Mutagenic effects of selected toxicants found in Wisconsin's ground water. [DNR-038] 1990

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Madison, Wisconsin: Wisconsin Department of Natural Resources, 1990

Wisconsin Groundwater Management Practice Monitoring Project No. 35

GROUNDWATER
Wisconsin's buried treasure

Wisconsin Department of Natural Resources

January 10, 1990

MEMORANDUM

TO: Wisconsin Department of Natural Resources
Groundwater Management Section

FROM: Lorraine F. Meisner, Ph.D.
Cytogenetics Section
State Laboratory of Hygiene
University of Wisconsin

RE: **Final Progress Report of FY 1988-89 Study:**

MUTAGENIC EFFECTS OF SELECTED TOXICANTS FOUND IN WISCONSIN'S GROUND WATER

Contents

Introduction

The purpose of this project was to continue to test the mutagenic effects of herbicides and pesticides by adding different concentrations of these compounds to human lymphocyte cultures, and scoring for chromosome damage in treated compared to control cultures. It was also an important objective to test the results of different combinations of pesticides and herbicides to check for possible interactive effects in production of chromosome damage. The final goal of this research was to be able to relate these in vitro results to what could be expected from comparable in vivo exposures; only by performing parallel animal experiments and comparing the results to the in vitro studies will it be possible to develop a model which will enable extrapolation of data from short term in vitro studies to enable human risk assessment. Therefore, mouse experiments were initiated in the Spring of 1989 in which mice were chronically exposed via their drinking water to atrazine with and without linuron. The final results are presented here.

Methods

In our previous reports, we described the methods used for the human lymphocyte cultures, including the methods of exposing the cells to the herbicides. In the course of our earlier experiments, we observed that there might be a possible synergistic effect between combination exposures involving atrazine and linuron. Therefore, in order to evaluate the significance of this finding relative to actual in vivo exposures, a 30 and 90 day mouse feeding experiment was initiated using linuron and atrazine.

The mouse feeding experiment was set up with eight mice in each of the following treatment groups:

1. Negative control group: These mice received no herbicides in their drinking water. Instead their water contained only 2 ml/L acetone, as this concentration of acetone was used to solubilize the herbicides fed to the mice in the herbicide-treatment groups.

2. Positive control group: These mice received 20 ppm cyclophosphamide in their drinking water (cyclophosphamide is water soluble and did not require a solvent).

3. Atrazine group: These mice received 20 ppm atrazine in their drinking water. The purpose was to see if we could duplicate the results of our previous mouse feeding experiments in which the same concentration of atrazine was used. Another purpose was to provide baselines for comparison to the atrazine/linuron combination used in the present experiment.

4. Linuron group: These mice received in their drinking water 2 ppm linuron dissolved in acetone.

5. Combination group: These mice received 10 ppm atrazine plus 1 ppm linuron, both added simultaneously to their drinking water.

6. Bear Creek group: The mice in this group received their water from a refrigerated supply which originated from a well from Bear Creek (this well is now sealed off and not available for human consumption because of the high level of contaminants found in the water). The purpose of this group was to test the effects of chronic ingestion of contaminated water previously consumed by humans.

7. Cottage Grove group: These mice received contaminated water from a DNR sampling well in Cottage Grove, which is being tested for the same reason as group 6.

The mice in each of these groups were given the control or test chemical in their drinking water. At the end of 30 days, four mice in each group were sacrificed in order to permit assessment of chromosome damage in their bone marrow cells and also to permit examination of any other parameters that might be affected by the treatments used. The remaining mice in each group were allowed to drink their contaminated or control water for a total period of 90 days, at which time they were sacrificed in order to permit evaluating longer term chronic exposures.

