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Flow cytometric assay for in vivo genotoxic effects of pesticides in Green frogs (*Rana clamitans*)

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Abstract

Frogs from farming regions in Quebec suffer a suite of physical and physiological problems associated with the use of agricultural pesticides. Flow cytometry was used to compare incidence of abnormal DNA profile, half-peak coefficient of variation (*CV*), and variation in genome size (pg DNA per haploid nucleus) between Green frogs (*Rana clamitans*) from such farming areas (corn and potato fields) and control sites dissociated from agricultural practices, to infer possible genomic manifestations of pesticide use. There was a significant increase in abnormal DNA profile in individuals from corn fields relative to the control sites ($P<0.05$). In all comparisons, adult frogs showed greater *CV*s than did juveniles ($P<0.0001$). Among adults, *CV*s were higher for samples taken from both potato and corn fields relative to the control samples ($P<0.005$), while in juveniles, only individuals from corn plots showed elevated *CV*s relative to controls ($P<0.05$). Juveniles showing physical deformity had significantly higher *CV*s than normal individuals ($P<0.05$), although there were no similar correlations with physiological disruption. Mean *C*-value (variation in DNA content) was different between adults and juveniles in all treatments ($P<0.0001$), but there were no significant differences in mean *C*-value and variance of such among similar age classes between treatments. The different classes of DNA damage found in this study are reflective of either acute or cumulative pesticide toxicity, and are exhibited by both sick and apparently normal individuals. We therefore believe flow cytometry to be a powerful technique for the measurement of pesticide-induced genomic disruption in amphibian populations. © 1997 Elsevier Science B.V.

Keywords: Flow cytometry; Pesticide; Amphibian; Genotoxicity; Aneuploidy

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1. Introduction

The use of flow cytometry (FCM) in identifying the cytological results of exposure to environmental mutagens is in its infancy. Nevertheless, early work has shown FCM to be a powerful tool for identifying and measuring aneugenic and clastogenic effects of environmental contaminants on the vertebrate genome (Deaven, 1982; McBee and Bickham, 1988; Bickham et al., 1988; Lamb et al., 1991; Fernandez et al., 1993). Although potential sources of error within the technology have not been fully evaluated (Vindeløv et al., 1983; Tiersch and Wachtel, 1993), the rapidity, accuracy and relative inexpense of FCM compared with other cytological methods make it well-suited for screening large numbers of samples. Thus, FCM represents: (a) a method by which populations may be surveyed for genetic damage when no physical or physiological manifestations exist; and (b) a source of complimentary/comparative data when such manifestations are apparent.

Amphibians, with their semi-permeable skins and biphasic life cycles, are particularly vulnerable to toxins in the environment (Harfenist et al., 1989; Bishop, 1992; Clark, 1992; Mahaney, 1994). For many species, this means potential exposure to contaminants in the aquatic medium during critical embryonic, larval and juvenile stages, as well as direct and indirect exposure during later aquatic/terrestrial adult stages. Pesticide application on croplands conjoining aquatic habitats that are utilised by breeding amphibians exemplifies this dual risk, and provides an ideal opportunity to assess the extent of the hazard. Severe chemical shocking can occur in such situations, wherein high concentrations of toxins find their way into breeding habitat either directly through application and runoff, or indirectly through the water table.

Despite widespread acknowledgement of their vulnerability and potential role as indicator organisms (Powell et al., 1982; Hall and Henry, 1992), amphibians have been the focus of comparatively little of the study devoted to the effects of environmental contaminants on vertebrates (Bishop, 1992). Moreover, in comparison with the number of laboratory studies of these effects, field studies have been few, and comprehensive ones even fewer (Harfenist et al., 1989).

Physical deformity and physiological stress have been reported in Green frogs (*Rana clamitans*) occupying ponds exposed to agricultural pesticides in the St. Lawrence River valley of Québec (Bonin et al., 1997; Ouellet et al., 1994). During the course of that investigation, blood samples taken from both sick and healthy frogs were analysed by FCM. We compared the incidence of aneuploidy, mosaicism, and other abnormal DNA profiles in pesticide-exposed and control populations. We also compared the coefficient of variation (*CV*) in DNA fluorescence as a measure of intraindividual genome size variability within and among pesticide-exposed and control populations for individuals exhibiting normal DNA profiles. Our assumption was that clastogenic effects of pesticide exposure would result in higher individual *CV*s for normal DNA profiles, as reported by Bickham et al. (1988) for turtles. The variation in DNA content (*C*-value; pg DNA per haploid genome) among individuals of different populations was also analysed, with the assumption that pesticide-exposed populations would exhibit greater variation. In addition, we

attempted to correlate these DNA parameters with physical deformity and physiological stress observed in the sampled frogs.

