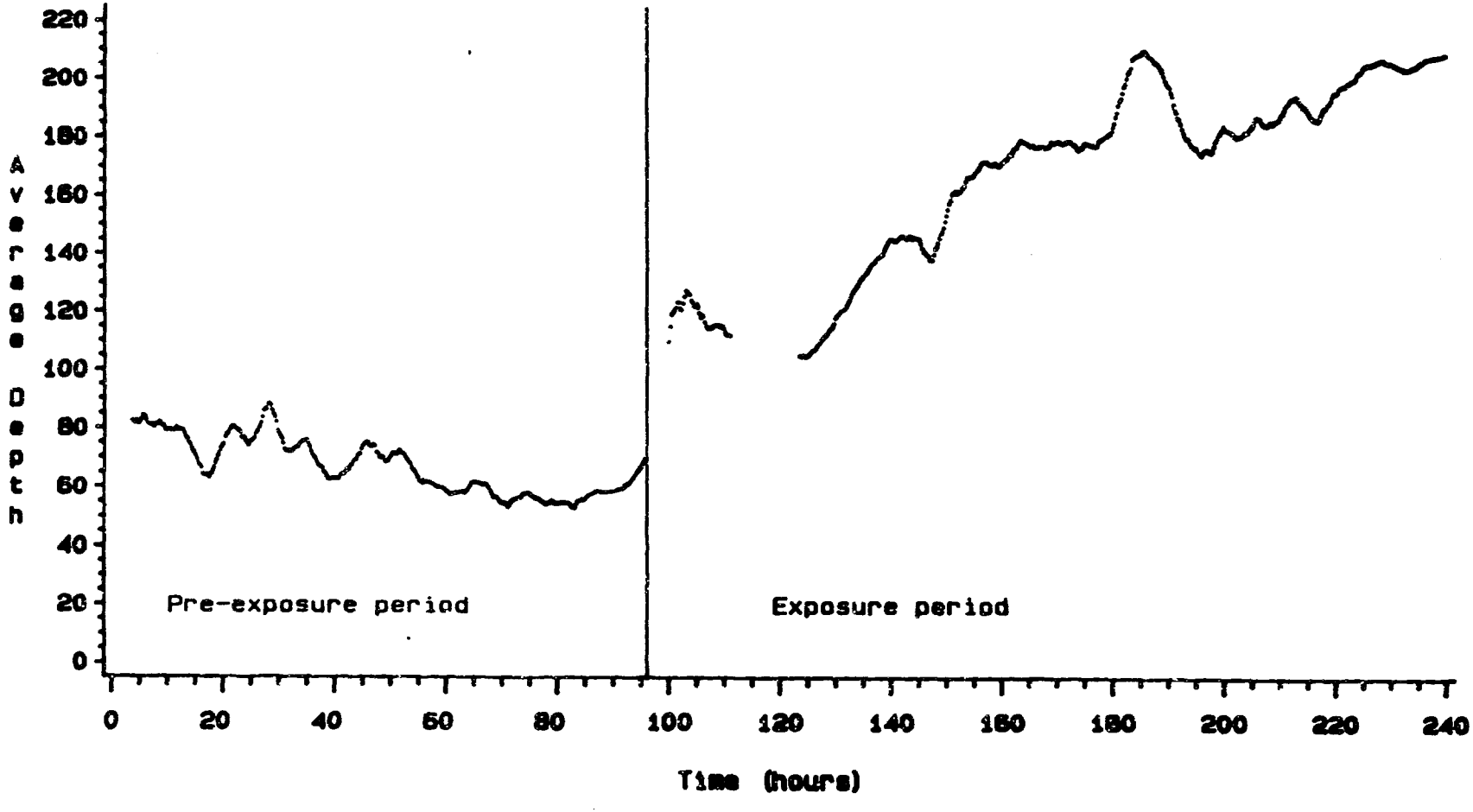


Figure 12. Plot of Average Depth (Moving Average) vs. Time, Fish #1, 0.613 mg/L TNB.

