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ECOTOXICOLOGY IN TROPICAL REGIONS

Risk assessment of agriculture impact on the Frío River watershed and Caño Negro Ramsar wetland, Costa Rica

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Abstract The Caño Negro Ramsar wetland is a conservation area of great natural and societal value, located in the lower part of the Frío River watershed in the north of Costa Rica. Its aquatic ecosystems may be considered vulnerable to pollution due to recent changes in land use toward agriculture. In 2011 and 2012, quarterly sampling was done at ten sites located in the middle and lower sections of the Frío River Basin that pass through crop areas and later drain into Caño Negro wetland. Pesticide residues, nitrates, sediment concentrations, and diversity of benthic macroinvertebrates and fish biomarkers were studied in the selected sites. Additionally, risk of toxicity was calculated in two different ways: (1) by using a ratio of MEC to hazard concentrations threshold for 5% of species (HC_5) to calculate a risk quotient (RQ), and (2) by using a ratio of MEC to available ecotoxicity data of native fish and cladocera for diazinon and ethoprophos, to obtain a risk quotient for native species (RQns). Results indicated that three out of the ten sites (rivers Thiales, Mónico, and Sabogal) showed variable levels of pollution including six different active ingredients (a.i.) of pesticide formulations (herbicides ametryn, bromacil, and diuron; insecticides cypermethrin, diazinon, and ethoprophos). Moreover, potential adverse effects on fishes in Thiales and Mónico rivers were indicated by cholinesterase (ChE) inhibition and glutathione S-transferase (GST) enhancement. Risk evaluations indicated pesticide residues of ametryn, bromacil, and ethoprophos to be exceeding the limits set by MTR, also RQ was high (>1) in 70% of the positive samples for diuron (most frequently found pesticide in water samples), cypermethrin, diazinon, and ethoprophos, and RQns was high for diazinon. Therefore, these substances might be of major concern for the ecological health of aquatic ecosystems in the middle basin of the Frío River. The most critical site was Mónico River, which had the highest pollution (75% detection samples with 3–5 a.i.) and highest calculated risk (RQ > 1 in 75% of the samples). This is also the river that most directly drains into the protected wetland. Even though pesticide pollution in this area is not as severe as in other parts of Costa Rica, it is imperative that measures are taken, particularly in the surroundings of Mónico River, in order to diminish and mitigate possible detrimental effects to biota in Caño Negro Ramsar Site.

Keywords Costa Rica · Caño Negro · Wetland · Pesticides · Ecotoxicology · Risk

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Introduction

Although wetland protection is mandatory in at least 159 countries including Costa Rica, wetlands are under pressure of drainage, and pollution with pesticides and nutrients related to agriculture (Verhoeven and Setter 2009). Ecologically, these environments are characterized by supporting a high biodiversity. Their topographic gradients, hydrologic properties, and seasonal cycles propitiate high productivity which in turn results in high diversity of flora and fauna, resident and migrant (Bacon 1997). Many of the resources provided by wetlands have long been used by human populations. Access to water, food, and recreation are among the most

evident services provided by wetlands (Carbonell 1997). Neotropical wetlands vary enormously in size and species composition due to their long latitudinal and altitudinal ranges which favors high biodiversity and endemism (Carbonell 1997). In this region, agriculture is one of the most important human activities associated with wetlands. Its expansion along with the use of pesticides represents a major pressure for these environments (Wittmann et al. 2015). This is evidenced by the presence of agrochemical compounds found in them (Laabs et al. 2002; de la Cruz et al. 2015).

The Frío River Basin, located in the northern plains of Costa Rica, holds 1281 ha of orange, 541 ha of rice, and 3771 ha of pineapple plantations (UNA 2012). The normal climate pattern is rainy most of the year, with less rainfall in March–April and July–August. Annual precipitation is 2000–3000 mm (Rojas et al. 2011). Caño Negro wetland is one of the main drainages in the Frío River Basin (Ramírez-Granados 2013). During the last three decades, these zones have experienced a process of agriculture conversion, from self-sustaining cultures or small scale farms (e.g., grains, coffee, cacao) to more extensive plantations for exportation products (e.g., orange, cassava, and pineapple) that had diverse social, economic and environmental outcomes in the region (Granados et al. 2005). Intensive livestock production still remains, which has led to decreased forest coverage (Villacis et al. 2003). All these factors have been accompanied with changes in the hydrodynamic conditions in the Frío River and Caño Negro. Environmental impacts are occurring due to sedimentation, dredging and reduction of natural water bodies, rapid decline of forest cover, and changes in land use from wetland to grass and agricultural areas (Solano 2002; Solano and Salas 2011). Yet, chemical impacts in the river and in Caño Negro due to pesticides have not been published to date.

Pesticide use in Costa Rica has doubled since the 1970s. Estimated values for 2006 were 19.3 kg of a.i./cultivated ha (Ramírez et al. 2009). Studies on the detection of pesticide residues and impacts on biodiversity have been conducted in various watersheds of the Pacific and Caribbean slopes, from mountain streams (Fournier et al. 2010) to the lowlands (Echeverría-Sáenz et al. 2012; Castillo et al. 2000, 2006) near conservation areas (de la Cruz et al. 2015; Mena et al. 2014a, 2014b) and near coasts (Castillo et al. 2014; de la Cruz et al. 1998; Carazo and Acuña 2009). However, no such studies have been done in the surroundings of the Caño Negro area until now. This is the first scientific report on water quality of the Frío River Basin and the Caño Negro wetland including the assessment of chemical pollution and the evaluation of biological impacts.

Caño Negro Wildlife Refuge (≈10,000 ha) is located in the lower part of the Frío River Basin, where 10% of the conservation area is flooded. In 1991, the refuge was declared a Ramsar wetland of international value, because it has the largest lagoon in the northern part of the country and high diversity of resident and migratory birds (IUCN 2006). It is also the habitat of an emblematic (representative) fish, the tropical gar *Atractosteus tropicus*, which has been considered a living fossil that reaches its southernmost geographic distribution limit in Costa Rica (Bussing 1998).

