Acute toxicity and cholinesterase inhibition of the nematicide ethoprophos in larvae of gar *Atractosteus tropicus* (Semionotiformes: Lepisosteidae)

Freylan Mena Torres[1], Sascha Pfennig[1,2], María de Jesús Arias Andrés[1], Gabriel Márquez-Couturier[3], Adrían Sevilla[4] & C. Maurizio Protti Q[4].

1. Laboratorio de Estudios Ecotoxicológicos del Instituto Regional de Estudios en Sustancias Tóxicas (IRET). Universidad Nacional de Costa Rica, Heredia, Costa Rica. Postal address: 86-3000-Heredia; marias@una.ac.cr, sascha_pfennig@yahoo.de, fmena@una.ac.cr
2. Abteilung Fischkrankheiten und Fischhaltung, Institut für Parasitologie, Stiftung Tierärztliche Hochschule, Hannover, Germany
3. División de Ciencias Biológicas, Universidad Juárez Autónoma de Tabasco, Km. 0.5 de la carretera Villahermosa - Cárdenas, entronque con Bosques de Saloya, Villahermosa, Tabasco, México; gmctabasco@hotmail.com
4. Laboratorio de Recursos Naturales y Vida Silvestre (LARNAVISI), Escuela de Ciencias Biológicas, Universidad Nacional de Costa Rica, Heredia, Costa Rica. Postal address: 86-3000-Heredia; mprotti@una.ac.cr, asevilla@una.ac.cr

Received 18-II-2011. Corrected 11-V-2011. Accepted 13-VI-2011.

Abstract: Biomarkers are a widely applied approach in environmental studies. Analyses of cholinesterase (ChE), glutathione S-transferase (GST) and lipid peroxidation (LPO) are biomarkers that can provide information regarding early effects of pollutants at different biochemical levels on an organism. The aim of this study was to evaluate the biomarker approach on a Costa Rican native and relevant species. For this, larvae of gar (*Atractosteus tropicus*) were exposed to the organophosphorus nematicide, ethoprophos. Acute (96hr) exposure was conducted with pesticide concentrations ranging from 0.1μg/L to 1 500μg/L. The 96hr LC_{50} calculated was 859.7μg/L. After exposure, three biomarkers (ChE, GST and LPO) were analyzed in fish that survived the acute test. The lowest observed effect concentration (LOEC) regarding ChE activity inhibition was 50μg/L. This concentration produced a significant inhibition ($p<0.05$) of the enzyme by 20%. The highest concentration tested without showing any effect on ChE activity and therefore considered as no observed effect concentration (NOEC) was 10μg/L. Ethoprophos concentration of 400μg/L caused a ChE inhibition by 79%. In this study, no significant variations ($p>0.05$) in GST activity and LPO were observed in *A. tropicus* larvae after exposure to ethoprophos. Rev. Biol. Trop. 60 (1): 361-368. Epub 2012 March 01.

Key words: gar, *Atractosteus tropicus*, ethoprophos, cholinesterase inhibition, glutathione S-transferase, lipid peroxidation.

The application of large amounts of agricultural pesticides is a serious environmental problem in Costa Rica, that has increased its imports during the last 30 years (Ramírez *et al*. 2009). In this period, it was found that insecticides/nematicides make up about 10% of the total amount of active ingredients (a. i.) being imported. Of these, ethoprophos was among the top ten pesticides imported relative to quantity (Ramírez *et al*. 2009). The intensity and volume of pesticide use, related to the expansion of intensive agriculture, therefore represents a serious threat for biodiversity, especially in fragile and complex systems such as wetlands.

Tropical ecosystems harbor much of the world's biodiversity, and are subjected to very high land conversion rates due to agriculture. Conservation and management of biodiversity urgently needs information regarding impacts at the ecotoxicological level on native organisms, so that the sensitivity of individual species as

well as of ecosystems can be characterized. More importantly, information regarding species-specific sensitivity to common pesticides and pollutants is an essential tool for establishing limits and regulations on pollutants released to the environment through agricultural activities. In this regard, most of the studies concerning species-specific ecotoxicology, and its use to establish limits and regulations have been carried out in North America and Europe (US-EPA 1986, OECD 2007). Some studies has been carried out in South America and Mexico (Akaishi *et al*. 2002, de la Torre *et al*. 2005, Azevedo *et al*. 2009, González-Mille *et al*. 2010), but information is scarce for Central America.

