Sublethal effects and tissue uptake of 1,4-dioxane

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I. SUMMARY

This report summarizes the laboratory investigation portion of a comprehensive environmental study of the Honey Creek watershed conducted on behalf of Gelman Sciences, Inc. (GSI). This laboratory investigation, and a previous field investigation, were undertaken to define the environmental characteristics of the Honey Creek watershed and to assess whether the chronic exposure of Honey Creek to 1,4-dioxane has resulted in observable abnormalities in the biota of the stream. The field investigation is summarized in a report entitled "An Evaluation of the Ecological Impact of Long-Term Chronic Exposure of the Biota of Honey Creek to 1,4-Dioxane" (Wiley and Diana 1989). The laboratory investigation reported here generally assesses sublethal effects and tissue uptake for fish and aquatic invertebrate species exposed to 1,4-dioxane.

Work in regard to this laboratory investigation was conducted under a research agreement, dated December 7, 1988, with the University of Michigan; a copy of which has been included in the Appendices. Analytical laboratory services were provided by Ann Arbor Technical Services (herinafter ATS).

II. INTRODUCTION

Toxicological studies of the effects of dioxane on terrestrial vertebrates are numerous (see Hartung 1987). Air breathing animals readily absorb 1,4 dioxane in proportion to its concentration in the atmosphere and through the skin following dermal application (Fairley et al. 1934, Young et al. 1978, Marzulli et al. 1981). Subsequent metabolic pathways, and tissue distributions have been quantified for several mammalian species (Woo et al. 1977, Braun and Young 1977). Yet for aquatic animals, there has not been even a simple demonstration that dioxane occurring in an aqueous medium is absorbed and present in an exposed organism's tissues. Likewise, while the toxic effects of dioxane in terrestrial vertebrates has been well studied, toxicity to aquatic organisms is poorly understood.

Several studies of the acute toxicity of 1,4 dioxane to aquatic animals suggest 24 and 48 hr LC50's (or EC50's) for freshwater organisms range from 250 to greater than 1000 ppm (Meier 1986). On the basis of acute bioassays with Ceriodaphnia and Pimephales notatus (Meier 1986), Michigan Department of Natural Resources (MDNR) has identified 0.36 ppm as the Aquatic Chronic Value (the maximum acceptable concentration protecting aquatic biota from chronic toxicity) for 1,4 dioxane (Masterson-MDNR/TCES 1986). However, there is neither any published data available on effects of chronic exposures, nor on sublethal effects of acute exposures. Both topics are of some interest in the context of the dioxane contamination of Honey Creek, since historically exposures have been both long-term (chronic) and at levels far below those associated with lethality in acute exposure experiments (Wiley and Diana 1989).

This report summarizes the results of several laboratory studies designed to address the following specific issues:

1. Is dioxane absorbed by aquatic invertebrate and vertebrate animals living in dioxane contaminated water?

2. If dioxane is absorbed, how are internal concentrations related to ambient concentrations?

3. If dioxane is absorbed, does uptake vary between different species?

4. Are there lethal effects associated with chronic (30-day) exposures to concentrations substantially below the LC50?

5. Are there sublethal metabolic effects associated with chronic (30-day) exposures to concentrations substantially below the LC50?

6. What ambient concentrations produce short-term sublethal metabolic responses in aquatic organisms, and therefore may be considered "detectable" from the organisms' perspective?

In the present studies, fish and invertebrates were exposed to sublethal concentrations of 1,4 dioxane at various levels over a 30+ day period. After the chronic exposure, routine metabolic rate of each species was tested to assess sublethal responses. The concentration of dioxane in the body of laboratory exposed invertebrates was determined to evaluate tissue uptake and potential for bioconcentration. Body burdens of fish living in contaminated regions of Honey Creek were also determined. Finally, the instantaneous metabolic response to dioxane of heptageniid mayflies was used to determine metabolic costs associated with exposure to dioxane, and to estimate the thresholds for detection of dioxane by these organisms. In order to make this study directly applicable to Honey Creek, native organisms were used rather than typical laboratory bioassay species.

III. METHODS

A. Laboratory Studies

A flow-through dosing system at the University of Michigan's School of Natural Resources wet lab facility was used to expose six species (three mayflies [Insecta: Ephemeroptera] and three fishes) to various dioxane concentrations for 30+ days. Dioxane was introduced to calibrated flows of diluting water (typically 6 ml's per minute) via syringe pumps. Achieved average concentrations ranged from 0.66 to 210 ppm (Table 1). Animals were held in 10 gallon aquaria fitted with standpipes to maintain a constant volume of 20 to 25 liters. All tanks were aerated. Because of the large amount of dioxane contaminated effluent produced by the flow-through system (approximately 8 gallons per tank per day), it was necessary to maximize the number of taxa exposed per tank. In each holding tank a suspended inner plexiglass chamber separated the insects from the fish, allowing all six species to be exposed in each tank. Individual species of fish or invertebrates were not separated from each other, and all taxa received identical dosing treatments.

All species were held without food on a photoperiod sequence of 14 hours of dark followed by 10 hours of light. Temperature was maintained at 10 Celsius (°C) throughout the period of dosing and metabolic rate measurement to retard development of the insects and reduce the chances of premature emergence of adults.

Fish used in this study were the common shiner (Notropis cornutus), the white sucker (Catostomus commersoni), and the bluegill (Lepomis macrochirus). Insect taxa included Stenonema, Leptophlebia, and Stenacron. Mortality estimates during the first

thirty days of dioxane exposure were made from direct counts of individual organisms.

Tests for sublethal stress associated with chronic dioxane exposure were made by examining routine metabolic rate of randomly selected individuals from each of the dosing tanks. Respiration rate measurements took an additional two weeks to complete, so the total exposure time prior to chronic metabolism evaluation varied somewhat among individuals (35 to 50 days). During this period, flow-through dosing continued. Animals were tested in uncontaminated water for effects of chronic dioxane exposure.

Instantaneous exposures of animals to 1,4-dioxane, and simultaneous measurement of metabolic rate, were used to determine the stress related to exposure, and the detection limits of the organisms. Insects were exposed to concentrations of 0, 30, 60, 125, 250, 640, 1025 or 2050 ppm dioxane. Insects were placed in the micro-respirometer and then following 30 minutes of acclimation to air-saturated water, they were dosed with dioxane while their respiration rate was continuously recorded. Sequences in which dioxane concentrations were presented were varied to avoid bias. Metabolism is expressed, for these experiments, as a proportion of the rate prior to exposure.

1,4-dioxane concentrations in the tissues of chronically exposed invertebrates were determined (see below). Total period of exposure prior to tissue analyses ranged from 35 to 41 days for the mayfly samples.

Metabolic rate measurements of individual insects were made using a Yellow Springs Instrument Company Model 5300 biological oxygen monitor equipped with a clark-style polarographic microprobe and 600 microliter respirometery chamber developed by Instech Laboratories Inc. Measurements were made in batch mode (stirred, sealed chamber) with uptake rates determined from the last 6 minutes of a (typically) 15-minute recording of oxygen uptake. Probe consumption was estimated to be less than 1 percent of insect metabolism and therefore was ignored (Instech Laboratories Inc.). Metabolic rate estimates (mg oxygen per hour) were standardized to a body weight of 1.5 mg prior to statistical analyses of dioxane effects. Standardization was based on a regression analysis of the relationship between weight and respiration using data from all tanks and all species (respiration = 0.545 dryweight $^{0.694}$).

Routine respiration rates of fishes were measured using a flow-through respirometry system (20 to 40 ml/min) employing adjustable length chambers (Wike 1987). Oxygen concentrations were monitored using a YSI model 58 digital oxygen meter (precision ± 0.01 ppm). Fish were allowed to acclimate to the respirometery chambers for a minimum of 1 hour prior to measurement. Several replicate determinations were then made over the next 1 to 2 hour period. After termination of the metabolism experiments, flow rate was determined by measuring discharge into a graduated cylinder for one minute. Metabolic rates (mg of oxygen consumed per g body weight per hour) were corrected for weight-related differences in metabolism by substituting weight$^{0.8}$ into the above equation (Brett and Groves 1979).

