SUSCEPTIBILITY OF *CHORDODES NOBILII* (GORDIIDA, NEMATOMORPHA) TO THREE PESTICIDES: INFLUENCE OF THE WATER USED FOR DILUTION ON ENDPOINTS IN AN ECOTOXICITY BIOASSAY

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ABSTRACT

The increased use of pesticides during recent years necessitates a reevaluation of the effect of those compounds by extending the range of nontarget species commonly used in risk assessment. In the present work, we thus determined the impact of the pesticides glyphosate, carbendazim, and malathion on the parasite *Chordodes nobilii* in both natural and reconstituted freshwater as the assay medium and tested the sensitivity of three of this species's ecologically relevant parameters—*e. g.*, embryo nonviablity and the infective capability of

larvae exposed for 48 or 96 h either *in ovo* or after hatching via the infection index mean abundance—to compare those parameters to data from previous trials with reconstituted freshwater. In natural-freshwater assays, at environmentally relevant concentrations, all three pesticides inhibited the preparasitic-stage endpoints; with carbendazim being the most toxic pesticide and the subsequent infectivity of larvae exposed *in ovo* the most sensitive endpoint. In general, the 50%-inhibitory concentrations assayed in reconstituted freshwater were higher than those obtained in natural freshwater, indicating a certain protective effect; whereas the maximal toxicity of the three pesticides in both aqueous environments was essentially similar. The sensitivity of *C. nobilii* to these agents demonstrated that this species is one of the most susceptible to toxicity by all three pesticides. These findings with the assay methodology provide relevant information for a future assessment of the risk of toxicity to aquatic ecosystems and furthermore underscore the need to include parasitic organisms among the nontarget species canvassed. We also recommend that in the bioassays in which the risk assessment is carried out, water from a nontarget species's natural environment be used in parallel in order to obtain more conclusive results.

CAPSULE

We underscore the necessity to include parasites among the biota bioassayed and also recommend the use of natural freshwater as the dilution medium in bioassays for the evaluation of pesticide ecotoxicity.

Key words

Assay medium; Bioassay; Freshwater; Infectivity; Parasites; Pesticides

1. INTRODUCTION

In a country such as Argentina with an extensive agricultural activity, as would be predicted, the intensive cultivation has resulted in a drastic increase in the amount of pesticide application. Moreover, the global market for pesticides is expected to grow in the immediate future (Binimelis et al., 2009; de Gerónimo et al., 2015; Lantieri et al., 2011; Mugni et al., 2010; Ossana and Salibian, 2013).

The use of pesticides carries the risk of contaminating the surrounding water bodies, mainly through runoff or leaching. Mugni et al. (2011a, b) observed ephemeral pulses of toxicity resulting from the field application of glyphosate (GLP) among other pesticides and concluded that runoff was a greater source of toxicity to the surrounding environment than direct exposure to aerosol fumigation.

Since pesticide application generates risks for the aquatic biota, pesticide-induced effects on nontarget organisms are seen to constitute a general environmental hazard, in Argentina as well as in the rest of the world (Miracle et al., 2011; Vazquez et al., 2012; Villaamil Lepori et al., 2013). Therefore, a priority should be extended to the inclusion of those nontarget species not usually utilized for the evaluation of sensitivity to pesticides, and with an emphasis on the biota that is representative of given region of cultivation as well as on the stages in the life cycle of each taxon that is the most susceptible to a particular compound.

Among the aquatic biota that are sensitive to pesticides is the gordiid *Chordodes nobilii*, a representative of Nematomorpha—the horsehair worms, one of the three completely parasitic phyla, similar to nematodes. This species is a denizen of the Sauce-Grande basin (Province of Buenos Aires, Argentina), an area within the pampas predominantly devoted to agriculture and cattle-raising (Aduriz et al., 2003; de Villalobos et al., 2005). A salient characteristic of the gordiids is their capability of parasitizing adjoining terrestrial regions since, while the juveniles parasitize mainly land insects, the free stages in the life cycle (microscopic eggs, larvae, and adults with body lengths from a few centimeters to more than 2 m) inhabit the adjoining freshwater. With respect to the gordiids, three toxic pesticides—GLP, malathion (MTN), and carbendazim (CBZ)—along with the metals cadmium and chromium and the detergent sodium dodecyl sulfate have been evaluated in bioassays for effects on the preparasitic life stages of *C. nobilii*, by means of a specific protocol for both stages. In laboratory experiments, the susceptibility of the gordiid embryos and larvae to environmentally relevant concentrations of those agents have been demonstrated in assays involving reconstituted freshwater RFW (Achiorno et al., 2008, 2009, 2010, 2015). These experiments pointed to the need to reevaluate the effect of biotoxic agents in bioassays on parasites, as had been expressed by different authors—*e. g.*, Thomas et al., 2006; Poulin, 1999; Sures et al., 2017; Vidal-Martinez et al., 2009. Accordingly, the inclusion of parasites in monitoring the environmental impact of pesticides is essential because those taxa outnumber free-living organisms and are fundamental constituents of sound ecosystems through their participation in food-web dynamics and their effect in altering the energy flow through those systems, thus constituting a principal component in shaping the composition of the community (Blanar et. al., 2009).

In designing bioassays, the medium must insure an accurate standarization and high reproducibility and not influence the health and performance of organisms being tested, so that reconstituted water is usually employed (Loureiro et al., 2011). These features enable a reproducible repetition by other investigators in different laboratories, but can make the extrapolation to natural environments of the results obtained difficult, and even questionable. Since the composition of the natural water is a major source of variation in the physical-chemical properties of pesticides and normally influences the behavior of the aquatic organisms as well, that environmental matrix, as a consequence, will necessarily modify the toxicity of those compounds. Therefore, in order to make experimental results mirror the

process that occurs in the wild more realistically, the water from the environment to be tested can be used as the medium in laboratory bioassays. Nevertheless, only few studies have evaluated the toxicities of different contaminants by comparing the results of experiments containing natural and reconstituted water in the bioassays. The reports of Jonczyk et al. (2001) and Zhang et al. (2012), however, constitute two notable exceptions.