Prior to, and at various intervals during the course of the experiment, the water given to the mice groups was tested. In particular, the water from the contaminated wells was tested to make sure that bacterial breakdown of some of the contaminants of this water does not result in changing their composition from the beginning to the end of the experiment. This testing was done in the Water Bacteriology Section of the State Laboratory of Hygiene, and both samples from contaminated wells were found to be free of bacteria which might change the composition of the water. In addition, the samples containing atrazine and linuron were also tested in the Environmental Sciences Section of the State Laboratory of Hygiene; the purpose of this testing was to ensure that the water was in solution at the required concentration. These tests revealed that atrazine was present in the mouse drinking water at 20 ppm as planned, but that there was no linuron detected in the water samples which were supposed to contain 2 ppm of linuron. Instead, the GC Mass Spectrometer yielded a very large unidentifiable peak, distinct from the peaks seen when linuron standards are analyzed. This indicated that the water which had been stored for several days prior to testing no longer contained the linuron which had been dissolved in it, but rather contained a metabolite of linuron, and judging from the single peak seen, only one major metabolite was present. Throughout the course of this experiment, the water samples were continually tested to make sure that the concentrations remained stable. This included testing aliquots from the Bear Creek and Cottage Grove water supplies stored in the refrigerator for this experiment.

Previous Methodology

At the conclusion of the 30 and 90 day feeding experiments, four of the mice in each group were sacrificed to permit: (1) removal of blood through cardiac puncture to permit peripheral blood cultures, (2) extraction of bone marrow cells from femurs, and (3) removal of the mouse thymus and spleens for weighing and for obtaining lymphocytes for cultures. After weighing

the spleens and thymuses, the cells were removed and put into 72 hour lymphocyte cultures to permit comparison of the chromosome damage of the mice exposed in vivo to that which we had previously seen in the human lymphocytes exposed in vitro. The cells which were extracted from the bone marrow were immediately placed into a medium containing a mitotic inhibitor (Colcemid) and were harvested by standard procedures four hours later to permit evaluation of cells which were dividing in the body (without any artifacts introduced by even a short term culture). Livers and kidneys from all of the mice were preserved in formalin for future studies.

Results

The cytogenetic results of dividing cells from the bone marrow and spleen of mice treated for 30 and 90 days are shown in Tables 1 and 2. As can be seen, the frequency of chromatid breakage is not statistically different from control for all the treated groups of mice. This is not unexpected because chromatid breakage partly reflects acute toxicity, and all of the treatments used were at concentrations well below toxic levels.

Although chromosome-type aberrations (translocations) which reflect genuine genetic damage were increased in the cyclophosphamide group treated for 30 days, as would be expected with exposure to a known mutagen, the aberrations were not statistically elevated in the 90-day treatment group. This pattern persists with the other treatment groups which had statistically elevated results in the 30-day treatments and lack of statistically elevated results in the 90-day treatment groups (atrazine, atrazine plus linuron and Cottage Grove). This would imply that any initially observed genetic damage is now no longer observable, possibly due to increased death of cells which have become multiply damaged with repeated exposure, while multiply damaged cells (as seen in the 30-day group) had a greater chance of being scored.

Initially, at the 30-day exposure period, the mitotic indices of the treated groups were equivalent to or higher than the controls. At 90 days the mitotic indices are all lower than the controls (atrazine and linuron being statistically lower then controls). This may imply that damaged cells were not being replaced and/or that the chemicals were causing a cellular growth inhibition. Our earlier experiments suggested that by 90 days bone marrow cell replacement was decreased in controls, which thus showed a lower mitotic index than in the present experiment. Unfortunately, the small size of the present experiment can result in distortions due to the small number of animals studied, so the control mitotic index numbers could have been artificially distorted.

The results of the 90 day feeding trial concerning the weights of the bodies and organs and the corresponding organ indices (organ weight divided by body weight) are shown in Tables 3 and 4. Despite much variability, Table 4 suggests that the cyclophosphamide and Bear Creek treatment groups had the greatest weight gain, while the other groups had decreased weight gains when compared to controls. The heaviest animals were observed to have the greatest amount of fat deposition, so the increased weight gains could represent, at least in part, increased fat deposition in more compromised and therefore less active animals.

The most interesting result obtained relates to the water consumption of the mice. As can be seen from Tables 5 and 6 and Figure 2, the atrazine/linuron combination treatment group drank considerably more water. This group happens to also have statistically significant increases in kidney index and splenic index, meaning not only that their body weight gain is even less than calculated, but 1) that the increased kidney weight may reflect toxic effects due to the increased consumption of water containing the combination of atrazine and linuron, and 2) that increased spleen weight may reflect accumulation of damaged blood cells. Histological studies will have to be done on the kidneys to determine whether fibrosis is the cause of the observed increase in weight.