B. Field Studies

Specimens of fish were collected by electroshocking from Honey Creek in April 1989 for analyses of dioxane tissue content. Fish were taken from stations HT1-3, HT1-4 and HT1-5; all stations found to have dioxane concentrations greater than 0.02 ppm at least once during extended monitoring in Fall 1988 (Wiley and Diana 1989). Whole fish were killed, ground and homogenized. A subsample (< 1 g) was then analyzed for 1,4 dioxane.

C. Dioxane Analysis

All concentrations of 1,4 dioxane were measured by ATS. This included water from the dosing system, body concentrations of insects exposed in the system, and body concentrations of the fish collected from Honey Creek. Analytical laboratory services provided by ATS used protocols and procedures developed specifically for analysis of 1,4-dioxane associated with water and soil samples and biological tissues. These protocols have been developed by ATS in concert with GSI and have been included for reference in the Appendices.

D. Statistical Analysis

The statistical methods used for analyses in this report varied. They included testing of uptake effects by regression, testing of metabolic rates by Analysis of Variance, and other general statistical tests. Results were considered significant at an alpha of 0.05.

IV. RESULTS

A. Laboratory Exposures to Dioxane

The laboratory flow-through system was originally configured to provide six different levels of dioxane exposure and two control treatments. Subsequent difficulties with the dilution system resulted in an over-dosing of the lowest level treatments, and effectively reduced the exposure range to four distinct concentrations (Table 1). Data and results from tanks 2, 3 and 6 were combined for the purposes of this report, and represent an average exposure concentration of approximately 3 ppm. Despite this difficulty, the exposure regimes achieved represented a range in dioxane concentrations of almost 2.5 orders of magnitude, all below the lowest LC50 for an aquatic organism (Meier 1986).

A further complication arose with respect to the control tanks. Based upon chemical analyses at the very end of the laboratory study, the control tanks became contaminated with dioxane. This almost certainly occurred during the final week or two of the study, after the initial 30 day exposure and mortality estimate, and during the period when fish were routinely being moved in and out of tanks to the respirometer. Since there is therefore some ambiguity with respect to the exposure experienced by the animals held in the control tanks, these results have been excluded from the principal analyses reported below.

Table 1: Chronic exposure concentrations (ppm). Nominal concentrations were the volumes used in all statistical analyses.

Tank #	Calculated Concentration	Concentration Checks 3/13/89	3/17/89	Average Observed Concentration	Nominal Concentration
1	218.00	180.00	240.00	210.00	210.00
2	2.30	1.60	1.40	1.50	1.00
3	0.05	1.30	0.87	1.08	1.00
4	0.00	--	--	--	0.00
5	96.00	81.00	86.00	83.00	83.00
6	0.80	5.50	3.70	4.60	1.00
7	0.03	0.62	0.70	0.66	0.50
8	0.00	--	--	--	0.00

Note: All concentrations in parts per million (ppm) unless otherwise specified.

B. Mortality Due to Chronic Exposures

Substantial mortality occurred during the chronic exposure period in both species of heptageniid mayflies and for the bluegill. However, no correlation between mortality in these or the other species and dioxane concentration was found (Table 2). Thus it is concluded that the mortality observed was not related to chronic dioxane exposure.

Bluegill mortality was high in most of the tanks. Excessive handling mortality in field collected bluegills has been a frequent problem at the U of M laboratory when the fish are taken at low winter temperatures (Diana, personal communication). At low temperatures these warmwater fish appear to be unable to recover from slight abrasions and scale loss experienced during collection and rapidly develop fungal infections. Most of the mortality observed occurred within the first week of the experiment.

Mortality was also high among the insects, particularly the heptageniids. In this case much of the mortality appeared to be the result of unexpected predation by Leptophlebia on the smaller heptageniid nymphs. Densities were extremely high in the holding tanks because of the relatively large amount of tissue required to analyze for dioxane.

Despite cold water temperatures and short day-lengths, some emergence (and considerable failed-emergence) occurred among the Leptophlebia and accounted for most of the apparent mortality in this group. All of the mayflies in the first control tank (Tank #5) were lost near the end of the thirty day exposure when a white sucker breached the inner container and consumed its entire contents.

TABLE 2- MORTALITY DATA FOR 30 DAY CHRONIC EXPOSURES.

cs=common shiner, ws=white sucker, bg=bluegill
lp=Leptophlebia sp. sa=Stenonema sp. Stenonema

	Tank	cs	ws	bg	lp	sa	se	Mineral Conc.	x
Initial Count	1	10	10	10	60	60	70	2100	-
	2	10	5	10	60	40	40	10	-
	3	10	10	9	60	40	40	10	-
	4	10	5	10	60	40	40	00	-
	5	10	10	10	60	60	70	830	-
	6	10	5	10	60	40	40	10	-
	7	10	10	10	60	40	40	05	-
	8	12	8	11	80	40	25	00	-
30 Day Count	1	11	10	3	57	[illegible]	[illegible]	[illegible]	
	2	6	5	8	23	26	6	10	-
	3	10	10	2	48	20	21	10	-
	4	9	5	1	-	-	-	00	-
	5	10	10	2	57	41	28	830	-
	6	9	5	9	47	34	44	10	-
	7	9	10	1	54	24	32	05	-
	8	11	8	7	40	25	6	00	-
% Mortality	1	20	0	0	5	32	57	[illegible]	22
	2	40	0	20	62	35	85	10	40
	3	0	0	70	20	50	47	10	32
	4	10	0	80	-	-	-	00	30
	5	0	0	00	22	32	60	[illegible]	34
	6	10	0	10	22	15	0	10	9
	7	10	0	90	10	40	20	05	22
	8	11	-	36	50	16	76	00	37
Mean Mortality		14	0	30	26	30	46		

C. Tissue Uptake

1,4 dioxane was recovered from the tissues of both the laboratory exposed mayflies, and the fish collected from contaminated reaches of Honey Creek (Table 3). Tissue concentrations, reported as mg dioxane per kg fresh tissue (ppm), were highly correlated with ambient dioxane concentrations to which animals were exposed ($r=0.94$, $a < 0.0001$; Figure 1). These values undoubtedly reflect equilibrium conditions with respect to dioxane uptake given the length of exposure in both the laboratory and field. Combining fish and insect data, this relationship was best described by the following regression equation :

$$Ct = 26.7 + 1.85\ Cw - 26.2FISH \qquad (1)$$

($r^2 = 0.97$, $F = 452$, $a < 0.0001$; slopes for both Cw and FISH > 0 at $a < 0.0001$)

where Ct is the tissue concentration of dioxane (ppm wet weight); Cw is the water concentration (ppm) and FISH is a dummy variable with the value of 1 if the species is a fish and zero if it is an insect. This regression model indicates that tissue concentration is proportional to ambient water concentration, and that fish had a significantly lower equilibrium tissue concentration than did the mayflies.

Table 3. Tissue uptake data from field and laboratory studies. Tissue concentrations are given in mg dioxane per kg fresh weight. hg=heptageniidae; lp=Leptophlebia; bg=bluegill; ws=white sucker; cs=common shiner; cc=creek chub; sc=mottled sculpin; ps=pumpkinseed; gs=green sunfish. All invertebrate tissue concentrations are from composite samples; fish values are from a mixture of individual and composite samples.

tissue conc ppm	water conc ppm	exposure type	species
360.00	210.0	lab	hg
490.00	210.0	lab	lp
160.00	83.0	lab	hg
150.00	83.0	lab	lp
41.00	1.0	lab	hp
47.00	1.0	lab	lp
40.00	1.0	lab	hg
39.00	1.0	lab	lp
19.00	0.5	lab	hg
14.00	0.5	lab	lp
0.03	4.000E-3	Honey crk.	cs
0.01	4.000E-3	Honey crk.	cs
0.02	4.000E-3	Honey crk.	cs
0.05	4.000E-3	Honey crk.	cs
0.04	4.000E-3	Honey crk.	cs
0.05	0.013	Honey crk.	gs
0.04	0.013	Honey crk.	cs
0.09	0.013	Honey crk.	bg
0.08	0.013	Honey crk.	gs
0.04	0.02	Honey crk.	ps
0.02	0.02	Honey crk.	sc
0.02	0.02	Honey crk.	cc
0.02	0.02	Honey crk.	cs
0.02	0.02	Honey crk.	bg
0.04	0.02	Honey crk.	bg
0.03	0.02	Honey crk.	bg
0.04	4.000E-3	Honey crk.	ws
0.03	4.000E-3	Honey crk.	sc
0.02	4.000E-3	Honey crk.	sc
0.02	4.000E-3	Honey crk.	bg
0.02	4.000E-3	Honey crk.	bg

