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ENVIRONMENTAL IMPACT OF INSECTICIDES APPLIED ON BIOTECH SOYBEAN CROPS IN RELATION TO THE DISTANCE FROM AQUATIC ECOSYSTEMS

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Abstract—Aquatic environments located in areas cultivated with biotech soybean were studied. Water and sediment samples were analyzed for insecticides, acute toxicity, genotoxicity, detoxification biomarkers, and fish diversity. Samples were taken in the core area of soybean cultivation in Argentina; all measures were related to the distance between the crops and the streams sampled. Endosulfan ($\alpha + \beta$) concentrations as high as 553.33 μg/kg were found in sediments from environments located at 0.15 m from treated fields. Ethoxyresorufin-*O*-deethylase (EROD) activity and cytochrome P4501A1 (CYP1A1) gene expression in fish showed the highest correlation with the environmental concentration of endosulfan. These biomarkers and mortality of amphipods significantly correlated with the concentration of endosulfan in water and sediment, which correlates inversely with the distance between the crop and streams. The differences with respective controls disappear at distances greater than 5 m. The fish diversity was significantly lower from distances between the margin of the stream and soybean crops, not exceeding 2 m. Environ. Toxicol. Chem. 2010;29:1907–1917. © 2010 SETAC

Keywords—Biotech soybean Insecticides Biomarkers Fish diversity Crop-stream distance

INTRODUCTION

In Argentina the area planted with soybeans from the years 1992 to 2008 showed an exponential growth, with an approximate annual growth factor of 1.15. Since the introduction of Round-up Ready soya (resistant to Round-up®, Monsanto) in 1996, the area planted increased from four million hectares to 17 million hectares. A total of 95% of this area corresponds to a transgenic variety of glyphosate tolerant soybean, which is cultivated by direct sowing [1].

A 5% increased occurred over the cropped surface area during the 2005/2006 period, due to increased cultivation south of Cordoba, north of La Pampa, west of Buenos Aires, as well as in the middle of the Negro River valley where the Patagonian soja's project is located. The majority (85%) of the planted area occupies the provinces of Buenos Aires, Cordoba, and Santa Fe, in the so-called core area. This is related to linear growth in productivity per hectare, in some cases exceeding 3,000 kg/ha. Relative to pesticides, insecticides are the focus of our work in the present study. Soy is attacked by a great diversity of defoliating caterpillars (Lepidoptera) during the growing season, whereas during the fruiting stage increased populations of bugs (Hemiptera) pose a serious threat to the crop because of their large effect on yield and quality of the seed. These stages are between the months of January and April. In this period the three most common pests found are: Isoc or loopers *Rachiplusia nu* (Lepidoptera, Noctuidae) normally controlled with the synthetic pyrethroid cypermethrin. The green bug, *Nezara viridula* (Hemiptera, Pentatomidae) is generally controlled with the organochlorine insecticide endosulfan and the *Epinotia aporema* borer outbreak, (Lepidoptera, Tortricidae) typically controlled by the organophosphate insecticide chlorpyrifos. Also, mixtures of endosulfan or chlorpyrifos are often used simultaneously with cypermethrin [2].

In Argentina in 2004 the National Secretary of Water Resources (2004) fixed the following levels of water column quality guidelines for endosulfan: <7 μg/L and cypermethrin and chlorpyrifos: <0.6 μg/L (www.obraspublicas.gov.ar). No guideline levels exist for these pesticides in benthic sediments. Very few studies have evaluated in a systematic way the effect of using these pesticides on aquatic ecosystems located in the so-called core area.

Work by Jergentz et al. [3] provides information on streams crossing soybean-growing areas near the city of Arrecifes (Buenos Aires): concentrations of the insecticide endosulfan ($\alpha + \beta$) as high as 318 and 43 μg/kg of suspended material. The present study reported a significant decrease in the number of macroinvertebrates with respect to the unpolluted streams in the same region. Our laboratory data shows that endosulfan is lethal for individuals of *Hyalella curvispina* at concentrations close to 1 μg/L [4]. Additionally, endosulfan can be rapidly bioaccumulated from sediment by rooted aquatic plants such as *Vallisneria spiralis* that reaches steady-state within 24 h [5]. In another study, Jergentz et al. [6] found for the same streams concentrations of chlorpyrifos and cypermethrin as high as 150 and 46 μg/kg of suspended solids (SS), respectively, and 100% mortality of *Hyalella curvispina* in field-condition toxicity tests. The authors relate the presence of these insecticides to runoff causes; however, they remark that other potential routes of pesticide entry into surface waters, such as spray drift, should also be investigated to provide data for a thorough risk assessment of pesticides in this region. Furthermore, the potential effects on fish are unknown in this area, although fish kills have been observed in the past. In this process it should

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relate well with the proximity of the soybean crop to aquatic environments.

The intent of the present study was to evaluate the impact of aerial spraying of insecticides at varying distances from aquatic organisms, present in different streams located in the core zone. The aerial application accounts for 30 to 70% of land applications. We propose that the effects are a function of the proximity of crops or the existence of a buffer zone between themselves and water bodies. We wanted to prove that the lack of it facilitates the incorporation into water of insecticides applied to the crop and thus affects the organisms present. Stress indicators were used in two different levels of organization: one suborganismal through the use of biomarkers and the other at the community level through the identification of fish diversity.

MATERIALS AND METHODS

Study area

The study area included the so-called core area of soybean cultivation in Argentina. This region saw increased acreage and productivity per hectare in the country. Located in the agricultural region of the humid Pampa, the core area is characterized by soil Mollisols of the suborder Argiudolls, subgroup typic Argiudolls. Clay–silt–sand content was 17, 57, and 26%, respectively. Mollisols are commonly the very dark colored, humus-rich surface horizon in which bivalent cations are dominant on the exchange complex and the grade of structure ranges from weak to strong. These are the mineral soils of the steppes [7]. This area is crossed by numerous streams of first and second order, and closely related to the surrounding land by runoff phenomena common after heavy rainfall (rainfall >100 mm/d). The average grade in the region is 2.5%. Due to the large size of cultivated areas, pesticide application is done by aerial spraying.

Sampling area

All sampled streams were no more than 5 m wide and 0.5 m deep, that is, narrows and shallows. All were included in cultivated areas. The maximum distance between the treated field and each stream was 20 m. The buffer zone was cover only for riparian vegetation no more than 20 cm high. The water and sediment samples were taken at 24 h postaerial application of insecticides to any sampling point, where no rainfall occurred for 15 d prior. This was to avoid a possible effect of contamination of water bodies due to runoff. The aerial application was conducted by aircraft such as AirTractor Piper Bravo 300, most of the agricultural aircraft were equipped with rotary atomizers like Micronair AU5000, and each atomizer can handle a flow of up to 23 L/min. The fumigation and weather conditions were: airplane speed 150 km/h, flight height 1.5 to 2 m, wind speed 8 to 11 km/h, air temperature 28 to 30°C, ground level relative humidity 40 to 45%, and swath width 30 m. The implementation of insecticides is done, in some cases, before flowering or when the presence of the pest is noted, according to the number of caterpillars present per meter and percentage of damage observed on soy. The recommended application doses are: endosulfan 35% (w/v) 1 to 3 L/ha, chlorpyrifos 48% 1 to 2 L/ha, Cypermethrin 25% (w/v) 100 to 160 cm^3/ha.

