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Effect of pesticides used in banana and pineapple plantations on aquatic ecosystems in Costa Rica

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Abstract

Current knowledge on fate and effect of agricultural pesticides comes is mainly from temperate ecosystems. More studies are needed in tropical systems in order to assess contamination risks to non-target endemic tropical species from the extensive use of pesticides e.g. in banana and pineapple plantations. In this study, acute laboratory toxicity tests with organophosphate pesticides ethoprophos and chlorpyrifos were conducted on two Costa Rican species, cladoceran *Daphnia ambigua* and fish *Parachromis dovii*. Tests showed that chlorpyrifos was more toxic than ethoprophos to *D. ambigua* and *P. dovii* and that *D. ambigua* was also more sensitive than *P. dovii* to both pesticides. Additionally, bioassays were performed by exposing *D. magna* and *P. dovii* to contaminated water collected from the field. Chemical analyses of field water revealed that fungicides were generally the most frequent pesticide group found, followed by insecticides/nematicides and herbicides. The bioassays and values obtained from the literature confirmed that *D. magna* was more sensitive to pesticide contamination than *P. dovii* and that *D. ambigua* was more sensitive than *D. magna*, suggesting that the native cladoceran is a more suitable test species than its temperate counterpart. Species sensitivity distributions showed no significant difference in sensitivity between tropical and temperate fish and the arthropod species exposed to chlorpyrifos in this study. Choline esterase activity (ChE) was measured in *P. dovii* in laboratory tests in order to assess the applicability of this biomarker. ChE inhibition in *P. dovii* was observed in the laboratory at levels below the LC_{10} of both ethoprophos and chlorpyrifos, confirming that ChE is an efficient biomarker of exposure. Both indigenous Costa Rican species used in this study were found to be suitable standard tropical test species. Further studies are needed to investigate how protective the safe environmental concentrations, derived from LC_{50} of native tropical species, are for protecting tropical aquatic natural communities.

Key words

Acute toxicity, Bioassays, Chlorpyrifos, ChE inhibition, Ethoprophos, Tropical aquatic ecosystems

Introduction

Costa Rica is the second largest banana producer in the world. Commercially grown bananas are among the world's most pesticide intensive crops. Approximately, 45 kg of active ingredient (a.i.) per hectare per year are used in the Costa Rican banana plantations (Castillo *et al.,* 1997; Ramírez, 2010; REPCar, 2011; Castillo *et al.,* 2012; Bravo *et al.,* 2013). The most important group of pesticides used are fungicides, followed by a wide range of insecticides, nematicides and herbicides (see (Castillo *et al.,* 1997; Ramírez, 2009) for a review). Currently, pineapple and rice are increasing their production area. Pineapple production uses an average of 30 kg of a.i. of pesticides per hectare per year and currently the crop is grown

over 45000 ha (Castillo *et al.,* 2012). This together with the banana plantation, these crops potentially brings along more environmental pollution and health risks (Barraza *et al.*, 2011).

Two widely used organophosphate (OP) pesticides are chlorpyrifos and ethoprophos. Plastic bags that are used to protect banana bunches are usually impregnated with chlorpyrifos (1% mass concentration equals 0.7 kg a.i. ha^{-1} y^{-1} (Matlock Jr and de la Cruz, 2002). Ethoprophos is one of the most commonly used nematicides to protect the root systems of banana and pineapple plants. In banana plantations, it is applied a maximum of three times a year (16-21 kg a.i. ha^{-1} y^{-1}; (Matlock Jr and de la Cruz, 2002)). Together with carbamate pesticides, OPs are neurotoxins, which act by inhibiting the cholinesterase enzymes (ChE) acetylcholinesterase (AChE) and butyryl cholin esterase (BChE) in the nervous system. Inhibition of ChE has been widely used to assess the effects of anticholinesterase compounds on wildlife (Thompson, 1999). In fish, inhibition of 20% or more is well accepted as indicator of exposure to OPs (Varó *et al.*, 2007).

The river Rio Madre de Dios (RMD) in the Atlantic lowlands of Limon, Costa Rica (Fig. 1) receives drainage water from banana plantations, pineapple and rice fields before it flows into a coastal lagoon, which is part of a protected coastal wetland with a large biodiversity. Due to the high rain precipitation in this region, with an average of 3.6 m per year (Waylen *et al.,* 1996), the intensive use of pesticides and fertilizers in these extensive agricultural systems may reach the RMD river and its lagoon trough run-off and endanger the non-target fauna such as fish and invertebrates. Several massive fish kills have been observed in these water courses, especially after heavy rains and are believed to be caused by pesticide run-off. Generally, fish species such as guapote (*P. dovii*), black surfperch (*Embiotoca jacksoni*), tilapia (*Oreochromis niloticus*) and other cichlid species have been found among the dead fish (Pais, 2009; Pais, 2010).