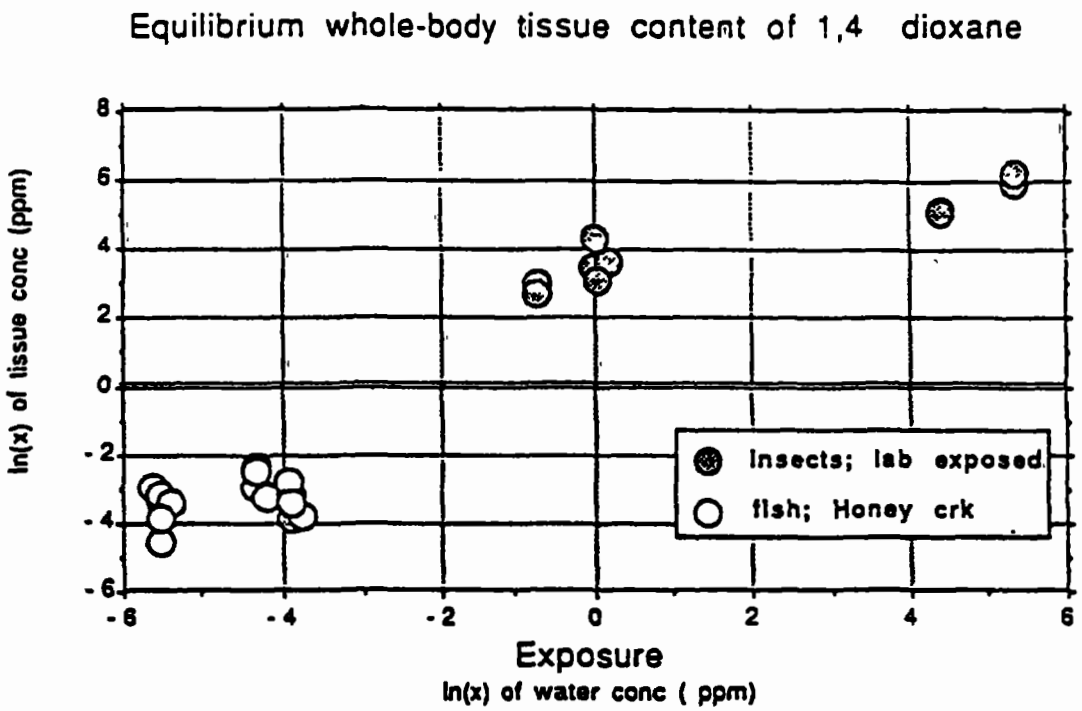


FIGURE 1-Equilibrium Whole-body Tissue Content of 1,4-dioxane

Assuming dioxane dynamics are adequately explained by a simple single compartment model, with first order depuration kinetics (Young et al. 1977), the bioconcentration factor (BCF) under steady state conditions is given by (Spacie and Hamelink 1985):

$$BCF = ku/kd; \quad \text{where } Ct = ku/kd * Cw \qquad (2)$$

and ku and kd are the rate coefficients for uptake and depuration, respectively. Again combining the fish and insect data, and forcing a least squares fit through the origin, BCF is estimated from these data as follows:

$$Ct = 2.001 * Cw \qquad (3)$$

Since the slope of this regression was 2.001, (significantly higher than 0.75, the fraction of water in an animal's body), it is concluded that the dioxane uptake coefficient is roughly twice the first order depuration coefficient.

D. Sublethal Effects of Chronic Exposure

Chronic exposure to dioxane had statistically significant effects on the metabolism of both insects and fish. These metabolic rate responses are most reasonably compared to the body burden of dioxane, since this represents the most precise estimate of chronic exposure. As tissue concentration of dioxane increased, the metabolic rate of mayflies tended to decrease (Figure 2; for corresponding tissue concentrations see Table 3). When the data from all three species were combined, the differences between treatments were statistically significant (ANOVA, $p<0.05$). The three species of insects individually showed generally similar responses, although effects were statistically significant only for Stenonema nymphs.