2. Materials and methods

Several populations occupying ponds or ditches on or adjacent to plots of either potatoes or corn, and three control populations from non-agricultural areas, were used in the analysis (Table 1). Second and third-year adult individuals were caught by hand or dipnet in July 1993, 1–5 days following the application of pesticides: carbofuran (Furadan®) on corn, and azinphos-methyl (Guthion®) on potatoes. Various other pesticides had been applied to the crop sites prior to our collection (Table 2). Newly metamorphosed juveniles, present in the ponds as larvae during pesticide applications, were collected by dipnet in late August, 1993.

Water samples were also collected from the sites while we collected adult frogs. Two test parameters of water genotoxicity were evaluated using cultured trout (*Oncorhynchus mykiss*) hepatocytes (Gagné and Blaise, 1995):

1. DNA integrity was evaluated by alkali precipitation (Olive et al., 1988);
2. chromatin damage was estimated by relative amounts of DNA repair as determined by the incorporation of labelled nucleotides (Snyder and Matheson, 1985).

Blood samples for FCM were taken in the field from decerebrated animals (according to Canadian Council of Animal Care guidelines) as follows: from a cardiac puncture, 20 μl of blood were collected in a microhaematocrit capillary tube and suspended in 200 μl of a sodium-citrate/sucrose freezing solution containing dimethylsulphoxide (DMSO) as anticoagulant (Tank et al., 1987; Lowcock et al., 1991). Samples were flash-frozen in liquid nitrogen in the field, transported to the laboratory of the Redpath Museum, McGill University, and stored in an ultracold freezer at −80°C until use.

Table 1
Site characteristics of samples used in flow cytometric analysis

Site no.	Crop	Longitude	Latitude	Habitat
1	potato	71°49′ W	45°14′ N	Pond
2	potato	73°13′ W	46°02′ N	Pond
3	corn	73°33′ W	45°46′ N	Ditch
4	corn	73°33′ W	45°46′ N	Pond
5	corn	73°33′ W	45°46′ N	Ditch
6[a]	none	73°15′ W	45°47′ N	Sand pit
7[a]	none	71°58′ W	45°16′ N	Pond
8[a]	none	73°41′ W	45°36′ N	Ditch

[a]Control sites.

Prior to FCM, samples were thawed rapidly in warm water to 4°C, and then stained with 750 μl amphibian propidium iodide (API; Tank et al., 1987) for 3–4 h using frequent agitation. To control for error due to variance in sample channel number, we added 40 μl of prepared *Xenopus laevis* blood from a single individual (Ward's Scientific; 2c DNA = 6.2 pg) to each sample as an internal standard prior to staining. All samples were filtered through a Nytex membrane (40 μm pore size) before FCM analysis. DNA profiles from propidium iodide-stained erythrocyte nuclei were generated on an EPICS Profile flow cytometer (Coulter Electronics, Hialeah, FL). The flow cytometric analysis of all samples was performed over 3 days, using a random assortment of sample tubes each day. We found it unnecessary to adjust the tonicity of the sheath fluid in the EPICS Profile as described by Licht and Lowcock (1991) for the EPICS V.

Red fluorescence values were collected from at least 15 000 nuclei per sample and plotted as histograms by the MDADS computer system (Coulter Electronics). Half-peak coefficient of variation (*CV*) was determined for each histogram. The *C*-value of each sample utilised in the analysis of normal profiles was calculated through linear comparison of sample peak channel number with channel number of the internal standard (STD) as follows

$$C\text{-value of sample} = \frac{C\text{-value of STD} \times \text{sample channel no.}}{\text{STD channel no.}}$$