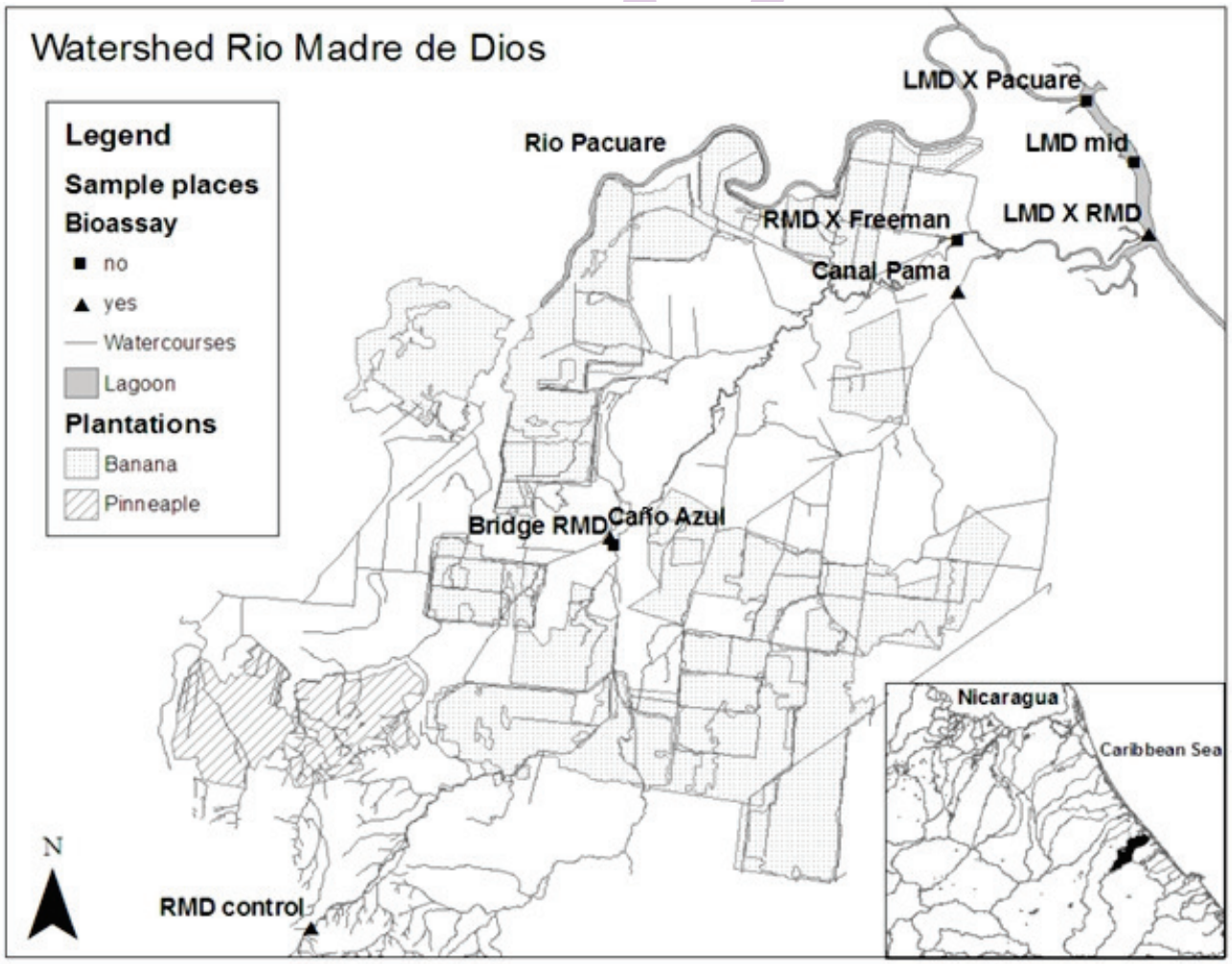

Fig. 1 : Sampling sites in watershed Rio Madre de Dios (14758 ha), Limon, Costa Rica. RMD is Rio Madre de Dios and LMD is Laguna Madre de Dios. RMD control was used as control site and together with Bridge RMD, Canal Pama, LMD X RMD they were used for the bioassays and ChE analyses. Caño Azul, RMD X Freeman, LMD mid and LMD x Pacuare were used for water quality and pesticide residue analyses only

Besides acute mortality, pesticides may also cause reproduction failure and other chronic effects, which could lead to a decline in fish populations. Furthermore, pesticide pollution may result in acute and chronic effects on macroinvertebrates (Castillo *et al.,* 2000), which could lead to changes in the benthic community structure and species richness (Pringle and Ramírez, 1998; Castillo *et al.,* 2006), and in turn change the food web structure.

Despite the high use of pesticides in Costa Rica and other tropical countries and their possible environmental impact, there is still relatively little knowledge about the fate and toxicity of pesticides in tropical aquatic ecosystems compared to temperate systems (Castillo *et al.,* 1997; Lacher and Goldstein, 1997; Mortensen *et al.,* 1998; Pringle and Ramírez, 1998; Robinson *et al.,* 1999; Matlock Jr and de la Cruz, 2002; Kwok *et al.,* 2007; Daam and Van den Brink, 2010). For instance, Maltby *et al.* (2005) collected toxicity data from different databases and only 11% of the species originated from tropical regions, while more than 50% were temperate species.There is an on-going debate on whether species sensitive distributions (SSDs) derived from temperate toxicity benchmark values (i.e. LC50 or NOEC values) can be used to assess the contamination risks for tropical species as well or reversely if it is necessary to calculate benchmark values only based on native tropical species.

A few recent studies have found no significant difference between the slope of SSD curves based on toxicity data for tropical and temperate species, suggesting that SSDs obtained based on temperate species could be applied to protect tropical species (Dyer *et al.,* 1997; Maltby *et al.,* 2005; Kwok *et al.,* 2007; Daam and Van den Brink, 2010; Rico *et al.,* 2010). In line with this, Daam *et al.* (2008) found no significant difference in species sensitivity at the community level for chlorpyrifos between temperate and tropical aquatic ecosystems. For some pesticides, including chlorpyrifos, data are available for the comparison of temperate and tropical species. For other pesticides such as ethoprophos, however, this is not the case, and therefore temperate data must be used to perform an ecological risk assessment for tropical species.

The aim of present study was to understand the effects of pesticides in tropical aquatic ecosystems by conducting standardized laboratory acute toxicity tests with chlorpyrifos and ethoprophos on two Costa Rican indigenous *cladoceran* species, *Daphnia ambigua* and *Parachromis dovii*. Furthermore, water samples were collected in the field to measure pesticide residue levels in rivers and creeks receiving agricultural runoff. Standard toxicity bioassays were performed under laboratory conditions using these field collected water samples on *Daphnia magna* and *P. dovii*. ChE inhibition assays were performed in the laboratory on *P. dovii* to evaluate the use this biomarker for exposure assessment. Furthermore, we compared the differences in sensitivity of these two tropical species (fish and crustacean) to their temperate counterparts *Oncorhynchus mykiss* and *D. magna*. A temperate and tropical species sensitivity distribution calculation was used to evaluate to what extent these tropical species are suitable for toxicity testing and environmental risk assessment in tropical regions.

Materials and Methods

Test species : Two cladoceran species (Crustacea) tropical *Daphnia ambigua* (Scourfield, 1947) and temperate *Daphnia magna* (Straus, 1820) and the guapote fish (Cyclidae) *Parachromis dovii* (Günther, 1864) were used as test organisms. The fish were cultivated and kept under a natural light : dark cycle (approximately 12:12 hr) in the laboratory at the Biology Department of the National University (UNA), Heredia, Costa Rica. Aged tap water (24 hr) with a temperature of 18-25°C was used in the fish tanks. The guapote fish were fed a mixture of fish, soya, cornflour, shrimps and soya oil, supplemented with vitamins and minerals. Fry were also fed with *Artemiasalina* and cultivated zooplankton. After 12 to 18 days, the fish were transferred for acclimatization for at least 24hr to the laboratory of ecotoxicological studies (ECOTOX) of IRET (Central American Institute for Studies on Toxic Substances), UNA Universidad Nacional, Heredia, Costa Rica. They had a mean length of 7.3 ± 0.5 mm and a weight of 8.8 ± 1.0 mg. *D. ambigua* and *D. magna* were cultured in laboratory of ECOTOX. The crustaceans were kept in glass beakers of 200 ml in a temperature controlled room (18-22°C) with a light : dark cycle of 16:8 hr. They were fed yeast, cereal leaves, tetramin (0.5 ml l^{-1}), Se^{2+} (5.2 ml l^{-1}), B_{12} (0.095 ml l^{-1}), *Chlorella* sp. (0.275 ml l^{-1}) and *Selenastrum* sp. (0.57 ml l^{-1}). Only neonates from adults that already had one or more clutches were used for the tests. Neonates between 24 and 72 hr old were taken for acclimatization (<24 hr) and used for testing.