In view of the above considerations, we were interested in determining whether the susceptibility of *C. nobilii* to pesticides under the standard conditions normally used in bioassays with that species were overestimated relative to the response that would occur in natural water as the assay medium. The objective of the present experiments was therefore to determine under standard laboratory conditions the toxicity of the three pesticides to *C. nobilii* in water from the worm's natural environment in comparison with the standard form of reconstituted water normally used in such laboratory bioassays.

2. MATERIALS AND METHODS

2.1. Test organisms

Adults from both sexes of *C. nobilii* were collected from streams within the Sauce-Grande basin in Sierra de La Ventana, a locality of the pampas plain, Buenos Aires, Argentina (38° 09” S, 61° 48” W). In the laboratory, individuals from each stream were kept in separate containers with aerated dechlorinated tap water, at a room temperature of 23 ± 1 ºC. After mating, the females were kept individually in separate containers for oviposition, which consisted in egg strings of *ca*. 0.5 mm in width. Because the bioassays were performed with embryos (mainly in the blastula stage) and larvae, the egg strings were separated into two groups. One group was used immediately in an embryo assay, while the other was maintained in running dechlorinated water under the same conditions until reaching the free-larva stage for larval assays.

2.2. Chemicals

We evaluated: GLP, in a formulation like that of Roundup™ at 35.2% (w/v), expressed as the acid equivalent; MTN, 100% pure; and CBZ, technical grade, 98% pure. The pesticides used in this study were obtained from Félix Menendez SRL and from the Servicio Nacional de Sanidad y Calidad Agroalimentaria de Argentina (SENASA).

2.3. Test solutions

In the bioassays used in these experiments, the dilution medium was natural freshwater (NFW) from the Sauce Grande River, where *C. nobilii* is usually found. In each assay, water from that natural source was collected at remote distances from the cultivated area and kept under refrigeration for no longer than two days before use in the experiments, with a sample first being removed for characterization of the physicochemical parameters—*i. e.*, the range of values for the pH, hardness, and conductivity of the water used in the bioassays. The values obtained were: pH between 7.9 and 8.7, hardness between 30 and 90 mg/L of $CaCO_3$, and conductivity between 95.1 and 495 µSiemens; which data are to be compared with those from our previous work at between 7.6 and 8.0, 160 and 180 mg/L of $CaCO_3$, and 816 and 1180 µSiemens, respectively.

For all the assays, a stock solution of each of the pesticides was first prepared in double-distilled water at the nominal concentrations (mg/L) of 370.5 for GLP, 2.5 for CBZ, and 100.0 for MTN. The concentrations assayed after dilutions from these stocks with the NFW water to be used in the assay were in a range between 0.07 and 6 mg/L for GLP, 0.01 and 0.32 mg/L for CBZ, and 0.05 and 0.3 mg/L for MTN. The analyte concentrations of the stock solutions (mg/L) used in the assays were: for GLP, 334.1 in RFW or NFW with embryos and in RFW with larvae plus 390.2 in NFW with larvae; for CBZ, of 2.8 in RFW and NFW with embryos, and 2.35 in RFW and NFW with larvae; and for MTN, 73 in RFW with embryos and larvae in NFW with embryos, and, 157 in NFW with larvae. The concentrations of the stock solutions were determined by ion-exchange chromatography with the columns AG4-AS4. The effective concentrations determined in each bioassay were found to be at values deviating from the nominal concentrations by no more than 12% in each treatment. For the purpose of comparison, the values used in the figures, however, were the nominal concentrations. Each assay had two types of control where no pesticide was added, one each of the natural and the reconstituted water. The equivalence in the values obtained with these dual controls verified that neither form of water caused any effect on the system in the absence of a pesticide. The RFW used for dilution of stock solutions in the previously published assays (Achiorno et al., 2008, 2009, 2015), and for the RFW controls in the present work, was of the following chemical composition (mg/L, as determined after Weber, 1993): $NaHCO_3$, 192; $CaSO_4 2H_2O$, 120; $MgSO_4$, 120; KCl, 8 at a pH of 7.6–8.0 and a hardness as $CaCO_3$ of 160–180 mg/L.

Each concentration including the controls was tested in triplicate in a rearing chamber at 23 ± 1 ºC, under semistatic conditions, and in the dark to avoid photodegradation (Rueppel et al., 1977).

The data analyzed in this study correspond to a total of 12 bioassays. The experimental protocol used to assess the effects of the three pesticides in NFW was the same as those that had been used previously for RFW (Achiorno et al., 2008, 2009, 2015)—there, in a partial life-cycle bioassay with the preparasitic stages of *C. nobilii* exposed during embryogenesis and/or the larval-growth phases. The

193 protocol used in this and the previous studies cited is outlined in the following
194 section.

195

196 **2.4. Experimental design**

197 **2.4.1 Embryo bioassays**

198 We selected pieces of egg strings having embryos mainly in the blastula
199 stage. The experimental protocol consisted of three consecutive periods.

200

201 (1) Exposure: The egg strings were cut into segments of about 3 mm in length,
202 each one containing approximately 4,000 eggs. Each egg segment was cut to
203 increase the exposed surface area and randomly placed in a 1.5-ml container,
204 resulting in an egg density of approximately 2,500–3,000 eggs mL. The exposure
205 to the pesticides lasted for 96 h with 0.75 ml of the assay medium being replaced
206 daily, this effecting a partial renewal. After exposure, the experimental medium
207 was replaced in its entirety by the control medium.

208

209 (2) Postexposure: The containers with embryos previously exposed to the pesticide
210 were periodically checked by light microscopy until the appearance of free-living
211 larvae. Then, a sample of randomly selected individuals was removed from each
212 replicate; and the proportion of free-living larvae, ready-to-hatch larvae within the
213 egg capsules, and nonviable embryos, (NVEs) with collapsed envelopes and
214 patches of amorphous material, was determined, up to a total of 100 individuals
215 monitored. The embryo bioassays were considered valid when the proportion of
216 NVEs in the untreated groups was <10%.