We controlled for variations in flow cytometric conditions by using only those profiles whose internal control *CV* fell between 2.65 and 2.75, as this control sample had been taken from the same individual *X. laevis* in all cases. All subsequent statistical comparisons were made between the *R. clamitans* values taken from the accepted profiles. To conduct intergroup comparisons of characteristic *CV*s, the data were first evaluated qualitatively, and histograms with multiple peaks or other asymmetries were removed from the data set so that *CV* comparisons were conducted between only those individuals with normal, symmetrical DNA profiles (Bickham et al., 1988). Abnormal profiles (Fig. 1) are reflective of aneuploid mosa-

Table 2
Pesticides used at the study sites and approximate application dates

Crop	May	June	July	August
Potato	Linuron Metribuzin Phorate	Azinphos-methyl Cypermethrin Deltamethrin Oxamyl Mancozeb Metalaxyl Chlorothalonil		Diquat
Corn	Atrazine Glyphosate Butylate		Carbofuran	

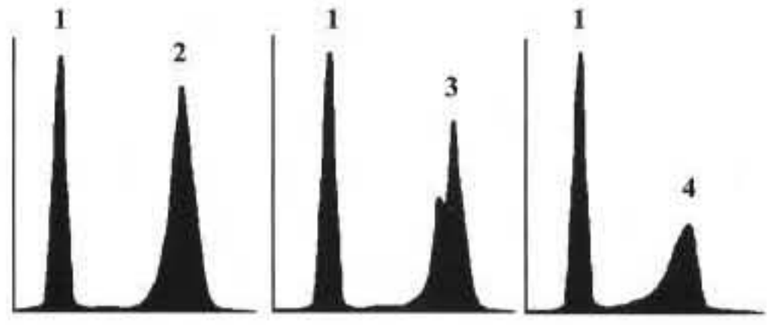

Fig. 1. *Rana clamitans* DNA profiles encountered in flow cytometric analysis. In all cases, peak 1 represents internal *Xenopus laevis* control whose CV fell between 2.65 and 2.75. Peak 2 represents normal sample peak used in statistical comparisons of CV values. Peaks 3 (aneuploid) and 4 (asymmetric) represent profiles which were removed from quantitative analysis, but which were used in qualitative analysis of abnormal profiles.

icism or chromosomal fragmentation phenomena that were inconsistent with the nature of our hypothesis regarding *CV*s, but which were considered as other possible manifestations of pesticide exposure (Table 3).

Non-parametric tests were used for all comparisons between *R. clamitans* samples (Sokal and Rohlf, 1981; Daniel, 1990). Mann–Whitney *U*-test tests were used for *CV* and *C*-value comparisons between two groups, and the Kruskal–Wallis test was used for similar comparisons between more than two groups. We used one-tailed tests where it was hypothesised that a particular sample would exhibit higher *CV*, and two-tailed tests where there was no directional hypothesis. A Fisher Exact test was used to evaluate the effect of intraerythrocytic parasites on DNA profiles, as well as to compare the frequency of various DNA profiles between groups.

3. Results

Nine aneuploids, one polyploid and 18 individuals with abnormal DNA profiles were encountered (Table 3). There was a significant difference in the frequencies of normal and abnormal DNA profiles between corn and control (Fisher Exact test, $P = 0.03$), whereas there was no significant difference between potato and control

Table 3
Site-characteristic DNA parameters derived from FCM profiles

Crop	Age class	*C*-value[a]	DNA profile (*n*)			
		(pg)	Normal	Abnormal	Aneuploid	Polyploid
Potato	Adult	6.00 ± 0.24	11	2	1	0
Potato	Juvenile	6.36 ± 0.14	21	8	1	0
Corn	Adult	6.00 ± 0.22	16	8	6	0
Corn	Juvenile	6.34 ± 0.10	6	0	0	0
Control	Adult	6.21 ± 0.22	7	0	1	1
Control	Juvenile	6.33 ± 0.17	9	0	0	0

[a]Mean ± SD.

($P > 0.05$) and potato and corn ($P > 0.05$). Abnormal profiles were more frequent in populations exposed to pesticides.

Within each treatment, *CV*s of adults were greater than juveniles (Mann–Whitney *U*-test, $P < 0.0001$), suggesting that these two groups should be treated independently. There were no differences among populations receiving the same treatment (Kruskal–Wallis, $P > 0.05$ in all comparisons), therefore, we combined populations receiving the same treatments so that test groups for interpopulation comparisons comprised separate adult and juvenile samples from combined corn plots, combined potato plots and combined control ponds.

