BIOLOGICAL MONITORING OF ENVIRONMENTAL GENOTOXICITY IN SOUTHWESTERN ONTARIO

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INTRODUCTION

Although southwestern Ontario is not as densely populated as other areas of Ontario and has a relatively modest industrial base, the close geographic proximity to Detroit and its environs has resulted in high levels of both atmospheric (Czuczwa and Hites, 1986; Armito, 1989; Edgerton et al., 1989; Eitzer and Hites, 1989; Kelly et al., 1989; Kelly et al., 1991) and aquatic pollution (Chau et al., 1985; Comba and Kaiser, 1985; Hamdy and Post, 1985; Pugsley et al., 1985; Thornley and Hamdy, 1984; Maccubbin et al., 1991; Manny and Kenaga, 1991; Carter and Hites, 1992; Ali et al., 1993). For instance, in 1987 power plants, steel mills, petroleum refineries, salt mines, chemical manufacturers and the car industry released 3 million cubic meters of effluent per day into the Detroit River by way of the Detroit Water Waste Treatment plant (EC and EPA, 1988). In addition, this region of Ontario is a prime agricultural area. Crops grown include cereal grains (e.g. corn, wheat, oats), fruits (e.g. apples, peaches, pears, plums), soya beans and a wide variety of garden vegetables (e.g. lettuce, radishes, tomatoes, potatoes). Consequently, a broad range of herbicides, insecticides and fertilizers are added to the chemical load of the area's atmosphere and waters (Manny et al., 1988). Supplementing the above we have the usual contributions to health-affecting agents from life-style activities (Meier, 1990; Randerath et al., 1992). Finally, there are contributions from accidental spills of hazardous agents (Edsall et al., 1988; Manny et al., 1988), through ground water contamination (EC and EPA, 1988) and through sewer overflows after heavy rainfalls (Pollman and Danek, 1988).

Many contaminants in our environment affect organisms directly, and rather rapidly, by causing physiological disturbances, abnormal development and/or a shortening of life span (Hontela et al., 1992; Folmar et al., 1993; Hall et al., 1993; Mercier and Robinson,

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1993). Some of these exogenous agents also affect the DNA of organisms causing induction of tumors and/or genetic mutations (West, 1988; Lin et al., 1990). Tumors, although they may take considerable time (decades in humans) to develop to a detectable level, are similar to non-DNA damaging agents in that they generally affect normal functions of organisms in the generation of exposure. The direct damage is limited to the individuals that are exposed to the contaminants. Gene mutations, on the other hand, are usually not detected in the generation of occurrence. Even in the case of dominant mutations, the novel phenotypes will generally be seen in the generation subsequent to the one in which the mutational event had occurred and the effects of recessive mutations will frequently be observed a number of generations later. These effects will usually include a reduction in fitness of the organisms, since most mutations whether dominant or recessive will be deleterious. Because damage to DNA is not immediately recognized at the organismic level, and yet may have broad ranging effects, it is essential that we develop a system which will enable us to **monitor changes in genotoxicity** of the environment.

Effective monitoring procedures, including those for genotoxicity, must involve assays that are highly sensitive, relatively straightforward to apply and inexpensive. Such procedures must include not only laboratory assays but tests that are applicable *in vivo* and on indigenous *sentinel* organisms. Towards this end procedures, used in monitoring genotoxicity levels in representatives of terrestrial and aquatic organisms in southwestern Ontario, are described. The former involved house mice, *Mus domesticus*, and the latter, either two species of fish, *Ameiurus nebulosus* (bullheads), and *Cyprinus carpio* (carp), for large bodies of water of the Huron-Erie corridor, or two species of amphibians, *Rana clamitans* (green frog) or *Bufo americanus* (American toad), for creeks, ponds and drainage ditches. The mice gave information on genotoxicity levels resulting primarily from contaminants in the atmosphere, whereas fish and amphibians monitored genotoxicity of ponds, rivers and lakes.

Of the numerous short term tests for detecting genotoxicity two were selected at the beginning of the study because of their sensitivity and ease of application in the mouse. These were the **sister chromatid exchange (SCE) assay** (Kornberg and Freedlander, 1974; Latt et al., 1979, 1981; Block, 1982; Knuutila, 1982) and the **micronucleus (MN) assay using peripheral blood erythrocytes** (see Heddle, 1973; Salamone and Heddle, 1983; Schlegal and Macgregor,1982). Because these assays require cells that are mitotically active and have relatively large chromosomes, they were found not to be suitable for the two species of fish used in our study. Both species had small chromosomes and the erythrocytes, the cells of choice, were not mitotically active. As a result we turned to a third analysis system for the fish and amphibians, the **single cell DNA electrophoresis** or **'comet assay'** (Singh et al., 1988; Tice et al., 1990).

The three procedures used to monitor the genotoxicity of the atmosphere and the waters of southwestern Ontario, as modified from published procedures, are described together with some preliminary data in support of the applicability of these assays.

MONITORING ASSAYS

Sister Chromatid Exchange (SCE) Assay

The **SCE assay** was selected at the outset because it had been used extensively on mice and because of its sensitivity. The basis for this assay is the exchange of chromosomal material during chromosomal replication. Each chromosome is composed of two DNA strands. On replication each strand serves as a template for the formation of a new strand. This results in two double-stranded structures or chromatids. The two chromatids share a single centromere and are genetically identical. At anaphase the centromere divides and the

Table 1. Preparation of chromosomes for the Sister Chromatid Exchange Assay in mice

1. 5-bromo-3′-deoxyuridine (BrdU) is incorporated into the mouse DNA by implanting a 50 mg paraffin-coated tablet subcutaneously between the scapulae after the mouse was anesthetized with avertin (4 g tribromoethanol in 2.5 ml amylene hydrate).
2. Twenty-four hours later 4 μg/g colchicine are injected intraperitoneally.
3. Two hours later the mouse is sacrificed by cervical dislocation and the femurs are removed.
4. The bone marrow cavity is flushed into 37°C 0.075M KCl (Gibco) and the cells are incubated in this hypotonic solution for 22 minutes after which the suspension is vortexed for 2 seconds.
5. The cells are then fixed in a cold methanol-acetic acid solution (3:1) two times with centrifugation after each fixing stage.
6. After fixation the cell suspension is applied dropwise to slides and these are air dried and allowed to age for 24 hours in the dark.
7. The slides are then placed in a Hoechst 33258 fluorescent stain (10 μg/ml) dissolved in distilled water for 15 minutes.
8. The slides are then placed in staining jars containing Sorensen's buffer (pH 8.0) and exposed to fluorescent lights for about 23 hours.
9. At the end of this period a slide is removed stained with Fisher's Giemsa in Gurr buffer (Gurr Biological Reagent 065568, pH 8.0) for 8 minutes and the chromatids are examined for differential staining. If the chromatids are differentially stained then the rest of the slides are processed.
10. If there is no differential staining of chromatids, the remaining slides are exposed to the fluorescent lights for an additional 10 minutes and staining with giemsa is repeated.
11. Twenty-five differentially stained cells from each mouse are examined for sister chromatid exchanges.

sister chromatids separate. Occasionally breaks will occur in the arms of both sister chromatids and a switching or exchange of segments takes place. This is the sister chromatid exchange (SCE) phenomenon. An SCE can be detected by labelling the new DNA strands as they are being formed with 5-bromo-3'-deoxyuridine (BrdU). After two replications, this results in one chromatid with BrdU incorporated in both of its strands and the other sister chromatid with BrdU in only one strand. After treatment with the fluorescent stain, Hoechst 33258, exposure to light and staining with Giemsa, the chromatid with BrdU incorporated in both strands will appear less intense than its counterpart which has BrdU incorporated in only one strand. Any exchanges between the sister chromatids can, as a result, be readily detected.