Atractosteu tropicus inhabit the San Juan River, its tributaries in Northern Costa Rica and the wetlands of the Caño Negro National Wildlife Refuge, where it is considered an ensign species. However, the intensive pesticide use in agriculture at the surroundings of the refuge, especially in orange and pineapple crops carry a potential hazard to the gar and other fish populations inhabiting the refuge (Soto & Ramírez 2002). Criticisms regarding the environmental damage caused by pesticide use in the Caño Negro National Wildlife Refuge have been published in the local press (Chacón 2009, Barquero 2010, Hernández 2010). However, until now, *A. tropicus* has not been considered a sentinel species for monitoring the effects of environmental pollution in the area. In this regard, studies developed with other species of gar fish have demonstrated the potential of these fish for monitoring the effects of pollutants, including pesticides, heavy metals and endocrine disruptors (Osborne & Rulifson 2010, Aguilera *et al*. 2010). These studies have evaluated the bioaccumulation of metals and the response of biomarkers.

Biomarker evaluation allows for early detection of pollutant impacts on organisms. Biomarkers, such as the inhibition of cholinesterase (ChE) activity are popular and widely used tools in the field of environmental toxicology, mainly with the goal of estimating the effects of pollutants at the sub-individual level (Triebskorn *et al*. 2001, Guilhermino 2006, Sanchez *et al*. 2007, Almeida *et al*. 2010). Inhibition of ChE activity is one of the best characterized biomarkers and has been intensively used in environmental studies, showing a specific response to organophosphate (OP) and carbamate pesticides (Gruber & Munn 1998, Thompson 1999, Pathiratne *et al*. 2008, Mdegela *et al*. 2010). The performance of ChE inhibition as a biomarker has rendered it as a potential substitute of chemical analysis used to detect exposure to OP and carbamate pesticides (Peakall 1992). Considering the intensive use of organophosphates in Costa Rican agriculture and the use of this kind of pesticides in the Caño Negro area (Soto & Ramírez 2002), ChE stands out as an ideal biomarker to evaluate agriculture-related pollution effects in the area. Further, it would be useful to trace a link between pesticide use, detection of residues in the environment and their toxic effects.

Biotransformation and oxidative stress biomarkers such as Glutathione S-transferase (GST) and lipid peroxidation (LPO) are also widely applied in environmental studies (Mdegela *et al*. 2006, Guilhermino 2006, Sanchez *et al*. 2007); these markers are more unspecific, but still useful signals of environmental stress as they might respond to a broad diversity of pollutants.

In Costa Rica, the process of induced breeding of *A. tropicus* has been achieved twice with promising results, and with the objective of producing fingerlings for aquaculture and for reintroduction in areas where wild populations has been considerably diminished (Protti *et al*. 2010). Considering the availability of *A. tropicus* larvae from controlled spawns and the ecological relevance of this species, the production of ecotoxicological information on this fish would facilitate, its use as a sentinel species in ecologically vulnerable areas. The objective of this study was to evaluate the acute toxicity and the effects at the sub-individual level of the insecticide/nematicide ethoprophos on the Costa Rican indigenous fish *A. tropicus*.

MATERIALS AND METHODS

Fish used in the assays were bred in the Laboratory of Natural Resources and Wildlife (LARNAVISI); toxicity tests and biomarker analyses were carried out at the laboratory of Ecotoxicology (ECOTOX), both laboratories are located at the Campus Omar Dengo, National University of Costa Rica. A batch of five-days old individuals (wet wight=0.031±0.0014g; total length=17.42±0.58mm; mean±standard deviation) fed only once before assays was used. Breeding of fish; toxicity testing and biomarker analyses were undertaken between May and August, 2009.

For acute 96-horus assay, fish were placed into glass containers (one individual/container) that held 100mL of filtered (MILLIPORE) and UV-treated (PURA) water. After a 24hr acclimation period, a volume of ethoprophos stock solution (79.84mg a.i./L) was added to each container to obtain the nominal concentrations of each assay. The concentrations used in the assays were 0.1, 1, 10, 50, 100, 200, 300, 400, 500, 600, 700, 900 and 1500μg/L. Ten replicates were used per concentration, assays were static and during the exposure fish were not fed. After 96hr, deaths were recorded and surviving fish were retrieved, placed in 1.5mL microtubes and stored at -20°C for biomarker analyses.

