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Identification lethal and sub lethal concentrations (LC_{50}) of Organophosphate (OP) pesticide Diazinon using an endemic species (Yucatan Molly, *Poecilia velifera* Regan 1914) as a potential biomonitor for the intensive agricultural activities of Southeastern Mexico.

Authors:
Francisco Ucan-Marin[* 1,2], Víctor Cobos-Gasca[3] and Roberto C. Barrientos-Medina[3]

Institution:
1. Aquatic Toxicology, Aquaponika Ltd. 126-340 Parkdale Ave. Ottawa Ontario, Canada K1Y 1P2.

2 . Chemistry and Environmental Toxicology, Department of Chemistry, Carleton University. 203 Steacie Building Ottawa, Ontario, Canada K1S 5B6.

3. Cuerpo Académico de Ecología Tropical, Depto. de Ecología. Facultad de Medicina Veterinaria y Zootecnia, Campus de Ciencias Biológicas y Agropecuarias, Universidad Autónoma de Yucatán. Km. 15.5 Carretera Mérida-Xmatkuil, Yucatán, México.

Corresponding author:
Francisco Ucan-Marin

ABSTRACT:

Organophosphate (OP) pesticides are commonly used in agriculture; this group of compounds includes very toxic chemicals. Diazinon (IUPAC name: O,O-Diethyl O-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate, INN-Dimpylate) is used often in the Yucatan Peninsula, Mexico. Regular tropical rain-floods and the Yucatan's karstic topography allow Diazinon to be incorporated quickly into the freshwater watersheds and other aquatic ecosystems surrounding agricultural areas. This dispersion process has various negative consequences for the aquatic ecosystems. In the present study we used the Yucatan Molly (*Poecilia velífera*) a native and endemic fish of Southeastern Mexico as a biomonitor for the first time to assess some of the basic gaps in the Diazinon toxicity data. 96 juvenile fish (fry) were exposed to two time exposure-observations; for acute (24 hours) and chronic exposures (10 weeks). Three Diazinon doses were added as follows: 0.01, 0.02 and 0.04 mg/l (and a duplicated control group). The results showed that the acute dosed group has 100% mortality. Fish exposed to a 0.01 mg/l did not have any observable effects. The LC_{50} value calculated during this experiment for Yucatan Mollies exposed to dissolve Diazinon is extremely toxic at 0.02 mg/L. These results confirm, that the sensibility of *Poecilia velifera* as a native bioindicator for pesticides; and compared with other published LC_{50} data appears to be the most sensitive. Further studies are recommended to continue the study on the Yucatán Molly physiology; this fish has the potential to become a reliable sentinel for the aquatic ecosystems in the Yucatan Area, Mexico.

Keywords:

Agriculture, Diazinon, LD_{50}, Yucatan Molly, Biomonitor, Yucatan Mexico, Ecotoxicology

Email Id:
francisco_ucanmarin@carleton.ca,

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INTRODUCTION

The Yucatan Peninsula in located in Southeastern Mexico, and is an intensive production area for citrus and horticultural farms, most of the farming is developed through small groups called "*Ejidos comunitarios*" (Community Cooperatives). Agricultural activities in this region includes: citrus production, mango and papaya farming, vegetables and herbs, but also intensive cattle and horse ranching. Organophosphate (OP) pesticides are used openly for infestation management and applied intensively. It has been noticed that farmers and agricultural workers in the Yucatan area do not use proper protection during pesticide applications. Furthermore, there are no enforced disposal regulations for the remaining chemicals or containers. Diverse factors of management and inadequate disposal protocols have contributed to leaking and accumulation of pesticides in sites near water sources. Therefore the potential of pesticides entering the aquatic ecosystems increase the possibilities of endocrine disruptions effects in wildlife, and eventually the surrounding human settlements.

