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aquatic environments with the alkaline single cell gel
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Caged amphibian tadpoles and in situ genotoxicity monitoring of aquatic environments with the alkaline single cell gel electrophoresis (comet) assay

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Abstract

In previous studies we demonstrated that indigenous amphibian tadpoles are suitable organisms for monitoring small bodies of water (e.g., creeks, ponds, and drainage ditches) using the alkaline single cell gel electrophoresis (SCG) or 'comet' assay. This approach involves detection, under alkaline conditions, of cell DNA fragments which on electrophoresis migrate from the nuclear core, resulting in a 'comet with tail' formation. However, although often plentiful, tadpoles are not present in all aquatic environments. Both larger bodies of water (e.g., lakes and rivers) and those impacted upon heavily by man (e.g., bodies of water near industrial sites, on landfills, and in urban areas) often do not support amphibian tadpole populations. An alternative approach to the collection of indigenous tadpoles is to place caged tadpoles at these sites for short term exposures to environmental contaminants. To determine the feasibility of such an approach, *Rana clamitans* (green frog) and *Bufo americanus* (American toad) tadpoles were housed in cages at 11 sites in southwestern Ontario (Canada). In a preliminary experiment, we found that tadpoles caged at a polluted reference site (Tallgrass Prairie Heritage Park in Windsor, Ontario) for either 7 or 14 days showed significant ($P < 0.05$) increases in DNA damage, relative to tadpoles caged in the laboratory in dechlorinated water. As a result we routinely used a 7 day exposure time. Significantly ($P < 0.05$) increased levels of DNA damage, relative to their controls, were observed in tadpoles caged at three sites along two creeks draining a large petrochemical installation south of Sarnia, Ontario; at two sites in the Tallgrass Prairie Heritage Park; and at a site along the Ecarte Channel which is part of the St. Clair River. The DNA damage levels of animals caged in Lake St. Clair, in the Trenton Channel of the Detroit River, at a landfill site, and in two creeks in the city of Windsor did not differ significantly ($P > 0.05$) from their controls. This study demonstrates that caged tadpoles are suitable for monitoring most bodies of fresh water, particularly those aquatic habitats mentioned above where indigenous tadpoles are not present. A combined approach of collecting indigenous tadpoles and using caged tadpoles should provide a sensitive system for aquatic genotoxicity monitoring. © 1998 Elsevier Science B.V.

Keywords: DNA damage; Aquatic genotoxicity monitoring; Alkaline comet assay; Alkaline single cell assay; *Rana clamitans*; *Bufo americanus*; Tadpole; Cage

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1. Introduction

Genotoxic agents continue to be deposited into the environment creating a need for sensitive assays to monitor their accumulation and impact. We recently reported on an in situ assay used to quantify genotoxicity of small bodies of water (e.g., creeks, ponds, and drainage ditches) in southwestern Ontario using four species of amphibian tadpoles and the alkaline single cell gel electrophoresis (SCG) or 'comet' assay [1,2]. In these studies indigenous tadpoles were collected from various sites. However, although often plentiful, tadpoles were not present at all aquatic sites sampled. In particular, larger bodies of water (e.g., lakes and rivers) and bodies of water heavily impacted by man (e.g., bodies located near industrial sites, on landfills, and in urban areas) often do not support amphibian tadpole populations. To address this problem we proposed using tadpoles housed in cages at such sites to monitor short term exposure to environmental contaminants.

A survey of recent literature indicated the broad range of organisms and of situations in which cages have been used for aquatic monitoring. These include: Atlantic tomcod (*Microgadus tomcod*) [3] and whitefish (*Coregonus lavaretus*) [4] for monitoring the impact of effluents of pulp and paper mills; rainbow trout (*Oncorhynchus mykiss*) in monitoring the genotoxicity of a polluted tributary flowing into the River Po in Italy [5]; channel catfish (*Ictalurus punctatus*) in determining the toxicity of an abandoned petroleum refinery [6]; oysters (*Crassostrea virginica*) in quantifying the toxic potential of dredged sediments [7]; and crustaceans (*Gammarus pulex*) in monitoring the genotoxicity of water receiving agricultural runoff [8]. Twenty years ago, caged tadpoles (*Rana temporaria* and *Bufo bufo*) were also used in toxicity studies of several pesticides in small bodies of water [9,10] and recently, *Rana clamitans* tadpoles have been used to determine the persistence and accumulation of the pesticide mexacarbate in an aquatic habitat [11]. The present study involves a more comprehensive approach using caged tadpoles to monitor both large and small bodies of water receiving genotoxicants from industrial, agricultural, and/or urban sources.

In situ cage studies have some advantages over both field and laboratory approaches. Field studies

can be hampered by the mobility of the sentinel organism (not a factor with tadpoles) or, as in this study, by the absence of suitable indigenous animals. Laboratory studies typically do not accurately reflect the complexity of natural habitats. The use of cages avoids these problems and also presents a number of advantages. Firstly, the researcher has knowledge of the history of the organisms being used and can choose individuals at similar developmental stages with a common genetic background, thus reducing interindividual variability prior to exposure. As a result, comparisons among animals housed in cages at different sites may be more informative than comparisons among field samples collected at these sites. Secondly, the site and length of exposure are precise, allowing greater geographic and temporal resolution. The former is particularly important in larger bodies of water. Finally, chronic endpoints can be readily studied since the maintenance requirements (cost and time) are insignificant compared to laboratory exposures.

The tadpoles used in this study include *R. clamitans*, the green frog, and *Bufo americanus*, the American toad. *R. clamitans* is one of the more common amphibians in southwestern Ontario and is ideal for biomonitoring because of its long tadpole lifespan and year round availability. Tadpoles of this species are large (length of 55–80 mm at transformation), appear in early spring and overwinter before transforming [12]. The *B. americanus* tadpoles are much smaller, between 22 and 28 mm at transformation, appear in early spring and complete their metamorphosis by the middle of June [12]. Tadpoles of both species are often found in large numbers so that a single collection can provide enough animals for several dozen cages.

