Embryotoxicity and teratogenicity of pesticide indoxacarb to sea urchin (*Strongylocentrotus intermedius*)

H. Wang, H. H. Huang, J. Ding and Y. H. Wang

**ABSTRACT**

Sperm cell and embryo toxicity tests using the sea urchin *Strongylocentrotus intermedius* (*S. intermedius*) were performed to assess the toxicity of indoxacarb, a new widely used insecticide. New toxicity data for indoxacarb expressed as median effective concentration (EC$_{50}$) were reported for the sea urchin species. When sperms and cells were exposed to the pesticide before fertilization, no significant inhibition in the fertilization success of *S. intermedius* (up to 40 mg/L) was observed. Developmental toxicity of the pesticide displayed a significant dose-related increase of larval malformations and differentiation arrest at concentrations of 0.1 mg/L to 40.0 mg/L at each cleavage, including the 2-cell stage, 4-cell, blastula, gastrula, prism and 4-arm pluteus stages. It seems that 4-arm pluteus is the most sensitive to indoxacarb with the EC$_{50}$ of 3.73 mg/L, two times less than that of the first cleavage stage. All these results indicate that more attentions should be paid to the potential marine pollutions caused by this pesticide indoxacarb.

**Key words** | embryotoxicity, indoxacarb, *Strongylocentrotus intermedius*

**INTRODUCTION**

As a new broad-spectrum indeno–oxadiazine foliar insecticide, indoxacarb has been used on a broad range of crops for the control of lepidoptera and other pests in the codex system. It acts by inhibiting sodium ion entering the nerve cells, and in this way results in the paralysis and death of target insect pests (*Wing et al. 2000*). However, the selective toxicity of indoxacarb between target insects and non-target organisms is still in debate (*Tsurubuchi & Kono 2003; Silver & Soderlund 2005*). Therefore, some efforts have been made towards studying the toxicity effects of indoxacarb on different terrestrial organisms, such as the fish, rat, bird, non-target plants, as well as the estuarine or marine aquatic invertebrates like *Mysidopsis bahia* and *Crassostrea virginica* (*Hetrick et al. 2005*). Based on these data, indoxacarb has been considered as moderately or very highly toxic to the freshwater invertebrates or marine invertebrates (*Dias 2006*), although it is designed by the U.S. Environmental Protection Agency (EPA) to be a ‘reduced-risk’ pesticide and is regarded as an organophosphate (OP) replacement. Although some work has been carried out on the toxic study of indoxacarb, little information is available about its toxicity to most marine organisms, especially at the early development of embryos of these organisms. Therefore, an evaluation of the risk associated with the occurrence of indoxacarb in marine environment is urgently needed.

Marine invertebrate embryos and larvae, in particular with bivalves and sea urchins, have been used for decades as sensitive, simple, and reliable tools for assessing and monitoring the marine pollutions (*Bellas et al. 2003*). The bioassays with embryos of bivalves and echinoderms are useful tools for environmental risk assessments (*Berias et al. 2005*), due to the fact that embryos are more sensitive to
toxicants than adults (Marin et al. 1991; Ringwood 1991). Sea urchins are one of the traditional key model organisms in the early developmental and molecular biology studies, which includes the study of the mechanisms of fertilization and egg activation, cleavage, gastrulation, and the regulation of differentiation in the early embryo. Likewise for several decades the sea urchin embryo toxicity test has been utilized in evaluating a number of xenobiotics and their future in the marine ecosystem (Bay et al. 1993).

Sea urchin Strongylocentrotus intermedius (S. intermedius, Echinodermata, Echinoidea) (Agassiz 1863), as one of good contamination indicator species, mainly inhabits in the northwest Pacific region of Asia (Balakirev et al. 2008). The species are usually found from the littoral and upper sublittoral zone to a depth of 25 m, playing key ecological roles in the general functioning of ecosystems by removing algal communities or by preventing their establishment, leading to dramatic changes in the structure of benthic assemblages.

In the present work, we have conducted bioassays with early developmental stages of the intermediate (short-spined) sea urchin S. intermedius to evaluate the toxic effects of indoxacarb on the development of sea urchin embryos.

MATERIALS AND METHODS

Biological material

Experiments were performed from July 20th to August 5th, 2008. Mature S. intermedius (diameter = 3.5 ± 0.5 cm, about 2 years old) were fed in our lab. Before experiment, the sea urchins were fed with natural algae and acclimated for one week in flow-through filtered natural seawater (FSW) (0.45 μm filter) system at 13 ± 1°C in the pond with 70 liters.