The range of concentrations of ethoprophos used as treatments were estimated from preliminary assays with other native freshwater species (not included). In this study, several treatments, in a wide range of concentrations, were used in order to obtain enough treatments with surviving fish for biomarker analyses.

Whole-fish samples were used for biomarker analyses due to the small size of organisms which made difficult to obtain specific tissues. Samples were prepared according to Monteiro *et al*. (2007). Briefly, for homogenization, 1mL of 0.1M potassium phosphate buffer (pH 7.2) was added to each microtube. Fish were then homogenized for 10s using an ultrasonic homogenizer (Branson Sonifier 450). Samples were centrifuged at 8 160g for 5min. Supernatants were transferred to new microtubes and used for analysis of biomarkers. Samples were kept on ice during the whole process.

Protein contents of all samples were determined according to the Bradford method (Bradford 1976) adapted to microplate (Bio-Rad Laboratories Inc. 2005). Bovine g-globulin (Bio-Rad) was used as the protein standard.

ChE activity was measured using the Ellman method (Ellman *et al*. 1961), adapted to microplate (Guilhermino *et al*. 1996). Because no differentiation between acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was determined, the activity measured was considered as total ChE. Ellman assay was conducted by exposing the samples to a reaction solution (75mM acetylthiocholine and 10mM 5,5'-dithiobis-(2-nitrobenzoic acid)). Change of absorbance at 412nm was followed in a microplate reader (MultiSkan Ascent, Thermo Electron Corporation) for 15min. ChE activity was calculated for a linear period of the reaction and then expressed as U/mg, U=Nmol/min.

Glutathione S-transferase (GST) activity was determined by the method of Habig *et al*. (1974), based on the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene. The assay was conducted in a microplate as described by Booth *et al*. (2000), and the reaction was monitored at 340nm during three minutes. GST activity was expressed as U/mg, U=Nmol/min[1].

Lipid peroxidation (LPO) was measured as described by Torres *et al*. (2002). Level of LPO was expressed as Nmol of thiobarbituric acid-reactive substances (TBARS) per milligram of protein.

Reagents (analytical grade): 5,5'-Dithiobis(2-nitrobenzoic acid (DTNB), (2-Mercaptoethyl) trimethylammonium iodide (thiocholine iodide), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), Tris-HCl, *Diethylene triamine pentaacetic acid (DTPA),* buthylated hydroxytoluene (*BHT*) dipotassium phosphate and monopotassium phosphate, Bradford reagent and the Bovine g-globulin.

SPSS statistics 17.0 software was used for data analysis. Probit regression was used to calculate the 96hr LC_{50}. Differences among treatments were tested using ANOVA followed by the Tukey HSD post-hoc test. Kruskal-Wallis non-parametric test was used when samples did not meet the normality and/or homogeneity of variance. In all cases, fish were analyzed individually and differences were considered significant at a probability level of $p<0.05$. Figures are presented as means with their respective standard errors.

RESULTS

Acute toxicity is one of the most relevant endpoints in ecotoxicological studies, and is also an important parameter to characterize the sensitivity of species to xenobiotics. Ethoprophos has a high toxic effect on *A. tropicus* larvae. The determined 96hr LC_{50} of ethoprophos for *A. tropicus* was 859.7μg/L (95% confidence limits=659.3-1170.6μg/L).

Organophosphorous pesticides affect the nervous system of organisms by inhibiting the enzyme ChE. In this study, ChE was significantly inhibited ($p<0.05$) in *A. tropicus* larvae exposed to ethoprophos at a concentration of 50μg/L, value representing the lowest observed effect concentration (LOEC). At this concentration, enzyme activity was inhibited by 20% whereas at a concentration of 400μg/L of the pesticide, inhibition of the enzyme reached 79% (Fig. 1). At concentrations below 50μg/L, no ChE inhibition was observed and 10μg/L was determined as the no observed effect concentration (NOEC).

Oxidative stress and biotransformation markers are known to respond to a diverse range of environmental stressors. In this study, GST and LPO measured in whole-organism samples did not revealed a significant response ($p>0.05$) after exposure to ethoprophos compared to the control groups. GST activity was low in the whole-organism samples (Fig. 2A). A similar result was observed for LPO determinations (Fig. 2B). Even considering the low levels of response measured, no significant difference was observed among treatments.