As mentioned, our previous work demonstrated that indigenous tadpoles are suitable for monitoring genotoxicity of small bodies of water which are subject to contaminant inputs from industrial and agricultural sources [1,2]. Laboratory studies have also shown that amphibians generally are sensitive organisms, suitable for the detection of genotoxic agents [13–18].

In this study, we again used the alkaline SCG assay [17] to quantify DNA damage in tadpoles. This technique was originally developed by Rydberg and Johanson [19] who used isolated cells in a microgel

to determine DNA damage. Modifications by Ostling and Johanson [20], involving electrophoresis under neutral conditions, permitted the detection of double-stranded DNA breaks. Subsequently, detection of single-stranded breaks and alkali-labile damage was made possible by Singh et al. [21] who performed the electrophoresis under alkaline conditions (see McKelvey-Martin et al. [22] and Fairbairn et al. [23] for comprehensive reviews of this assay).

Information is reported on the levels of DNA damage in *R. clamitans* and *B. americanus* tadpoles housed in cages for 24 h, 7 days, and 14 days at a polluted reference site within the City of Windsor (Tallgrass Prairie Heritage Park). Subsequently, the study was extended to 7 day exposures at 10 additional sites in southwestern Ontario.

2. Materials and methods

2.1. Chemical reagents

The low melting agarose (electrophoresis purity quality) was obtained from BioRad (Mississauga, Ontario, Canada). Calcium- and magnesium-free phosphate buffered saline (PBS) was purchased from Gibco-BRL (Grand Island, NY). Hank's balanced salt solution, disodium ethylenediamine-tetra-acetate (Na_2 EDTA), tris(hydroxy-methyl)aminomethane hydrochloride (Tris), dimethylsulfoxide (DMSO), ethidium bromide, Na sarcosinate, and Triton X-100 came from Sigma (St. Louis, MO). Fully frosted microscope slides and number one cover glasses were supplied by Fisher (Toronto, Ontario, Canada).

2.2. Monitoring sites

See Fig. 1 for the location of the various monitoring sites in southwestern Ontario (Canada). The specific location of the sites and a brief description are as follows.

(1) Trenton Channel—cages were placed a few metres from shore in the shallow, relatively still water of an inlet (width 50 m), approximately 100 m from the main flow of the Detroit River, at the southern end of the channel.

(2) Turkey Creek—cages were placed a few metres from shore (creek width 30 m) in shallow, slow

moving water, 500 m upstream of its junction with the Detroit River.

(3) Tallgrass Prairie Heritage Park—one set of cages was placed in the centre of a large, irregularly shaped (maximum diameter 150 m), shallow pond located among prairie grasses and primary growth. A second set of cages was placed in a shallow ditch (width 1 m), adjacent to a gravel road and 150 m from the abovementioned pond. The ditch and the pond were not directly connected.

(4) Malden Landfill—cages were placed a few metres from the edge of a large, circular (100 m diameter), shallow pond located on the landfill site and surrounded by secondary growth.

(5) Little River—cages were placed a few metres from the edge of the river (width 10 m) in slow moving water, a few metres in depth, 500 m downstream of the Little River waste water treatment plant (WWTP) and 1 km upstream of its junction with the Detroit River.

(6) Lake St. Clair—the cage was placed in shallow water, sheltered by a partial concrete barrier, at the southeastern tip of Peche Island, which is located at the head of the Detroit River in Lake St. Clair.

(7) Ecarte Channel—cages were placed a few metres from the channel shoreline (channel width 50 m) in shallow, slow moving water, adjacent to a gravel road and 5 km downstream of its junction with the main channel of the St. Clair River.

(8) Talfourd Creek site #1 (downstream)—the cage was placed a few metres from shore (creek width 10 m) in shallow, slow moving water, beneath a highway overpass, near its junction with the St. Clair River.

(9) Marsh Creek—cages were placed in this shallow, slow moving creek (width 1 m), adjacent to a paved road and 500 m upstream of its junction with Talfourd Creek.

(10) Talfourd Creek site #2 (upstream)—cages were placed in the centre of this shallow, slow moving creek (width 5 m), beneath a highway overpass and 5 km upstream of its junction with the St. Clair River.

2.3. Animals

Approximately 200 *R. clamitans* tadpoles were obtained in a single collection in the early summer of