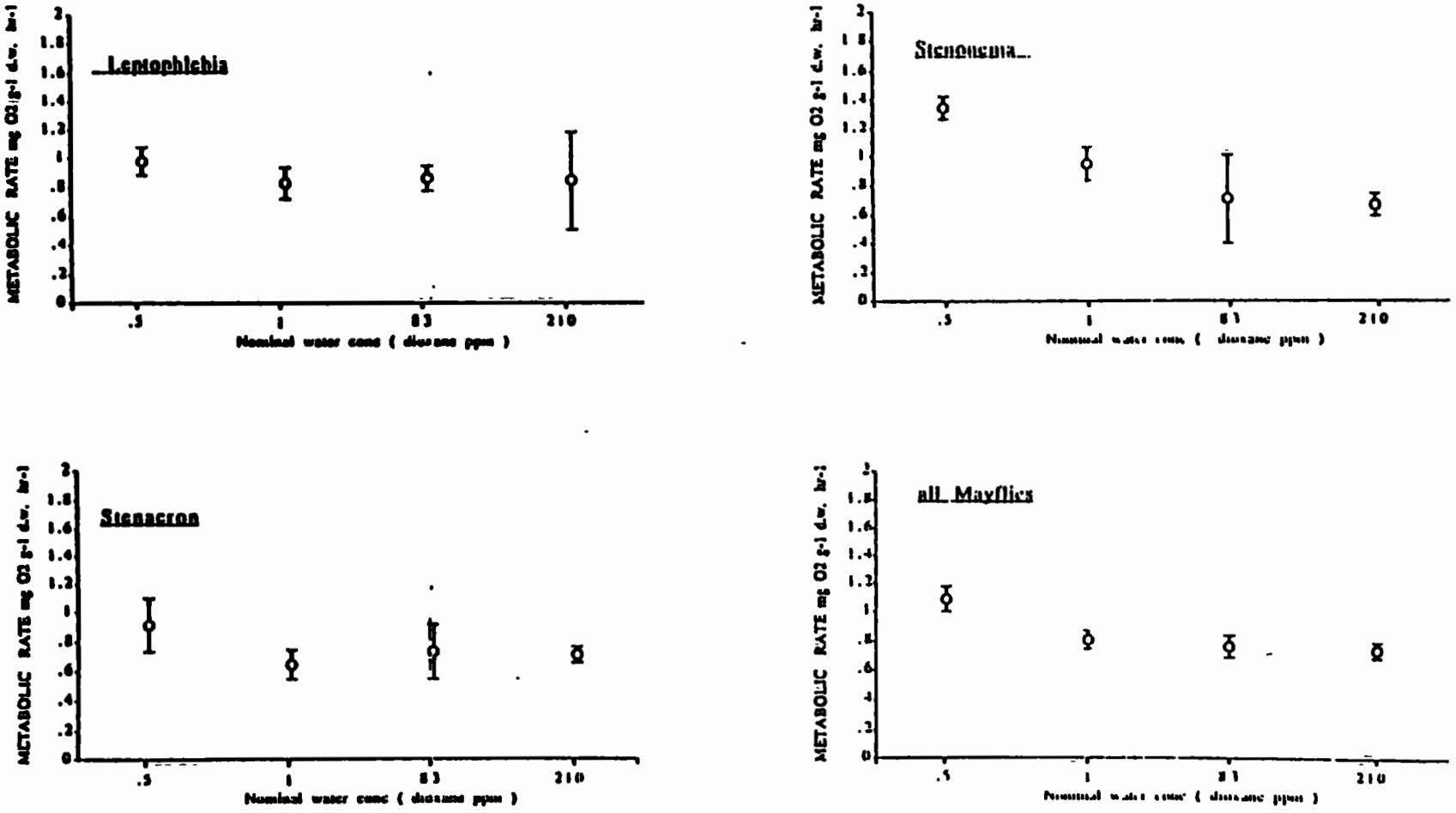


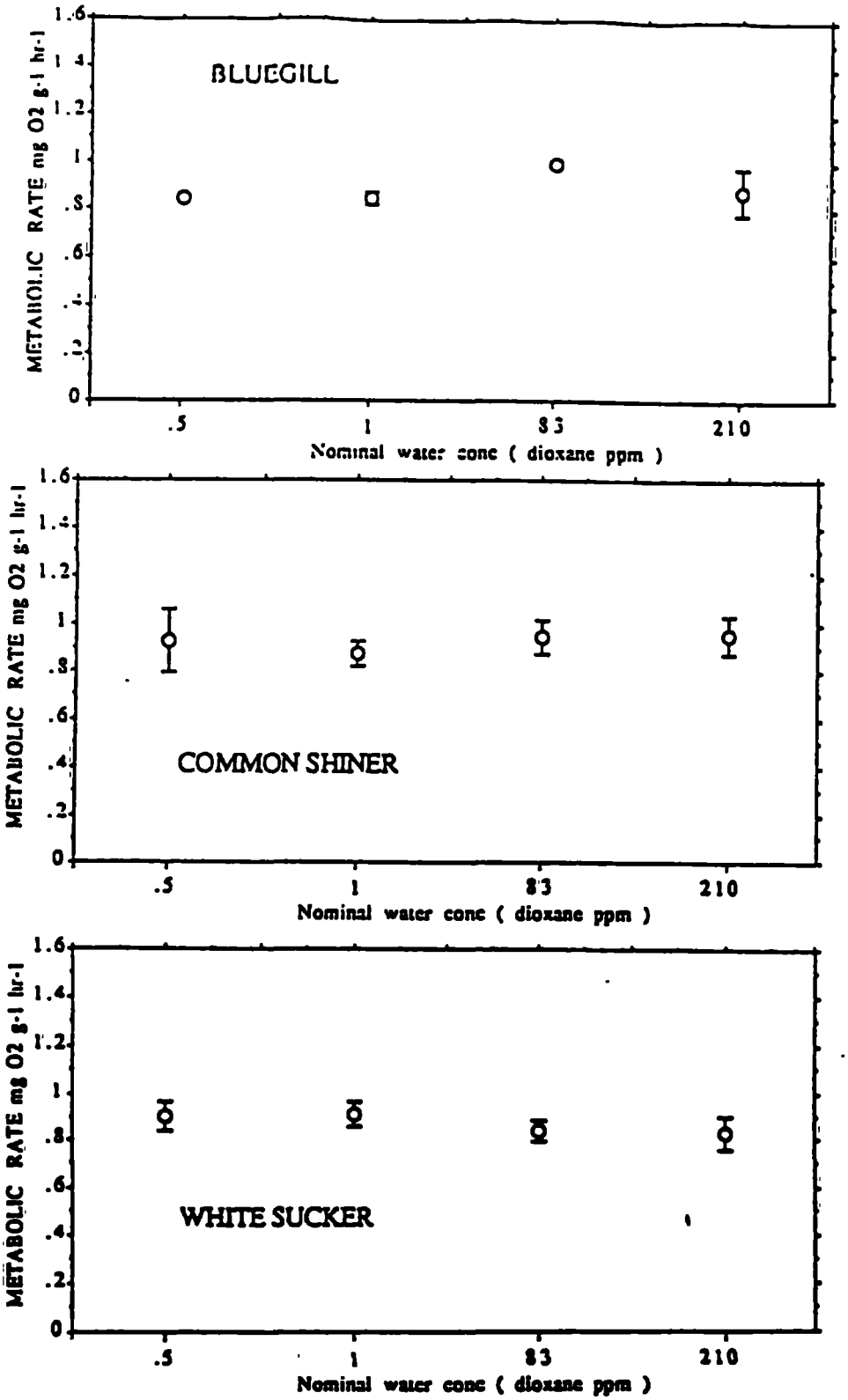
FIGURE 2-Nominal 1,4-Dioxane Concentration in Water versus Metabolic Rate for Mayfly Species.

Note: These were the water concentrations the mayflys were exposed to prior to metabolic rate measurements, as metabolic rate was determined in clean water.

The extent of metabolic suppression varied between species; in the most significant cases respiration rates declined as much as 50 percent. Metabolic rates measured were all well within the ranges reported in other studies on normal, uncontaminated mayflies (Fax et. al. 1937; Wingfield 1939; Erikson 1964).

The three species of fish did not show similar responses to dioxane exposure. It was not possible to compare metabolic rate to body burden, as body burden was not determined for fishes. However, metabolic rate generally increased with chronic exposure to dioxane. Internal physiological differences among species did not allow an overall comparison of fish. The data could be examined in two ways; either among exposure treatments, or between treated and control groups.

In analyses excluding control groups, bluegills and white suckers had significant changes in metabolic rate with different exposure treatments. However, suckers decreased in metabolism with increasing exposure, while bluegills increased (Figure 3). Common shiners had a non-significant effect of exposure. There were no control data for bluegill, as mortalities prevented analysis of control fish for metabolism. The other two species had significant treatment effects when compared to controls, but again the direction of change differed (Table 4). Shiners had a significant increase in metabolism with exposure above control, while suckers had a significant decrease.



Note: These were water concentrations fish were exposed to prior to metabolic rate measurements; metabolic rates were determined in clean water.

FIGURE 3- Nominal 1,4 Dioxane Concentration in Water versus Metabolic Rates for Fish Species.

TABLE 4-SUBLETHAL EFFECTS OF CHRONIC DIOXANE EXPOSURE
ANOVA's of metabolic rate response to exposure treatments. S=statistically significant at $\alpha<0.05$; NS=not statistically significant.

SPECIES		TREATMENT 1	2	3	4	5	ANOVA (CONTROLS EXCLUDED)
WHITE SUCKER	$\bar{x}$	0.841	0.900	0.910	0.840	0.830	S
	sd	0.092	0.055	0.051	0.047	0.064	
	n	4.000	5.000	14.000	8.000	5.000	
	Water Conc. (ppm)	0.000	0.500	1.000	83.000	210.000	
COMMON SHINER	$\bar{x}$	0.720	0.930	0.870	0.940	0.950	NS
	sd	0.085	0.127	0.051	0.072	0.077	
	n	5.000	8.000	15.000	8.000	4.000	
	Water Conc. (ppm)	0.000	0.500	1.000	83.000	210.000	
BLUE GILL	$\bar{x}$	-	0.850	0.850	0.990	0.880	S
	sd	-	-	0.240	0.010	0.092	
	n	-	1.000	13.000	2.000	2.000	
	Water Conc. (ppm)	-	0.500	1.000	83.000	210.000	
LEPTOPHLEBIA	$\bar{x}$	-	0.990	0.830	0.860	0.850	NS
	sd	-	0.207	0.350	0.184	0.344	
	n	-	4.000	10.000	3.000	9.000	
	Tissue Conc. (ppm)	-	14.000	43.000	150.000	490.000	
STENACRON	$\bar{x}$	-	0.920	0.650	0.730	0.710	NS
	sd	-	0.412	0.306	-0.423	0.155	
	n	-	5.000	10.000	5.000	9.000	
	Tissue Conc. (ppm)	-	19.000	41.000	160.000	360.000	
STENONEMA	$\bar{x}$	-	1.350	0.950	0.710	0.670	S
	sd	-	0.163	0.364	0.309	0.234	
	n	-	5.000	9.000	5.000	10.000	
	Tissue Conc. (ppm)	-	19.000	41.000	160.000	360.000	
COMBINED MAYFLIES	$\bar{x}$	-	1.09	0.810	0.780	0.740	S
	sd	-	0.332	0.350	0.305	0.265	
	n	-	14.000	29.000	15.000	28.000	
	Tissue Conc. (ppm)	-	16.500	42.000	155.000	425.000	

E. Instantaneous Effects

Instantaneous exposure to dioxane in the water resulted in a significant reduction in the metabolic rate of heptageniid mayflies (Figure 4). When metabolism was compared to the initial level (control level for that animal), there was a significant decrease in metabolism with increasing exposure. Increased concentrations of dioxane resulted in larger decreases in metabolism, with maximum suppression in the 40 to 50 percent range at concentrations over 100 ppm. The metabolic responses observed seemed to be somewhat dependent upon length of exposure, and upon the total accumulated dose during the experiment.