Latt et al. (1981) have succinctly outlined the advantages and disadvantages of this assay. They claim that it is excellent for detecting the effects of alkylating agents such as ethyl methanesulfonate and mitomycin C on DNA molecules; that it can detect the effects of both direct-acting agents and those requiring metabolic activation; that it appears extremely sensitive, since even very low concentrations of an agent will result in increased SCEs; that the assay can be used to detect DNA damage in a broad range of organisms and tissues; and finally, that the test appears to give few false positives. On the negative side: the test appears insensitive to compounds causing double-stranded DNA breaks; the assay requires cells undergoing mitosis; the chromosomes must be large enough so that exchanges can be visible with an optical microscope; and the process of SCE formation is not understood. The steps involved in this assay are given in Table 1.

Micronucleus (MN) Assay for Peripheral Blood

The **micronucleus assay for peripheral blood** was selected as the second monitoring system, even though it is considered less sensitive than other assays by some (Baucknecht et al., 1984; Tice et al., 1987), because it involves very little tissue preparation, and because scoring is faster and easier than with other cytological genotoxicity assays (Heddle, 1973;

Table 2. Preparation of slide for the Micronucleus Assay using peripheral blood of the mouse. The following is a modification of a procedure obtained from Dr. R. Tice (personal communication)

1. Peripheral blood is obtained from the mouse by inserting a heparinized capillary tube into the suborbital sinus.
2. A drop of blood is spread uniformly on a microscope slide and the preparation is air-dried.
3. The slide is fixed in absolute methanol for 15 minutes.
4. After 24 hours of aging the slide is stained with acridine orange (0.06125 mg/ml of pH 7.4 phosphate buffer) for 3 to 4 minutes, then rinsed with the buffer and soaked in the buffer for 6 minutes.
5. The spread is covered with a coverslip and examined under fluorescence microscopy. acridine orange discriminates between DNA which fluoresces yellowish-green and RNA which fluoresces red.
6. The slides are scored for the number of micronucleated polychromatic erythrocytes (PCES) in 1000 PCES, the number of micronucleated normochromatic (NCES) per 1000 NCES and the percentage of PCES in 1000 erythrocytes.
7. PCES are erythrocytes which have just been released into the bloodstream. because they have some RNA in the cytoplasm they have a red fluorescence. NCES are PCES after the RNA has been broken down. The cytoplasm in these cells does not fluoresce. the former have a life span of up to 72 hours in the mouse and the latter about 30 days.
8. If either the fluorescence or background are not suitable, the slides can be destained with absolute alcohol and restained.

Salamone and Heddle, 1983). The **MN assay** is based on some DNA being excluded from the nucleus of a cell and retained in the cytoplasm. The DNA could be a fragment of a chromosome without the centromere or it could be an entire chromosome which had somehow become detached from the spindle fibers. In mature mammalian erythrocytes, these pieces of DNA or micronuclei remain in the cell while nuclei are normally extruded. To detect micronuclei, a DNA sensitive stain such as acridine orange is used.

Tice and Ivett (1985) summarized the positive features of the bone marrow micronucleus assay. The following apply to the peripheral blood adaptation. Micronuclei can be identified readily in anucleated erythrocytes and metaphase preparations are not required. In addition because the animals do not need to be sacrificed to obtain blood samples, repeated bleedings are possible permitting continuous monitoring of mice during long term exposure studies. Furthermore, erythrocytes in the blood stream can be classified into two types: **polychromatic (PCE)** and **normochromatic (NCE)**. Since PCEs are converted to NCEs in about 72 hours of their formation in the bone marrow, the time of exposure can also be divided into two periods: a recent period, probably the last 3 or 4 days, and an earlier about 35 day period since PCEs have a lifespan of 30-35 days.

Details of the micronucleus procedure as applied to peripheral blood in mice are given in Table 2.

Single Cell DNA Electrophoresis (SCDE)

Because the chromosomes in the two fish species selected as target organisms in this study were too small and numerous to be tracked and because we were not able to induce micronuclei in the erythrocytes of peripheral blood in either of these species, another assay, **single cell DNA electrophoresis (SCDE)**, was used. This assay, as mentioned earlier, has been found to be very sensitive and capable of detecting genetic damage even in nondividing cells regardless of the size of the chromosmes (Singh et al., 1988). The **SCDE assay** involves suspending nucleated cells in agarose, lysing the cells, subjecting the cells to electrophoresis and then staining for DNA. The electrophoresis will separate any small fragments of DNA which have broken off from the nuclear core. The core, because of the size of the DNA

Table 3. Detailed description of the Single Cell DNA Electrophoresis Procedure as modified from a protocol developed by Singh et al (1988)

1. Blood is added to 10% Hank's balanced salt solution and a dilution is made so that a single field at 400X will have 3 or 4 well dispersed cells.
2. Five hundred microliters of the erythrocyte suspension are then mixed with 1 ml of low melting (37°C) agarose.
3. Five hundred microliters of the erythrocyte-agarose suspension are then pipetted on a fully-frosted slide and overlayed with a coverslip.
4. After placing the slide on ice for 15 minutes to complete the polymerization of agarose, the coverslip is removed and the slide is lowered into a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Na sarcosinate) with pH 10.0 and incubated at room temperature in the dark for 1.5 hours.
5. The slide is then drained of lysing solution and covered with an alkaline buffer (0.3 N NaOH, 1 mM EDTA) for 15 minutes to allow the DNA to unwind.
6. The slide is then subjected to 265-270 mA at 25 V in the alkaline buffer in the dark at 3°C for 20 minutes. the current can be adjusted by changing the buffer level.
7. After electrophoresis, the slide is placed in neutralizing buffer (0.4 M Tris, pH 7.5) in the dark for 5 minutes. the buffer is replaced once and drained.
8. The slide is then overlayed with 60 μl of ethidium bromide (10 mg/ml) and covered with a coverslip and stored overnight.
9. The next day the slide is examined at 400X with a fluorescent microscope equipped with a B excitation filter, a Y544 supplementary excitation filter and a 530 mm barrier filter.
10. Routinely 25 nonoverlapping DNA masses are measured as they come into view in a linear scan. a ratio of DNA length (core + tail) to DNA width is used in all analyses.

molecules forming it, remains relatively immobile. This results in the formation of a fast moving "tail" and a large nuclear core. This formation is frequently referred to as a "comet". The length and density of the tail reflect the amount of DNA damage that the cell has sustained (Singh et al., 1991; Olive et al., 1992).

See Table 3 for a more detailed description of the protocol for the SCDE assay.

OTHER ASPECTS OF THE MONITORING SYSTEMS USED

In situ Monitoring

An effective monitoring system must be such that it considers the effects of all of the agents that can cause damage and excludes those that do not reach the target tissue or are inactivated by the organisms of concern. A suitable surveillance system should, therefore, involve the use of organisms at the site that is to be monitored. This has a number of advantages over laboratory assays. For instance, the presence of toxic agents, as detected by various chemicophysical analyses or even biological assays under laboratory conditions, need not affect organisms of concern, if these organisms do not come in contact with the agents, or more specifically, if the agents do not reach the appropriate target sites within the organisms for any number of reasons (e.g. detoxification, preferential excretion, lipid sequestering). Conversely, certain agents may be undetectable in nonbiological or biological laboratory assays and yet have a significant effect on organisms of concern (e.g. agents at very low concentrations; complex mixtures in which components may act additively or even synergistically). The environment may, moreover, provide conditions under which certain agents can produce an effect, conditions that either cannot be duplicated in the laboratory or are so subtle as to be unrecognized by the investigator. In addition, indigenous organisms *in situ* may spend their entire life in a particular environment and as a result have the potential

to show the effects of a weak agent after long term exposure. Finally, organisms *in situ* can provide continuous monitoring of a particular environment.

Selection of Sentinel Organisms

To monitor the terrestrial environment, house mice (*Mus domesticus*) were selected as the target organism because they are ubiquitous, and are frequently associated with such human habitats as houses, barns, corn cribs, etc. Adequate sized samples can generally be obtained especially in southwestern Ontario, where numerous cribs, filled with corn grown in the area, provide an excellent habitat for mice. House mice are also easily maintained in the laboratory under regulated conditions and genetically fixed strains are available for controlled experiments. For monitoring purposes, mice can readily be housed in small containers filled with corn. Such cages may be placed outdoors, in orchards, etc., or indoors in industrial settings without causing much commotion, since the animals can live on the corn without need for attention for four to five weeks. Finally, mice are good indicators of the effect that the environment has on humans, since mice have activation and detoxification enzymes similar to those present in humans.