Chemicals : The tested pesticides were chlorpyrifos (CAS nr. 2921-88-2, Dr. Ehrenstorfer, purity 98.4 ± 0.5%) and ethoprophos (CAS nr. 13194-48-4, Dr. Ehrenstorfer, purity 91%). All tests were conducted in the ECOTOX laboratory. Stock solutions of the pesticides were made by diluting chlorpyrifos in acetone (1:1) and ethoprophos in deionized water from a Milli-Q water purification system (Millipore). Test concentrations for fish were prepared by diluting the stock solution with carbon filtered, UV treated water. For daphnids, hard reconstituted water was used, which was prepared by adding KCl (0.008 g l^{-1}), $MgSO_4$ (0.12 g l^{-1}), $CaSO_4.H_2O$ (0.12 g l^{-1}) and $NaHCO_3$ (0.192 g l^{-1}) to deionized water (Dutka, 1989), with a conductivity of 3.96 ms cm^{-1} and a pH of 8. For control, same water was used as for the dilutions. The maximum amount of acetone used in the concentration ranges was also added to the acetone control.

A range finding test was performed with fish to determine the concentration range of chlorpyrifos for the final test. The concentration ranges for the other test were based on earlier experiences at the ECOTOX laboratory or by using the geometric mean of LC_{50} values present in the EPA database (www.epa.gov/ecotox).

Acute toxicity test set up: All tests were performed in triplicate (n=3) with 10 individuals per treatment and performed according to adapted standard protocols of OECD (1992) and conducted at an ambient temperature (24.9 ± 1.2°C) and a light:dark cycle of 12:12 hr. Crustaceans were exposed in 150 ml medium water in a glass beaker covered with parafilm, without aeration and food. Ten fish were put in each beaker glass with 200 ml of aerated water and were not fed during the tests. Each beaker was continuously aerated during the tests using air pumps connected to a glass Pasteur pipette. After two days, 150 ml of test water was replaced by new stock solution. Oxygen concentration (Wissenschaftlich-Technische Werkstätten (WTW) GmbH Oxi325), pH (Corning pHmeter 220), conductivity (WTW cond 315i), water temperature (WTW cond315i) and air temperature (Digital thermometer Cresta) were recorded at 24, 48, 72 and 96 hr. This was done for one randomly picked replicate of each concentration. Water samples of the stock solutions and of one random replicate per concentration at the end of each test were taken for pesticide concentration analyses to the chemical laboratory (LAREP) of IRET, Heredia, Costa Rica. For control and acetone control, only one replicate of each was collected for pesticide analysis.

Mortality was monitored every 24 hr and was defined for fish as the lack of gill movement. A *Daphnia* individual was considered dead if the animal did not visually move after one minute of stimulation, using a magnifying lens. Dead animals were removed from the test immediately.

Environmental sampling : Eight sites within the watershed Rio Madre de Dios located in the Caribbean lowlands of Costa Rica downstream from large banana and pineapple plantations (Fig. 1) were sampled during rainy season (8-10-2009). One sample of surface water was collected from each site for pesticide analysis. The water samples were collected in a pre-washed 4 l capacity amber glass bottles, which were rinsed once with surface water. At four sites (Fig. 1), additional water samples were taken for laboratory bioassays. The bottles were kept on ice during their transport to the laboratory (approx. half a day). A site situated upstream of most of the plantations was assumed to be relatively unpolluted and therefore chosen as a reference. The site near the bridge over the "Rio Madre de Dios" (Bridge RMD) was chosen as a relative uncontaminated site but was surrounded by plantations and used as an experimental treatment for the bioassay. Another site, the "Canal Pama" channel, collects drainage water from various banana plantations and was chosen for the bioassay because of high fish mortality that has been reported from this site. The last site of four selected sites for the bioassays was located at the entrance of the river into the lagoon (RMD X LMD). At all sites oxygen concentration (WTW Oxi325), pH (WTW pH320), conductivity (WTW cond 315i) and water temperature (WTW cond 315i) were measured.

Bioassays : For the bioassays (n=3), i.e. laboratory toxicity tests carried out with water from the four sites described above, *P. dovii* (72 hr, semi static with a 75% water exchange after every 24 hr) and *D. magna* (48 hr, static) were used. *D. magna* was used instead of *D. ambigua* due to availability at that moment. For both species, ten individuals were exposed to 200 ml of field water obtained from each site. Besides the field samples and the field control, a negative control (n=3) with carbon-filtered, UV treated water for fish and reconstituted water for *Daphnia* (as described above) was also included in the experimental setup. The tests were conducted at ambient temperature (24.7 ± 0.9°C). Mortality was recorded every 24 hr for *Daphnia* and fish.

Cholinesterase assay : Surviving fish from toxicity tests and bioassays were analysed for cholinesterase (ChE) inhibition. At the end of each toxicity tests, ten fish were randomly chosen from three replicates of each test concentration and from each of the bioassay tests. When the number of surviving fish was less than ten, remaining fish were used. The fish were rinsed with deionised water and kept frozen individually in Eppendorf tubes at -18°C. Whole fish samples were thawed and homogenized in 1 ml ice-cold phosphate buffer (0.1 M, pH 7.2) using a Branson SLPt sonifier. Samples were then centrifuged at room temperature (Eppendorf 5415c, 10,000 rpm, 5 min). Supernatants were transferred to new Eppendorf tubes and stored at -18°C until further analysis. Protein concentration in the samples were determined in triplicate by the Bradford method (Bradford, 1976) adapted to microplate (Bio-Rad Laboratories 2005). Bovine γ-globulin was used as standard. Dilutions were made to normalize protein content to approximately 0.5 mg ml^{-1}. Dye reagent and protein standard were purchased from Bio-Rad. The ChE activity was determined at room temperature (25°C) by the method of Ellman *et al.,* (1961) adapted to a microplate (Lopes *et al.,* 1996; Mena *et al.,* 2012). Acetylthiocholine (Sigma-Aldrich) hydrolysis was measured as an increase of absorbance at 414 nm caused by the reaction of thiocholine with DTNB (Sigma-Aldrich) to produce yellow 5-thio-2-nitro-benzoic acid anion. Absorbance was measured at an interval of 2.5 min for 15 min. For the determination of ChE activity, change in absorbance between 5 and 10 min was calculated. A Thermo Multiskan Ascent microplate reader was used for both enzymatic and protein determinations. ChE activities were calculated as nmol of hydrolyzed substrate per min and mg of protein. The results were expressed as units (U) per mg of protein (U = n mol min^{-1}).