Interspersed control measurements involved re-introductions of uncontaminated, saturated water after a series of dioxane exposures. When the dioxane exposures had been of a relatively short duration (15 to 30 mins) the interspersed control treatment often resulted in a markedly elevated respiration rate, often exceeding the initial uncontaminated rates.

If sequential exposures were of a longer duration, the interspersed controls usually resulted in only a minor recovery of the respiration rate. Thus the sequentially measured responses to dioxane concentrations measured in these experiments are not entirely independent. For this reason parametric statistical tests have not been applied to these data, but instead the results are presented as a series of Tuckey box plots (Figure 4) which clearly illustrate metabolic suppression in response to instantaneous dioxane exposure.

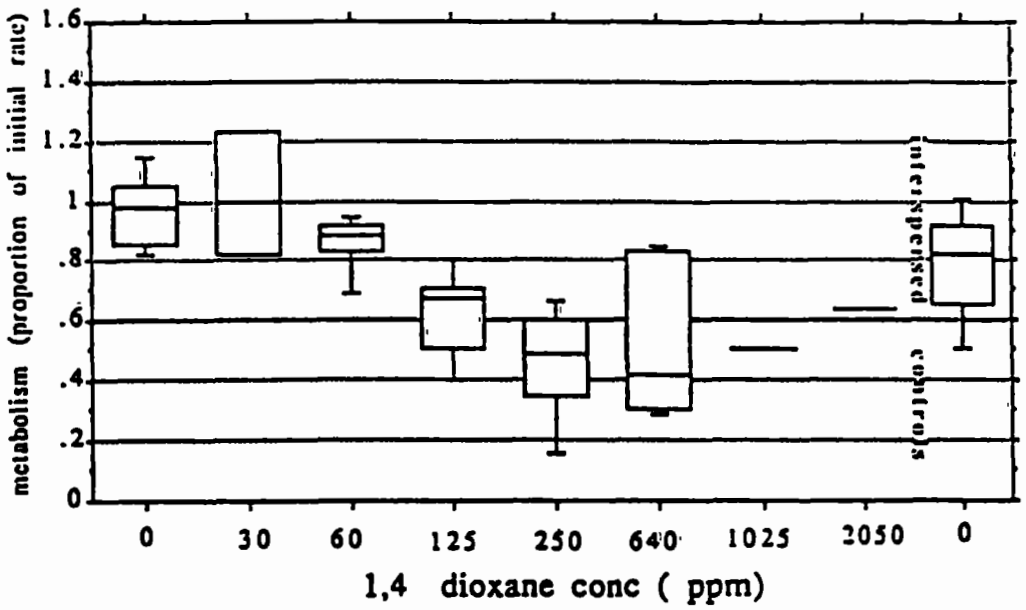


FIGURE 4-Metabolic Rate of Heptageniid Mayflies versus 1,4-Dioxane Concentrations in Water under instantaneous Exposures.

V. DISCUSSION

Exposures of aquatic animals to sublethal concentrations of a toxicant may result in variable outcomes. At very low concentrations, a suppression of metabolism often occurs (Webb 1978). As concentrations increase, metabolic rate may elevate substantially as the metabolic costs of homeostatic regulation increase. In some instances, a toxicant may interfere with oxygen uptake (or carbon dioxide elimination) and actually reduce metabolic rate at high concentrations. It is believed that the former increase in oxygen consumption should occur in response to high concentrations of 1,4-dioxane, as it has not been shown to interfere with metabolite transfer.

Metabolism is a fairly sensitive indicator of physiological condition. In fishes, the maximum metabolic rate can be as much as 25 times higher than standard metabolism at the same temperature. In aquatic insects, the range in routine metabolism is somewhat lower, but it still can vary by a factor of 4 to 5. Many other processes also affect metabolism directly. For example, an increase in temperature of 10 degrees Celsius can increase standard metabolism of fish 2 to 4 times, depending on the species (Brett and Groves 1979). Therefore it is expected that relatively large changes in metabolism should occur if an animal is highly stressed by a toxicant. Small changes in metabolism (less than 40 percent) indicate relatively little stress.

The present study was conducted to evaluate the possible effects of sublethal exposure of aquatic organisms to 1,4-dioxane. As these organisms were exposed to 1,4-dioxane for 30+ days to testing, the effects measured are obviously of less severity than in acute lethal tests.

Previous toxicological studies have reported both increases and decreases in metabolism related to exposure to sublethal concentrations of various toxic substances (Mount 1962, Dowden 1966, Mayer 1970). It is not surprising then, that a conditioned optimum (lowered metabolic rate) was observed in response to low levels of dissolved dioxane. Such conditioned optima have been found in many sublethal studies with a variety of aquatic animals (Brett and Groves 1979). Often the pattern of metabolic suppression is reversed as toxicant levels begin to approach lethal levels, and metabolism increases dramatically as the costs of homeostatic regulation increase.

Such patterns, however, are not universal and vary according to the specific toxic action of a substance (Spacie and Hamelink 1985). In some studies of organophosphates, for example, significant effects on whole fish metabolism were not found until concentrations reached acutely lethal levels (Waiwood and Johansen 1974, Spacie and Hamelink 1985). In the present study, dramatic increases in respiration rate were not seen, suggesting concentrations did not approach dangerous levels, and the exposed animals were not greatly stressed.

The biological significance of the responses observed is more difficult to evaluate. The percent change in routine metabolism observed in the chronically exposed fish were relatively small, typically no more than 10 to

15 percent. It is concluded that the level of variation in routine metabolism observed is minor, and would seem unlikely, for example, to result in detectable changes in the growth or survival of these fishes. In fact, regular seasonal changes in temperature or daily changes in feeding and activity undoubtedly produce much larger metabolic responses (Brett and Groves 1979).

The metabolic responses of the mayflies were more marked, up to 50 percent reductions in routine metabolism observed in both the instantaneous and chronic exposure experiments. It is interesting in this context to note that 1,4-dioxane has been reported to have narcotizing effects in terrestrial vertebrates at concentrations approaching the LC50 (NIOSH 1977). If metabolic suppression were sufficient to reduce growth rates, life cycle timing may conceivably be altered in these univoltine species. Based upon the results reported here, long-term exposures exceeding 100 ppm may result in ecologically significant reductions in the metabolic rate of these univoltine mayflies.

Ambient 1,4-dioxane concentrations in Honey Creek are 2 to 3 orders of magnitude lower than those used in the present laboratory studies. There is no evidence, therefore, from the current study that 1,4-dioxane has had any significant impact on the fauna of Honey Creek. This is consistent with earlier field studies, which showed no statistically significant effect of 1,4-dioxane on faunal diversity in the watershed (Wiley and Diana 1989).

VI. SUMMARY AND CONCLUSIONS

Chronic exposures of fish and aquatic insects to 1,4-dioxane in the laboratory provided an opportunity to examine the effects of sub-acute concentrations on mortality and routine metabolism. Further, controlled exposure rates and tissue analyses led to a first estimate of an aquatic bioconcentration factor for dioxane. On the basis of these studies, the following conclusions are offered:

1. Aquatic animals do absorb dioxane from the water, and this results in measurable body burdens of dioxane in fish and insects.

2. Aquatic organisms appear to absorb dioxane into their tissues at a rate roughly proportional to its environmental concentration, and bioconcentrate it somewhat above ambient water levels.

3. There was no significant difference in dioxane uptake rate between different species of mayfly, or different species of fish. There was, however, a small but statistically significant difference between the uptake rate of insects and fish.

4. No mortality of aquatic insects or of fish could be associated with chronic exposures to 1,4 dioxane within the range of concentrations examined (up to 210 ppm).