The aquatic environment was divided into two classes: large bodies of water such as the Detroit River and Lake St. Clair and small bodies such as creeks, drainage ditches and ponds. For monitoring the former we used two species of fish, the brown bullhead (*Ameiurus nebulosus*) and the carp (*Cyprinus carpio*). These organisms were selected because both species have been described as common in the Lake Huron-Lake Erie corridor and because of their feeding habits. The bullhead is a benthic feeder and as a result is exposed to chemicals which have accumulated in the sediment. Its tendency to be localized in its activities makes it an excellent choice for assessing local environmental conditions. Moreover, this species appears sensitive to contaminants in the environment. In contaminated situations these fish have shown an increased incidence of papillomas, liver tumors and distorted barbels (Metcalfe, 1990). The carp feeds on detritus found both in the sediment and in the water column. In addition both species of fish can be housed in cages which may be placed even at sites where these species are normally absent.

Target organisms for the smaller bodies of water were two species of tadpoles, *Rana clamitans,* the green frog, and *Bufo americanus,* the American toad. Both are found in large numbers in many of the creeks and ponds in southern Ontario. The clamitans tadpoles are especially valuable as monitors because amphibians of this species spend the winter in the tadpole stage. Tadpoles of the other species transform in a single summer. Tadpoles of both species can be placed in cages to monitor bodies of water where they are not normally found.

The map in Figure 1 indicates the areas from which the sentinel species were collected.

Approach to Terrestrial Monitoring

For the terrestrial studies, baseline values were established as reference points for both the **SCE** and **MN assays** by using inbred strains of mice (BALB/ByJ and C3H/HeJ) and wild mice which had been maintained in the laboratory for several months. SCE and MN values obtained from samples of mice freshly collected from corn cribs were then compared with the baseline values as well as with one another. The mice in the present study came from corn cribs because of the ease with which large samples could be collected and because the corn cribs form identical microcosms throughout the study area. The animals were collected as the cribs were being emptied. These populations, their habitats and the collecting procedures are described elsewhere by Petras and Topping (1981).

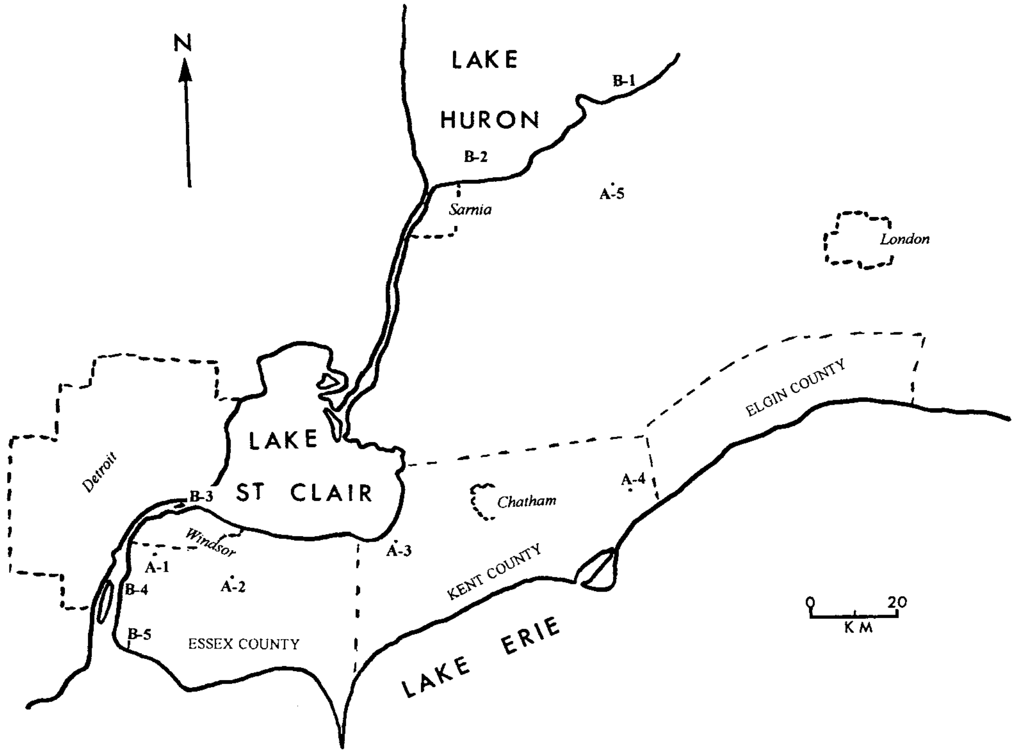

Figure 1. A map of the study area in southwestern Ontario. The counties named are ones from which the mice for the SCE study came. The sites marked with an **A** were the sources of the amphibians (**A-1 Windsor-Ojibway, A-2 Essex west, A-3 Tilbury north, A-4 Highgate, A-5 Arkona**) and those marked with a **B** were the sites where the fish were collected (**B-1 Port Franks, B-2 southern Lake Huron, B-3 Detroit River at Peche Island, B-4 Detroit River at La Salle, B-5 Big Creek**).

Since the monitoring of wild mice was dependent on the emptying of cribs, the timing of which was determined by the farmer and out of our control, a second approach was sought. After considering a number of options, we used large galvanized metal garbage cans (90 liters) and smaller plastic pails (22 liters) filled with dried field corn at outdoor and indoor sites, respectively. The dried corn provided nutrients, water and cover for the mice. The larger containers could house 6 to 8 mice for almost four months without intervening care. These containers were placed in agricultural settings where they were left undisturbed for six weeks. The smaller plastic containers held 5 or 6 mice for about five weeks without the need for any further care. These plastic containers were placed at a number of industrial sites and in situations where the larger containers could not be used (e.g. on a spraying rig).

C3H/HeJ and BALB/ByJ mice were used in laboratory experiments to determine the sensitivity of the assay, to serve as controls in the various SCE and MN studies, and as monitoring organisms in the cage studies.

Approach for Aquatic Monitoring

Fish were bled via cardiac puncture either in the field or shortly after they were brought to the laboratory. The fish survived the bleeding without any discernable ill-effects; in some

Table 4. SCE responses of wild and C3H/HeJ male mice and their variances to different doses of cyclophosphamide. Bartlet's test of homogeneity of variances shows that the differences are not significant

Mice	CP level (mg/kg)	Number of mice	SCE values	Variances
Wild offspring	0.000	8	6.236	1.929
	0.045	8	5.348	0.915
	0.450	8	7.883	2.561
	4.500	9	18.078	4.796
C3H	0.000	8	4.185	1.494
	0.045	8	4.338	0.423
	0.450	8	6.234	1.467
	4.500	8	12.634	6.549

cases individuals were rebled three or four times. Because DNA damage could not be detected in bullheads or carp using the **SCE** or **MN assays**, **single cell DNA electrophoresis** was used. Since no information was available on the suitability of this assay in detecting DNA damage in fish, the published procedures were modified (Pandrangi et al. 1994, submitted) using bullheads obtained from a fish hatchery. As these fish showed very little spontaneous DNA damage they were used in a series of experiments with a known clastogen, cyclophosphamide. These experiments were used to establish the optimum bleeding time after exposure, the sensitivity of fish to different concentrations of the cyclophosphamide, and the types of measurements to be made of the DNA patterns observed. In addition, some of the wild fish were kept in the laboratory over an extended period of time to determine if the fish, which initially showed high levels of genetic damage as detected by SCDE, would return to a reduced level or perhaps even to a background level of damage. Finally, freshly caught bullheads from a number of different localities were examined for DNA damage. Carp were also used to establish background levels and to determine the levels of DNA damage in fish from several geographic sites.