Pesticide analyses : For determination of pesticides concentration, water was extracted by solid phase extraction (SPE) and pesticides were analyzed by gas chromatography with mass detector (GC-MS) and by liquid chromatography with photodiode array detector (LC-PDA). In the field samples, 16 pesticides were analyzed: ametryn, bromacil, carbofuran, carbaryl, chlorothalonil, chlorpyrifos, diazinon, difenoconazole, diuron, epoxiconazole, ethoprophos, fenamiphos, hexazinone, metalaxyl, terbufos, triadimenol. They were arranged in five pesticide groups (insecticides, nematicides, herbicides,

fungicides and insecticides/ nematicides) based on Tomlin (2003). The SPE cartridges (200 mg ENV+/6 ml Isolute or C18 500mg 3ml^{-1}) were previously conditioned with ethyl acetate (5 ml, analytical grade of Fluka Pestanal), methanol (10 ml analytical grade of J.T. Baker hplc solvent), deionized water (10 ml) and methanol (5 ml). The water samples (1l) were passed through the cartridges, than they were dried by centrifugation (2 min at 5000 rpm) and then by vacuum for 8 min. For GC-MS analyses, the pesticides were eluted from the cartridges with two times 6 ml ethyl acetate for 6 ml cartridges and 3 ml ethyl acetate for 3 ml cartridges. These samples were either diluted with acetone : cyclohexane (1:9) or concentrated to approximately 0.1 ml with nitrogen gas and filled up with acetone : cyclohexane (1:9) to a final volume of 1 ml. In a parallel extraction step for LC-PDA analysis with the SPE cartridges (200 mg ENV+/6 ml Isolute), elution was performed with 2 x 5 ml of methanol. This extracts were evaporated at 30-350ºC under nitrogen current to 0.05 ml, and reconstituted in 1 ml methanol / water (40:60) mixture.

The GC-MS extracts were analyzed by gas chromatography with mass spectrometer with an Agilent 7890A GC and 5975C MS (Agilent Technologies, Palo Alto, USA), in synchronous SIM Scan mode, a CTC Combipal autosampler (CTC Analytics AG, Switzerland), and a capillary column BP35 (Agilent Technologies, Palo Alto, USA) (25 m x 0.25 mm x 0.25 µm). The data acquisition was carried out using Chemstation software and NIST05 Mass Spectral Database. The temperature program was 90ºC for (1 min) to 210ºC with 20ºC min^{-1}, and then up to 300ºC with 4ºC min^{-1}, the interface was held on 280ºC, the ion source on 230ºC, the injector on 230ºC. The sample (1 µl) was injected in splitless mode. Pesticides 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 (125 mm x 3 mm x 5 µm particle size, Merck, Germany). 50 µl of extracts were analyzed. 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^{-1}. 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 5 min. The total run time was 45 min. Identification was performed using retention time and the UV-spectra of the pesticides included in the analysis. Quantification was performed by the external standard method. Good recovery yields were obtained for all pesticides between 77 and 105%.

Data evaluation : To calculate LC_{50} and LC_{10} values and their 95% confidence limits, log-logistic analyses were performed using the statistical software Genstat, 12th edition (Lawes Agricultural Trust, VSN International Ltd.). The LC_{50} concentrations were converted from µg l^{-1} into molarity (mol l^{-1}) by using molecular weight (chlorpyrifos: 350.6 g mol^{-1} and ethoprophos: 242.3 g mol^{-1} (Tomlin, 2003)). All calculations were done using nominal concentrations. Adjustments for control mortality were made by the program. The following formula, modified from Schroer *et al.* (2004), was used to construct a dose response curve.

$$y\,(conc) = \frac{1\text{-}c}{1 + e^{\,-b(\ln conc - a)}} \qquad (1)$$

Where 'y' is the fraction of dead animals (dimensionless), (conc) is the applied dose in µg l^{-1} on basis of the nominal concentrations at t = 0 and the parameters are: a (ln LC_{50}), b (slope in l µg^{-1}) and c (fraction of background effect or mortality in the control). The parameters were provided by the Genstat program.

From the bioassay results, box plots were created using statistical program PASW (SPSS version 18). To detect spatial variation in effects among the different sampling sites, a one-way ANOVA with a LSD post-hoc test was conducted in PASW.

Comparison of temperate versus tropical species sensitivities : For comparison of sensitivity of temperate versus tropical species to ethoprophos and chlorpyrifos, species sensitivity distribution curves (SSDs) were plotted using previously reported geometric mean LC_{50} values of the temperate species rainbow trout *Oncorhynchus mykiss* and *D. magna.* A temperate and tropical SSD for fish for chlorpyrifos was plotted using the ETX 2.0 software (Van Vlaardingen 2004) that uses the method of Aldenberg and Jawarska (2000) to generate the SSD distribution, the derived hazard concentrations HC_5 (hazard concentration that affects 5 % of the species), HC_{50} and their 95% lower and upper confident limits. The SSD is described by a statistical cumulative frequency distribution of either LC_{50} or NOEC values, based on a log normal distribution. Acute toxicity data (LC_{50}, 96 hr) from the EPA database (www.epa.gov/ecotox) were used here to construct the SSDs. If multiple LC_{50} data were available for one species, the geometric mean was taken. Since only two species were available between 23.5N-23.5S (classification for tropical species by (Kwok *et al.,* 2007)), all species between 35N and 35S, with at least one distribution limit within the tropical region, were used. Other species outside this range were categorised as temperate. Overlap was allowed outside the range of 23.5N-23.5S. Using these criteria, 12 tropical and 13 temperate fish species were available to construct SSDs for chlorpyrifos. For ethoprophos, only seven different species were available of which none could be classified as tropical and only three as temperate according the criteria above, therefore no SSD was made. The temperate and tropical distributions were compared on significant slope differences using a Kolmogorov-Smirnov test (sign. level 0.05) (PASW statistics 18).

Results and Discussion

The analytical recovery for chlorpyrifos was 99.8% and for ethoprophos 97.9%. In the tests performed with *D. ambigua,* hardly any dissipation of the pesticides was observed. This was in

contrast to the tests performed with *P. dovii*, where dissipation was up to 99.3%.The fish systems were aerated for 96 hr, which could have led to high volatilization of both chemicals during the test since both chemicals have a relatively high Henry's law constant (0.478 Pa m^3 mol^{-1} at 25ºC for chlorpyrifos and 0.0135 Pa m^3 mol^{-1} at 25ºC for ethoprophos (Tomlin, 2003). For both species, nominal values were used for further calculations.

All mortality levels in the controls were below 20% (maximum acceptable level set by the (OECD, 1992) for *Daphnia*) except for the test with *D. ambigua* and ethoprophos,in which 33% mortality was observed in the control. These test results are used for further calculations because they still yielded a clear dose response relationship but the results should be interpreted with caution.

Results of acute toxicity tests showed that *D. ambigua* was more sensitive to both organophosphates (OPs) compared to *P. dovii* and that chlorpyrifos was more toxic to these two species than ethoprophos, although they have a similar mode of action (i.e. both are neurotoxins that cause inhibition of AChE) (Table 1 and Fig. 2). The difference in toxicity of two pesticides and in species sensitivity have been reported earlier for other OPs (Persoone *et al.,* 1985, cited in (Varó *et al.,* 2000)). The difference in sensitivity may be the result of differences in enzymatic expression or metabolism (Boone and Chambers, 1997). Furthermore, arthropods crustaceans are usually the most sensitive taxonomic group to insecticides, i.e. compared to vertebrates and other aquatic groups (Maltby *et al.,* 2005; Daam and Van den Brink 2010). The variation in toxicity between two compounds could also be explained by difference in chemical structure leading to different physico-chemical properties such as solubility and hydrophobicity and thus to difference in uptake and elimination of the compound by the individuals.