In total, 13 sites were selected for sampling in the core zone. Seven of them were in the province of Buenos Aires in an area of approximately 1536 km^2, bordered by the cities of Salto, Rojas, Arrecifes, and Pergamino. Four samples were taken in the Córdoba province and two in Santa Fe. Water samples were taken just above the bottom sediments, with a horizontal sampler of polyvinyl chloride Van Dorn's type (Wildco®, Wildlife Supply) and placed in glass bottles to reduce phthalate adsorption. At each sampling site pH, conductivity, dissolved oxygen, temperature, salinity, and turbidity were measured using a multiparameter probe Horiba U10 model. Laboratory determinations included total dissolved solids, total SS, and volatiles as described by the American Public Health Association (APHA) [8]. The sediments were sampled with a hand shovel, carefully collecting the first 5 cm of silt-clay fraction, placed in polypropylene bags. Sediments were characterized by determination of total organic carbon (TOC), sulfides, pH, and total ammonia, according to APHA methodology [8]. All samples were immediately refrigerated at 4°C until transportation to the laboratory, where they were stored at −20°C or used for conducting toxicity testing within 24 h of arrival. Figure 1 shows schematically the area covered by samples and in Table 1 the geographic coordinates of each point sampled are referenced, determined using a Garmin GPS II (global positioning system).

The sampling points were differentiated mainly by the proximity of soybean crops to the margins of the streams and the presence of crops on one or both sides of the aquatic environments.

Fish collection and diversity index

Based on preliminary sampling, it was determined that four passes (bank to bank) of a 10-m area with a 0.2×0.2 cm mesh seine with a blocking net of similar construction downstream to prevent escape was sufficient to sample 80% of the fish species present. At each sampling site fish were collected and counted and classified taxonomically. Diversity index was calculated according the Brillouin formula:

$$B = \frac{1}{N}\log\left(\frac{N!}{N_1!N_2!N_i!}\right)$$

Where N is the total number of individuals and N_1, N_2, N_i the individuals number for each species [9]. Healthy individuals of *Bryconamericus iheringii* were captured. In the field, fish were weighed and standard and total lengths were recorded. Liver and gills were removed, placed in cryovials, and immersed in a portable liquid nitrogen thermos. Once in the laboratory they were transferred to a −80°C freezer until analysis.

Toxicity tests

Acute toxicity assessment of water samples was performed with the green alga *Scenedesmus quadricauda* and the following native organisms: the microcrustacean *Daphnia spinulata*, the amphipod *Hyalella curvispina*, and the Poeciliid *Cnesterodon decemmaculatum*. Tests were performed following the U.S. Environmental Protection Agency (U.S. EPA) [10] and the Organization for Economic Cooperation and Development (OECD) [11] guidelines for receiving waters.

Algal toxicity tests

The strain *S. quadricauda* CCAP 276-21 was used in 96-h algal toxicity tests. Algal stock cultures were maintained in modified Detmer's nutrient medium (pH 7.5) under controlled conditions in an orbital shaker, Forma® (ThermoFisher Scientific), incubator chamber, at 22 ± 1°C, 3,000 lux/cm^2 of continuous cool-white fluorescent lighting, and 100 rpm [12]. The inoculants were prepared from these cultures to provide an initial cell density of 5×10^4 cell/ml in treated and control

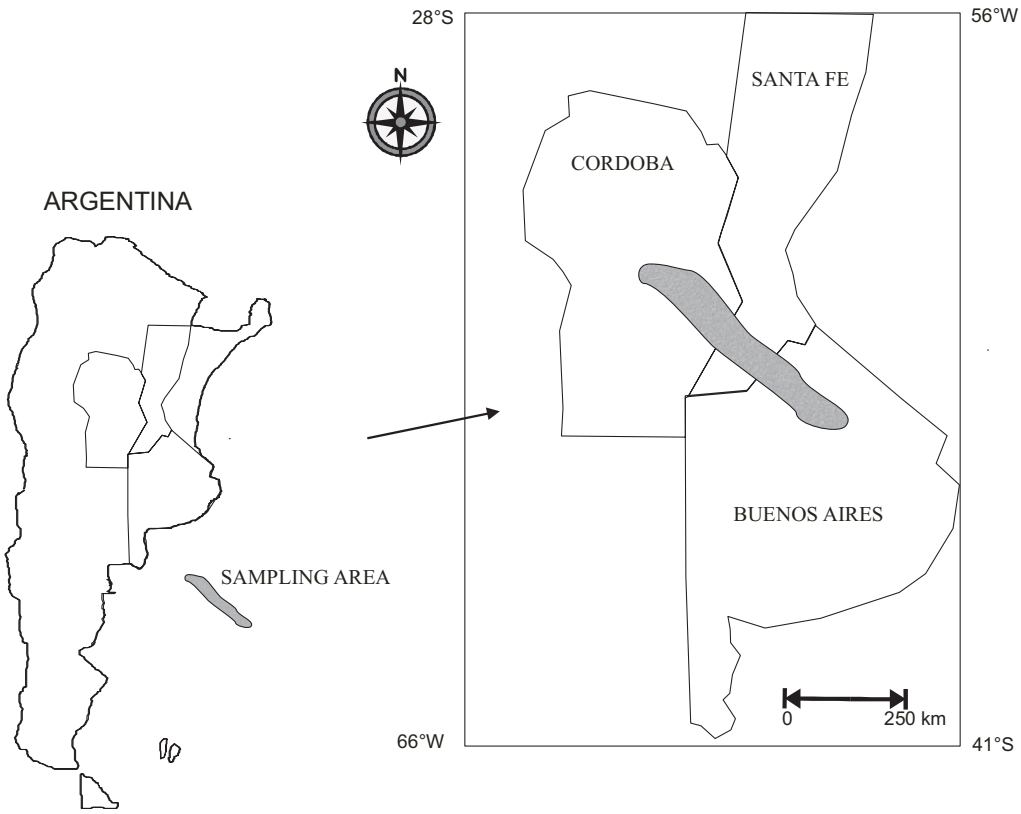

Fig. 1. Region under study in the core area, occupying the provinces of Buenos Aires, Cordoba, and Santa Fe, having 85% of the area planted with soy in Argentina.

flasks. Test solutions consisted of enriched stream water samples with algal medium nutrient solutions. Control cultures were incubated in the same medium. Control and treated cultures were grown under the same conditions of temperature, photoperiod, and shaking and were carried out in triplicate. Chlorophyll *a* was measured by a TD 700 Turner fluorometer (Turner Designs). Definitive protocols for toxicity testing using this species are described in Sáenz et al. [13].

Microcrustacean toxicity test

Tests were performed with laboratory cultures of *D. spinulata*, a native cladoceran widespread in freshwater environments of Buenos Aires Province. A 48-h static test was conducted at $21 \pm 1^{\circ}C$ in the dark. Twenty-four-hour-old organisms placed in artificial freshwater (pH, 7.87; total hardness, 95.87 mg CO_3Ca/L; conductivity, 475 μS/cm; and alkalinity, 189.37 mg CO_3Ca/L) served as controls. Definitive protocols for toxicity testing using these species are described in Alberdi et al. [14].

Amphipod toxicity tests

The native amphipod *H. curvispina* was selected as the test organism. Ten-day-old individuals were chosen because of their relative sensitivity (96-hLC50 median lethal concentration = 0.31 mg potassium dichromate/L) and size appropriate for test handling [15]. Age control was made using the equation $TL = 0.71 + t\ 0.037$ where *TL* is the total length in mm and *t* is the time in days. The water quality for controls was: hardness, 82 mg CO_3Ca/ L; conductivity, 500 μS/cm; pH, 8.3; and alkalinity, 212 mg CO_3Ca/L. A 96-h static test was carried out in darkness at $21 \pm 1^{\circ}C$, with 40 amphipods per water sample. Definitive protocols are described in Di Marzio et al. [16].