217

218 (3) Exposure of *Aedes aegypti* (Diptera) larvae to *C. nobilii* larvae: The third
219 experimental period was started when the proportion of free-living larvae in the
220 sample was >50%. The viability of *C. nobilii* larvae was determined by evaluation
221 of their infective capability as quantified by the infection index mean abundance,
222 IIMA—*i. e.*, the total number of *C. nobilii* parasites observed in the *A. Aegypti*
223 larvae divided by total number of those hosts examined (Bush et al., 1997). *Aedes*
224 *aegypti* larvae reared in the laboratory were placed in containers (N = 30 for each
225 treatment) with 12 ml of well water. The samples from each replicate obtained in
226 the postexposure experimental period were added to the containers at the same
227 time, thus insuring a similar exposure rate for all the potential host larvae. At 72 h
228 thereafter, the mosquito larvae were fixed in 70% (w/v) aqueous ethanol and then
229 dissected under the light microscope to search for *C. nobilii* larvae in the body

cavities. This examination was performed for each replicate during the postexposure period.

2.4.2 Larva bioassays

The survival of *C. nobilii* larvae as an endpoint was evaluated as their capacity to infect *A. aegypti* larvae because of the infrequent movements of the *C. nobilii* larvae even when viable. The experimental protocol consisted of two consecutive periods. In the first, egg-string segments of about 3 mm in length containing 50% *C. nobilii* free-living larvae were exposed to a given pesticide for 48 h with partial renewal of the solution after 24 h., as described under (1) in the Embryo bioassays section above. In the second period, *A. aegypti* larvae were exposed to free-living larvae of *C. nobilii* for 72 h in control media under the same conditions as described under (3) in that same section. These bioassays were considered valid when the IIMA in the control group was >2.

2.5. The endpoints

The endpoints evaluated in these experiments were the NVEs; the IIMA for the larvae that hatched from exposed eggs (IIMA-E); that same index for the directly exposed hatched larvae (IIMA-L), and the 50%-inhibitory concentration (IC_{50}) for each of those respective endpoints. The IC_{50} concentrations were calculated from the nominal pesticide concentrations by the linear-interpolation method recommended by the Environmental Research Laboratory of the United States Environmental-Protection Agency (Norberg-King, 1993) by means of the software ICp (Versión 2.0).

2.6 Statistical analysis

Significant differences between the controls and groups treated with pesticide and evaluated in NFW were determined by the one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc*–comparison test. The Shapiro-Wilk test and the Levene median test were used to assess normality and homogeneity of variance of the data, respectively. An arc-sine transformation was applied to the IIMA. Finally, to compare the results obtained in both media and in view of the great variability observed for the infective capability owing to the progenitor females originating the hatch (Achiorno et al., 2017), at the moment of making the comparisons between the comparable concentrations for the endpoints, we generated new indices. For the proportion of NVEs and the infection indices, IIMA-E and IIMA-L, the data obtained were expressed as a percent of the respective control values for each assay to give the modified indices NVEc, IIMA-

Ec, and IIMA-Lc respectively. These data were analyzed by either the Student t test or the Wilcoxon test. To test for concentration dependency in the effects of the pesticides, regression analyses were performed with the exclusion of the control groups in order to prevent the data scatter caused by the wide separation of those values from the data of the experimental groups, thus disrupting an estimation of the differential effect of pesticide concentration on the dependent variable (Zar 2010). The significance level was set at $p < 0.05$. Statistical analyses were performed with the InfoStat software (Di Rienzo et al., 2016).

3. RESULTS

3.1. Pesticide effect in natural freshwater

Although the effect of a given pesticide was evaluated in only the NFW medium in each bioassay; as stated above, no significant differences were observed between the two controls with RFW and NFW run at same time. Therefore we can discard an effect of the nature of the water on any parameter except the action of the pesticides.

3.1.1. Embryos

Fig. 1 illustrates the increase in NVEs as a function of CBZ concentration thus demonstrating the frank inhibition of embryonic development caused by the pesticide, an effect that is statistically significant at a concentration of 0.04 mg/L or greater and reaches a maximum of 100% nonviability at 0.08 mg/L. Moreover, the narrow concentration range within which this compound acts between the threshold and plateau value is most notable. Furthermore, CBZ exhibited a sublethal action in that, at the lower concentrations, the larvae that managed to emerge from the treated egg strings exhibited a significantly reduced infectivity of the potential *A. aegypti* hosts relative to the infective capability of the control larvae, as visualized in the decline in the IIMA-Ec values at increased concentrations of CBZ. In contrast, the embryonic development of *C. nobilii* was not inhibited in a consistent fashion by GLP (Fig. 2), while the modest inhibition by MTN never reached a level of statistical significance (Fig. 3). Nevertheless, with these latter two pesticides, as with CBZ, the hatched larvae from exposed eggs had lower infectivities than the respective controls, an inhibition that became statistically significant at values of between 0.7 and 1 mg/L of GLP, and between 0.05 and 0.75 mg/L of MTN.

Table 1 lists the IC_{50} values for the inhibition of the embryonic-developmental endpoints (IIMA-E, IIMA-L, and NVEs) by the three agrochemicals tested. The IC_{50} for the NVEs is presented for only CBZ because that pesticide alone caused an inhibition of embryonic development. On the basis

of the IC_{50} values indicated in the table, the order of toxicity of the three pesticides for the endpoint IIMA-E is seen to be CBZ > MTN > GLP.

3.1.2. Larvae

Figs. 1–3 demonstrate a significant reduction in larval survival after exposure to all three pesticides because that diminution occurred at the lowest concentration tested for each compound. The IC_{50} values summarized in Table 1 indicated that the order of toxicity for the three pesticides of this endpoint, IIMA-L, was the same for the larvae as for IIMA-E —namely, CBZ > MTN > GLP.

In the presence of NFW, both MTL and CBZ exerted a concentration-dependent reduction in the viability endpoints tested although the increase in the NVE index upon elevations in the concentration of MTN were not significantly different from the control values. Likewise, GLP exerted a concentration-dependent inhibition of both the IIMA-E and IIMA-L indices. On the basis of the results summarized in Table 1 and Figs. 1–3, we conclude that the *C. nobilii* embryos are more sensitive to the deleterious effects of CBZ and MTL than to those of GLP.

3.2. Comparison of the effect of the water in the bioassay medium (NFW vs. RFW)

A comparison of the data from Fig. 4 and the IC_{50} values for the endpoints listed in Table 1 reveals that the toxicity of the three pesticides was lower in the presence of NFW than in RFW and that this difference reached up to an order of magnitude with GLP. The sole exception to this pattern was with the endpoint IIMA-E for MTN, where no difference was observed (Table 1).