For *P. dovii* no toxicity data have previously been reported for both OP compounds. The LC_{50} value of *P. dovii* reported in this paper for ethoprophos is similar to the earlier reported LC_{50} values for same species and compound (96 hr LC_{50}: 54, 158 and 220 µg l^{-1}). The fish used in these experiments were older and therefore possibly less sensitive, which could explain the slightly higher LC_{50} values. Compared to the standard temperate test species on *O. mykiss* (geometric mean 96 hr LC_{50} was 19 µg l^{-1} for chlorpyrifos (n=13) and 3153 µg l^{-1} for ethoprophos (n=6)), *P. dovii* was less sensitive for chlorpyrifos and more sensitive to ethoprophos, both within an order of magnitude.

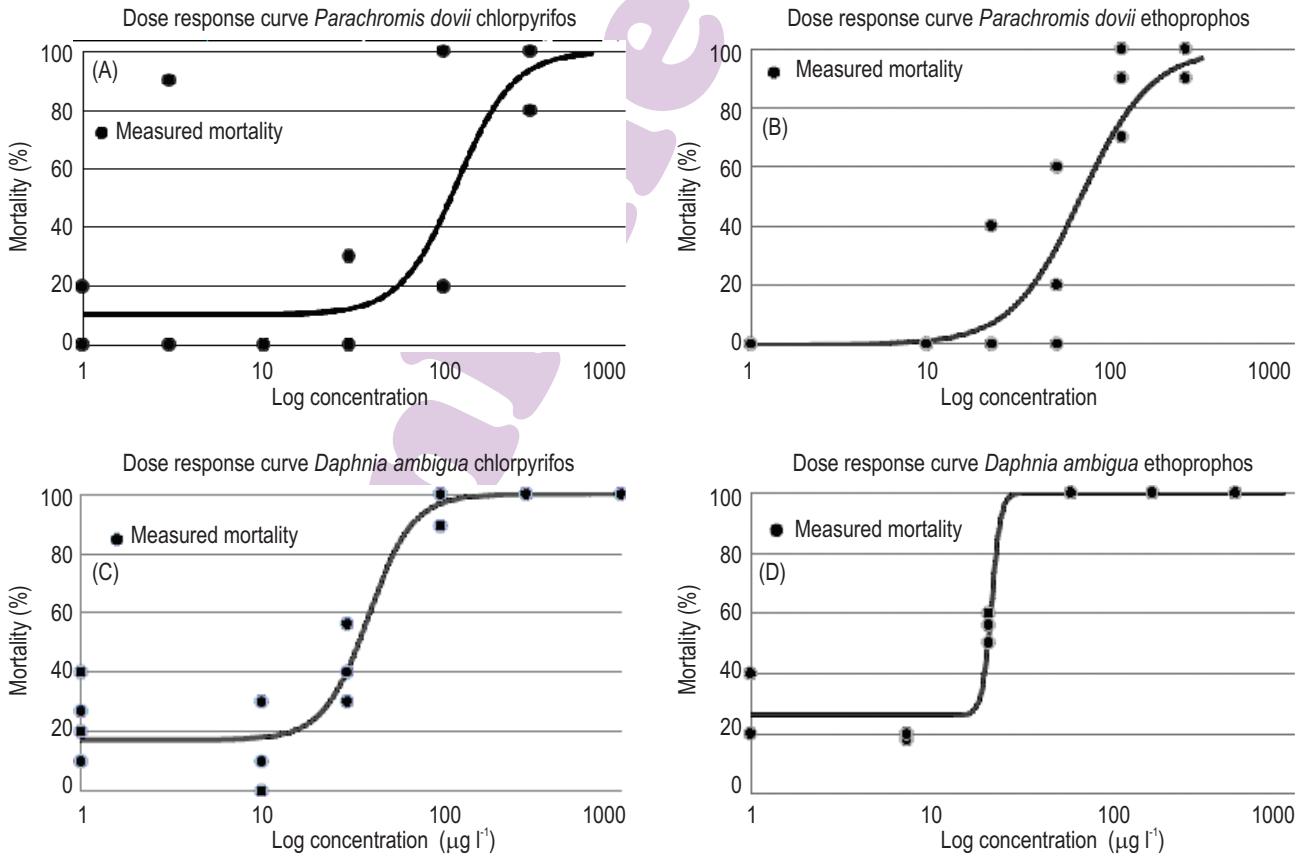

Fig. 2 : Dose response curves for *Parachromis dovii* (A and B) and *Daphnia ambigua* (C and D) and for chlorpyrifos (A and C) and ethoprophos (B and D) (n=3 with 10 individuals per replicate)

Table 1 : Results of acute toxicity tests for chlorpyrifos and ethoprophos with *Parachromis dovii* and *Daphnia ambigua*

		LC_{10} (µg l^{-1}) (95% CI)	LC_{50} (µg l^{-1}) (95% CI)	LC_{50}(mol l^{-1})
Parachromis dovii	Chlorpyrifos	53 (29, 98)	117 (87, 158)	$3.3*10^{-7}$
	Ethoprophos	73 (43, 125)	242 (179, 328)	$1.0*10^{-6}$
Daphnia ambigua	Chlorpyrifos	0.021(0.013, 0.034)	0.039 (0.029, 0.053)	$1.1*10^{-10}$
	Ethoprophos	7.01 (6.66, 7.37)	7.63 (7.30, 7.97)	$3.1.10^{-8}$

The SSD (Fig. 3) shows that the LC_{50} of *P. dovii* falls within the confidence limits (95%) of tropical HC_{50} but outside the range of the temperate. Based on this SSD, tropical fish species were indicated as less sensitive to chlorpyrifos, yet no statistical difference was detected between the temperate and tropical distributions (p=0.975). This is consistent with a previous comparison study for fish performed by Dyer *et al.* (1997) who found no difference between tropical and temperate sensitivity for carbaryl, lindane, malathion, pentachlorophenol and phenol. Moreover, Rico *et al.* (2010) (methylparathion) and Rico *et al.* (2011) (malathion and carbendazim) showed that three indigenous tropical fishes and freshwater invertebrates species of the Amazon were not more sensitive than their temperate counterparts.

Castillo *et al.* (1997) recommended the guapote fish as a tropical freshwater standard test species because of its economic value and/or sensitivity. Its economic value is considerable since it is popular in local fisheries and sport fishing (tourism) and is used for human consumption. In addition, this fish species is easy to cultivated, lay a substantial number of eggs (800-1000) and is therefore suitable for toxicity testing. However, the results show that the guapote fish is not particularly sensitive for the tested compounds. Therefore, in order to protect 95% of tropical fish species from chlorpyrifos, an uncertainty factor of 100 should be applied to its LC_{50} to obtain a threshold value that lies within the confident limits of tropical HC_5 (Fig. 3). More toxicity tests need to be conducted to reveal its sensitivity to other toxicants.