DISCUSSION

Evaluation of toxicological parameters in tropical species is essential if the purpose is to characterise the impact of pollutants on the environment. The 96hr LC_{50} value calculated for ethoprophos on *A. tropicus* demonstrated a high toxicity of the pesticide for this species according to data from the Pesticide Action Network (PAN) of North America.

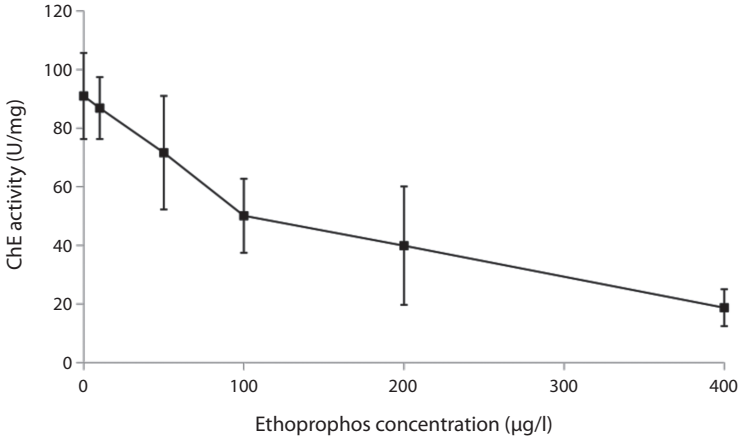

Fig. 1. Cholinesterase activity in *A. tropicus* larvae exposed to ethoprophos. Activity measured in whole-organism samples. Values are expressed as Mean±standard error.

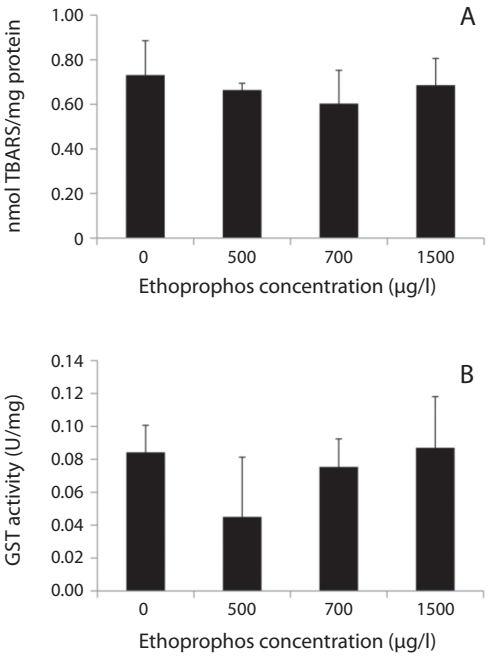

Fig. 2. Oxidative stress and biotransformation markers measured in *A. tropicus* larvae exposed to ethoprophos. **(A)** Lipid peroxidation (LPO); and **(B)** Glutathione S-transferase (GST) activity. Markers measured in whole-organism samples. Values are expressed as Mean±standard error.

Compared to other species commonly used in environmental studies (*Cyprinodon variegatus* (LC_{50}=958μg/L); *Oncorhynchus mykiss* (LC_{50}=700μg/L), *A. tropicus* is sensitive to ethoprophos (PAN Pesticides Database 2010).

An inhibition of 20% in ChE activity is considered a reliable indicator to diagnose exposure to organophosphates and carbamates, whereas enzyme inhibition of over 50% can be considered as an evidence that exposure to these pesticides caused the mortality in fish kill scenarios (Peakall 1992, Thompson 1999). At 50μg/L ChE activity was inhibited by 21%, but no mortality was observed at this concentration. These results show the suitability of the biomarker as an early warning signal; although other indigenous fish species should be tested regarding their sensitivity to anti-ChE pesticides, in order to produce a reliable species sensitivity distribution for the neotropical environment.

Determinations of oxidative stress and biotransformation markers were not able to distinguish effects of exposure, even at the highest test concentration of 1 500μg/L of the pesticide. Measurements should be done in specific tissues (liver or gills), as they are the primary organs involved with the pollutants entering the organism (Giaria *et al*. 2008). Also, gills and liver are frequently used in studies with more consistent results for these biomarkers (Li *et al*. 2008, Dinu *et al*. 2010). In this case, the small size of the fish made it impossible to sample these organs, but it should be considered in future experiments.