Nevertheless, a statistical analysis of the data for the endpoints at equal concentrations of a given pesticide in the presence of NFW versus RFW (Table 2) revealed that a difference in the endpoint NVEc occurred with respect to the action of CBZ for the three highest concentrations tested with no difference obtaining for the effect of MTN at any concentration. In contrast, the effect of GLP on this endpoint was the same in either aqueous environment. Those high concentrations of CBZ, moreover, produced a complete developmental inhibition in NFW. The endpoint IIMA-Ec was significantly different at but a single concentration of the pesticides GLP and CBZ, whereas MTN had a significant differential effect at three out of the five concentrations tested. Finally, the nature of the water in the assay affected the endpoint IIMA-Lc significantly in at least three of the concentrations tested for all three pesticides.

The results summarized in Table 2 comparing the actions of the pesticides in NFW *versus* RFW thus indicated that the effect of the nature of the water depended on the pesticide in question. GLP produced both a sublethal alteration, as assessed by the endpoint IIMA-Ec, and a lethal action, as evidenced in the endpoint IIMA-Lc, in both those types of water. In contrast, no evidence was obtained for a consistent action of this herbicide on embryonic development (endpoint NVEc). With CBZ, the results were more complex: A definite inhibition of embryonic development occurred, as assessed by the endpoint NVEc, in the presence of NFW; whereas a sublethal effect, as manifest in the infectivity of the larvae hatched from exposed embryos (endpoint IIMA-Ec), did not evidence any consistent differences between assays in the two types of water. On the contrary, the action of this pesticide on larval survival (endpoint IIMA-Lc) proved to be considerably blunted in RFW at the upper five out of the six concentrations of the pesticide tested (Tables 1 and 2). With MTN, the nature of the water in the assay caused a significant shift in the sublethal endpoint (IIMA-Ec) because three of five concentrations compared evidenced significant differences (Table 2); while the presence of NFW reduced the pesticide's effect on the lethal endpoint (IIMA-Lc), and did so markedly (Table 1), which difference in action was statistically significant at all three concentrations tested (Table 2). For the other two compounds, with the exception of the embryonic inhibition generated by CBZ, when significant differences resulted between the two media, the action of the pesticides was decreased if the assay medium was NFW as opposed to RFW, with this difference being highly significant with GLP.

4. DISCUSSION

With respect to the objectives proposed, we can conclude that the susceptibility of the ecologically relevant parameters of *C. nobilii* (NVE, IIMA-E, and IIMA-L), has been verified by the present results, and we would emphasize that the concentrations at which the effects were observed were within the ranges established for the pesticides in the environment—*i. e.*, between 0.10 and 4.5 mg/L for GLP (Giesy et al. 2000, Mugni et al. 2011a,b), 1.40 and 1.79 mg/L for MTN (Giri et al., 2012), and 0.021 and 10.13 mg/L for CBZ (Andreu Sanchez 2008, Suárez-Serrano et al., 2010). The susceptibility of *C. nobilii* to these agents demonstrated in this work underscores the necessity to include parasitic organisms in general and this species in particular within the gamut of nontarget and indigenous species considered in the pesticide-risk assessment for aquatic ecosystems.

In determining whether differences exist in the action of pesticides on embryonic development and larval survival according to the assay medium employed, the use of IC_{50} could either under- or overestimate the effect. With the IC_{50} obtained in these tests we could conclude that the nature of the aqueous medium most likely affected the results. A comparison between comparable

concentrations of the three pesticides, however, fully confirmed that preliminary conclusion for the IC_{50} of MTN and CBZ, though not for the IC_{50} of GLP. Nevertheless, after having made comparisons between equivalent concentrations, we are left within the conclusion that, although the effect of the pesticides was generally lower in NFW, we could not establish a overall pattern in the toxicity of those compounds according to the nature of the aqueous environment.

To the best of our knowledge, literature data that might be used for a comparison with our findings on the toxicity of these pesticides in the presence of two different types of water are extremely sparse, with those authors that did compare the inhibitions obtained not presenting those results at comparable concentrations, as was done in the present work. We therefore cannot compare all our observations with those of other authors for the pesticides evaluated here.

Among the studies that assessed the effect of pesticides in natural media, van Wijngaarden et al. (1998) used pond water in one of the bioassays to determine the action of CBZ on invertebrates; but, unlike the present approach, the objective of those authors was not in comparing the toxicity over backgrounds of different types of water in the biossay. For *Daphnia magna* they obtained (in mg/L) an LC_{50} value between 0.091 and 0.399 at 48 and 96 h, respectively. The data from our assays with NFW, however, indicated that *C. nobilii* was susceptible to the deleterious effects of that fungicide at a concentration that was an order of magnitude lower than the value reported for that crustacean (Table 1).

Only few studies in the literature have investigated the effect of the nature of the diluent used on the subsequent action of toxic agents in model systems apart from the publications of Wan et al. (1989), Jonczyk et al. (2001), and Zhang et al. (2012). Among those articles, the first evaluated the effects of reconstituted water and four natural sources of water for the dilution of GLP on the acute toxicity of the pesticide on two species of fish. The authors reported a notable variation in the 96-h LC_{50} values attained for the same fish species when different types of water were used in the bioassays, that the 96-h LC_{50} values for those different types of water could vary by an order of magnitude, and that the hardness and pH of the water appeared to be the key parameters causing the variation of those values. Our studies also demonstrated that the IC_{50} could differ by an order of magnitude between different types of water; but though a difference in hardness and conductivity is a reasonable consideration, the subsequent bioassay did not indicate significantly different values at comparable concentrations of that herbicide in the two types of water used by us (Table 2). Zhang et al. (2012), evaluating the toxic effect of metals using two freshwater sources for preparing stock solutions—and either RFW or river water for dilution—indicated that the results obtained with RFW would still mirror the effects of exposure to the metal under natural conditions. This conclusion is similar to ours only with respect to the existence of a systematic difference in the inhibition at equivalent concentrations of the test agent, though in our experiments we did document statistically significant differences when particular concentrations were evaluated. Finally, among the three, Jonczyk

et al. (2001) conducted a study comparable to the present work, but using different types of seawater and evaluating the toxic effect of a metal. Those authors found that the toxicity of $CuSO_4$, measured as the IC_{25} and IC_{50}, was lower with artificial seawater than with natural water as the diluent. Despite the major differences in composition of the two natural types of water, this finding contrasts with these present results since we found that in the majority of the samples the toxicity of the three compounds was greater in the artificial water.