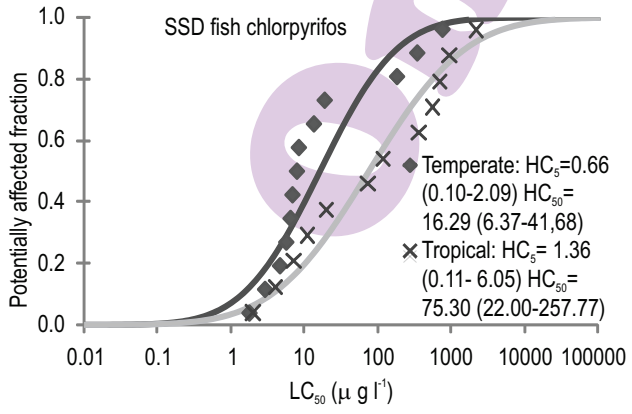

Fig. 3 : Temperate and tropical species sensitivity distribution (SSD) for chlorpyrifos and fish

The LC_{50} of *D. ambigua* for chlorpyrifos was higher than the earlier published LC_{50} value of 0.035 µg l^{-1} (95% CI 0.032-0.037 µg l^{-1}) (Harmon *et al.,* 2003). Ethoprophos showed a steep dose response curve, which indicates that a small change in concentration can result in a large change in the mortality response (Fig. 2D). This was illustrated by LC_{10} value that was very close to the LC_{50} value (Table 1). *D. ambigua* was 10 to 100 times more sensitive than the standard temperate test species, *D. magna* (geometric mean of LC_{50}: 0.8 µg l^{-1} for chlorpyrifos (n=2) and 63.9 µg l^{-1} for ethoprophos (n=2)). *D. ambigua* LC_{50} values for chlorpyrifos were found lowest among all the arthropods and fish species in de EPA database. Tropical HC_5 for chlorpyrifos derived by Maltby *et al.* (2005) from an SSD with acute toxicity data for arthropods had a value of 0.06 µg l^{-1} (95% CI 0.002-0.16) and was not statically different from the temperate value (0.13 µg l^{-1}). The lower confidence level of HC_5 derived from an SSD gives a conservative estimate of the ecosystem threshold concentrations (Maltby *et al.,* 2005). With a LC_{50} value below the tropical HC_5, *D. ambigua* is a sensitive species, therefore they could be used as a tropical freshwater indicator species instead of *D. magna* in tropical risk assessments. Furthermore, this species is easy to culture and is suitable for routine toxicity tests (Harmon *et al.,* 2003). Moreover, *Daphnia* sp. are taxonomic stable, sensitive to wide range of chemicals and widely distributed (Sarma and Nandini, 2006).

The physico-chemical parameters of the surface water measured at the field sites showed natural fluctuations in pH and conductivity; i.e. higher pH and conductivity levels towards the sea. In the tropics, it should be taken into account that warmer water can contain less oxygen. In Canal Pama, the lowest oxygen concentration (1.84 mg l^{-1}) was measured, dead fish were observed and a change in water colour (from brown to green) was observed within few hours, indicating a rapid algal bloom. With five different pesticides (difenoconazole, epoxiconazole, ethoprophos, fenamiphos, triadimenol) detected, second highest number of pesticides were detected in Canal Pama (Table 2). A few days before the analysis it had rained heavily (local meteorological data), probably causing runoff of pesticides and nutrients from many banana plantations that drained into Canal Pama and at this time fish mortality was observed. The highest concentration was found for the nematicide fenamiphos (2 µg l^{-1})(Table 2), which was lower than the acute 96 hr LC_{50} for fish, crustacean and algae but slightly higher than 48 hr LC_{50} for aquatic invertebrates (EU FOOTPRINT). The death of fish at the time of sampling (a few days after the first fish mortality) was probably caused due to

Table 2 : Concentrations (µg l^{-1}) of different pesticides per sample site found during one sampling time

Pesticides	RMD control	Bridge RMD	Caño Azul	Canal Pama	RMD X Freeman	LMD X RMD	LMD mid	LMD X Pacuare
Ametryna			0.5			traces	0.06	0.06
Carbaryl			0.4					
Carbofuran			5.0					
Chlorpyrifos			traces					
Chlorothalonil					0.05	traces	0.07	0.05
Diazinon			0.05					
Difenoconazol			0.8	0.3				
Diuron			2.0			0.1	0.1	0.2
Epoxiconazol			0.6	0.6	0.1	0.2	0.3	0.2
Ethoprofos			0.1	traces		0.9	0.9	1.0
Fenamiphos				2.0				
Metalaxyl			traces					
Triadimenol				0.07				

combination of multiple stressors i.e.; low oxygen concentration combined with pesticide exposure (Fig. 4).

Most pesticides (ametryn, carbofuran, carbaryl, chlorpyrifos, diuron, difenoconazole, epoxiconazole, ethoprophos, fenamiphos) were found in Caño Azul (Table 2) as was hypothesized due to the extensive drainage of banana and pineapple plantations but no dead fish or algae blooms were observed here. Carbofuran (5.0 µg l^{-1}) and diuron (2.0 µg l^{-1}) were found at high concentrations. At the control site of Rio Madre de Dios (RDM control) no pesticides were found confirming its suitability as use as a control (Table 2). From the Caño Azul to the crossing of RMD with Freeman (RMD X Freeman), the concentration and the number decrease from eight pesticides to one. The decrease of pesticides can be explained by biological and chemical degradation, such as photolytic degradation, vaporization and sorption to organic matter. Compared to temperate systems, these processes can be faster due to the tropical conditions (see (Daam and Van den Brink 2010) for a review), though many of the detected pesticides are known to be persistent in water. In lagoon, the amount and concentration of the pesticides increased probably due to entry of Canal Pama and other side streams. Especially, ethoprophos (0.9 - 1.0 µg l^{-1}) and epoxiconazole (0.2 - 0.3 µg l^{-1}) has been found consistently throughout the whole lagoon.It should be noted that one single pesticide and physico-chemical parameter analysis is not sufficient to obtain a full overview of pesticide concentration dynamics due to the spatial and temporal variation in applications and rain events. Currently, a long-term monitoring program (by the TROPICA project and IRET) is running, which aims at the determination of pesticide concentrations in water and sediment in this watershed and evaluates the overall risks of pesticides to native species in this ecosystem. This will give important insight to the dynamics in such systems.