5. Statistically significant sublethal depressions of routine metabolism following chronic (30+ days) exposure were found in at least one species of fish (white sucker) and one species of mayfly (Stenonema); another fish (bluegill) showed elevated metabolic rates at higher exposures although the data for this species are weak. All of these changes in metabolism, except for Stenonema, were low in magnitude and probably biologically unimportant. Threshold concentrations for these responses appeared to be in the vicinity of 1 ppm for Stenonema and 80 ppm to greater than 200 ppm for other mayfly and fish species.

6. Metabolic rate suppression of heptageniid mayflies was observed in response to instantaneous exposures to 1,4-dioxane. Thresholds for statistically significant reductions were in the 60 to 125 ppm range. The animals in essence can "detect" water levels down to 60 ppm.

7. The results of these laboratory investigations provide no evidence that past or present exposure to 1,4-dioxane has resulted in a negative impact to the biota of Honey Creek. This corroborates earlier field studies which also found no demonstrable effect of 1,4-dioxane on the biological communities of Honey Creek.

VII. LITERATURE CITED

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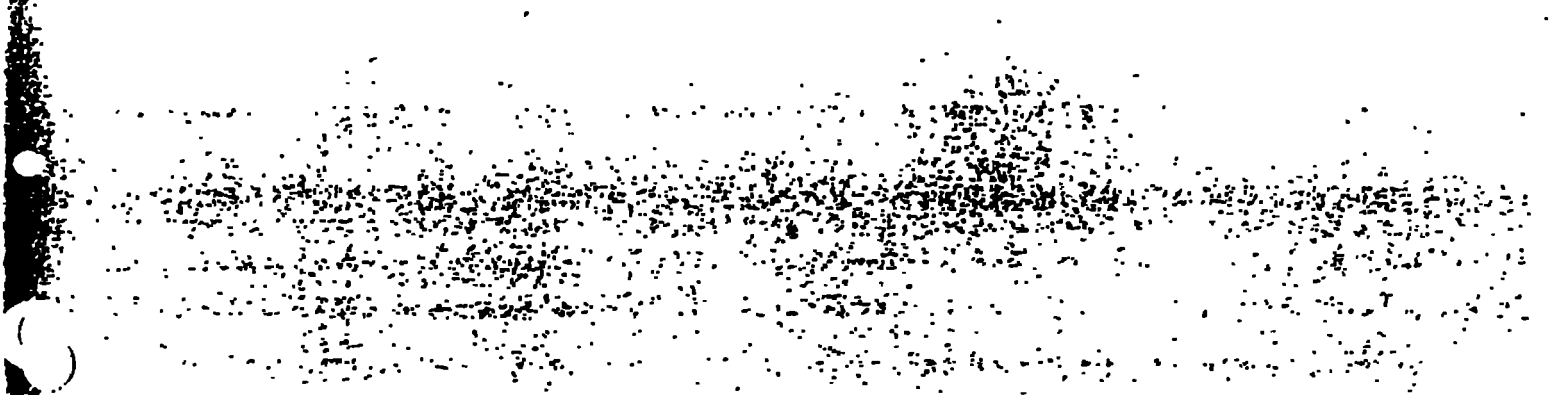
APPENDIX A

UNIVERSITY OF MICHIGAN AGREEMENT



THE UNIVERSITY OF MICHIGAN
ANN ARBOR, MICHIGAN 48109-1092

OFFICE OF CONTRACT ADMINISTRATION
(313) 763-2193

341 WEST ENGINEERING BUILDING

December 22, 1988

James Braithwaite, President
Braithwaite Consultants, Inc.
3928 Varsity Drive
Ann Arbor, MI 48108

Reference: Research Agreement Dated December 7, 1988

Dear Mr. Braithwaite:

Enclosed herewith for your file is a fully executed copy of the above referenced agreement.

Sincerely,

Paul J. Stemple, Manager

PJS:rmk

Enclosure

ROUNDTABLE RESEARCH AGREEMENT

THIS AGREEMENT effective this 7th day of December, 1988, by and between BRAITHWAITE CONSULTANTS, INC. (hereinafter "Sponsor") and the REGENTS OF THE UNIVERSITY OF MICHIGAN, a non-profit educational institution (or its agent) of the State of Michigan (hereinafter "University").

WHEREAS, the research program contemplated by this Agreement is of mutual interest and benefit to University and to Sponsor, will further the instructional and research objectives of University in a manner consistent with its status as a non-profit, tax-exempt, educational institution, and may derive benefits for both Sponsor and University through inventions, improvements, or discoveries;

NOW, THEREFORE, in consideration of the promises and mutual covenants herein contained, the parties hereto agree to the following:

ARTICLE 1 - DEFINITIONS

As used herein, the following terms shall have the following meanings:

1.1 "Project" shall mean the description of the project as described in DRDA 89-1296 under the direction of Michael Wiley as Project Director entitled *Sublethal Effects and Tissue Uptake of 1,4 Dioxane.*

1.2 "Contract Period" is November 1, 1988, through December 31, 1989, unless earlier terminated pursuant to this Agreement.

1.3 "University Intellectual Property" shall mean individually and collectively all inventions, improvements or discoveries which are conceived or made (i) by one or more employees of University; or (ii) jointly by one or more employees of University and by one or more employees of Sponsor in performance of the Project during Contract Period.

ARTICLE 2 - RESEARCH WORK

2.1 University shall use reasonable efforts to perform such Project substantially in accordance with the terms and conditions of this Agreement.

2.2 In the event that the Project Director becomes unable or unwilling to continue Project, and a mutually acceptable substitute is not available, University or Sponsor shall have the option to terminate said Project.

ARTICLE 3 - REPORTS AND CONFERENCES

3.1 Written program reports shall be provided by University to Sponsor periodically and a final report shall be submitted by University at the conclusion of the Contract Period.

3.2 During the term of this Agreement, representatives of University may meet with representatives of Sponsor at times and places mutually agreed upon to discuss the progress and results as well as ongoing plans, or changes therein, of Project to be performed hereunder.

ARTICLE 4 - COSTS, BILLINGS, AND OTHER SUPPORT

4.1 It is agreed that total costs to Sponsor hereunder shall not exceed the sum of Sixteen Thousand Six Hundred Dollars ($16,600). Payment shall be made by Sponsor within thirty days of receipt of monthly invoices for actual charges incurred.

4.2 University shall retain title to any equipment purchased with funds provided by Sponsor under this Agreement.

4.3 In the event of early termination of this Agreement by Sponsor pursuant to this Agreement, Sponsor shall pay all costs accrued by University as of the date of termination, including non-cancellable obligations, which shall include all non-cancellable contracts and fellowships or postdoctoral associate appointments called for in Project, incurred prior to the effective date of termination. After termination, any obligation of Sponsor for fellowships or postdoctoral associates shall end no later than the end of University's academic year during which termination occurs.

ARTICLE 5 - PUBLICITY

Sponsor will not use the name of University, nor of any member of University's Project staff, in any publicity, advertising or news release without the prior written approval of an authorized representative of University. University will not use the name of Sponsor, nor any employee of Sponsor, in any publicity without the prior written approval of Sponsor.

ARTICLE 6 - PUBLICATIONS

Sponsor recognizes that under University policy, the results of University Project must be publishable and agrees that researchers engaged in Project shall be permitted to present at symposia, national, or regional professional meetings, and to publish in journals, theses or dissertations, or otherwise of their own choosing, methods and results of Project, provided, however, that Sponsor shall have been furnished copies of any proposed publication or presentation at least one month in advance of the submission of such proposed publication or presentation to a journal, editor, or other third party. Sponsor shall have one month after receipt of said copies, to object to such proposed presentation or proposed publication because there is patentable subject matter which needs protection. In the event that Sponsor makes such objection, said researcher(s) shall refrain from making such publication or presentation for a maximum of six months from date of receipt of such objection in order for University to file patent application(s) with the United States Patent and Trademark Office or foreign patent office(s) directed to the patentable subject matter contained in the proposed publication or presentation.