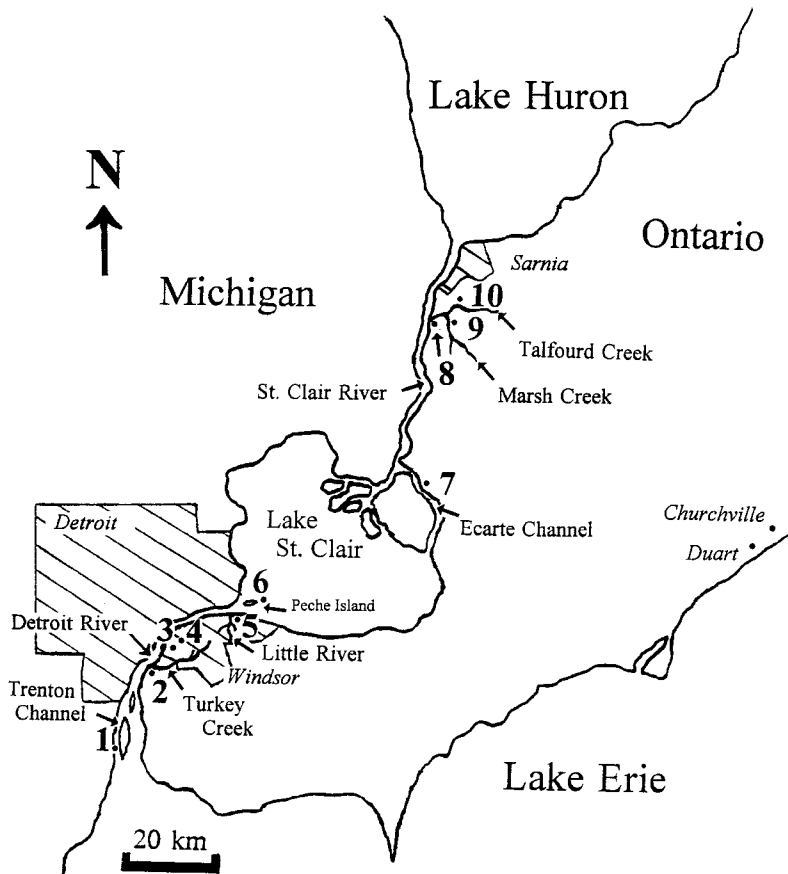


Fig. 1. Map of caged tadpole sites in southwestern Ontario. Cage sites: (1) Trenton Channel, (2) Turkey Creek, (3) Tallgrass Prairie Heritage Park, (4) Malden Landfill, (5) Little River, (6) Lake St. Clair, (7) Ecarte Channel, (8) Talfourd Creek, (9) Marsh Creek, (10) Talfourd Creek site #2 (upstream). Cities and towns listed in italics.

Table 1

Detection of DNA damage in erythrocytes of *R. clamitans* tadpoles placed simultaneously in cages at the Tallgrass Prairie Pond and removed after different durations of exposure

Exposure period	Caged group			Control group ^b (dechlorinated water)		Caged:control DNA ratio ^c	<i>P</i> ^d
	Surviving number	Number processed	DNA length: width ratio ± SEM ^a	Number processed	DNA length: width ratio ± SEM ^a		
24 h	8/8	7	1.513 ± 0.102	4	1.203 ± 0.098	1.26	> 0.05
7 days	5/8	5	2.231 ± 0.075	5	1.587 ± 0.109	1.41	< 0.01
14 days	6/8	6	1.791 ± 0.147	4	1.220 ± 0.085	1.47	< 0.05

^aRatios based on 25 cells/tadpole.

^bDNA length:width ratios of the three control groups were significantly different ($P < 0.05$) and thus the ratios were not pooled for statistical analyses.

^cRatio of DNA length:width ratios in caged tadpoles to DNA length:width ratios in control tadpoles.

^dLevel of significance between caged and control groups based on Student's one-tailed *t*-test.

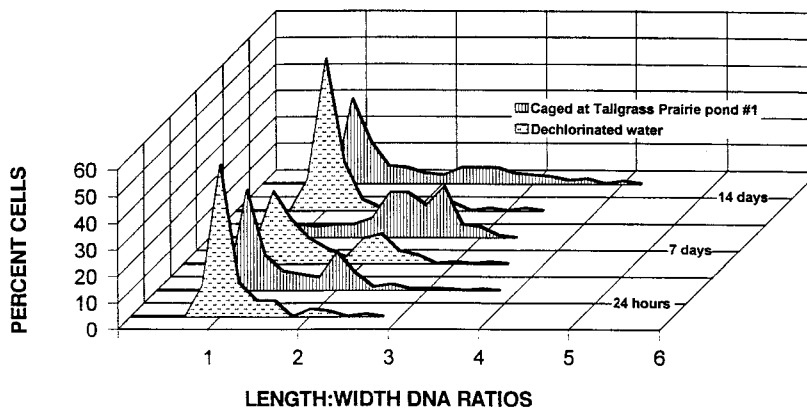


Fig. 2. Distribution of DNA damage (based on length:width ratios of DNA patterns) observed at the cellular level in *R. clamitans* tadpoles housed simultaneously in cages in the Tallgrass Prairie pond and removed after variable periods of exposure. Tadpoles caged in dechlorinated water in the laboratory are given for comparison.

1994 from a ditch located 1 km west of Churchville, Ontario (Fig. 1). This ditch traverses cultivated fields and is adjacent to a gravel road. Approximately 1000 *B. americanus* tadpoles were obtained in a single collection in the same summer from a pond 5 km east southeast of Duart, Ontario (Fig. 1). This pond

is used by livestock and is surrounded by pasture and cultivated fields. The animals from these two collections were used for all subsequent studies both in the cages and as controls. In the laboratory, the tadpoles were housed in polypropylene containers (29 × 19 × 13 cm) with water from the collection site. Plant

Table 2

Detection of DNA damage in erythrocytes of *R. clamitans* tadpoles housed in cages for 7 days at nine sites in southwestern Ontario

Location	Surviving number	Number processed	DNA length:width ratio ± SEM*	Caged:control DNA ratio**	P***
Marsh Creek	5/8	4	2.782 ± 0.386 ^a	1.63	a vs. i < 0.001
Talfourd Creek site #2 (upstream)	6/8	5	2.715 ± 0.060 ^b	1.59	b vs. i < 0.001
Ecarte Channel	8/8	7	2.701 ± 0.152 ^c	1.58	c vs. i < 0.001
Tallgrass Prairie ditch	8/8	5	2.522 ± 0.123 ^d	1.48	d vs. i < 0.001
Talfourd Creek site #1 (downstream)	8/8	6	2.447 ± 0.091 ^e	1.43	e vs. i < 0.001
Malden Landfill	7/8	6	1.675 ± 0.084 ^f	0.98	f vs. i > 0.05
Trenton Channel	8/8	6	1.449 ± 0.111 ^g	0.85	g vs. i > 0.05
Little River	8/8	8	1.385 ± 0.088 ^h	0.81	h vs. i > 0.05
Control**** (dechlorinated water)		21	1.709 ± 0.032 ⁱ		
Lake St. Clair	7/8	4	1.507 ± 0.022 ^j	1.00	j vs. k > 0.05
Control**** (dechlorinated water)		5	1.500 ± 0.065 ^k		

*Ratios based on 25 cells/tadpole.