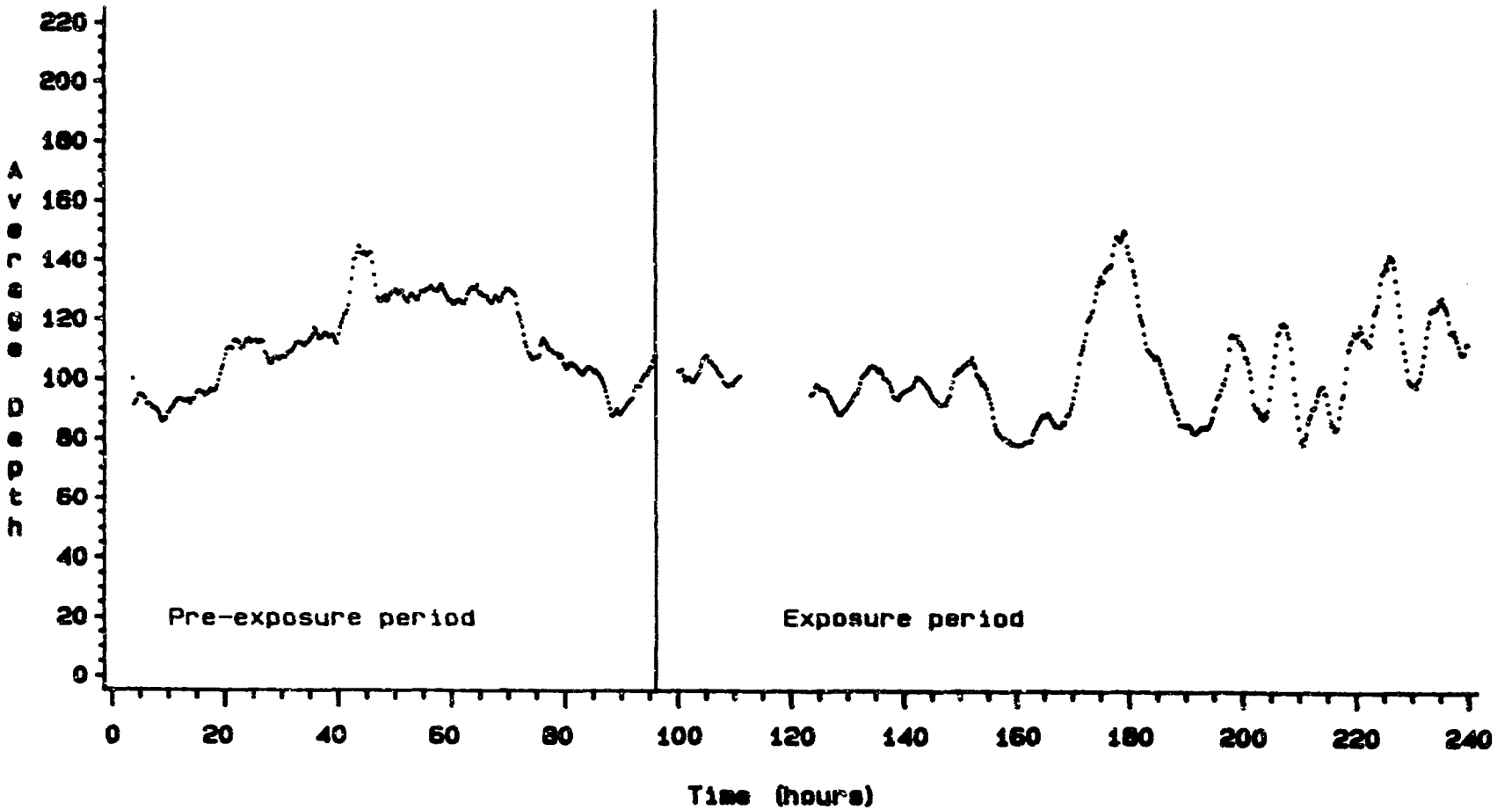


Figure 13. Plot of Average Depth (Moving Average) vs. Time, Fish #25, Control, TNB.

TABLE 5. DIFFERENCES BETWEEN PRE-EXPOSURE AND EXPOSURE MAXIMA IN THE SOLVENT VENTILATORY TEST (TRITON X-100 AND ACETONE)

Parameter	Nominal Concentration Triton X-100 (µg/L)+ Acetone (µg/L)	Mean Differences Between Maxima[a]	Standard Deviation
Average Depth	10+395	-31.73	14.68
	5+198	-17.62	14.40
	0+0 (control)	-9.45	3.88
Ventilatory Rate	10+395	-7.13	4.60
	5+198	0.86	27.53
	0+0 (control)	-13.25	13.30
Cough Rate	10+395	0.08	1.17
	5+198	-0.03	0.11
	0+0 (control)	-0.03	0.29
Percent Movement	10+395	2.50	5.78
	5+198	-0.35	1.62
	0+0 (control)	-1.23	0.91

a. Mean value (exposure maxima - pre-exposure maxima) for all fish at each treatment level (N = 5).

TABLE 6. DIFFERENCES BETWEEN PRE-EXPOSURE AND EXPOSURE MINIMA IN THE SOLVENT VENTILATORY TEST (TRITON X-100 AND ACETONE)

Parameter	Nominal Concentration Triton X-100 (µg/L)+ Acetone (µg/L)	Mean Differences Between Minima[a]	Standard Deviation
Average Depth	10+395	-31.23	12.46
	5+198	-26.81	14.91
	0+0 (control)	-18.58	5.84
Ventilatory Rate	10+395	-7.19	3.73
	5+198	-6.95	3.07
	0+0 (control)	-8.13	2.81
Cough Rate	10+395	-0.16	0.38
	5+198	-0.12	0.17
	0+0 (control)	-0.03	0.06
Percent Movement	10+395	-0.73	1.30
	5+198	-0.18	0.33
	0+0 (control)	-0.15	0.24

a. Mean value (exposure minima - pre-exposure minima) for all fish at each treatment level (N = 5).

Chlordane static acute test conditions varied slightly from nominal levels. The average test temperature was 20.5°C, rather than the target temperature of 22°C. The reduced temperature probably did not affect the sensitivity of the bluegills to chlordane, since it has been found that the acute toxicity of chlordane to bluegills does not change significantly between 18°C and 24°C.[23] Test concentrations dropped substantially over the 96 hours of the test (Table 7), except in the positive control, which maintained a stable measured concentration for the duration of the test. This suggests that the chlordane loss over 96 hours in the other treatments was due to uptake by the fish.

TABLE 7. STATIC ACUTE TEST CHLORDANE CONCENTRATIONS

Nominal Concentration (µg/L)	Mean Measured Concentration (µg/L)	Initial (0 hr)	Final[a] (72 hr)
100	63.9	103.6	24.2
56	41.7	75.0	8.4
32	23.8[b]	-	-
18	13.4[b]	-	-
10	8.8	17.6	BDL[c]
Water Control	<5.0	BDL	BDL
Solvent Control	<5.0	BDL	BDL
Positive Control (56 µg/L)[d]	72.9	73.8	72.0

a. Test continued for 24 hr after final sample set taken.
b. Value not measured, calculated using the nominal concentration ratio.
c. Below detection limit (0.5 µg/L).
d. Treatment contained no fish.