Among adults, *CV*s were significantly higher for potato vs. controls (Mann–Whitney *U*-test, $P < 0.005$) and corn vs. controls (Mann–Whitney *U*-test, $P < 0.005$); there was no significant difference for potato vs. corn. In juveniles, only corn *CV*s were significantly higher than controls (Mann–Whitney *U*-test, $P < 0.05$).

Physical deformities (ectromelia and ectrodactyly) and illness were apparent only in juveniles, and *CV*s of deformed individuals ($n = 3$) were significantly higher than those of non-deformed individuals from potato site 2 ($n = 18$; Mann–Whitney *U*-test, $P < 0.05$). In contrast, there was no similar correlation with physiological disruption; neither individuals with degenerative liver (hepatic lipidosis), kidney damage, or general systemic illnesses had greater *CV*s than apparently healthy animals (Mann–Whitney *U*-test, $P > 0.05$ in all comparisons).

Of 16 adults from various locations for which both DNA and blood smear analyses were available, ten carried intraerythrocytic parasites. However, *CV*s were no different among carriers and non-carriers, and carriers did not exhibit abnormal DNA profiles (Mann–Whitney *U*-test, $P > 0.05$).

Mean *C*-value was significantly different between adults and juveniles (Mann–Whitney *U*-test, $P < 0.0001$), with juveniles having greater mean *C*-values in each case. Comparisons of adult or juvenile *C*-values between sites of same treatment revealed no significant differences (Mann–Whitney *U*-test, $P > 0.05$), nor was there any difference in *C*-value between the two treatments and control (Kruskal–Wallis, $P > 0.05$). Individuals characterised by deformities, intraerythrocytic parasites, or physiological stress showed no significant differences in mean *C*-value (Mann–Whitney *U*-test, $P > 0.05$). The characteristic *C*-value of *R. clamitans* from several widespread populations in eastern North America (6.0–6.6 pg; L.A. Lowcock, personal observation) was more or less maintained. The variability in *C*-values exhibited by our pesticide-exposed populations was no greater than previously reported values for many other amphibian species (Table 2; Olmo and Morescalchi, 1978; Licht and Lowcock, 1991).

A genotoxic effect was detectable in all water samples, with those sites subjected to pesticides receiving the highest genotoxicity ratings (Bonin et al., 1997).

4. Discussion

The presence of different classes of DNA profile abnormality, as identified by

FCM, allows inference into the general mechanisms of pesticide-induced DNA damage at our study sites. Aneuploid mosaicism implies an acute effect early in development resulting in two viable cell lineages of differing DNA content. As in our study, Bickham et al. (1988) encountered aneuploid mosaicism and other abnormal DNA profiles. We follow their lead in excluding such individuals from quantitative analysis, and our data imply that the observed elevated frequencies of these irregularities are the likely result of aneugenic and clastogenic effects brought about through mitotic interference by environmental contaminants (Krepinsky and Heddle, 1983; Galloway, 1994). The presence of two abnormal profiles (i.e. one polyploid and one aneuploid) in the control sites supports spontaneous variation in ploidy in amphibian populations, a well documented phenomenon (e.g. Lowcock and Licht, 1990).

This study confirms the expectation that *CV* of DNA profiles is elevated in individuals exposed to pesticides. A similar hypothesis, of increased *CV* in erythrocytes of turtles exposed to low-level radiation in effluent ponds of nuclear power plants, was also proven by FCM (McBee and Bickham, 1988; Bickham et al., 1988). Elevated *CV*s emanate from cell populations of relatively widely-varying DNA content within an individual, and are most often a result of chromosomal aberrations caused by clastogenic agents (Galloway, 1994). It is unlikely that significant numbers of circulating erythrocytes had been formed subsequent to the most recent pesticide exposure (Schultz et al., 1993), and as such the effects we have observed are probably reflective of sub-chronic exposure.