In general, the most frequently found pesticide groups in this study were fungicides followed by herbicides and insecticides / nematicides (Table 2), which is in accordance with the findings of Castillo *et al.* (1997). Earlier sampling in the catchment showed that 67% of the samples taken from RMD, the following seven pesticides were frequently found in water and/or sediment: chlorothalonil, bromacil, difenoconazole, diuron, ethoprophos, esfenvalerate and cypermethrin. In this study, bromacil, esfenvalerate and cypermethrin were not found in any of the samples. In general, epoxiconazole and ethoprophos were most frequently detected at respectively six and five of the eight samples, while only traces of chlorpyrifos were detected at Caño Azul. This reflects the wide use of ethoprophos and its mobility through the watershed, possible due to its high solubility (700 mg l^{-1} at 20ºC (Tomlin, 2003)). Robinson *et al.* (1999) found that 30% of the applied ethoprophos runs off into rivers while the runoff potential of chlorpyrifos is low (Ware and Racke, 1993). Although chlorpyrifos is widely used in the fruit protection bags, its leaching potential due to rain events could be minimal because of its low solubility (1.4 mg l^{-1} at 25ºC (Tomlin, 2003)) and because the product is the applied to the inside of the bag, thus with limited exposure to rain. When reaching the water column, the compound likely to bind to organic material in the sediment or living animals and may bioaccumulate in the biota (Varó *et al.*, 2000; Varó *et al.*, 2002). On the other hand, it is rapidly metabolized into less toxic molecules and eliminated from the body (Ware and Racke, 1993; Barron and Woodburn 1995). More research is required to determine the fate of chlorpyrifos in the plastic banana protection bags.

In Central America, maximum environmental concentrations found in water, sediment and biota were higher for chlorpyrifos (water 0.18 µg l^{-1}; sediment 34.2 µg kg^{-1}; biota 8 µg kg^{-1} (Castillo *et al.*, 1997)) than for ethoprophos (water 0.10 µg l^{-1} (Unpublished data); sediment 1.98 µg kg^{-1} (Robinson *et al.*, 1999). Ethoprophos concentrations measured in this study (max concentration in water 1µg l^{-1}) were 10 fold higher than previous reported concentration while chlorpyrifos concentrations were under the detection limit in this study. Calculating the risk quotient (maximal environmental concentration/predicted no effect concentration for most sensitive species in this study (LC_{50}

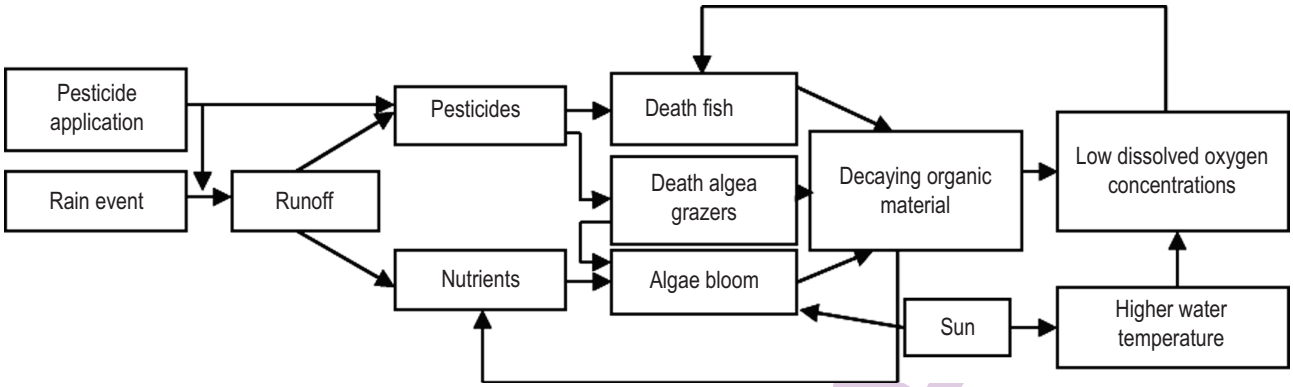

Fig. 4 : Conceptual model of environmental processes possibly leading to fish kills after a rain event

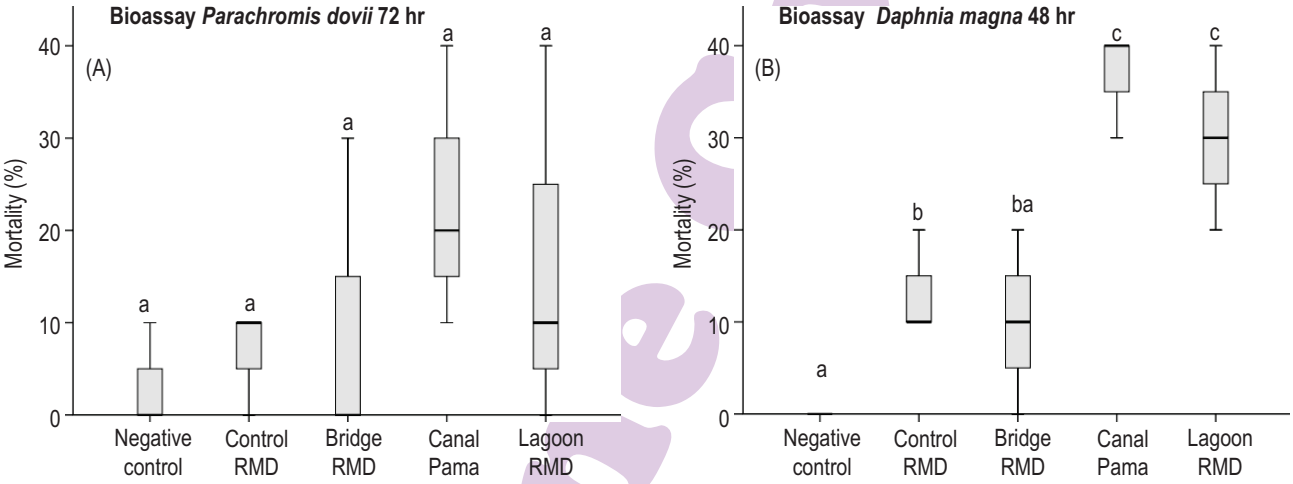

Fig. 5 : Bioassays for (A) *P. dovii* (72 hr) and (B) *D. magna* (48 hr) showing mortality per sample site in the watershed Rio Madre de Dios. Values are mean of three replicates ± SD. Differences in letters indicate sites with significant difference (α =0.05)

divided by a safety factor 10) gives a rough estimate that there is a substantial risk in tropical ecosystems for both compounds with risk quotients of 16 and 46 for ethoprophos and chlorpyrifos respectively. Besides acute effects, chronic effects may occur at lower concentrations.

Fig. 5 shows the difference in response of the bioassays performed with *P. dovii* and *D. magna* among the sampled sites. Mortality of both species was below 40% at all sampling sites. For fish, no significant difference (p=0.482) in mortality between sites could be detected. Significant differences in mortality among sites (p=0.001) were found for *D. magna*. The LSD post-hoc test showed that the observed mortality at Bridge RMD was significantly different from Canal Pama (p=0.001) and lagoon (p=0.007) but similar to both controls. RMD and Lagoon RMD were not significantly different (p=0.290) but differed in observed mortality with all other sites.

For *D. magna* a higher mortality in the most polluted sites and difference among sample sites could be detected (Fig. 5). Therefore, it proved to be a good indicator of pollution and is useful for rapid detection of environmental contamination. The toxicity test, showed that *D. ambigua* was more sensitive than *D. magna* for the evaluated compounds. Since it is an indigenous tropical species, it might be even more suitable as an indicator species in the tropics and replace *D. magna* in a tropical test battery.

