Genetic and Biochemical Biomarkers in the Macrophyte *Bidens laevis* L. Exposed to a Commercial Formulation of Endosulfan

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Received 5 October 2012; revised 28 November 2012; accepted 7 December 2012

ABSTRACT: Previous studies in the wetland macrophyte *Bidens laevis* L have demonstrated that the insecticide endosulfan induces a high frequency of somatic chromosome aberrations in anaphase–telophase (CAAT) but no DNA changes as determined by the single cell gel electrophoresis (Comet) assay. Thus, cytogenetic biomarkers appear to be more sensitive to the toxic effects of the insecticide than the DNA molecule in the studied species. For this reason, the goals of this study were to use cytogenetic biomarkers—CAAT and abnormal metaphase—and defense biomarkers such as the activity of the antioxidant enzymes—guaiacol peroxidases (POD), glutathione reductase, and microsomal and cytosolic (m- and c-) glutathione-*S*-transferase (GST)—to evaluate in *B. laevis* effects caused by a commercial formulation of endosulfan. The frequency of CAAT was increased at 5, 10, 50, and 100 μg/L endosulfan with respect to the negative controls by 3.1, 2.5, 2.5, and 3.2-fold, respectively while the frequency of abnormal metaphases was also increased at the same concentrations by 3.5, 2.8, 3.2, and 11.3-fold, respectively. In addition to these aneugenic effects, other abnormalities such as C-mitosis and chromosome clumping were observed at 10 μg/L endosulfan. On the other hand, POD induction at 0.02, 0.5, 5, and 10 μg/L and m-GST inhibition at 0.5, 10, and 50 μg/L in plants exposed during 24 h to endosulfan were observed but all of these responses were highly variable. In conclusion, only cytogenetic biomarkers like CAAT in *B. laevis* can serve potentially as early warning systems to detect environmentally relevant concentrations of endosulfan in aquatic ecosystems. © 2013 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2013.

Keywords: genotoxicity; chromosome aberrations; commercial formulation of endosulfan; wetland macrophytes; peroxidases

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Contract grant sponsor: CONICET
Contract grant number: PIP 112-200-801-02190 2008
Contract grant sponsor: UNMdP
Contract grant number: EXA 515/10
Contract grant sponsor: 2011 SETAC/Procter and Gamble Fellowship for Doctoral research in Environmental Sciences (to Débora Pérez)
Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/tox.21836

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INTRODUCTION

It has been suggested that the progress of environmental toxicology requires the development of a battery of biomarkers to evaluate chemical hazards (Ernst and Peterson, 1994; Brain and Cedergreen, 2008), and that no biomarker by itself can offer a complete solution. Only a multiparametric approach, including both physiological, general stress and more specific biomarkers can adequately contribute to ecotoxicological diagnostics (how precisely an effect can be identified and/or characterized) (Ferrat et al., 2003; Brain and Cedergreen, 2008).

Compared to animals, biomarkers in plants have been less explored, and limited examples have been reported in the literature. Notwithstanding, plant biomarkers have demonstrated utility as biomonitoring agents and for the elucidation of modes of action (Ferrat et al., 2003; Brain and Cedergreen, 2008).

In the biomarker selection, sensitivity is the key factor, because the greater the biomarker sensitivity to the xenobiotic exposure, the earlier will be its response avoiding occurrence of deleterious effects on the organism or the population (den Besten and Munawar, 2005). In this regard, a good biomarker is the one that is strongly dependent on concentration and exposure time to a xenobiotic stress (Enrst and Peterson, 1994). Biomarkers of defense can only indicate the presence of certain contaminants in the environment (e.g., antioxidant enzymes, phase I and phase II detoxification enzymes). In contrast, biomarkers of damage can reveal significant impairments to one or more features that compromise the performance of the organism, for example, genetic biomarkers such as DNA fragmentation and chromosome aberrations (De Lafontaine et al., 2000).

Growth, photosynthesis, total chlorophyll, and respiration are the variables most commonly used to assess the effects of contaminants on aquatic plants. Growth as an endpoint provides full integration of many physiologic variables and a biological link to resource management. Because suborganismal responses lead to organismal responses, it follows that organism growth is not an extremely sensitive indicator of contaminant exposure (Siesko et al., 1997). However, it has been well established that the early warning systems, for example, enzymatic and genetic biomarkers are the most sensitive, showing a fast response to xenobiotic exposure (Menone and Pflugmacher, 2005; Brain and Cedergreen, 2008).