Conclusions

Our previous studies testing the genetic effects of pesticides on human lymphocytes exposed in vitro demonstrated increased chromatid breakage with atrazine but not linuron, with the greatest increase seen when the two were used together (each at half of their original concentrations to enable demonstration of possible synergisms). However, despite the apparent synergistic effect seen in vitro, no such synergism was evident in the in vivo experiments, possibly because it was masked by the random fluctuations associated with the small numbers of animals used, or perhaps it was due to some biological effect which we could not measure. The latter possibility is suggested by the unexpected increase in water consumption by the mice receiving the atrazine-linuron combination, since this group consumed more water than any of the other groups in both the 30 and 90 day experiments (Tables 5 and 6, Figures 1 and 2). Yet this increased water consumption was associated with the second lowest weight gain at 90 days; although the atrazine group had a slightly lower weight gain (9.20 vs. 9.28 grams weight gain in the combination group), the combination group had the highest weight of livers, kidneys, and spleens of any of the groups, indicating some significant toxic effects had occurred, such as fibrosis, which led to increased weight of these organs.

The fact that the most marked biological effects were observed in the combination group which consumed only 11 ppm pesticide, compared to the atrazine group which received 20 ppm (and especially when compared to the 20 ppm of the mutagen cyclophosphamide consumed by the positive control group), points to the possibility that chemical combinations may be of greater importance than higher levels of single chemicals. Thus, not only did the water ingestion increase in the combination linuron-atrazine group, it was also up in the cyclophosphamide group (which also had increased liver, spleen and kidney weights), as well as the Bear Creek and Cottage Grove water groups (with the Bear Creek group showing increased liver and spleen weights, and Cottage Grove showing increased kidney weights associated with decreased 90 day weight gain as compared to the controls). The fact that water consumption was increased in the positive control and in the groups receiving the combinations, including the combinations which were present in the Bear Creek and Cottage Grove water, suggests that there may be biological mechanisms leading animals to increase their water consumption when they are ingesting more contaminants, perhaps as a means of metabolic compensation for the contaminant load leading to the urge to drink more water to dilute it out. If such a mechanism is operative in human populations as well, this would mean that water consumption of persons drinking from contaminated wells would actually increase,

so that basing regulatory standards on estimated water consumption would lead to an underestimation of the actual exposure levels.

Although the last part of the experiment did not show in vivo increases in chromosomal damage, it did show other biological effects from drinking contaminated water, and it further suggests that interactions between contaminants may be more important than currently imagined.

This raises much concern towards the health of Wisconsin's residents, which may be affected 10 to 20 or more years down the road by increasing numbers of concentrated water pollutants and associated low dose accumulations of cytogenetic damage. Interestingly, herbicide exposure has been linked to an increased incidence on non-Hodgkins lymphoma, especially with farm herbicide usage. Also, an increase in leukemia has been observed in Midwestern farmers suggesting that chronic exposures in humans might be capable of leading to cumulative genotoxicity.

We are very grateful for the added limited funding provided, which helped us to uncover a potential health hazard. Unfortunately, we have just started opening a door to pertinent information concerning Wisconsin's drinking water suggesting that much more work has to be done along the lines described here.

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TABLE 1

Bone Marrow Analysis From Mice Treated for 30 and 90 Days

	# of Mice	# of Cells	% Chromatid Breaks	% Translocations	% Mitotic Index
30-Day Treatment					
Control	4	180	11.7±6.8	15.7±3.5	0.6±0.2
Cyclophosphamide	4	200	11.0±4.8	33.5±10.0*	1.0±0.2*
Atrazine	4	200	10.0±2.8	25.5±5.0*	0.7±0.3
Linuron	3	150	10.7±1.2	20.0±5.3	1.2±0.2*
Atrazine + Linuron	3	150	10.7±4.2	22.0±2.0*	0.9±0.1*
Bear Creek (Lorge)	4	200	6.5±6.4	14.0±4.0	0.9±0.6
Cottage Grove (Morril)	2	100	9.0±4.2	24.0±0.0*	0.6±0.4
90-Day Treatment					
Control	6	300	5.0±4.9	12.3±6.6	1.2±0.6
Cyclophosphamide	3	150	6.0±4.0	14.0±5.3	0.7±0.5
Atrazine	4	200	10.5±6.0	15.5±9.7	0.4±0.2**
Linuron	3	150	5.3±4.2	10.7±5.8	0.2±0.1**
Atrazine + Linuron	3	143	9.1±1.0	12.5±1.3	0.5±0.7
Bear Creek (Lorge)	4	200	7.0±4.8	16.5±6.4	1.0±0.6
Cottage Grove (Morril)	2	100	9.0±4.2	10.0±7.1	0.7±0.7

*, ** Significantly different as compared to control at $P<0.5$, $P<0.1$ respectively.