Chemical

The (S)-methyl 7-chloro-2, 5-dihydro-2-[(methoxycarbonyl)[4(trifluoromethoxy)phenyl]amino]carbonyl indeno[1,2-e][1,3,4]oxadiazine-4a(3H)-carboxylate indoxacarb (CAS No: 144171-61-9) was purchased from Sigma Chemical Co. (St. Louis, MO) (Table 1).

Stock solutions were prepared by dissolving indoxacarb in dimethyl sulfoxide (DMSO, analytical purity) firstly. Since indoxacarb is not readily soluble in water, subsequently, the mixture was dissolved in the FSW. The final test solution contained no more than 0.1% DMSO. Then the stock solution was stored at 4°C for 1 h before the beginning of the experiments (Bellas et al. 2005). A solvent control containing 0.1% DMSO was also tested for its toxicity. The range of optimal concentration was determined by the previous test of the compound, which was 0 (control), 0.1, 2.0, 5.0, 10.0, 20.0 and 40.0 mg/l. Five replicates of each experimental concentration and five negative controls were conducted using the FSW, and all the tests were repeated five times on five different batches. All solutions were kept at 18.5°C throughout the experiment. All glassware was acid-washed (HNO₃ 10% volume) and rinsed with acetone and distilled water before the experiments. Physicochemical conditions of the experiments were as followed: salinity is 32.90 ± 0.23 ppt, dissolved O₂ is 6.55 ± 0.35 mg/L, and pH is 8.2 ± 0.05 (mean ± SD, n = 15) during the whole 5 days.

Table 1 The chemical indoxacarb used for toxicity analyses

<table>
<thead>
<tr>
<th>Common name</th>
<th>CAS-Reg. No.</th>
<th>Purity (%)</th>
<th>M_w (g/mol)</th>
<th>Structure</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indoxacarb</td>
<td>144171-61-9</td>
<td>99</td>
<td>527.83</td>
<td><img src="image" alt="Structure" /></td>
<td>Sigma Chemical Co. (St. Louis, MO)</td>
</tr>
</tbody>
</table>
Stage specificity of *S. intermedius*

To identify the most sensitive developmental stage of *S. intermedius* to indoxacarb, the embryos were exposed to indoxacarb for six developmental stages: 2-cell, 4-cell, blastula, gastrula, prism and 4-arm pluteus stages. After 90 hour post-fertilization (hpf), larvae were fixed and the first 100 larvae were counted for the normal developmental embryos only. The concentrations and controls were run in quintuplicate and repeated at least five times with different batches.

Embryotoxicity experiments

Gametes were harvested and embryos were reared as described by Pagano et al. (1986). Spawning was induced in sea urchin by injection of 1 ml of 0.5 M KCl (3.75 g of KCL in 100 ml of distilled water) through the perioral membrane. Eggs were transferred into a measuring cylinder FSW. A few drops of dry sperm were collected directly from the gonad with a Pasteur pipette, added to the egg suspension and carefully stirred to allow fertilization success (assessed by the percentage of eggs showing a fertilization membrane) and egg density. The observation of the fertilization membrane in more than 98% of the eggs confirmed that the fertilization was successful and then the embryos were distributed in the rearing media immediately after the fertilization.

For each experiment, six individual females were selected for their appropriate egg quality (no immature forms, no dilapidation and no fertilized eggs) and amount. Males were selected with good sperm motility (checked under microscope) and high amount. Then, the best male and female gametes were pooled and filtered through nylon cheesecloth (*D* = 200 μm for eggs and 50 μm for sperm). The egg suspension (stock solution) was diluted in order to obtain the final concentration of 250-300 eggs/mL.

A batch of fertilized eggs was exposed to the indoxacarb experimental concentrations (0.1, 2.0, 5.0, 10.0, 20.0 and 40.0 mg/L) in order to follow the effects of indoxacarb during different stages of the embryonic development of *S. intermedius*. Aliquots of the egg suspensions containing approximately 100 embryos were fixed at various times after fertilization, and the percentage of embryos at 2-cell, 4-cell, blastula, gastrula, prism and 4-arm pluteus stages was recorded.