The growing area of rice, orange, and pineapple mentioned above will most likely increase the runoff of nutrients, pesticide residues, and sediments into the surface waters and may affect the protected area. Studies conducted in similar ecosystems in Brazil, south of Florida (USA), and the north Pacific coast of Costa Rica have determined significant pesticide residues including organophosphates, pyrethroids, triazines, uracils, and substituted ureas. In addition, effects on ChE inhibition, reduction in the consumption rate of *Daphnia magna*, and risk of toxicity for arthropods and primary producers have been observed (de la Cruz et al. 2015; Schuler and Rand 2008; Laabs et al. 2002). In this study, we present results of water quality in several rivers in the drainage area to Caño Negro. We determined the presence of pesticides in the waters, performed an acute mortality toxicity test with the insecticide diazinon in a native cladoceran, and an effect assessment with fish based on biochemical responses related to oxidative stress. We also assessed the diversity of the community of aquatic invertebrates present in sites upstream and downstream of crop areas. The evaluation of toxicity risk, invertebrate community, and chemical characterization data were compared in order to characterize the influence of pesticides in the ecosystem. The results aim to aid in identifying the environmental risks of pesticides to the aquatic ecosystems of the Frío River and Caño Negro wetland.

Materials and methods

Study area

Ten sites of the Frío River Basin were sampled: Frío River (FR1, FR2, and FR3), Samen River (SR1, SR2), Thiales River (TR1, TR2), Celeste River (CR), Mónico River (MR), and Sabogal River (SR) (Fig. 1, Table 1). These sampling points in the medium and lower parts of the Frío River Basin cover about 60% of the total catchment area. The study period (January, April, July, and October 2011; February, May, and August 2012) coincided with a strong La Niña event, which led to drier conditions from March until October 2011. The precipitation deficit during this time period (compared to average local precipitation in the same months) reached almost 80% in some areas and months (IMN 2011).

Physical and chemical analysis

Quarterly water samples were taken for water quality analysis as shown in Table 1: sites downstream from crop areas (TR2,

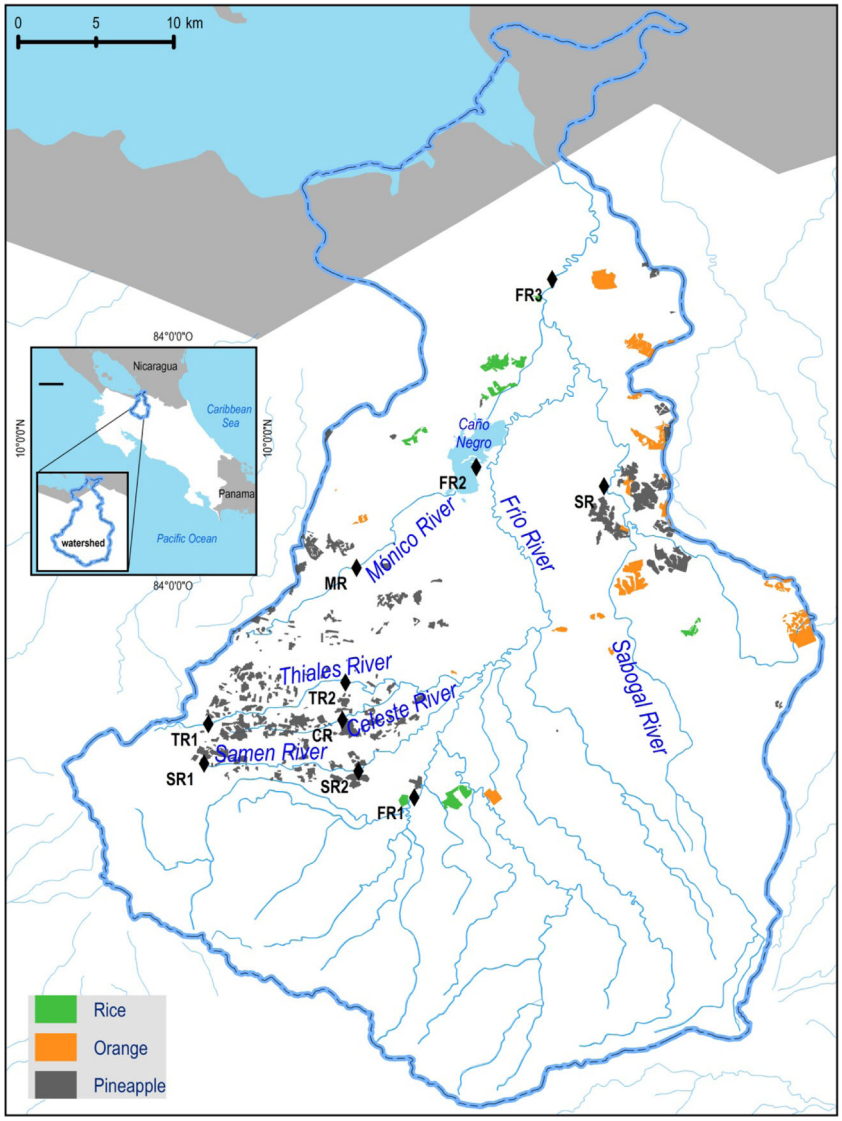

Fig. 1 Study sites at Frío River Basin in Costa Rica; location of orange, pineapple, and rice plantations, 2011–2012

MR, SR, and FR2) were sampled seven times between January 2011 and August 2012; one additional sample was taken at MR in October 2010. The rest of the sites were sampled four times in 2011.

Physicochemical water parameters including temperature (°C), pH, conductivity (μS/cm), and dissolved oxygen (mg/L) were measured in situ with a HACH HQ40d multi probe. Total suspended solids (TSS, mg/L) and nitrates (NO_3, mg/L) were determined according to the ASTM (1999) and APHA (2005) Standard Methods, respectively.