**Ratio of the DNA length:width ratios in caged tadpoles to the DNA length:width ratios in control tadpoles.

***Level of significance between caged and control groups based on Student's one-tailed *t*-test.

****Pooled ratios of laboratory maintained tadpoles processed with each run except for the Lake St. Clair sample. Because the control ratios of individual runs did not differ significantly ($P < 0.05$), they were pooled.

*****Laboratory maintained tadpoles processed with tadpoles caged in Lake St. Clair. Ratios were significantly different ($P < 0.05$) from the remaining controls and thus were not pooled with them. The Lake St. Clair animals were processed 4 weeks after the first group.

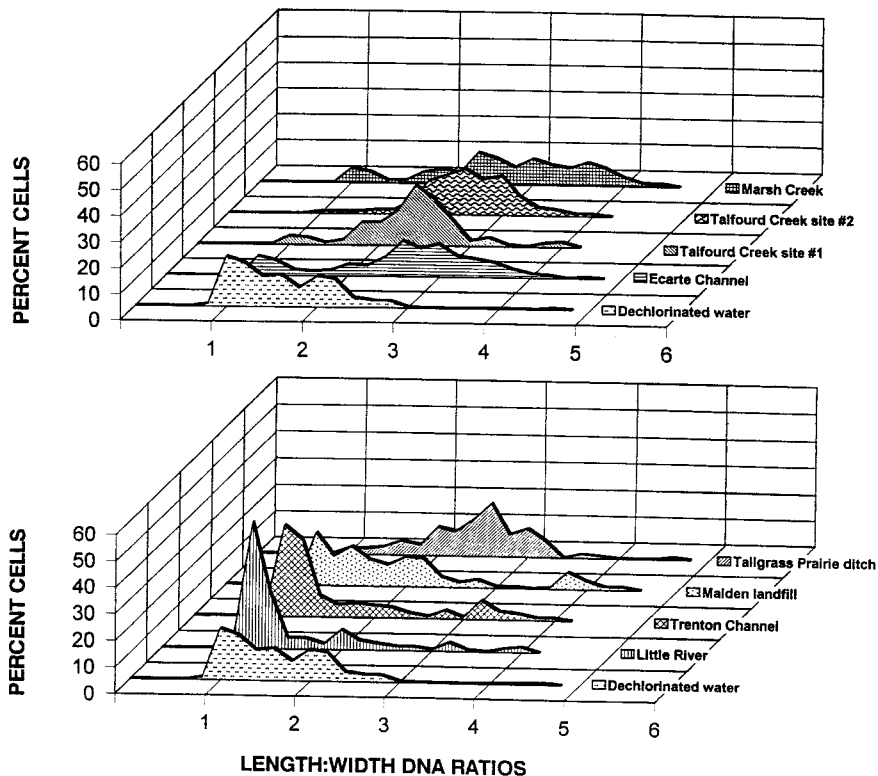


Fig. 3. Distribution of DNA damage (based on length:width ratios of DNA patterns) observed at the cellular level in *R. clamitans* tadpoles housed in cages at eight sites in southwestern Ontario for 7 days. Tadpoles caged in dechlorinated water in the laboratory are given for comparison in both panels.

material from the collection site was added to the containers as a food source. The background level of DNA damage in a subset of these animals was determined within 5 days of their capture; the remainder were transferred to dechlorinated water and fed Wardley shrimp pellets (Wardley, Secaucus, NJ)

ad libitum. The dechlorinated water was completely changed and the containers rinsed every 48 h.

2.4. Alkaline SCG assay

The procedure used was basically that described by Singh et al. [21,24]. Modifications due to the

Table 3

Detection of DNA damage in erythrocytes of *B. americanus* tadpoles placed simultaneously in cages in the Tallgrass Prairie Pond and removed after different durations of exposure

Exposure period	Surviving number	Number processed	DNA length:width ratio \pm SEM ^a	Caged:control DNA ratio ^b	<i>P</i> ^c
24 h	16/16	8	1.329 \pm 0.075	0.95	> 0.05
7 days	10/16	9	1.741 \pm 0.145	1.25	< 0.05
14 days	8/16	8	1.716 \pm 0.084	1.23	< 0.05
Control ^d (dechlorinated water)		12	1.398 \pm 0.089		

^aRatios based on 25 cells/tadpole.

^bRatio of DNA length:width ratios in caged tadpoles to DNA length:width ratios in control tadpoles.

^cLevel of significance between caged and control groups based on Student's one-tailed *t*-test.

^dPooled ratios of laboratory maintained tadpoles processed with each run did not differ significantly (*P* > 0.05) and were thus pooled.

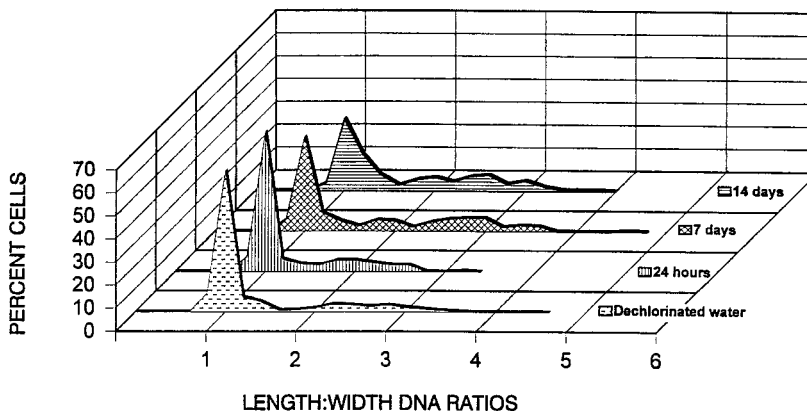


Fig. 4. Distribution of DNA damage (based on length:width ratios of DNA patterns) observed at the cellular level in *B. americanus* tadpoles housed simultaneously in cages in the Tallgrass Prairie pond and removed after variable periods of exposure. Tadpoles caged in dechlorinated water in the laboratory are given for comparison.