The chlordane static acute test resulted in a 96 hour LC50 of 56 µg/L (95% confidence limits: 49-64). This value is close to the value of 59 µg/L (95% confidence limits: 50-71) for bluegills at 22°C used in conjunction with the chronic test with chlordane.[10] This suggests that the bluegills used in the previous chronic test and the present ventilatory test had similar sensitivity to the toxic effects of chlordane. Exposure concentrations for the present study are shown in Table 7. The intermediate concentrations of 13.4 µg/L and 23.8 µg/L were calculated on the basis of the nominal:measured concentration ratio.

A preliminary chlordane ventilatory test was conducted with use of the anterior and posterior electrode arrangement, but could not be successfully completed because distortions of the ventilatory signals were too great for computer analysis to accurately depict changes in ventilatory signals. Fish position in reference to the electrodes and insufficient grounding of the test

system contributed to the ventilatory signal distortion. With introduction of the newly designed test chamber (Fig. 2) and complete grounding of all water flows including drains, the ventilatory signal was much more stable and reflected the actual ventilatory patterns.

Chlordane ventilatory test concentrations are given in Table 8, while results of the chlordane ventilatory test are given in Tables 9 and 10. No statistically significant differences from the controls were seen in any treatments for any of the ventilatory parameters. No consistent trends in the maxima and minima were observed to be related to increasing chlordane concentrations. A slow decrease of ventilatory rate and depth was seen in the controls over the entire ventilatory test (pre-exposure and exposure periods). It appeared as though the bluegills were still slowly acclimating to the test conditions at the end of the test. The high standard deviation for the mean values indicates the highly variable responses of bluegills in both the solvent and chlordane tests.

TABLE 8. VENTILATORY TEST CHLORDANE CONCENTRATIONS

Nominal Concentration (µg/L)	Mean Measured Concentration[a] (µg/L)	Initial (day 1)	Final (day 6)
15.00	8.91	10.00	7.76
7.50	4.46	4.95	3.91
3.75	2.78	3.26	2.28
1.88	1.51	1.27	1.73
0.94	0.88	1.01	0.74
Control	BDL[b]	BDL	BDL

a. Measured values were corrected for an 82% spike recovery.
b. Below detection limit (0.04 µg/L).

TNB RESULTS

The TNB dynamic acute test concentration data are reported in Table 11. Mean measured concentration values were based on initial samples of all test tanks (two replicates) and one sample from each treatment on day 1. A diluter malfunction within the last 16 hours of the test reduced the toxicant concentrations by an overall 40 percent. These values, obtained at 96 hours, were not used to calculate the mean measured concentrations. The TNB flow-through acute test established a 96-hour LC50 for the bluegill of 0.57 mg/L. The 95 percent confidence limits were 0.50 mg/L to 0.65 mg/L. The bluegills used were slightly more sensitive to TNB under flow-through conditions then the reported static acute toxicity of TNB to bluegills (96-hour LC50 of 0.85 mg/L, 95 percent confidence limits of 0.52 to 1.38).[11] The toxicity of TNB to bluegills generated in this test was used to establish the treatment levels in the TNB ventilatory test. The top concentration of the ventilatory test was set at the LC50 to ensure a response from the ventilatory system.

TABLE 9. DIFFERENCES BETWEEN PRE-EXPOSURE AND EXPOSURE MAXIMA IN THE CHLORDANE VENTILATORY TEST

Parameter	Mean Measured Chlordane Concentration (µg/L)	Mean Differences Between Maxima[a]	Standard Deviation
Average Depth	8.91	-26.96	10.32
	4.46	1.37	16.25
	2.78	-1.91	8.25
	1.51	-37.71	40.28
	0.88	-14.90	9.14
	BDL[b]	-40.18	34.85
Ventilatory Rate	8.91	-1.51	44.51
	4.46	-11.68	39.36
	2.78	-6.59	26.82
	1.51	-18.08	12.21
	0.88	-12.57	16.71
	BDL	-7.00	16.04
Cough Rate	8.91	-0.51	1.35
	4.46	-0.44	0.34
	2.78	-0.29	0.23
	1.51	-0.24	0.23
	0.88	-0.03	0.13
	BDL	-2.00	3.41
Percent Movement	8.91	-2.51	20.71
	4.46	-4.12	4.67
	2.78	-1.59	1.87
	1.51	-2.56	7.53
	0.88	-0.98	1.19
	BDL	-4.67	3.60

a. Mean value (exposure maxima - pre-exposure maxima) for all fish at each treatment level (N = 5).
b. BDL - Below detection limit (0.4 µg/L).