Furthermore, some contradicting results has been reported about the response of GST and LPO in fish towards ChE inhibiting pesticides. In the case of organophosphates, Almeida *et al*. (2010) found no significant response of these biomarkers in *Dicentrarchus labrax* after exposure to fenitrothion (OP). On the other hand, inhibition of GST was observed in *Pseudorasbora parva* exposed to methomyl (carbamate) (Li *et al*. 2008). It also should be considered that the activities of GST and LPO vary widely between fish species (Solé *et al*. 2006, Sanchez *et al*. 2007, Azevedo *et al*. 2009, Oliveira *et al*. 2010). Nevertheless, characterization of these non-specific biomarkers is necessary if the species is going to be used in field monitoring, considering that in the environment, organisms would be exposed to unknown mixtures of pollutants.

Our findings that *A. tropicus* larvae are highly sensitive to ethoprophos support the use of this species as a sentinel organism for the potential effects of pesticide exposure for wetlands embedded in a matrix of intensive agriculture. Cholinesterase activity in *A. Tropicus* larvae also resulted to be a sensitive biomarker of exposure to organophosphates. Future studies should be conducted with organisms of a larger size in order to characterize accurately the response of biomarkers in specific organs. The possibility of using blood samples in future biomarker studies should be evaluated

as a non destructive technique which would be especially important working with valuable and endangered species. Further investigation should be conducted to evaluate the sensitivity of *A. tropicus* to other pollutants to increase the value of the species as a tool field monitoring.

ACKNOWLEDGMENTS

Three anonymous reviewers contributed with their corrections, comments and suggestions to this article. Sascha Pfennig is grateful with the German Academic Exchange Service (DAAD) for his Ph.D. scholarship.

RESUMEN

El proceso de reproducción inducida de *Atractosteus tropicus* es útil para la acuicultura y la reintroducción en zonas donde las poblaciones silvestres se han reducido considerablemente. En larvas de esta especie se evaluó la toxicidad aguda, así como la respuesta de tres biomarcadores: actividad colinesterasa (ChE), actividad de Glutation S-transferasa (GST) y peroxidación de lípidos (LPO). Asimismo, se realizaron exposiciones agudas (96hr) a etoprofos (nematicida organofosforado), en donde se utilizaron concentraciones entre 0.1μg/L y 1 500μg/L del nematicida. La concentración letal 50 (LC50) calculada fue de 859.7μg/L; la máxima concentración sin efecto en los organismos (NOEC) 10μg/L y la concentración más baja en la cual se observó algún efecto (LOEC) 50μg/L. A esa concentración, el efecto observado fue una reducción significativa ($p<0.05$) en la actividad de la ChE. Una concetración de etoprofos de 400μg/L causó una inhibición del 79% en la actividad ChE. La actividad GST y la LPO no mostraron una respuesta significativa ($p>0.05$) luego de la exposición de los organismos a etoprofos.

Palabras clave: pez gaspar, *Atractosteus tropicus*, etoprofos, inhibidores de la colinesterasa, glutatión S-transferasa, peroxidación lipídica.

REFERENCES

Aguilera, C., R. Mendoza & R. Chacón. 2010. Physiological response of alligator gar (*Atractosteus spatula*) to pollution, p 13. *In* A. Ferrara & Q. Fontenot (eds.). Resúmenes III Reunión Internacional sobre la Investigación y Manejo de Lepisostéidos. Nicholls State University, Thibodaux, Louisina, USA.

Akaishi, F., H. Silva de Assis, S. Jakobi, D. Eiras-Stofella, S. St-Jean, S. Courtenay, E. Lima, A. Wagener, A. Scofield & C. Oliveira-Ribeiro. 2002. Morphological and neurotoxicological findings in tropical freshwater fish (*Astyanax sp*.) after waterborne and acute exposure to water soluble fraction (WSF) of crude oil. Arch. Environ. Contam. Toxicol. 46: 244-253.

Almeida, J., C. Oliveira, C. Gravato & L. Guilhermino. 2010. Linking behavioural alterations with biomarkers responses in the European seabass *Dicentrarchus labrax* L. exposed to the organophosphate pesticide fenitrothion. Ecotoxicology 19: 1369-1381.

Azevedo, S., A. Serafim, R. Company, E. Braga, D. Fávaro & M. Bebianno. 2009. Biomarkers of exposure to metal contamination and lipid peroxidation in the benthic fish *Cathorops spixii* from two estuaries in South America, Brazil. Ecotoxicolgy 18: 1001-1010.