Fish toxicity tests

Fish toxicity tests were performed with the species *C. decemmaculatum*, a native member of the Family Poeciliidae that is widespread in the temperate water region throughout Buenos Aires Province. Ninety-six-hour semistatic tests were conducted. Fish captured in an artificial freshwater pond were

Table 1. Geographic coordinates of sampling sites, Argentina

Site	Buenos Aires		Córdoba		
Cañete (S1)	33°58′0.56″S	60°16′24.85″W	San Francisco (S8)	31°34′55.08″S	62°16′48.68″W
Maguire (S2)	34°2′37.47″S	60° 8′23.28″W	Bell Ville (S9)	32°34′15.16″S	62°42′18.57″W
Site 3 (S3)	33°57′49.94″S	60°16′41.78″W	Bell Ville (S10)	32°36′9.22″S	62°40′19.00″W
Site 4 (S4)	33°57′59.34″S	60°31′49.74″W	Marcós Juarez (S11)	32°47′47.82″S	62° 8′2.75″W
Site 5 (S5)	33°58′18.45″S	60°37′3.19″W		Santa Fe	
Site 6 (S6)	34° 5′38.54″S	60°38′32.33″W	Arteaga (S12)	33° ′05.50″S	61°46′37.00″W
Site 7 (S7)	34°15′23.47″S	60°32′9.04″W	Arequito (S13)	33°7′50.64″S	61°39′11.97″W

acclimated in groundwater (pH, 7.9; conductivity, 400 μS/cm; dissolved oxygen [DO] concentration. 8.20 mg/L; hardness, 98 mg $CaCO_3$/L) for 20 d under environmental test conditions. Tests were made using groundwater in glass aquaria at $21 \pm 1°C$ and a photoperiod of 12:12 h light:dark. The final volume was 2 L in order to obtain a loading rate of 0.5 g of fish/L. Samples and the control were in quadruplicate. Ten 1-month-old fish were used for each sample. Mortality was recorded every 24 h. The definitive protocol is described in Di Marzio et al. [17].

Sediment tests

Sediment toxicity was assessed with *H. curvispina* in a 10-d test. Sediment test conditions are summarized as follows: sampled sediment/control sediment; homogenized 50 g/250-ml dry weight; 10 animals per test chamber, eight replicates; 10 to 15 d old. The overlying water was aerated and cleaned every 24 h. Mortality and growth were recorded at the end of exposure. Sediment of an unpolluted stream containing less than 3% organic matter was used as the control in the experiments. Sediment organic matter was measured according to the APHA [8]. Further details of the protocol used are in U.S. EPA methods [18] and Di Marzio et al. [17].

Pesticides analysis

Samples were extracted twice with methanol (1:4) in an ultrasonic bath (Testlab®) for 60 min and then passed through C18 columns (Supelco). The pesticide analysis was performed after elution of the C18 columns with 2 ml hexane followed by 2 ml dichloromethane. Final volume was adjusted to 1 ml with N_2. The sample extracts were injected into a Shimadzu gas chromatograph (GC) 17A V 1.3 model, fitted with standard electron capture detector (ECD). A second analysis, to confirm the identity of the compounds, was performed using a GC with mass spectrometer QP 5050A and Mass Spectrometry Workstation Class 5000 (Shimadzu GC/MS) according to U.S. EPA method SW-846/8081B [19]. The GC/MS confirmation was accomplished by analyzing the same extract that was used for GC/ECD analysis and the extract of the associated method blank. Target compounds were endosulfan (α, β, sulfate), Chlorpyrifos, and α-cypermethrin. Quantification limits were 5 μg/kg (dry wt) and 0.5 μg/L for endosulfan and chlorpyrifos and 10 μg/kg for α-cypermethrin. Quantification was done using an external standard method purchased from Supelco. Laboratory quality assurance control included laboratory blanks and surrogate recovery spikes and were used to estimate the quality of the analytical data. Spiked sediments and water were recovered at 85 to 110% and 90 to 120, respectively.

EROD activity

Ethoxyresorufin-*O*-deethylase activity was measured according to the Association Française de Normalisation (AFNOR) method [20] with few modifications. Individual liver samples were thawed on ice and then homogenized in 100 mM phosphate–phenyl-methyl-sulfonyl fluoride (PMSF) 0.2 mM buffer (PB), pH 7.8 (1:4) using Eppendorf-fitting pestles. The homogenate was centrifuged at 9,000*g* for 20 min at 4°C and the postmitochondrial supernatant (PMS) collected for immediate use. Protein content of the PMS was determined by Quant-It™ Protein assay kit and Qubit™ fluorometer (Invitrogen/Molecular Probes). The reaction mixture contained PB, PMS, and 7-ethoxyresorufin. The reaction was initiated by adding nicotinamide adenine dinucleotide phosphate (NADPH) and incubating at room temperature ($21 \pm 1°C$) for 2 min. Fluorescence was measured with a Turner TD 700 fluorometer at excitation/emission wavelengths of 530/590 nm. The EROD activity was expressed as picomoles of resorufin produced per milligram of total protein per minute (pmol R mg $Prot^{-1}$ min^{-1}). Betanaphtoflavone was used as a positive control.

RNA extraction and isolation

Total RNA was isolated from liver samples using RNAeasy Midi kit from Qiagen that is suitable for 20 to 250 mg of liver tissue (Tecnolab). Liver samples (50 mg) were disrupted and homogenized in a highly denaturing guanidine isothiocyanate-containing buffer using Eppendorf-fitting, ribonuclease-free pestles. The lysate was centrifuged for 10 min at 5,000*g* and the supernatant carefully transferred to a new tube. Ethanol was added to provide appropriate binding conditions and the sample was then applied to the column containing a silica-gel-based membrane where total RNA binds and contaminants are efficiently washed away. High-quality RNA was then eluted in RNase-free water. Total RNA and possible DNA coextracted were estimated by fluorometric method using Quant-iT RNA and dsDNA BR assay kits and Qubit™ fluorometer (Invitrogen / Molecular Probes). Contaminating genomic DNA, if found, was removed using DNase I amplification grade (Invitrogen / Molecular Probes).

Design of oligonucleotides

The oligonucleotides were obtained from Operon and their sequence is detailed in Table 2. We chose the pairs of primers in the conserved region of vertebrate CYP1A sequences as proposed previously by Chaty et al. [21]. Primers actin-forward (ACT-F) and actin-reverse (ACT-R) were also used as coamplification of a standard gene.

OneStep RT-PCR

Reverse transcription (RT) and polymerase chain reaction (PCR) were carried out sequentially in the same tube using the One-Step RT-PCR kit from Qiagen. The protocol was optimized for 1 pg to 2 μg of total RNA as final concentration. The RNase inhibitor was added in the master mix at 5 to 10 units/reaction. Polymerase chain reactions were performed in an

Table 2. Primers used for polymerase chain reaction amplification of *Bryconamericus iheringii* cDNA corresponding to CYP1A1 and actin gene products.

Primer name	Oligonucleotide sequences	Origin	References
CYP1A1-F3	GGCTSCCWGGACCRAAGCCCC	Several vertebrates	[21]
CYP1A1-R3	CCGTAYTCWGGNGTCAWGTC		
CYP1A1-F4	SACTGCCATGARCRAGCGCTA		
CYP1A1-R4	TTCTCSCCNTCNWGCTTGTT		
ACT-F	GGAYGAYATGGAGAARATCTGG	Vertebrates and invertebrates	[21]
ACT-R	CCTGYTTGCTGAYCCACATCTG		

iCycler (Biorad) thermal cycler. The protocol conditions basically consist of 30 min for RT at 50°C, then the initial PCR activation step at 95°C, where HotStarTaq polymerase is activated, RT inactivated, and the cDNA template is denatured. Cycling parameters were as follows: 30 to 40 cycles of heat denaturation at 94°C for 30 s, annealing between 55 and 65°C for 30 s, polymerization or extension at 72°C for 1 min, and 10 min as final extension at 72°C. Aliquots (10 μl) of each reaction were separated by electrophoresis on 1% agarose gel in TAE (TRIS 40 mM, acetic acid 1 mM, ethylenediaminetetraacetic acid [EDTA] 40 mM) buffer, gels were stained with ethidium bromide, and the PCR products were visualized under ultraviolet (UV) light using a transiluminator. Digitalized gel images were analyzed measuring optical density (OD) for each amplimer's band by Image-Pro Plus V4.0 (Media Cybernetics). The OD ratio between CYP1A1 and Actin expression was used as a semiquantitative endpoint.