Endosulfan is an organochlorine insecticide belonging to the cyclodiene subgroup, which has been detected in soil, sediment, invertebrates, fish, and macrophytes (Nowak et al., 1995, Menone et al., 2000). It is the only organochlorine insecticide still in use around the world for protecting cereal, vegetable, and fruit crops from a variety of insects (Jergentz et al., 2004; Kumar and Philip, 2006). Although at the fifth meeting of the Stockholm Convention on Persistent Organic Pollutants the decision was taken to prohibit its use by 2012 (IISD, 2010), several countries obtained permission for extending its use for additional time (SENASA, 2012). The commercial formulations of endosulfan consist of a mixture of α- and β-endosulfan isomers in a ratio 7:3, respectively (Kumar and Philip, 2006). In addition, the formulations could contain epichlorohydrin as a stabilizer, a known genotoxic chemical (Koplan, 2000). For this reason, larger genotoxic effects would be expected in these formulations when compared to the endosulfan alone exposure. According to the EPA, endosulfan concentrations above 0.22 μg/L (acute) and 0.056 μg/L (chronic) will have an adverse impact on the health of aquatic organisms (Mersie et al., 2003). In biomonitoring studies, Silva et al. (2005) reported concentrations of 0.2–1.1 μg/L in surface water close to paddy fields in Argentina, while in studies from USA run-off waters endosulfan ranged from 1 to over 100 μg/L (Mersie et al., 2003).

The negative effects of endosulfan have been demonstrated in the aquatic macrophyte *Myriophyllum quitense*, in which the antioxidant biomarkers glutathione-*S*-transferase (GST), glutathione reductase (GR), and catalase (CAT) increased in activity at 5 μg/L (Menone et al., 2008), and in the wetland species *Bidens laevis*, in which the cytogenetic biomarkers chromosome aberrations in anaphase–telophase (CAAT) and abnormal metaphases increased in frequency between 5 and 100 μg/L in root cells (Pérez et al., 2008, 2011). On the other hand, no DNA damage, as determined by the single cell gel electrophoresis (Comet) assay has been observed (Pérez et al., 2010). The fact that endosulfan induced high frequency of CAAT and no DNA fragmentation in the previously mentioned studies indicates that cytogenetic biomarkers are sensitive to aneugenic compounds like cyclodiene insecticides, they are also more adequate for evaluating endosulfan genotoxicity in *B. laevis*. For this reason, the goals of this study were to use cytogenetic biomarkers—CAAT and abnormal metaphases—and defense biomarkers like antioxidant enzymes—activities of guaiacol-peroxidases (POD), GR, and microsomal as well as cytosolic (m- and c-) GST—to evaluate effects caused by a commercial formulation of endosulfan in *B. laevis*. Toward this end, we established a concentration-response relationship of cytogenetic biomarkers and concentration- and time-response relationships of antioxidant biomarkers.

MATERIALS AND METHODS

Material Handling

Seeds of *B. laevis* were collected in La Brava lake (37° 53′ S, 57° 59′ W), Argentina, in May 2010 to perform the experiments. Seeds were sterilized in a 30% solution of commercial bleach (DEM Argentina, 5.5 g/L) during 5 min, rinsed several times in distilled water, and placed in Petri dishes with moist filter paper for germination. Seedlings were transferred to soil-containing pots and grown during 2 months in a greenhouse until exposure.

For all experiments, the experimental unit was one plant and the number of replicates per treatment was six. Seedling roots were carefully rinsed in water and immersed in glass flasks containing 330 mL of a defined treatment solution, detailed below. A photoperiod of 12 h light/12 h darkness and a room temperature of 22°C were set up.

Experimental Solutions and Treatments

Experiment 1: Concentration-Response of Cytogenetic Biomarkers in B. laevis Exposed to the Commercial Formulation of Endosulfan

The stock solution contained 3500 mg/L of active ingredient of endosulfan in the commercial formulation (hereafter, endosulfan), with 500 μL of ''Endosulfan 35 Nufarm'' (35%, hexacloro-endometileno bicicloheptenо-bis (oximetileno)-sulfite, Nufarm S.A.) in 50 mL dimethyl sulfoxide (DMSO). Endosulfan treatments were prepared by diluting the corresponding volume of stock solution in Hoagland medium to a final volume of 1000 mL. The six final exposure solutions contained 0.02, 0.5, 5, 10, 50, and 100 μg/L of active ingredient in the commercial formulation and 0.004% of DMSO. In addition, three controls were used: two negative controls (a) Co-1, that consisted of Hoagland solution and (b) Co-2, in which DMSO was added to the Hoagland solution at 0.004% (similar to the concentration of DMSO used for the endosulfan exposures), and one positive mutagenic control (Co+) consisting of 10 mg/L of methylmethanesulfonate.

Because CAAT and abnormal metaphases are biomarkers of damage, a 48 h exposure was carried out, followed by a recovery period of 24 h in Hoagland solution to allow the completion of the cellular cycle in the exposed cells (Grant and Owens, 2002).