Table 4

Detection of DNA damage in erythrocytes of *B. americanus* tadpoles housed in cages for 7 days at five sites in southwestern Ontario

Location	Surviving number	Number processed	DNA length:width ratio \pm SEM ^a	Caged:control DNA ratio ^b	<i>P</i> ^c
Tallgrass Prairie ditch	6/12	6	2.192 \pm 0.165	1.47	< 0.001
Talfourd Creek site #2 (upstream)	8/12	7	1.990 \pm 0.168	1.33	< 0.001
Turkey Creek	8/12	8	1.708 \pm 0.175	1.14	> 0.05
Little River	9/12	8	1.459 \pm 0.091	0.98	> 0.05
Malden Landfill	9/12	9	1.407 \pm 0.070	0.94	> 0.05
Control ^d (dechlorinated water)		18	1.492 \pm 0.040		

^aRatios based on 25 cells/tadpole.

^bRatio of DNA length:width ratios in caged tadpoles to DNA length:width ratios in control tadpoles.

^cLevel of significance between caged and control groups based on Student's one-tailed *t*-test.

^dRatios of laboratory maintained tadpoles processed with each run did not differ significantly (*P* > 0.05) and were thus pooled.

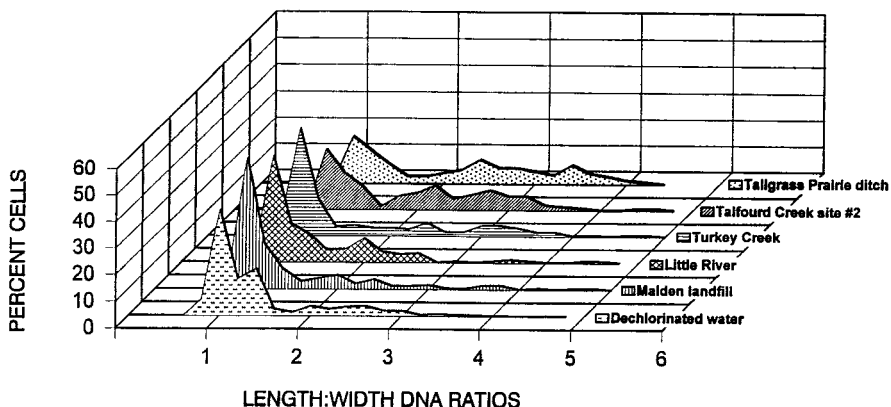


Fig. 5. Distribution of DNA damage (based on length:width ratios of DNA patterns) observed at the cellular level in *B. americanus* tadpoles housed in cages at five sites in southwestern Ontario for 7 days. Tadpoles caged in dechlorinated water in the laboratory are given for comparison.

Table 5
DNA damage in erythrocytes of individual *R. clamitans* tadpoles placed in cages at various sites in southwestern Ontario

Exposure site and duration	Animal number	Caged group DNA length: width ratio \pm SD	Animal number	Control group DNA length: width ratio \pm SD
Tallgrass Prairie pond—24 h	1	1.870 \pm 0.501	1	1.094 \pm 0.128
	2	1.558 \pm 0.622	2	1.285 \pm 0.291
	3	1.076 \pm 0.111	3	0.998 \pm 0.070
	4	1.764 \pm 0.444	4	1.433 \pm 0.473
	5	1.287 \pm 0.348		
	6	1.489 \pm 0.681		
	7	1.548 \pm 0.563		
Tallgrass Prairie pond—7 days	1	2.088 \pm 0.707	1	1.862 \pm 0.584
	2	2.356 \pm 0.431	2	1.727 \pm 0.708
	3	2.459 \pm 0.356	3	1.311 \pm 0.387
	4	2.147 \pm 0.363	4	1.348 \pm 0.419
	5	2.103 \pm 0.523	5	1.686 \pm 0.591
Tallgrass Prairie pond—14 days	1	1.890 \pm 0.733	1	1.117 \pm 0.182
	2	1.986 \pm 0.945	2	1.145 \pm 0.213
	3	1.276 \pm 0.469	3	1.146 \pm 0.467
	4	2.303 \pm 0.740	4	1.472 \pm 0.589
	5	1.763 \pm 0.965		
	6	1.530 \pm 0.451		
Marsh Creek—7 days	1	3.640 \pm 0.384	1	1.688 \pm 0.542
	2	3.104 \pm 0.504	2	1.639 \pm 0.569
	3	1.838 \pm 0.553	3	1.711 \pm 0.517
	4	2.545 \pm 0.504		
Talfourd Creek site #2—7 days	1	2.682 \pm 0.440	1	1.800 \pm 0.629
	2	2.737 \pm 0.665	2	1.668 \pm 0.543
	3	2.702 \pm 0.292	3	1.617 \pm 0.533
	4	2.913 \pm 0.412	4	1.749 \pm 0.488
	5	2.539 \pm 0.542		
Ecarte Channel—7 days	1	2.936 \pm 0.860	1	1.436 \pm 0.504
	2	3.291 \pm 0.781	2	1.791 \pm 0.612
	3	2.371 \pm 0.594		
	4	2.320 \pm 0.859		
	5	2.848 \pm 0.772		
	6	2.931 \pm 0.686		
	7	2.212 \pm 0.682		
Tallgrass Prairie ditch—7 days	1	2.818 \pm 0.496	1	1.613 \pm 0.367
	2	2.745 \pm 0.699	2	1.883 \pm 0.531
	3	2.135 \pm 0.575	3	1.763 \pm 0.479
	4	2.409 \pm 0.653	4	1.938 \pm 0.756
	5	2.501 \pm 0.377		
Talfourd Creek site #1—7 days	1	2.496 \pm 0.454	1	1.744 \pm 0.427
	2	2.708 \pm 0.553	2	1.661 \pm 0.418
	3	2.141 \pm 0.359		
	4	2.362 \pm 0.474		
	5	2.292 \pm 0.664		
	6	2.681 \pm 0.537		
Malden Landfill—7 days	1	1.561 \pm 0.429	1	1.712 \pm 0.485
	2	1.402 \pm 0.521	2	1.384 \pm 0.343
	3	1.661 \pm 0.356		
	4	2.000 \pm 0.410		
	5	1.630 \pm 0.493		
	6	1.797 \pm 0.763		
Trenton Channel—7 days	1	1.934 \pm 0.532	1	1.883 \pm 0.425
	2	1.287 \pm 0.375	2	1.700 \pm 0.488