In the amphibian tadpoles, the **SCDE assay** was used in much the same way as in the fish, except that no "hatchery-bred" organisms were available. The tadpoles came from a number of different sites in southwestern Ontario. A sample of tadpoles from each area was processed within several days of being brought to the laboratory. Because no changes in the level of DNA damage were observed for several weeks after the animals were brought into the laboratory, the tadpoles did not need to be processed immediately on being collected. A large sample of tadpoles was obtained from a site near Highgate, Ontario. Some of these were maintained in the laboratory and monitored for an extended period of time. The length of the DNA tail decreased to a stable level by 4 months. These animals were then used in determining the sensitivity of tadpoles to known genotoxicants. These laboratory-maintained tadpoles also served as controls in other studies.

Because of the size of the tadpoles, bleeding by cardiac puncture was not feasible. Instead the animals were decapitated and the bodies were placed in Hank's balanced salt solution. Enough erythrocytes leaked into the Hank's solution to run the assay.

RESULTS

Terrestrial Environment - SCE Results in Mice

Since the sensitivity of the SCE assay in inbred laboratory mouse strains has been well established, our first concern was the sensitivity of the wild mice to known mutagens

and whether wild mice because of their genetic heterogeneity showed a greater variance in response to a mutagen than did mice of an inbred strain. Table 4 shows that laboratory-bred offspring of wild mice responded to a range of cyclophosphamide (CP) doses just as did mice of the inbred strain C3H. In fact the wild offspring responded more strongly to the higher doses of CP. A comparison of the variances in the two groups of animals, using Bartlet's test of homogeneity of variances, showed no significant differences.

The monitoring of natural populations of mice for genetic damage using the SCE assay was begun in 1983 and continued into 1987. The objective was to detemine whether seasonal and/or geographic patterns existed. As described earlier, the mice for this work came from corn cribs. The results are summarized in Table 5. Although there was some variation from year to year because of weather conditions, the spraying of herbicides usually began in early May and continued into June. In all cases the SCE levels observed during the spraying period were higher than in the prespraying period (6 of 7 comparisons were signficantly higher; $p = 0.05$) and in most cases there was a decrease during the post-spraying period (in 3 out of 5 cases the drop was significant; $p = 0.05$). In the one instance where there was no decrease, the results could be due to an engine repair shop located within 40 meters of the crib. Exhaust, from motors being repaired, might have contributed to the pollution of the area.

Although an earlier study (Nayak and Petras, 1985) suggested a geographic pattern with higher SCE values in the western part of the study area and lower in the east, no such pattern was evident from these SCE data.

Industrial Settings and SCE Levels

The three industrial sites selected for this study were a plastics-molding plant, a diesel engine repair shop and a chrome plating plant. The plastics-molding plant was included because such chemicals as polyvinyl chloride (Anderson et al., 1981), propylene oxide (Hogsted et al., 1983) and ethylene oxide (Hardin et al., 1983) associated with the plastics industry have been found to be mutagenic and SCE inducers. The diesel engine repair garage was chosen because the Ames Salmonella Test has shown that such products of combustion as 9-nitroanthrocene and 1-nitropyrene caused marked induction of revertants (Pitts et al., 1982). Finally, a chrome plating plant was monitored because hexavalent chromium has caused gene mutations (Ohno et al., 1982; Bianchi et al., 1983).

Table 5. SCE levels and standard errors of the means in wild male mice collected from corn cribs in three regions of southwestern Ontario before, during and after spraying of crops with herbicides. The numbers in parentheses are the sample sizes

	Locality	Prespraying	Spraying	Post-spraying
1987	Essex County	5.25 ± 0.39 (16)	6.63 ± 0.44 (10)	5.09 ± 0.83 (6)
	Kent County	4.95 ± 0.51 (12)	6.07 ± 0.28 (17)	5.01 ± 0.27 (7)
1986	Essex County	4.64 ± 0.22 (20)	6.76 ± 0.16 (34)	6.01 ± 0.24 (32)
	Kent County	5.69 ± 0.41 (27)	5.86 ± 0.38 (17)	
1985	Essex County	5.16 ± 0.16 (30)	7.07 ± 0.43 (24)	7.21 ± 0.84 (10)
	Kent County		5.92 ± 0.36 (37)	
	Elgin County		4.74 ± 0.31 (7)	
1994	Essex County	5.70 ± 0.19 (28)	6.35 ± 0.17 (32)	
	Kent County	4.65 ± 0.43 (19)	6.66 ± 0.24 (38)	4.89 ± 0.18 (30)
	Elgin County		6.62 ± 0.46 (20)	
1983	Pooled	5.99 ± 0.32 (12)	9.22 ± 0.86 (10)	

Table 6. Mean SCE values and standard errors of the means in male C3H mice placed in cages partially filled with corn in three different industrial settings

Site	No. of mice	Mean SCE/cell ± SEM
Controls (University housed)	6	3.61 ± 0.041
Plastics molding plant	5	5.63 ± 0.276*
Diesel repair garage	6	7.05 ± 0.532*
Chrome plating plant	7	5.47 ± 0.021

*Deviation from controls significant at the 5% level.

In each case six C3H mice were placed in a 22 liter plastic pail, three-quarters filled with ears of dried corn. A plastic container with mice was placed in each of the work environments for two weeks. An identical container with mice was kept as a control in the animal facility at the University of Windsor.

The results are summarized in Table 6. The SCE counts in mice housed in the plastics molding plant and the repair garage showed modest but significant increases above the controls ($p < 0.05$).

Outdoor Population Enclosures

The outdoor enclosures were the 90 liter galvanized garbage cans half filled with corn. These containers were placed in three regions of Essex County: southwest - Amherstburg, central -Town of Essex and northeast - near Tilbury in the winters of 1985 through 1987. The results summarized in Table 7 show no geographic differences. Table 8 presents the SCE data for mice housed at a single site over an eight month period. The SCE levels observed in June were higher than those seen in any other period, with the lowest coming in November. These results are consistent with those obtained for the corn crib mice.

To determine whether herbicide spraying had an effect on the SCE values in mice, animals were placed in 22 liter plastic pails and attached to a spraying rig just above the nozzles. The results are summarized in Table 9. There was a gradual increase in SCEs which was correlated with the length of exposure . Both the 8 hour and the 3 day exposure differed significantly from the 2 hour exposure ($p < 0.05$).

Terrestrial Monitoring - MN Data

In laboratory experiments genetic damage through MN induction could be detected in both inbred and wild mice. The MN data for freshly caught wild mice collected in the summers of 1986 and 1987 are summarized in Table 10. Very little variability was observed; no temporal or geographic patterns are evident. Mice placed in enclosures also did not show either seasonal or geographic patterns with the MN assay.

Aquatic Monitoring Using Fish

Hatchery-bred bullheads were injected with a range of cyclophosphamide (CP) doses to determine the SCDE response of the fish. The results are summarized by Figure 2. CP was dissolved in dimethylsulfoxide in concentrations ranging from 1.25 to 20 mg/kg body weight. Even the lowest concentration showed a significant increase in DNA damage ($p < 0.05$).

To examine the applicability of SCDE technique to natural populations, freshly caught bullheads were assayed. These fish, from six different sites, showed a wide range of

Table 7. Mean SCE values and standard errors of the means for inbred mice placed in 90 liter containers filled with corn. The containers were placed at three different regions of Essex County in southwestern Ontario. The West and Central sites were separated by 17 km and the Central and East by 32 km

Site	No. of mice	Mean SCE/cell
Sampling date: Dec. 12, 1985		
West (Laramie farm)	5 C3H	4.14 ± 0.080
Central (McKim farm)	4 C3H	2.97 ± 0.155
East (Trudell farm)	5 C3H	4.28 ± 0.125
Controls	4 C3H	4.02 ± 0.245
Sampling date: Dec. 10, 1986		
West (Laramie farm)	7 C3H	3.62 ± 0.151
Central (McKim farm)	6 C3H	4.38 ± 0.351
East (Boide farm)	3 C3H	4.17 ± 1.097
Controls	5 C3H	4.33 ± 0.259
Sampling date: Mar. 17, 1987		
West (Laramie farm)	5 BALB/c	4.12 ± 0.188
Central (McKim farm)	4 BALB/c	3.98 ± 0.380
East (Boide farm)	1 BALB/c	4.27
Controls	5 BALB/c	4.13 ± 0.206

DNA damage (Figure 3). Bullheads that came from Big Creek, Hamilton Harbour and the Detroit River gave DNA length to width ratios of 3.38 to 4.45. Bullheads from the southern part of Lake Huron, from a small lake near Peterborough and from a fish farm in upstate New York gave ratios of 1.28 to 1.38.