The first cleavage occurred in most cases 80–90 min after fertilization at 18.5°C for *S. intermedius*. The rate and nature of the first cleavage, namely the proportions of undivided cells and normal doubled cells in each test water set, were checked after some adequate time. After successive divisions and developmental changes, the ratios of blastula to gastrula were observed. Lastly, the proportions of normal plutei, retarded, or larvae were checked at 48 or 72 h after fertilization. 100–120 eggs or embryos were fixed with 5% formaldehyde at the time of these observations.

After incubation with different concentrations of indoxacarb solutions, one or a half drop of formalin was added and the percentage of normal developed embryos from 2-cell to 4-arm pluteus larvae stages was recorded. In this work the ‘abnormal’ embryos were defined as the mortal, delayed and malformed embryos. Mortal embryos/larvae were embryos that stopped development and never reached the next stage. Delayed embryos/larvae were those underwent a delay in their development compared with the controls. Malformed embryos/larvae were those with defective characteristics typically exhibiting as misshapen spicules, missing arms or incomplete guts, etc.

Statistical analysis

Results were presented as mean ± standard deviation (S.D.). Significant differences were evaluated by means of one-way ANOVA followed by the Tukey–Kramer test for the concentration-response curves. Stage exposure experiments were analyzed using two-way ANOVA with Tukey Test for pairwise multiple comparison. Comparisons were also made among concentrations across stages using a Tukey Test. The EC₅₀ and the 95% confidence intervals (95% CI) were calculated according to the Bliss probit analysis using response and toxicant concentration data for all solutions (Bliss 1935).

RESULTS

The embryotoxicity effect of pesticide indoxacarb was evaluated using bioassay species in the aquatic ecosystem.
In these assays, the embryos and larvae of sea urchin which are frequently used in ecotoxicological tests were used, as they are highly sensitive to low doses of toxic chemicals. Presently, toxicity experiments were conducted to the 2-cell, 4-cell, blastula, gastrula, prism and 4-arm pluteus stages of a new species sea urchin S. intermedius using the 95% CI values for the EC50 estimates, and the most sensitive stage of S. intermedius to the pesticide indoxacarb was determined.

The stage of cell division

The EC10, EC50 and EC90 values for the first cleavage are 5.20 (0.38–7.76), 9.70 (4.97–14.64) and 18.09 (12.70–139.52) mg/L, respectively. For the second cleavage (4-cell) they are 2.27 (0.00–4.80), 6.04 (0.05–10.38) and 16.12 (9.56–60.14) mg/L, respectively (Table 2). The EC10, EC50 and EC90 values of the blastula stage are 3.25, 7.12 and 15.60 mg/L, in which stage the 95% confidence intervals were not calculated, and for the gastrula stage the EC50 are 1.80 (0.29–3.11), 4.84 (2.57–6.87) and 12.99 (8.77–38.98) mg/L, respectively. For the pluteus stage, the EC50 are 1.77 (0.08–3.48), 5.18 (1.74–7.62) and 15.11 (9.96–67.43) mg/L, respectively. During the whole embryo development, an enhancing toxic effect appeared, and the most sensitive developmental stage to indoxacarb is 4-arm pluteus of S. intermedius. According to these results as well as the recognized guidelines (Kamrin 1997), the pesticide of indoxacarb could be defined as 'very high toxic' to organisms indicated by its LC50 value which is less than 100.

The abnormal rate of the embryos

The embryos developed with good synchrony in negative controls. While with the increase of the indoxacarb concentration, a normal synchrony of the cellular division disappeared as shown by the retard rates of each stage (data not shown). From the first cleavage to the 4-arm pluteus, even at a low concentration (0.1 mg/L) of indoxacarb used, an abnormal development of the embryos began to appear. The abnormal rates increased by about 40% from the 2-cell to 4-arm pluteus stages as shown in Figure 1. On the 2-cell stage, a serious developmental abnormality emerged when 0.1 mg/L of indoxacarb was added, and its abnormal rate was 20.8% ± 6.4% as compared to the control whose abnormal rate is 6.9% ± 1.4%. The higher concentration (10 mg/L) would cause an irreversible impairment of the embryo development when more than 70% embryos were abnormal. Especially on the 4-arm pluteus stage the average

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**Table 2** | Different EC10, EC50 and EC90 values of indoxacarb to different developmental stages of sea urchin S. intermedius