Even though complex experimental systems such as micro- and mesocosms are essential for the effective assessment of higher-tier ecologic risks from pesticides; for the purpose of basic regulation, acute ecotoxicity information, such as is presented in the work reported here, is still a fundamental prerequisite for making first-tier risk assessments (Hayasaka et al. 2012). Thus, in view of the findings from the present studies, we conclude that, for the purpose of extrapolating experimental results in the laboratory to the circumstance in a natural environment, special attention should be paid to the possible interference in the action of an agent by the type of water used for dilution and in a bioassay. Jonczyk et al. (2001) in their investigations considered the advantages and disadvantages of natural versus synthetic water: For example, natural water could contain microelements that are required for the maintenance and healthy development of organisms as well as possess the additional advantage of being ecologically relevant because such natural water, when used in bioassays, would come from environments that could contain organisms or substances that could consume or alter the toxic agent(s) being evaluated. The disadvantage would be the normal variation in quality of natural water over time since that water might contain, among other undesirable components, pathogens or predators that could significantly influence the data obtained in bioassays. In contrast, the use of synthetic water offers the advantage of being absolutely standardized, thus eliminating the possibility of the presence of those types of natural environmental contaminants in addition to enabling a comparison of experimental results among different laboratories. The major disadvantage, however, in the use of synthetic water is that that aqueous medium may not enable a faithful reflection of what occurs in the natural environment.

Clément (2000), who evaluated the influence of the quality of the dilution medium on the ecotoxicologic response of test organisms using microbioassays, concluded that the employment of a standard medium in bioassays generally resulted in a risk evaluation that was slightly overprotective, which conclusion coincides with the flaw in risk assessment that is associated with a consideration of only the IC_{50} values obtained with a toxic agent. Alternatively, the use of an assay medium with the water of the aquatic system under study would enable the establishment of more realistic limits for the concentrations of the toxic agent in the environment under investigation. Nevertheless, the effect of the natural dilution medium is expected to be moderate. We agree with the points made in the two reports cited above and with the conclusions of Clément (2000). As a consequence, we consider that the use of the water from a natural environment for bioassays is

fundamental in order to determine the true ecologic impact of pesticides in the real-life situation and thus from those results establish more accurate limits in the use of such compounds so as to enable a more dependable protection of key nontarget biota.

5. CONCLUSIONS

The results of this study provide essential information regarding the sensitivity of three ecologically relevant endpoints of the parasite *C. nobilii* to three pesticides, thus demonstrating that these organisms are susceptible to those agents, in concentrations that can be found in the environment and underscoring the necessity to include such organisms among the group of nontarget biota normally bioassayed.

Our results add substantially to those obtained by the only few studies in the literature that have investigated the effect of the nature of the dilution water used on the subsequent action of toxic agents in model systems; moreover, our methods may provide useful information for the assessment of the risk of sensitivity to toxic agents in aquatic ecosystems in general. We must emphasize that, in addition to the IC_{50} values for the different endpoints evaluated, the relative response of the endpoints under consideration to the absolute concentrations used in a given treatment between different assay protocols must also be considered in a statistical comparison. Finally, the results of this work would indicate that, although the effect of dilution water on the toxicity of a given pesticide can be negligible—and certainly is with certain compounds—we nevertheless believe that the complementary use of NFW along with the RFW in a toxicity assay may well facilitate a more complete and comprehensive conclusion about the effect of a toxic agent under investigation since with certain pesticides a differential toxicity does occur.

ACKNOWLEDGEMENTS

We thank Dr. Juan García (CEPAVE, CONICET) for providing us with *Aedes aegypti* (Diptera) larvae. Dr. Donald F. Haggerty, a retired academic career investigator and native English speaker, edited the final version of the manuscript.