Bullheads from the Big Creek area were divided into two groups, small (weighing less than 70 g) and large (weighing more than 90 g). The size should reflect the age of the fish and therefore, the duration of exposure. No significant differences were observed in the ratios.

Some of the bullheads that came from Big Creek were maintained in the laboratory in dechlorinated water for at least three months. These gave ratios which approached those of the controls (Figure 4) suggesting that the baseline levels in wild bullheads and the hatchery-bred fish were of the same order of magnitude.

Results for the carp were similar to the bullhead data. Carp from Big Creek gave ratios averaging about 4.49, while carp from Lake Huron and controls gave values of 1.36

Table 8. SCE counts and standard errors of the means in inbred mice housed in outdoor enclosures on a farm in southern Essex County

	C3H mice		DBA/2 mice	
Date of sample	No.	SCE counts	No.	SCE counts
November 4	8	3.64 ± 0.13		
March 9	8	4.12 ± 0.33	6	4.68 ± 0.47
April 19	8	4.33 ± 0.25	6	5.34 ± 0.31
May 16	7	4.62 ± 0.23	6	4.79 ± 0.35
June 22	8	5.65 ± 0.17	6	5.82 ± 0.29
July 26	13	4.14 ± 0.20	14	3.86 ± 0.12

Table 9. SCE values and standard errors of the means in male C3H mice that were placed in 22 liter pails partially filled with corn. The pails were attached to a sprayer while the farmer was spraying young corn plants with a herbicide

Duration of exposure	No. of mice	SCE counts and SEM
2 hours	6	3.98 ± 0.321
8 hours	6	5.55 ± 0.158
3 days*	6	5.69 ± 0.427

*Mice were exposed for about 10 hours per day.

and 1.17, respectively (Figure 5). Big Creek carp, maintained in the laboratory in dechlorinated tap water for 3 months, gave ratios similar to that seen in the bullheads.

Monitoring Small Bodies of Water Using Tadpoles

SCDE results of tadpoles from four ponds and a drainage ditch are seen in Figure 6. The Windsor Ojibway pond is located near the western limits of the city, next to a prairie grassland preserve and a conservation area. There are also a few small industries and some residences nearby. With the huge River Rouge industrial complex only about 6 km to the northwest, across the Detroit River, this site receives considerable industrial atmospheric fallout. The Essex west sample came from a ditch draining a farm area and about 12 residences. The Tilbury north pond is located on the edge of a large marshy conservation area and is adjacent to a railroad line. The remaining two ponds, Highgate and Arkona, are in agricultural areas with woodlots nearby. All five sites gave ratios greater than 2.0.

Since there were no suitable laboratory controls for comparison, a baseline, against which the population data could be compared, was established by maintaining some tadpoles from the Essex west drain and the Highgate pond in the laboratory for an extended period of time. After four months the ratios in tadpoles from both sites were substantially reduced. The values dropped from 2.5 to about 1.2. The lower value should approach the ratios that would be expected from spontaneous levels of DNA damage. If this assumption is valid, then all areas sampled showed considerable levels of DNA damage with the Ojibway and Arkona sites showing the most and the Guestwood site (Essex west) the least.

The laboratory-maintained Highgate tadpoles were used in several experiments. One involved placing tadpoles in water containing different concentrations of methyl methanesulfonate (MMS) for a 24 hour period. Figure 7 summarizes the results of this study. Animals

Table 10. Results of micronucleus frequencies and standard errors of the means observed in polychromatic and normochromatic erythrocytes of peripheral blood obtained from the suborbital sinus of mice. The last column is a measure of the formation of new erythrocytes

Location	No.	Mean MN-NCE per 1000 NCE	Mean MN-PCE per 1000 PCE	Mean percent PCE per total
Essex County				
Prespraying	41	0.70 ± 0.019	1.32 ± 0.036	2.97 ± 0.056
Spraying	42	1.00 ± 0.020	1.45 ± 0.028	1.33 ± 0.024
Kent/Elgin counties				
Prespraying	23	0.89 ± 0.067	1.64 ± 0.060	2.42 ± 0.090
Spraying	9	1.70 ± 0.161	1.12 ± 0.141	3.34 ± 0.613
Controls	32	1.14 ± 0.026	1.95 ± 0.049	1.87 ± 0.025

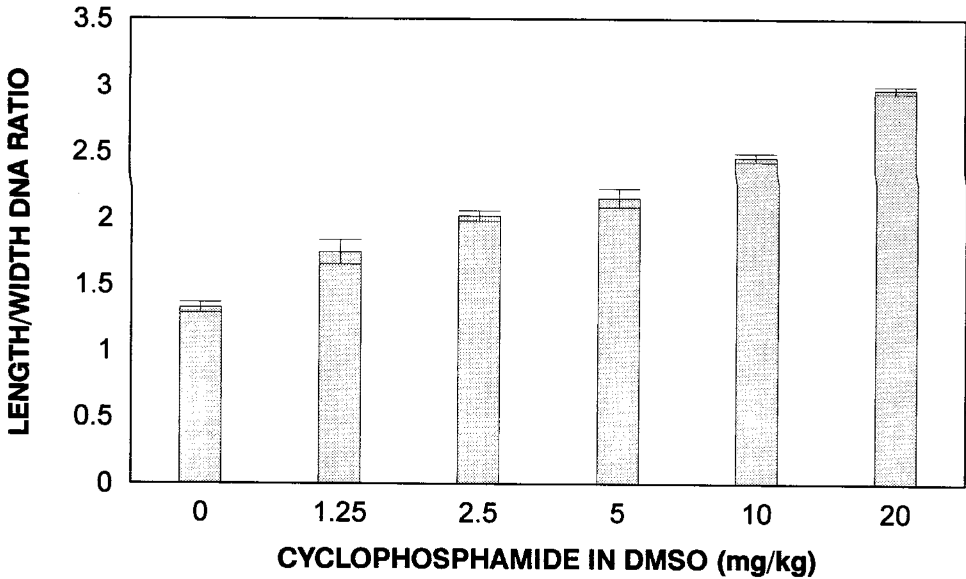

Figure 2. The SCDE ratios and standard errors of the means in bullheads injected with different doses of cyclophosphamide dissolved in dimethyl sulfoxide (DMSO).

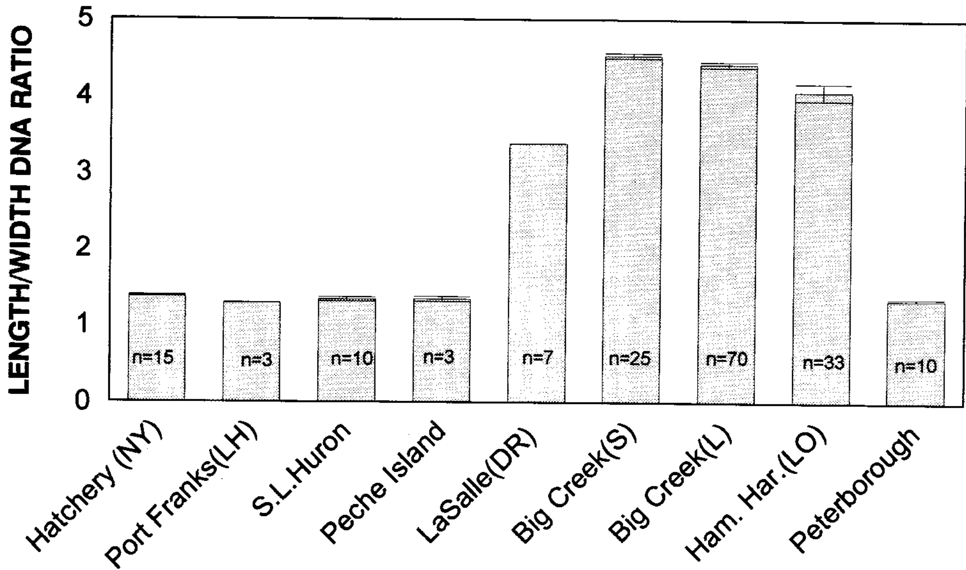

Figure 3. The SCDE values and standard errors of the means for bullheads collected from sites in southern Ontario. The ratios were compared to data from bullheads obtained from a fish hatchery. Two of the sites are from Lake Huron (Port Franks and S.L. Huron), two from the Detroit River (Peche Island and La Salle), one from the western region of Lake Erie (Big Creek), one from the western part of Lake Ontario (Hamilton Harbour) and one from the Peterborough area north of Lake Ontario.