Single-cell electrophoresis assay

Gills were homogenized in phosphate-buffered saline (PBS) and centrifuged at 500*g* to remove gill filaments and other tissues. Individually suspended gill tissue cells were obtained from the supernate. Cell viability was determined using 0.4% of Trypan blue. A single-cell electrophoresis assay (SCEA) was carried out according to Di Marzio et al. [22]. Basically, the gels were composed of three layers of agarose. The suspensions of gill cells were diluted (1:2) with 1% low-melting-point agarose (LMPA), giving a final agarose solution of 0.66%, and 80 μl of the cell suspension was transferred to a slide having a thin layer of solidified 1% agarose. The slides were covered with a coverslip and left on ice for 10 min to allow the second layer of agarose to solidify. The coverslip was gently removed and 80 μl of 0.5% LMPA was spread over the second layer. A coverslip was placed on top of this third layer and the agarose solidified. This last coverslip was removed and each slide was immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris [pH 10)] 1% N-laurylsarcosinate, 1% Triton X-100, and 10% dimethylsulfoxide [DMSO]) to remove proteins and lipids. Lysis time was 60 min. Slides were then placed in an electrophoresis tank and covered with electrophoresis buffer (300 mM sodium hydroxide [NaOH], 1 mM Na_2EDTA, pH 13.5) for 25 min at room temperature to allow DNA unwinding. Electrophoresis (300 mA, 30 min, 1 V/cm) was then performed in the same buffer. The slides were washed once for 10 min in neutralization buffer (0.4 M Tris, pH 7.5). Before analysis the slides were stained with 30 μl of 20-mg/ml ethidium bromide. The slides were observed using an epifluorescence microscope (Nikon Eclipse 600) with a dichroic filter (excitation filter, BP 510-550 nm; suppression filter, BA 590 nm) linked to an image analysis system (Image-Pro Plus V4.0, Media Cybernetics). The DNA migration was measured as: % tail DNA (the percentage of DNA that migrated from the nucleus in the direction of the anode expressed as 100 − head % DNA). Hydrogen peroxide (H_2O_2) and PBS were used as positive and negative controls, respectively. Experiments were performed in triplicate and 900 cells were analyzed per each fish. Only comets with similar size and shape nuclei were scored to avoid heterogeneous response of differing cell types present in the cell suspension [22].

Statistical analysis

Amphipod mortalities recorded in sediment tests were compared by Kruskal–Wallis analysis of variance (ANOVA) and median test at $p < 0.05$ [23,24]. The EROD activity, genotoxicity, and gene expression were compared by one-way statistical ANOVA in conjunction with Dunnett's test, considering LF site as a negative control. A square root transformation was necessary to tail DNA (%) data to stabilize the variances and approximate a normal distribution. Site samples were analyzed by principal component analysis (PCA) mainly to explore the degree of correlation of EROD, genotoxicity, and gene expression parameters with the final concentrations of insecticides and distance between crop and streams. One matrix was constructed using these variables and the 13 site samples as the horizontal rows of the PCA data matrix. All statistical analysis was performed using Statistica v. 8 (StatSoft).

RESULTS

Table 3 shows the values of the physicochemical parameters determined at each sampling site. The concentration of SS varied over one order of magnitude between the different streams under study, ranged from 17.8 to 250 mg/L, and 90% percentile equal to 220 mg/L. Similarly, the percentage of organic matter in the SS varied between 0.22 and 3.08%, and 90% percentile equal to 2.64%. The sediments showed the following ranges for the determined parameters: TOC 30 to 55 mg/g dry weight, $N\text{-}NH_4^+$ 1.8 to 5.7 mg/L, sulfides 7 to 25 μg/L, pH 6.4 to 6.7.

Table 3. Physicochemical parameters of water at each sample and sites description

Site	Ph	Conductivity μS/cm	Turbidty NTU	Salinity 0/00	TDS mg/L	SS mg/L	VSS mg/L	Off-crop distance m	Soy crop
S1	7.01	1010	29	0.04	707	20.8	0.55	2	Om
S2	7.12	1150	63	0.05	805	29.4	1.01	1.7	Om
S3	7.59	2200	23	0.1	1540	17.8	0.89	0.15	Bm
S4	8.74	2970	785	0.14	2079	140	2.38	10.00	Om
S5	7.64	970	150	0.06	679	66.7	2.67	0.25	Bm
S6	8.02	3330	36	0.16	2331	26.67	1.36	15.00	Om
S7	8.67	7200	100	0.39	5040	40	0.48	5.00	Bm
S8	8.11	700	500	0.05	490	130	2.33	5.00	Om
S9	8.42	5390	500	0.29	3773	134	2.97	1.00	Bm
S10	8.84	1160	999	0.05	812	220	4.33	20.00	Bm
S11	6.7	980	391	0	686	100	1.7	1.20	Bm
S12	7.95	575	270	0.02	402.5	110	1.59	0.50	Bm
S13	7.72	1140	999	0.08	798	250	3.65	10.00	Bm
LF	8.31	811	51	0.01	540	40	5	—	—

All samples had dissolved oxygen concentrations greater than 5 mg/L. Bm = soy crop in both margins of the stream, Om = one margin. NTU = nephelometric turbidity units, TDS = total dissolved solids, SS = suspended solids, and VSS = volatile suspended solids. (—) = no crop.

Table 4. Endosulfan concentrations in sediment and water of the sampling sites, Argentina

Site	Description	μg/kg dry wt	SD	μg/L	SD
S1	Cañete	106.67	15.28	1.90	0.14
S2	Maguire	134.50	29.26	0.80	0.28
S3	+Maguire	553.33	145.72	20.00	1.41
S4	Pergamino 1	16.44	2.80	1.10	0.14
S5	Pergamino 2	403.33	45.09	14.00	1.41
S6	Pergamino 3	12.00	2.44	1.05	0.07
S7	Salto	50.67	5.55	1.05	0.22
S8	San Francisco	43.76	3.74	3.60	0.57
S9	-Bell Ville	200.70	25.92	8.80	1.70
S10	Bell Ville	22.71	3.20	1.20	0.28
S11	Marcos Juárez	230.66	20.94	7.50	2.12
S12	Arteaga	359.33	61.98	11.50	0.71
S13	Arequito	76.59	8.50	1.50	0.01
Las Flores	Control	ND	—	ND	—

SD = standard deviation, ND = not detectable. (—) = no data.

In water column and sediment samples, the presence of endosulfan sulfate, chlorpyrifos, or cypermethrin was not determined, but the insecticide endosulfan ($\alpha + \beta$) was identified in most of the studied samples, as shown in Table 4.

Water samples from all sites sampled did not result in acute toxicity to exposed organisms representing different trophic levels. Sediment samples from sites S1–S3, S5, S9, S11, and S12 caused mortalities between 65 to 100%. The remaining samples were not toxic, in terms of mortality or growth, comparing the total length reached by the exposed organisms with respect to the control group (Table 5).