ARTICLE 7 - INTELLECTUAL PROPERTY

7.1 All rights and title to University Intellectual Property under Project shall belong to University and shall be subject to the terms and conditions of this Agreement.

7.2 Rights to inventions, improvements and discoveries, whether or not patentable or copyrightable, relating to Project made solely by employees of Sponsor shall belong to Sponsor. Such inventions, improvements, and discoveries shall not be subject to the terms and conditions of this Agreement.

7.3 University will promptly notify Sponsor of any University Intellectual Property. If Sponsor directs that a patent application or application for other intellectual property protection be filed, University shall promptly prepare, file, and prosecute such U. S. and foreign application in University's name. Sponsor shall bear all costs incurred in connection with such preparation, filing, prosecution, and maintenance of U. S. and foreign application(s). Sponsor shall cooperate with University to assure that such application(s) will cover, to the best of Sponsor's knowledge, all items of commercial interest and importance. While University shall be responsible for making decisions regarding scope and content of application(s) to be filed and prosecution thereof, Sponsor shall be given an opportunity to review and provide input thereto. University shall inform Sponsor of all developments with respect to such application(s) and shall promptly supply to Sponsor copies of all papers received and filed in connection with the prosecution thereof in sufficient time for Sponsor to comment thereon.

7.4 If Sponsor elects not to exercise its option as described below or decides to discontinue the financial support of the prosecution or maintenance of the protection, University shall be free to file or continue prosecution or maintain any such application(s), and to maintain any protection issuing thereon in the U. S. and in any foreign country at University's sole expense.

ARTICLE 8 - GRANT OF RIGHTS

University grants Sponsor the first option, at Sponsor's sole selection, for either a limited non-exclusive, royalty-free license, or for consideration, an exclusive license with a right to sublicense on terms and conditions to be mutually agreed upon to University Intellectual Property. The option shall extend for a period of sixty days from the termination date of the Agreement.

ARTICLE 9 - TERM AND TERMINATION

9.1 This Agreement shall become effective upon the date first written above and shall continue in effect for the full duration of the Contract Period. The parties hereto may, however, extend the term of this Agreement for additional periods as desired under mutually agreeable terms and conditions which the

parties reduce to writing and sign. Either party may terminate this agreement upon ninety days prior written notice to the other.

9.2 In the event that either party commits any breach of or default in any of the terms or conditions of this Agreement, and fails to remedy such default or breach within ninety days after receipt of written notice thereof from the other party, the party giving notice may, at its option and in addition to any other remedies which it may have at law or in equity, terminate this Agreement by sending notice of termination in writing to the other party. Such termination shall be effective as of the date of the receipt of such notice.

9.3 No termination of this Agreement, however effectuated, shall release the parties from their rights and obligations accrued prior to the effective date of termination.

ARTICLE 10 - INDEPENDENT CONTRACTOR

10.1 University shall be deemed to be and shall be an independent contractor and as such University shall not be entitled to any benefits applicable to employees of Sponsor;

10.2 Neither party is authorized or empowered to act as agent for the other for any purpose and shall not on behalf of the other enter into any contract, warranty or representation as to any matter. Neither shall be bound by the acts or conduct of the other.

ARTICLE 11 - INSURANCE AND INDEMNIFICATION

11.1 University warrants and represents that University has adequate liability insurance, such protection being applicable to officers, employees, and agents while acting within the scope of their employment by University. University has no liability insurance policy as such that can extend protection to any other person.

11.2 Each party hereby assumes any and all risks of personal injury and property damage attributable to the negligent acts or omissions of that party and the officers, employees, and agents thereof.

11.3 Sponsor understands that the University is an educational institution created under Article 8, Section 5 of the Michigan Constitution and operated pursuant to authority conferred by the State of Michigan. As a state institution the University is prohibited from lending the credit of the state pursuant to Article 9 of the Michigan Constitution. Sponsor acknowledges that this Agreement does not confer upon Sponsor any right of claim of indemnification by the University, either express or implied.

ARTICLE 12 - GOVERNING LAW

This Agreement shall be governed and construed in accordance with the laws of the State of Michigan.

ARTICLE 13 - ASSIGNMENT

13.1 This Agreement shall not be assigned by either party without the prior written consent of the parties hereto.

13.2 This Agreement is assignable to any division of Sponsor, any majority stockholder of Sponsor, or any subsidiary of Sponsor in which fifty-one percent of the outstanding stock is owned by Sponsor.

ARTICLE 14 - AGREEMENT MODIFICATION

Any agreement to change the terms of this Agreement in any way shall be valid only if the change is made in writing and approved by mutual agreement of authorized representatives of the parties hereto.

ARTICLE 15 - NOTICES

Notices hereunder shall be deemed made if given by registered or certified envelope, postage prepaid, and addressed to the party to receive such

Preliminary toxological studies (Meier 1987; Hartung 1987) suggest that acute lethal levels of Dioxane are quite high (>100 ppm) for the few species of fish and aquatic invertebrates examined to date. We propose that sublethal impacts of Dioxane be explored in the laboratory, utilizing the species from local streams most likely to be intolerant to organic pollutants. Since Dioxane is not highly toxic, if population and community-level perturbations do occur, the mechanisms most likely to be involved would be energetic and ecological in nature, and should appear as sublethal effects in laboratory exposure. Fry's paradigm (Fry 1947) provides a conceptual framework useful in this regard: sublethal toxological stresses should show up in increased metabolic costs and decreased metabolic scope. This increase in metabolism can be measured in the laboratory and used as an indication of relative stress imposed by exposure to varying concentrations of a potential contaminant (in this case, Dioxane).

Specifically, we propose that several fish and ephemeropteran species which appeared to be sensitive to Dioxane be exposed in laboratory under controlled conditions to varying concentrations of Dioxane for varied periods of time. Species to be examined include 3 fishes: the white sucker (Catostomus commersoni), common shiner (Notropis cornutus), and the stoneroller (Campostoma anomalum); and species from the following four genera of mayflies: Stenonema, Stenacron, Leptophlebia and Isonychia. Metabolic and behavioral responses will be measured and compared to that of unexposed organisms. For initial studies, we suggest concentrations of 0.1, 1 and 10 ppm. Such experiments

would address the following points:

1. Is Dioxane detectable by the fish or invertebrates at concentrations in the range of 0.1-10 ppm?

2. Is there any evidence of sublethal stress (metabolic rate elevation) at these concentrations?

3. If there is a measureable metabolic response, does acclimation occur with prolonged exposure?

Each of these questions is pertinent to understanding past and potential future impacts of the Dioxane discharge. If no evidence for sublethal effects are found in the 0.1 - 10 ppm range, concentrations can be increased until the threshold for a response is identified. Conversely, if sublethal stress is identified, continued testing at reduced concentrations can again identify threshold levels.

Protocols

Laboratory studies of sublethal effects on mayfly nymphs (Ephemeropterans) will use a behavioral regulation assay designed to identify incipient lethal levels of oxygen described in detail by Wiley and Kohler (1980). Increases in basal metabolic rates will result in shifts in incipient lethal levels and in proportion exposed ratios. Tests will be run at three concentrations and with instantaneous, short-term (1 hr) and long-term (2 week) exposures to Dioxane.

Studies with fish will utilize flow-through respirometry with similar exposure and concentration regimes. All studies will be performed at 20 C, and with sufficient replication to adjust metabolic rates for size-specific differences.

Dioxane concentrations of test solutions will be analyzed by Ann Arbor Technical Services, 6540 Jackson Road, under a contract

between them and the sponsor. Timely evaluation of water samples is important for the toxicology work. AATS will provide a protocol for the preparation of stock solutions of Dioxane. The Dioxane concentration of these solutions is a critical variable, and analyses will be made by AATS and provided to UM within a week of sample submission. Water from each aquarium used for experiments will also be evaluated for Dioxane, and analyses will be provided by AATS within four weeks.

In addition to sublethal effects studies, several species of mayfly will also be exposed in the laboratory to varying concentrations of Dioxane for 1 to 3 months. Water concentrations of Dioxane from these exposures will be analyzed at AATS as described above. In addition, invertebrate samples will be provided to AATS for body burden analysis. These samples will be frozen or delivered fresh for analysis by AATS prior to submission.