<table>
<thead>
<tr>
<th>Stage</th>
<th>EC10 (mg/L)</th>
<th>EC50 (mg/L)</th>
<th>EC90 (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-cell</td>
<td>5.20 (0.38–7.76)</td>
<td>9.70 (4.97–14.64)</td>
<td>18.09 (12.70–139.52)</td>
</tr>
<tr>
<td>4-cell</td>
<td>2.27 (0.00–4.80)</td>
<td>6.04 (0.05–10.38)</td>
<td>16.12 (9.56–60.14)</td>
</tr>
<tr>
<td>Blastula</td>
<td>3.25 (n.c.)</td>
<td>7.12 (n.c.)</td>
<td>15.60 (n.c.)</td>
</tr>
<tr>
<td>Gastrula</td>
<td>1.80 (0.29–3.11)</td>
<td>4.84 (2.57–6.87)</td>
<td>12.99 (8.77–38.98)</td>
</tr>
<tr>
<td>Prism</td>
<td>1.77 (0.08–3.48)</td>
<td>5.18 (1.74–7.62)</td>
<td>15.11 (9.96–67.43)</td>
</tr>
<tr>
<td>4-arm Pluteus</td>
<td>0.99 (0.27–1.80)</td>
<td>3.73 (2.21–5.00)</td>
<td>14.01 (10.28–24.86)</td>
</tr>
</tbody>
</table>

The 95% confidence intervals (95 CI) are given in brackets. n.c.: not calculated.

**Figure 1** | Percentage of normal embryos of S. intermedius for stage-specific exposure to indoxacarb of different concentrations. Black: 0.1 mg/L (low), gray: 2 mg/L (moderate), white: 5 mg/L (high). Error bars represent the standard deviations (n = 3). a denotes a significant difference of toxic effects appeared as compared to the low concentration (p < 0.05). b denotes a significant difference compared to the low and moderate concentration or to low concentration only.
abnormal rate was as high as about 90%, when most of the cells were cytolytic. And when the pesticide was at higher concentration (up to 20 mg/L), all the fertilized eggs became dead or moribund. These results show that with the concentration increase of indoxacarb, the rate of abnormalities increases.

**Stage specificity of S. intermedius**

To determine which developmental stage was more sensitive to the impact of indoxacarb, the stage-specific exposures were conducted using *S. intermedius*. Embryo responses were normalized to controls and analyzed for significant differences either between stages, within stages, or across stages at each concentration which are shown as bar graphs in Figure 1. (Bellas et al. 2003) As can be seen from the figure, all biological responses were significantly different from those of the controls, and the abnormal rates between the low (0.1 mg/L), moderate (2.0 mg/L) and high (10.0 mg/L) concentrations are also significantly different. In Figure 1, the significant differences between the abnormal rates of each concentration within stages are noted with either an (a) or a (b) indicating significant difference when compared to the low concentration (a) or when compared to both the low and moderate concentrations (b).

**DISCUSSION**

Indoxacarb is a novel broad-spectrum oxadiazine insecticide and registered for using against a wide variety of lepidopteron pest insects, as Homoptera and Coleoptera. Mammalian toxicity studies of this pesticide were extrapolated from laboratory studies on rats. However, the importance of the endpoint to the effects of indoxacarb on wildlife populations is limited certain (U.S. EPA 2000; Dias 2006). But some data have shown that many non-target organisms such as *Apis mellifera* (Honey bee) have been affected by the drug (U.S. EPA 2000). It was also found to be moderately or very highly toxic to freshwater and estuarine/Marine fish and invertebrates both from literatures and this work as shown in Table 3.

In the present work, we found the EC 50 of indoxacarb was 3.73 mg/L at the endpoint of 4-arm pluteus (50 h), which was similar with its acute toxicity to *Daphnia*

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**Table 3 | Toxicity of indoxacarb to various species in freshwater and estuarine/marine obtained from both literature and this work**