The EROD activity of fish sampled in eight of the 13 sites under study was significantly ($p < 0.05$) higher for those caught in the stream considered control. These sites were in the province of Buenos Aires S1, S2, S3, S5, and S7, in Cordoba S9 and S11, and S12 in Santa Fe (Fig. 2). However, the relative gene expression was significantly different ($p < 0.05$) only in sites S3, S5, S11, and S12 (Table 6). All of these sites were bordered by soybean crop on both margins and the distance from soy crops and streams was less than 1.5 m.

Genotoxic effects, determined in gill cells of *B. iheringii*, were significantly higher ($p < 0.05$) for fish caught at the sampling stations S3, S5, and S12 (Fig. 3).

Table 5. Mortalities of *Hyalella curvispina* exposed to the sediment samples

Sample	Mortality	SD	Total length (μm)	SD
Control	7.50	7.07	1059.75	30.78
S 1	70*	5.34	1120	20.43
S 2	65*	9.16	1230	24.49
S 3	100*	—	—	—
S 4	6.25	7.44	1062.31	47.89
S 5	100*	—	—	—
S 6	8.75	6.41	1070.00	70.10
S 7	8.75	11.26	1128.44	83.27
S 8	6.25	9.16	1024.06	9.72
S 9	70*	7.07	1144.25	85.44
S 10	12.5	11.65	1142.38	90.49
S 11	80*	8.86	1021.88	7.76
S 12	100*	—	—	—
S 13	5	7.56	1061.25	3.11
LF	5.00	7.56	1050.94	19.41

Data are average values expressed in percentage. SD = standard deviation, LF = Las Flores. (—) = no data or all data 100%.
* Significantly different with respect to the control LF at $p < 0.05$.

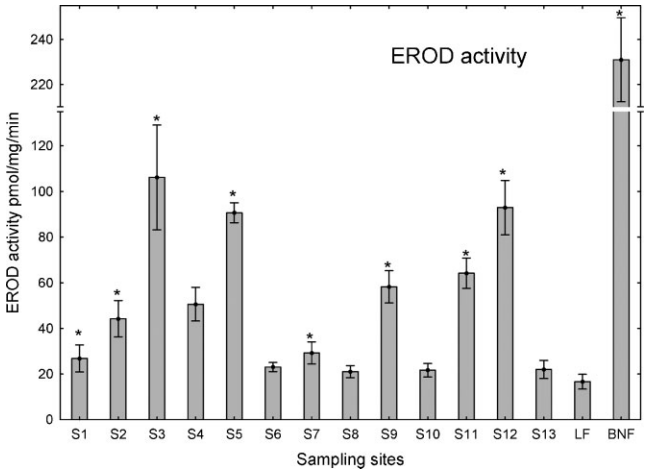

Fig. 2. Ethoxyresorufin-*O*-deethylase activity expressed in pmol/mg protein/min of *Bryconamericus iheringii* sampled at Buenos Aires (S1–S7), Córdoba (S8–S11), and Santa Fe (S12–S13), provinces. *Significantly different at $p < 0.05$ (ANOVA = analysis of variance, Dunnett's test) with respect to fish present at Las Flores (LF) stream. BNF = beta-naphtoflavone positive control.

The fish diversity was significantly different from distances between the margin of the stream and soybean crops, not exceeding 2 m (Fig. 4).

DISCUSSION

There was a relationship between the distance of the crop to the margins of streams, the relative values obtained in each biomarker used, and fish diversity. Integrating all the results presented by PCA, we saw that two components of PCA analysis (Fig. 5) explained more than 90% of the variance. The first component would define the variability, taking into account the total endosulfan concentrations for all samples. The second component could be interpreted as a measure of the variability of the distance between crop and aquatic environments. Table 7 shows the partial correlation coefficients between the variables from the PCA. The distance was negatively correlated with other measured parameters, having a stronger correlation with the concentration of endosulfan in water and sediment. Among the biomarkers, EROD activity showed the highest correlation coefficients with the concentration of insecticide in both arrays (Table 7). Mortality of amphipods exposed to sediment factors showed correlations

Table 6. Ratios of reverse transcriptase-polymerase chain reaction products intensities (CYP1A1/ACT) from Las Flores site (LF) and ethoxyresorufin-*O*-deethylase's positive sites for Buenos Aires (sites 1-5 and 7), Córdoba (sites 9 and 11), and Santa Fe (site 12) provinces, Argentina

	CYP1A1/ACT	SD	SEM	Off-crop distance (m)
Site 1	0.262	0.15	0.07	2
Site 2	0.154	0.10	0.04	1.7
Site 3	1.29 *	0.20	0.09	0.15
Site 5	1.184 *	0.17	0.08	0.25
Site 7	0.158	0.07	0.03	5
Site 9	0.324	0.09	0.04	1
Site 11	0.47 *	0.18	0.08	1.2
Site 12	0.5 *	0.22	0.10	0.5
LF control	0.172	0.11	0.05	No crop

Average values from five animals were used in each group. SD = standard deviation, SEM = standard error of the mean.

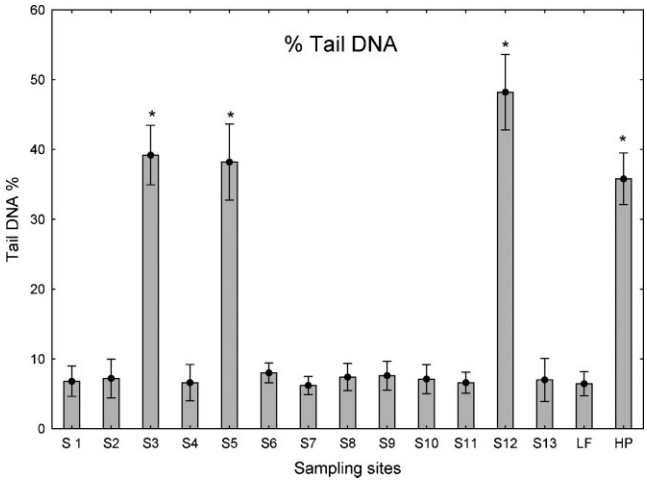

Fig. 3. Tail DNA of gill cells of *Bryconamericus iheringii* sampled at Buenos Aires (S1–S7), Córdoba (S8–S11), and Santa Fe (S12–S13), provinces. *Significantly different at $p < 0.05$ (ANOVA = analysis of variance, Dunnett's test) with respect to the fish present at Las Flores (LF) stream. HP = hydrogen peroxide, positive control.

higher than 0.8 regarding endosulfan in water and sediment concentrations (Table 7).

The EROD and gene expression biomarkers and mortalities of amphipods significantly correlated with the concentration of endosulfan, which correlates inversely with the distance between the crop and streams (Table 8). The differences with respect to controls were no longer statistically significant at distances greater than 5 m.