Barquero, M. 2010. Piña sorteó la crisis, pero arrecia crítica ambiental. La Nación (20/04/2010.), San José, Costa Rica. (Downloaded: 10 december 2010, www.nacion.com).

Bio-Rad Laboratories Inc. 2005. Quick Start Bradford Protein Assay; Instruction manual. Bio-Rad Laboratories, Berkeley, California, USA.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem.72: 248-254.

Booth, L., V. Heppelthwaite & A. McGlinchy. 2000. The effect of environmental parameters on growth, cholinesterase activity and glutathione S-transferase activity in the earthworm (*Apporectodea caliginosa*). Biomarkers 5: 46-55.

Chacón, V. 2009. Expansión piñera entre principales amenazas: Lanzan voz de alerta por Humedal Caño Negro. Semanario Universidad (10/11/2009.), San José, Costa Rica. (Downloaded: 10 december 2010, www.semanario.ucr.ac.cr).

de la Torre, F.R., L. Ferrari & A. Salibián. 2005. Biomarkers of a native fish species (*Cnesterodon decemmaculatus*) application to the water toxicity assessment of a peri-urban polluted river of Argentina. Chemosphrere 59: 577-583.

Dinu, D., D. Marinescu, M. Munteanu, A. Staicu, M. Costache & A. Dinischiotu. 2010. Modulatory Effects of Deltamethrin on Antioxidant Defense Mechanisms and Lipid Peroxidation in *Carassius auratus gibelio* Liver and Intestine. Arch. Environ. Contam. Toxicol. 58: 757-764.

Ellman, G., D. Courtney, V. Andres Jr. & R. Featherstone. 1961. A new and rapid colorimetric determination of

acetylcholinesterase activity. Biochem. Pharmacol. 7: 88-95.

Giaria, L., E. Simonia, M. Manerab & B. Dezfuli. 2008. Histo-cytological responses of *Dicentrarchus labrax* (L.) following mercury exposure. Ecotoxicol. Environ. Saf. 70: 400-410.

González-Mille, D., C. Ilizaliturri-Hernández, G. Espinosa-Reyes, R. Costilla-Salazar, F. Díaz-Barriga, I. Ize-Lema & J. Mejía-Saavedra. 2010. Exposure to persistent organic pollutants (POPs) and DNA damage as an indicator of environmental stress in fish of different feeding habits of Coatzacoalcos, Veracruz, Mexico. Ecotoxicology 19: 1238-1248.

Gruber, S. & D. Munn. 1998. Organophosphate and Carbamate insecticides in agricultural waters and Cholinesterase (ChE) inhibition in common carp (*Cyprinus carpio*). Arch. Environ. Contam. Toxicol. 35: 391-396.

Guilhermino, L., M. Lopes, A. Carvalho & A. Soares. 1996. Inhibition of acetylcholinesterase as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere 32: 727-738.

Guilhermino, L. 2006. The use of biomarkers to assess the effects of environmental contamination in coastal and estuarine ecosystems: what questions remain? An example of the Portuguese NW coast. ICES CM 1:17.

Habig, W., M. Pabst & W. Jakoby. 1974. Glutathione S-Transferases: The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249: 7130-7139.

Hernández, C. 2010. Caño Negro denuncia peligro en refugio. La Nación (20/04/2010), San José, Costa Rica. (Downloaded: 10 december 2010, www.nacion.com).

Li, H., H. Jiang, X. Gao, X. Wang, W. Qu, R. Lin & J. Chen. 2008. Acute toxicity of the pesticide methomyl on the topmouth gudgeon (*Pseudorasbora parva*): mortality and effects on four biomarkers. Fish Physiol. Biochem. 34: 209-216.

Mdegela, R., M. Braathen, D. Correia, R. Mosha, J. Skaare & M. Sandvik. 2006. Influence of 17α-ethynylestradiol on CYP1A, GST and biliary FACs responses in male African sharptooth catfish (*Clarias gariepinus*) exposed to waterborne Benzo[a]Pyrene. Ecotoxicology 15: 629-637.

Mdegela, R., R. Mosha, M. Sandvik & J. Skaare. 2010. Assessment of acetylcholinesterase activity in *Clarias gariepinus* as a biomarker of organophosphate and carbamate exposure. Ecotoxicology 19: 855-863.