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TABLE 2

Spleen Lymphocyte Analysis From Mice Treated for 30 and 90 Days

	# of Mice	# of Cells	% Chromatid Breaks	% Translocations	% Mitotic Index
30-Day Treatment					
Control	2	50	8.0	4.0	0.5
Cyclophosphamide	4	100	6.0±2.8	1.0±1.4	1.4±0.7
Atrazine	4	100	2.0±2.8	1.0±1.4	2.2±1.3
Linuron	4	100	12.0±5.7	1.0±1.4	3.8±0.6
Atrazine + Linuron	4	100	1.0±1.4	2.0±0.0	1.4±0.3
Bear Creek (Lorge)	4	100	6.0±0.0	1.0±1.4	2.2±2.3
Cottage Grove (Morril)	4	100	10.0±8.5	3.0±1.4	0.6±0.3
90-Day Treatment					
Control	4	200	9.5±4.1	1.5±3.0	1.1±0.9
Cyclophosphamide	4	200	6.5±3.0	6.5±4.4	0.7±0.4
Atrazine	4	200	6.5±1.9	6.0±2.8	1.2±1.2
Linuron	4	200	7.5±3.0	6.5±1.9*	0.5±0.4
Atrazine + Linuron	4	200	6.0±2.8	3.0±2.6	0.5±0.7
Bear Creek (Lorge)	4	200	6.0±1.6	2.0±2.3	1.2±1.0
Cottage Grove (Morril)	4	200	8.0±1.6	4.0±2.3	0.6±0.1

* Significantly different as compared to control at $P<0.05$.

Table 3

ORGAN WEIGHTS AND ORGAN WEIGHT INDICES FOR MICE ON 90 DAY FEEDING TRIAL[a]

	Weights of livers (mg)	Liver Index	Weights of Kidneys (mg)	Kidney Index
Control	1687.3±448.8	44.36±1.53	354.1±98.3	9.30±0.92
Cyclophosphamide	1718.1±275.7	40.72±1.57+	417.1±45.4	9.95±0.71
Atrazine	1485.2±94.4	43.16±5.64	334.2±33.6	9.64±0.59
Linuron	1417.8±287.1	41.65±4.45	329.9±31.9	9.79±0.44
Atrazine + Linuron	1644.9±358.6	51.37±7.33	397.3±47.6	12.49±1.02++
Lorge	1781.6±269.9	46.00±4.02	369.1±31.1	9.59±0.82
Morril	1689.3±200.5	47.86±10.24	414.8±49.0	11.70±2.41

a Organ weight indices were calculated as mg organ wt/g body wt.
o Only based on 3 mice.
+ Significantly higher results compared to controls at $P<0.05$
++ Significantly higher results compared to controls at $P<0.01$

Table 4

ORGAN WEIGHTS AND ORGAN WEIGHT INDICES FOR MICE ON 90 DAY FEEDING TRIAL[a]

	Weights of spleens (mg)	Splenic Index	Weights of Thymuses (mg)	Thymic Index	Body Weight (gm)	Weight Gain (gm)
Control	102.3±6.5	2.81±0.61	57.9±14.8	1.53±0.22	37.98±9.70	12.08
Cyclophosphamide	119.3±8.8+	2.88±0.52	48.1±7.9	1.18±0.32	42.35±7.96	14.03
Atrazine	105.5±15.5	3.06±0.54	46.9±16.9	1.36±0.46	34.75±3.77	9.20
Linuron	102.3±14.3	3.03±0.31	45.0±25.2	1.28±0.55	33.83±4.40	10.63
Atrazine + Linuron	121.0±11.3+	3.81±0.35+	41.5±13.7	1.31±0.45	31.83±2.92	9.28
Lorge Bear Creek	118.0±11.2o	2.96±0.19o	45.4±15.1	1.16±0.27	38.73±4.59	13.48
Morril	105.0±6.9	2.94±0.26	57.7±26.4	1.63±0.83	36.08±5.11	10.98

a Organ weight indices were calculated as mg organ wt/g body wt.
o Only based on 3 mice.
+ Significantly higher results compared to controls at $P<0.05$
++ Significantly higher results compared to controls at $P<0.01$