In the case when the two banks of streams are bordered by crops, the aerial application of insecticides would include them as part of the cultivated area. Insecticides are more or less bioavailable in relation to the concentration of SS and organic matter content. Furthermore, the concentration of SS present in water bodies can reduce the toxicity of endosulfan to factor increases close to five times one order of magnitude of the SS [4]. Moreover, streams that are in such shallow (<0.5 m) and with low current (0.1–0.3 m^3/s) environments have intimate contact between the sediment, water column, and biota. In this scenario, if we consider the aerial application of endosulfan at doses between 1 to 3 L/ha of commercial formulation 35% (w/v) on a body of water of 0.3 m average depth, this will result in an insecticide concentration of 300 μg/L. However, a value of log K_{OC} for endosulfan of 4.09 and the fraction of organic carbon in SS and sediments, approximately equivalent to 5%, results in a K_d near 600. Taking 0.1 g/L as the average concentration of SS in the environments studied, we obtain nominal concentrations of endosulfan of 0.18 μg/ml in water and 112 μg/g in the SS. Preliminary studies on endosulfan spraying artificial lagoons yield values close to those mentioned above (W. Di Marzio, unpublished data). This partition to the solid matrix can be accelerated by using an antievaporating agent in aerial application. If the relative humidity is below 60%, it is recommended to use oils that provide this function (e.g., soybean oil) to prevent loss by volatilization of the drops applied on the crop. Thereby the insecticide can enter the water, contained in a hydrophobic matrix that can easily adsorb the material in suspension, and thus precipitate the sediment rapidly. Following spray application, Wan et al. [25] measured endosulfan ($\alpha + \beta$) concentrations in farm ditch sediments in Canada ranging from 5 to 2,461 μg/kg. Leonard et al. [26] measured up to 48 μg/kg of total endosulfan ($\alpha + \beta$) in sediments of the Namoi River in Australia. Antonious and Byers [27] studied the fate and movement of endosulfan under field conditions and reported the vertical movement of endosulfan through the vadose zone at a concentration of 630 μg/L.

Jergentz et al. [3] performed in situ bioassays with amphipods observing toxic effects, according to the authors, associated with chlorpyrifos and endosulfan adsorbed to particulate matter. However, the animals used were kept in chambers of 1,500 μm mesh, closed at the ends, with the base in contact with sediments. Thus, that the field effect could be caused by pesticides associated with the superficial sediments. In the present study the sediments belonging to sites S1–3, S5, S9, and S11–12 were relatively toxic to amphipods compared to a control site, resulting in 100% mortalities some cases; it is possible that this mortality could be related to the concentration of endosulfan in those sediments (Table 5, Fig. 5). As mentioned above, cypermethrin and chlorpyrifos were not detected in samples of water or sediment. During the sampling period

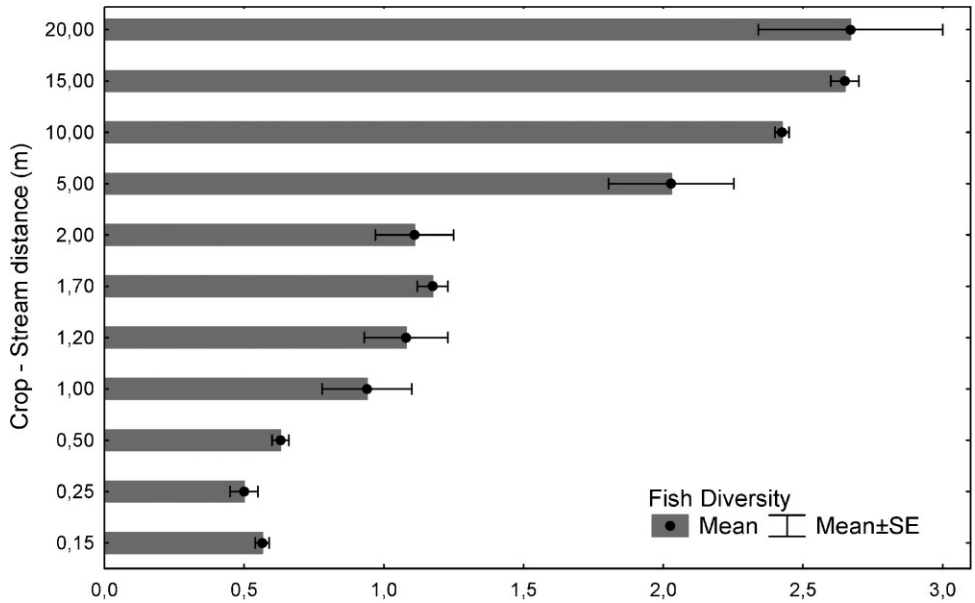

Fig. 4. Fish diversity at each sampling site. SE = standard error of the mean.

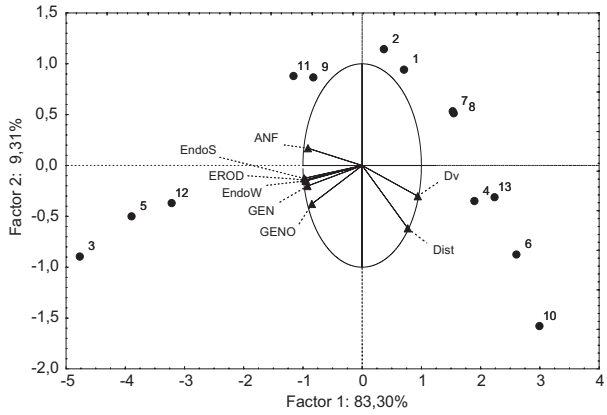

Fig. 5. Biplot of two-dimensional scattergram corresponding to the distributions of principal components parameters of each sampled site and the measured variables. The two components, factors 1 and 2, explained more than 90% of total variance. EROD = ethoxyresorufin-*O*-deethylase activity; ANF = amphipod mortality; EndoS = endosulfan sediment concentrations; EndoW = endosulfan water concentrations; GEN = gene expression; GENO = genotoxicity.

(summer 2006–2007) the high temperatures limited the application of cypermethrin due to its greater volatility and was replaced by endosulfan. The application of the latter was predominant, regardless of the type of pest present between the months of January to March.

Endosulfan can also be incorporated by runoff but the spray drift is a major source of pesticide exposure in off-crop habitats, neighboring streams, and other water bodies [28]. Jergentz et al. [6] found that the highest chlorpyrifos concentration in the suspended particles was detected in a period without runoff events. The authors concluded that the likely alternative source in this case was airplane fumigation, resulting in insecticide deposition on the water surface and subsequent sorption to suspended particles and sediments. It is a general management practice in the region under study to spray the soybean fields by air after the plants reach a certain size because airplanes cause less damage to the crop than vehicles. Aquatic environments are included in the application area, especially if there is no separation with the crop, and/or a buffer zone does not exist between them. Wang and Rautmann [28] concluded that wind speed, relative humidity (weather condition), nozzle type, and spray pressure (spray practice) are the main factors influencing spray drift. The influence of each has a strong relationship with the distance between sprayed field and surface waters. Wind speed has a strong positive influence far away from fields, whereas close to fields the effect diminished substantially. Very close to field margins (3 m or less) the effect of wind speed is negative and increasing values cause a reduction of spray drift.

Table 8. Regression parameters obtained considering biomarkers

Y	*X*	Intercept	Slope	Adjusted R^2	F	*p* value
EndoS	1/distance	64.38	82.38	0.864	77.57	0.00001
EndoW	1/distance	2.51	2.88	0.813	53.19	0.00002
EROD	EEC	23.57	0.156	0.857	72.67	0.00004
GEN	EEC	-0.013	0.002	0.88	93.81	0.00001
GENO	EEC	2.63	0.073	0.719	31.70	0.0001

EROD = ethoxyresorufin-*O*-deethylase activity; GEN = gene expression; GENO = genotoxicity. Endosulfan concentrations in water (EndoW) and sediment (EndoS), EEC = environmental endosulfan concentration, and distance crops – stream.

According to Wang and Rautmann [28], spray pressure has a marked (positive) effect on this variable in the proximity of field borders, in the range of 2 to 15 m, whereas at larger distances spray drift is not affected by spray pressure.

Aerial application (AA) and runoff (RO) are the two sources of insecticides in streams from treated fields. From the information generated in other studies and the data obtained in our study, considering the design's sample in which runoff events were avoided, we assume that AA was $\gg$RO, so that the environmental endosulfan concentration (EEC) (water + sediment) was based on aerial application. In this regard, and given the proximity of crops to streams, the spray pressure would be the variable that most influences the contribution of AA, which varies depending on the mode of application and the nozzle type. The latter determines the mean diameter of the droplets of spray related with drift postimplementation. From these considerations the results of this study allow us to establish the relationship between the EEC and the distance between the crop/edge of streams and the EEC with the biomarkers studied, as presented in Table 8.