Diazinon (O,O-diethyl O-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] phosphorothioate), is an organophosphate insecticide, it has agricultural and commercial uses, and is used in Southeastern Mexico to control a wide diversity of insects including aphids, beetles, scales, pill bugs, and others (Cobos-Gasca 1995; Cox 1992). Diazinon is a compound of large variety of applications soluble in water, and produces a chemical half-life recorded up to 12 weeks in water (Blackburn *et al..,* 1988). In addition, Diazinon does not settle permanently in sediments; as a result, it is easily hydro transported which facilitates its presence in aquatic ecosystems (Blackburn *et al.*, 1988). Diazinon permanency in the aquatic ecosystems has been associated directly with rain-runoff and its unofficial unregulated disposal, where it has been noticed to settle on freshwater ecosystems (Bailey *et al.*, 2000). Diazinon has been previously reported as a dangerous chemical able of acute toxicity and sub-lethal effects in fresh water fish and other aquatic organisms (Banaeea *et al.*, 2011). The mode of action of OP chemicals it is associated with the inhibition of acetyl cholinesterase, and oxidative stress (Ozcan and Demet 2007). Diazinon disrupting effects on aquatic organisms has been widely documented in freshwater organisms such as snail (*Gillia altilis*), largemouth bass (*Micropterus salmoides*) and rainbow trout (*Oncorhynchus mykiss*), among others (Robertson and Mazzella 1989; Pan and Dutta 1998; Beauvais *et al.,* 2000).

Mexico's Yucatan geography and hydrological conditions create a unique aquatic underground environment (Figure 1), and also a variety of unique endemic species of fish (Figure 2) and aquatic ecosystems. Due to this unique karstic ground (limestone bedrock) characteristic, the leaching and transporting of contaminants such as pesticides and fertilizers into groundwater do not favor soil absorption (Pacheco and Cabrera 1996). In the Yucatan, underground water is the only source for human consumption since rivers are almost not existent. The underground water table is close to the surface in the north and near the coasts, but in the higher elevations of the middle and south of the Peninsula water is too far below the surface for access by hand-dug wells; nevertheless, the ancient Maya maintained dense populations in this area for centuries using reservoirs and underground tanks (chultuno'ob). Therefore, the assessment and monitoring of water and environment quality through biomonitoring is highly significant for a local social, economic and community context.

Previous regional studies has assessed the effect of OP pesticides in estuarine ecosystems aquatic macro-fauna such as; shrimps from genus *Penaeus* (Acosta-Maya *et al.*, 1997), and mosquito fish *Gambusia yucatana* (Rendón von-Osten *et al.*, 2005). Diazinon has also been repeatedly detected in

Figure 1 Cenote (in Spanish) or Sinkhole a common freshwater ecosystem in the Yucatan area of Mexico (Credit; Francisco Ucan-Marin).

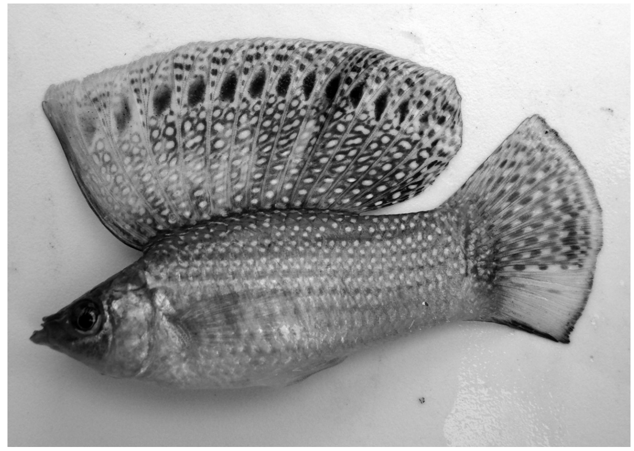

Figure 2. Yucatan Molly (*Poecilia velifera*, male) a native fish of Southeastern Mexico (Credit: Lizbeth Chumba-Segura)

horticultural water supplies (underwater sinkholes) in the Yucatan region (Cobos-Gasca *et al.*, 1997). One of the most common observable fish surrounding the areas of concern is the Yucatan molly (*Poecilia velifera*, Regan, 1914). The Yucatan molly (Figure 2) belongs to the Poeciliidae family, and is an endemic species to the Yucatan Peninsula, Mexico (Miller 1983; Miller 2005; Hankison *et al.*, 2006). This fish was introduced internationally for two main reasons; first, as a valuable aquarium specie, and as a biological control against mosquitoes larvae (Courtenay and Meffe 1989; Lever 1996).