<table>
<thead>
<tr>
<th>Species</th>
<th>Study type</th>
<th>Concentration (mg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Freshwater fish</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Oncorhynchus mykiss</em> (Rainbow trout)</td>
<td>Acute toxicity LC 50</td>
<td>0.65</td>
<td>Wing et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>Chronic LOEL/NOEL</td>
<td>0.25/0.15</td>
<td></td>
</tr>
<tr>
<td><em>Cyprinus carpio</em> (Carp)</td>
<td>Acute toxicity LC 50</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td><em>Ictalurus punctatus</em> (Channel catfish)</td>
<td>Acute toxicity LC 50</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td><strong>Estuarine/Marine Fish</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cyprinodon variegatus</em> (Sheepshead minnow)</td>
<td>Acute toxicity LC 50</td>
<td>&gt;0.37</td>
<td>Hetrick et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>Chronic LOEL/NOEL</td>
<td>0.042/0.017</td>
<td></td>
</tr>
<tr>
<td><strong>Freshwater aquatic invertebrates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Daphnia carinata</em></td>
<td>Acute Toxicity EC 50</td>
<td>2.94</td>
<td></td>
</tr>
<tr>
<td><em>Daphnia magna</em> (Water flea)</td>
<td>Acute Toxicity EC 50</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chronic LOEL/NOEL</td>
<td>0.19/0.075</td>
<td></td>
</tr>
<tr>
<td><strong>Estuarine/Marine aquatic invertebrates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Crassostrea virginica</em></td>
<td>Acute toxicity EC 50</td>
<td>0.203</td>
<td></td>
</tr>
<tr>
<td><em>Mysidopsis bahia</em></td>
<td>Acute toxicity EC 50</td>
<td>0.0542</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chronic LOEL/NOEL</td>
<td>0.0407/0.0184</td>
<td></td>
</tr>
<tr>
<td><em>S. intermedius</em> (Sea urchin) 4-arm pluteus</td>
<td>Acute toxicity EC 50 (50 h)</td>
<td>3.73</td>
<td>This work</td>
</tr>
</tbody>
</table>
carinata in freshwater (EC50 was 2.94 mg/L, Hetrick et al. 2005). Compared with the acute toxicity to other estuarine/marine aquatic invertebrates reported by Wing et al. (2000) and Hetrick et al. (2005), the toxicity of indoxacarb was also high to the embryo and larvae of sea urchin. Therefore, the possible contamination caused by the use of this pesticide should be of concern.

Among the various methods employed for monitoring the marine environmental pollution, the sea urchin system might be one of the most important bioassays (Kobayashi & Okamura 2002; Bellas 2006). The sea urchin embryo appears to be an appropriate in vitro screening test for predicting the potential teratogenicity without resorting to more complex systems which are usually employed in chronic toxicity testing. This model also proves to be multi-informative, because it permits an investigation about the toxicity of the molecules tested throughout embryogenesis. Sea urchin gametes have been demonstrated to be convenient for investigating the cytotoxicity of many chemicals (Epel et al. 2006). Therefore, in this work, a sea urchin test system has been selected for the purpose of testing indoxacarb to marine environmental invertebrates.

Up to now, to our best knowledge, the study reporting the effects of indoxacarb on the early embryo development of sea urchin is still unavailable. Our present investigation found that indoxacarb could disrupt the embryonic and larval development of S. intermedius to different extent. The results showed that indoxacarb exhibited no evident toxic effects on the fertilization of eggs, and the fertilization rate kept about 100% at each experimental concentration of the pesticide for all the tested and control samples. It is also interesting that the compound showed a very slight toxic effect on the initial cleavage stage of the cell. The reason might be due to the fact that in this stage the eggs were well protected by the fertilized membrane, and also the physiological and metabolic activities of the eggs cells are in a state of latency (Ozretic et al. 1998). All this makes this stage the most resistant phase in the sea urchin embryonic life cycle. But after this developmental stage, the profound damages could be observed at all stages of the embryonic and larval development even under low concentrations of the pesticide used.

A further analysis of the hydrophobicity described by log P (the logarithm of the octanol water partition coefficient) would broaden our understanding about the toxicity of indoxacarb. Log P can, generally, well describe the bioavailability of a chemical to organism, reflecting the ability of a compound to form non-covalent interactions with its environment, to dissolve and persist in water or in a lipidic environment, or to permeate the phase interfaces (Pontolillo & Eganhouse 2001). And normally, larger log P indicates a stronger ability of the chemical to permeate the cell membrane of an organism and, therefore, much more easily to interact with its target in the organism. Presently, the log P value of indoxacarb is 4.60, which indicates that it is very hydrophobic. Therefore, we speculated that the toxicity of this compound may be directly related to the membrane-crossing ability caused by its high hydrophobicity. This consideration was supported by the fact that the 4-arm pluteus was the most sensitive stage to the toxicity of indoxacarb during the whole embryo development. The possible reason of this toxicity was that indoxacarb acts by inhibiting the entry of sodium ion into specific nerve cells, resulting in the paralysis and death of the target animals (Zhao et al. 2005; Salgado & Hayashi 2007). All above results suggest that more attentions should be given to the potential marine pollutions caused by the pesticide indoxacarb.

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