The potential relationships to species diversity is not included because we are assuming that the EEC is related to the aerial application and postapplication within 48 h of the occurrence. However, the diversity indices recorded in this study are related to stream/crop distance (Fig. 4) and would reflect the effect of the sustained implementation of recent years on this variable. In this regard, a close relation between the distance of the crops to streams and fish diversity was observed (Fig. 5). While no deaths or acute effects occurred on the water samples tested, the species diversity was significantly different between different sampling sites. This was reflected in the number of species (Table 9) and, in the evenness component of the fish communities, heterogeneity related with the distribution of specific abundances (data not shown). The fish diversity for the control stream was similar to that reported by Di Marzio et al. [29].

Figure 6 represents the concentrations of endosulfan, in water and sediment, as a function of distance between streams

Table 7. Correlation matrix of principal component analysis variables, correlation factors between paired variables

	EndoS	EROD	GENO	GEN	ANF	Dist	EndoW	Dv
EndoS	1.00	0.94	0.86	0.94	0.88	−0.75	0.96	−0.87
EROD	0.94	1.00	0.86	0.88	0.84	−0.64	0.92	−0.86
GENO	0.86	0.86	1.00	0.79	0.71	−0.45	0.82	−0.70
GEN	0.94	0.88	0.79	1.00	0.79	−0.59	0.93	−0.79
ANF	0.88	0.84	0.71	0.79	1.00	−0.70	0.82	−0.87
Dist	−0.67	−0.64	−0.45	−0.59	−0.77	1.00	−0.63	0.91
EndoW	0.96	0.92	0.82	0.93	0.82	−0.73	1.00	−0.86
Dv	−0.87	−0.86	−0.70	−0.79	−0.87	0.91	−0.86	1.00

Biomarkers EROD = ethoxyresorufin-*O*-deethylase activity; GEN = gene expression; GENO = genotoxicity. Endosulfan concentrations in water (EndoW) and sediment (EndoS); Dist = distance crops – stream; ANF = amphipod mortality; Dv = fish diversity.

Table 9. Freshwater species found at each sampling site widespread in an agricultural area close to 30,000 km^2 in three provinces, Argentina

Specie	LF	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13
Asiphonichthys stenopterus	+													
Oligosarcus jenynsi	+	+	+	+	+	+	+	+	+		+		+	
Diapoma terofali	+				+				+					
Pseudocorynopoma doriai	+		+		+			+	+		+		+	
Astyanax eigenmanniorum	+	+	+		+		+	+	+		+		+	
Astyanax fasciatus	+	+	+		+			+	+	+	+	+	+	
Bryconamericus iheringi	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hyphessobrycon anisitsi	+	+	+		+			+			+		+	
Hyphessobrycon meridionalis	+				+			+	+	+	+			
Cheirodon interruptus	+	+	+	+	+	+	+	+	+	+	+		+	
Hoplias malabaricus	+	+	+		+		+	+	+		+		+	
Characidium fasciatum	+				+								+	
Curimata gilberti	+													
Heptapterus mustelinus	+	+	+		+			+	+				+	
Pimelodella laticeps	+				+									
Rhamdia sapo	+	+	+		+		+	+	+		+		+	
Corydoras paleatus	+	+	+		+		+	+	+	+	+		+	
Loricariichthys anus	+	+	+		+		+	+	+		+		+	
Ancistrus cirrhosus	+		+		+		+	+					+	
Hypostomus commersoni	+		+		+		+		+		+			
Jenynsia lineata	+				+						+			
Cnesterodon decemmaculatus	+	+	+	+	+	+	+	+	+		+	+	+	+
Phalloceros caudimaculatus	+	+			+		+	+	+				+	
Synbranchus marmoratus	+	+		+	+			+	+		+	+	+	+
Gymnogeophagus australis	+		+	+	+									
Cichlasoma facetum	+		+	+	+			+			+		+	+
Crenicichla sp.	+													

and crops. This exponential behavior in the ambient concentration of endosulfan, presumably derived from aerial application, was described previously by Ernst et al. [30], who found the following concentrations of the insecticide as a function of the distance between the sprayed field and the studied pond water: 607 to 670, 503 to 535, 91 to 62, and 28 to 12 μg/L for 0, 3, 10, and 30 m. The results of that study indicated an obvious concern, in that fish mortality was never less than 80%, even at distances of up to 200 m downwind. Also, they noted that under normal spraying conditions, using conventional delivery technology, unacceptable deposits, or those that would cause 50% mortality in an exposed population, occur at distances of 10 m. The authors recommended that an appropriate buffer zone should exceed 200 m and that endosulfan should not to be used near water without further refinement of buffer zone requirements.

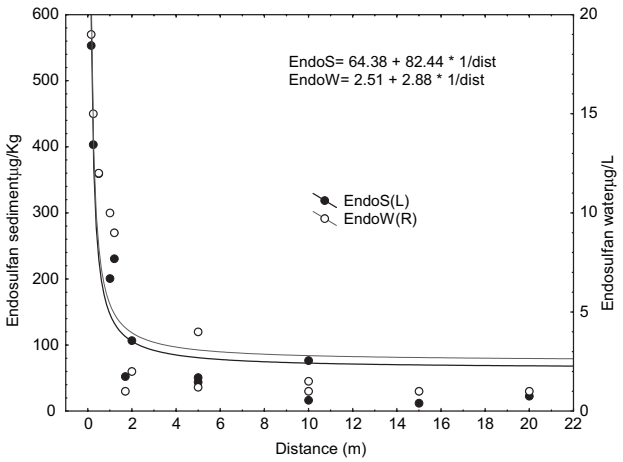

Fig. 6. Relations between endosulfan concentrations in water and sediment samples with the distance between the crop and stream. EndoS = endosulfan sediment concentrations; EndoW = endosulfan water concentrations.

The inherent capacity of a pesticide-stressed aquatic community to recover is considered important for the authorization and risk assessment of these chemicals. Ecological effects of modern agrochemicals are typically limited to brief episodes of increased mortality or reduced growth that are qualitatively similar to natural disturbance regimes. The long-term ecological consequences of agrochemical exposures depend on the intensity and frequency of the exposures relative to the rates of recovery of the exposed populations [31]. Recently, Brock et al. [32] discovered that treatment-related effects on macroinvertebrates were expected to become less pronounced as a larger proportion of the ditch surface area remained unsprayed. Their results suggest that overall insecticide effects may be smaller and ecological recovery faster in systems where uncontaminated refuges are present, despite the presence of local patches with relatively high exposure concentrations. However, in aquatic environments such as those studied here, where virtually all sections of the streams are included in an area planted with soybeans, this recovery can be greatly reduced by determining the loss of species and therefore reducing the species diversity and without doubt related to the proximity of the crop to the stream (see Fig. 7). Jergentz et al. [6] proposed as a strategy, suitable under local conditions, for improving water quality and protecting the water resources, vegetated wetlands as one potentially effective means of mitigating the risk associated with agricultural pesticide contamination. We propose that initially the crops should start 5 m away from water bodies and that this separation may or may not be vegetated. This would be a starting point to control the excessive cultivation and associated pesticide spraying.