Pesticide residue samples were taken in 4-L glass bottles stored at 4–6 °C and processed before 48 h. Unfiltered water samples were extracted using solid-phase extraction (SPE) Isolute Env + 200 mg, 6 mL (Biotage, Sweden). Pesticide residues were identified and quantified using gas chromatography with mass detection (GC-MS) for nonpolar pesticides and high performance liquid chromatography (HPLC) with diode array detection for polar pesticides (LC-PDA). A GC-MS with an Agilent 7890A GC and 5975C MS (Agilent Technologies, Palo Alto, USA), in synchronous SIM and Scan mode, a CTC Combipal autosampler (CTC Analytics AG, Switzerland), and a capillary column BP35 (Agilent Technologies, Palo Alto, USA) (25 m × 0.25 mm × 0.25 μm), were used. The data acquisition was carried out using Chemstation software and NIST08 Mass Spectral Database. The temperature program was 90 °C for (1 min) to 210 °C with 20 °C/min, and then up to 300 °C with 4 °C/min, the interface was held at 280 °C, the ion source at 230 °C, the injector at 230 °C. The samples (2 μL) were injected in pulsed splitless mode. Ethion was used as internal standard, and pesticide concentrations were quantified by external calibration.

LC-PDA analyses were performed using a Shimadzu HPLC LC-10AD with an SPD-M10A diode array detector (Shimadzu, Kyoto, Japan). The chromatographic column was a LiChroCART HPLC RP-18e column

Table 1 Description of sampling sites and timeline in Frío River Basin, Costa Rica

Sites	FR1	FR2	FR3	SR1	SR2	CR	TR1	TR2	MR	SR
Location	Middle catchment area, near village	Caño Negro wetland	Downstream of protected area	Upstream of crops	Downstream of crops	Downstream of volcano	Upstream of crops	Downstream of crops	Downstream of crops	Downstream of crops
Land slope	Level	Level	Level	Moderately inclined	Level	Level	Very gently inclined	Level	Level	Very gently inclined
Bottom substrate	Cobbles, gravels, sand	Silt	Cobbles, gravels, sand, silt	Blocks, stones, cobbles, gravels	Cobbles, gravels, sand	Blocks, stones, cobbles, gravels	Cobbles, gravels, sand	Cobbles,gravels, sand, silt	Silt	Silt
Riparian vegetation cover (%)	10–50	10–50	10–50	>80	50–80	50–80	50–80	10–50	<10	10–50
Depth (m)	1	2.5–6	2–6	0.5–1.5	0.3–1.5	0.6–2	0.2–0.8	0.3–1	0.3–1.65	0.6
Chemical analysis 2010									X	
2011	X	X	X	X	X	X	X	X	X	X
2012		X						X	X	X
Benthic macro invertebrates 2011				X	X		X	X		
Fish biomarkers 2012								X	X	X

(125 mm × 3 mm × 5-μm particle size, Merck, Germany) and held at 40 °C. The mobile phase consisted of 20 mM sodium acetate in ultra-pure water/methanol 56:44 (solvent A) and methanol (solvent B). Mobile phase was delivered at a flow rate of 0.5 mL/min. Gradient elution program started from 100% of solvent A, decreased to 50% A in 15 min and held for 5 min; decreased to 20% A in 5 min and held for 5 min; and finally increased to 100% A in 5 min and held for 5 min. The total run time was 45 min. Volume injection was 50 μL. Identification was performed using retention time and the UV-spectra of the pesticides included in the analysis. Quantification was performed by external calibration.

The analyses included a list of 38 pesticides used in crops like pineapple, rice, orange, and banana. Quantification limits for detected substances were as follows: ametryn (0.04 μg/L), bromacil (0.05 μg/L), cypermethrin (1 μg/L), diazinon (0.01 μg/L), diuron (0.04 μg/L), ethoprophos (0.1 μg/L), and propiconazole (0.2 μg/L). The list of analyzed pesticides includes around 28% of those commonly used on pineapple, rice, and orange crops; glyphosate, carbendazim, and mancozeb are the most used a.i., but were not analyzed in this study because the methodology required is not in service at our laboratory.

Toxicity risk assessment

In order to evaluate the risk of the presence of pesticides in the collected water samples, two different evaluations were executed:

1. Risk quotient (RQ): The risk for each detected active ingredient was calculated by the comparison between its MEC and the 5% hazard quotient HC_5 as a risk index ($RQ = MEC/HC_5$). RQ values >1 were classified as high risk of toxicity, values from 0.5 to ≤1 as moderate, and values ≤0.5 were considered of low risk. HC_5 refers to the hazardous concentration to 5% of species 95% of the time, which is derived from species sensitivity distributions (SSDs).

SSDs were used to interpret the results of toxicity tests obtained with single species and to determine an ecotoxicity threshold. This reflects the concentration at which a significant risk to the community of species is reached (Del Signore et al. 2016; Posthuma et al. 2002).

To construct the SSD curves, single-species chronic toxicity data were collected from open access existing toxicity databases ECOTOX (http://www.epa.gov/ecotox/), OPP Pesticide Ecotoxicity Database (http://www.ipmcenters.org/ecotox), EPA-RED and other EPA documents (USEPA 1981, 1992, 2005) and publications (Kussatz et al. 2001; Maltby et al. 2005; Van den Brink et al. 2006; Wilson and Wilson 2010; Arias-Andrés et al. 2014; Diepens et al. 2014;

Pathiratne and Kroon 2016). In this study, two different taxonomic groups were selected to generate the SSD, primary producers (diatoms, green algae, cyanobacteria) for herbicides, and arthropods (shrimps and water fleas) for insecticides.

Other criteria such as test endpoint and duration were used to select the species toxicity data. Used endpoints were mainly the NOEC for growth, mortality and reproduction of arthropods, and population growth for plants and algae. If few chronic toxicity data were available, the lowest observed effect concentration (LOEC) was also used. Selected test duration was 1 to 28 days for arthropods, >6 days for macrophytes, and 2 to 10 days for algae. When more than one toxicity value within the time span and with similar endpoints was reported for a species, then the geometric mean was calculated. Each species was used only once in the distribution, and in cases with multiple acceptable toxicity values, the lowest value was selected.