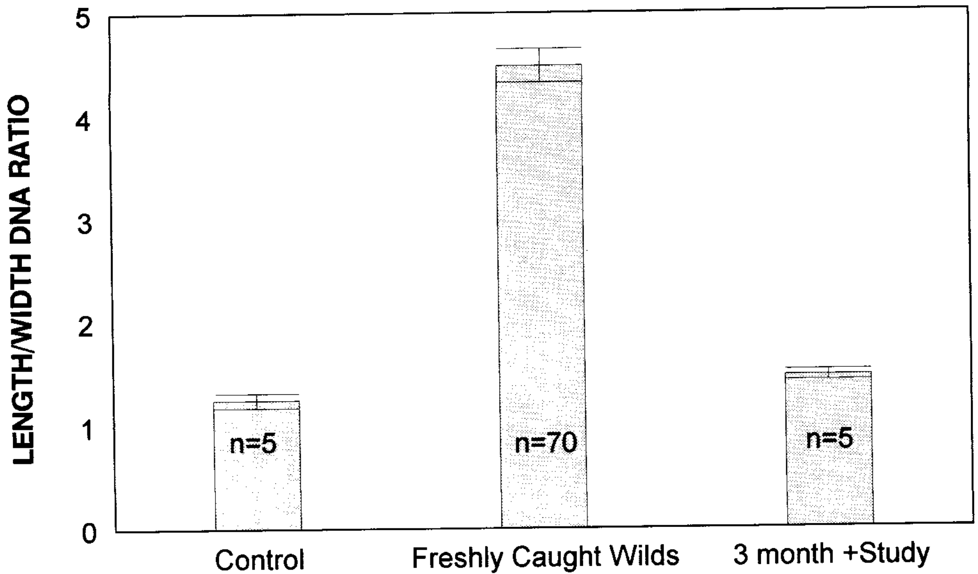

Figure 4. SCDE values in bullheads from Big Creek which had been maintained in the laboratory for three months. They are compared to values obtained in the hatchery fish and freshly caught Big Creek bullheads.

placed in water containing 50 mg/l MMS all died. At 25mg/l none of the preparations showed intact DNA cores; the DNA was completely fragmented into granules. At 12.5 mg/l of MMS five of nine animals showed no DNA cores, two of the remaining four had DNA cores with distinct tails in relatively few cells. The other two animals also showed considerable damage but there were a good number of cells with intact DNA cores and tails. Between 0 and 12.5

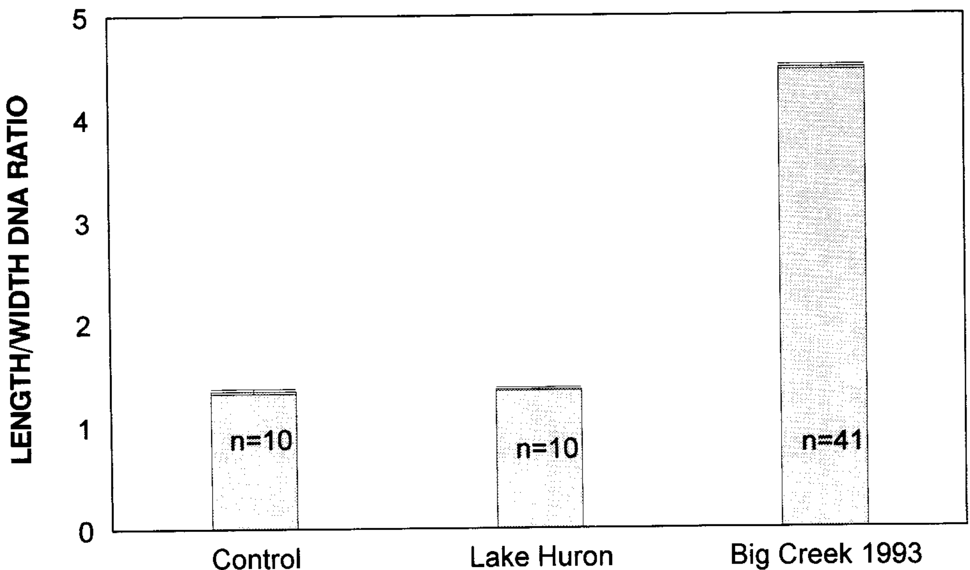

Figure 5. SCDE data for carp from Lake Huron and Big Creek.

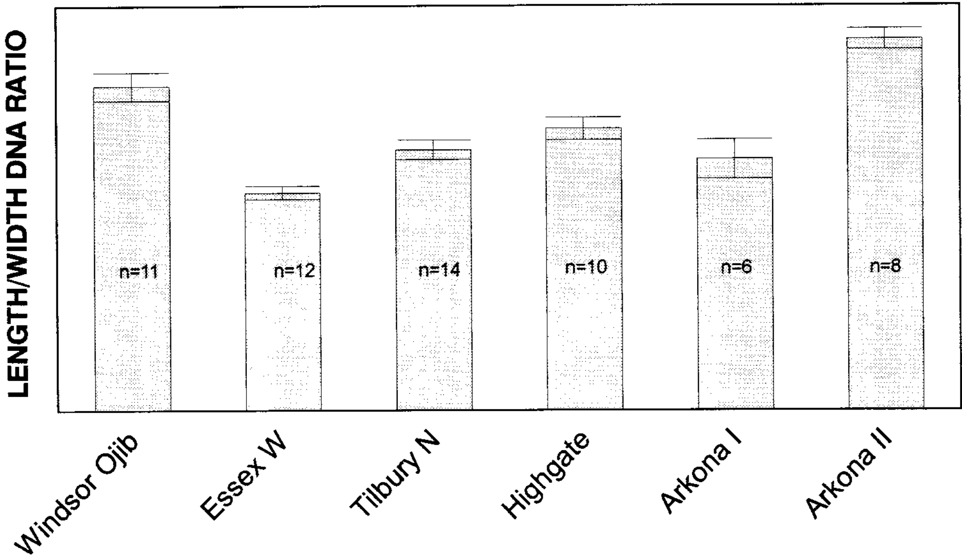

Figure 6. Geographic patterns of *Rana clamitans* tadpoles in southwestern Ontario. The two Arkona samples came from the same pond but were run a week apart.

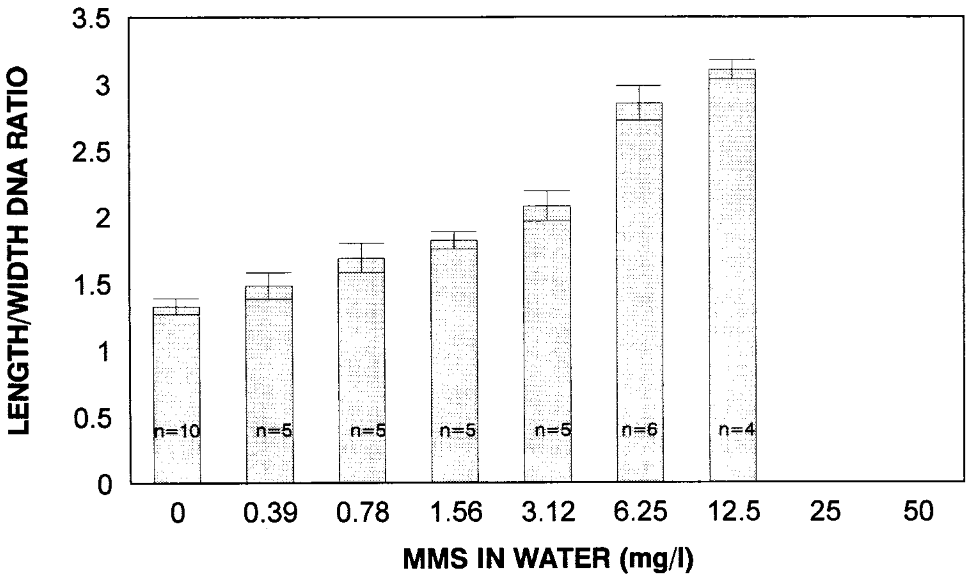

Figure 7. SCDE ratios and standard errors of the means for *Rana clamitans* tadpoles kept in water with methyl methanesulfonate (MMS) for 24 hours. Animals in water with 50 mg/l of MMS did not survive and those in 25 mg/l did not give intact DNA cores.