Sample Preparation and Microscopic Observations. Root tips (1 cm long) were fixed in ethanol:glacial acetic acid (3:1, v/v) during 24 h and maintained in 70% alcohol in a refrigerator until analysis. Then, they were macerated in 1 M HCl at 60°C during 10 min and stained with Feulgen reagent for 2 h in darkness, squashed in a solution of 1–2% carmine in 45% acetic acid, and observed in an optic microscope Olympus BH2 at 400× and 1000× magnification. The mitotic index was calculated as the number of cells at any stage of mitosis per 1000 observed cells per plant, in one slide and was only used to indicate if the number of mitotic cells was high enough to carry out the analysis of chromosome aberrations, rather than as a quantitative measure of toxicity. Two hundred cells in anaphase–telophase were observed to detect CAAT, if any, in individual roots in one or more (if necessary) slides per plant. Data were expressed in terms of the median of chromosome aberrations per 200 cells in anaphase–telophase. Two aberration types were scored according to whether they were indicative of either (1) spindle disturbance or aneunogenesis (including vagrant and laggard chromosomes) or (2) clastogenicity (including bridges and fragments). In addition, metaphases with noncongregated chromosomes at the metaphasic equator were considered abnormal (abnormal metaphase). One hundred metaphases were analyzed; the number of abnormal metaphases detected was expressed in terms of the median per 100 cells in that mitotic stage. During the process, various other types of chromosomal abnormalities like C-mitosis and chromosome clumping were also observed and scored but not statistically analyzed because these abnormalities appeared principally in only one treatment.

Experiment 2: Concentration-Response of Antioxidant Biomarkers in B. laevis Exposed to the Commercial Formulation of Endosulfan

Seven treatments were used: a negative control that consisted of Hoagland solution with 0.004% DMSO (similar to the concentration of DMSO used for the pesticide exposures) and six concentrations of active ingredient in the commercial formulation, detailed in Experiment 1: Concentration–Response of Cytogenetic Biomarkers in B. laevis Exposed to the Commercial Formulation of Endosulfan section. Because antioxidant enzymes are biomarkers of defense, a 24 h exposure was carried out.

Enzyme Preparation. Preparation of microsomal (m-) and cytosolic (c-) extracts were carried out according to Pflugmacher and Steinberg (1997). Approximately 2–3 g of roots were ground to a fine powder with mortar and pestle under liquid nitrogen and then 3 mL sodium-phosphate buffer (0.1 M, pH 6.5), containing 20% glycerol, 14 mM DTE, 1 mM EDTA was added. Cell debris was removed by centrifuging at 10,000 × *g* for 10 min. The supernatant was centrifuged at 100,000 × *g* for 60 min to obtain the membrane fraction, named as the microsomes. The microsomes were resuspended in sodium phosphate buffer (20 mM, pH 7.0) containing 20% glycerol and 1.4 mM DTE and homogenized in a glass potter. Solid ammonium sulfate was added to the supernatant to achieve 35% saturation. After centrifugation at 20,000 × *g* for 20 min, the pellet was discarded and ammonium sulfate was added to the supernatant to 80 % saturation. After centrifugation at 30,000 × *g* for 30 min, the pellet (containing the cytosolic proteins) was resuspended in sodium phosphate buffer (20 mM, pH 7.0) and desalted by gel filtration through NAP-10 columns.

Enzyme Activity Measurement. All enzyme measurements were carried out in triplicate. POD measurement in the cytosolic protein fraction using guaiacol as substrate was performed as described by Drotar et al. (1985). Determination of m- and c-GST activity with the model substrate 1-chloro-2, 4-dinitrobenzene was carried out according to Habig et al. (1974). GR activity was measured in the cytosolic protein fraction spectrophotometrically via the reduction in absorbance of NADPH in the reaction mixture at 340 nm, using oxidized glutathione as substrate, according to Tanaka et al. (1994). Protein determination in the

TABLE I. Mitotic index, median abnormal metaphases, and median chromosome aberrations in anaphase–telophase (CAAT) in roots of *Bidens laevis* exposed to endosulfan (μg/L)

			CAAT[a]				
			Aneugenic		Clastogenic		
	Mitotic Index Median (ID)	Abnormal Metaphases Median (ID)	Laggards	Vagrants	Bridges	Fragments	Total Median (ID)[b]
Co-1	6.5 (7.1)	4.0 (2.0)	22	0	5	0	6.0 (2.0)
Co-2	4.2 (1.3)	3.0 (3.0)	25	0	5	0	5.0 (4.0)
Co+	6.8 (0.7)	9.0 (1.0)*	53	6	11	2	14.0 (6.0)*
0.02	6.8 (0.9)	11.0 (3.0)*	23	5	9	0	5.0 (7.0)
0.5	4.6 (0.4)	4.0 (2.0)	32	3	4	0	8.0 (2.0)
5	6.0 (4.1)	10.5 (5.5)*	58	15	12	2	15.5 (7.7)*
10	6.2 (2.3)	8.5 (4.7)*	56	11	14	2	12.5 (2.5)*
50	6.9 (2.6)	9.5 (12.7)*	57	8	14	0	12.5 (4.7)*
100	6.7 (1.2)	34.0 (26.0)*	64	5	11	0	16.0 (6.0)*