Table 5 (continued)

Exposure site and duration	Animal number	Caged group DNA length: width ratio \pm SD	Animal number	Control group DNA length: width ratio \pm SD
Little River—7 days	3	1.138 \pm 0.110		
	4	1.465 \pm 0.634		
	5	1.368 \pm 0.337		
	6	1.502 \pm 0.379		
	1	1.417 \pm 0.483	1	1.555 \pm 0.582
	2	1.498 \pm 0.550	2	1.960 \pm 0.610
	3	1.243 \pm 0.381		
	4	1.240 \pm 0.490		
Lake St. Clair—7 days	5	1.161 \pm 0.203		
	6	1.924 \pm 0.643		
	7	1.210 \pm 0.226		
	8	1.397 \pm 0.457		
	1	1.513 \pm 0.606	1	1.699 \pm 0.525
	2	1.481 \pm 0.659	2	1.424 \pm 0.386
	3	1.469 \pm 0.366	3	1.500 \pm 0.457
	4	1.565 \pm 0.500	4	1.312 \pm 0.414
		5	1.566 \pm 0.454	

Summaries of these data are presented in Tables 1 and 2.

uniqueness of the biological material studied and to the equipment available were relatively minor. The modified procedure was described fully by Ralph et al. [17].

Blood samples were collected from *R. clamitans* tadpoles by decapitation followed by immediately placing the animals in a 10% solution of Hank's balanced salt solution for 2 min. Blood samples were collected from *B. americanus* tadpoles by mincing them with scissors in a 10% solution of Hank's balanced salt solution and leaving the tadpoles in this solution for 10 min. All animals were treated individually. Microscopic examination showed that the vast majority of cells collected were erythrocytes. Erythrocytes were chosen because they are nucleated in amphibians. Serial dilutions were made so that three or four cells would be seen without crowding in a single field at 400 \times magnification. The appropriate erythrocyte suspension was then mixed with 0.5% low melting agarose and this suspension was pipetted onto fully frosted slides and covered with coverslips. The slides were stored at 3°C for 20 min to allow complete polymerization of the agarose. A single layer of agarose was used as described by Pandrangi et al. [25].

After the agarose polymerized, the coverslips were removed and the slides were lowered into freshly

made lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 10% DMSO, 1% Na sarcosinate, 1% Triton-X 100, pH 10.0) and incubated at room temperature in the dark for 2 h. After lysing, the slides were drained and placed in the alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na₂EDTA, pH 13.0) at 3°C for 15 min to form single-stranded DNA. For electrophoresis, the power supply was set at 25 V and the current adjusted to 265–270 mA by slowly changing the buffer level in the tray. Slides were routinely exposed to this current in the dark at 3°C for 20 min. After electrophoresis, the slides were placed in a staining tray and covered with a neutralizing buffer (0.4 M Tris, pH 7.5) in the dark for 5 min. This last step was repeated. The slides were then drained and overlaid with ethidium bromide and covered with coverslips. The slides were examined the next day at 400 \times using an Olympus RH-2 epifluorescent microscope. All slides were coded and examined randomly. Routinely, 25 cells were examined per animal. The length and width of the DNA masses were measured using an ocular micrometer disk. The length:width ratios were used in all comparisons. Under these conditions, DNA patterns from *R. clamitans* and *B. americanus* tadpoles with a ratio of one have DNA lengths of approximately 40 and 30 μ m, respectively, and those

Table 6

DNA damage in erythrocytes of individual *B. americanus* tadpoles placed in cages at various sites in southwestern Ontario

Exposure site and duration	Animal number	Caged group DNA length: width ratio \pm SD	Animal number	Control group DNA length: width ratio \pm SD
Tallgrass Prairie pond—24 h	1	1.221 \pm 0.363	1	1.054 \pm 0.147
	2	1.225 \pm 0.402	2	1.312 \pm 0.394
	3	1.143 \pm 0.301	3	0.182 \pm 0.391
	4	1.465 \pm 0.529	4	1.065 \pm 0.131
	5	1.776 \pm 0.563		
	6	1.160 \pm 0.420		
	7	1.376 \pm 0.616		
	8	1.263 \pm 0.517		
Tallgrass Prairie pond—7 days	1	2.294 \pm 1.025	1	1.161 \pm 0.698
	2	1.326 \pm 0.559	2	1.934 \pm 0.866
	3	1.975 \pm 0.863	3	1.719 \pm 0.896
	4	2.403 \pm 0.929	4	1.337 \pm 0.638
	5	1.241 \pm 0.503		
	6	1.917 \pm 0.777		
	7	1.472 \pm 0.873		
	8	1.734 \pm 0.707		
	9	1.308 \pm 0.508		
Tallgrass Prairie pond—14 days	1	1.779 \pm 0.711	1	1.117 \pm 0.147
	2	1.617 \pm 0.743	2	1.565 \pm 0.689
	3	1.642 \pm 0.652	3	1.495 \pm 0.757
	4	1.922 \pm 0.904	4	1.832 \pm 0.996
	5	1.910 \pm 0.802		
	6	1.244 \pm 0.421		
	7	1.980 \pm 0.694		
	8	1.633 \pm 0.748		
Tallgrass Prairie ditch—7 days	1	2.100 \pm 0.936	1	1.514 \pm 0.512
	2	2.682 \pm 0.943	2	1.621 \pm 0.655
	3	2.317 \pm 0.763	3	1.457 \pm 0.671
	4	2.097 \pm 1.001	4	1.518 \pm 0.586
	5	2.452 \pm 0.964		
	6	2.501 \pm 0.725		
Talfourd Creek site #2—7 days	1	1.872 \pm 0.743	1	1.232 \pm 0.491
	2	2.047 \pm 0.610	2	1.534 \pm 0.656
	3	2.217 \pm 0.737	3	1.456 \pm 0.657
	4	1.518 \pm 0.520	4	1.657 \pm 0.757
	5	1.968 \pm 0.975		
	6	2.802 \pm 0.970		
	7	1.504 \pm 0.654		
Turkey Creek—7 days	1	2.226 \pm 1.059	1	1.439 \pm 0.621
	2	1.430 \pm 0.749	2	1.409 \pm 0.625
	3	2.005 \pm 1.204	3	1.601 \pm 0.772
	4	1.277 \pm 0.474	4	1.442 \pm 0.596
	5	2.580 \pm 0.935		
	6	1.469 \pm 0.700		
	7	1.415 \pm 0.653		
Little River—7 days	8	1.263 \pm 0.480		
	1	1.321 \pm 0.522	1	1.241 \pm 0.512
	2	1.134 \pm 0.343	2	1.167 \pm 0.457
	3	1.403 \pm 0.420	3	1.674 \pm 0.747
	4	1.676 \pm 0.437		
5	1.685 \pm 0.537			