AATS will also provide complete QA/QC data of Dioxane analyses for our use in publication. The lab is certified by the state and by EPA for chemical analyses. Dioxane analysis is a specialized methodology, and is considered a trade secret by AATS. We will not be able to publish details of this analysis, only analytical results and accuracy. We do not consider this a limit to publication of the toxicology data.

The proposed study is a basic toxicology work, to be done mainly by an M.S. candidate in Natural Resources. It will serve as her thesis work and be published as a thesis. We also anticipate that the results will be published soon after

Water from all exposures will be collected and stored. This contaminated water will be picked up by the sponsor for disposal.

References

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Meier, P.G. 1986. Acute toxicity of 1,4 Dioxane to Ceriodaphnia dubia and Pimephales promelas. Institute of Environmental and Industrial Health, School of Public Health, University of Michigan, Ann Arbor.

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Proposed Budget

Direct costs (including personnel and supplies)	$10,000
Indirect costs (66%)	$ 6,600
Total costs	$16,600

Budget Notes

This budget is proposed based on approximate calculations for each category. The sponsor is not interested in the breakdown of costs into budget category, but only into direct costs ($10,000) and indirect costs ($6,600). Other modifications in the budget categories may be made without sponsor approval.

APPENDIX B

ANN ARBOR TECHNICAL SERVICES
CHEMICAL ANALYSIS PROTOCOLS

ATS TEST METHOD SUMMARY
Revision 4
Date 1 Jan 89

Gas Chromatography/Mass Spectrometry for 1,4-Dioxane in Water

1.0 SCOPE AND APPLICATION

1.1 This method is used to determine 1,4-dioxane at residue levels in drinking water, groundwater, natural surface waters, and industrial wastewaters.

1.2 Under optimum conditions, the method detection limit is 1 ug/l, based on a 10 milliliter sample size.

2.0 SUMMARY OF METHOD

2.1 The sample is mixed with 4.0 grams of Na_2SO_4 in a demountable purge chamber, outfitted with a needle-type sparger. Nitrogen is bubbled through the sample and a portion of the analyte is partitioned from the aqueous phase into the gas phase. The gas phase is swept through a sorbent column capable of trapping the analyte. After purging is completed, this trap is heated rapidly and backflushed with helium to desorb the analyte into a gas chromatograph (GC). The analyte is separated from possible interfering compounds by the GC and detected by a mass spectrometer (MS).

2.2 Identification of the analyte (qualitative analysis) is performed by comparing GC retention time and the background corrected characteristic spectral masses with those of authentic standards.

2.3 Quantitative analysis is performed by GC/MS using selected ion current (SIM) profile areas. Calibration is done using both the external standard method and an isotopically labeled internal standard.

2.4 Quality is assured through reproducible calibration and testing of the purge and trap and GC/MS systems.

ATS TEST METHOD SUMMARY
Revision 0
Date 1 Jan 89

Gas Chromatography/Mass Spectrometry for 1,4-Dioxane in Biological Tissues

1.0 SCOPE AND APPLICATION

1.1 This method is used to determine 1,4-dioxane at residue levels in biological tissue samples.

1.2 Under optimum conditions, the method detection limit is 10 ug/kg, based on a 2 gram sample size.

2.0 SUMMARY OF METHOD

2.1 A ground tissue sample is mechanically homogenized with 10 ml of laboratory pure water in a high speed tissue blender. The homogenized sample is transferred along with 4.0 g Na_2SO_4 into a specially designed purge chamber, where it is agitated to form a free-flowing slurry. Nitrogen is bubbled through the sample slurry at 40°C, and a portion of the analyte is partitioned from the slurry into the gas phase. The gas phase is swept through a sorbent column capable of trapping the analyte. After purging is completed, this trap is heated rapidly and backflushed with helium to desorb the analyte into a gas chromatograph (GC). The analyte is separated from possible interfering compounds by the GC and detected by a mass spectrometer (MS).

2.2 Identification of the analyte (qualitative analysis) is performed by comparing GC retention time and the background corrected characteristic spectral masses with those of authentic standards.

2.3 Quantitative analysis is performed by GC/MS using selected ion current (SIM) profile areas. Calibration is done using both the external standard method and an isotopically-labeled internal standard.

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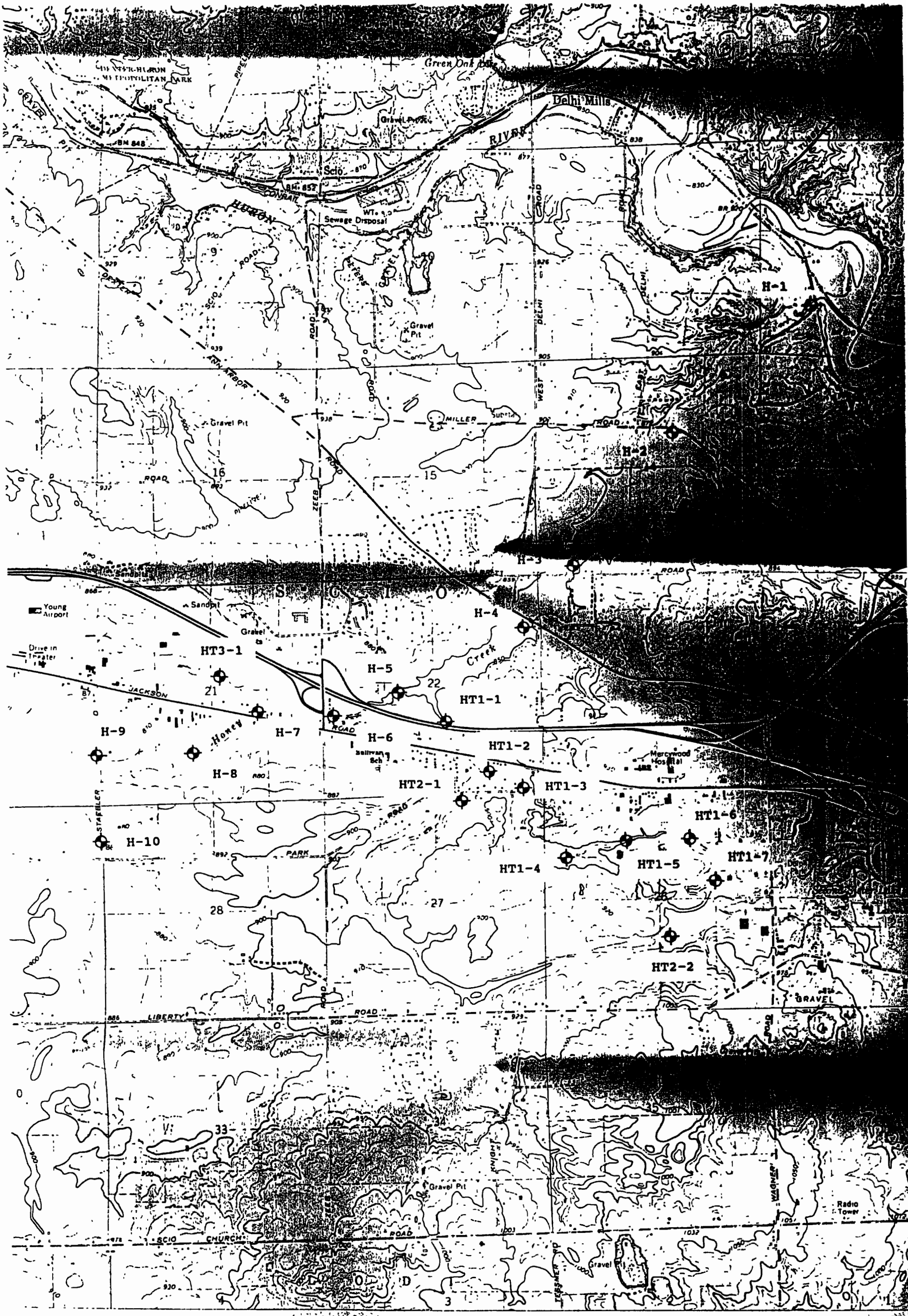
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