The number of data points for the construction of the SSD was between five and nine; this is still low and has associated uncertainties, however, the uncertainties in HCx decrease when the number of data points is greater than four (Del Signore et al. 2016; Van den Brink et al. 2009). The amount of chronic toxicity data available for the herbicides ametryn and bromacil and the insecticide ethoprophos were low, but sufficient to generate SSDs for primary producers and crustaceans. Six chronic toxicity data were available for ametryn and eight for bromacil in 12 species (supplemental Table S2). These included seven species of green algae, two species of diatoms, one species of blue green algae, and two species of aquatic monocots (duckweed and tapegrass). Toxicity values for ethoprophos were available for five species of crustaceans, two shrimps and three cladocera (Table S2). HC_5s for the herbicide diuron and the insecticides cypermerthryn and diazinon were already constructed and calculated (Van den Brink et al. 2006; Maltby et al. 2005). In the case that the reported HC_5s from the literature were derived from acute toxicity endpoints, values were divided by a safety factor of 10. The HC_5 and their 95% confidence intervals were calculated using the CADDIS data analysis from EPA software (EPA 2005) obtained from CADDIS home page (http://www3.epa.gov/caddis/da_advanced_2.html).

2. Risk Quotient for native species (RQns): We performed a lower tier risk assessment of insecticides found in water samples with native species. For this, we compared the measured environmental concentrations (MECs) and available ecotoxicity data of native fish and cladocera to the insecticide ethoprophos (Mena et al. 2012, Mena et al. 2014a, 2014b; Diepens et al. 2014; Arias-Andrés et al. 2014). Furthermore, acute toxicity tests were performed in the laboratory with the insecticide diazinon and a culture of *Simocephalus semiserratus* (cladocera) isolated from the study area. Only the sensitivities of ethoprophos and diazinon were analyzed by this method since ecotoxicity data for native species were available for just these two pesticides.

Cladocera culture conditions and pesticide solution preparation followed procedures described in Arias-Andrés et al. (2014). Toxicity tests were performed based on standard OECD (2004) methods with modifications as in Arias-Andrés et al. (2014). Briefly, stock and working solutions from analytical grade pesticide reagents (Sigma-Aldrich, >90% purity) were prepared by IRET's Laboratory of Pesticide Residue Analysis, UNA, using acetone as solvent. Aliquots of stock solutions were weighed using volumetric flasks to prepare working solutions in purified water (Milli-Q Plus System, Millipore Co.). The working solutions were used to prepare the test concentrations in moderately hard reconstituted water (MHRW). A solvent control was also used in toxicity tests to ensure that the observed toxicity was not caused by the solvent. Tests were performed at 20 °C, with ten individuals (of less than 24 h) per replicate of each treatment. An average LC50-48 h was obtained based on three independent repetitions of the acute test.

In order to be conservative, RQ_{ns} were calculated using the maximum MEC of these two insecticides detected in surface water and the ecotoxicity data described in Table 3 according to the formula RQ_{ns} = [MEC/LC50 or NOEC] × safety factor. A safety factor of 10 and 100 (European Commission 2002) was applied when NOEC and acute (LC50) values were used, respectively. Ethoprophos and diazinon sensitivities were analyzed by this method since ecotoxicity data for native species were available.

Biological evaluation in situ

Quarterly sampling periods (January, April, July, and October 2011) were executed to evaluate the benthic macroinvertebrate communities in the Samen (SR1, SR2) and Thiales (TR1, TR2) rivers. These analyses were performed upstream (SR1 and TR1) and downstream (SR2 and TR2) of crop areas to investigate the impact of these activities on the biological community. Sampling dates included dry and rainy seasons, as well as the transition period. Benthic macroinvertebrates were collected by sampling with a triangular kick net (250 μm mesh) over a 5-min period within all available microhabitats and then sorted in the field. Organisms were preserved in ethanol (95%) and stored in ethanol 70% prior to identification to family or genus level, when possible. Generated data included taxa richness; abundance; diversity; percentage of Ephemeroptera, Plecoptera, and Trichoptera taxa in each macroinvertebrate sample (%EPT); and Biological Monitoring Working Party index, modified for Costa Rica (BMWP-CR; Springer et al. 2010; MINAE-S

2007). ANOVAS were used to compare diversity, richness, %EPT, and BMWP-CR results among sites and sampling dates. Also, simple regression analyses were used to calculate relations between diversity measures and BMWP-CR with all measured physical-chemical parameters. Total abundance of intolerant species to organic pollution between upstream and downstream from crops sites was compared with a paired *t* test. Analyses were done using PAST-3 statistical package (University of Oslo).

Biomarkers were measured in fish exposed in situ: juveniles (2.5–3 cm long) of a native fish, *Parachromis dovii* (Cichlidae), were placed in cages built with PVC pipe frames (25 × 30 × 15 cm) and 4 mm mesh and exposed to river water at sites TR2, MR, and SR during February, May, and August 2012. These three sites were chosen for biotoxicity assessment of fishes because they were located downstream of crops and before drainage into Caño Negro wetland. Five fish per cage and two cages per site were fixed from the shore and suspended at mean water level. A control group of ten fish was kept in a container filled with filtered UV-treated water (Millipore®) and aeration during the exposure period. All the exposed fishes and control group were dissected after 48 h. Samples of brain, lateral muscle, and liver of individual fish were collected, transported to the laboratory in liquid nitrogen, and then stored at −80 °C until analysis.