Studies assessing the toxicity of Diazinon to aquatic fauna had documented that this pesticide is able to cause neurotoxic effects on fish (Dias-Assis *et al.*, 2012). However, the importance of the present study is the original contribution of lethal and sub lethal data assessing this native and endemic species. The values do not only contribute to aquatic toxicology knowledge, but also add the proposition to use the Yucatan Molly as a biomonitor of pesticide activities surrounding agricultural communities across the southeastern Mexico.

MATERIALS AND METHODS

Gravid female Yucatan mollies were captured from freshwater near the port of Celestun, in the State of Yucatan, Mexico, and kept under laboratory conditions. Fish tanks were used for the acclimation, and 15 days after collection, 134 fingerlings were hatched and were fed with commercially available food for four weeks. The bioassay to assess the toxicity of Diazinon was conducted by selecting 96 juveniles (fry), of similar length and weight, and distributed randomly sex independent into four tanks with 20 liters of freshwater. The conditions were: temperature, 26.9 ± 0.1°C; saturation of dissolved oxygen, 62 ± 0.1%; electric conductivity, 468.61 ± 0.1 µS /cm and pH of 7.40 ± 0.1 units. The experiment had four treatments 0.04 mg/l, 0.02 mg/l, and 0.01 mg/l of commercially available Diazinon (Dragon®) and a control group.

Mortality was first observed after 24 h and data were adjusted accordingly with two regression models: binomial logit model (Collet 2003) and the probit model (Finney 1971), with the help of STATGRAPHICS package. This statistical procedure consent the estimation of regression parameters by maximum likelihood method and use the percentage of deviance explained as a measure of fit for comparing the models, and estimates the median lethal concentration (LC_{50}). The bioassay was sustained up to 10 weeks of exposure, where concentration levels were applied in order to study severe chronic effects, and a constant concentration of pesticide in the tanks were monitored. Mortality data obtained after 10 weeks, excluding the highest concentration, were analyzed using repeated measures analysis of variance (ANOVA), considering weeks as repeated sampling units and using as response variable the number of dead organisms (base-10 log transformed) and concentrations as treatments employing Tukey-test as multiple comparison procedure to distinguish the dose effects, including the control group (Kuehl 2001). Repeated measure ANOVA were carried out with PAST software (Hammer *et al.*, 2001), version 2.14 and for all statistical analyses, the significant level of 5% was considered as appropriated. Finally, to aid in the interpretation of results, standardized mortality was calculated according to the following expression (Raymond 1985):

$$M_E = \frac{M_T - M_C}{100 - M_C}$$

Where ME is the standardized mortality, MT is the death occurred in each dose and MC the mortality that occurred in the control group. This expression allows separating the mortality caused by the pesticide of natural mortality.

RESULTS

Within the first 24 h of exposure, all organisms exposed to the highest concentration (0.04 mg/l) perished. Diazinon effects on the exposed fish began to be noticeable at the moment of the first exposure, where erratic swimming behavior and disruption in the posture were clearly observable. Fish exposed started to swim close to the bottom of the tank, revolving around a single point, with the head close to the bottom and the body placed in perpendicular to it. Fish groups exposed at 0.02 and 0.01 mg/l had mortalities of 16% and 52% (Figure 3), and control registered no deaths or changes in the swimming behavior. Regression analyses revealed that both; the logit model and probit models, are appropriate to describe the relationship between concentration and mortality at 24 hours of exposure. Both models were highly significant (P < 0.001). Probit model had greater percentage of deviance in the data (98.84%) when compared to logit model (97.89%). According to the probit model, estimates of the regression parameters are β_0=2.4246 and β_1= 127.93, both significantly different from zero according to the confidence limits at 95% (Table 1). This means that in the modeling of mortality by effect of diazinon the intercept (β_0) should be considered as an intercept, as a measure of the response obtained in the absence of pesticide (natural mortality), and that the association between pesticide dose and mortality is direct and significant, given that mortality is increased nearly 124 units (on average) by each increase in the applied dose of diazinon.