Table 6 (continued)

Exposure site and duration	Animal number	Caged group DNA length: width ratio \pm SD	Animal number	Control group DNA length: width ratio \pm SD
Malden Landfill—7 days	6	1.869 \pm 0.671		
	7	1.219 \pm 0.346		
	8	1.361 \pm 0.378		
	1	1.266 \pm 0.524	1	1.407 \pm 0.594
	2	1.714 \pm 0.543	2	1.817 \pm 1.014
	3	1.534 \pm 0.624	3	1.672 \pm 0.847
	4	1.219 \pm 0.296		
	5	1.376 \pm 0.585		
	6	1.609 \pm 0.702		
	7	1.182 \pm 0.248		
	8	1.593 \pm 0.541		
	9	1.168 \pm 0.265		

Summaries of these data are presented in Tables 3 and 4.

with ratios of three have DNA lengths of approximately 120 and 90 μ m, respectively. Cell viability was found to be more than 95% using the trypan blue exclusion technique.

2.5. Caged animals

R. clamitans tadpoles were maintained in the laboratory in dechlorinated water for 10 weeks and *B. americanus* tadpoles for 6 weeks before the onset of testing. Cages were constructed from two sheets of plastic mesh (40 \times 25 cm, 2 \times 2 mm holes) sown together with fishing line. Each cage contained a mesh metal ring (1 \times 1 cm holes) to maintain its shape. The cage diameter was approximately 15 cm. Vegetation from each site was placed in the cages as a food source and pebbles were added to keep the cage submerged. Cages were anchored at each site, above the sediment, either with metal rods or by attaching them with a cord to vegetation on shore. Once removed, animals were maintained in water from the test site until bled, which occurred within 3 days of removal. Caged tadpoles maintained in the laboratory in dechlorinated water served as controls. Each electrophoretic run included animals of both exposed and control groups.

2.6. Statistical analysis

An alpha level of 0.05 was used to determine significance in all statistical analyses. Different groups were compared using a Student's one-tailed

t-test. The comparisons were based on the mean values of individual animals.

3. Results

Freshly caught *R. clamitans* tadpoles collected from the Churchville ditch gave DNA length:width ratios of 2.082 ± 0.082 SE ($n = 10$) and *B. americanus* tadpoles from the Duart pond gave ratios of 2.071 ± 0.068 ($n = 11$).

Normally, all surviving tadpoles were processed immediately upon removal from their cages. The only exceptions were eight of the 16 *B. americanus* tadpoles from the 24 h exposure group (Table 1), and the occasional animal which died awaiting processing. Also, some were not typed because the slides were found unscorable because of technical difficulties (e.g., the agarose did not adhere properly to the slide during processing).

To determine the appropriate duration of exposure to environmental contaminants, groups of *R. clamitans* tadpoles caged simultaneously in the Tallgrass Prairie pond were assayed for DNA damage at different times. The tadpoles were removed after 24 h, 7 days, and 14 days of exposure. In all three exposures, tadpoles caged at this site gave greater DNA length:width ratios than tadpoles caged in dechlorinated water in the laboratory (Table 1). However, only tadpoles caged for 7 or 14 days gave significantly greater ($P < 0.01$ and $P < 0.05$, respectively)

In spite of its numerous positive features, the use of the SCG assay in situ using sentinel organisms has been limited (see Tice [27] and Ralph and Petras [1] for further discussion). Recently, we described the adaptation of the alkaline SCG assay for amphibian tadpoles [17] and the application of this assay for in situ studies using four species of amphibians [1,2]. In these studies we collected tadpoles indigenous to selected sites in southwestern Ontario. However, since tadpoles were not present in all aquatic habitats sampled, cages containing tadpoles were placed at some of these sites in the current study. The sites included small bodies of water (e.g., creeks, ponds, and drainage ditches) associated with industrial areas (sites #2, 8–10), a landfill (site #4), and a WWTP (site #5). To examine the suitability of caged tadpoles for monitoring larger bodies of water (e.g., lakes and rivers), we placed cages in the Trenton (site #1) and Ecarte (site #7) Channels, which are considered polluted, and in Lake St. Clair (site #6), which is considered relatively 'pristine'. Finally, for comparison with indigenous animals subjected to chronic exposure to environmental contaminants, cages were placed at two sites in the Tallgrass Prairie (site #3) where we had previously collected tadpoles with high levels of DNA damage.