Co-1: Hoagland solution; Co-2: Hoagland solution + dimethyl sulfoxide; Co+: methyl methanesulfonate (10 mg/L).
ID, Interquartile distance; aneugenic, laggards and vagrants; clastogenic, bridges and fragments; total, aneugenic + clastogenic.
[a] 1200 anaphase–telophase cells analyzed per treatment.
[b] Kruskal Wallis p value = 0.0036.
* Significantly different from Co-2 ($p < 0.05$).

microsomal and cytosolic fractions was carried out according to Bradford (1976) using bovine serum albumin as standard. Enzymatic activities are reported in nanokatals per milligram of protein (nkat/mg prot), where 1 kat is the conversion of 1 mol of substrate per second.

Experiment 3: Time-Response of Antioxidant Biomarkers in B. laevis Exposed to the Commercial Formulation of Endosulfan

Taking into account that responses of antioxidant biomarkers are faster than genetics ones, a time-response relationship was established. A 10 μg/L endosulfan concentration was chosen according to the results obtained in the concentration-response relationships from the present work (positive for antioxidant biomarkers as well as for C-mitosis and chromosome clumping). Four different exposure times to endosulfan: 6, 12, 24, and 48 h and five negative controls (time 0, 6, 12, 24, and 48 h) were tested. The controls consisted of Hoagland solution with 0.004% DMSO (similar to the concentration of DMSO used for the pesticide exposures).

Enzyme preparation and activity measurements were carried out as detailed in Experiment 2: Concentration–Response of Antioxidant Biomarkers in *B. laevis* Exposed to the Commercial Formulation of Endosulfan section.

Statistical Analyses

Normality and homogeneity of variances were verified by Shapiro-Wilk and Levene tests, respectively. Nonparametric tests were applied because assumption of homogeneity of variance was not meet. Therefore, datasets were described using the median, as measure of central tendency, and the interquartile range (ID) as measure of statistical dispersion. In this regard, the Kruskal-Wallis nonparametric test was applied and, *a posteriori,* differences among treatments were tested by the Dunn test (Zar, 1999). Statistical analyses were carried out using Infostat Software Package (Grupo InfoStat, 2008), with a 0.05% significance level.

RESULTS

Concentration-Response of Cytogenetic Biomarkers in *B. laevis* Exposed to the Commercial Formulation of Endosulfan

Mitotic index in control plants varied from 5.2 to 10.8, high enough to score the required number of cells for the CAAT assay. This parameter did not show differences among treatments ($p > 0.05$) (Table I). Total CAAT frequency was not significantly different between Co-1 and Co-2 and between Co-2 and 0.02 and 0.5 μg/L endosulfan. The positive control showed statistically significant higher CAAT frequency than the Co-2 ($p < 0.05$) (Table I). Similarly, a significant increased frequency of CAAT in plants exposed from 5 to 100 μg/L endosulfan in comparison with Co-1 and Co-2 ($p < 0.05$) was observed. Aneugenic CAAT (laggards and vagrants) were more frequent than clastogenic CAAT (bridges and fragments) ($p < 0.05$). The frequency of abnormal metaphases was not significantly different between Co-1 and Co-2, but it was significantly different between Co-2 and Co+ ($p < 0.05$) and between the following endosulfan concentrations 0.02, 5, 10, 50, and 100 μg/L and Co-2 ($p < 0.05$) (Table I). Other abnormalities like C-mitosis and chromosome clumping appeared mainly at 10 μg/L endosulfan (Table II). Various CAAT, abnormal metaphases, and C-mitosis are shown in Figure 1.

TABLE II. Total number of C-mitosis and chromosome clumping per 100 cells in metaphase, in roots of *Bidens laevis* exposed to endosulfan (μg/L)

	Controls						Endosulfan (μg/L)											
	Co-1		Co-2		Co+		0.02		0.5		5		10		50		100	
Plant Number	Cm	Cc	Cm	Cc	Cm	Cc	Cm	Cc	Cm	Cc	Cm	Cc	Cm	Cc	Cm	Cc	Cm	Cc
1	0	0	0	0	0	0	11	0	0	0	2	0	4	6	0	0	0	0
2	0	0	3	0	0	0	4	0	0	0	0	0	2	17	0	0	0	0
3	0	0	0	0	0	0	3	0	0	1	0	0	8	13	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	2	7	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	5	13	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	170	0	2	5	0	0	0	0

Co-1: Hoagland solution; Co-2: Hoagland solution + dimethyl sulfoxide; Co+: methyl methanesulfonate (10 mg/L); Cm: C-mitosis; Cc: chromosome clumping.