Cholinesterase activity (ChE) was measured in the brain and muscle samples, while glutathione S-transferase activity (GST), catalase activity (CAT), and lipid peroxidation (LPO) were measured in liver samples. Methods for sample preparation and biochemical measurements are described in Mena et al. (2014a, 2014b). Briefly, protein content in sample homogenates was determined by the method of Bradford (1976) using γ-globulin as a standard. ChE activity was measured using the method of Ellman et al. (1961), using 1 mM acetylthiocholine and 0.1 mM 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB) as a substrate and conjugate, respectively; reaction was measured at 415 nm during 15 min and expressed as nanomoles per minute per milligram protein. GST activity was determined as described by Habig et al. (1974), exposing samples to 1 mM CDNB and 1 mM GSH and monitoring the reaction at 340 nm for 3 min; activity was reported as nanomoles per minute per milligram protein. Lipid peroxidation was measured by the thiobarbituric acid reactive species (TBARS) assay (Oakes and Van Der Kraak 2003) and expressed as nanomoles of TBARS per milligram of protein. CAT activity was measured according to Aebi (1974) by the decrease in absorbance at 240 nm during 20 s due to H_2O_2 consumption and expressed as micromoles per minute per milligram protein. Biomarker data were transformed to generate an integrated biomarker response (IBR) according to Beliaeff and Burgeot (2002) and Devin et al. (2014). As suggested by these authors, circular permutations in the order of biomarkers were created during the calculation to generate a set of IBR values for every sample. This allowed a comparison between sites and the control. Differences in each biomarker among sites were tested with a one-way ANOVA coupled with a Dunnet test to compare every site to the control. Post hoc tests were ran looking for inhibition of ChE and inhibition or induction of the other three biomarkers compared to the controls. Differences in IBR values were assessed with a Kruskal-Wallis test coupled to a pairwise comparison. Analyses were done using IBM® SPSS® Statistics 22 software (trial version).

Results

Chemical analysis and toxic risk estimations

Mean concentrations of nitrates (Table S1 in supplementary data) were low at all sites (<0.5–0.8 mg/L) according to the limits established for the water quality environmental protection in Costa Rica (MINAE-S 2007). Suspended solids at site FR2 (Table S1), located in the conservation area exceeded the limit of 25 mg/L established for the protection of aquatic communities in Costa Rica (MINAE-S 2007).

Regarding pesticide residue contamination, 10 of the 53 surface water samples were positive for six different pesticides; residues were detected only at three sites, TR2, MR, and SR, all downstream from crop areas (Table 2). Pesticide residues were detected in 75% of the surveys carried out at MR and in 28.6% of the surveys in TR2 and SR. Mónico River showed the highest contamination in terms of number of pesticides and concentrations. The second highest concentrations were found at site SR influenced by pineapple and orange crops and at TR2, which is directly affected by a drainage canal from a pineapple packing plant. The herbicide diuron was the most frequently detected pesticide (80% of all positive samples), and ametryn and bromacil were present in approximately half of these samples. Insecticides were present only once (cypermethrin) or twice (ethoprophos and diazinon) during the sampling period, mostly in MR and TR2 (Table 2).

Also, calculated chronic risk (RQ; Table 2) for the individual herbicides was moderate to high for diuron (RQ = <0.9–4.5), and from low to moderate for ametryn (RQ = 0.3–0.8) and bromacil (RQ = 0.1–0.5) particularly in MR, followed by TR2 and SR. The chronic risk of insecticides for crustaceans was high for cypermethrin in MR (RQ = 5000) and diazinon (RQ = 1.2) in TR2.

The RQ_{ns} of the insecticides calculated using field measured concentrations and ecotoxicity data from species that are native to the study area indicate risks of acute toxicity only for the cladoceran *Simocephalus semiserratus* in TR2 by maximum concentrations of diazinon (RQ_{ns} = 2), but not for ethoprophos. No risk of acute toxicity were calculated in relation to the concentrations of ethoprophos quantified in this

Table 2 Pesticide (a.i.) residue concentrations (μg/L) in surface water from the studied rivers

Sites (+/n)	FR1 (0/4)	FR2 (0/7)	FR3 (0/4)	SR1 (0/4)	SR2 (0/4)	CR (0/4)	TR1 (0/4)	TR2 (2/7)		MR (6/8)		SR (2/7)	
								a.i.	RQ	a.i.	RQ	a.i.	RQ
Herbicides (HC_5)													
Ametryn (0.065)	nd	nd	nd	nd	nd	nd	nd	0.05	0.8	<0.04	0.3[a]	0.04	0.6
								0.05	0.8	0.04	0.6		
Bromacil (0.606)	nd	nd	nd	nd	nd	nd	nd	nd		0.09	0.1	nd	
										0.14	0.2		
										0.30	0.5		
										0.30	0.5		
Diuron (0.044)	nd	nd	nd	nd	nd	nd	nd	<0.04	0.5	0.04	0.9	0.07	1.6
										0.08	1.8		
										0.10	2.3		
										0.11	2.5		
										0.13	3.0		
										0.20	4.5		
Insecticides (HC_5)													
Cypermethrin (0.0004)	nd	nd	nd	nd	nd	nd	nd	nd		2	5000		
Diazinon (0.026)	nd	nd	nd	nd	nd	nd	nd	0.03	1.2	<0.01	0.2[a]		
Ethoprophos (0.160)	nd	nd	nd	nd	nd	nd	nd	nd		<0.10	0.3[a]		
										0.20	1.3		

Positive of total samples ratio (+/n), HC_5 (μg/L), and chronic toxicity risk (RQ) also included. "Positive of total samples ratio" refers to number of samples with pesticide detection over total number of samples collected at each site

nd no pesticides detected

[a] Pesticide concentrations were reported as traces, and therefore, RQ was calculated with the concentration value that represents half of the detection limit

study for native fishes *A. aeneus*, *A. tropicus*, or *P. dovii* (Table 3).