TABLE 10. DIFFERENCES BETWEEN PRE-EXPOSURE AND EXPOSURE MINIMA IN THE CHLORDANE VENTILATORY TEST

Parameter	Mean Measured Chlordane Concentration (µg/L)	Mean Difference Between Minima[a]	Standard Deviation
Average Depth	8.91	-14.01	8.24
	4.46	9.18	12.52
	2.78	5.08	10.63
	1.51	-11.65	11.99
	0.88	-13.54	14.05
	BDL[b]	-5.77	15.24
Ventilatory Rate	8.91	-6.06	20.22
	4.46	-5.97	2.55
	2.78	-6.48	2.53
	1.51	-5.51	3.10
	0.88	-10.81	13.76
	BDL	-7.56	2.70
Cough Rate	8.91	-0.17	0.08
	4.46	-0.07	0.09
	2.78	-6.48	0.10
	1.51	-0.01	0.06
	0.88	-0.08	0.16
	BDL	-0.05	0.07
Percent Movement	8.91	-1.85	2.73
	4.46	-0.54	0.58
	2.78	-0.78	0.56
	1.51	-0.48	0.81
	0.88	-0.32	0.35
	BDL	-0.68	0.31

a. Mean value (exposure minima - pre-exposure minima) for all fish at each treatment level (N = 5).
b. BDL - Below detection limit (0.4 µg/L).

TABLE 11. DYNAMIC ACUTE TEST TNB CONCENTRATIONS

Nominal Concentration (mg/L)	Mean Measured Concentration N = 3 (mg/L)	Range (mg/L)
3.00	3.07	3.00-3.11
1.50	1.29	1.22-1.33
0.75	0.69	0.63-0.73
0.38	0.34	0.31-0.39
0.19	0.14	0.13-0.16
Control	BDL[a]	BDL

a. Below detection limit (0.10 mg/L).

The TNB concentration data for the ventilatory test are given in Table 12. Results of the test are given in Tables 13 and 14. There were no statistically significant differences from the controls found for any ventilatory parameter when the minima data were analyzed (Table 14). The maxima data (Table 13) showed statistically significant differences from the controls at some treatment levels for all the ventilatory parameters except ventilatory rate. Ventilatory rate maxima had a large mean increase at the highest toxicant concentration over the controls; however, the great variability of bluegill responses within the treatment caused the treatment effects to be insignificant when compared to the controls.

TABLE 12. VENTILATORY TEST TNB CONCENTRATIONS

Nominal Concentration (mg/L)	Mean Measured Concentration N = 4 (mg/L)	Range (mg/L)
0.600	0.613	0.600-0.641
0.300	0.279	0.253-0.301
0.150	0.128	0.121-0.137
0.075	0.061	0.057-0.064
0.038	0.034	0.031-0.038
Control	BDL[a]	BDL

a. Below detection limit (0.02 mg/L).

TABLE 13. DIFFERENCES BETWEEN PRE-EXPOSURE AND EXPOSURE MAXIMA IN THE TNB VENTILATORY TEST

Parameter	Mean Measured TNB Concentration (mg/L)	Mean Differences Between Maxima[a,b]	Standard Deviation
Average Depth	0.613	74.13[c]	50.36
	0.279	42.03[c]	16.98
	0.128	12.06[c]	22.70
	0.061	11.37	13.38
	0.034	8.07	25.11
	BDL[d]	-8.30	19.59
Ventilatory Rate	0.613	23.28	35.06
	0.279	-23.23	16.64
	0.128	-13.16	7.95
	0.061	-4.88	20.57
	0.034	-8.75	6.98
	BDL	-7.51	15.89
Cough Rate	0.613	4.71[c]	2.80
	0.279	0.16	0.37
	0.128	-1.41	1.59
	0.061	-0.10	0.24
	0.034	0.02	0.22
	BDL	0.29	0.69
Percent Movement	0.613	14.39[c]	7.96
	0.279	1.10	5.72
	0.128	-3.58	2.05
	0.061	-1.01	1.09
	0.034	-0.05	2.58
	BDL	-0.54	2.00