TABLE 5

Water Consumption in Mice Treated 30 Days

Treatment	Water/Day/ Mouse (mls)	Water/Day/ gm BW	Pesticide Concentration (µg/ml)	Pesticide/ Day/Mouse (µg)
Control	5.1±0.7	0.172±0.023		
Cyclophosphamide	5.9±0.5^{++}	0.190±0.017^{+}	20	117.24
Atrazine	6.3±0.6^{+++}	0.187±0.017^{+}	20	126.50
Linuron	5.0±0.4	0.161±0.014	2	10.056
Combination	6.7±0.6^{+++}	0.189±0.018^{+}		
Atrazine			10	67.08
+ Linuron			1	6.708
Bear Creek (Lorge)	5.5±0.5^{+}	0.174±0.015		
Alachlor			0.18	0.9979
Atrazine			0.066	0.3659
Metolachlor			0.0038	0.0211
Cyanazine			0.012	0.0665
Cottage Grove (Morril)	5.4±0.7	0.170±0.021		
Alachlor			0.0024	0.0129
Atrazine			0.0600	0.3229
Metolachlor			0.0100	0.0538
Cyanazine			0.0032	0.0172
Metribuzine			0.00025	0.0013
Linuron			<0.0008	0.0043

$^{+}$, $^{++}$, $^{+++}$ Significantly different as compared to control at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$; respectively.

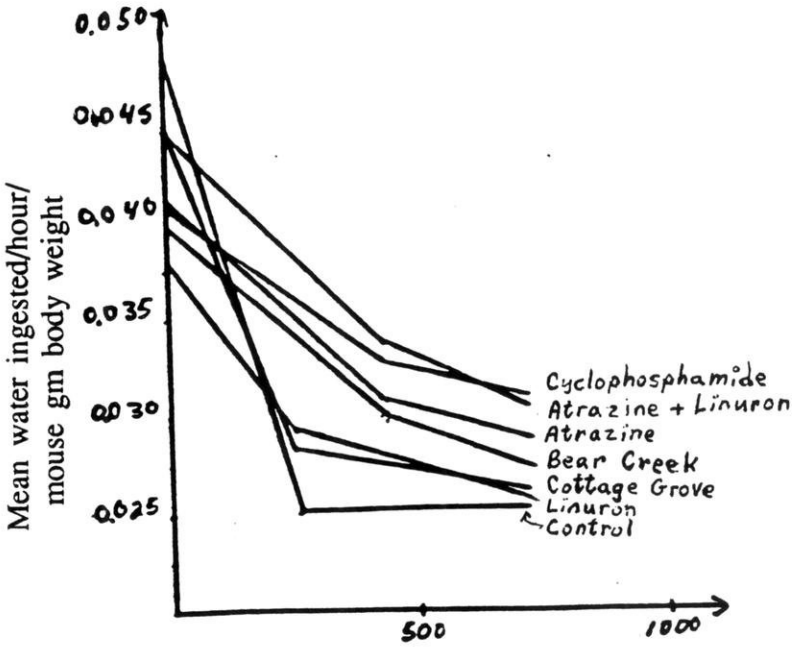
TABLE 6

Water Consumption for Mice Treated 90 Days

Treatment	Water/Day/ Mouse (mls)	Water/Day/ gm BW	Pesticide Concentration (µg/ml)	Pesticide/ Day/Mouse (µg)
Control	4.4±0.5	0.115±0.013	0	
Cyclophosphamide	5.3±0.7^{+++}	0.125±0.016^{++}	20.0	105.48
Atrazine	4.3±0.8	0.123±0.022^{+}	20.0	85.56
Linuron	4.4±0.8	0.130±0.023^{+++}	2.0	8.82
Combination	5.6±0.6^{+++}	0.176±0.019^{+++}		
Atrazine			10.0	56.16
Linuron			1.0	5.612
Bear Creek (Lorge)	5.2±0.6^{+++}	0.135±0.014^{+++}		
Alachlor			0.18	0.9374
Atrazine			0.66	3.4373
Metolachlor			0.038	0.1979
Cyanazine			0.12	0.6250
Cottage Grove (Morril)	5.0±0.5^{+++}	0.140±0.015^{+++}		
Alachlor			0.0024	0.0121
Atrazine			0.0600	0.3028
Metolachlor			0.0100	0.0505
Cyanazine			0.0032	0.0161
Metribuzine			0.00025	0.0013
Linuron			<0.0008	0.0040