As with the study of Bickham et al. (1988), our data also demonstrate *CV* increase with age class in both pesticide-exposed and control populations. In the case of the pesticide-exposed individuals, we hypothesise a concomitant effect between cumulative toxicity and naturally occurring variation resulting from temporally accrued DNA instability and replication error, the latter effect having sole influence upon the control populations. The use of half-height *CV* eliminates individual micronuclei from subsequent analysis, as their small size and limited fluorescence places them outside the electronically gated area of measurement generated by the flow cytometer (Schultz et al., 1993). Within the electronically gated area around the DNA profile histograms are normal nuclei, or abnormal nuclei which have either lost or acquired measurable amounts of chromosomal material through micronucleus formation and fusion (Krepinsky and Heddle, 1983). Elevated *CV*s may therefore be viewed as complementary measures of the same clastogenetic phenomenon as are estimated by micronucleus frequency (Krauter et al., 1987; Risley and Pohorenec, 1991; Rudek and Rozek, 1992; Krauter, 1993; Schultz et al., 1993), and as such, may also give a more accurate measure of the total extent of nuclear disruption, which is likely underestimated through measures of micronucleus frequency alone (Krepinsky and Heddle, 1983).

The fact that *C*-values of juveniles were higher than those of adults in all cases including controls was not surprising in light of similar changes studied in *X. laevis* (Fritz et al., 1990). Increases in larval cellular DNA content are induced by gene amplification for gene products involved in differentiation during metamorphosis

(Fritz et al., 1990), and as with other metamorphic phenomena, such as the change-over of erythropoietic centres, and a shift in haemoglobin characteristics, increased cellular DNA from metamorphosis-induced gene amplification can be expected to persist for at least a short period post-metamorphosis. This phenomenon does not appear to be accentuated in our pesticide-exposed populations, and we therefore believe it to be unrelated to pesticide exposure.

4.1. Known toxicological effects of pesticides in the study sites

Our intention in this paper was to demonstrate the existence of, and ability to screen for, genotoxic effects of pesticide exposure, not to infer effects of specific chemicals. However, in light of the results, it is worth briefly reviewing the relevant toxicologies of the main pesticides used on our study fields: Guthion® (the organophosphate, azinphos-methyl); and Furadan® (the dimethylcarbamate, carbofuran).

Azinphos-methyl (potato fields), a non-systemic insecticide and acaricide, has been shown to have both short- and long-term effects on amphibians. Sanders (1970) reported a 96-h LC_{50} of 0.13 ppm in static bioassays utilising *Bufo woodhousii* larvae; the tadpoles went through a period of irritability followed by a loss of equilibrium, and finally death. On cropland, an application rate of 1.8 kg ha^{-1} resulted in an eventual 100% mortality of *Rana catesbeiana* (Mulla, 1962), but at rates of 0.11 and 0.45 kg ha^{-1}, no mortality was observed in cages containing *Bufo boreas* and *Scaphiopus hammondi* adults (Mulla et al., 1963). Nevertheless, increased incidence of chromosomal abnormalities have been reported in cultured Chinese hamster cells exposed to this compound at concentrations of 60 ppm or more (Worthing and Hance, 1991). Azinphos-methyl has 'long persistence' after application (Worthing and Hance, 1991), and is thus a prime candidate for study of long-term or cumulative toxic effects.

At very low concentrations, the insecticide carbofuran (corn fields) suppressed muscle contraction evoked by nerve or muscle stimulation in *Rana pipiens*. Response to indirect nerve stimulation was suppressed more effectively than response to direct muscle stimulation (Takeno et al., 1977). These signs of acute intoxication are characteristic of the acetylcholinesterase inhibition of many carbamates, although we have evidence that certain Ranids are resistant to such effects (Bonin et al., 1997). Although considerable experimental evidence indicates that, as a class, methyl- and dimethylcarbamates are not mutagenic, weak mutagenicity in cultured rodent cells has been reported for carbofuran (Baron, 1991). The general and severe toxic effects of carbofuran exposure have led to a recent call by the World Wildlife Fund for a total ban on the use of this compound, which is ingested by migratory birds passing through agricultural areas where it is in widespread use (World Wildlife Fund, 1994).

A suite of pesticides (Table 2) applied at varying concentrations and frequencies in our test plots, may have had a possible influence on the frog populations. Many of these chemicals are known to be highly toxic, mutagenic, or both (Worthing and Hance, 1991). In addition, many chemicals used on nearby crops or in past crop

rotations on the same fields are highly toxic but of unknown persistence, while others have been shown to be highly toxic but exhibit extremely low environmental persistence (Worthing and Hance, 1991). Regardless of the differing contamination characteristics of the corn and potato fields, similar genetic manifestations were picked up by the FCM analysis. As such, it is impossible to differentiate between the various aspects of contamination (i.e. recent vs. cumulative exposure, crop rotation, synergistic effects, etc.) and their specific effects without controlled in vivo toxicological studies. Similarly, the water genotoxicity values calculated from samples taken at the time of frog collection represent a point in time, and shed no light on the long-term genotoxicity of the study sites.