The estimate of LC_{50} was 0.0189 mg / l (0.0160 to 0.0231 mg / l, 95% confidence), statistically similar to the value of intermediate concentration used

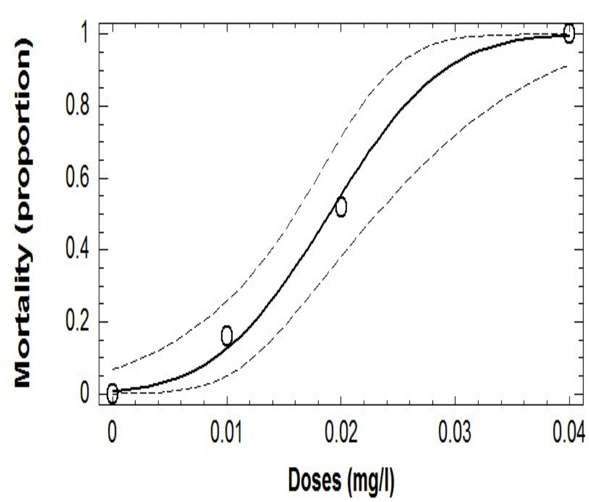

Figure 3. Relationship between the concentration of diazinon and mortality of Yucatan Molly *Poecilia velifera*, according to the probit regression model. The 95% CI for the regression curve is also included (dotted lines).

Table 1. Estimates of the regression parameters according to the probit regression model.

Parameter	Estimation	SE	CI 95%
β_0	-2.42	0.43	-4.46, -0.39
β_1	127.93	26.97	11.87, 243.98

SE = standard error, CI = confidence interval

(0.02 mg / l), which will cause a mortality of 52%. Comparing selected data (Table 2) as average for lethal concentration obtained in this bioassay for freshwater fish (Figure 4), shows that Yucatan Molly tolerance to Diazinon exposure is clearly lower. The repeated measures ANOVA revealed significant differences between treatments ($F= 1164$, $P<< 0.05$ with 2, 29 df). In fact, the three treatments differ in terms of mortality (Tukey's $P< 0.05$, in all cases): the two sub lethal concentrations causes mortalities of 9 and 48% respectively, compared with control (Figure 4). At the intermediate concentration, which produces five times greater mortality than the lowest concentration (0.01 mg/ l), swimming disruption was observed after three weeks of exposure. In the fourth week we observed that one fish had damage in the orbits and visible spine paralysis, which prevented movement and feeding. The fish died a few hours after this behavior first appeared. At the seventh week another fish was observed also with severe

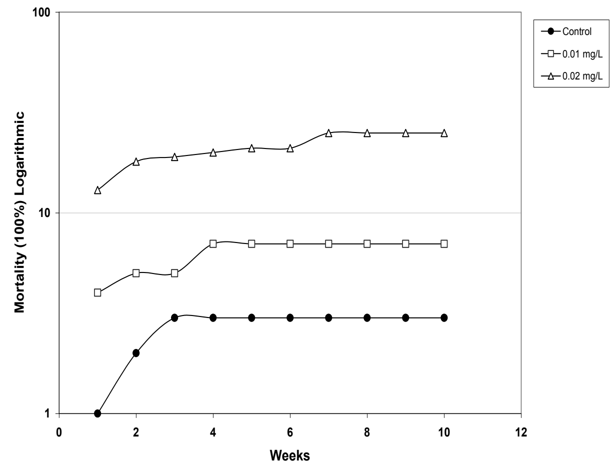

Figure 4. Yucatan molly (*Poecilia velifera*) accumulative mortality for each treatment during the 10 weeks of exposure to Diazinon.

spinal paralysis. In both cases, fish were not able to feed for themselves and perished.