Concentration-Response of Antioxidant Biomarkers in *B. laevis* Exposed to the Commercial Formulation of Endosulfan

The concentration-response of POD, m- and c-GST, and GR are shown in Figure 2. A significant induction of POD activity at 0.02, 0.5, 5 and 10 μg/L endosulfan with respect to the negative control ($p < 0.05$) [(Fig. 2(A)] was observed. This activity peaked at 5 μg/L and then declined, reaching values similar to the negative control in plants exposed to 50 and 100 μg/L endosulfan. The m-GST activity showed a significant inhibition in plants exposed to 0.5, 10, and 50 μg/L endosulfan with respect to the control ($p < 0.05$) [Fig. 2(B)]. On the other hand, the c-GST and GR did not change their activities in the exposed plants with respect to the control [Fig. 2(C,D)], respectively ($p > 0.05$).

Time–Response of Antioxidant Biomarkers in *B. laevis* Exposed to the Commercial Formulation of Endosulfan

The time-response of POD, m- and c-GST, and GR are shown in Figure 3. POD activity was significantly induced at 6, 12, 24, and 48 h [Fig 3(A)] whereas a significant inhibition of m-GST activity at 12 h with respect to its control was found ($p < 0.05$) [Fig. 3(B)]. Cytosolic-GST activity did not change at any time (Fig. 3C) ($p > 0.05$). Inhibition of GR activity at 24 h with respect to its negative control was found [Fig. 3(D)] ($p < 0.05$).

DISCUSSION

Cytogenetic Biomarkers

The application of biomarkers is an important approach for investigating the causal relationship between exposure to environmental pollutants and the observation of long-term effects in individuals and populations. The use of a battery of biomarkers in field monitoring has been increasing over the past 15 years. Genotoxicity biomarkers are now considered to be an integral part of this approach because exposure to genotoxic agents may exert damage beyond that of individuals and may be detected through several generations (Frenzilli et al., 2009). In this study, it was demonstrated that ingredients (endosulfan or excipients) contained in the commercial formulation Endosulfan 35 Nufarm interacts with the mitotic spindle, as evidenced by (a) the increment in frequencies of laggards and vagrants in comparison to bridges and fragments, (b) the increase in frequency of abnormal metaphases.

There is a lack of information in the literature concerning genotoxicity mechanisms of pesticides. However, Grant (1978) reported that, in general, pesticides induce specific types of chromosomal abnormalities (chromosome clumping, contraction, stickiness, paling, fragmentation, dissolution, chromosome and chromatid bridges, C-mitosis, and endopoliploidy) and that the agrochemicals interfering with the spindle mechanism possess only a very mild clastogenic effect. In this study, plants of *B. laevis* exposed to endosulfan showed aneugenic aberrations; the low frequency of clastogenic aberrations—brigdes and fragments—could be due to normal breakage-fusion-bridge cycles that can occur in mitotic cells, similar to the process described in other species (McClintock, 1941 in Rieger et al., 1976). C-mitoses and chromosome contraction are always present together because both abnormalities proceed from a single process which is the lack of formation of the mitotic spindle. This fact could explain the observation of C-mitosis and chromosome clumpling mainly at 10 μg/L endosulfan. In particular, C-mitoses are rare (Rank, 2003) and exhibit a typical threshold reaction and never occur unless concentration of the spindle disrupter exceeds a critical value (Rieger et al., 1976), meaning that in this case may be 10 μg/L endosulfan was the threshold value. The appearance of typical C-metaphases after complete spindle inactivation can vary, as chromosomes may be: (a) distributed over the entire cell, (b) clumped together in the cell center, (c)