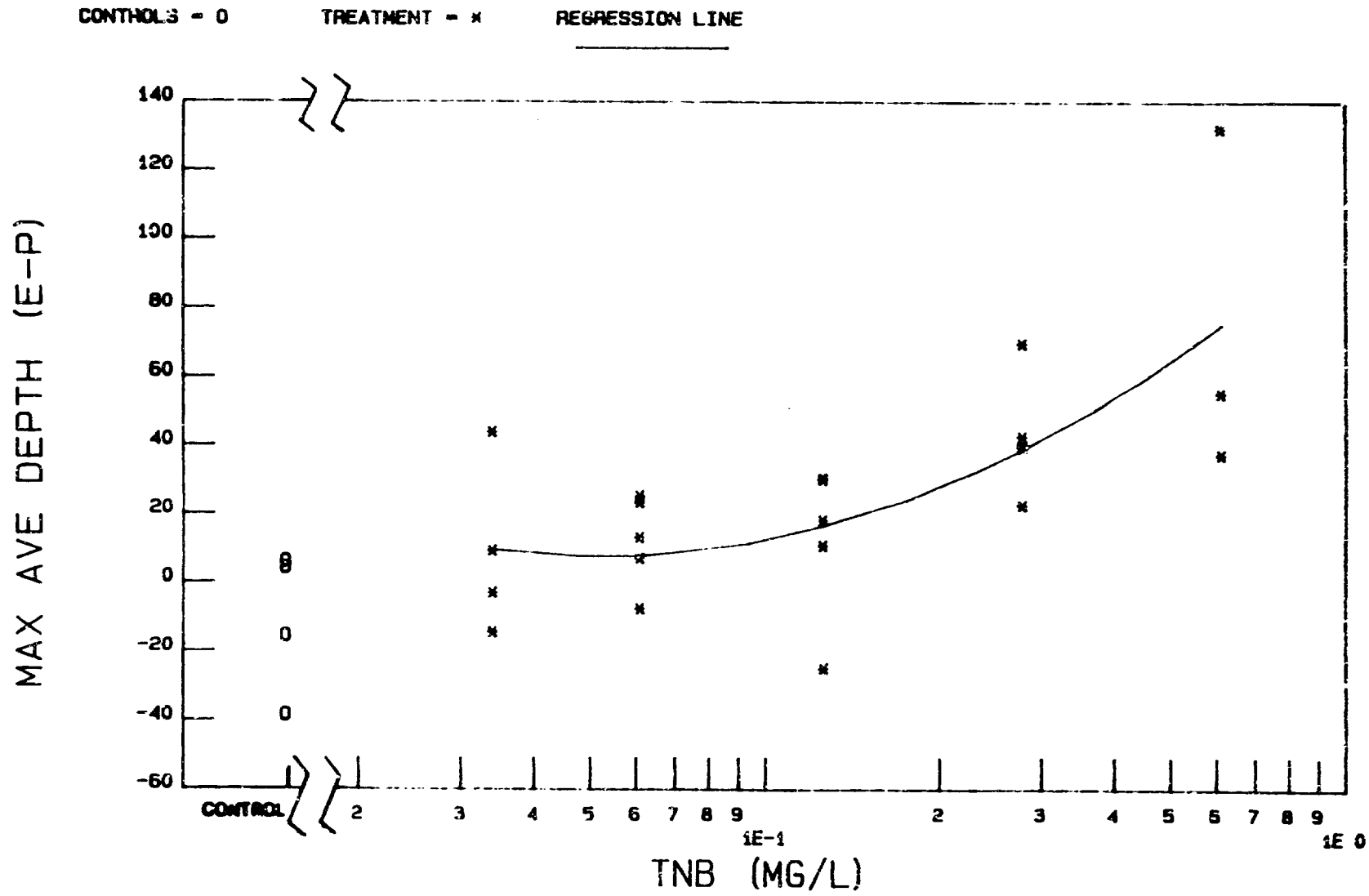
a. Mean value (exposure maxima - pre-exposure maxima) for all fish at each treatment level.
b. N = 5 for all treatments except 0.613 mg/L (N = 3) and 0.034 mg/L (N = 4).
c. Significantly different from control ($p < 0.05$).
d. BDL - Below detection limit (0.020 mg/L).

TABLE 14. DIFFERENCES BETWEEN PRE-EXPOSURE AND EXPOSURE MINIMA IN THE TNB VENTILATORY TEST

Parameter	Mean Measured TNB Concentration (mg/L)	Mean Differences Between Minima[a,b]	Standard Deviation
Average Depth	0.613	-25.94	67.22
	0.279	15.93	28.50
	0.128	4.97	13.49
	0.061	-15.46	31.11
	0.034	2.11	18.89
	BDL[c]	-12.04	8.58
Ventilatory Rate	0.613	-6.58	4.14
	0.279	-14.56	8.48
	0.128	-11.13	4.05
	0.061	-7.83	5.55
	0.034	-8.93	5.89
	BDL	-6.89	3.71
Cough Rate	0.613	-0.47	0.18
	0.279	-0.27	0.09
	0.128	-0.11	0.14
	0.061	-0.09	0.14
	0.034	-0.12	0.27
	BDL	-0.18	0.17
Percent Movement	0.613	-0.45	0.12
	0.279	-0.58	0.52
	0.128	-0.04	0.75
	0.061	-0.21	0.13
	0.034	-0.54	0.53
	BDL	-0.44	0.52

a. Mean value (exposure minima - pre-exposure minima) for all fish at each treatment level.
b. N = 5 for all treatments except 0.613 mg/L (N = 3) and 0.034 mg/L (N = 4).
c. BDL - Below detection limit (0.020 mg/L).

The most sensitive ventilatory parameter was average depth, which showed significant effects at 0.128 mg/L and above. The average depth exposure minus pre-exposure maximum for each fish was plotted against TNB treatment (Fig. 14). The controls were plotted on the same graph for reference. The increase in the average depth maxima with increasing TNB concentration is apparent, but there is also substantial variability in bluegill response within each treatment.



(E-P) - EXPOSURE MINUS PRE-EXPOSURE MAXIMA.
TNB CONCENTRATIONS FROM 0.034 TO 0.613 MG/L.
EFFECTS (P<0.05) AT 0.128 MG/L AND ABOVE.

Figure 14. TNB Ventilatory Test; Average Depth Maxima.