After only 24 h of exposure, *R. clamitans* (green frog) tadpoles placed in the Tallgrass Prairie pond had increased (but not significantly) DNA damage relative to controls. The DNA ratios were, however, significantly higher after 7 and 14 day exposures (Table 1). *R. clamitans* tadpoles housed in another cage placed 150 m away in the Tallgrass Prairie ditch also showed significantly increased DNA ratios after a 7 day exposure (Table 2). This area, in Windsor, Ontario, is within 2 km of possible sources of contaminants including a petrochemical installation, a WWTP, and a closed sanitary landfill (Malden Landfill). As well, the industrial complexes (oil refineries, steel mills, foundries, and auto manufacturers) in Detroit, MI, 4 km to the west, may contribute contaminants through atmospheric deposition. This has been shown to be an important pathway for the introduction of some toxic substances into the Great Lakes [28]. Finally, internal combustion engine emissions associated with the large populations of Detroit and Windsor (approximately 4 million and 200 000 citizens, respectively) may likewise release genotoxi-

cants into the area. There is, however, no direct evidence of the specific contribution of any of these sources to the genotoxicant load (see Edsall et al. [29] and Manny et al. [30]).

Samples of tadpoles of *R. clamitans* and *Rana pipiens* (leopard frog), collected in 1994 and 1995 from the Tallgrass Prairie pond used in the current study, gave DNA ratios ranging from 2.175 to 2.487 (three samples totalling 32 tadpoles) (1). *R. pipiens* tadpoles collected in 1994 and 1995 from the Tallgrass Prairie ditch gave DNA ratios ranging from 2.650 to 2.956 (5 samples totalling 45 tadpoles). The *R. pipiens* data are used for comparison because no *R. clamitans* were found in this ditch [1]. The DNA ratios obtained using caged *R. clamitans* tadpoles, therefore, appear to be comparable to those of indigenous samples from these sites.

The levels of DNA damage among *R. clamitans* tadpoles placed in cages at other sites in the city of Windsor (Malden Landfill and Little River) did not differ significantly from controls (Table 2). The low DNA ratios in tadpoles caged at the Malden Landfill site, a closed sanitary landfill, suggested that there was little if any leaching of genotoxicants into the pond. Unfortunately, to the best of our knowledge, no specific information regarding the levels of genotoxicants at the landfill site is available. The cage in Little River was located downstream of the Little River WWTP, which treats mainly domestic sewage and discharges from a few small industrial plants. Johnson and Kauss [31], on examining contaminant levels in water, suspended solids, and bottom sediments of 13 tributaries on the Canadian side of the St. Clair River, the Detroit River, and Lake St. Clair, found the levels of some industrial organic compounds (hexachlorobenzene, hexachlorobutadiene, and phenols) and pesticides (dichlorodiphenyltrichloroethane (DDT) and its metabolites, dieldrin, and lindane) to be detectable but, in general, lower in Little River than in other tributaries. However, inorganics (aluminum, arsenic, cadmium, chromium, copper, iron, lead, mercury, nickel, and zinc) frequently exceeded provincial water quality objectives (PWQOs) and/or dredging guidelines (DGs), but again, these were not disproportionately high relative to other tributaries.

R. clamitans tadpoles placed in cages along Marsh Creek and at two sites in Talfourd Creek gave DNA

The DNA ratios of *B. americanus* tadpoles caged at other sites in the city of Windsor (Malden Landfill, Little River, and Turkey Creek) did not differ significantly from dechlorinated water controls, though there was an increase in the DNA ratios of animals caged at Turkey Creek (Table 4). Turkey Creek receives water from a series of drains and ditches in south Windsor that may receive genotoxic inputs from sources such as a large metal recycling yard, several auto manufacturing plants, and some farmland [31]. Johnson and Kauss [31], sampling in Turkey Creek, found phenols, inorganic compounds (aluminum, arsenic, cadmium, chromium, copper, iron, lead, mercury, nickel, and zinc) and some pesticides (lindane, endrin, endosulphan, dieldrin, and DDT metabolites) frequently present and, in some cases, exceeding PWQOs and/or DGs. However, these values were not disproportionately high relative to other tributaries.

Finally, *B. americanus* tadpoles placed at the upstream Talfourd Creek site showed significantly increased levels of DNA damage relative to the dechlorinated water controls (Table 4), which is not unexpected based on the contaminant data discussed above.

Based on caged:control ratios, the levels of DNA damage in both *R. clamitans* and *B. americanus* caged simultaneously at the Tallgrass Prairie ditch, Little River, and the Malden Landfill are comparable (Tables 2 and 4). By the same comparison, *R. clamitans* caged at the Tallgrass Prairie pond (all exposure times studied) and in Talfourd Creek (upstream site) had higher levels of DNA damage than *B. americanus* caged at the same time at these sites (Tables 1–4). This observation that *R. clamitans* tadpoles may be more sensitive to environmental contaminants is similar to the pattern seen with tadpoles collected from the wild [2].

At three of the nine sites (Marsh Creek, Trenton Channel and Ecarte Channel) where both species were caged, *B. americanus* tadpoles did not survive a 7 day exposure whereas *R. clamitans* did. No attempt was made to house *B. americanus* in cages at either the downstream Talfourd Creek site or in Lake St. Clair. In contrast, of the 11 sites chosen for *R. clamitans*, only those caged at Turkey Creek did not survive a 7 day exposure. In general, caged *B. americanus* tadpoles do not appear to be as hardy as

R. clamitans, which is not unexpected considering their size and their absence from turbulent waters.

5. Conclusion

Caged *R. clamitans* and *B. americanus* tadpoles appear to be suitable organisms for monitoring genotoxicity of aquatic habitats. The caged animals complement the use of indigenous organisms. Caged tadpoles may be especially useful, as shown by cages placed in streams draining the 'Chemical Valley', in determining the impact of genotoxic agents released from industrial sources. Both species can be used but, the larger *R. clamitans* tadpoles may be more suitable because they can be found throughout the year, appear to be more sensitive, and survive better in turbulent waters. Finally, the combination of caged tadpoles and/or indigenous tadpoles and the SCG assay should result in a genotoxicity monitoring system that is applicable to most freshwater habitats.

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