GRAPHICS

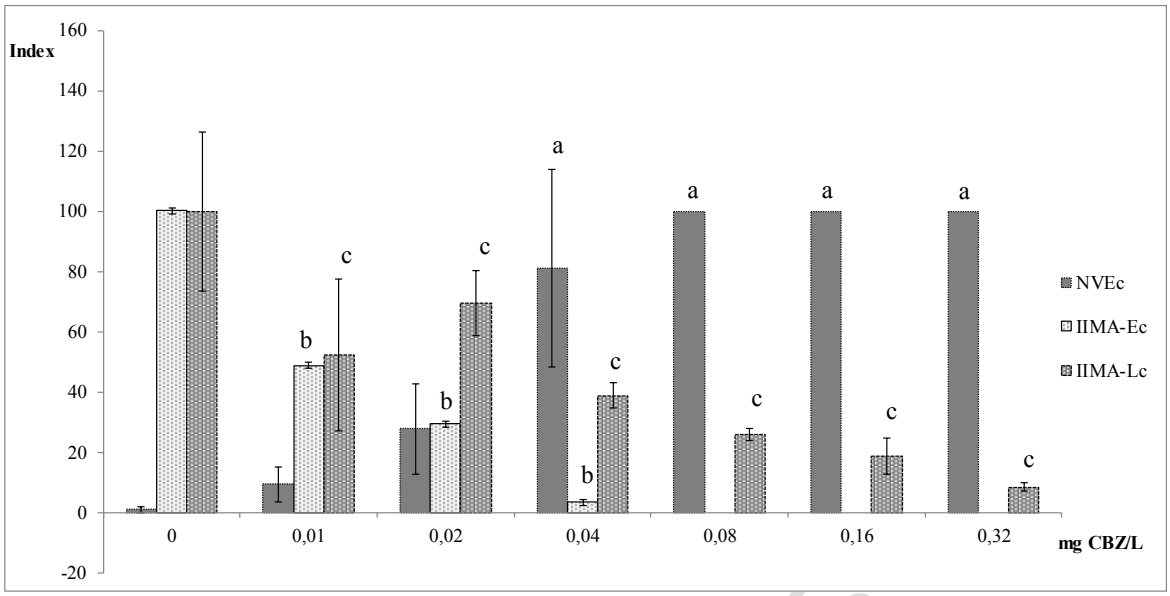

Fig. 1. Effect of carbendazim (CBZ) on the embryonic (E) and the hatched larval (L) stages of *Chordodes nobilii* in the presence of natural freshwater as the assay medium. The index of nonviable embryos (NVEc) pertains to the embryos and the infection index mean abundance (IIMA) of the two respective types of larvae tested—*i. e.*, the larvae emerging from exposed embryos (IIMA-Ec) and the larvae exposed after hatching (IIMA-Lc). In the figure, these indices are plotted on the ordinate as a function of each of the concentrations of CBZ in mg/L tested on the abscissa. Each index is expressed as the mean percent of the respective control value ± the standard deviation in brackets. Where no brackets are present the deviation was too small to plot. Key to the bar code: black, NVEc; light gray, IIMA-Ec; dark gray, IIMA-Lc. The respective statistically significant differences are indicated as: a for NVEc, b for IIMA-E, and c for IIMA-L.

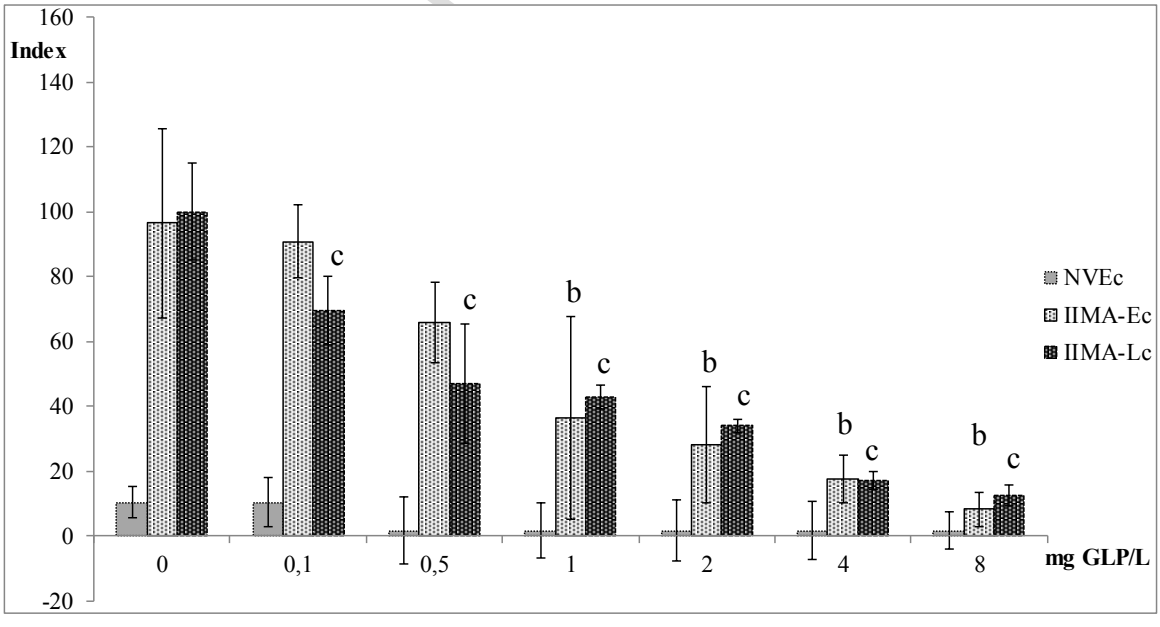

Fig. 2. Effect of glyphosate (GLP) on the embryonic (E) and the hatched larval

524 stages of *Chordodes nobilii* in the presence of natural freshwater as the assay
525 medium. In the figure, the IIMA indices and the bar code are the same as in Fig. 1.
526 These indices are plotted on the ordinate as a function of each of the concentrations
527 of GLP in mg/L tested on the abscissa. Each index is expressed as the mean percent
528 of the respective control value ± the standard deviation in brackets. Where no
529 brackets are present the deviation was too small to plot. The indications of
530 statistical comparisons are the same as in Fig. 1.

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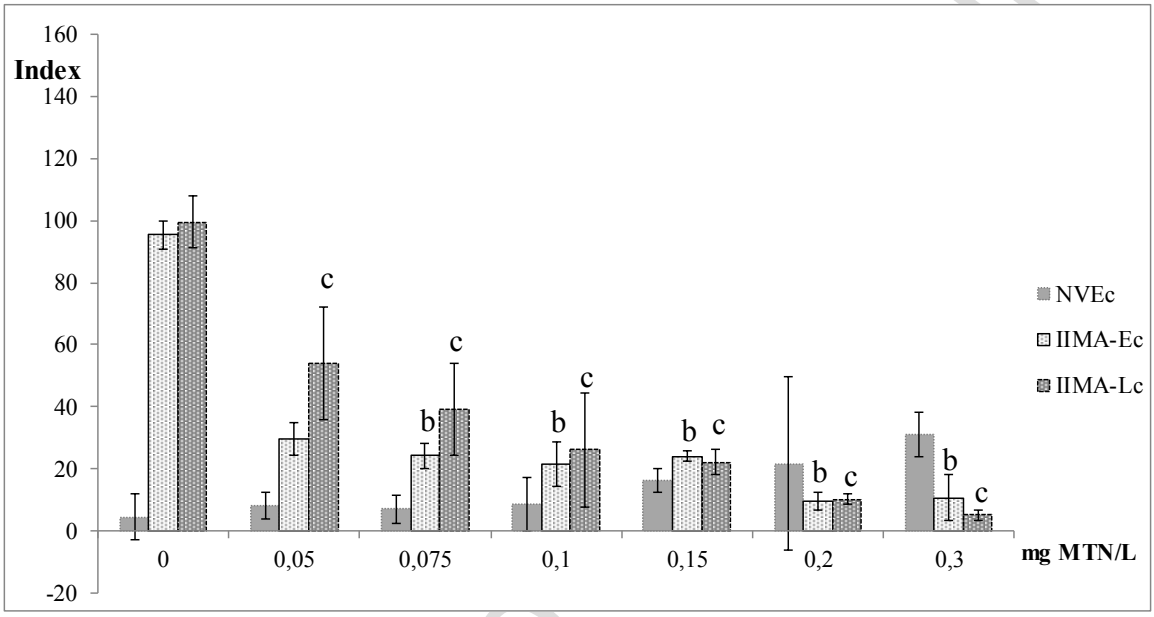

532
533 **Fig. 3.** Effect of malathion (MTN) on the embryonic (E) and the hatched larval (L)
534 stages of *Chordodes nobilii* in the presence of natural freshwater as the assay
535 medium. In the figure, the IIMA indices and the bar code are the same as in Fig 1
536 These indices are plotted on the ordinate as a function of each of the concentrations
537 of MTN in mg/L tested on the abscissa. Each index is expressed as the mean
538 percent of the respective control value ± the standard deviation in brackets. Where
539 no brackets are present the deviation was too small to plot. The indications of
540 statistical comparisons are the same as in Fig. 1.