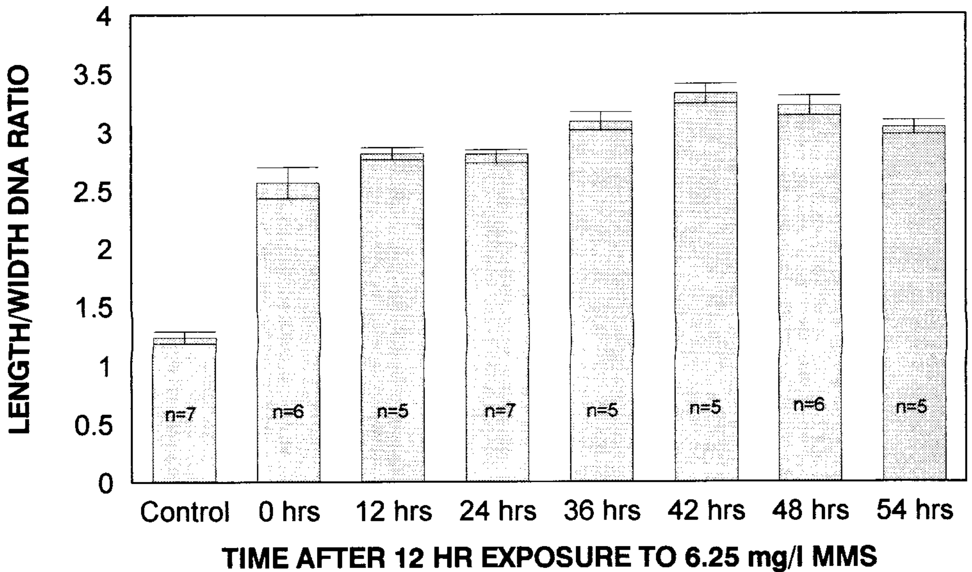

Figure 8. SCDE ratios and standard errors of the means for *Rana clamitans* tadpoles exposed to 6.25 mg/l methyl methanesulfonate in water for 12 hours. The tadpoles were bled at different times after the exposure period. In the post-exposure period the tadpoles were kept in distilled water.

mg/l MMS, there was a gradual increase in DNA damage as determined from tail length. This information was used for the second experiment in which the tadpoles were placed in water containing 6.25 mg/l MMS for 12 hours. At the end of this period some tadpoles were removed from the MMS solution and bled immediately, while the rest were placed in distilled water for varying lengths of times. Figure 8 shows that considerable damage was observed immediately after the end of the exposure period and the damage persisted for the 54 hours of the study with the peak being reached at 42 hours after the exposure period. This suggests that tadpoles do not need to be bled immediately on being brought into the laboratory. There is at least a 24 hour period of grace after the tadpoles are placed in distilled water. Usually, however, tadpoles are left in pond water until tested.

DISCUSSION

Since humans have been extremely effective in releasing into the environment a broad spectrum of agents affecting organisms, the need for suitable monitoring systems to detect changes in genotoxicity levels in the environment is obvious. This contribution is concerned primarily with describing the application of several assays which have been developed to monitor the genetic damage in organisms. The three assays, **sister chromatid exchanges**, **micronuclei in erythrocytes** and **single cell DNA electrophoresis (comet)** have been used in three different classes of vertebrates, a mammal, two species of amphibians and two species of fish. We have examined samples of indigenous animals from natural populations, laboratory animals placed in enclosures which in turn were placed at sites to be monitored and animals exposed to genotoxic agents under laboratory conditions.

In selecting a test system, we considered the criteria that were described succinctly by Giesy and Hoke (1989) in their rationale for species and assay selection in the assessment of sediment for toxicity. Of the criteria they suggested, the following are applicable to any biological situation. The ideal bioassay system should involve organisms that are easily maintained in the laboratory, whose response under controlled conditions should be predictable, and that respond to many different types of toxicants. The assays, according to Giesy and Hoke, should be related to ecologically and biologically relevant processes and should be applicable to a number of different organisms and environments. They should be "rapid, replicable, inexpensive and easily implemented so that large areas of concern can be surveyed rapidly with good resolution" (Giesy and Hoke, 1989). In addition, these authors suggest that the assays should be sufficiently sensitive to not only identify problems areas but also quantify the seriousness of the problem. Although Giesy and Hoke were concerned with monitoring the general toxicity of sediment, their criteria for suitable assays and their experimental approach can readily be applied to monitoring genotoxicity in any environmental situation.

The monitoring procedures used in this study meet most of the criteria proposed by Giesy and Hoke. The organisms selected as sentinels are abundant in the study area, have specific characteristics which make them good candidates for *in situ* monitoring and are easily maintained in the laboratory. The assays are looking at specific critieria, criteria that give information about genetic damage. They can be used as *in vivo* procedures thus making use of the organism's physiology to activate and detoxify chemicals. This should make the tests more powerful and rigorous. The three tests used appear, at least in part, complimentary. The **SCDE assay** does not have any special requirements and as a result can probably be used in most organisms, while the **SCE** and **MN procedures** require mitotically active cells with the SCE also requiring chromosomes that are rather large. Although, the relative sensitivities of the **SCDE** and **SCE procedures** have not as yet been thoroughly determined, the **SCDE assay** appears more sensitive to some agents (Betti et al., 1994). Moreover, the **SCE test** will not, for example, detect damage caused by ionizing radiation in mice whereas the former will (Singh et al., 1988). The **MN assay** appears to be the least sensitive.

At the outset we began with two assays, assays which had already been developed in laboratory studies using among other organisms, inbred strains of mice. We found that wild mice responded, based on SCE and MN results, as readily to known mutagenic agents as did the inbreds. Studies on the corn crib mouse populations support the view that the **SCE assay** is more sensitive to genotoxicants than **MN assay**. In fact, the **MN assay** gave no valuable information in the mouse population studies. The **SCE assay** gave results which suggest an association between DNA damage and herbicide spraying practices. This hypothesis is supported by two cage studies, one involving the large galvanized containers and the other the smaller plastic pails. The former were placed on a farm and the latter were attached to a spraying rig. Both groups of mice showed increases in DNA damage during the spraying periods. However, when attempts were made to induce DNA damage in mice under laboratory conditions, using herbicides that were being sprayed in the area, no significant increases in SCEs were seen (unpublished data). The herbicides that were tested included alachlor (2-chloro-2′,6′-diethyl-N-(methoxymethyl)acetanilide), bentazon (3-isopropyl-2,1,3-benzothiadiazin-(4)-one-2,2-dioxide), 2,4-D (2,4-dichlorophenoxyacetic acid), Mecoprop (d,l-2-(4-chloro-o-tolyloxy)-propionic acid) and dicamba (3,6-dichloro-o-anisic acid). Two of these have been known to affect DNA in other studies. Alachlor has been found tumorgenic in mice and 2,4-D has significantly increased SCE values in human lymphocytes (Korte and Jalal, 1982). There is no obvious explanation for the laboratory findings.

Population cage studies also indicate that the genetic makeup of the mice does not appear to be very important in the response to environmental genotoxicants; both wild and inbred mice, although radically different in their genetic heterogeneity, show the equivalent heterogeneity or variance in response. In the laboratory, wild mice showed a slightly greater response to cyclophosphamide than did the inbreds, especially at higher doses.