Monteiro, M., C. Quintaneiro, A. Nogueira, F. Morgado, A. Soares & L. Guilhermino. 2007. Impact of chemical exposure on the fish *Pomatoschistus microps* Krøyer (1838) in estuaries of the Portuguese Northwestcoast. Chemosphere 66: 514-522.

OECD. 2007. Guidance Document on the Validation of (Q) SAR Models. Organization for Economic Cooperation and Development. Environmental Health and Safety Publications. Series on Testing and Assessment, Paris, France.

Oliveira, M., I. Ahmad, V. Maria, M. Pacheco & M. Santos. 2010. Monitoring pollution of coastal lagoon using *Liza aurata* kidney oxidative stress and genetic endpoints: an integrated biomarker approach. Ecotoxicology 19: 643-653.

Osborne, J.H. & R.A. Rulifson. 2010. Mercury concentrations in the muscle tissue of Longnose Gar *Lepisosteus osseus* in coastal North Carolina with additional contributions to the Life History, p 39. *In* A. Ferrara & Q. Fontenot (eds.). Resúmenes III Reunión Internacional sobre la Investigación y Manejo de Lepisostéidos. Nicholls State University, Thibodaux, Louisina, USA.

PAN Pesticides Database. 2010. Chemical search. (Downloaded: 12 August 2010, http://www.pesticideinfo.org/List_AquireAll.jsp).

Pathiratne, A., L. Chandrasekera & P. De Seram. 2008. Effect of biological and technical factors on brain and muscle cholinesterases in Nile tilapia, *Oreochromis niloticus*: implications for biomonitoring neurotoxic contamination. Arch. Environ. Contam. Toxicol. 54: 309-317.

Peakall, D. 1992. Animal biomarkers as pollution indicators. Chapman and Hall, London, England.

Protti, Q.M., G. Márquez & A. Sevilla. 2010. Adaptación tecnológica para el control de la reproducción, larvicultura, alevinaje y engorde del pez gaspar *Atractosteus tropicus* (Pisces: Lepisosteidae), Informe final. Escuela de Ciencias Biológicas, Universidad Nacional, Heredia, Costa Rica.

Ramírez, F., F. Chaverri, E. de la Cruz, C. Wesseling, L. Castillo & V. Bravo. 2009. Importación de plaguicidas en Costa Rica, periodo 1977-2006. Universidad Nacional, IRET, Heredia, Costa Rica.

Sanchez, W., S. Aït-Aïssa, O. Palluel, J. Ditche & J. Porcher. 2007. Preliminary investigation of multi-biomarker responses in three-spined stickleback (*Gasterosteus aculeatus* L.) sampled in contaminated streams. Ecotoxicology 16: 279-287.

Solé, M., J. Kopecka & L. García de la Parra. 2006. Seasonal variation of selected biomarkers in sand

gobies Pomatoschistus minutes from the Guadalquivir estuary, Southwest Spain. Arch. Environ. Contam. Toxicol. 50: 249-255.

Soto, L. & F. Ramírez. 2002. Diagnóstico del uso de plaguicidas en la Cuenca del Río Frío. Estudio realizado para PROUDESA, en el proyecto "Restauración de la Cuenca del Río Frío", con el apoyo de UNOPS/PNUD. Escuela de Ciencias Ambientales, Instituto Regional de Estudios en Sustancias Tóxicas, Universidad Nacional, Heredia, Costa Rica.

Thompson, H. 1999. Esterases as markers of exposure to organophosphates and carbamates. Ecotoxicology 8: 369-384.

Torres, M., C. Testa, C. Gáspari, M. Masutti, C. Panitz, R. Curi-Pedrosa, E. Almeida, P. Di Mascio & D. Filho. 2002. Oxidative stress in the mussel *Mytella guyanensis* from polluted mangroves on Santa Catarina Island, Brazil. Mar. Pollut. Bull. 44: 923-932.

Triebskorn, R., J. Bohmer, T. Braunbeck, W. Honnen, H. Kohler, R. Lehmann, A. Oberemm, J. Schwaiger, H. Segner, G. Schuurmann & W. Traunspurger. 2001. The project VALIMAR (Validation of biomarkers for the assessment of small stream pollution): objectives, experimental design, summary of results, and recommendations for the application of biomarkers in risk assessment. J. Aquat. Ecosyst. Stress. Recov. 8: 161-178.

US-EPA. 1986. United States Environmental Protection Agency. Quality criteria for water. Office of water regulations and standards, Washington D.C., USA.