DISCUSSION

Given the intensive use of pesticides in Yucatan, several evaluations of the quality of groundwater have been done; mainly assessing areas specialized in growing citrus and vegetables (Cobos-Gasca *et al.*, 1997; Santos-Vázquez 1989; Cabrera *et al.,* 1992). These studies have shown that the presence of these contaminants in the aquifer is linked to the rainfall season. During this event the rain wash and carry the all sort of organic compounds

Table 2. Comparative levels of LC_{50} (lethal concentration, 50%) of Diazinon (mg /l) for some freshwater fish.

Species	LC_{50}	Time of exposure	Reference
Channa punctata (Bloch, 1793)	11.00	96 h	Robertson and Mazella, 1989
Carassius auratus (Linnaeus, 1758)	9.01	96 h	Turner L, 2002
Oreochromis niloticus (Linnaeus, 1758)	7.83	96 h	Giron-Perez *et al.,* 2007
Cyprinus carpio (Linnaeus, 1758)	4.97	96 h	Turner L, 2002
Cyprinodon variegatus (Lacepède, 1803)	1.47	96 h	Turner L, 2002
Oncorhynchus mykiss (Walbaum, 1792)	5.52	24 h	Robertson and Mazella, 1989
Lepomis macrochirus (Rafinesque, 1819)	0.76	24 h	Robertson and Mazella, 1989
Micropterus salmoides (Lacepède, 1802)	0.09	24 h	Robertson and Mazella, 1989
Danio rerio (Hamilton, 1822)	2.52	96 h	Turner L, 2002
Poecilia sphenops (Cuvier, 1846)	1.65	96 h	Turner L, 2002
Gambusia affinis (Baird and Girard, 1853)	1.27	48 h	Turner L, 2002
Poecilia reticulata (Peters, 1859)	0.08	96 h	Turner L, 2002
Poecilia velifera (Regan, 1914)	0.02	24 h	* *Present study*

Table 3. Qualitative descriptors for categories of fish and aquatic invertebrate toxicity (from Zucker, 1985)

LC_{50} or EC_{50}	Category description
< 0.1 ppm	Very highly toxic
0.1- 1 ppm	Highly toxic
> 1 ppm	Moderately toxic
> 10 < 100 ppm	Slightly toxic
> 100 ppm	Practically non-toxic

passing over calcareous soil, which cause difficulty of absorption into the subsoil. If a compound is exposed directly over the karstic soil, the heat and light of the tropics are a decisive factor to their breakdown. Also, due to the edaphological characteristics of the area, these do not allow bacterial enzymes to speed the breakdown of Diazinon. However, since the pesticide is stored in cold water underground, the persistence of Diazinon is an environmental concern. Toxicology studies assessing other native fish species to the Yucatan Peninsula, the mosquito fish *Gambusia yucatana* (Rendón von-Osten *et al.*, 2005) determined, granted midsize lethal concentration (LC_{50} of 0.085 mg/l for chlorpyrifos, 17.79 mg/l for glyphosate, 0.636 mg/l for carbofuran and 0.011 mg/l for a mixture of chlorpyrifos and glyphosate). Chlorpyrifos (IUPAC name: O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) after 96 h of exposure had LC_{50} values of 0.085 mg/l in mosquito fish (Rendón von-Osten *et al.*, 2005).

Our results showed that Diazinon is extremely toxic to the Yucatan molly fry, with high probability to cause mortality as these chemicals enter the surrounding aquatic ecosystems (wells, watersheds and sinkholes) where frequent use is registered for this pesticide (Cobos -Gasca *et al.*, 1997). Differences in LC_{50} values among different species may be due to physiological and ecological factors, although most fish are sensitive to Diazinon, it is known that fish living in fresh and hard (calcium-bicarbonate-containing) waters are more resistant to those who live in saltwater environments (Banaeea *et al.*, 2011).