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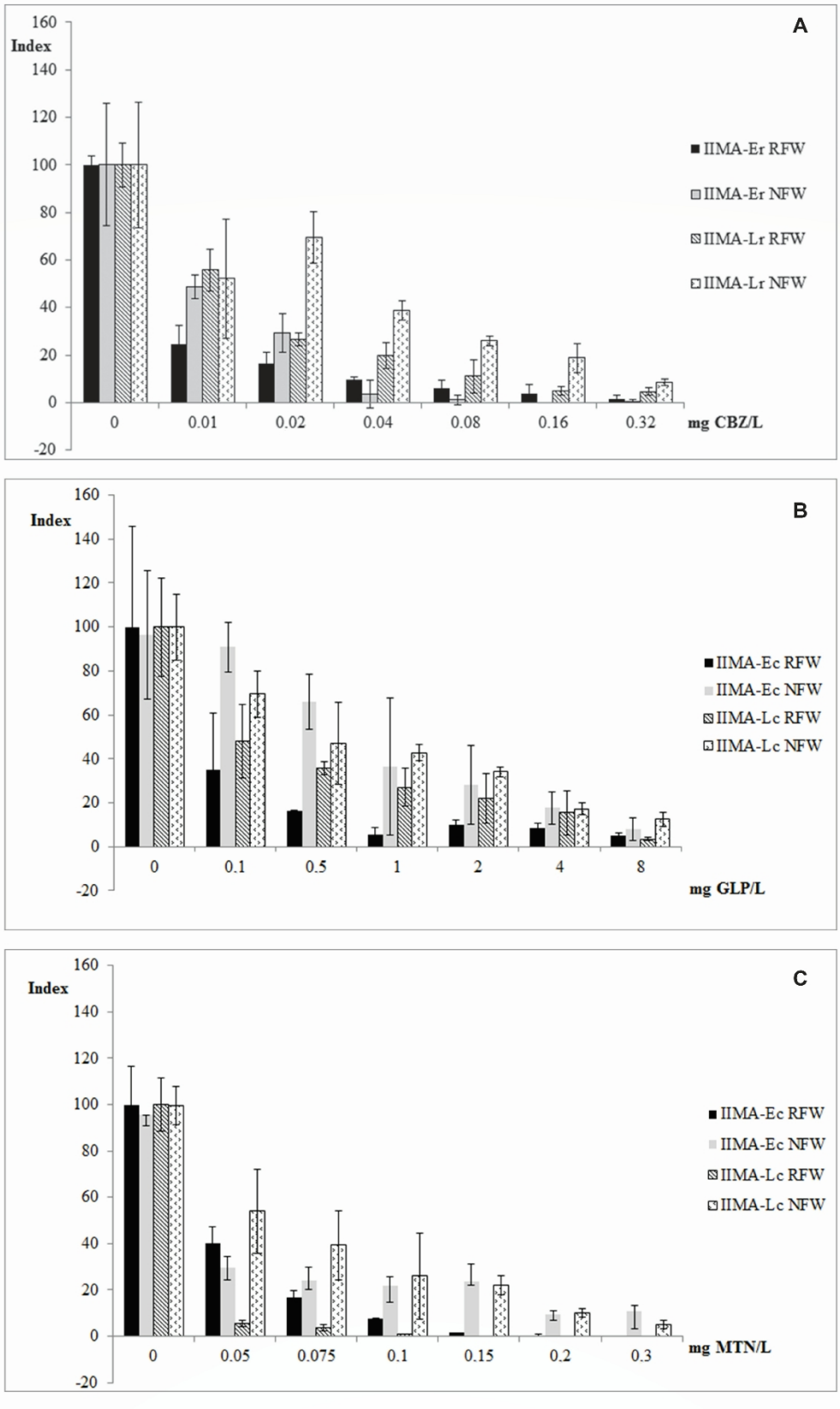

Fig. 4. Effect of CBZ (Panel a), GLP (Panel b) and MTN (Panel c) on the embryonic stages (E) and the hatched larvae (L) of *Chordodes nobilii* in the presence of natural freshwater (NFW) or reconstituted freshwater (RFW) as the

assay medium. In the figure, the IIMA indices are the same as in Fig. 1 in either NFW or RFW and are plotted on the *ordinate* as a function of the concentration of the respective pesticide, on the *abscissa*. Each index is expressed as the mean percent of the respective control value. Key to the bar code: black, IIMA-Ec RFW; light gray, IIMA-Ec NFW; white with fine stripes, IIMA-Lc RFW; white with coarse stippling, IIMA-Lc NFW

TABLES

Table 1. Inhibition of *Chordodes nobilii* embryonic-developmental endpoints by agrochemicals acting in natural or reconstituted freshwater

Pesticide*	Endpoint**	IC_{50} (mg/L)	LC (mg/L)
CBZ/NFW	NVE	0.032	0.022-0.071
	IIMA-E	0.011	0.007-0.021
	IIMA-L	0.028	0.012-0.057
CBZ/RFW	IIMA-E	0.007	0.006-0.009
	IIMA-L	0.011	0.006-0.015
GLP/NFW	IIMA-E	0.570	0.334-1.262
	IIMA-L	0.357	0.055-1.261
GLP/RFW	IIMA-E	0.054	0.027-0.355
	IIMA-L	0.067	0.037-0.547
MTN/NFW	IIMA-E	0.031	0.020-0.295
	IIMA-L	0.089	0.044-0.151
MTN/RFW	IIMA-E	0.030	0.023-0.038
	IIMA-L	0.019	0.019-0.020

*GLP, glyphosate; CBZ, carbendazim; MTN, malathion; NFW, natural freshwater; RFW, reconstituted freshwater (data from Achiorno et al., 2008, 2009, 2015 used for the present calculations of the IC_{50} and LC values)

**IIMA-E, infection index for larvae hatched from exposed eggs; IIMA-L, infection index for directly exposed hatched larvae; NVE, index of nonviable embryos.