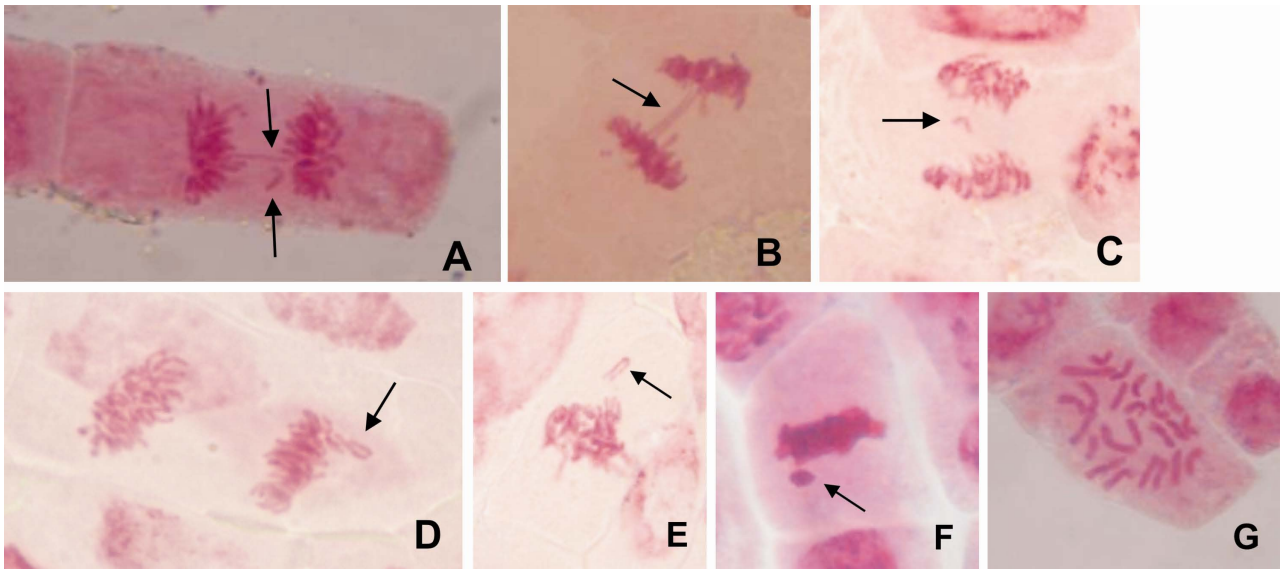

Fig. 1. Chromosome abnormalities in root cells of *Bidens laevis* exposed to endosulfan (×1000). (A) Bridge and fragment; (B) bridge; (C) laggards; (D) vagrant; (E) chromosome noncongregated; (F) chromosome clumping; (G) C-mitosis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

arranged in a star-shape configuration, or (d) strewn around the peripheral regions of the cell (Rieger et al., 1976). Our results are similar to those reported for other organochlorine pesticides that showed C-mitotic effects in terrestrial plants, for example, isomer γ of hexachlorocyclohexane (HCH), aldrin, heptachlor, and endrin in *Pisum sativum* and *Lens sculenta* (Jain and Sarbhoy, 1987), dieldrin in *Crepis*, and HCH in *Allium*, *Vicia faba*, *Zea mays*, *Triticum*, *Hordeum*, *Secale* (Grant, 1978). The response of this type of abnormality was not linear. Therefore, C-metaphases cannot be considered as optimal biomarkers, but they contribute to increase the knowledge about the genotoxicity of this pesticide.

Noteworthy, a lower frequency of CAAT in plants exposed to endosulfan in comparison to that reported with the use of the active ingredient alone (Pérez et al., 2011) was found. In the fish *Channa punctatus*, the LC50 at 96 h for endosulfan was 7.75 μg/L while the value for the commercial formulation was 3.07, indicating a more toxic effect for the commercial products (Pandey et al., 2006). There are no available literature that compares sublethal effects of the active ingredient of endosulfan with its commercial formulations. Although, for some pesticides it has been established in animals that commercial formulations can be more toxic than the active ingredients by themselves (Jemec et al., 2007; Contardo-Jara et al., 2009), many other pesticides show considerably lower formulation-specific toxicity relative to the parent active ingredient. In our study, a possible antagonistic effect of stabilizers and other additives in the formulation with α- and/ or β- isomers can be hypothesized.

Enzymatic Biomarkers

The 24-h exposure of *B. laevis* to endosulfan caused changes in the tested antioxidant enzymatic protection system, denoting a possible oxidative stress effect in the cells, as it was previously observed in the aquatic macrophyte *Myriophyllum quitense* exposed to endosulfan (Menone et al., 2008). GSTs are often measured for xenobiotics that tend to induce oxidative stress (Pflugmacher, 2004; Lei et al., 2006), or in pesticide crop or weed metabolism studies (Brain and Cedergreen, 2005; Sergiev et al., 2006). It is well known that the main metabolite of endosulfan is endosulfan-sulfate, which originates by conjugation with sulfate instead of glutathione. Therefore, changes in GST in *B. laevis* exposed to endosulfan can be due to the antioxidant role of this enzyme, as it functions in plants as glutathione peroxidases to directly detoxify membrane lipid peroxides (Marrs, 1996; Cummins et al., 1999). On the other hand, and for any given toxin, GST activity can be regulated differently in different species. A study on four species of algae exposed to pyrene showed large species-specific differences for differential compound tolerance (Lei et al., 2006). This could explain inhibition of m-GST in *B. laevis* but induction of m- and c-GST in *M. quitense* (Menone et al., 2008). No reproducibility of m- and c- GST and GR results between concentration and time relationships was observed, indicating that these enzymes are not good biomarkers of endosulfan in *B. laevis*. On the other hand, previous investigations have shown the capacity of POD to be induced in a dose-dependent manner in aquatic plants exposed to different pollutants like aromatic ring structures and sulfonylurea herbicide (Byl et al., 1994) and