The abnormal behavioral responses (loss of equilibrium, hanging vertically in the water, rapid gill movement, erratic swimming, swimming at the water surface, and staying motionless on the aquarium bottom) of the Yucatan Molly in the present study are similar behavioral responses observed with the guppy [*Poecilia reticulate*] (Viran *et al.,* 2003), freshwater catfish [*Heteropneustes fossilis*] (Saha and Kaviraj 2003) and young mirror carp [*Cyprinus carpio*] (Calta and Ural 2004). Nevertheless, the physiological response has been extensively analyzed, where it has been recently observed by Ucan-Marin *et al.,* (2012) that the Brain Acethyl cholinesterase is the main enzyme affected by OP insecticides. Briefly, the mechanism of action of OP insecticides is based on the irreversible inhibition of Brain Acethylcholinesterase (AChE) which leads to the accumulation of ACh in synapses resulting in an initial over-stimulation of neurotransmission followed by depression of neurotransmission, paralysis, and eventual death (Pope 1999). The disruption of AChE activity either above or below 50% of normal has been considered as a valid indicator of adverse effects (De Marco *et al.*, 2002). The loss of mobility and lack of coordination in animals previously exposed to pesticides is often attributed to a decrease in the activity of brain AChE (Arufe *et al.*, 2007). Yet, the same effect was observed when brain AChE activity is increased, Zatta *et al.,* (2002) studied mice exposed to aluminum and reported that brain AChE increased activity also has the effects of paralysis and lack of control in the mobility.

One of the possible mechanisms of action of Diazinon is centered on its potent ability to open g-aminobutyric acid (GABA)-gated ClK channels (Campbell 1989) in both invertebrates and vertebrates. Ucan-Marin *et al.,* (2012) studied salmon smolts and observed that behavioral symptoms similar to those associated with Diazinon are observed when AChE disruption is present, where either an elevated or depressed significantly brain AChE activity can be a response to stress (Nijholt *et al.*, 2004). Diazinon can be metabolized to diazoxon by cytochrome P-450 monooxygenase (Hogan and Knowles 1972). This compound, which is a potent inhibitor of the brain acetylcholine esterase (Ucan-Marin *et al.*, 2012), is usually not detectable *in vivo* because of its rapid hydrolysis, catalyzed by the oxonase, to 2-methyl-6-isopropyl-4-pyrimidinol (pyrimidinol). The monooxygenase-catalyzed reaction accomplishes also an oxidative ester cleavage (Sultatos 1991). So, the production of diazoxon is accompanied by the concurrent formation of other metabolites, including pyrimidino1 (Fuji and Asaka 1982). Finally, Diazinon can also be a substrate for the glutathione S-transferase, which cleaves an ethyl group from the phosphate and gives rise to the S-ethyl-glutathione conjugate (de Bruijn I and Hermens 1991). The remaining portion of the OP may be hydrolyzed by phosphodiesterases to give pyrimidinol and other products. Since monooxygenases are present in fish with very different levels, it is likely that the rate of formation of the oxon from diazinon as well as from other organophosphothionates, acts in combination with the AChE affinity to cause the species-specific toxicity of OPs among fish (Keizer *et al.*, 1995).

In-vivo evaluation of Yucatan molly as biomarkers is highly significant due to the capacity to react to a real environmental exposure. *In-vitro* studies do not assess the specifics and co-factor triggered responses, in fact the main drawback of *in-vitro* toxicity tests is their apparently lower sensitivity compared to fish (Castano *et al.*, 2003; Segner 2004) which restricts their use as alternative to the acute fish tests. Because of its aquatic distribution capabilities, Diazinon affects a wide range of non-target organisms like invertebrates, mammals, birds, and fish; but especially animals inhabiting aquatic ecosystems (Burkepile *et al.*, 2000). During the present study, the Yucatan Molly was used as the most sensible fish for monitoring the presence of Diazinon near extensive and intensive agricultural activities.

CONCLUSION

In the present study we obtained an LC_{50} of 0.02 mg/L (24h) for young (fry) Yucatan molly *Poecilia velifera* (Regan 1914*)* and due to its high sensibility to Organophosphate compounds (OP) can be used as a bioindicator for the presence of pesticides in the aquatic ecosystems in the Yucatan Peninsula, Mexico. *P. velifera* is an exceptional sensible fish able to physiologically react to very low concentrations of Diazinon present in their ecosystems. Since Diazinon is used today in diverse agricultural activities in Latin America, therefore the use of *P. velifera* as a first class biomarker could improve the time, precision and costs of monitoring practices.

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