In the laboratory toxicity tests with different concentrations of ethoprophos and chlorpyrifos, there was a significant treatment effect on ChE activity analysed in whole fish tissue (Fig. 6A and 6B) (one-way ANOVA). Post-hoc comparisons showed that for ethoprophos the three highest concentrations were not significantly different, and for chlorpyrifos 10 and 30 µg l^{-1}, and the highest two concentrations were not significantly different (Fig. 6). The unexpected high fish mortality rate (33%) at 3 µg l^{-1} chlorpyrifos concentration (Fig. 2A) is probably due to other factors, such as biological effects or a fungal infection, than the pesticides toxicity since high ChE activity does not reveal any inhibition. Fig. 6 shows

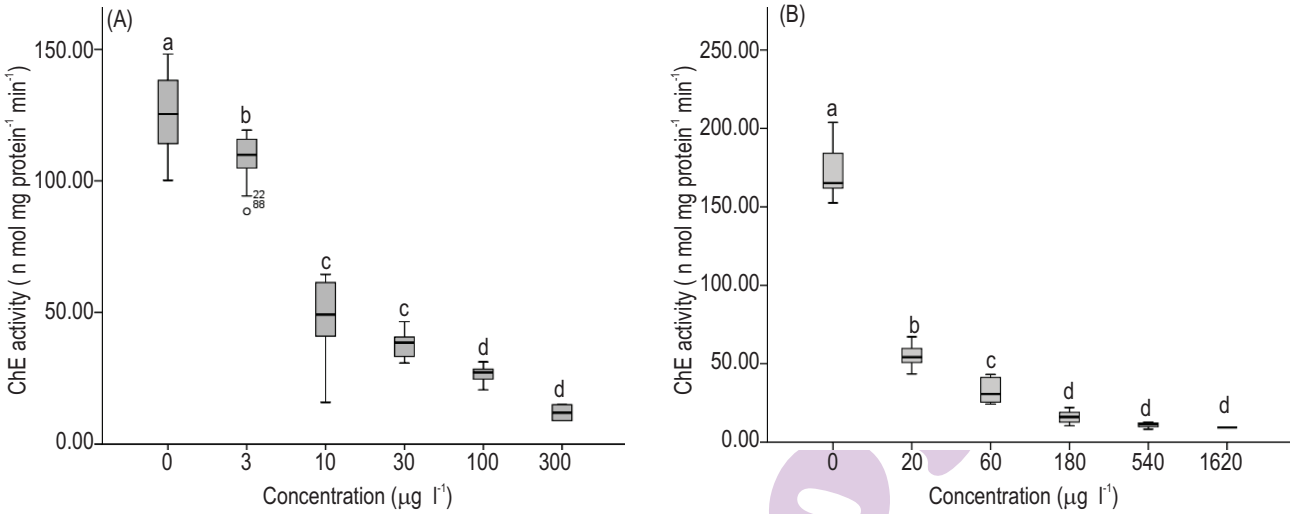

Fig. 6 : ChE inhibition in P. *dovii* after 96 hr exposure to (A) chlorpyrifos and (B) ethoprophos. Values are mean of three replicates ± SD. Difference in letters indicate that the treatments were significantly different from each other (alpha =0.05)

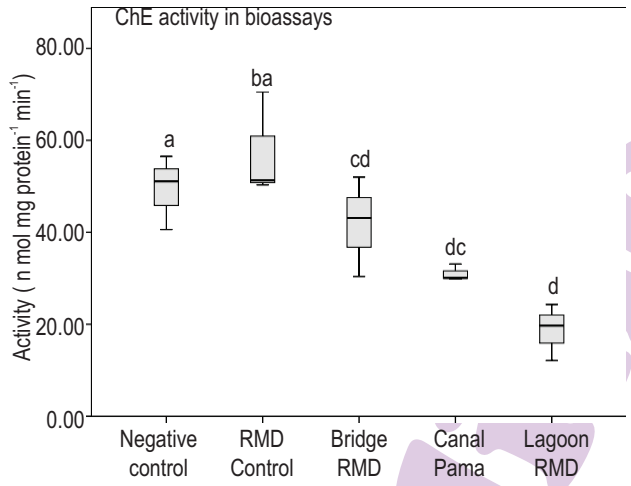

Fig. 7 : ChE inhibition in *P. dovii* after 72 hr exposure to surface water taken from watershed Rio Madre de Dios. Values are mean of three replicates ± SD. Differences in letters indicate that the treatments were significantly different from each other (alpha =0.05).

that the amount of ChE decreased with increasing concentrations with significant difference for ethoprophos between the control and the first concentration and for chlorpyrifos between the first and second concentration. This and the threshold value of 20% inhibition (Varó *et al.,* 2007) indicates that inhibition begins for ethoprophos at 20 µg l^{-1} (32%) and for chlorpyrifos at 10 µg l^{-1} (38%). For both pesticides no immobile or dead fish were observed at these concentrations while ChE inhibition showed already sub lethal effects. Therefore, this biomarker could be used as an early warning indicator. Since ChE recovery was low or even assumed to be irreversible it can provide long-term exposure information instead of a snapshot by chemical analysis of water samples. Moreover, inhibition of ChE might be linked to different indirect effects at individual level, such as changes in behaviour, reproduction and feeding success, which could affect populations in long term.

ChE activities measured in the bioassay (Fig. 7) shows that ChE activity measured in the negative control of the toxicity tests was significantly similar to ChE activity levels measured at the field control site (RMD control). ChE activity in fish exposed to water from Bridge RMD and Canal Pama did not differ from each other and were both significantly lower than control. However, Canal Pama was similar to Lagoon RMD. The strongest ChE inhibition was found in the Lagoon RMD, where only one OP could be detected. This was surprising since there was also a higher dilution in the lagoon with incoming seawater from the Carribean Sea and from other adjacent streams. These other streams, however, could bring other OPs and carbamates that we did not look for in our chemical analyses. During the bioassays, no fish mortality was observed, the ChE inhibition however, showed a clear response i.e. 37% inhibition in Canal Pama and 62% inhibition in the Lagoon RMD. This again indicates the usability of this biomarker as an early warning indicator of exposure to OPs and carbamates. Yet, the majority of the pesticides found consisted of fungicides and herbicides, which have a different mode of action than OPs and carbamates. Therefore, a combination with other biomarkers and/or toxicity tests is needed in order to detect pesticide contamination and asses its environmental risks in this watershed.

In conclusion, the proposed tropical species were similar or more sensitive than their temperate counterparts. More toxicity data are needed about to evaluate possible similarities or respectively differences between temperate and tropical species to chemical contamination. Further studies are also needed to investigate how protective safe environmental concentrations, derived with LC_{50}s of temperate species, are for protecting tropical

aquatic natural communities. Both recommended tropical species were found suitable for toxicity testing, and might be used as a part of a tropical test battery and environmental risk assessment in tropical regions.

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