4.2. Possible toxicological effects observed in this study

Various problems were encountered. The first of these were possible toxic manifestations, such as limb deformities, that reflect exposure to pesticides during critical developmental stages. Liver and kidney degeneration may also result from effects of either or both acute and chronic toxicity. Non-specific response to contaminants, e.g. physiological stress from either acute or chronic toxicity, may also result in increased susceptibility to opportunistic infections from bacterial or fungal agents.

The correlation between high *CV*s (and hence putative DNA damage) and hind-limb deformity, does not necessarily indicate a causal link, although this remains a possibility. It does suggest, however, that high exposure levels during critical cell division stages of development has contributed to both phenomena. Interestingly, a collection of 21 newly metamorphosed Blue-spotted salamanders (*Ambystoma laterale*) collected from a ditch bordering a cornfield in Cumberland Co., Nova Scotia, also displayed a high incidence (11 individuals, 52.3%) of hind-limb deformity (split limbs, polydactyly), and also included two individuals that proved to be somatic intersexes (L.A. Lowcock, personal observation).

There was no correlation between physiological effects (illness, etc.) and amount of putative DNA damage, demonstrating that there exists a number of independent, specific and non-specific, acute and chronic effects of exposure to the pesticides used in our study plots.

4.3. Genotoxicity

Although high *CV*s in adults and juveniles clearly demonstrate persistent and ongoing DNA damage in pesticide-exposed populations, there are other relevant manifestations of genotoxicity revealed by FCM.

The numerous abnormal DNA profiles encountered are consistent with the clastogenic generation of micronuclei, a common occurrence after chemical exposure in amphibians (Krauter et al., 1987; Krauter, 1993; Fernandez et al., 1993). However, in FCM, micronuclei themselves are small enough to be gated out, hidden by internal standards, or lost in sample 'noise' (Schultz et al., 1993). A standard test for micronuclei, conducted on blood smears during intense periods of erythropoi-

esis, is a simple and reliable method for evaluating in vivo genotoxic effects of freshwater pollutants (Krauter et al., 1987; Schreiber et al., 1992; Krauter, 1993; Fernandez et al., 1993; Weller et al., 1993), and we suggest that it would be informative to conduct such tests in conjunction with the complimentary *CV* data generated from FCM studies.

4.4. Sources of error

Elevated *CV*s may have been influenced by the presence and degree of intra-erythroparasitism, i.e. amount of parasite DNA present in samples, and not actual DNA damage. Our controlled test of *CV* between infected and normal individuals negates this particular source of error in the interpretation of our results. In addition, it is possible that profiles which we interpreted as having elevated *CV*s may have actually been stable aneuploids for one of the smaller metacentric to submetacentric chromosomes in the *R. clamitans* genome ($2n=26$). Aneuploid cell populations involving these elements may have thus been characterised by *C*-values beneath the threshold required to define a separate DNA content peak (relative to the normal cell population) during analysis, and as such would appear as a single peak having elevated *CV*. Finally, increased *CV* and false aneuploidy have been correlated with a number of factors, including sampling more than a single tissue type and their differential staining properties (Tiersch and Wachtel, 1993). We feel that our collection methodology (i.e. cardiac punctures) and use of half-peak *CV* both minimise the potential influence of this type of error.

5. Conclusions

There is an established link between *CV* increase and both chemical and radiation exposure. In our study, juvenile frogs were visibly affected by pesticide application; physical effects occurring during early developmental stages were further correlated with clastogenic effects, and thus may have been directly associated with DNA damage. Physiological deterioration appears to result from either acute or cumulative toxicity. Apparently healthy adults in our test populations also showed hidden genotoxic effects, and thus, we believe FCM to be a powerful tool that may be employed not only to assess degrees of genetic damage in affected populations, but to screen apparently healthy or suspected vulnerable populations for incipient effects related to the presence of a wide variety of environmental contaminants.