¶Pesticide concentration producing a 50% inhibition of the endpoint tested

†Limits of confidence

Table 2. Statistical comparison between the action of a given pesticide in the presence of natural *versus* reconstituted freshwater

Pesticide¶	Endpoint†	Concentrations (mg/L) of pesticides compared with respect to toxicity between natural and reconstituted freshwater					
GLP‡		0.07	0.40	0.75	1.5	3	6
	IIMA-Ec	---	*	---	---	---	---
	IIMA-Lc	---	*	---	*	---	*
CBZ		0.01	0.02	0.04	0.075	0.16	0.32
	NVEc	---	---	---	*	*	*
	IIMA-Ec	*	---	---			
	IIMA-Lc	---	*	*	*	*	*
MTN		0.05	0.075	0.1	0.15	0.3	
	NVEc	---	---	---	---	---	
	IIMA-Ec	---	*	---	*	*	
	IIMA-Lc		*		*	*	

¶GLP, glyphosate; CBZ, carbendazim; MTN, malathion

†IIMA-Ec, percent infection index for larvae hatched from exposed eggs compared to control values; IIMA-Lc, percent infection index for directly exposed hatched larvae compared to control values; NVEc, index of nonviable embryos compared to control values

‡GLP did not consistently alter the endpoint NVEc under either assay condition.

*A statistically significant difference between the action of the pesticide at the indicated concentration in natural *versus* reconstituted freshwater, according to analysis by either the Student t test or the Wilcoxon test. The concentrations indicated in black were not tested.

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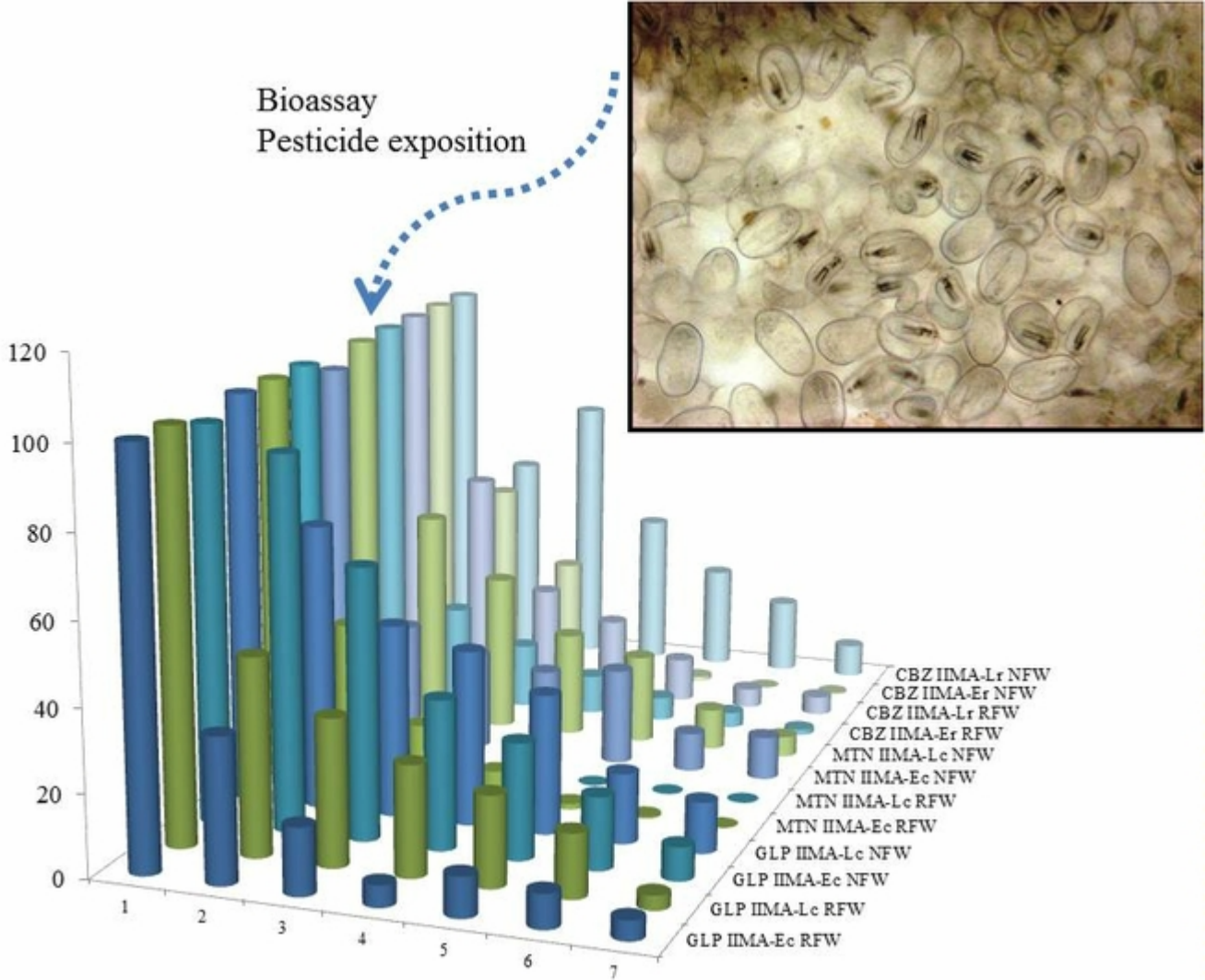
HIGHLIGHTS

- ✓ To evaluate pesticide effects on nontarget species and representative biota of each region is urgent.
- ✓ The inclusion of parasites in monitoring the environmental impact is essential.
- ✓ In designing bioassays, the medium should insure an accurate standarization and enable an extrapolation of the results to the environment.


Bioassay
Pesticide exposition
120
100
80
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CBZ IIMA-Lr NFW
CBZ IIMA-Er NFW
CBZ IIMA-Lr RFW
CBZ IIMA-Er RFW
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