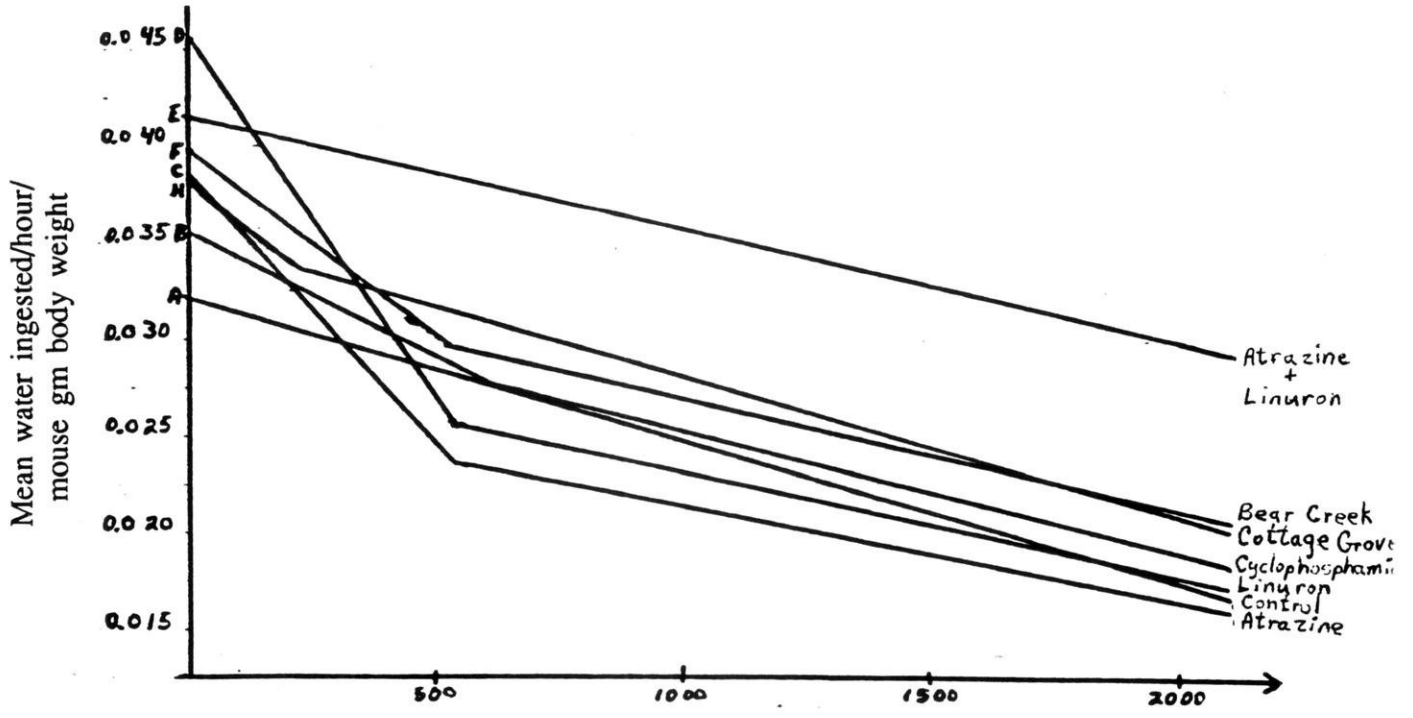
$^{+}$, $^{++}$, $^{+++}$ Significantly different as compared to control at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$; respectively.

FIGURE 1: Water consumption for 30-day mouse treatment groups



Time (In hours)

FIGURE 2: Water consumption for 90-day mouse treatment groups



Time (In hours)

050867- Mutagenic Effects of Selected Toxicants Found in Wisconsin's Ground Water