Biological evaluation

Reference sites (SR1 and TR1) did not differ significantly from downstream sites (SR2 and TR2) in terms of macroinvertebrate diversity ($F = 0.80$; $df = 3$; $p > 0.05$), %EPT ($F = 0.99$; $df = 3$; $p > 0.05$) or BMWP-CR indices ($F = 0.20$; $df = 3$; $p > 0.05$). However, it is noteworthy that four of the eight upstream/downstream comparisons had a lower BMWP-CR at the downstream site (Table 4). According to the classification of water quality proposed by the BMWP-CR index, only one sample was classified as "bad, very contaminated" water (Jan 2011, at SR2), while nine samples were "regular, moderately contaminated" (all four sites) and six were "good quality waters" (all four sites) (Table 4). BMWP-CR did not differ among sampling months either ($F = 0.84$; $df = 3$; $p > 0.05$), but diversity was significantly higher in October than in January and July ($F = 4.12$; $df = 3$; $p < 0.05$). Simple regression analysis showed a moderately strong inverse relationship between diversity and the pH

Table 3 Sensitivity of native aquatic species to insecticides found in the Frío Watershed

Target group	Substance	Species	Effect	Endpoint	μg/L	Source
Fish	Ethoprophos	*Astyanax aeneus*	Mortality	96-h LC50	250–500	Mena et al. (2014b)
		Parachromis dovii	Mortality	96 h LC50	242 (179–328)	Diepens et al. (2014)
		Atractosteus tropicus	Mortality	96 h LC50	859.7 (659.3–1170.6)	Mena et al. (2012)
			Cholinesterase inhibition	LOEC	50	Mena et al. (2012)
				NOEC	10	Mena et al. (2012)
Cladocera	Ethoprophos	*Simocephalus semiserratus*[a]	Mortality	48 h LC50	10.6 ± 2.1	Arias-Andrés et al. (2014)
			Reproduction inhibition	NOEC	4	Arias-Andrés et al. (2014)
	Diazinon	*S. semiserratus*	Mortality	48 h LC50	1.5 ± 0.3	This study

In the endpoints values: ( ) numbers in paragraphs indicate 95% confidence interval of mean; mean ± SD

[a] Identified before as *S. serrulatus*

Table 4 Benthic macroinvertebrates abundance, diversity (Shannon H), %EPT, and water quality indices (BMWP-CR) reported for the SR1, SR2, TR1, and TR2 sites in four sampling periods of 2011

Abundance	SR1	SR2	TR1	TR2	Diversity	SR1	SR2	TR1	TR2
January	110	196	237	231	January	2.6	2.5	2.5	2.7
April	44	193	277	290	April	2.6	2.7	2.9	2.8
July	159	322	91	273	July	2.3	2.9	2.7	2.8
October	155	173	262	305	October	2.9	3.2	2.9	2.9
%EPT	SR1	SR2	TR1	TR2	BMWP-CR[a]	SR1	SR2	TR1	TR2
January	52	41	57	49	January	94	57	91	80
April.	40	49	49	52	April	79	78	111	77
July	52	58	48	47	July	91	101	63	103
October	33	53	49	67	October	101	108	113	75

[a] BMWP-CR: <15 = extremely contaminated waters; 16–35 = bad quality waters, very contaminated; 36–60 = contaminated waters; 61–100 = regular quality waters, moderately contaminated; 101–120 = good quality waters; >120 = excellent quality waters

($r = -0.66$, $p = 0.006$). None of the other measured physical and chemical parameters were significantly related to diversity or BMWP-CR. Pesticide residues were not detected in Samen and Thiales rivers (SR1, SR2, TR1, TR2) during the macroinvertebrate sampling period; however, pesticides were detected at TR2 in 2012.

In terms of community structure taxa such as Isopoda, *Cloeodes* sp. (Baetidae), *Perigomphus* sp. (Gomphidae), *Polyplectropus* sp. (Polycentropodidae), *Dryops* sp. (Dryopidae), *Hexacylloepus* sp., *Phanocerus* sp. (Elmidae), Ptilodactylidae, *Dixella* sp. (Dixidae), and Dolichopodidae were present at one or both reference sites but were absent from both downstream sites. Also, *Hexanchorus* sp., *Macrelmis* sp. (Elmidae), *Baetodes* sp. (Baetidae), and *Nectopsyche* sp. (Leptoceridae) were very abundant in sites upstream of crops and significantly less abundant in downstream sites (Table S3, supplementary information). Total abundance of the abovementioned taxa was significantly higher in upstream than in downstream sites (paired $t = 8.13$; $p < 0.0001$).

A significant inhibition of ChE activity was found in muscle of *P. dovii* exposed at sites TR2 (16.4% compared to control) and MR (17.6% compared to controls) during February 2012 (supplemental Fig. S1, A). During the same period, GST activity significantly increased (45.2% compared to controls) in fish exposed at MR (Fig. S1, C). As well, the five biomarkers measured for each site in every period during the study in 2012 were integrated into IBRs to have a unified value of the biochemical responses. This resulted in the following IBRs for each site and period: TR2 (2.31 in February, 0.65 in May, and 2.22 in August), SR (1.21 in February, 5.54 in May, and 4.19 in August) and MR (5.25 in February, 1.43 in May, and 0.02 in August). Out of these IBR values, the ones calculated for February at MR and in May at MR and SR were significantly higher than controls. During this study, we observed that three of the biomarkers measured varied significantly ($p < 0.05$) in their level (range of units) observed among the sampling periods. This based on a comparison of control fish among periods. The magnitude of the variation was as big as double for muscle ChE and triple for LPO and CAT (Fig. S1).

Discussion

Chemical analysis and toxic risk estimations

Agrochemicals used in agricultural areas of pineapple, rice, and orange may be reaching the aquatic ecosystems of the Frío River watershed and the Caño Negro wetland, either dissolved in water or associated with soil particles as a result of the runoff. Also, the presence of packing plants in the area can be associated with residues found at TR2; pesticide residue occurrence in other fruits packing plants is high (Castillo et al. 2000). Pesticide use (in kg a.i./ha/year) for the three main crops in the Frío River watershed has been estimated to be 25.2 for pineapple (Bravo et al. 2013), 16.2 for rice (Ledezma 2010), and 5.2 for orange (Ramírez 2014).

Pesticide levels detected in water in some of the sampling sites exceed national and international regulations. The maximum concentrations of ametryn, bromacil, and ethoprophos found in the field exceeded 5, 44, and 3 times, respectively, the limits set by MTR regulation for the protection of aquatic ecosystem (RIVM 2015). Also, cypermethrin concentration in surface water exceeded once the maximum acceptable value of the Environmental Quality Standard (EQS) for the protection of aquatic organisms by over 22,000-fold (RIVM 2015). The presence of these substances in such concentrations might pose a danger for the aquatic biota of the sites evaluated, and this could ultimately be of major concern once the water reaches Caño Negro Ramsar wetland.