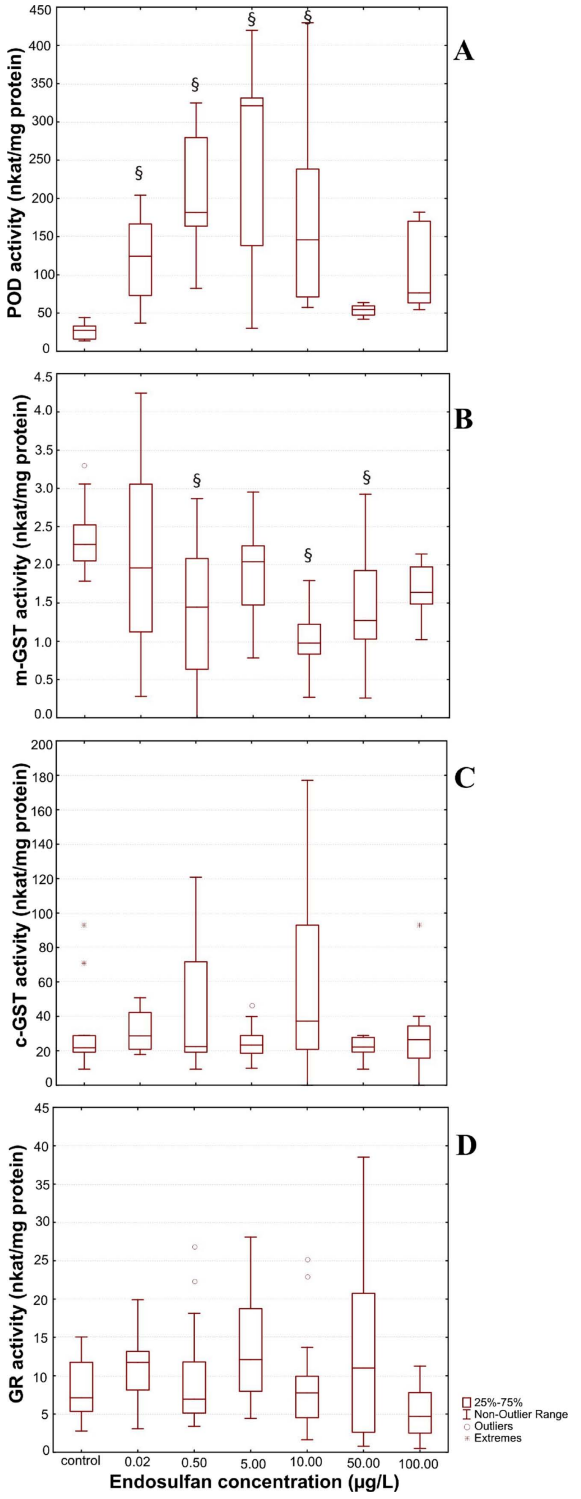

Fig. 2. Median of antioxidant enzyme activities in roots of *Bidens laevis* exposed to endosulfan. POD, guaiacol peroxidase; m-GST, microsomal glutathione-*S*-transferase; c-GST, cytosolic glutathione-*S*-transferase; GR, glutathione reductase. §Significantly different from the control ($p < 0.05$). $n = 6$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