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Appendix

Individual DNA characteristics listed by site

Site no.	Sample	Age class[a]	*CV*	*C*-value (pg)	DNA profile[b]
Control					
8	L4V03	j	2.24	6.44	n
	L4V04	j	3.32	6.47	n
	L4V05	j	3.34	6.27	n
	L4V09	j	2.70	6.51	n
	L4V10	j	2.79	6.44	n
	L4V12	j	3.57	6.40	n
	L4V14	j	2.73	6.26	n
	L4V15	j	2.16	6.40	n
	L4V19	j	2.93	5.94	n
7	S5V02	a	3.38	6.08	n
	S5V04	a	4.12	6.45	n
6	V3V01	a	3.09	6.20	n
	V3V05	a	3.43	5.95	n
	V3V07	a			p
	V3V09	a			an
	V3V10	a	3.48	6.45	n
	V3V17	a	3.78	6.07	n
	V3V20	a	4.07	6.14	n
Potato					
2	A5V02	j			a
	A5V13	a	3.74	6.20	n
	A5V18	a			a
	A5V20	a	4.69	5.95	n
	A5V24	j			a
	A5V27	j			a
	A5V29	j			a
	A5V35	a			an
	A5V36	a	4.96	6.20	n
	A5V37	a			a
2	A5V43	j			a
	A5V44	j	2.82	6.46	n
	A5V45	j	2.51	6.53	n

Site no.	Sample	Age class[a]	*CV*	*C*-value (pg)	DNA profile[b]
	A5V46	j	2.83	6.28	n
	A5V47	j	2.66	6.39	n
	A5V48	j	3.15	6.52	n
	A5V49	j	3.40	6.27	n
	A5V50	j			a
	A5V51	j	2.73	6.57	n
	A5V52	j	2.54	6.40	n
	A5V53	j	4.82	6.20	n
	A5V54	j	4.59	6.27	n
	A5V55	j			a
	A5V56	j	2.50	6.29	n
	A5V57	j	2.55	6.39	n
	A5V58	j	2.24	6.39	n
	A5V60	j	3.73	6.31	n
	A5V61	j	2.30	6.36	n
	A5V62	j	4.87	6.57	n
	A5V63	j	2.90	6.30	n
	A5V64	j			a
	A5V81	j			an
	A5V82	j	2.50	6.32	n
	A5V83	j	4.98	6.45	n
	A5V84	j	3.64	6.41	n
	A5V85	j	4.15	5.97	n
1	S3V01	a	5.47	5.47	n
	S3V02	a	5.15	6.20	n
	S3V03	a	5.33	5.59	n
	S3V04	a	5.50	5.47	n
	S3V05	a	5.99	5.83	n
	S3V06	a	3.50	6.08	n
	S3V14	a	6.99	6.33	n
	S3V15	a	3.72	6.20	n
Corn					
3	M1V01	a	4.86	6.20	n
5	M2V01	a			an
	M2V02	a			an
	M2V03	a			an
	M2V05	a			a
	M2V06	a	5.47	6.07	n
	M2V07	a			a
	M2V08	a			a
	M2V09	a	6.23	6.01	n
	M2V10	a			an
4	M3V02	a	5.55	5.77	n
	M3V03	a			a
	M3V04	a	4.12	5.69	n
	M3V05	a	4.41	6.20	n
	M3V06	a			an
	M3V07	a	3.09	6.20	n
	M3V08	a	5.87	6.08	n

Site no.	Sample	Age class[a]	*CV*	*C*-value (pg)	DNA profile[b]
	M3V09	a			a
	M3V10	a	3.90	5.89	n
	M3V11	a			an
	M3V12	a			a
	M6V01	a	4.86	5.95	n
	M6V03	a	6.26	5.41	n
3	M7V01	a	4.33	5.95	n
	M8V01	a	4.88	6.08	n
	M8V02	a	3.92	6.26	n
	M8V03	a			a
	M8V09	a			a
	M8V10	a	5.40	6.20	n
	M8V11	a	4.26	6.08	n
4	M9V01	j	2.74	6.45	n
	M9V03	j	3.17	6.45	n
	M9V04	j	3.59	6.20	n
	M9V05	j	4.89	6.37	n
	M9V06	j	3.98	6.34	n
	M9V08	j	3.88	6.26	n

[a]a, adult; j, juvenile.
[b]n, normal; a, abnormal; an, aneuploid; p, polyploid.

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