Herbicides were the group of pesticides more frequently detected (Table 2) in the Frío watershed. Presence of these compounds has also been observed in other tropical wetlands

associated with agricultural areas such as those from Lake Okeechobee to Everglades National Park (Ramsar site) in southern Florida (USA) (Schuler and Rand 2008) and the Pantanal wetland in Mato Grosso, Brazil (Laabs et al. 2002), both located downstream of intensive agricultural areas with frequent pesticide use. Ametryn, for example, has been found in higher (maximum of 1 μg/L in the Everglades) and lower concentrations (maximum of 0.082 μg/L in Pantanal) than the ones reported in Frío River (0.8 μg/L). In the Everglades study, diuron and bromacil were also present in more than 80% of the samples, in concentrations ranging 0.59–76 and 0.81–14 μg/L, respectively. Back in Costa Rica, but in the Caribbean region, at sites downstream of pineapple and banana plantations, ametryn, bromacil, and diuron have been also found at elevated concentrations and high frequencies (Echeverría-Sáenz et al. 2012, 2016; Rämö et al. 2016; Arias-Andrés et al. 2016). In those studies, the risk for primary producers has been estimated as very high (Rämö et al. 2016; Arias-Andrés et al. 2016). In our study, diuron and bromacil concentrations were much lower (maximum 0.2 and 0.3 μg/L, respectively); however, herbicides were detected in 19% of the samples collected and we obtained estimations of moderate and high risk for chronic effects related to herbicides, mostly due to diuron. This should be considered as an existing threat for the primary production and the stability of the Caño Negro wetland.

Regarding insecticides, cypermethrin was the compound with the higher risk estimation during this study. The levels found in water might cause acute and chronic effects to sensitive species of these ecosystems, such as Cladocera and Insecta (Antwi and Reddy 2015; Christensen et al. 2005). There are indications that a short-term (1-h) exposure to the pyrethroid fenvalerate (up to 0.01 μg/L) may significantly retard growth and emergence period of insect larvae (Liess and Schulz 1996). Pyrethroids, such as fenvalerate and cypermethrin, have similar fate in the environment and mechanisms of toxicity to organisms, being cypermethrin more acutely toxic than fenvalerate (Solomon et al. 2001).

Aside from cypermethrin, two organophosphates (diazinon and ethoprophos) were also present in water, and these compounds are frequently found in samples from agricultural areas in Costa Rica (Echeverría-Sáenz et al. 2012, 2016; Rämö et al. 2016; Arias-Andrés et al. 2016). The evaluation of the acute risk posed by ethoprophos on one native Cladocera species, *S. semiserratus* (Arias-Andrés et al. 2014), and two local and representative fish species, *Astyanax aeneus* (Mena et al. 2014a, 2014b) and *Atractosteus tropicus* (Mena et al. 2012), inhabiting the Frío River watershed (Bussing 1998) rendered no concerning results. Therefore, we estimated the risk for mortality events within this crustacean and these fish populations to be low based on the residue concentrations found.

However, we did find chronic risks based on the HC_5 analyses derived from the SSD curves (Table 2).

Diazinon residue levels found in this study might be a threat for native Cladocera such as *S. semiserratus*, as this insecticide has been shown to cause acute effects on this species (Arias-Andrés et al. 2014). Additionally, effects on crustaceans (*Ceriodaphnia dubia*, *D. magna*) and other arthropods have also been reported (Burkelipe et al. 2000).

The chronic risk estimated for pesticides present in water highlighted that the herbicide diuron and the insecticides cypermethrin, diazinon, and ethoprophos might be of major concern for the ecological health of aquatic ecosystems in the middle basin of the Frío River. These pesticides have also been indicated to have risky pesticides in other aquatic ecosystems of Costa Rica (Rämö et al. 2016; Arias-Andrés et al. 2016; Echeverría-Sáenz et al. 2012, 2016). Most of the contaminated sites were located downstream of crops in the middle of the basin. Meanwhile, pesticides were not detected at sites located upstream of the farming areas or near the mouth of Frío River, where there are fewer crops and sites are situated inside or below extensive wetlands. This could suggest that lagoon ecosystems would be degrading or trapping chemicals and/or accumulating them in sediments, both interesting topics to study. It is well known that degradation of some agricultural pesticides occurs actively in wetlands (Poissant et al. 2008) due to processes like the indirect photolysis of herbicides in shallow depths exposed to sunlight throughout the water column (Miller and Chin 2005). In addition, wetlands can improve water quality through processes of sedimentation, nutrient transformation, and reduce biological pollution (Knox et al. 2008).

Biological evaluation

Families which showed the highest abundances throughout the study sites such as Elmidae (Coleoptera), Simuliidae (Diptera), Baetidae and Leptohyphidae (Ephemeroptera), Perlidae (Plecoptera), Hydropsychidae, Leptoceridae, and Philopotamidae (Trichoptera) are considered moderately to highly sensitive to organic pollution (MINAE-S 2007; Sermeño-Chicas et al. 2010; Hilsenhoff 1988; Roldán-Pérez 2003). High abundances of such families suggest moderately good conditions (in terms of organic pollution) in the four sites investigated in this study, reflected by the BMWP-CR and %EPT indices. It is noteworthy that even though diversity and biotic indices did not vary significantly among upstream and downstream sites, the structure of the community did present changes in terms of the presence and absence (or abundance) of specific taxa (Table S3). For example, some of the taxa that were present at the reference sites and were absent or had significantly lower abundances in sites downstream from crops such as Isopoda, *Polyplectropus* sp. (Polycentropodidae), *Dixella* sp. (Dixidae), Dolichopodidae,

and *Nectopsyche* sp. (Leptoceridae) have been classified by Liess and Von der Ohe (2005) as species at risk of being affected by pesticides (SPEAR). Furthermore, Gomphidae, Baetidae, and Polycentropodidae are taxa which were reported to have higher relative sensitivity to organophosphates (Rico and Van den Brink 2015).