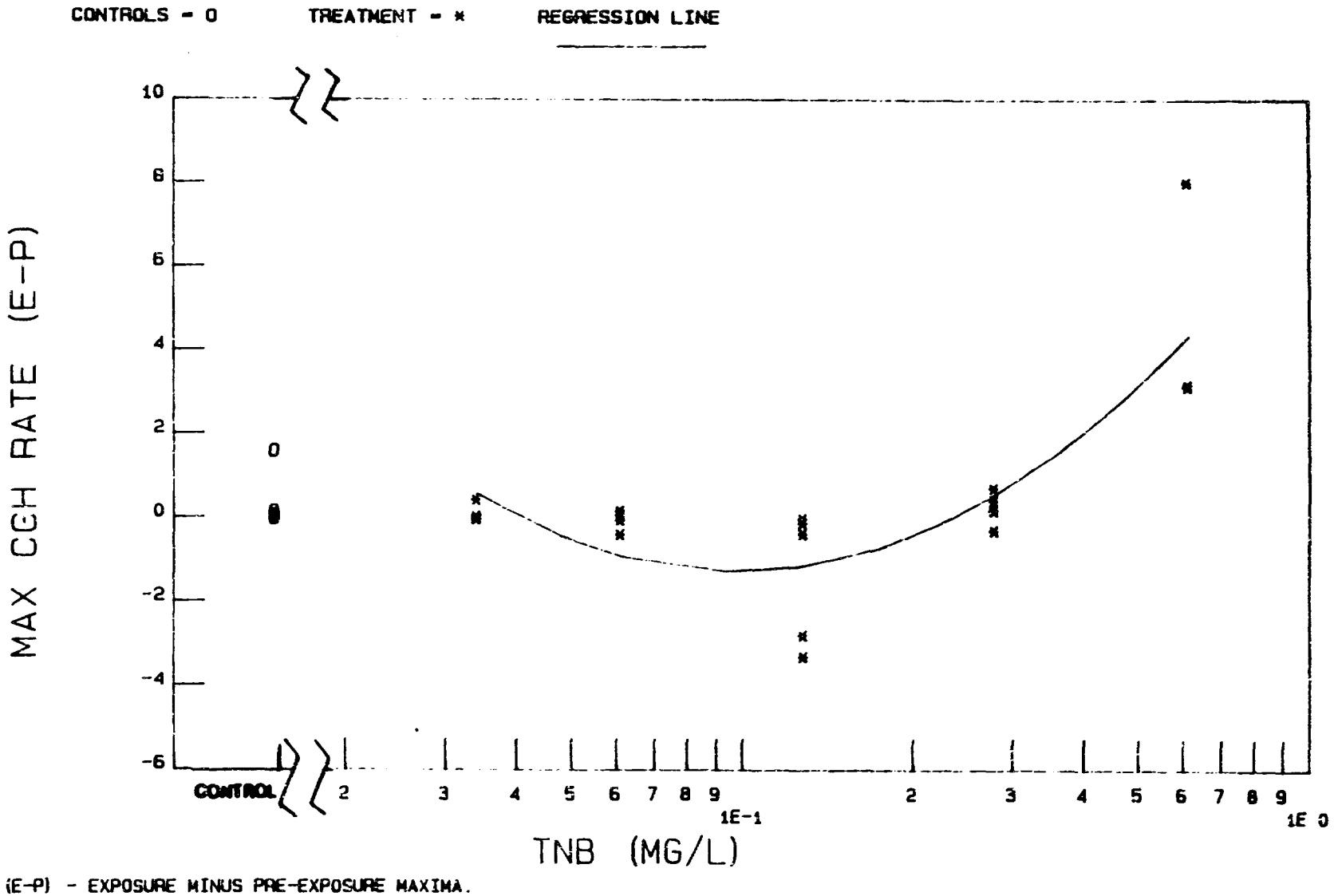
Cough rate and movement were also affected by TNB exposure (Table 13, Figs. 15 and 16), but only at the highest TNB concentration (0.613 mg/L). For ventilatory depth, cough rate, and movement (Figs. 14-16), the R-square values for the quadratic models were low. Especially for cough rate, a threshold for effects seems to occur at 0.128 mg/L, so a quadratic model may be inappropriate. Increased variability between fish in movement responses was evident at higher TNB concentrations (Fig. 16, 0.279 mg/L and 0.613 mg/L). There may have been some interaction between cough and movement counts. During the computer analysis of the ventilatory signals, a rapid variation in number and amplitude of ventilatory peaks could possibly be counted as movement. When a great number of coughs are generated together, the computer analysis may count this series of peaks as movement. Conversely, movement peaks could be counted as coughs if the 15-second ventilatory pattern did not meet the computer's criteria for movement.

TOXICITY TEST COMPARISONS

A summary of relevant acute and chronic toxicity data for chlordane and TNB is given in Table 15. The acute toxicity of chlordane to bluegills was similar for the test conditions used in the ventilatory and chronic[10] toxicity tests. However, the highest concentration tested in the ventilatory study (8.91 µg/L) did not elicit a response in any of the four parameters monitored. The reported chlordane chronic effect concentration of 1.22 µg/L[10] was a factor of 7 below the highest chlordane ventilatory concentration tested. The sensitivity of the chlordane ventilatory test might have been greater if the cough counting accuracy had been better during the test. There was also the possibility that the exposure to low concentrations of chlordane was not sufficiently long to cause a ventilatory response. Drummond and Carlson[2] reported that some toxicants such as endrin (which, like chlordane, is an organochlorine pesticide) may require up to 10 days of exposure to elicite a ventilatory response at chronic toxic concentrations. In contrast to the chlordane test results, the no effect-effect range of the TNB ventilatory test was very similar to the reported no effect-effect ranges of the TNB early life stage tests.[11] Also, the bluegills tested in the present study were only slightly more sensitive to TNB than were bluegills tested previously in this laboratory under static exposure conditions.