CONCLUSION

The amount of pesticide that enters aquatic environments is related to the distance separating the treated fields from adjacent aquatic habits. Higher concentrations of insecticide were related to the smaller separation between them. This effect would

Fig. 7. Images showing the proximity between crops and stream environments. Photos corresponding to site S5 (left) and S12 (right).

disappear when the distance is greater than 5 m. Fish that live in streams located within this distance show values significantly different from those considered controls for the biomarkers evaluated. Within these, EROD activity and CYP1A1 gene expression showed the highest correlation with the total ambient concentration for endosulfan, the only quantified insecticide of the three insecticides potentially applied in the area under study. Moreover, the fish diversity in the environments studied also significantly correlated with the distance. The reduction of fish diversity in environments closer to soybean crops reflect a cumulative effect of environmental stress in terms of the quantity of pesticides applied related to the intensive cultivation of soybeans. Therefore, the requirement of a separation line equal to or greater than 5 m between the crop and the margin of the aquatic environment would contribute substantially to reducing the incorporation of chemicals in ecosystems and their associated impact.

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REFERENCES

1. Pengue WA. 2005. Transgenic crops in Argentina: The ecological and social debt. *Bull Sci Technol Soc* 25:314–322.
2. Aragón J, Molinari A, Lorenzatti S. 1998. Manejo integrado de plagas de soja. In Giorday L, Baigorri H, eds, *El cultivo de soja en la Argentina* Instituto Nacional de Tecnología Agropecuaria. Editar, Marcos Juárez, Córdoba, Argentina, pp 247–288.
3. Jergentz S, Pessacq P, Mugni H, Bonetto C, Schulz R. 2004. Linking in situ bioassays and population dynamics of macroinvertebrates to assess agricultural contamination in streams of the Argentine pampa. *Ecotoxicol Environ Saf* 59:133–141.
4. Di Marzio W, Sáenz M, Alberdi J. 2007. Relación entre la ecotoxicidad del insecticida endosulfan y la concentración y naturaleza de sólidos en suspensión. Resúmenes, VIII Congreso SETAC LA, Montevideo, Uruguay, October 8-11, p 74.
5. Di Marzio WD, Sáenz ME, Alberdi JL, Tortorelli MC, Nannini P, Ambrini G. 2005. Bioaccumulation of endosulfan from contaminated sediment by *Vallisneria spiralis*. *Bull Environ Contam Toxicol* 74:637–644.
6. Jergentz S, Mugni H, Bonetto C, Schulz R. 2004. Runoff-related endosulfan contamination and aquatic macroinvertebrate response in rural basins near Buenos Aires, Argentina. *Arch Environ Contam Toxicol* 46:345–352.
7. U.S., Department of Agriculture. 2003. *Keys to Soil Taxonomy*, 9th ed. USDA Natural Resources Conservation Service, Washington, DC, USA.
8. American Public Health Association. 1992. *Standard Methods for the Examination of Water and Wastewater*, 19th ed. Washington, DC, USA.
9. Krebs CJ. 1989. *Ecological Methodology*. Harper Collins, New York, NY, USA.
10. U.S. Environmental Protection Agency. 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. EPA-821-R- 02-012. Washington, DC.
11. Organisation for Economic Co-operation and Development. 1981. Alga growth inhibition, *Daphnia* sp. acute immobilisation, Fish acute toxicity tests. *Guidelines for Testing of Chemicals No. 201-3*. Paris, France.
12. Walsh GE. 1988. Principles of toxicity testing with marine unicellular algae. *Environ Toxicol Chem* 7:979–987.
13. Sáenz ME, Alberdi JL, Di Marzio WD, Tortorelli MC. 1996. *Protocolo estandarizado de ensayos de toxicidad con las algas de agua dulce Scenedesmus acutus y Scenedesmus quadricauda* Resúmenes, X Congreso de Toxicología, Buenos Aires, Argentina, September 18-20, p 32.
14. Alberdi JL, Di Marzio WD, Sáenz ME, Tortorelli MC. 1996. *Protocolo para evaluación de la toxicidad aguda con Daphnia spinulata (Cladocera)* Resúmenes X Congreso de Toxicología, Vol 1, Buenos Aires, Argentina, September 18–20.
15. Simionato E, Alberdi WD, Sáenz ME, Tortorelli MC, Di Marzio WD. 1997. *A native South American amphipod: Hyalella curvispina used in ecotoxicological bioassays. Growth and sensitivity. Abstracts.* 18th Annual Meeting of SETAC, San Francisco, CA, USA, November 16–20, p 295.
16. Di Marzio WD, Sáenz ME, Alberdi JL, Tortorelli MC. 1996. *Protocolo para evaluar la toxicidad aguda sobre Cnesterodon decemmaculatus (Pisces, Poeciliidae)* Resúmenes, X Congreso de Toxicología, Vol 1, Buenos Aires, Argentina, September 18–20, p 31.
17. Di Marzio WD, Sáenz ME, Alberdi JL, Tortorelli MC. 1999. Assessment of the toxicity of stabilized sludges using *Hyalella curvispina* (Amphipod) bioassays. *Bull Environ Contam Toxicol* 63:654–659.
18. U.S. Environmental Protection Agency. 2000. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. EPA 600/R-99/064. Duluth, MN, USA.
19. U.S. Environmental Protection Agency. 2008. Test Methods for Evaluating Solid Waste, Physical/Chemical Methods, final up-date IV of SW-846. EPA-HQ-RCRA- 2006-0833. Washington, DC, USA.
20. Association Francaise de Nornalisation. 2001. Méthode de mesure de l'activité enzymatique EROD chez le poisson d'eaux douces et marines. AFNOR NF T 90-385, Saint-Denis La Plaine, France.
21. Chaty S, Rodius F, Vasseur P. 2004. A comparative study of the expression of CYP1A and CYP4 genes in aquatic invertebrate (freshwater mussel, *Unio tumidus*) and vertebrate (rainbow trout, *Oncorhynchus mykiss*). *Aquat Toxicol* 69:81–93.
22. Di Marzio WD, Saenz ME, Lemiere S, Vasseur P. 2005. Improved single cell gel electrophoresis assay for the earthworm *Eisenia foetida*. *Environ Mol Mutagen* 46:246–252.
23. Sparks T. 2000. *Statistics in Ecotoxicology*. John Wiley & Sons, New York, NY, USA.
24. Zar JH. 2010. *Biostatistical Analysis*. Pearson, Boston, MA, USA.

25. Wan MT, Szeto S, Price P. 1995. Distribution of endosulfan residues in the drainage waterways of the Lower Fraser Valley of British Columbia. *J Environ Sci Health (B) Pest Food Contam Agric Wastes* 30:401–433.
26. Leonard AW, Hyne RV, Lim RP, Pablo F, Van den Brink PJ. 2000. Riverine endosulfan concentrations in the Namoi River, Australia: Link to cotton field runoff and macroinvertebrate population densities. *Environ Toxicol Chem* 19:1540–1551.
27. Antonious Gf, Byers ME. 1995. Fate and movement of endosulfan under field conditions. *Environ Toxicol Chem* 16:644–649.
28. Wang M, Rautmann D. 2008. A simple probabilistic estimation of spray drift—Factors determining spray drift and development of a model. *Environ Toxicol Chem* 27:2617–2626.
29. Di Marzio WD, Tortorelli MC, Freyre LR. 2003. Diversidad de peces en un arroyo de llanura. *Limnetica* 22:73–78.
30. Ernst WR, Jonah P, Doe K, Julien G, Hennigar P. 1991. Toxicity to aquatic organisms of off-target deposition of endosulfan applied by aircraft. *Environ Toxicol Chem* 10:103–114.
31. Barnthouse LW. 2004. Quantifying population recovery rates for ecological risk assessment. *Environ Toxicol Chem* 23:500–508.
32. Brock TCM, Roessink I, Belgers JDM, Bransen F, Maund SJ. 2009. Impact of a benzoyl urea insecticide on aquatic macroinvertebrates in ditch mesocosms with and without non-sprayed sections. *Environ Toxicol Chem* 28:2191–2205.