Other authors have reported similar results; for example, Castillo et al. (2006) indicated that macroinvertebrate community structure was the most reliable endpoint when assessing the impacts of banana production in the Caribbean region of Costa Rica. Also, Kohlmann et al. (2015) reported lower abundances or absence of some of the abovementioned taxa in their study in Dos Novillos River, and—as well as in the present study—the authors did not find EPT or taxa richness to be statistically different among their sampling sites.

Echeverría-Sáenz et al. (2016) reported that habitat parameters (such as type of water body and vegetation on the river bank), physical-chemical parameters (depth, pH, dissolved oxygen, conductivity) and pesticide residues, played a very important role in the distribution of macroinvertebrates along agricultural polluted sites in the Caribbean region of Costa Rica.

Pesticide residues were not detected in water at the time of the macroinvertebrates samplings. Thus, it is not possible to associate our findings with this variable. Nevertheless, pesticides were detected at site TR2 later on, suggesting that pesticide contamination at (at least) this site is plausible. Consequently, pesticide pollution should not be discarded as a possible factor affecting the macroinvertebrate community composition in this study area. Nonetheless, there is still a great amount of information regarding sensitivity to pollutants that needs to be generated for tropical ecosystems, since many of the organisms identified in this study are not found in temperate countries databases, and could not be compared to taxa from Liess and Von der Ohe (2005) or Rico and Van den Brink (2015).

In the present study, only pH was significantly associated with macroinvertebrate diversity. This parameter has been related to diversity in other studies (Petrin et al. 2007; Braukmann 2001; Guérold et al. 2000). However, all those papers referred to diminished biodiversity with increasing acidification of the surface waters, rather than slight alcalinization, which is the case for our study sites (pH 7.1–8.08). According to Chapman (1996) and MINAE-S (2007), this range of pH values supports healthy aquatic communities and is not anticipated to harm or significantly alter aquatic biota composition.

With respect to habitat parameters, lower percentage of riparian vegetation in the downstream sites (Table 1) could have also acted as an influencing factor in the distribution of macroinvertebrates and the absence of sensitive taxa. Lorion and Kennedy (2009) suggested that deforestation of the riparian buffers can alter the taxonomic composition of benthic macroinvertebrate assemblages, reducing diversity and eliminating the most sensitive taxa.

Regarding the ChE inhibition observed in fish exposed at two of the studied sites, this response is known to be related with the exposure to organophosphates and carbamates insecticides (Thompson 1999). Nonetheless, other pollutants and even environmental conditions have been proven to affect this enzyme (Moreira et al. 2010; Lionetto et al. 2011), and this should be considered when multiple pollutants and stress factors affect one ecosystem. In this study, two organophosphate compounds (ethoprophos and diazinon) were detected in water samples, but not at the same time ChE inhibition was observed in fish. It is known that some biochemical responses such as ChE inhibition may be observed in one organism even when the compound effector is no longer detectable in the environment (Wijeyaratne and Pathiratne 2006; Van Cong et al. 2008). This might explain the lack of alignment between the effects observed in fish and the pesticides detected in water. In addition, due to methodological limitations, not all pesticides used in agriculture of the area were analyzed. The ChE inhibition observed in fish exposed at MR, Diepens et al. (2014) reported that ethoprophos, at a concentration of 20 μg/L, significantly inhibited the ChE activity in fingerling of *P. dovii*. In this study, that compound was detected at MR but not at a concentration as high as that. However, the inhibition of the enzyme might be indicating that this pesticide and/or other cholinesterase inhibitors could be reaching concentrations higher than the ones observed in the point sample.

GST induction was also observed once in *P. dovii* exposed during this study. This enzyme is involved in the metabolism of xenobiotic compounds; a change in its activity in an organism has been associated with environmental exposure to pollutants of various chemical groups, including pesticides (Lenártová et al. 1997). About the variation in the levels of some biomarkers observed between sampling periods, a similar observation was described by Pfennig (2006) in the case of muscle ChE for another fish species (*Astyanax aeneus*). In that case, the change was related to the size of the fish. In our study, for each sampling period, a group of fish from the same brood and similar size was distributed in cages at the three sites and the control. However, distinct fish broods were used due to the different times of the year in which the experiments were conducted. Thus, it is not possible to compare biomarker variation between sampling periods, but comparisons of effects were possible between fish during the same sampling period.

As an outcome of this study, significant individual and integrated biomarker responses were observed in fish. We suggest that these signals be interpreted as an early warning of the presence of pollutants in the water as several substances capable of eliciting these responses were present in the area.

Conclusions

Residues of agricultural pesticides in concentrations that represent a risk to biota of rivers and lagoon ecosystems were detected in three studied sub-basins of the Frío River. According to a risk evaluation of the pesticide residues found in the Frío River Basin, there is a high to moderate chronic risk of toxicity of insecticides (cypermethrin, diazinon, and ethoprophos) to target group(s) of arthropods, and of diuron to plants and algae. The risk of toxicity for the herbicides ametryn and bromacil is moderated to low. Thus, it is important to conduct toxicity tests that focus on chronic exposure effects on reproduction and feeding rate of fishes and crustaceans, as well as the effects caused by the exposure to a mixture of a.i. with different mechanisms of action.

The results of the in situ biological evaluation were less conclusive, yet the biomarker response showed slight indications of being related to higher loads of pesticides. This seems to be a threat to Caño Negro Ramsar site by the entry of toxic substances to the wetlands in the lower part of the catchment area, especially through the Mónico River. Agricultural expansion of crops with high application rates of pesticides without practices of soil conservation and protection of riparian vegetation are likely to have an impact on the biological resources in the conservation area. A more sound approach would be to promote and maintain a mosaic of various productive activities such as the alternation of agricultural plots, forest, and wooded pastures. Also, large areas of monoculture should be discouraged. This, together with the protection and restoration of riparian forests, could be a way to reduce the impact on the Ramsar wetland in the Caño Negro Wildlife Refuge.

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