Although, because of the chromosomal properties of the fish species selected, the **SCE** and **MN assays** could not be used on the bullheads and carp, this does not apply to all fish. The SCE procedure has been used on fish exposed to Rhine River water (Alink et al., 1980) and by Kligerman (1979) who studied SCE induction in the eastern mudminnow by known mutagens. The **MN assay** has also been used on a number of fish including: the marine *Genyonemus lineatus* (white crocker) and *Paralabrax clathratus* (kelp bass) (Hose et al., 1987), the *Umbra limi* or central mudminnow (Walton et al., 1984) and the eastern mudminnow, *Umbra pygmaea* (Hooftman and deRaat, 1982).

The **SCE** and **MN assays** have, likewise, been used in amphibians. Laboratory experiments by Chakrabarti et al. (1984) showed that DNA damage could be detected using the SCE assay on the common Indian toad *Bufo melanosticus.* Siboulett et al., (1984) found that X-irradiation and five chemical agents including benzo(a)pyrene and caffeine increased the MN counts in larvae of the newt *Pleurodeles waltl.* Similar results were obtained by Jaylet et al. (1986) and Grinfield et al. (1986). The **MN assay** has also been used to detect the effects of gamma rays from a cesium source on the erythrocyte chromosomes of *R. catesbiana* tadpoles (Krauter et al., 1987). Some preliminary data are also available on the application of the **SCE** and **MN assays** in amphibians of southwestern Ontario. Both of these assays have been used on *R. pipiens* and *R. clamitans* adults (Mazak, unpublished data: Inman, unpublished data). A comparison involving the simultaneous application of the three assays to adult frogs should be done.

As mentioned earlier, the chromosomal properties of the two species of fish forced us to turn to a third assay, **single cell DNA electrophoresis**. Based on the experiments with low concentrations of known mutagens, there is no doubt that this is a highly sensitive assay in at least some fish and amphibians.

One of the concerns with applying the **SCDE test** to samples of natural populations was the establishment of the baseline level of DNA damage in both the fish and amphibians. This was accomplished by housing some of the wild animals in the laboratory for an extended period of time. In the case of the bullheads and carp, the fish were maintained in dechlorinated water for 3 months. At the end of this period those fish showed DNA damage that was similar in magnitude to that of the hatchery-bred bullheads. A similar approach was used in the amphibians. A considerable reduction in DNA damage was noticed after the tadpoles had been kept in purified water for a four-month period. Unfortunately there are no laboratory bred or maintained amphibian stocks against which the four-month values could be compared. The amphibian tadpoles used in laboratory experiments were tadpoles from the Highgate pond, maintained in the laboratory for at least four months. The **SCDE assay** has not been used on samples of natural populations of mice as yet. Studies comparable to the SCE and MN work are planned.

The results seen in samples of natural populations in all three species strongly suggest that *in situ* monitoring using indigenous organisms should not be ignored mainly because we do not know the full range of factors that affect the stability of DNA in these organisms. Under these circumstances, a battery of agents may impinge on the target organisms resulting not only in additive effects but also synergistic interactions. In both cases undetectable trace level chemicals could be involved. In addition, underlying the chemical effects may be physical stresses operating on the organisms of concern. Such stresses could make organisms more susceptible to genotoxicants. The converse could also occur, that is, environmental or chemical factors inhibiting the damaging action of

a particular agent. *In situ* testing also takes into account the availability of an agent to the target organisms. Existence of a known genotoxicant in the environment will not result in DNA damage, if it does not get to the target sites within the organism. Conversely even though an agent may be at a low concentration in the environment, bioaccumulation in prey may expose the predator to high concentrations of the toxicant. *In situ* monitoring, therefore, overcomes a large number of variables, in determining whether a particular environmental situation is harmful to a species.

Both the **SCE** and **SCDE assays** should be included in a battery of tests determining the toxicity of the environment. They should be considered as strong candidates for keystone assays in monitoring genetic damage because they can be used in *in vivo*, *in situ* studies on large sample sizes. Certainly the **SCDE assay** has considerable potential for use in monitoring the environment with indigenous animals because it can be used on virtually any organism and any tissue. If necessary both assays can and have been used on cell cultures. Although the **MN asssay** has been ignored in this discussion, it too has a role if the environment is heavily polluted. Its main advantage is the ease with which the slides can be prepared and typed. In the case of fish, amphibians and some mammals (e.g. the mouse) peripheral blood can be used as can of course other tissues. Its disadvantage is the lack of sensitivity when low concentrations of agents are encountered.

CONCLUSIONS

Tests to monitor genotoxicity should be part of the battery of tests to monitor the environment for general environmental toxicity. Two of the tests, **sister chromatid exchanges** and **single cell DNA electrophoresis**, used in this study should be included because they have the necessary sensitivity, the applicability to a wide spectrum of organisms, the technical ease of application and low costs per run. The third test, the **micronucleus assay on peripheral blood**, although not as sensitive as the others, could be useful in circumstances where the genotoxicity is high. Where possible, these tests should be used *in situ* on indigenous organisms.

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APPENDIX

The three monitoring systems, **sister chromatid exchanges, micronucleus assay in peripheral erythrocytes** and **single cell DNA electrophoresis**, can easily be used on large sample sizes. The chemicals needed are inexpensive and the technical assistance needed is at the level of a competent graduate of a technology program. The procedures are very straightforward and do not require individuals with advanced background. The individual must, however, be meticulous in following the procedures and consistent in scoring slides. Third year undergraduate students in a biology program have become highly proficient in using these assays.

The **sister chromatid exchange (SCE)** assay is the most time consuming because of the number of steps involved. The implantation of 5-bromo-3'-deoxyuridine (BrdU) beneath the skin above the scapulae will take about 90 minutes for 20 mice. The most labour-intensive segment of the procedure involves the removal of femurs, placing the bone marrow into a hypotonic solution and fixing the tissue. The first part of this step requires at least two people. This phase takes about 4 hours for 20 animals. The preparation of slides is

done the next day and take about two hours. Before exposure to fluorescent lighting the slides are allowed to 'age' overnight. After a 23 hour exposure to fluorescent lights, a slide is removed and stained. If differential staining is observed, the rest of the slides are stained. If not then the remaining slides are exposed for an additional 10 minutes and another slide is stained. Usually by this time differential staining has been acheived and the rest of the slides are stained. The staining procedure takes about one hour. The SCE assay requires five days for processing of 20 samples and then another 12 to 15 hours to score the slides. This is usually done over a three day period and can overlap with the next run. Twenty samples can easily be processed and scored in a five day work week.

The equipment required for the **SCE assay** includes a compound microscope with high quality objectives and condenser, staining jars and a fluorescent light chamber. The chemicals and disposables are relatively inexpensive except for the BrdU which costs $2.20 per tablet. The cost for supplies is less than $3.00 per sample.

The **micronucleus (MN) assay** on peripheral blood is a very simple technique. The animals are bled, a drop of blood is spread on a slide, the slide is fixed and the stained. Twenty mice can be bled and the blood processed in 60 minutes. It usually take between 15 and 25 minutes to score a slide. If staining is done with acridine orange then an epifluorescence microscope is required. For some of the other stains sometimes used an ordinary compound microscope is suitable. In our laboratory the acridine orange is used because it appears to produce fewer artifacts than some of the other stains. The cost per sample is less than 30 cents.

Devolpments concerned with the automation of MN counting are in progress. See, for further information, Castelain et al., (1993), Grawe et al., (1993) and Hyashi (1992).

On the first day the **single cell DNA electrophoresis** or **'comet' assay** involves, typically, obtaining the blood sample, suspending it in agarose, lysing the cells and the electrophoresis. This should take about five hours for a sample of 12 individuals. In our laboratory, the slides are scored the next day and it takes from 15 to 25 minutes to a score each slide. A run of 12 animals can be completed in two days. The cost of supplies per sample is sligthly less than $1.00. In addition to an epifluorescence microscope about $3500 of equipment is required. This includes a power supply, electrophoretic chambers, water bath and pipettors.

For large scale operations there is available a computer-coordinated scoring system. See the contribution of Dr R. Tice in this symposium for a description of such a system.