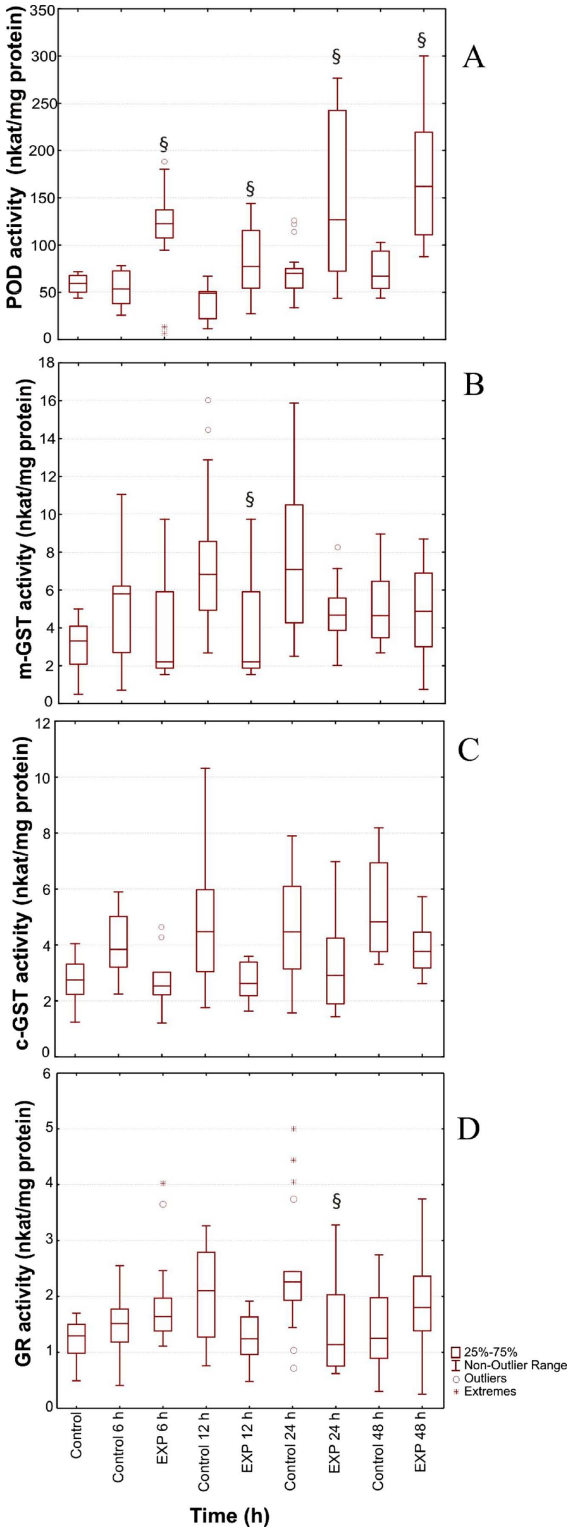

Fig. 3. Median of antioxidant enzyme activities in roots of *Bidens laevis* exposed to 10 μg/L endosulfan. POD, guaiacol peroxidase; m-GST, microsomal glutathione-*S*-transferase; c-GST, cytosolic glutathione-*S*-transferase; GR, glutathione reductase. §Significantly different from the control ($p < 0.05$). $n = 6$. EXP: exposed to endosulfan. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

various metals (Teisseire and Guy, 2000; Nimpstch et al., 2005; Ding et al., 2007). In addition, POD is ubiquitous in plants, protects cells from free radical oxidation, and responds before classical measures of plant stress are apparent (Byl et al., 1994) making it a very good biomarker candidate to assess the presence of pesticides in aquatic ecosystems. In *B. laevis* exposed to endosulfan, POD showed the typical concentration-response relationship in which induction takes place at relatively low concentrations of the xenobiotic and, after a threshold is reached, the activity decreases. POD was the only enzyme that presented induction from 6 to 48 h of exposure; however, due to its high variability more studies are necessary to propose its use as a biomarker.

Biomarkers should react to environmentally realistic concentrations, showing a good dose-response to different levels of pollution (Au, 2004). In our study, biomarkers such as CAAT showed an increment, dependent on the concentrations of the environmentally relevant range of the pesticide tested. Levels from 0.2 to >100 μg/L have been reported in run-off waters from several parts of the world (Antonious and Byers, 1997; Mersie et al., 2003; Silva et al., 2005) suggesting that pulses of endosulfan in the field can potentially induce acute effects, as chromosomal aberrations observed in this investigation.

Based on the knowledge that biomarkers should go beyond visible and morphological parameters, establish such processes and products of plants that enable early recognition of xenobiotic stress in a dose- or time-dependent manner, and be observable earlier than visible damage (Ernst and Peterson, 1994 in Brain and Cedergreen, 2008), our results can alert about possible morphological effects. In particular, somatic chromosomal aberrations like CAAT can conduce to damage of tissues and reduce the viability of individuals that finally can reduce the reproductive success (Bickham et al., 2000).

CONCLUSION

Because chemical contamination can negatively affect population survival and/or development by the induction of mutations (some of which can occur in reproductive tissues and potentially transmitted between generations), as well as nongenetic modes of toxicity, biomonitoring programs should include a selected battery of biomarkers that respond to environmentally realistic concentrations. Changes in cytogenetic parameters in the emergent aquatic macrophyte *B. laevis* exposed to endosulfan were observed, allowing the identification of the most appropriate biomarkers to be used in future field studies. Chromosomal aberrations (CAAT in this work) in this sensitive macrophyte could offer a good complement to chemical analysis for the evaluation of pollution in freshwater ecosystems, but further studies will be necessary to assess if similar responses occur in *B. laevis* exposed to other pesticides or toxic compounds in the field.

Results showed in this work are parts of the PhD Thesis of Dra. Débora J. Pérez and PhD Thesis of Lic. Germán Lukaszewicz. The authors thank also to the anonymous reviewers for helpful comments and discussion.

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