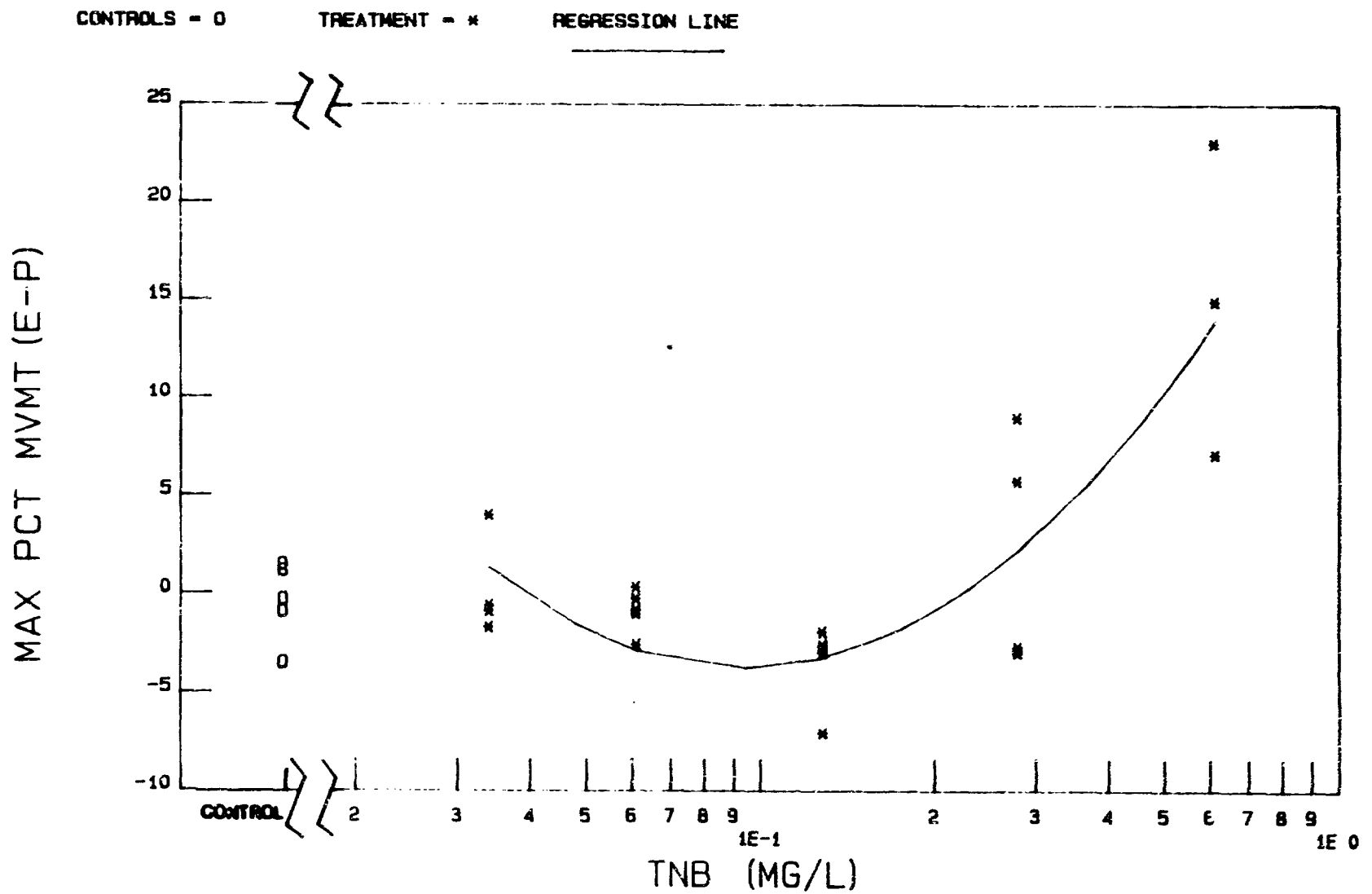
CONCLUSIONS AND RECOMMENDATIONS

The automated ventilatory monitoring system may have potential as a short term chronic toxicity screening test. The continuous monitoring aspect of the system gave a large data base from which toxicant effects could be determined, and the test duration of 13 days allows for relatively rapid evaluation of a toxicant for chronic effects. The ventilatory monitoring system recorded significant responses from bluegills exposed to TNB concentrations at concentrations known to cause chronic toxic effects. Although chlordane caused no significant ventilatory responses at concentrations up to 7 times the reported chronic effect concentration, the lack of response may have been due to poor accuracy in cough monitoring or an insufficient period of exposure.



(E-P) - EXPOSURE MINUS PRE-EXPOSURE MAXIMA.
TNB CONCENTRATIONS FROM 0.034 TO 0.613 MG/L.
EFFECT (P<0.05) AT 0.613 MG/L ONLY.

Figure 15. TNB Ventilatory Test; Cough Rate Maxima.



(E-P) - EXPOSURE MINUS PRE-EXPOSURE MAXIMA.
TNB CONCENTRATIONS FROM 0.034 TO 0.613 MG/L.
EFFECT (P<0.05) AT 0.613 MG/L ONLY.

Figure 16. TNB Ventilatory Test; Percent Movement Maxima.

TABLE 15. TOXICITY TEST COMPARISONS FOR TECHNICAL CHLORDANE AND TNB

Test Material	Test Type	Fish	Length of Exposure (days)	Test End Point	Results (95% Confidence Limits) (µg/L)	Ref.
Technical chlordane	Static acute	Bluegill	4	LC50	59 (50-71)	10
Technical chlordane	Static acute	Bluegill	4	LC50	56 (49-64)	This Report
Technical chlordane	Partial chronic	Bluegill	270	Lowest effect concentration[a]	1.22	10
Technical chlordane	Ventilatory	Bluegill	6	No effect - effect range[b]	>8.91[c]	This Report
TNB	Static acute	Bluegill	4	LC50	850 (520-1,380)	11
TNB	Flow through acute	Bluegill	4	LC50	570 (500-650)	This Report
TNB	Early life stage	Rainbow trout	71	No effect - effect range	80-170	11
TNB	Early life stage	Fathead minnow	32	No effect - effect range	80-120	11
TNB	Ventilatory	Bluegill	6	No effect - effect range	61-128	This Report

a. Lowest concentration found to cause major chronic effects.
b. Range from the highest no-effect concentration to the lowest concentration tested causing a significant difference ($P < 0.05$) from the control.
c. No significant effect ($P < 0.05$) at highest concentration tested.

The accuracy of the computer analysis of the ventilatory patterns of bluegills was excellent for ventilatory rate and average ventilatory depth. The accuracy of the movement parameter was judged to be acceptable. The accuracy of the cough rate analysis did not remain consistent over the three ventilatory tests. The TNB cough rate accuracy was good, while the chlordane and solvent test accuracies were poor. The variation in analysis between tests was possibly due to the lower ventilatory signal amplitude of the smaller bluegills used in the first two tests. Monitoring several ventilatory parameters increased the overall sensitivity of the monitoring system. No effect on ventilatory rate was found at 0.613 mg/L TNB, while significant changes in ventilatory depth occurred at a TNB concentration nearly five times lower. Cough rate and percent movement changed significantly, but only at acutely toxic TNB concentrations.

The ventilatory monitoring system was predictive of chronic toxicity for TNB, but not for chlordane. The chlordane test should be repeated using larger bluegills to improve the cough rate response, and the exposure period should be extended from 6 to 10 days. Due to the variability in bluegill responses to toxicants, a greater number of fish at each treatment level would be helpful in establishing a toxicant effect. Bluegills used in ventilatory tests should be uniform in size and greater than 50 millimeters long. More complete isolation of the test chambers from routine laboratory activities would reduce the variability of the ventilatory signals caused by external disturbances. Testing other compounds should continue to establish the screening capabilities of the ventilatory monitoring system.

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APPENDIX A

STANDARD OPERATING PROCEDURE FOR DETERMINATION OF CHLORDANE IN WATER BY GAS CHROMATOGRAPHY/ELECTRON CAPTURE DETECTOR*

1. APPLICATION AND SCOPE

Developed for determining concentration of chlordane in well water.

a. Concentration Range: 0.5 to 100 µg/L in water.

b. Detection Limit: Lower detection limit in water is 0.5 µg/L.

2. SUMMARY OF METHOD

Ten milliliters of a water sample are extracted with 1 mL hexane spiked with 6 ppb phorate as an internal standard. The hexane layer is then analyzed by a gas chromatograph equipped with a electron capture detector.

3. HAZARDS

Chlordane is a severe poison and under suspicion as a cancer-causing agent. An analyst should avoid all direct contact and any inhalation of its toxic vapors.

4. INTERFERENCES

Chlorinated compounds with similar gas chromatographic retention times could interfere. However, in present applications, no interferences were found.

5. APPARATUS AND MATERIALS

a. Instrumentation

(1) Hewlett-Packard Model 5830A Gas Chromatograph equipped with a electron capture detector.

(2) Glass chromatographic column, 6 feet in length, 1/4 inch, O.D. and 2 mm I.D. packed with 3 percent OV-1 on 80/100 mesh Chromsorb W AW.

b. Operating Parameters

(1) Flowrate: 30 mL/minute Argon/Methane carrier gas
(2) Oven Temperature: 200 C isothermal for 10 minutes
(3) Injection Port Temperature: 250 C
(4) Electron Capture Detector Temperature: 300 C
(5) Chart Speed: 0.5 cm/minute

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* Source: Analytical Chemistry Section, US Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, Frederick, MD.

c. Glassware and Hardware

(1) Test tubes with screw caps and teflon liners size 125 mm x 16 mm
(2) Pipets (1 mL, 2 mL, 5 mL, 10 mL, 15 mL, and 20 mL sizes) volumetric class A

(3) Pasteur pipets
(4) 100 mL volumetric flasks class A
(5) 10 µL Hamilton syringe Model 701

d. Chemicals

(1) Hexane, pesticide grade
(2) Chlordane, technical standard
(3) Deionized/distilled water
(4) Phorate, technical standard

e. Extraction: Ten-milliliter aliquots of samples and working stocks are placed into acid-washed and acetone-washed 15 mL glass vials. One milliliter of hexane (spiked with phorate) is added to each sample and working stock. All vials are capped tightly and shaken vigorously by hand for 2 minutes and allowed to stand for 5 minutes to allow the layer to separate. The hexane layer (top) is ready for gas chromatograph analysis.

f. Calculations: The retention time of each peak is the identifier for the analyst. Any deviation 0.1 minute from this time is considered subject to comparison with the internal standard (if the chlordane peaks are earlier or later than expected, so should be the internal standard). Chlordane is a mixture of chlorinated compounds. In performance of this analysis three of the chlordane peaks at the end of the chromatogram are compared to the internal standard peak.

When the chlordane peaks and internal standard peak are identified, the peak areas are converted to a ratio. Thus:

$$\text{(R) Ratio} = \frac{\text{peak areas of chlordane}}{\text{peak area of internal standard}}$$

These calculations are made on the chromatographic printout. The R-Value is identical to instrument response (R).

$$R = \frac{\text{area chlordane}}{\text{area internal standard}} = \text{instrument response}$$

g. Plot of Standard Curve: The ratios, R, for all working standards, are plotted against the concentration of chlordane in the working standards. The plot can be produced manually with graph paper, or made rapidly by a computer. Both methods yield a slope (m) and intercept (b), and the equation of a straight line. With the computer regression analysis, the values of chlordane concentration can be determined from areas for samples.

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